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PHOSPHOPEPTIDE PROBES IN RAW MILK AND DERIVED PRODUCTS

Candidate

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To my husband

To my parents

ABSTRACT

Bioactive peptides can be produced *in vitro* by fermentation of milk inoculated with starter or non-starter cultures or by digestion of milk proteins with one or more proteolytic enzymes. *In vitro* casein hydrolysis by gastrointestinal enzymes is also capable of generating bioactive peptides.
Our study has focused on the fraction of bioactive peptides called casein phosphopeptides (CPPs). The negatively charged side chains of phosphoserines make the phosphorylated residues capable to chelate macroelements, such as Ca, Mg and Fe, as well as oligoelements, such as Zn, Ba, Cr, Ni, Co and Se, as suitable metal ion carriers from which derive their biological activity.

CPPs are also resulted very good markers of raw material quality, process and authenticity of dairy products beyond their bioactivity.
Native and partially dephosphorylated CPPs are markers of:

1) Raw milk from infected subjects with high somatic cell count (SCC);

2) Heat treatment intensity of commercial milks;

3) Parmigiano Reggiano (PR) cheese at different age of ripening: younger cheeses (up to 15 months) were distinguished from those older than 26 month;

4) Adulterated water buffalo milk (or ovine and caprine) with cheaper bovine milk used for producing cheese made from single-species milk.

In milk with high SCC, the majority of the peptides identified by LC-ESI-Q-TOF-MS originated from α_{s1}-, α_{s2}-, β- and κ-casein (CN), as a result of degradation by plasmin (PL) and enzymes of somatic cells. Moreover, seventeen native and twenty partially dephosphorylated CPPs were identified through selective enrichment on hydroxyapatite (HA) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. The degree of phosphorylation was well correlated with the alkaline phosphatase (ALP) activity of milk.

The CPP enrichment on HA of heat-treated milk samples has allowed detection of CPPs, ordinarily suppressed by the co-existence of non-phosphorylated peptides. Most CPPs had C-terminal lysine probably derived from the proteolytic activity of endogenous PL present in milk. α_{s1}-, α_{s2}- and β-CN are preferentially hydrolyzed based on their affinity for PL, according to the decreasing order: β- > α_{s2}- > α_{s1}-CN. Several phosphopeptides belonging to the β-CN family and to the α_{s2}-CN were detected in raw and heat treated milk. A synthetic peptide analogue of β-CN (f1-28) 4P was used as internal standard to measure the rate of proteolysis of β-CN. β-CN was found to have released ~4.3% of the β-CN (f1-28) 4P, in raw milk. Moreover, CPPs underwent partial lactosylation by milk heating or drying. Lactosylated CPPs, β-CN (f1-29) 4P, (f1-28) 4P, (f1-27) 4P and α_{s2}-CN (f1-21) 4P, (f1-24) 4P, were observed especially in liquid and powder milk. Raw and thermized/pasteurized milk did not contain lactosylated peptides/proteins whereas they were specific of intensely heated milks. The limit of detection for spiked raw or pasteurized milk with Ultra High Temperature milk was ~10%.

Using MALDI-TOF, pH 4.6-soluble CPPs of different cheese varieties, including PR, selectively enriched on HA were identified. The rate of proteolysis and dephosphorylation phenomenon is resulted to change in cheeses from raw or pasteurized milk. Heat treatments modified the peptide profile by increasing the content of larger peptides whereas the shorter CPPs, containing the multi-phosphorylated motif -SerP-SerP-SerP-Glu-Glu- more resistant to proteolytic enzymes, accumulated in long-ripened cheeses made from raw milk. The β-, α_{s1}- and α_{s2}-CN CPPs were isolated from PR cheese samples aged 5, 15, 26 and 35 months (mo). β-CN was hydrolyzed within the first 15 mo of ripening faster than α_{s1}- and α_{s2}-CN did. In contrast, α_{s1}-CN was more hydrolyzed in 26- and 35-mo-old cheeses than β-CN, and α_{s1}-CN (f67-79) 3P, α_{s1}-CN (f71-79) 1P, α_{s1}-CN (f64-74) 2P, α_{s1}-CN (f66-74) 3P and α_{s1}-CN (f67-74) 2P were detected just in these older samples. There was a significant hydrolysis of α_{s1}-CN at 26 mo of ripening; afterwards, the increase slowed down with time. On the other hand, the shorter CPPs, α_{s1}-CN (f65-74) 3P and α_{s1}-CN (f64-74) 3P, accumulated in the 35-mo-old PR cheese. Unlike cheese-milk, proteolysis proceeded in the PR cheese sequentially in the decreasing order: β- > α_{s1}- > α_{s2}-CN. On the basis of CPP monitoring, the findings have allowed to categorize PR cheese according to the age, *i.e.* young, within 5- and 15-mo-old, and old, aged more than 26 mo.

The MALDI analysis of proteotypic CPPs for bovine (B) and water buffalo (WB) milk has allowed to set up a proteomics methodology for the false-positive detection of B milk in genuine WB milk and derived cheese. Tryptic phosphopeptides β-CN (f1-25) 4P and β-CN (f1-28) 4P, selectively bound to HA, were used as markers of B and WB species, respectively. The actual percentage of adulterating B milk was calculated using a calibration plot with a detection limit of 0.5% *via* MALDI-TOF-MS analysis of species markers. The

detection of B β-CN (f1-25) 4P in CN digests of WB Mozzarella cheese samples have allowed to measure the percentage of B adulterant. The excellent specificity of proteotypic peptides opens the possibility to broaden the application to other species' milk or to any food protein of which is known the protein sequence.

RIASSUNTO

Peptidi bioattivi possono essere rilasciati *in vitro* dalle fermentazioni batteriche di colture, starter o non-starter, inoculate nel latte o dalla digestione delle proteine del latte mediante l'utilizzo di uno o più enzimi proteolitici. Anche la proteolisi *in vivo* per opera di enzimi gastrointestinali è in grado di rilasciare tali peptidi.
Il nostro studio si è focalizzato su una frazione di peptidi bioattivi chiamati caseinofosfopeptidi (CPPs). Le cariche negative delle catene laterali delle fosfoserine rendono i residui fosforilati in grado di chelare sia macroelementi, quali Ca, Mg e Fe, che oligoelementi, del tipo dello Zn, Ba, Cr, Ni, Co e Se, e quindi possono trasportare ioni metallici. Da ciò deriva la loro attività biologica.

Al di là della loro bioattività, i CPPs sono risultati buoni indicatori della qualità della materia prima, del processo produttivo e dell'autenticità del prodotto. I CPPs, nella loro forma nativa o parzialmente defosforilata, sono marcatori di:

1) latte crudo con un elevato numero di cellule somatiche (SCC), proveniente da animali infetti;

2) intensità del trattamento termico di latti commerciali;

3) formaggio Parmigiano Reggiano (PR), a diverse età di maturazione; pertanto, formaggi giovani (fino a 15 mesi) sono stati differenziati da quelli invecchiati per più di 26 mesi;

4) latte di bufala (o ovino e caprino) adulterato con latte bovino più conveniente, per la produzione di formaggi a singola specie.

Nei latti con alto SCC, la maggior parte dei peptidi, identificati mediante spettrometro di massa LC-ESI Q-TOF MS, derivava dalla proteolisi delle frazioni caseiniche, α_{s1}-, α_{s2}-, β- and κ-casein (CN), per azione della plasmina (PL) e degli enzimi delle cellule somatiche. Inoltre, in questi latti, sono stati identificati diciassette CPPs nella loro forma nativa e venti CPPs parzialmente fosforilati, grazie all'utilizzo di una tecnica di arricchimento selettiva, basata su microgranuli di idrossiapatite (HA) e l'analisi MALDI. È stato mostrato che il grado di fosforilazione delle frazioni caseiniche ben si correlava all'attività della fosfatasi alcalina (ALP) del latte.

L'arricchimento su HA dei CPPs di campioni di latte, trattato termicamente, ha consentito di rilevare peptidi fosforilati, il cui segnale di massa sarebbe stato soppresso dalla co-presenza di peptidi non-fosforilati. La maggior parte dei CPPs, rilevati nei campioni, derivava dall'azione proteolitica della PL endogena del latte. È stato mostrato che le frazioni caseiniche, α_{s1}-, α_{s2}-, and β-CN, venivano idrolizzate secondo l'ordine decrescente di affinità alla PL: β- > α_{s2}- > α_{s1}-CN. Diversi fosfopeptidi appartenenti alla β-CN e all'α_{s2}-CN sono stati rilevati nel latte crudo e nei campioni di latte trattato termicamente. Un fosfopeptide sintetico analogo alla β-CN (f1-28) 4P è stato usato come standard interno per misurare il livello di proteolisi della β-CN nel latte crudo. É risultato che è stato rilasciato dalla β-CN ~ il 4.3% del fosfopeptide β-CN (f1-28) 4P nel latte crudo. Inoltre, i CPPs subivano una parziale lattosilazione in funzione del trattamento termico. I CPPs lattosilati della β-CN, (f1-29) 4P, (f1-28) 4P e (f1-27) 4P e quelli dell'α_{s2}-CN, α_{s2}-CN (f1-21) 4P e (f1-24) 4P, sono stati rilevati principalmente nei latti in polvere. Il latte crudo e quello termizzato/pastorizzato non contenevano peptidi o proteine lattosilate, il che rendeva questi peptidi specifici del trattamento termico. L'analisi dei CPPs del latte pastorizzato, in miscela con diverse percentuali di latte UHT, ha consentito di rilevare fino al 10% di aggiunta di latte UHT.

I CPPs estratti dalla frazione solubile a pH 4.6 di diverse varietà di formaggio, incluso il PR, sono stati selettivamente arricchiti su HA ed identificati mediante l'utilizzo del MALDI-TOF. I nostri risultati mostrano che il tasso di proteolisi e di defosforilazione variava nei formaggi prodotti da latte crudo o da latte pastorizzato. Il trattamento termico modificava il profilo fosfopeptidico, aumentando il contenuto dei peptidi più lunghi. I CPPs più corti, contenenti la sequenza aminoacidica -SerP-SerP-SerP-Glu-Glu- risultata più resistenti agli enzimi proteolitici, si accumululavano nei formaggi a lunga maturazione, a partire da latte crudo. I CPPs delle frazioni caseiniche, β-, α_{s1}- and α_{s2}-CN, sono stati isolati nei diversi campioni di PR a 5, 15, 26 e 35 mesi di stagionatura. I risultati hanno evidenziato che la β-CN era idrolizzata più velocemente nei primi 15 mesi rispetto all'α_{s1}- e all'α_{s2}-CN. Al contrario, α_{s1}-CN era idrolizzata più intensamente tra 26 e 35 mesi, per cui i CPPs, *i. e.* α_{s1}-CN (f67-79) 3P, α_{s1}-CN (f71-79) 1P, α_{s1}-CN (f64-74) 2P, α_{s1}-CN (f66-74) 3P e α_{s1}-CN (f67-74) 2P, sono stati rilevati esclusivamente nei campioni di formaggio a maggior tempo di stagionatura. Un significativo incremento dei CPPs dell'α_{s1}-CN è stato osservato nei formaggi a 26 mesi di stagionatura. Dopo il 26° mese, il numero dei CPPs non aumentava ulteriormente, sebbene l'accumulo dei CPPs più corti, quali α_{s1}-CN (f65-74) 3P and α_{s1}-CN (f64-74) 3P, si verificava principalmente nei formaggi a 35 mesi. A differenza del latte, nel PR, la proteolisi procedeva seguendo il seguente ordine: β- > α_{s1}- > α_{s2}-

CN. Il monitoraggio dei CPPs nei diversi campioni di PR ci ha consentito di caratterizzare i campioni di PR in funzione dell'età, distinguendo i formaggi più giovani, di 5 e 15 mesi, da quelli maturati più di 26 mesi.

Infine, l'analisi MALDI dei CPPs proteotipici del latte bovino (B) e di bufala (B) ha permesso di mettere a punto una metodologia analitica per la rilevazione di falsi-positivi per aggiunte di latte B in latte WB, usato per la produzione di formaggi fatti da un'unica specie. I CPPs triptici, B β-CN (f1-25) 4P e WB β-CN (f1-28) 4P, selettivamente arricchiti su HA, sono stati usati come marcatori delle due specie. La percentuale di aggiunta del latte B in latte WB è stata calcolata utilizzando la curva di calibrazione dei due CPPs, costruita grazie all'analisi MALDI. Il limite di rilevabilità è stato dello 0.5%. La presenza di B β-CN (f1-25) 4P nei digeriti triptici della CN, estratta dalla Mozzarella di bufala, ha permesso di determinare l'aggiunta percentuale di latte B. L'eccellente specificità della tecnica consente di estendere la sua applicazione ad altre specie lattifere o ad altre proteine alimentari delle quali si conosca la sequenza proteica.

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0 PREFACE

This PhD thesis contains the results of research undertaken at the Department of Food Science, University of Naples – Federico II, under the encouraging supervision of the Professor Francesco Addeo. The thesis work has been developed from May 2010 to February 2013. During the last six months of PhD, the candidate has carried out a skilled training on high throughput quantitative proteomics at Proteomics and Molecular Cell Dynamics Group of University College London (UCL) under the supervision of the Professor Jasminka Godovac-Zimmermann.
The dissertation consists of four main results relatives to casein phosphopeptides isolated from raw milk and derived products and their role as markers of raw material quality, process and authenticity of dairy products. The list of publications produced during the last three years is reported at the bottom of the thesis. Three scientific papers and two book chapters have already been published and included as appendices.

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1 STATE OF ART

An impressive increase of data is supporting the bioactive role of food proteins and derived peptides beyond their nutritional impact. Specific biological properties relative to the different dietary proteins make these components potential ingredients of functional or health-promoting foods. The absorption of intact food proteins in the human gastrointestinal tract is widely limited by their molecular size. Most of them shows specific biological activity after the gastrointestinal digestion. Actually, many of protein properties are attributed to peptides encrypted in their inherent amino acid sequence, exerting biological functions after their release from the intact protein. They are 3 to 20 residue long peptides although some have been reported to have >20 amino acid residues. These peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion, fermentations or food processing. Various physiologically functional peptides are reported to exert a wide range of functions including antihypertensive, antioxidative, opioid agonistic, immunomodulatory, antimicrobial, prebiotic, mineral binding, antithrombotic and hypocholesterolemic activities. For instance, purified hydrolysates from cooked eggs release seven ACE-inhibitory peptides, five tripeptides (VDF, LPF, MPF, IPF, and TTI) and two pentapeptides (YTAGV, ERYPI), exhibiting *in vivo* antihypertensive activity (Majumder & Wu, 2009) as well as many ACE-inhibitory peptides are originated from food sources such as soybean, mung bean, sunflower, rice, corn, wheat, buckwheat, broccoli, mushroom, garlic, spinach and wine (Guang & Phillips, 2009). Small peptides and free amino acids (FAA) in mackerel hydrolysates exhibit strong antioxidant activity *in vitro* (Wu et al., 2003) as well as this activity is exhibited by papain hydrolysates of porcine myofibrillar proteins (Saiga et al., 2003). Moreover, there are two different groups of proteins that can interact with minerals. To the first group belong the proteins with specific binding site(s) for mineral ions, such as lactoferrin and α-lactalbumin (α-La) from milk and ovotransferrin from egg yolk. The other group includes proteins rich in acidic residues binding positive charges of metals through electrostatic interactions, such as casein (CN), the major fraction of milk proteins, and phosvitin, the major fraction of hen egg yolk phosphoproteins. The presence of highly acidic clusters in food proteins explains their powerful ability to sequester divalent cations (up to 60 iron atoms per phosvitin molecule, Webb et al., 1973) and their resistance to proteolytic enzymes. Therefore, an elevated number of phosphate groups is often considered nutritionally negative due to lowering the bioavailability of iron, as occurs in the case of phosvitin (Ishikawa et al., 2007). The apparent absorption of cations (calcium, magnesium, iron etc) from a yolk protein-based diet is found to be lower than that from CN, attributing this effect to the higher resistance of yolk phosvitin to proteolytic action (Ishikawa, et al., 2007).

1.1 Casein phosphopeptides

CN, representing 80% of total milk proteins, consists of four α_{s1}-, α_{s2}-, β-, and κ-CN families in the approximate ratio 38:11:38:13, respectively. These fractions exhibit different level of phosphorylation: the maximum level of phosphorylation is 13 phosphate groups for bovine α_{s2}-CN, 9 for α_{s1}-CN, 5 for β-CN and 2 for κ-CN. A direct relationship between the phosphorylation level and mineral chelating ability has been already reported (Kitts, 1994) so that their ability follows the order α_{s2}-CN > α_{s1}-CN > β-CN > κ-CN. The importance of phosphorylated residues for the CN functionality (*e.g.* the formation of micelles and the binding of Ca^{2+}) is emphasized by the high conservation of the phosphorylation sites (Fiat & Jollès, 1989). CN phosphorylation is a post-translational event that occurs during the transfer of the completed polypeptide chains from the smooth endoplasmic reticulum to the mammary gland Golgi apparatus, where most of phosphate incorporation presumably takes place (Mercier, 1981). Evidence supports that the polypeptide chain of CN is synthesized prior to the incorporation of phosphate and that a pool of non-phosphorylated or partially phosphorylated CN exists in the mammary gland (Singh et al., 1967). Casein kinase in the lactating mammary gland is believed to be the enzyme responsible of the phosphorylation of partially phosphorylated CN, by using ATP as a phosphate donor in the presence of divalent cations (Mg^{2+}, Ca^{2+}, Mn^{2+} or Co^{2+}). Casein kinase is an enzyme with a higher specificity for dephosphorylated CN.

All the phosphorylated hydroxy-amino acid residues in CN from different species occur in tripeptide sequences Ser/Thr-X-A, when X represents any amino acid residue, but Pro, and A an acidic residue such as SerP, Glu or Asp (Mercier, 1981). The occurrence of the consensus sequence is a necessary but not sufficient condition for the CN phosphorylation. Possible factors of constraint are involved such as different intrinsic properties of phosphate acceptor residues and acidic determinants, the characteristics of the local environment in terms of overall charge and hydrophilicity, secondary structure, steric hindrance and insufficient available pool of casein kinase(s) (Mercier, 1981).

Bioactive peptides with the three-phosphorylated motif, -SerP-SerP-SerP-Glu-Glu-, called casein phosphopeptides (CPPs), are released by *in vivo* and/or *in vitro* enzyme-mediated proteolysis of CN and show multifunctional bioactivities. The negatively charged side chains of phosphoserines make the

phosphorylated residues capable of chelate macroelements such as Ca, Mg and Fe, as well as oligoelements such as Zn, Ba, Cr, Ni, Co and Se. The Ca-binding activity of the CPPs may be further influenced by amino acids around the phosphorylated binding sites (Meisel, 1998). It involves phosphoserine residues, as well as the free carboxyl groups of Glu; a Ca^{2+} bridge results to be formed between SerP and Glu. The hydrophobic tail protects this complex from further interactions and hence prevents formation of insoluble calcium phosphate (Meisel, 1998). Moreover, the glutamic acid may catalyze the formation of CN micelle through the bridge SerP-Ca (Dijk, 1990). According to the theory of van Dijk (Dijk, 1990), the elimination of Glu residues adjacent to SerP might cause the loss of the peptide ability to form Ca complexes. In addition, the negative charges of phosphate groups and side chains make CPPs resistant to the gastrointestinal enzymatic digestion and thus suitable as carriers for metal ions (FitzGerals, 1998). Therefore, CPPs are reported to enhance intestinal absorption of Ca^{2+}, to exert anticarie (Iijima et al., 2010), cytomodulatory (Meisel & FitzGerald, 2003), immunoenhancing activity (Otani et al., 2000).

1.2 Casein hydrolyzed and CPPs

Peptides with various bioactivities can be produced *in vitro* i) by fermentation of milk inoculated with starter or non-starter cultures; ii) by digestion of milk proteins with one or more proteolytic enzymes or *in vivo* by gastrointestinal enzymes.

(i) Bioactive peptides can be generated by the starter and non-starter bacteria used in the manufacture of fermented dairy products. For instance, the proteolytic system of lactococci is able to degrade CN using cell-wall bound proteinases (PrtP), releasing oligo-, tri-, dipeptides and/or amino acids, which support their own growth. Oligopeptides, containing 4 up to 35 amino acid residues, are taken up by the oligopeptide transport system (Opp), while the ion-linked transporters (DtpT and Dpp) facilitate the uptake of di- and tripeptides (Mark et al., 2005). Internalized peptides are further hydrolyzed into amino acids by a large array of intracellular peptidases (Figure 1).

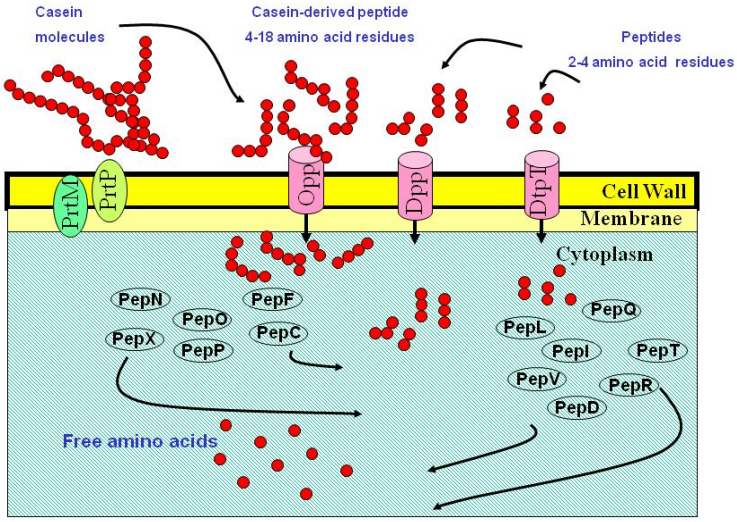

Figure 1. A simplified model presenting proteolysis, transport, peptidolysis and regulation of the proteolytic system of *Lactococcus lactis* on casein breakdown (Savijoki et al., 2006). Intracellular peptidases, PepO and PepF are endopeptidases, PepN/PepC/PepP are general aminopeptidases, PepX is X-prolyldipeptidyl aminopeptidase, PepT is tripeptidase, PepQ is prolidase, PepR is prolinase, PepI is proline iminopeptidase, and PepD and PepV are dipeptidases D and V. The role of PepL, PepI, PepQ, PepT, PepV, PepR, PepD and peptidolytic cycles are depicted schematically (various alternative routes of breakdown are possible for most peptides).

The first intracellular enzymes acting on internalized oligopeptides are endopeptidases, (PepO and PepF), general aminopeptidases (PepN/PepC/PepP) and X-prolyl dipeptidyl aminopeptidase (PepX) (Savijoki, et al., 2006). Di- and tripeptides generated by these enzymes are further subjected to additional cleavage by the tripeptidase, PepT, and dipeptidases, PepV and PepD (Figure 1). Other peptidases with high substrate specificity include: PepA, which liberates N-terminal acidic residues from 3 to 9 residue long peptides; PepP,

which prefers tripeptides carrying proline in the middle position; PepR and PepI, which act on dipeptides containing proline in the penultimate position; PepQ, which cleaves dipeptides carrying proline in the second position and PepS, which shows preference for peptides containing two to five residues with Arg or aromatic amino acid residues in the N-terminal position (Christensen et al., 1999; Kunji et al., 1996; Fernandez-Espla & Rul, 1999; Savijoki et al., 2006). In a comprehensive review of literature, no enzyme with carboxypeptidase (CPase) activity has been reported for lactococci (Léonil, 2000; Kunji et al., 1996), while activity of *Lactobacillus (Lb) helveticus* CPases were deduced from the identification of several N- and C-terminally truncated peptides (Caira et al., 2003). In addition to the proteolytic systems, lactococcal cytoplasm peptidases, released consequently to cell autolysis, can further contribute to the higher levels of proteolysis into the curd/cheese (Crow et al., 1995). In this manner, bacteria proteinases play a primary role, as they are able to generate specific bioactive peptides. Proteins released in cheese from a starter-based thermophilic lactic acid bacteria (LAB), such as *Lb. helveticus*, *Lb. delbruecki* subsp. *lactis* and *Streptococcus salivarius* subsp. *thermophilus* and *Propionibacterium freudenreichii*, have been identified using 2D-PAGE and mass spectrometry (MS) analysis (Gagnaire et al., 2004). Similarly, bioactive peptides have been determined using high performance liquid chromatography (HPLC) and offline matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Saz & Marina, 2008). The combination of the LAB bacteria and proteolytic enzymes could serve to increase the range of bioactive peptides.

(ii) *In vitro* enzymatic hydrolysis can produce a wide range of bioactive peptides. Endopeptidase P shows a broad specificity and preferentially cleaves hydrophobic, especially aromatic, residues. Trypsin specifically hydrolyses peptide bonds just after Lys or Arg residues of β-CN A^1 and A^2 variants (11 and 4, respectively), κ-CN A and B (9 and 5), α_{s2}-CN A (23 and 6) or α_{s1}-CN (14 and 6). We have demonstrated that CN hydrolysates by action in succession of pepsin and trypsin do not contain peptides with molecular mass greater than 2431 Da (Table 1). A high number of peptides with antimicrobial, anti-hypertensive and opiod-like activity has been identified, some of which exactly matched those described in the literature for potential bioactivity (Table 1). Moreover, different CPPs derived from β-CN, α_{s1}-CN and α_{s2}-CN are released by sequential digestion of milk proteins, using the combined action of PT (Table 1).

Table 1. Identity of bioactive peptides found in the PT digests of milk protein powder sample. The bioactivity of the peptides from which they derive and the references are reported below.

Identity of bioactive peptides	Bioactivity	References	PT peptides
β-CN (f60-70); (f59-61); (f59-64); (f60-68)	Opiod	Teschemacher et al., 1997; Meisel, 1986	(f58-68); (f59-63); (f59-62)
β-CN (f74-76)	ACE-Inhibitory	Ondetti & Cushman DW, 1982; Bruneval et al., 1986; Meisel, 1993	(f69-80); (f71-80); (f75-80)
β-CN (f80-90); (f84-86)			(f80-88); (f81-89); (f81-88); (f81-92); (f81-93); (f81-94)
β-CN (f108-113)			(f108-103)
β-CN (f140-143)			(f139-142)
β-CN (f177-183)			(f177-183); (f177-184); (f179-182)
β-CN (f169-174)			(f169-176); (f172-176)
β-CN (f193-198)			(f193-199)
β-CN (f193-202)			(f193-202)
β-CN (f1-25)4P	Mineral carrier, immunomodulatory, cytomodulatory	Sato et al., 1986; Reynolds, 1997; Meisel & Olieman, 1998; Hala et al., 1998; Hal et al., 1999	(f12-17) 1P; (f12-25) 3P; (f12-25) 4P; (f15-25) 2P; (f15-25) 3P; (f19-25) 1P; (f19-25) 2P
β-CN (f29-41)1P	Mineral carrier	FitzGerald, 1998	(f33-42) 1P; (f33-43) 1P; (f33-44) 1P
β-CN (f84-86)	ACE-Inhibitory	Ondetti & Cushman DW, 1982; Bruneval et al., 1986; Meisel, 1993	(f80-87); (f81-88); (f81-89); (f81-92); (f81-93); (f81-94)
α_{s1}-CN (f23-34)			(f24-32)
αs_1-CN (f25-27)			(f25-31)
α_{s1}-CN (f90-96); (f90-95); (f91-95)	Opiod (agonist)	Loukas et al., 1983; Loukas et al., 1990; Pihlanto-Leppälä et al., 1994	(f92-95); (f91-95); (f90-95)
α_{s1}-CN (f142-147)	ACE-Inhibitory	Ondetti & Cushman DW, 1982; Bruneval et al., 1986; Meisel, 1993	(f142-145); (f143-146); (f143-150); (f144-149)
α_{s1}-CN (f157-164)			(f155-164)
α_{s1}-CN (f194-199)			(f194-199); (f197-199); (f193-199)
α_{s1}-CN	Mineral carrier	FitzGerald, 1998	(f110-119) 1P
α_{s1}-CN			(f41-55) 1P; (f68-79) 2P
α_{s2}-CN (f174-179)	ACE-Inhibitory	Ondetti & Cushman DW, 1982; Bruneval et al., 1986; Meisel, 1993	(f174-179)
α_{s1}-CN (f189-193)			(f189-193)
α_{s2}-CN	Mineral carrier	FitzGerald, 1998	(f138-146) 1P
α_{s2}-CN			(f124-137) 2P; (f126-136) 2P; (f126-137) 2P
κ-CN (f33-38)	Opiod (antagonist)		(f58-65) 1P

PT= Pepsin and Trypsin action; P= Phosphate group

β-CN resulted extensively hydrolyzed into a high number of oligopeptides by using proteases with well-known cleavage specificity. The choice of digestion enzyme needs to be evaluated carefully because it influences the final composition of hydrolysates.

The *in vitro* sequential use of pepsin, pancreatic proteases and extracts of human intestinal brush border membranes, mimicking the gastric, duodenal and jejuneal *in vivo* digestion of CN, respectively, exhibits considerable bioactive effects. A limited number of CN and whey protein peptides survives the *in vitro* simulated gastrointestinal digestion. The anionic character seems to confer a marked resistance to multi-CPP hydrolysis by endoprotease. Ten out of 19 CPP contain SerP available for binding minerals and four of these peptides, α_{s1}-CN (f57-90) 5P, α_{s1}-CN (f56-90) 5P, α_{s1}-CN (f55-76) 5P, β-CN (f1-52) 5P, are reported for the first time in the CN digests (Miquel et al., 2006). Other studies show that phosphorylated clusters are almost completely hydrolyzed and only β-CN (f1-25) 4P, 3P and 2P survive the simulated gastrointestinal digest of CN (Picariello et al., 2010).

Finally, the *in vivo* digestion of milk proteins takes place mainly in the stomach, under the action of pepsins, gastric digestive proteinases, able to digest ~20% proteins. Afterwards, the pepsin digests pass to the duodenum where peptides are further hydrolyzed by pancreatic enzymes. The digestion is completed by membrane proteases and a variety of peptidases embedded in the brush border of the small intestine and/or released by the intestinal microflora. The peptidases release an amino acid residue or a dipeptide from the N- and C-terminal side of oligopeptides (Kenny & Maroux, 1982; Yoshioka et al., 1987; Erickson et al., 1989). Phosphatase(s), located in the brush border of the apical membrane of enterocytes, act(s) in removing phosphate groups, thus promoting partial or full dephosphorylation of peptides. The phosphorylated sequence is responsible, at the intestinal pH, for binding Ca^{2+}, Zn^{2+} and Mg^{2+} and for its *in vivo* resistance to gastrointestinal proteases (Mellander & Folsch, 1972). Fe complexed to β-CN (f15-25) 4P is scarcely hydrolyzed throughout the digestion, suggesting that the coordination of iron ions to CPPs inhibits the action of both phosphatase and peptidases (Boutrou et al., 2010). Brush border alkaline phosphatase (ALP) activity could improve the absorption of complexed Fe by its release from CPPs. Also zinc absorption can be enhanced by the formation of complexes with CCPs, in particular β-CN (f1-25) 4P (Pérès et al., 1998). Some

CPPs, formed in the small intestine, are found in the feces of rats fed with a CN-based diet (Kasai et al., 1995). CPPs have been for the first time detected in human ileostomy fluid, confirming their ability to survive gastrointestinal passage into the human distal ileum (Meisel & Frister, 1988). The *in vivo* formation of bovine CPPs is demonstrated in the small intestinal fluid of minipigs after ingestion of a diet containing CN (Meisel & Frister, 1988) and in the stomach and duodenum after ingestion of milk or yogurt (Chabance et al., 1998).
The peptides seem to interact directly with the plasma membrane. One possible mode of CPP action on the transmembrane flux of calcium is that CPPs might insert themselves into the plasma membrane by forming some calcium selective channels or by acting as calcium-carrier peptides rapidly internalized via endocytosis or other processes (Ferraretto et al., 2001). The results of *in vivo* studies regarding intestinal CPP absorption are still too controversial. There are many factors that affect Ca availability, among which the co-present of various dietary compounds in the intestinal lumen. The most of diet components, reaching the ileum, make soluble calcium or keep it in solution. The *in vivo* survival of CPPs to the prolonged intestinal passage in the distal small intestine is a prerequisite for their function as bioactive substances (Meisel et al., 2003).
Whole casein or individual casein fractions are used as raw materials to obtain CPPs as dietary supplements. CPPs have already been produced on an industrial-scale and have been used for application as ingredients in both 'functional foods' and pharmaceutical preparations. On the other hand, CPPs have already approved as foods for specified health uses (FOSHU) in Japan (Shimizu, 2003). CPPs in commercial hydrolysed casein (Arla Foods Ingredients, Sweden) seem to help the absorption of chelated calcium, iron, copper, zinc and manganese in the intestine. Moreover, CPP-amorphous calcium phosphate (ACP) displayed anticariogenic effect when added to dentifrices or oral care products by localizing calcium and phosphate ions at the tooth surface. Similarly, it has been claimed that a chewing gum or other confectionery product containing a combination of CPP-ACP and sodium bicarbonate as active ingredients can provide dental health benefits (Luo SJ, Wong LL. Oral care confections and method of using. US Patent 6733818).

1.3 CPP detection in raw milk and dairy products

CPP bioactivity has encouraged their search in different dairy products. This study suggests that they are very good marker of raw milk quality, process and authenticity beyond their bioactivity. In particular, native and partially dephosphorylated CPPs are possible markers of:

5) Raw milk from infected subjects with high somatic cell count (SCC);

6) Heat treatment intensity of commercial milks

7) Parmigiano reggiano (PR) cheese at different age of ripening: younger cheeses (up to 15 months) from those older than 26 month;

8) Adulterated water buffalo milk (or ovine and caprine) with cheaper bovine milk used for producing cheese of single species.

1.3.1 Proteolysis and partial dephosphorylation of casein in sheep milk with high somatic cell count

Mastitis defined as "an inflammatory reaction of the mammary gland" (IDF. 1987) is the most important and costly disease in dairy production. While clinical mastitis (CM) is easier to detect, subclinical mastitis (SCM) is difficult to find because of the few literature data on small and ruminants and the lack of reliable diagnostic methods (Leitner et al., 2004). SCC is resulted the indicator of udder health more widely used by dairy cow, sheep and goat farmers. Herds with high SCC bulk milk are very likely to be affected by infecting pathogens present in the farm environment. The EU Directives don't establish threshold values for SCC in ewe and goat milk. Nevertheless, a limit of 1,500,000 cells/ml has been recommended for small ruminant milk (Barbosa et al., 1994). The milk of healthy ewes often contains more than 1,000,000 SCC/ml of milk (Bufano et al., 1996). Invasion of pathogens activates the immune defense in the udder, manifesting in milk the increase of somatic cells (SC) (Poutrel et al., 1981), predominantly white blood cells or leukocytes, including lymphocytes, polymorphonuclear neutrophils (PMNs) and macrophages. In nearly bacteria-free milk, macrophages (Concha et al., 1986) and epithelial cells (Leitner et al., 2000) are the predominant cell type (35-79%) followed by PMNs (3-26%), lymphocytes (10-24 %) and epithelial cells (2-15%) (Sohn et al., 2007). After pathogens invade the mammary gland, macrophages release chemo-attractants, which trigger the migration of PMNs from the blood toward the infection in the gland (Le Roux et al., 2003). Therefore, the

PMNs are increased from a basal level of 5-25% of the total cell population to approximately 90% (Silanikove et al., 2006). Upon host infection, PMNs ingest and destroy invading pathogens via reactive oxygen species and a wide range of proteolytic enzymes (Paape et al., 1979). Several studies have suggested that the dairy products made from high SCC milk showed a more intense proteolytic activity of lysosomal enzymes (Cooney et al., 2000). Enzymes, including neutral and acidic proteases, such as elastase (EC 3.4.21.36), G (EC 3.4.21.20), B (EC 3.4.22.1) and D (EC 3.4.23.5) cathepsins, originate from SC, as well as an ALP is present in substantial amounts in the PMN cytoplasm (Bank & Ansorge, 2001; Considine et al., 2004; Owen & Campbell, 1999). Two different mechanisms can be involved in the CN proteolysis by PMN enzymes: *contact proteolysis* and *proteolysis by internalization* (Le Roux et al., 2003).
The first one consists of direct proteolysis of milk protein. The PMN degranulation releases several proteolytic enzymes in milk. Proteolytic activity of neutral proteases such as elastase, collagenase, cathepsin G etc. (Owen & Campbell, 1999) is more relevant than that of acidic proteases such as cathepsins B, C, D or L as the latter are not at their optimal pH after degranulation (milk pH tends to neutrality). The main role of acid proteinases is in intracellular protein digestion, which occurs within the acidic environment of lysosomes. Nonetheless, these proteinases may degrade caseins in the neutral pH of milk if they are released at high enough concentrations or in the microenvironment of external periphery that allows the local maintenance of an acid pH.
The second one involves the phagocytic capacity of SC. Casein peptides are internalized in vesicles via endocytosis by milk PMNs. These vesicles would fusion with lysosomes where the acidic pH would permit an optimal function of acidic proteases. The resulting degradation products would be partly or completely released in milk (Le Roux et al., 2003).
Moreover, the transit of serum constituents, such as plasminogen and numerous other enzymes, from the blood toward the mammary gland into in the milk, causes a higher proteolytic activity during SCM compared to normal milk (Sordillo et al., 1997). Plasmin (PL) (EC 3.4.21.7), the predominant indigenous milk proteinase, originate from the blood and enter the gland, mixing with the milk. An increase of the milk SCC results in an 8-fold enhance in plasminogen activator activity per cell which in turn activate plasminogen into PL (Zachos et al., 1992). PL activity in milk is positively correlated with SCC. An increase of the SCC from 100,000 cells/ml to 1,300,000 cells/ml was associated with a 2.3-fold increase in PL (Politis et al., 1989). PL preferentially cleaves peptide bonds at C-terminal of Lys residues and, to a lesser extent, arginine Arg residues (Ueshima et al., 1996). β-CN is the preferred substrate for PL that partly converted native β-CN into pH 4.6 insoluble γ-CN and complementary soluble proteose peptones (PP) (Andrews et al., 1983). PP include peptides such as β-CN (f1-105/107) (component PP5), (f1-28) (component PP8-fast), (f29-105/107) 1P (component PP8-slow) (Andrews et al., 2006; Farrell et al., 2004). α_{s1}-CN (McSweeney et al., 1993) and α_{s2}-CN (Le Bars et al., 1989) are also susceptible to PL hydrolysis and the former gives rise to λ-CN (Aimutis et al., 1983). κ-CN appears to be quite resistant to PL action (Diaz et al., 1996), whereas cathepsins B and D produce the glycomacropeptide, κ-CN (f106-169). Moreover, ALP, released primarily from the liver and bone into the blood, is further transferred to milk at a higher rate because of damage to the blood-milk barrier by infection (Yang et al., 2011). ALP activity is significantly higher during SCM compared to normal milk (Yang et al., 2011; Mauriello et al., 2007). The increase of serum ALP in infected milk suggests its important role in the pathogenesis of the disease (Vangroenweghe, 2004) and as a marker of mastitis milk (Akerstedt et al., 2011). The strong level of casein dephosphorylation depends on the increased amount of ALP and these values are associated with a high SCC (Mauriello et al., 2007). Phosphorylation could occur simultaneously at many different sites, as previously observed for ovine and caprine β-CN (Chianese et al., 1992; Neveu et al., 2002). Moreover, the ALP activity could compromise the bioavailability of CPPs through the modulation of the phosphorylation level of CN.
Our study has concentrated on the casein proteolysis in milk of infected subjects by using a proteomic approach. Specific casein peptides and partially dephosphorylated CN/CPPs have been identified as indicators of SCM in infected milks.

1.3.2 Lactosylated CPPs as specific indicators of heated milks

Intense heating processes affect the bioactivity of CPPs, causing dephosphorylation of phosphoserine residues and formation of dehydroalanine (Meisel, 1991). Dehydroalanine may further react with Lys residues leading to lysinoalanine or with the thiol group of cystein to form lanthionine (De Koning & Van Rooijen, 1982). Hydrolysis of phosphoseryl esters and not β-elimination to dehydroalanine is considered the prevailing mechanism of heat degradation of CPPs (Meisel, 1991). Phosphoseryl residues, occurring in cluster sequence -SerP-SerP-SerP-Glu-Glu-, do not contribute preferentially to the formation of lysinoalanine, as shown by structural studies of Manson and Carolan (Manson W & Carolan T, 1980) while phosphoseryl residues which occupy isolated positions in the casein sequences are mainly involved in

lysinoalanyl formation. Moreover, in heat-treated liquid and powder milk, the ε-amino group of Lys or amino-termini residues can react with the carbonyl group of lactose with formation of lactosylated proteins/peptides. In the initial step of the Maillard reaction, lactosylation occurs during heating and, to a lesser extent, during milk powder storage (Guyomarc'h et al., 2000). Although determined for whey proteins and CN, lactosylation of CN peptides has not been deeply evaluated to distinguish various types of milk (Arena et al., 2011). It is known that the degree of lactosylation of β-CN increases steadily with temperature between 37 °C and 60 °C (Groubet et al., 1999). Lactose specifically binds to Lys_{34} in α_{s1}-CN and to Lys_{107} in β-CN under moderate heat treatment conditions (72–85 °C for 15–30 s). The number of binding sites increases to seven for α_{s1}-CN and five for β-CN after intensive treatment (142–145 °C for 2–5 min) (Scaloni et al., 2002). Especially in highly heated milk, casein proteolysis is greatly affected by the quality of raw milk. Raw milk of good quality has 1-2 mg/ml PP, which can increase during storage. A significant accumulation of PP5 has been reported for commercially packaged pasteurized milk (De Noni et al., 2007). Due to the specificity of PL, milk heating could induce lactosylation of the side chains of released peptide at basic C-terminal residues (mainly Lys or Arg). β-CN (f1-28) 4P produced by PL during milk storage ranges from 8% to 12% PP and matches the formation of γ_1-CN and β-CN (f29-209) (Andrews, 1978). Lysosomal enzymes of PMNs can contribute to the release of peptides in poor quality heated milks. Due to plethora of milk peptides, we have focused on signature CPPs released in definite number by milk proteinases.
The goal of our study is the detection of both native and lactosylated CPPs by direct MALDI-TOF analysis of CPPs *in situ* immobilized on hydroxyapatite (HA) microgranules. The comparison between the potentially lactosylable and actually lactosylated sites could serve to differentiate processed milks according to heat intensity. Moreover, the use of low value ingredients such as Ultra High Temperature (UHT) and milk powder in raw or pasteurized milk (PM) is strictly prohibited. Specific temperature/time combinations for the production of pasteurized and UHT milk are established by European Union Directives (92/46 CEE and 94/71 CEE). Nowadays, rapid methods are mainly based on the evaluation of furosine, a useful indicator of heat damage in processed milk. In our work, the lactosylated CPPs have been used for discriminating raw and pasteurized from UHT milk. Therefore, the finding of lactosylated β-CN (f1-28) 4P in mixtures of UHT and pasteurized milk has allowed to detect UHT milk in amounts not lower than 10%.

1.3.3 CPP profiling of Parmigiano Reggiano cheese at different ripening ages

The combined action of proteinases/peptidases and phosphatases plays an important role in cheese quality. Due to proteolytic activity, an elevated number of water-soluble peptides, most of which are multi-CPP, is released in cheeses, which significantly increases during ripening of cheese. In particular, chymosin, pepsin and other enzymes released by LAB autolysis are responsible for the progressive degradation of α_{s1}-, α_{s2}-, β- and κ-CN fractions even if they are inactivated by curd cooking at 55 °C in cooked cheeses. The enzymatic digestion of CN could be reduced by milk thermal treatment but PL activity seems to enhance likely owing to an increase in plasminogen activation at higher temperature (Richardson, 2006). Even if PM completely inactivates indigenous ALP, this enzyme is stable at temperatures slightly higher than those required to kill pathogens in milk (Kay & Graham, 1935). In cheeses made from PM, indigenous acid phosphatase or bacterial phosphatases are probably responsible for casein dephosphorylation while, in raw milk cheese, *e.g.* PR or Grana Padano (GP), ALP may play an important role (Pellegrino et al., 1997). In the cooked cheeses, molding causes a slow cooling of the rind from 55 °C to room temperature but the core remains at 52-56 °C for 8-10 h. For this reason, ALP is inactivated in the wheel core although its activity remains at $3x10^5$ mU/kg or more in the rind (Pellegrino et al., 1997). Anyway, several studies suggest that partial dephosphorylation of water-soluble CPPs occurs during cheese ripening (Ferranti et al., 1997; Singh et al., 1997; Silva et al., 1987).
Our study has focused on CPPs of PR, an cooked, semi-fat hard cheese made from raw, naturally skimmed cow's milk according to the EU Standards of Identity (Official Journal of the European Union C 87 of 16 April 2009, 2011/C 215/16). The PR cheese wheels have to ripen for at least 12 month (mo) but can be aged for 3 years or longer. During this long period of ripening, an intense proteolysis of casein fractions occurs in the cheeses. During cheese ripening, the number of whey starter thermophilic lactobacilli such as *Lb helveticus*, *Lb delbrueckii* subsp. *lactis* and *Lb delbrueckii* subsp. *bulgaricus* decreases (Cocconcelli et al., 1997; Zambonelli et al., 2002), whereas that of mesophilic heterofermentative LAB becomes dominant until 5 mo (Coppola et al., 2000). Mesophilic heterofermentative LAB belonging to *Lb. casei* are frequently detected in 12-mo-old PR cheese samples, suggesting that they play not only a passive role in releasing enzymes capable of hydrolyzing CN by cell autolysis but also an active role in the advanced stages of PR ripening (Gala et al., 2008) It is known that CN degradation produces high-molecular mass (HMM) peptides, soluble low-molecular mass (LMM) peptides and FAA. Parent β- and α_{s1}-CN and newly formed HMM peptides have been simultaneously detected, using gel isoelectric focusing (IEF) and immunoblotting with

polyclonal antibodies against the parent CN fraction (Addeo et al., 1995). PP (HMM CPPs) have been detected by HPLC to evaluate the age of packaged pasteurized milk (De Noni et al., 2007) or the proteolysis of semi-hard Herrgard cheese (Ardö et al., 2007). CPPs belonging to LMM peptides accumulate in the cheese owing to their well-known resistance to further breakdown into smaller fragments (Ferranti et al., 1997). For this reason, long-ripened PR cheese is considered a rich source of multi-CPPs, able to interact with colloidal calcium phosphate and to manifest anticariogenic properties in human and animal experiments (Rosen et al., 1984; Krobicka et al., 1987). CPPs binding calcium and phosphate buffer these ions when the increase of solubility of dental HA decreases pH. β-, α_{s1}-, α_{s2}- and κ-CN derived CPPs inhibit the adhesion of *Streptococcus mutans* to saliva-coated HA through their binding in solution (Schüpbach et al., 1996).
Several data on CPP enrichment from cheese are available in the literature. Collection of the Ba-CPP precipitates by the addition of an equal volume of 96% ethanol to the CPP solution allows the isolation of CPPs and the subsequent analysis by HPLC (Ferranti et al., 1997). An improvement in the recovery of singly and multiply CPPs in Herrgard, PR (Lund & Ardö, 2004) and Beaufort cheeses (Dupas et al., 2009) has been obtained using cation-exchange chromatography followed by immobilized metal-ion affinity chromatography (IMAC).
In the present work, CPP enrichment based on in-batch HA (Pinto et al., 2010) has been used for profiling CPPs from the pH 4.6-soluble fraction of PR at different periods of ripening: 5-, 15-, 26- and 35-mo. Such profiles greatly enable us to characterize the proteolytic events that occur during ripening of brine-salted hard cheese. Moreover, the MALDI analysis of CPPs bound to HA allows the distinction of young PR cheeses (5- to 15-mo-old) from those aged more than 26 month (mo).

1.3.4 Milk speciation through the monitoring of proteotypic CPPs

A fraudulent practice, commonly implemented by producers, is the substitution, partial or full, of water buffalo (WB) milk with cheaper and all year available bovine (B) milk. In particular, in the manufacturing of WB Mozzarella cheese, the use of B milk is drastically increasing in order to satisfy the growing demand of consumers, especially during the summer period corresponding to lean peak. The use of adulterant B milk can endanger the consumer safety because of possible occurrence of allergic reactions and stomach irritation to sensitive consumers. Currently, cheese made from non-bovine milk is considered adulterated when the error in the evaluation of the extraneous milk does not exceed 1% (European Commission, EC 213/2001. Off. J. Eur. Comm, 44, L37/31).
Recent researches have focused on the discovery of markers to detect adulterating milk of other species for the production of cheese made exclusively from WB, ovine or caprine milk. B milk adulterating WB milk has been recognized by HPLC analysis using β-lactoglobulin (β-Lg) A as B marker (Official Gazette of the Republic of Italy 11 June 1996. Off. J. Ital. Rep. 1996; 135: 12). Similar procedures have been developed for detecting B milk in binary (Official Gazette of the Republic of Italy 17 April 2003. Off. J. Ital. Rep. 2003; 90: 9.6) or tertiary milk mixtures by capillary electrophoresis (Herrero-Martinez et al., 2000). These methods give good results, as they are able to detect the presence of adulterant milk at levels as low as 0.5-1.0%, but they are very slow and laborious. Fast detection of B α-La and β-Lg in WB and ovine milk is reported to show a linear MALDI-TOF response up to a limit of 2% (Cozzolino et al., 2002). Among protein-based methods for the detection of adulterant milk, it is now widely recognised that CN is a more useful indicator than heat-sensitive whey proteins. Presently, IEF of CN is the European reference method for cow's milk detection (Commission Regulation No 273/2008, Official Journal of the European Union, L 88, 53-115). This reference method is based on the detection of hydrolysis product of B β-CN, γ_2- and γ_3-CN, by detecting low levels (0.5%) of added cow's milk. A version more simplified than IEF profile has been obtained using anti-peptide antibodies able to detect almost exclusively γ_2-CN, with a limit of detection (LOD) of 0.25% (Addeo et al., 2009). Recently, our strategy has combined MS and synthetic peptide analogues as internal standards (IS) to quantify species-specific CN peptides with a LOD of 0.5% (B milk) and of 1% (caprine milk) using either MALDI or the liquid chromatography–electrospray ionisation-mass spectrometry (LC-ESI-MS) analysis (Cuollo et al., 2010). The high degree of homology among the CN sequences of different species involves the careful selection of the proteotypic peptides. The amino acid substitutions occur also within the CPP sequence. Some sequences of CPPs, released from *in vitro* hydrolysis of milk different species, are different enough to be detected by MS techniques.
In our study, tryptic CPPs such as bovine β-CN (f1-25) 4P and water buffalo β-CN (f1-28) 4P have been identified and then used as markers of B milk contaminating WB milk, respectively. The method provides an alternative analytical means for detecting adulterated milks/cheeses with 0.5% LOD, using direct MALDI-TOF-MS analysis of HA-CPP complexes.

1.4 References

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2 AIM OF THE STUDY

The main aim of the work is the study of CPPs in different dairy products by an enrichment method based on HA. The CPP bioactivity is affected from their full or partial dephosphorylation, which could occur in milk of poor quality, in intensely heated milk or in long ripened cheese. Moreover, the research work has focused on on the discovery of markers of cheese-milk adulteration by mixing milks from different species through the monitoring of proteotypic CPPs.
Therefore, the work has achieved the following goals:

1) The identification of specific CN peptides and partially dephosphorylated CPPs as possible indicators of SCM in milk from infected subjects;

2) The identification of CPPs as suitable markers of the heat treatment intensity. The method has allowed to discriminate the UHT milk added to the pasteurized milk in amount up to 10%;

3) The CPP profiling of PR cheese at different age of ripening. The chromatographic procedure resulted useful to distinguish between younger cheese (up to 15 months) and that older than 26-mo;

4) The identification of proteotypic CPPs as markers of adulterated water buffalo milk (or ovine and caprine) with cheaper bovine milk used for producing cheese of single species.

The advantages of using signature phosphopeptides as analytical surrogates of the proteins are that (1) it is easier to separate and detect peptides than proteins, (2) structure of the CPPs does not alter during the analysis, (3) putative peptides suggested from databases can be easily recognized and synthesized for quantitative analysis.
The developed method could be applied in the molecular design and control of other dairy and non-dairy products.

2.1 Techniques of phosphopeptide detection and Workflow

The study of phosphorylated component has posed a difficult challenge in proteomic field. Phosphorylated peptides escape detection by MS analysis because of their substoichiometric concentration, poor ionization/detection efficiency and the low m/z values of MS2 fragment ions (Steen et al., 2006). The negative charge of phosphorylated residues affects ionization and detection efficiency of the phosphopeptides (they remain negatively charged even at pH 2), under acidic conditions used for MS experiments in positive ion mode (Steen et al., 2006). It is reported that their desorption/ionization efficiency is an order of magnitude lower than that recorded for the non-phosphorylated counterpart and it becomes increasingly difficult with the number of phosphate groups (Craig et al., 1994). Many MS-based methods have been developed for the identification of phosphorylated peptides and the assignment of phosphorylation sites. Multiply and singly, tryptic phosphopeptides can be simultaneously detected using MALDI-TOF and the location of phosphate groups can be obtained by a combination of tandem mass spectrometry (MS/MS) and computer-assisted database search programs (*e.g.* SEQUEST, Trademark, University of Washington, Seattle Wash) (McCormack et al., 1997; Eng et al., 1994). Direct analysis of phosphopeptides can use two orthogonal MS scanning techniques, both based on the production of specific marker ions at m/z 63 and/or 79, in the negative ion mode (Annan et al., 2001). These scanning methods combined with the ESI and nano-ESI-MS/MS allow to selectively detect and identify phosphopeptides even in complex proteolytic digests. An alternative strategy uses the precursor neutral loss scanning by using ESI-MS/MS. In this way, phosphopeptides can be recognised from neutral loss mass of m/z 98 (phosphoric acid is released from β-elimination of phosphoserine or phosphothreonine residues upon fragmentation in MS), while an additional fragmentation of the product of the precursor neutral loss can be obtained by multi-stage activation MS3 experiments (Schlosser et al., 2001). Anyway, the methods of phosphoproteins/phosphopeptides enrichment prior to MS analysis have been widely used in studies of phosphoproteome to greatly improve their identification and characterization. An ancient technique consisted of the phosphopeptide precipitation as insoluble barium salts and their recovery by centrifugation, as according to the Manson & Annan method (Manson & Annan, 1971). High-throughput phosphoproteome technologies currently rely on combining pre-separation of proteins, most commonly by high-resolution two-dimensional polyacrylamide gel, in-gel tryptic cleavage of proteins and subsequent MALDI-TOF or ESI-MS/MS mass spectrometry analysis of peptides (Thiyagarajan et al., 2009). Alternative methods are based on chemical derivatization. For example, β-

elimination reactions by strong base such as NaOH or $Ba(OH)_2$ are able to form dehydroalanine or dehydroaminobutyric acid by cleavage of the phosphoester bonds of phosphoserine and phosphothreonine, respectively. Dehydroalanine or dehydroaminobutyric acid are further able to react with different nucleophiles, such as ethanedithiol (EDT) or dithiothreitol (DTT). EDT or DTT-labeled amino acid residues can be linked to immobilizing agents or affinity tag. For instance, biotinylated residues can produce the ion fragments observed at m/z = 446 in the MS/MS spectra because of the facile cleavage of peptide bonds or can be captured by avidin purification (Oda et al., 2001). An interesting approach for high-throughput phosphoproteome quantitation employs isotope coded affinity tags (ICAT) (Gygi et al., 1999). This method involves derivatization of two distinct proteomes with the light and heavy versions of the ICAT reagent where eight hydrogen atoms in the linker arm of the light reagent (D0) have been substituted by eight deuterium atoms in the heavy reagent (D8). Although the derivatization methods are highly selective, they are not widely applied in phosphoproteome studies due to sample loss by the multiple reaction steps and unavoidable side reactions (Zhou et al., 2001). The affinity chromatography-based methods have been found to be advantageous for selective phosphopeptide enrichment. IMAC (with Fe^{3+}, Ga^{3+}, Ni^{2+} and Zr^{4+} metal ions) and metal oxide affinity chromatography (MOAC, with TiO_2, ZrO_2, Al_2O_3 and Nb_2O_5) have been widely used for the quantitative binding of phosphopeptides on resin or adsorbent. Iminodiacetic acid (IDA, a tridentate metal-chelator) or nitrilotriacetic acid (NTA, a quadradentate metal chelator) are often used as IMAC functional matrices. Multivalent metal ions (*e.g.* Fe^{3+}, Ga^{3+} and Al^{3+}) are usually bound to a chelating support to provide positive charges useful for the phosphopeptides enrichment (Posewitz & Tempst, 1999; Kokubu, 2005). The negatively charged phosphopeptides can also selectively interact with TiO_2 microspheres via bidentate binding at the dioxide surface (Pinkse et al., 2004; Rinalducci et al., 2006.; Thingholm et al., 2006). Phosphopeptides bound to affinity resins can be released by increasing either pH or the phosphate concentration in the buffer (Thingholm, et al., 2006; Zhou et al., 2007; Thingholm & Jensen 2009). The main problem associated with the chelating resins is the metal-ion leaching, which leads to CPP loss during the enrichment procedure. The selectivity of these methods was somewhat compromised by the detection of several acidic non-CPPs co-eluted by the resins (Dunn et al., 2010). To overcome this drawback, the carboxyl groups can be methyl esterified, which eliminates the non-specific adsorption of acidic peptides on IMAC (Moser & White, 2006). Considerable efforts have been expended to remove acidic non-CPPs by using aromatic modifier such as 2,5-dihydroxybenzoic acid (DHB) or phthalic acid in loading buffer (Thingholm et al., 2006). It has been found that aliphatic hydroxyl acid modified metal oxide works more efficiently and more specifically than aromatic modifiers in titania and zirconia MOC (Sugiyama et al., 2007). However, all affinity techniques developed for the current enrichment strategies of CPPs gave reproducible but incomplete results due to poor binding of low concentrations of CPPs and the insufficient recovery of multiple phosphorylated peptides (McLachlin & Chait, 2001). Recently, a robust and sensitive phosphoproteomic strategy (collectively abbreviated "TiSH") combines an initial TiO_2 pre-enrichment step (to eliminate most of non-phosphorylated peptides), a post-fractionation using sequential elution from IMAC (SIMAC) (to fractionate mono-phosphorylated peptides from multi-phosphorylated) and hydrophilic interaction liquid chromatography (HILIC) (to fractionate further the mono-phosphorylated peptides) prior to LC-MS/MS analysis (Engholm-Keller et al., 2012).

An easier procedure based on HA mocrogranules has been recently developed for specific phosphoprotein/CPP enrichment from complex mixtures (Pinto et al., 2010; Mamone et al., 2010). Salt of calcium phosphate in the form of HA (formula $[Ca_{10}(PO_4)_6(OH)_2]$), found in bone and tooth tissue, has been previously used to enrich bone proteins (Zhou et al., 1998). The phosphate groups of phosphoproteins/phosphopeptides interact with crystalline lattice Ca^{2+} more strongly than do the carboxyl groups (Kawasaki, 1991). Moreover, the higher phosphorylation level leads to tighter binding of the proteins/CPPs to HA so that the affinity of the multi-phosphorylated proteins/peptides for HA results significantly higher than that of the same components with lower phosphorylation (Schmidt et al., 2007). The procedure employs ceramic HA microgranules as a solid-phase adsorbent to efficiently capture phosphoproteins and CPPs through their phosphate groups while the non phosphorylated components are washed out using various buffers (Figure 2).

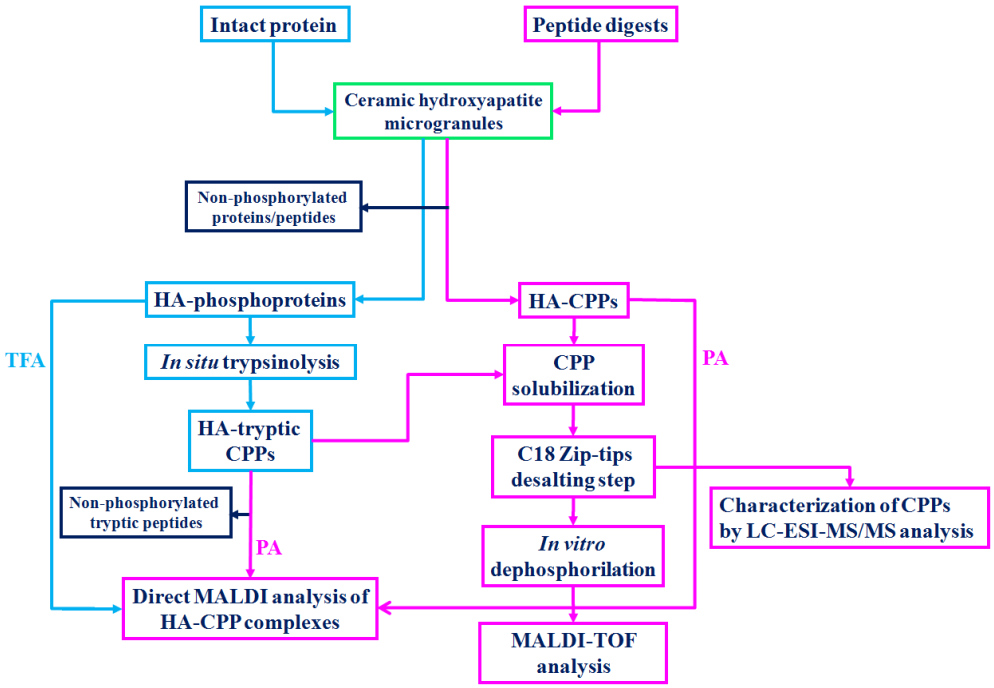

Figure 2. Workflow of the CPP enrichment technique based on HA microgranules. The samples were enriched and analyzed as follows. An aliquot of the solution containing casein and/or peptide mixture was mixed with the HA microgranules to capture the phosphorylated components. Another aliquot containing HA-bound casein or tryptic CPPs was spotted on the target plate, covered with the MALDI matrix solution containing exogenous acids (TFA for phosphoproteins, shown in blue; PA for CPPs, shown in violet) and the protein/peptide ions desorbed from the MALDI-TOF matrix. The HA-based protein/peptide enrichment procedure was tested using MALDI-TOF to discriminate the phosphoanalytes in two distinct mass ranges. The presence of phosphoanalytes in the mixture was verified through phosphate-specific reactions (*e.g.*, sensitivity to alkaline phosphatase). Moreover, HA-bound CPPs can be dissolved, desalted and analyzed by LC-ESI-MS/MS.

Phosphoproteins/CPPs immobilized on HA microgranules form a complex that can be loaded directly on the MALDI-TOF plate. Moreover, HA-trytic CPPs can be obtained by *in situ* digestion with trypsin; non-phosphorylated peptides were released in the flow-through while the dried HA-trytic CPP microgranules included in the co-crystallization matrix were analyzed directly by MALDI-TOF (Pinto et al., 2010). Sequencing by LC-ESI-MS/MS allows to confirm the CPP sequences as well as to establish their level of phosphorylation (Pinto et al., 2010).

Phosphoproteins/peptides compete for the C-sites on ceramic HA over a wide pH range (Schmidt et al., 2007) and both are eluted for MALDI analysis (Mamone et al., 2010; Addeo et al., 1977). In a previous article, CPPs immobilized on HA were progressively eluted with increasing phosphate buffers and then identified by off-line MALDI-MS (Mamone et al., 2010). Our procedure did not use elution because the HA-phosphocaseins/CPP complexes were directly solubilized by spotting on the MALDI plate matrix mixed to trifluoroacetic acid (TFA) (for caseins) or phosphoric acid (PA) (for CPPs) (Figure 2). PA enhances the detection of phosphopeptide ions by MALDI-TOF (Kjellstrom & Jensen, 2004). This procedure was accelerated by the removal of elution step, and CPP losses during elution were minimized. This method is useful for measuring the phosphorylation level of phosphoproteins/CPPs quickly, with less than 2 h including the readout of the MALDI spectra (excluding the trypsinolysis step).

In conclusion, the more important advantages of the procedure are the possibility of (1) detecting phosphorylated proteins/peptides even in complex mixtures, (2) determining phosphorylated sites and those dephosphorylated by phosphatase, (3) attaining information regarding weakly and heavily phosphorylated peptides and (4) the quickness and simplicity of the enrichment steps.

2.2 References

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3 RESULTS AND DISCUSSION

3.1 Topic 1: Proteolysis and partial dephosphorylation of casein in sheep milk with high somatic cell count

In the present work, the direct analysis of proteins by ESI-Q-TOF-MS has allowed to identify partially dephosphorylated CN/CPPs that are released in the milk of infected subjects with high SCC. In order to improve our knowledge of the peptide bonds that are hydrolyzed by PL and the dephosphorylated SerP sites, HA-enriched tryptic CPPs have been studied. These CPPs resulted useful for the unambiguous assignment of phosphorylation level of parent CN by MS. The presence of specific CN peptides and partially dephosphorylated CPPs may be possible indicators of SCM in milk.

3.2 Materials and methods 1

3.2.1 Chemicals

Milk from ovine Sarda breed was obtained from local dairy farms. A flock of 120 sheeps was milked once daily half-udder separately by the opposite half-udder, for a total of 240 samples. Milks with the three different levels of SC, SCC<500,000, 500,000<SCC<1,500,000 and SCC>1,500,000 were obtained from all of animals primiparous and homogeneous for lambing date, feeding scheme and stage of lactation. Upon milking, a sample was taken from the milk of each half-udder in order to determine the SCC (Fossomatic, Foss Electric, Hillerød, Denmark) while the rest was conserved in a plastic bag and stored at 4 °C up to SCC analysis. On the basis of the SCC, each sample of milk was then blended to have three bulk milks corresponding to the pre-established SCC thresholds. Isoelectric CN was obtained from skimmed milk after centrifugation at 3,000 x g for 10 min by the addition of acetate buffer according to the procedure of Aschaffenburg & Drewry (Aschaffenburg & Drewry, 1959). Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), potassium chloride (KCl), urea, TFA, acetonitrile (ACN) HPLC grade, 85% PA, ammonium bicarbonate (AMBIC), formic acid (FA), potassium phosphate monobasic (KH_2PO_4) and di-Sodium hydrogen phosphate anhydrous (Na_2HPO_4) were purchased from Carlo Erba (Milan, Italy). HA (Macro-Prep Ceramic Hydroxyapatite TYPE I) was supplied from Bio-Rad (Milan, Italy). Coomassie Brilliant Blue G-250 (CBB) and TPCK-treated trypsin from bovine pancreas was obtained from Sigma-Aldrich (Milan, Italy). DTT, Sodium acetate (AcNa) trihydrate, 3,3-diaminobenzidine, DHB, and nickel chloride hexahydrate ($NiCl_2$) were obtained from Fluka (Milan, Italy). All other reagents were of analytical grade and were used without further purification.
ALP was evaluated according to the spectrophotometric Official Method established by the Commission of the European Community (1991).

3.2.2 Electrophoresis and immunoblotting

IEF was performed on small, ultrathin-layer polyacrylamide gel (thickness, 0.25 mm). A pH gradient in the range of 2.5-6.5 was obtained by mixing Pharmalyte (GE Healthcare, Uppsala, Sweden) 2.5-5.0, 4.5-5.4 and 4.0-6.5 at a ratio of 1.6:1.4:1 (v/v/v). Protein separation was performed on the PhastSystem apparatus (Pharmacia, Uppsala, Sweden) according to the procedure previously described (Moio et al., 1989). After gel electrophoresis, the proteins were transferred to a nitrocellulose membrane by capillary diffusion. The present procedure differed from that described by Chianese (Chianese et al., 1992) since the blocking solution was replaced with PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4) containing 10% heat-inactivated horse serum (v/v).
Immunostaining was performed using anti-peptide antisera as the primary reagent and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG polyclonal antibodies as the secondary reagent (Bio-Rad, Hercules, CA). For CN detection, the nitrocellulose was placed in 10 mM Tris-HCl, pH 7.5, containing 0.5 mg/ml 3,3'-diaminobenzidine (Fluka, Buchs, Switzerland) and 0.06% (w/v) $NiCl_2$ hexahydrate. To initiate the reaction between the HRP-labeled goat anti-rabbit IgG antibodies and the 3,3'-diaminobenzidine, 20 µl 30% (v/v) hydrogen peroxide was added. Incubation of the nitrocellulose membrane was performed at room temperature with gentle shaking. The membrane was examined periodically until the protein band color was

converted to a red-brown (1–5 min). When the desired intensity of red-brown color was achieved, the reaction was stopped by rinsing several times the membrane in distilled water.

3.2.3 Synthetic peptides and production of the antisera

We chemically synthesized a number of peptides corresponding to their amino acid sequences. These peptides mimicked the sequence of the natural protein and were purified using reverse phase HPLC (Primm, Milan, Italy). A cysteine residue was added to each N- or C-terminal peptide for conjugation with ovalbumin according to the coupling procedure described by Mattson (Mattson et al., 1993). The cysteine was added to the N-terminal of the peptides corresponding to amino acids 195-209 and to the C-terminal peptides corresponding to amino acids 1-28 of β-CN. The cysteine was then added to the N-terminus of the α_{s1}-CN peptides containing the amino acid sequence 186-199 and to the C-terminus of the α_{s1}-CN peptides containing the amino acid sequence 1-22. The cysteine was added to the N-terminus of the peptide containing amino acids 193-207 of α_{s2}-CN and to the N-terminus of the peptides containing the amino acid sequence 92-111 of κ-CN. The peptides were quality-controlled using MALDI-TOF-MS. The ovalbumin conjugates were used to immunize two rabbits. Finally, the antisera were filtered through a 0.45 μm membrane (Millipore, Bedford, MA) and were divided into two 1 ml portions, which were stored at -20 °C until use.

3.2.4 HA-based enrichment of tryptic CPPs

Solutions of ovine CN (10 mg) from infected udders were loaded on HA (100 mg), previously equilibrated with the sample loading buffer (50 mM Tris-HCl, 0.2 M KCl, 4.5 M urea and 10 mM DTT, pH 8.0). The HA-bound proteins were incubated for 15 min at room temperature and centrifuged at 4,000 x g for 2 min. HA was successively washed with three different buffers: 1 mL of loading buffer, 1 mL of 50 mMTris-HCl, pH 8.0 (washing buffer), and 1 mL of buffer containing 20mM Tris-HCl, pH 8.0 and 20% ACN (v/v). The resin was washed with 1 mL of Milli-Q water and freeze-dried with a SpeedVac concentrator system (Thermo Electron, Milford, MA) (Pinto et al., 2010). The proteolysis/peptidolysis of HA-bound phosphoproteins/CPPs was performed *in situ* with trypsin, which was added to the suspension at an enzyme/substrate ratio of 1:50 (w/w) in 50 mM Tris-HCl buffer, pH 8.0 containing 0.2 M KCl, 4.5 M urea and 10 mM DTT at pH 8.0. The reaction was performed at 37 °C overnight. The reaction was stopped by centrifuging the HA-CPP microgranules at 4,000 x g for 2 min and the microgranules were washed as previously described. Finally, after washing with Milli-Q water, the HA microgranules were subsequently dried and analyzed by MALDI-TOF (Pinto et al., 2010).

3.2.5 MALDI-TOF MS analysis

The MALDI-TOF mass spectra were recorded using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Framingham, USA) in positive linear mode. All spectra were acquired in the range of 1–4 kDa for the peptides with the following settings: an accelerating voltage of 20 kV and a grid voltage 95% of the accelerating voltage, a guide wire of 0.05% and a delayed ion extraction time of 175 ns. The laser power was set immediately above the ion generation threshold to obtain peaks with the highest possible signal-to-noise ratio. All spectra were acquired with 200 shots in three replicates. The measured molecular masses with a ±0.5 Da tolerance, matching the calculated masses, enabled the identification of the ion signals. The MALDI matrix solution was freshly prepared before analysis. DHB (10 mg) was dissolved in 1 ml H_2O/ACN/PA (49/50/1) using an ultrasonic bath. HA-tryptic CPP complexes were deposited onto the MALDI plate and covered with the DHB matrix (0.5 μl), which promoted the analyte/matrix co-crystallization in the presence of 1% PA (Pinto et al., 2010).

3.2.6 LC-ESI-MS analysis

LC-ESI-MS analysis of whole CN was performed using an Agilent 1100 Series LC/MSD system with a single quadrupole mass detector (Agilent Technologies, Avondale, PA, USA) in the positive mode. The mass spectrometer was coupled to a Hewlett Packard LC system. Solvent A was 0.1% TFA (v/v) in ultrapure water and solvent B was 0.1% TFA (v/v) in ACN. A solution (200 μl) containing 1 mg CN sample/ml solvent A was loaded onto a Vydac C4 column (214TP54 5 μm, 250 mm × 2.1 mm internal diameter column, Vydac, Hesperia, CA, USA) with a detection wavelength of 220 nm. The elution program involved a gradient from

30 to 50% solvent B over 40 min and 50 to 100% B over 2 min at a flow rate of 1 ml/min. The optimum mass spectrometric parameters were as follows: capillary voltage, 4.0 kV; cone voltage, 80 V; heated N_2 gas (350 °C, 10 l/min); and compressed N_2 gas (13 psi). The mass spectra of the CN components were scanned from 1800 to 400 at a scan cycle of 5 sec/scan. The LC-MS data were acquired with the Hewlett Packard 1100 series ChemStation Chromatographic analysis software (Hewlett-Packard Company, Waldbronn, Germany).

3.2.7 ESI-Q-TOF-MS analysis

Native whole CN was analyzed on a CapLC nano-flow high-pressure pump system (Waters/Micromass, Manchester, UK) interfaced with an ESI-tandem MS. The mass spectrometer was the Q-TOF™ hybrid Q-TOF (Micromass Ltd., Manchester, UK) equipped with a Z-spray ion source in the positive ion mode (ESI-Q-TOF and MS/MS). Chromatographic separations were performed on a reversed phase Atlantis dC18 capillary column (75 µm i.d.). The mobile phase was water (A) and ACN (B) with 0.1% FA. A linear gradient from 5 to 70% B was applied to the pre-column and column over a 45 min period at a flow-rate of approximately 300 nl/min pre-column splitting using a pump operating at 5 µl min^{-1}. The source conditions were as follows: capillary voltage, 2.6 kV; cone voltage, 100 V; and RF1 lens, 40 V. External calibrations with apomyoglobin from equine skeletal muscle (A8673 Sigma Aldrich) and [Glu1]-Fibrinopeptide B (human) (GFP) (F3261, Sigma Aldrich) were performed over mass ranges of 600 to 2,000 and 400 to 1,600 m/z, respectively. All spectra were acquired with the TOF analyzer in "V-mode" (9.1 kV TOF) and the MCP voltage was set at 2000 V. The masses were calculated using the MaxEnt3 deconvolution algorithm. The raw data were processed using the MassLynx 4.0 ProteinLynx software.

3.3 Results and discussion 1

3.3.1 Identification of CN from healthy and infected ovine by LC-ESI-MS

Ovine CN fractions secreted from a healthy udder (SCC/ml, 46 x 10^3/ml), the contralateral infected udder (SCC/ml 4,897 x 10^3/ml) and an intermediate udder (SCC 1,041 x 10^3/ml) were simultaneously compared. The effect of the udder health on CN breakdown was preliminarily visualized using antibodies against the N-terminal β-, α_{s1}-, α_{s2}- and κ-CN peptides and against the C-terminal β- and α_{s1}-CN peptides (Figure 3).

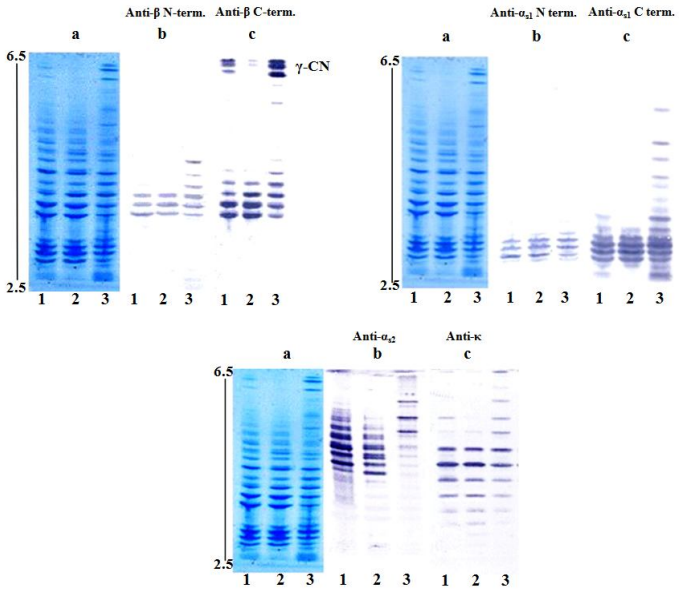

Figure 3. CBB stained SDS-PAGE gel (panel a) of ovine casein from the right and left udder with medium (lanes 1), normal (lane 2) and high (lane 3) SCC content. The other gels show an immunoblot of the same samples probed with polyclonal anti N-terminal β-, α_{s1}-, α_{s2}-CN peptide antibodies (all the panels b) and anti-C terminal β-, α_{s1}-CN and antiN-terminal κ-CN peptide antibodies (all the panels c).

The intensity of the three γ-CN bands is significantly higher in the milk with higher SCC content as visualized using antibodies against the C-terminal β-CN peptides (Figure 3, first gel, panel c). A number of major polypeptides identified using antibodies against the N-terminal β-CN peptides moved faster than the β-CN and slower than the α_{s1}-CN (Figure 3, first gel, panel b). These polypeptides, identifiable with PP, were produced by the action of proteases associated with a high SCC. The presence of a subclinical intramammary infection has been reported to involve an increase of the PP fraction and a decrease of β-CN (Martí-De Olives et al., 2011). High concentrations of PL in mastitic or high SCC quarter milk could explain this finding (Le Roux et al., 1995). Moreover, the immunoblots of high SCC milk showed both high and low mobility peptides using antibodies against the C-terminal peptides of the α_{s1}-CN (Figure 3, second gel, panel c). A possible explanation is that cathepsin D and other enzymes could hydrolyze the N-terminal region of the α_{s1}-CN, forming N-terminally truncated peptides, whereas the C-terminal portion remained intact. α_{s2}-CN and κ-CN fractions were probed using a specific anti-peptide antibody preparation for each peptide (Figure 3, third gel). The immunoblot profile of α_{s2}-CN in milk with high SCC significantly differed from the control CN for the presence of the three intense bands at a higher pI, whereas the intensity of the native α_{s2}-CN simultaneously decreased (Figure 3, third gel, panel b). The immunoblotting procedure with regard to the native κ-CN profiling gave results quite similar to the α_{s2}-CN as several bands were formed at the higher pI (Figure 3, third gel, panel c). There are little data available regarding the hydrolytic products of κ-CN. This finding confirms that the native κ-CN survives the enzymatic degradation, even in milk samples with high SCC. In conclusion, the higher the SCC, the more intense is the proteolytic phenomenon, except for κ-CN. Grieve & Kitchen reported that the results for CN degradation by leukocyte proteases were in the order α_{s1}- > β- >> κ-CN (Grieve & Kitchen, 1985). The propensity for degradation varied according to the CN fraction that was exposed to proteolysis and also varied with the incubation time (Haddadi et al., 2005). The results of this study support the previous findings that β-CN and α_{s2}-CN are more susceptible than α_{s1}-CN and κ-CN to degradation in the order β- > α_{s2}- > α_{s1}- >> κ-CN.

Although new electrophoretic bands have been observed as a result of enzymatic activity increase in high SCC milk, the exact identity of the bands remained unknown.

MS has provided a valid alternative to conventional electrophoretic and other descriptive methods. It has allowed to characterize the peptides derived from the hydrolysis and the dephosphorylation of ovine CN. LC-ESI-MS analysis was used to identify CN fractions and peptides in samples with low and high SCC (Figure 4).

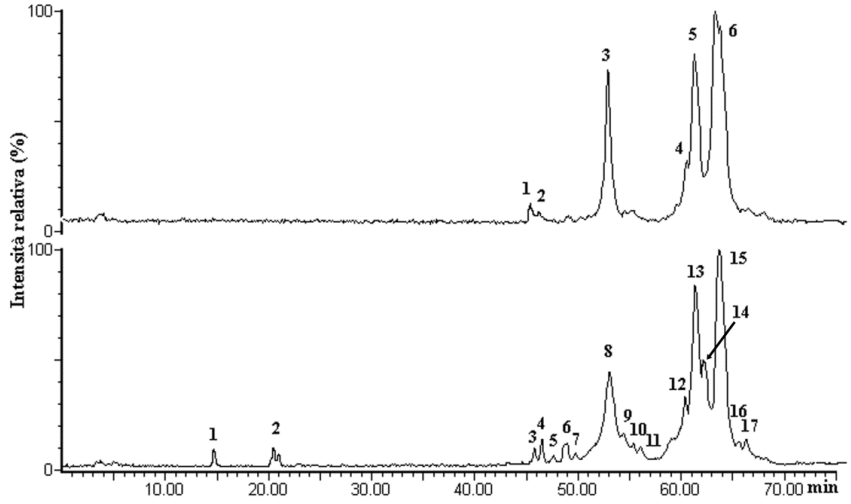

Figure 4. LC-ESI-MS profile of ovine casein fractions isolated from milk secreted by a healthy (a) and infected (b) udder.

Although the LC patterns of the CN samples with low (healthy) and high (infected) SCC seemed to be similar, a greater heterogeneity was observed (Figure 4a and 4b): the ovine CN sample from a healthy udder gives six peaks (Figure 4a), while there are seventeen peaks for the infected udder sample (Figure 4b). The comparison of CN fractions from healthy and infected udder and the relative abundance (RA) of the phosphorylated forms within each CN family are reported in Table 2.

Table 2. Main components of ovine casein fractions from a healthy and infected udder separated by LC (Figure 4a and 4b) and identified by online ESI-MS analysis.

Healthy milk	RA of the phosphorylated form	Infected milk	RA of the phosphorylated
α_{s2}(f1-208)-$_{(34-42)}$ 11P		α_{s2}(1-208)-$_{(34-42)}$ 11P	
α_{s2}(f1-208)-$_{(34-42)}$ 10P	*9P>8P>7P>*	α_{s2}(1-208)-$_{(34-42)}$ 10P	
α_{s2}(f1-208)-$_{(34-42)}$ 9P	*11P>10P*	α_{s2}(1-208)-$_{(34-42)}$ 9P	*11P>10P>9P>8P>7P*
α_{s2}(f1-208)-$_{(34-42)}$ 8P		α_{s2}(1-208)-$_{(34-42)}$ 8P	
α_{s2}(f1-208)-$_{(34-42)}$ 7P		α_{s2}(1-208)-$_{(34-42)}$ 7P	
α_{s2}(f1-208) 12P		α_{s2}(1-208) 12P	
α_{s2}(f1-208) 11P		α_{s2}(1-208) 11P	*12P>10P>*
α_{s2}(f1-208) 10P	*11P>12P≥10P>8P>9P>7P*	α_{s2}(1-208) 10P	*11P>9P>8P*
α_{s2}(f1-208) 9P		α_{s2}(1-208) 9P	*>7P*
α_{s2}(f1-208) 8P		α_{s2}(1-208) 8P	
α_{s2}(f1-208) 7P		α_{s2}(1-208) 7P	
α_{s1}(f1-199)-$_{(110-117)}$ 8P		n.d. (not detected)	
α_{s1}(f1-199)-$_{(110-117)}$ 7P	*6P>7P>8P*	α_{s1}(1-199)-$_{(110-117)}$ 7P	*6P>7P*
α_{s1}(f1-199)-$_{(110-117)}$ 6P		α_{s1}(1-199)-$_{(110-117)}$ 6P	
α_{s1}(f1-199)-$_{(Gln78)}$ 10P		n.d.	
α_{s1}(f1-199)-$_{(Gln78)}$ 9P	*8P>9P>10P*	α_{s1}(1-199)-$_{(Gln78)}$ 9P	*7P>8P>9P*
α_{s1}(f1-199)-$_{(Gln78)}$ 8P		α_{s1}(1-199)-$_{(Gln78)}$ 8P	
n.d.		α_{s1}(1-199)-$_{(Gln78)}$ 7P	
α_{s1}(f1-199) 10P		n.d.	
α_{s1}(f1-199) 9P	*7P>8P>9P*	α_{s1}(1-199) 9P	*8P>7P>9P*
α_{s1}(f1-199) 8P	*>10P*	α_{s1}(1-199) 8P	
α_{s1}(f1-199) 7P		α_{s1}(1-199) 7P	
β(f1-207) 6P		β(1-207) 6P	
β(f1-207) 5P		β(1-207) 5P	
β(f1-207) 4P	*5P>4P>6P*	β(1-207) 4P	*5P>4P>3P>*
β(f1-207) 3P	*>3P*	β(1-207) 3P	*6P>2P>1P*
n.d.		β(1-207) 2P	
n.d.		β(1-207) 1P	
κ(f1-171) 2P	*2P>1P*	κ(1-171) 2P	*2P>1P*
κ(f1-171) 1P		κ(1-171) 1P	

RA = the relative abundance of the components within each CN family

The disappearance of the fully phosphorylated forms of CN fractions and the appearance of those less phosphorylated is particularly evident for α_{s1}-CN and its deleted form α_{s1}-CN–Gln_{78} and for β-CN (Table 2). Moreover, a different RA was observed in the samples from healthy and infected milk. These results were confirmed by the measurement of ALP activity in the different milks with different SCC (Table 3).

Table 3. Measurement of ALP activity in milk samples with different SCC.

Milk Samples	SCC		ALP mU/l	
	Healthy	**Infected**	**Healthy**	**Infected**
IZ5073	79,000	1,041,000	767.0	1,697.2
IZ5073	273,000	1,054,000	460.0	1,763.4
IZ4346	46,000	4,897,000	308.5	2,051.2
IZ4741	67,000	2,040,000	947.9	2,652.9
OS6107	78,000	2,465,000	117.2	1,011.8
0S7339	118,000	13,341,000	2,548.1	11,492.0

In any case, the ALP activity is higher in milk from SCM than healthy controls. ALP can dephosphorylate CN based on its activity in the milk of SCM-affected cows. The ALP activity could explain the appearance of partially dephosphorylated CN fractions, such as β-CN 1P and 2P and α_{s1}-CN–Gln_{78} 7P and the concomitant disappearance of the α_{s1}-CN–Gln_{78} 10P in milk with high SCC. Therefore, the phosphorylation degree of β-CN varied in the range of 6P to 1P, whereas the β-CN peak area did not change significantly with respect to the control CN. In healthy milk, internally deleted counterpart α_{s1}-CN–(f110-117) shows a phosphorylation level lower than full-length α_{s1}-CN because of loss of deleted mono-CPP. Although the α_{s1}-CN–(f110-117) form had nine sites potentially phosphorylable, the α_{s1}-CN–(f110-117) 9P was not detected in any sample. Despite the different number of SCC, the proportion of native κ-CN 1P and 2P was similar. Moreover, in both milk samples, the quantity of full-length α_{s2}-CN was greater than the homologous internally deleted counterpart, although the deleted peptide, α_{s2}-CN (f34-42), did not contain any phosphorylated residue within its sequence. Notably, the internally deleted α_{s2}-CN lacks the Lys-containing peptide, which causes a less efficient interaction with PL and the full-length α_{s2}-CN. The proportion of native α_{s2}-CN globally decreased in the CN sample from the infected udder because of the modification of either partially dephosphorylated α_{s2}-CN (Table 2) or the degradation into shorter peptides (Table 4), such as the α_{s2}-derived peptides (f151-208), (f152-208), (f1-114) (3P-2P).

Table 4. Main peptides of ovine CN from a healthy and infected udder separated by LC (Figure 4a and 4b) and identified by online ESI-MS analysis.

Healthy milk	Infected milk	RA of the phosphorylated form
n.d. (not detected)	β(49-207)	
n.d.	β(49-198)	
n.d.	β(98-207)	
n.d.	β(1-105) 5P	
n.d.	β(1-105) 4P	
n.d.	β(1-105) 3P	*5P>4P>2P>3P*
n.d.	β(1-105) 2P	
n.d.	β(106-206)	
n.d.	β(106-207)	
n.d.	β(1-97) 5P	
n.d.	β(1-97) 4P	
n.d.	β(1-97) 3P	*4P>2P>5P>3P*
n.d.	β(1-97) 2P	
n.d.	β(170-207)	
n.d.	β(1-29) 4P	
n.d.	β(84-113)	
n.d	α_{s2}(1-114) 3P	
n.d.	α_{s2}(1-114) 2P	*2P>3P*
n.d.	α_{s2}(151-208)	
α_{s2}(f152-208)	α_{s2}(152-208)	
n.d.	α_{s1}(1-60) 3P	
n.d.	α_{s1}(1-60) 2P	
n.d.	α_{s1}(1-60) 1P	*2P>1P>3P>0P*
n.d.	α_{s1}(1-60)	
n.d.	α_{s1}(24-79) 6P	*4P>3P>5P>6P*
n.d.	α_{s1}(24-79) 5P	
n.d.	α_{s1}(24-79) 4P	
n.d.	α_{s1}(24-79) 3P	
n.d.	α_{s1}(24-72) 2P	
n.d.	α_{s1}(24-72) 1P	*0P>1P>2P*
n.d.	α_{s1}(24-72)	

RA = the relative abundance of the components within each CN family

β-, α_{s1}-, and α_{s2}-CN derived peptides were α_{s1}-CN (f24-72) (2P-0P), (f24-79) (6P-3P), (f1-60) (3P-0P), α_{s2}-CN (f151-208), (f152-208), (1-114) (3P-2P) and β-CN (f84-113), (f1-29) 4P, (f170-207), (f1-97) (5P-2P), (f106-206), (f106-207), (f1-105) (5P-2P) (f98-207), (f49-198), (f49-207), as shown in Table 4. The control sample had only an extra peptide, α_{s2}-CN (f152-208) (Table 4). PP, β-CN (f1-29) 4P and β-CN (f1-105) 5P, as well as β-CN (f1-97) 5P were PL-mediated CPPs. The non-phosphorylated counterparts, such as β-CN (f98-207), (f106-206), (f106-207) and (f98-207), were concurrently detected in the infected milk samples. Other peptides were released by PL action on Lys_{49}-Ile_{50}, Lys_{169}-Val_{170} and Lys_{113}-Tyr_{114} bonds of β-CN. Three peptides of α_{s2}-CN, *i.e.* α_{s2}-CN (f151-208), (f152-208), (1-114) 3P, originated from PL. All these peptides were justified by higher susceptibility of the β-CN and a_{s2}-CN to PL hydrolysis. The influx of PMNs into the milk due to the weakening of the milk-blood barrier caused the increase of SC proteinases such as cathepsin D (Capony et al., 1989). Partially purified cathepsin D from milk was reported to release two complementary peptide α_{s1}-CN (f1-23) and α_{s1}-CN (f24-199) (a_{s1}-I-CN) (McSweeney et al., 1995). The α_{s1}-I-

CN peptide is further degraded by cathepsin D *in vitro* (Larsen et al., 1996). Therefore, α_{s1}-CN (f24-79) is released by the co-presence of PL and cathepsin D.
Other enzymes are responsible for the cleavage of the peptide bonds Phe-Val, Gln-Met, Val-Pro, Leu-Val and Pro-Val in the target sequence. These results provide evidence that the CN degradation is ascribed to PL and proteases from the PMNs. Lysosomal proteinases, such as elastase and cathepsin B, almost certainly occur in milk from cows suffering from mastitis (Napoli et al., 2007; Considine et al., 2000; Considine et al., 2002). Cathepsin G preferentially cleaves the oxidized β-chain of insulin between Leu_{15} and Tyr_{16} (Blow et al., 1977), whereas elastase cleaves the bonds of non-charged aromatic amino acids.

3.3.2 Identification of HA enriched tryptic CPPs in ovine CN sample from high SCC milk

The objective of our study is to estimate the post-translational modifications of CN fractions in order to detect the markers of infected milk. Therefore, the HA-based method was applied to the tryptic digests of high SCC ovine CN (Pinto et al., 2010) to confirm the phosphorylation level of CN fractions. The direct analysis of the HA-bound CPPs, co-crystallized with MALDI matrix containing PA, produced the spectrum shown in Figure 5.

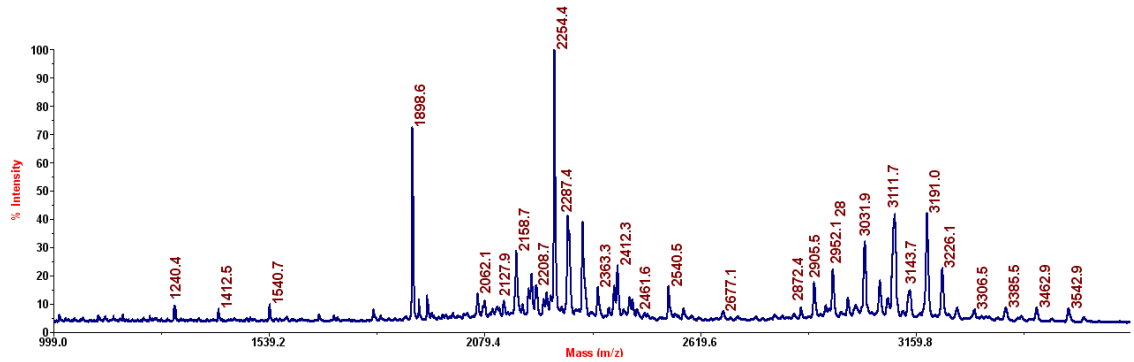

Figure 5. The MALDI partial view for the mass range 1.0–3.7 kDa for the HA-bound tryptic ovine CPPs from infected milk with an SCC of 7,750,000.

The identification of tryptic CPPs was obtained using the program FindPept (http://www.expasy.ch/tools/findpept.html) (Table 5).

Table 5. Tryptic CPPs detected by MALDI spectrum (Figure 5) and relative phosphorylation level. α_{s2}-CN derived CPPs with non-specific tryptic cleavage are also listed below.

Amino acid sequence of tryptic peptides	CPPs
REQEELNVVGETVESLSSSEESITHINK/(K)	β-CN (f1-28) 5P, 4P, 3P, 2P, 1P
(K)/FQSEEQQQTEDELQDK/(I)	β-CN (f33-48) 1P
KHKMEHVSSSEEPINISQEIYKQEK(N)	α_{s2}-CN (f1-25) 4P, 3P, 2P, 1P
KHKMEHVSSSEEPINISQEIYK/(Q)	α_{s2}-CN (f1-22) 4P, 3P, 2P, 1P
(K)/MEHVSSSEEPINISQEIYK/(Q)	α_{s2}-CN (f4-22) 4P, 3P, 2P
(R)/SSSEESAEVAPEEVK/(I)	α_{s2}-CN (f57-71) 4P, 3P
(R)/EQLSTSEENSKK/(T)	α_{s2}-CN (f126-137) 2P
(R)/EQLSTSEENSK/(K)	α_{s2}-CN (f126-136) 2P
(K)/ENINELSKDIGSESIEDQAMEDAK/(Q)	α_{s1}-CN (f35-58) 3P, 2P
(K)/DIGSESIEDQAMEDAKQMK/(A)	α_{s1}-CN (f43-61) 2P
(K)/SAEEQLHSMK/(E)	α_{s1}-CN (f115-124) 1P
(K)/AGSSSSSEEIVPNSAEK/(Y)	α_{s1}-CN (f62-78)-$_{Gln78}$ 6P, 5P, 4P
(K)/AGSSSSSEEIVPNSAEQK/(Y)	α_{s1}-CN (f62-79) 6P, 5P, 4P, 3P
Amino acid sequence of non-specific peptides	**CPPs**
KHKMEHVSSSEEPINISQEI Y(K)	α_{s2}-CN (1-21) 4P
(K)HKMEHVSSSEEPINISQEIY(K)	α_{s2}-CN (2-21) 4P
(K)MEHVSSSEEPINISQEIY(K)	α_{s2}-CN (4-21) 4P
(Y)SIRSSSEESAEVAPEEVK/(I)	α_{s2}-CN (54-71) 4P, 3P

The non-CPPs were absent after enrichment on HA, whereas the presence of partially dephosphorylated forms was confirmed, particularly in the high SCC CN samples. Fully and partially CPPs were detected as an effect of ALP activity, which shifts the peptide signals by multiples of 80 Da corresponding to the phosphate group (H_3PO_4) loss. These results demonstrated the efficacy of CPP enrichment on HA and suggested that the procedure will be of general use in phosphoproteome studies (Table 5) (Pinto et al., 2010). We have not attempted to identify the phosphorylated sites but to provide more information regarding the phosphorylation level of CPPs. The complementary information was acquired by the multiply phosphorylated peptides enriched on HA at low abundance, which can be lost during the ESI-MS analysis. The ESI-MS analysis of infected ovine CN detected six phosphorylated forms of β-CN with a molecular weight corresponding to β-CN 6P, 5P, 4P, 3P, 2P and 1P (Table 2). The presence of tryptic CPPs, such as β-CN (f1-28) (5P-1P) and β-CN (f33-48) 1P (Table 5), confirmed that β-CN was intensely dephosphorylated in milk with high SCC. Other partially dephosphorylated α_{s1}-CN and α_{s2}-CN CPPs were identified in high SCC milk samples. The tryptic CPPs, such as α_{s1}-CN (f62-79) (6P-3P) and its internally deleted form, α_{s1}-CN (f62-78)–Gln_{78} (6P-4P), bound HA together with α_{s1}-CN (f35-58) (3P-2P), α_{s1}-CN (f43-61) and α_{s1}-CN (f115-124) 1P (Table 5). The 80-Da multiple loss value confirmed the presence of α_{s1}-CN (9P-7P) and α_{s1}-CN (9P-7P)–Gln_{78}. In infected milk, the detection of α_{s1}-CN (f62-79) 3P suggested the presence of α_{s1}-CN 6P that was not detected by ESI analysis (Table 2). Moreover, the results of the ESI/MS analysis showed that native forms of α_{s1}-CN 10P and α_{s1}-CN–Gln_{78} 10P were suppressed by more abundant less phosphorylated species (Table 2). Similar observations can be made for α_{s2}-CN CPPs. Among the tryptic CPPs, three had a different N-terminal extension, 1-25, 1-22 and 4-22. α_{s2}-CN (f1-25) and (f1-22) were the most affected by dephosphorylation and showed a phosphorylation level from 4P to 1P. The non-specific tryptic cleavage of the C-terminal Tyr_{21}-Lys_{22} bond gave rise to α_{s2}-CN (f1-21) 4P, α_{s2}-CN (f2-21) 4P and α_{s2}-CN (f4-21) 4P, whereas cleavage at Tyr_{53}-Ser_{54} produced α_{s2}-CN (54-71) (4P-3P). In our study, we do not investigate the exact identities of the SC proteases or other proteases responsible for these hydrolysis products but we observe the released CPPs from enzymatic activity. The proteolytic release of peptides and the dephosphorylation of those phosphorylated occur almost simultaneously as demonstrated by the presence of CPPs shorter than the control CN (Table 4). However, the data suggest that a marked increase of SC in milk determines the CN modifications, which essentially consists of dephosphorylation and proteolysis. Notably, changes of milk casein have allowed to identify some signature peptides that are available for the early detection of SCM.

3.4 Conclusions 1

The CN proteolysis and dephosphorylation in milk of healthy and infected ewes have been well defined using different proteomic techniques. The results on CN phosphoproteome include also the detection of tryptic CPPs by enrichment on HA microgranules. In this manner, deeper information has been obtained regarding low-abundance, mono- and multi-CPPs. Follow-up work on healthy ovine milk, *i.e.* raw milk with low SCC, has confirmed that no sensible alteration of the CN sequence occurs compared to that observed in raw milk with high SCC. The overall data on the CN proteolysis have also provided at least one signature CPP from each CN fraction, excluded κ-CN. These signature peptides are particularly useful for evaluating the severity of the attack by proteolytic enzymes associated to a high level SC of milk. A low level of SC affords the formation of almost exclusively β-CN peptides by action of endogenous PL, followed by α_{s2}-CN and α_{s1}-hydrolytic products. κ-CN is the most resistant to proteolysis by PL and other enzymes associated to SC as its hydrolysis could cause destabilization of the CN micelles and the resulting coagulation of milk in the mammary gland. The influence of high levels of SC affects the CN micelle by proteolysis according the hydrolysis order, *i.e.* β-> α_{s2}-> α_{s1}->>> κ-CN. The assumption that high SCC of raw milk reduces the shelf-life and flavor quality of pasteurized fluid milk is substantiated by partially dephosphorylated CN and derived tryptic peptides. Moreover, the intense dephosphorylation reduces the bioactivity of CPPs relative to binding capacity of phosphate groups. From the economic point of view, processing the milk with a high SCC reduces the cheese yield. Therefore, bonus would be paid to dairy farmers able to supply milks with low levels of SC to the cheese factory.

3.5 References

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3.6 Topic 2: Lactosylated CPPs as specific indicators of heated milks

In our study, we have focused on the detection of heated milks through signature CPPs that undergo lactosylation once released by proteinases. The comparison between the native and lactosylated CPPs has been used to differentiate processed milks according to heat intensity. Lactosylation sites have been localized on peptide sequence by post-source decay (PSD) fragmentation. In this work, the lactosylated CPPs have allowed us to detect the addition of UHT milk, in amounts not lower than 10% for spiked raw or pasteurized milk with UHT milk. We suggest extending this approach to the analysis of any dairy product with suspected addition of UHT milk, milk protein or milk powder.

3.7 Materials and methods 2

3.7.1 Sample preparation

Raw milk was from local dairy farms. The pasteurized and UHT milks were purchased from a local store. Milk protein (casein + whey) powder and milk powder were supplied by Sacco Industry (Milan, Italy). The chemical composition of milk samples as well as the time/temperature combinations is reported in Table 6.

Table 6. Protein and lactose content in heat-treated commercial milk samples

Sample	% protein	% lactose	Heating treatment	Time/temperature combinations
Raw milk	3.2	4.9	No	No
Pasteurized milk	3.2	4.9	Pasteurization	15 sec/71.7 °C
UHT milk	3.1	4.9	Direct Ultra High Temperature processing	2 sec/135 °C
Milk protein powder	46	5	Membrane filtration*and Spray-drying	Air temperature at 200°C
Milk powder	36	56	Spray-drying	Air temperature at 200°C

** Concentrated commercial milk proteins were obtained by milk ultrafiltration on membrane*

Each sample, including raw, pasteurized and UHT milk, protein or milk powder in solution, was treated with HA as described below. Pasteurized milk was added of UHT milk to the final concentration from 90 to 1%.

3.7.2 HA-based phosphoprotein/CPP enrichment from various milk samples

HA (100 mg), previously equilibrated with the loading buffer (50 mM Tris-HCl, 0.2 M KCl, 4.5 M urea and 10 mM DTT, pH 8.0), were put in contact with proteins in solution (10 mg) or equivalent amounts of raw, pasteurized and UHT milk. The HA-bound proteins/CPPs were incubated for 15 min at room temperature and centrifuged at 4,000 x g for 2 min. The resin was successively washed and freeze-dried as above described. Solid milk samples (1 mg/ml) were solubilized in H_2O/ACN (50:50) solution with 0.1% TFA. Liquid milk samples (10 µL) were diluted 1:100 with the same solution. Each protein solution (0.5 µl) was loaded on the MALDI plate well and covered with DHB matrix (0.5 µl) for the preliminary analysis.The HA-bound CPPs were then analyzed by MALDI as described above.
In order to confirm the degree of lactosylation, the dried HA-bound CPPs (1 mg) were dissolved in a 5% aqueous PA solution (120 µl), desalted with a ZipTip C18 pipette tip and dephosphorylated with ALP according to the procedure previously described (Pinto et al., 2012). The HA-bound CPPs were also analysed by PSD-MALDI-TOF.

3.7.3 PSD-MALDI-TOF experiments

PSD fragment ion spectra were acquired after isolation of the appropriate precursor ions by using timed ion selection. In this manner, the spectra of lactosylated β-CN (f1-28) 4P (m/z 3,803 Da) and its unphosphorylated counterpart (m/z 3,483 Da) were acquired. Acquisition was performed with the following settings: accelerating voltage 20 kV, grid voltage 80% of the accelerating voltage, guide wire 0.02 and delayed time 100 ns. Each spectrum was the average of 300 shots in three replicates.

3.7.4 Synthesis of peptide analogues

The β-CN (1-25) 4P peptide was synthesized by solid-phase methods using the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy on a Pioneer peptide synthesizer (Synthesis System 9050 instrument; PE-Biosystems, Framingham, MA, USA). The level of the β-CN (f1-28) 4P was chosen as the index for quantification of β-CN proteolysis. The natural and the analogue synthesized peptides were used to generate a calibration curve (Table 7).

Table 7. The amino acid sequences of synthesized natural and modified peptides are shown. The differentiating Trp (W) and Leu (L) residues are underlined. Sp indicates phosphorylated serine residue.

Synthetic natural peptide	**[M+H]**	**Sequence**
β-CN (f1-28) 4P	3,478.5 Da	RE**L**EELNVPGEIVESpLSpSpSpEESITRINK
Synthetic modified peptide	**[M+H]**	**Sequence**
β-CN (1-25) 4P	3,196.3 Da	RE**W**EELNVPGEIVESpLSpSpSpEESITR

A constant concentration (10 μg/μl) of synthesized modified peptides was used to spike different solutions of synthesized natural peptides to create a calibration curve, with the natural/modified area ratio as a function of the concentration ratio of the corresponding peptides. The amount of β-CN (f1-28) 4P was obtained by spiking 10 μl of raw milk with 10 μg/μl IS and calculating the quantity of this peptide according to the equation of the calibration plot.

3.8 Results and discussion 2

3.8.1 MALDI analysis of protein lactosylation

A preliminary study was carried out to evaluate the lactosylation of intact proteins in the raw, pasteurized, UHT, milk protein powder and milk powder samples. Our results indicated that MALDI analysis was useful to identify lactosylation during heating or spray-drying through the monitoring of the molecular mass shift of +324 Da per lactose molecule. These post-translational modifications were clearly visualized by MALDI analysis especially for milk whey proteins: β-Lg and α-La (Pinto et al., 2012). Unmodified β-Lg A and B were still present in all samples except milk powder. Lactosylated proteins in pasteurized milk were not detected as well as in raw milk. β-Lg A and B was lactosylated as well as α-La (1 lactose residue/ molecule) in UHT milk. In two studies, Lys_{47} and Lys_{100} of β-Lg and Lys_{98}, Lys_{114} or Lys_{122} of α-La were lactosylated in UHT milk (Fogliano et al., 1998; Siciliano et al., 2000). The lactosylation of β-Lg A and B was higher in milk powder (6 lactose residues/molecule) than in milk protein powder (4 lactose residues/molecule). α-La was less lactosylated in the milk protein powder (3 lactose residues/molecule) than in milk powder (4 lactose residues/molecule). The different levels of lactosylation of proteins in the powder samples were probably due to the lower lactose content of ultrafiltered milk or whey. However, the Maillard reaction was incomplete even in the powdered milk preparations. The Amadori compound, the first intermediate in the Maillard reactions, is probably degraded by heat (Ledl & Schleicher, 1990) via the Strecker degradation (Yaylayan & Huyghues-Despointes, 1994). Thus, the lactosylation level of whey proteins increased steadily with temperature, progressively from pasteurization to spray-drying passing through to UHT. It seemed that

heating extensively enhanced lactosylation by exposing lysine residues on the surface of the proteins. The diffuse lactosylation of casein fractions expecially in the powdered samples due to the low resolution of MALDI, encouraged us to find the lactosylated markers of CPPs released in the different heat-treated milks.

3.8.2 CPP evaluation in raw milk by MALDI-TOF analysis in the 1.2–5 kDa range

Raw samples were examined by MALDI-TOF in the 1.2–5 kDa mass range before and after enrichment on HA (Figure 6).

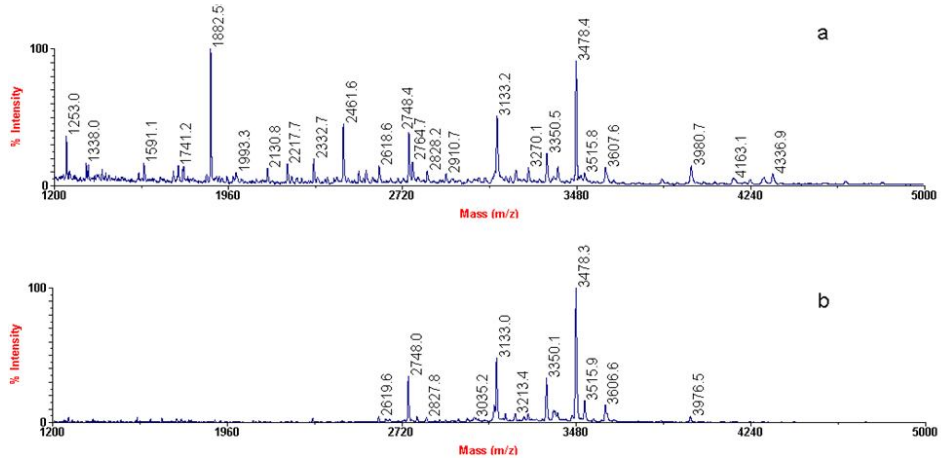


Figure 6. MALDI-TOF spectra of raw milk peptides in the 1.2–5 kDa mass range before (a) and after enrichment (b) using HA as concentrating probe.

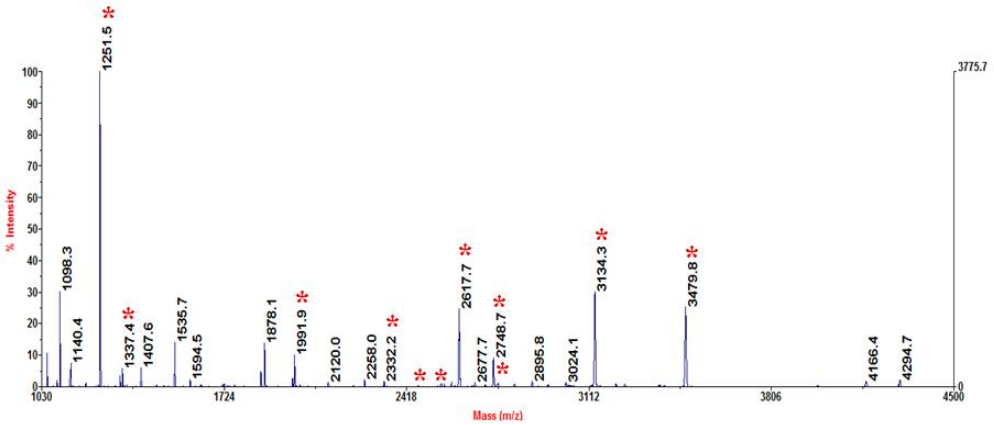
The peptides detected in raw milk are listed in Table 8. After enrichment on HA, only phosphorylated peptides were detected (Figure 6b, Table 8).

Table 8. List of the peptides identified in the MALDI spectra of raw milk (Figure 6) before and after enrichment on HA.

Peptide	Expected mass	Measured mass	Number of phosphate groups	N-terminal peptide bond cleavage	C-terminal peptide bond cleavage
α_{s2}-CN (f198-207)[a]	1,251.5	1,252.7	0P	Lys_{197}-Tyr_{198}	C-terminal peptide
α_{s1}-CN (f80-90)[a]	1,336.9	1,338.0	0P	Lys_{79}-His_{80}	Arg_{90}-Tyr_{91}
β-CN (f195-209)[a]	1,589.9	1,591.1	0P	Gln_{194}-Glu_{195}	C-terminal peptide
β-CN (f194-209)[a]	1,718.1	1,718.9	0P	Tyr_{193}-Gln_{194}	C-terminal peptide
β-CN (f193-209)[a]	1,881.3	1,882.5	0P	Leu_{192}-Tyr_{193}	C-terminal peptide
α_{s2}-CN (f150-165)[a]	1,991.3	1,992.9	0P	Lys_{149}-Lys_{150}	Lys_{165}-Lys_{166}
α_{s1}-CN (f181-199)[a]	2,129.0	2,130.9	0P	Ser_{180}-Asp_{181}	C-terminal peptide
α_{s1}-CN (f180-199)[a]	2,216.5	2,217.7	0P	Phe_{179}-Ser_{180}	C-terminal peptide
α_{s1}-CN (f4-22)[a]	2,235.6	2,235.6	0P	Lys_{3}-His_{4}	Arg_{22}-Phe_{23}
α_{s2}-CN (f189-207)[a]	2,331.9	2,332.6	0P	Lys_{188}-Ala_{189}	C-terminal peptide
α_{s1}-CN (f4-23)[a]	2,382.3	2,383.0	0P	Lys_{3}-His_{4}	Phe_{23}-Phe_{24}
α_{s1}-CN (f1-21)[a]	2,460.4	2,461.6	0P	N-terminal peptide	Leu_{21}-Arg_{22}
α_{s1}-CN (f4-24)[a]	2,529.4	2,529.6	0P	Lys_{3}-His_{4}	Phe_{24}-Val_{25}
β-CN (f29-48)[a,b]	2,560.6	2,561.4	1P	Lys_{28}-Lys_{29}	Lys_{48}-Ile_{49}
α_{s1}-CN (f1-22)[a]	2,617.1	2,618.6	0P	N-terminal peptide	Arg_{22}-Phe_{23}
α_{s2}-CN (f2-21)[b]	2,618.9	2,619.6	4P	Lys_{1}-Asn_{2}	Lys_{21}-Gln_{22}
α_{s2}-CN (f1-21)[a,b]	2,747.6	2,748.4	4P	N-terminal peptide	Lys_{21}-Gln_{22}
α_{s1}-CN (f1-23)[a]	2,763.5	2,764.7	0P	N-terminal peptide	Phe_{23}-Phe_{24}
β-CN (f184-209)[a]	2,910.5	2,910.8	0P	Arg_{183}-Asp_{184}	C-terminal peptide
α_{s2}-CN (f1-24)[a,b]	3,132.9	3,133.3	4P	N-terminal peptide	Lys_{24}-Asn_{25}
β-CN (f1-25)[b]	3,122.9	3,122.6	4P	N-terminal peptide	Arg_{25}-Ile_{26}
β-CN (f1-27)[a,b]	3,349.4	3,350.1	4P	N-terminal peptide	Asn_{27}-Lys_{28}
β-CN (f1-28)[a,b]	3,478.4	3,478.4	4P	N-terminal peptide	Lys_{28}-Lys_{29}
β-CN (f1-29)[a,b]	3,606.6	3,607.7	4P	N-terminal peptide	Lys_{29}-Ile_{30}
β-CN (f1-32)[a,b]	3,977.0	3,977.5	4P	N-terminal peptide	Lys_{32}-Phe_{33}

[a] *and* [b] *indicate the peptides identified in Figure 6a and 6b, respectively*

A high number of peptides belonging to the β-CN, α_{s2}-CN and α_{s1}-CN fractions were recorded in MALDI spectrum before enrichment (Figure 6a, Table 8). CPPs detected in the raw milk as well as in the other samples derived all from β-CN and α_{s2}-CN (Figure 6b, Table 8). α_{s1}-CN derived CPPs failed to form due to the internal location of the phosphorylation cluster. In contrast, they are released in long ripened cheeses (Ferranti et al., 1997). The most of peptides were released by PL-mediated hydrolysis of cleavage bonds containing N-terminal and C-terminal Lys or Arg. Other peptides with non-specific PL cleavage occurred probably because of the co-presence of active somatic cell proteinases. In order to verify the role played by proteolytic enzymes in CN hydrolysis, *in vitro* PL-mediated peptides were compared with those arose from raw milk (Figure 7).



** indicates the peptides detected also in the raw milk sample (Table 8)*

Figure 7. MALDI spectrum of peptides released by *in vitro* plasminolysis of bovine CN

The choice of PL as the proteolytic enzyme depends on its role played during milk storage. In the PL digest (Figure 7), there are many peptides detected in raw milk (Table 8), including β- and α_{s2}-CN CPPs that were released by PL. This agrees with the known activities of PL and other proteases associated with milk somatic cells. When CPP levels were higher than normal, milk was of poor quality because of higher activity of proteolytic enzymes.

3.8.3 MALDI-TOF analysis of different commercial products

The main objective of our work was to determine signature CPPs of heat-treated milk samples by using the HA-based procedure.
Pasteurized, UHT, milk protein and milk powder samples were examined by MALDI-TOF and CPPs were enriched on HA. Similarly to raw milk, the major CPPs detected in raw milk were identified also in the other commercial milk samples (Figure 6b, Table 8). Moreover, our results showed that in all the milk samples, both native and lactosylated forms of β-CN (f1-28) 4P and β-CN (f1-27) 4P were detected, except in raw and pasteurized milk (Figure 8).

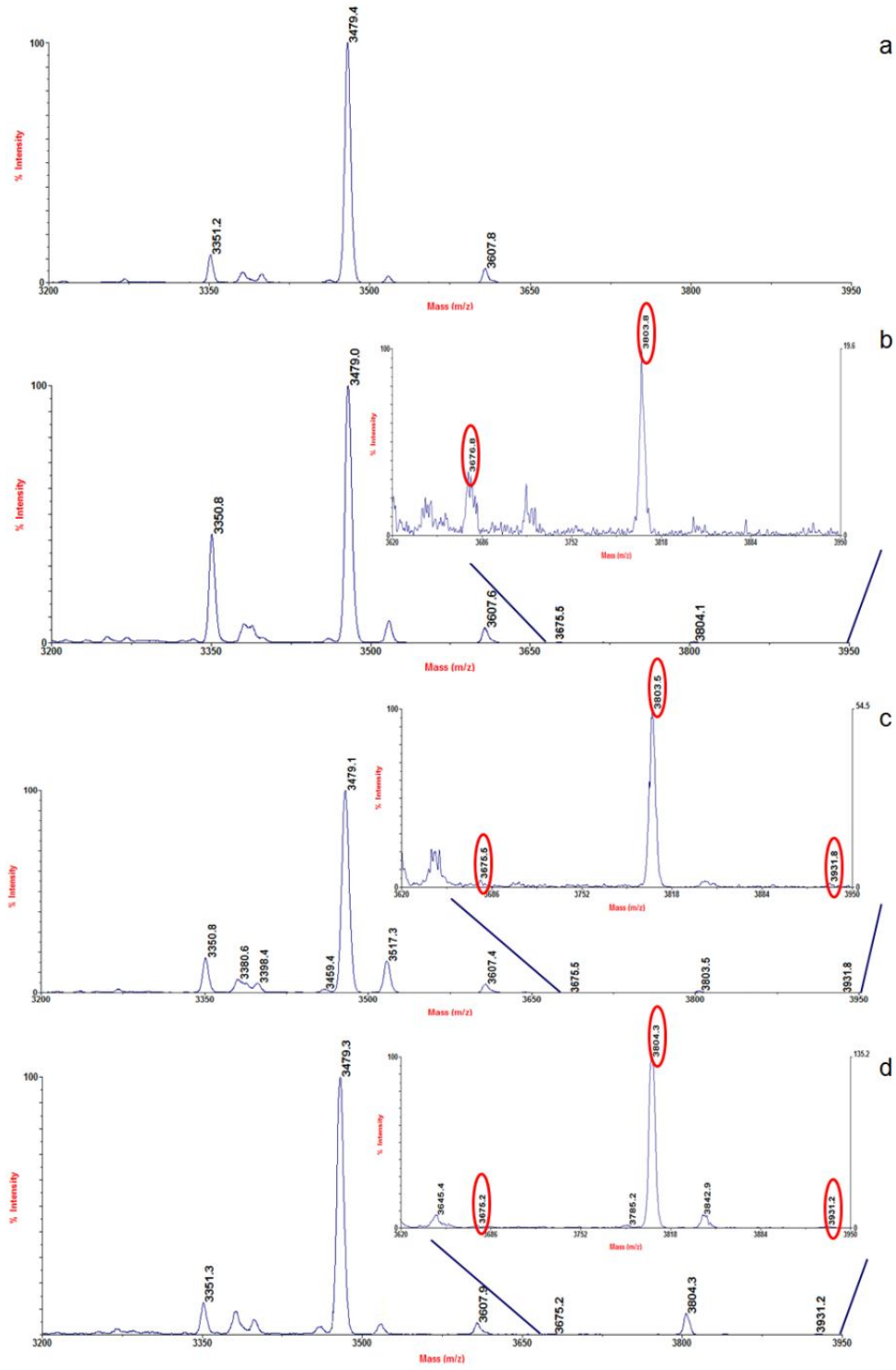


Figure 8. The MALDI spectra show mass signals of β-CN derived CPPs enriched on HA microgranules for samples of pasteurized milk (a), UHT milk (b), milk protein powder (c), and milk powder (d). The spectra are zoomed in the the m/z values in the 3,200–3,950 Da mass range. Mono-lactosylated CPPs labelled with a red circle correspond to β-CN (f1-27) 4P (m/z 3,675 Da), β-CN (f1-28) 4P (m/z 3,803 Da) and β-CN (f1-29) 4P (m/z 3,931 Da).

Therefore, none of these lactosylated CPPs occurred in the spectrum of pasteurized milk (Figure 8a). CPPs differed only in the level of lactosylation, which was extensive in milk powder, the most intensively treated sample. Milk powder contained all the lactosylated forms of β- and α_{s2}-CN derived CPPs as shown in Table 9.

Table 9. β- and α_{s2}-CN derived CPPs and relative lactosylated forms found in milk powder

Measured mass (MH^+) (Da)	Expected mass (Da)	CPP identification
3,607.9	3,606.6	β-CN (f1-29) 4P
3,931.2	3,930.8	β-CN (f1-29) 4P + 1 Lactose
3,479.3	3,478.4	β-CN (f1-28) 4P
3,803.3	3,802.9	β-CN (f1-28) 4P + 1 Lactose
3,351.3	3,350.2	β-CN (f1-27) 4P
3,675.2	3,674.5	β-CN (f1-27) 4P + 1 Lactose
2,748.3	2,747.5	α_{s2}-CN (f1-21) 4P
3,072.5	3,071.8	α_{s2}-CN (f1-21) 4P + 1 Lactose
3,132.8	3,133.0	α_{s2}-CN (f1-24) 4P
3,459.3	3,457.9	α_{s2}-CN (f1-24) 4P + 1 Lactose

The native and lactosylated α_{s2}-CN (f1-21) 4P co-exists in milk powder while the lactosylated forms of this CPP were not detected in the other samples. We have identified the lactosylated β-CN (f1-29) 4P as a signature CPP of milk proteins and powdered milk preparations because it was missing in raw, PM (Figure 8a), intensely PM (121 °C for 2–4 sec) (spectrum not shown) and UHT milk (Figure 8b). It seemed that the level of lactosylation in milk was closely related to the intensity of the heating process. Thus, lactosylated CPPs may serve as suitable chemical markers of the intensity of heat treatment.
The phosphorylation and lactosylation level of β-CN (f1-28) 4P was further confirmed by PSD-MS experiments (Figure 9).

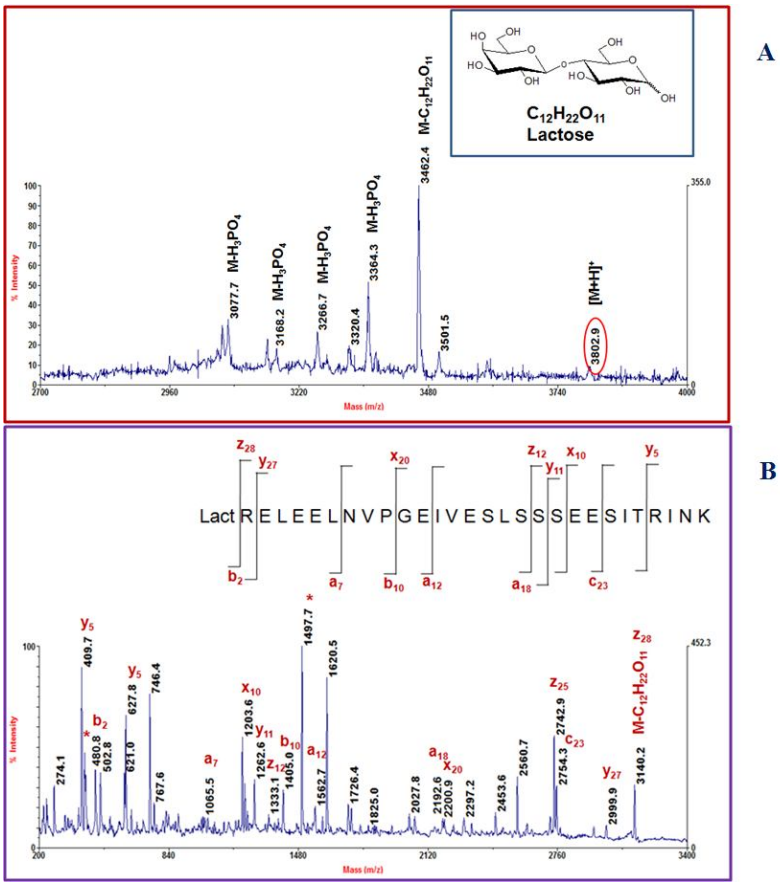


Figure 9. PSD-MALDI-TOF-MS/MS spectrum of lactosylated β-CN (f1-28) 4P at m/z 3,802.9 Da exhibiting a prominent neutral loss of 340 Da (one lactosyl group) from the precursor ion at m/z 3,802.9 Da. Neutral loss of 98 Da (H_3PO_4) is consistent with a peptide containing 4 phosphate residues (A). PSD MALDI-TOF MS/MS spectrum shows the fragmentation of lactosylated β-CN (f1-28) 0P at m/z 3,483 Da after dephosphorylation. The signal at m/z 3,140.2 Da confirms the loss of one lactose group (B).

The PSD spectrum of lactosylated β-CN (f1-28) 4P exhibited a prominent neutral loss of 340 Da (one lactosyl group) from the precursor ion at m/z 3,802.9 Da (Figure 9A). Moreover, the PSD spectrum of lactosylated β-CN (f1-28) 4P yielded a neutral loss of 98 Da (H_3PO_4), consistent with a peptide containing 4 phosphoserine residues (Figure 9A). The PSD spectrum of lactosylated β-CN (f1-28) 0P confirmed the loss of one lactose group (m/z 3,140.2 Da) from the peptide (Figure 9B).

3.8.4 Quantification of β-CN (f1-28) 4P in raw milk by MALDI-TOF-MS

Based on a set of naturally occurring peptides, β-CN (f1-28) 4P was chosen as it is the most abundant phosphorylated member that undergoes glycosylation during heat treatment. Specifically, a signature peptide, synthetic 25-residue analogue that was identical to the N-terminal 25-mer peptide except for the amino acid substitution $Leu^3 \rightarrow Trp^3$, was used as IS for the absolute quantification of the mono-lactosylated β-CN (f1-28) 4P (Table 7). The natural proteotypic peptide was mixed with IS in known concentrations and the MALDI intensity of the two co-occurring signals was measured (Figure 10).

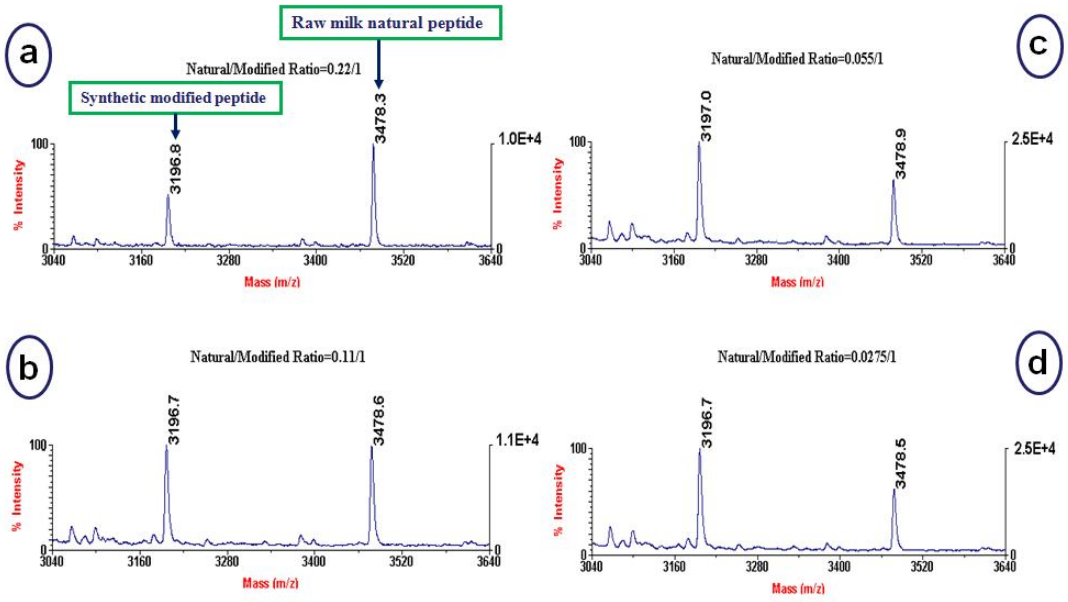


Figure 10. MALDI spectra of natural and modified peptide β-CN (f1-25) 4P in four binary solutions containing indicated peptide ratios. MH^+ 3,196.7 Da is molecular mass of the β-CN (f1-25) 4P peptide containing the $Leu^3 \rightarrow Trp^3$ modification; MH^+ 3,478.6 Da is molecular mass of the β-CN (f1-28) 4P natural CPP.

MALDI measurements performed on four binary solutions containing natural peptide/IS ratios of 0.22/1, 0.11/1, 0.055/1 and 0.0275/1 produced a line defined by the equation y= 6.2451x + 0.3635 (R^2=0.9855) (mean of 10 replicates). Using this approach, raw milk β-CN was found to have released ~4.3% of the β-CN (f1-28) 4P.

3.8.5 Specific detection of lactosylated CPPs

Raw milk is rejected by dairy processing plants whenever the sample is found to be contaminated with UHT or other heat-treated milk. The HA-based method could ensure that UHT milk and sterilized milk are not used for the production of pasteurized milk. To demonstrate this, aliquots of pasteurized milk were artificially spiked with 90, 70, 50, 30, 10, 5, or 1% UHT milk. The LOD was determined by MALDI searching for the lactosylated β-CN (f1-28) 4P, assumed as the signature CPP of UHT milk. The method did not discriminate UHT milk in amounts below 10%.
Using improved MS equipment, in terms of mass resolution and sensitivity, the LOD could be substantially lowered. Trypsinolysis of HA-bound CPPs, enhancing the signal of lactosylated β-CN (f1-25) 4P, could further lower the LOD value.

3.9 Conclusions 2

Our results have shown that the extent of the casein lactosylation is a function of the heating intensity. Moreover, the ability to dynamically characterize lactosylation of CPPs can be regarded as a model for studying the accessibility of lactose to casein micelles. To detect lactosylated CPPs by MALDI analysis, both native and lactosylated CPPs have been concentrated on HA microgranules. This peculiarity makes the HA-method able to detect low-abundance signature CPPs. Therefore, the results of our experiments have demonstrated that (i) native and lactosylated CPPs are equally bound to HA; (ii) CPPs have higher affinity for HA than non-phosphorylated peptides co-purifying with them using other methods; (iii) mono-lactosylated β-CN (f1-28) 4P represents a distinct signature peptide that is detected distinctly from the native counterpart, making possible the detection of not less than 10% UHT milk spiking raw or pasteurized milk.
However, the present procedure needs further signature peptides to distinguish thermized and pasteurized milks. Even with this limitation, the method could be applied at the molecular level to the design and control of any dairy product.

3.10 References

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3.11 Topic 3: CPP profiling of Parmigiano Reggiano cheese at different ripening ages

In the present work, CPP enrichment based on in-batch HA has been used for profiling CPPs from different varieties of cheese; particular attention has been given to the pH 4.6-soluble fraction of PR at different periods of ripening: 5, 15, 26 and 35 months (mo). The investigation has been mainly focused on CPPs derived from the enzymatic hydrolysis of cheese CN that are progressively shortened and dephosphorylated by the enzymes involved in ripening. Such profiles have greatly enabled us to characterize the proteolytic events that occur during ripening of brine-salted hard cheese. Moreover, the MALDI analysis of CPPs bound to HA allows the distinction of young PR cheeses (5- to 15-mo-old) from those aged more than 26-mo.

3.12 Materials and methods 3

3.12.1 Preparation of the pH 4.6-soluble fractions of cheese

Samples of mold-ripened Gorgonzola (GR) (2-mo-old), semi-cooked Asiago d'Allevo (AA) (3-mo-old), semi-hard, *pasta filata* Provolone del Monaco (PMC) (6-mo-old) and grated PR cheese (5-, 15-, 26-, 30 and 35-mo-old) (10 g) were suspended in 45 ml of 0.1 N HCl (Sforza et al., 2008). The suspension was homogenized for 1 min by an Ultra Turrax T50 (Janke & Kunkel IKA® Labortechnik, Staufen, Germany), adjusted to pH 4.6 with 1 M NaOH and then centrifuged at 4,000 x g for 10 min. After cooling, a thick layer of fat rose to the surface of the sample and was removed with the aid of spatula. The supernatant was filtered through filter paper; the resulting solution was adjusted to pH 7.0 with 1 M NaOH and freeze dried.

3.12.2 HA-CPP enrichment from various cheese samples

Lyophilized samples (10 mg) of the pH 4.6-soluble fraction of cheeses were dissolved in loading buffer (50 mM Tris-HCl, 0.2 M KCl, 4.5 M urea and 10 mM DTT, pH 8.0) and enriched on HA (100 mg) as above described. The dried HA-CPP complexes were loaded on MALDI plate, covered with DHB (10 mg/ml) and analysed by MALDI as already described.

3.12.3 LC-ESI-Q-TOF MS/MS analysis

HA-bound CPPs (1 mg) were dissolved in a 5% aqueous PA solution (120 µL), desalted with a ZipTip C18 pipette tip and analyzed using a CapLC nano-flow high-pressure pump system (Waters/Micromass, Manchester, UK) interfaced with a QTOF Micromass spectrometer (Waters/Micromass) operating in the positive ion mode. Chromatographic separations were performed on a reverse phase Atlantis dC18 capillary column (75 µm i.d.). The mobile phase was water (A) and ACN (B) with 0.1% FA. A linear gradient from 5% to 70% of B was applied to the pre-column and column over a 45-min period at a flow-rate of approximately 300 nl/min pre-column splitting using a pump operating at 5 µL min^{-1}. The source conditions were as follows: capillary voltage, 2.6 kV; cone voltage, 100 V; and RF1 lens, 40 V. Argon was used as the collision gas in the ESI-MS/MS experiments for peptide sequencing. Raw data were processed using MassLynx 4.0 ProteinLynx software.

3.13 Results and discussion 3

3.13.1 CPPs in a few cheese varieties

A preliminary study was carried out on CPPs of pH 4.6 soluble fractions of GR, AA and PMC 6-mo-old cheeses. CPP fraction of hard PR (30-mo-old) was compared to cited cheeses; a further study was then carried out by comparing the enriched CPPs of 5-, 15-, 26- and 35-mo-old PR cheeses.
Some CPPs enriched on HA were derived from the Lys-X or Arg-X cleavage by PL such as β-CN (f1-28) 4P (Lys_{28}-Lys_{29}) or β-CN (f1-29) 4P (Lys_{29}-Ile_{30}), α_{s1}-CN (f61-79) 5P (Lys_{79}-His_{80}) and α_{s2}-CN (f1-24) 4P (Lys_{24}-Asn_{25}). The native PL-derived CPPs were further hydrolyzed by cheese aminopeptidases and CPase into shorter peptides (Figure 11).

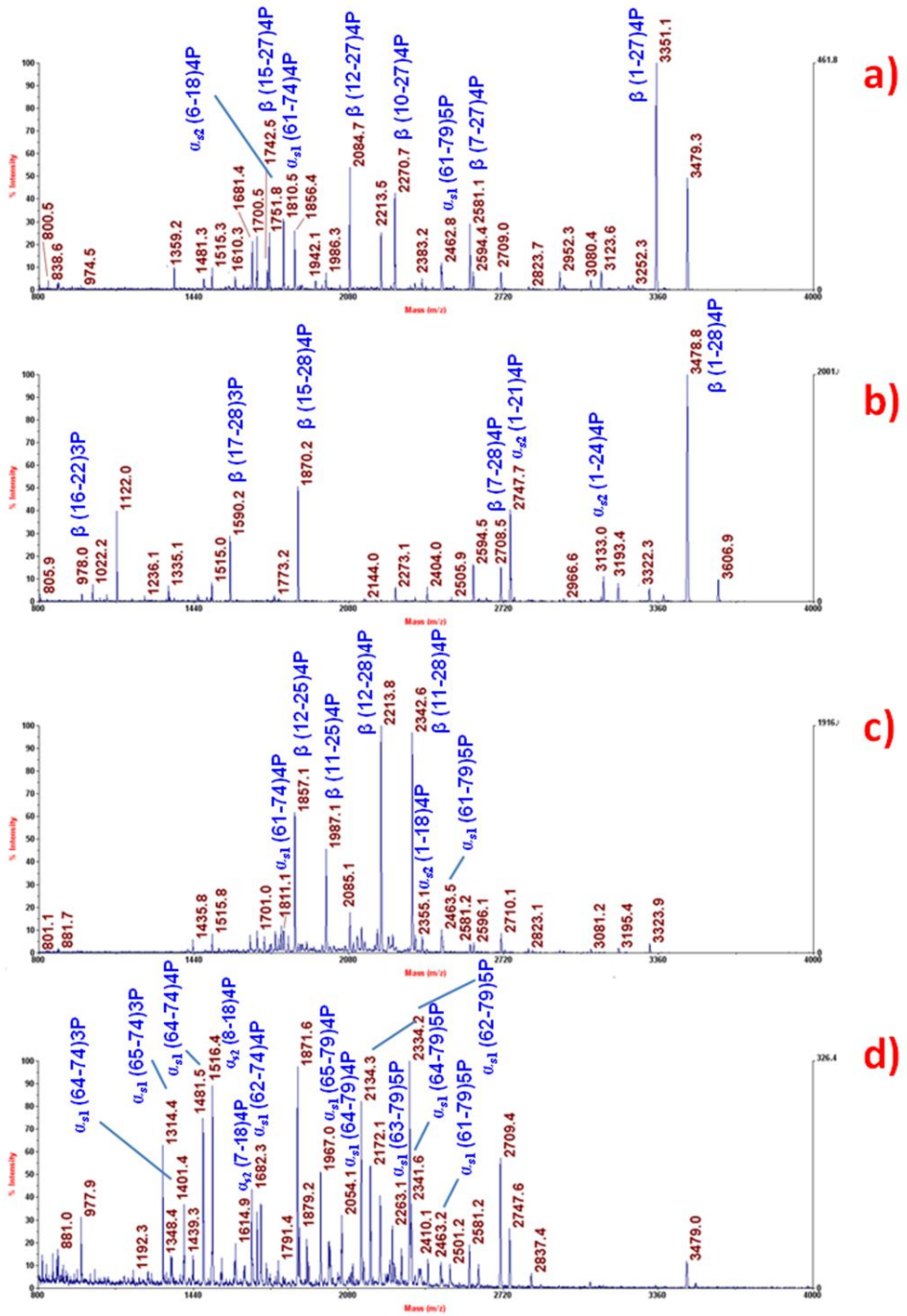

Figure 11. The MALDI spectra of CPPs isolated by the addition of HA to pH 4.6 soluble fractions of Gorgonzola (a), Asiago (b), Provolone del Monaco (c) and Parmigiano Reggiano (d) cheeses. The inset magnifies the m/z values in the lower molecular peptide mass range (0.8–4 kDa).

The most abundant CPPs of GR, AA and PMC cheeses derived all from the peptide β-CN (f1-28) 4P (3,478.4 Da) (Figure 12). β-CN (f1-28) 4P in AA cheese was the most abundant signal of the MALDI spectrum (Figure 11b and 12) and it results partially hydrolyzed into the shorter peptides β-CN (f7-28)4P (2,708.5 Da), β-CN (f15-28)4P (1,869.7 Da) and β-CN (f17-28) 3P (1,589.6 Da).

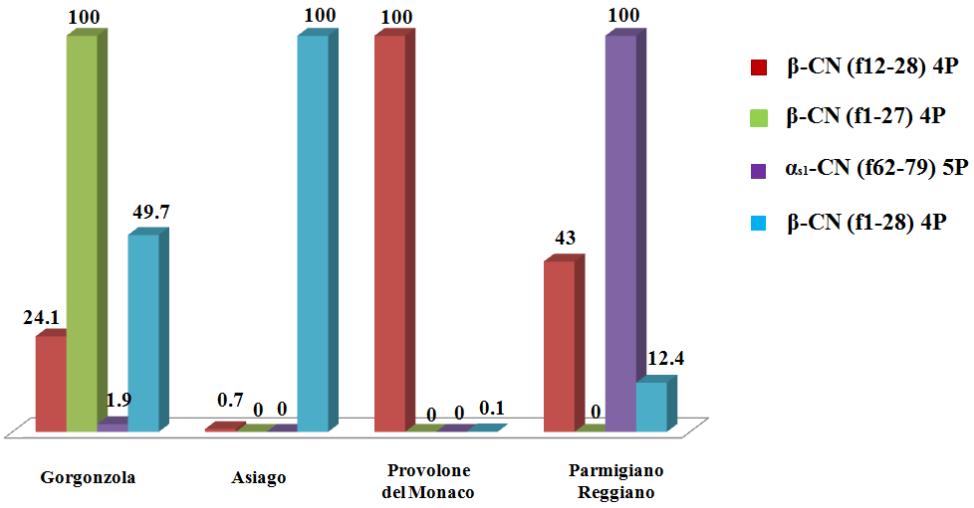

Figure 12. Histogram representation of CPPs at 100% relative intensity and their performance in four cheeses.

The β-CN hydrolysis was particularly evident in PMC cheese made from raw milk for which the PL-mediated β-CN (f1-28) 4P peptide, representing ~0.1% of the CPPs, was almost completely hydrolyzed into the shorter peptides β-CN (f11-28) 4P (2,341.1 Da), β-CN (f12-28) 4P (2,212.0 Da), β-CN (f11-25) 4P (1,985.7 Da) and β-CN (f12-25) 4P (1,856.6 Da) (Figure 11c and 12). In GR cheese, β-CN (f1-27) 4P (3,350.2 Da) was released from non-specific PL cleavage of the C-terminal Asn_{27}-Lys_{28} bond (Figure 11a and 12) or from the action of cheese CPases. β-CN (f1-27) 4P was further hydrolyzed into the shorter β-CN (f7-27) 4P (2,580.4 Da), β-CN (f10-27) 4P (2,270.0 Da), β- CN (f12-27) 4P (2,083.8 Da) and β-CN (f15-27) 4P (1,742.4 Da). Profile of long ripened PR cheese CPPs was dissimilar from the other cheeses for its higher content of CPPs derived from α_{s1}-CN (f61-79) 5P; α_{s1}-CN (f62-79) 5P (2,332.9 Da) was the most abundant CPP (Figure 11d and 12). In 30-mo-old PR, α_{s1}-CN CPPs originated for the greater parts from the internal regions of the amino acid sequence, namely from α_{s1}-CN (33-60) 3P and α_{s1}-CN (61-79) 5P whereby they were released by a more intense proteolytic activity. In PR cheese, 23 CN-derived CPPs derived from the internal region of α_{s1}-CN, *i.e.* α_{s1}-CN (f59-79) 5P, whereas they were not detected in AA (Figure 13).

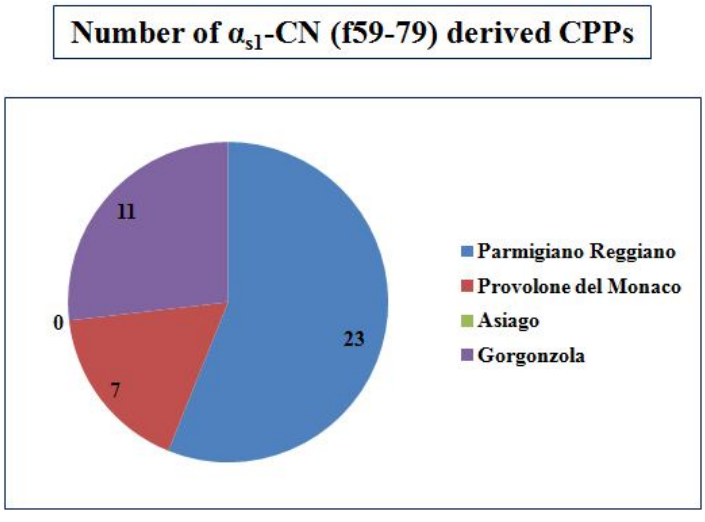

Figure 13. The number of CPPs derived from α_{s1}-CN (f59-79) 5P in PR, PMC, AA and GR cheeses.

The CPP profile depicts the mechanisms of both the proteolysis and dephosphorylation in a long ripened cheese. α_{s1}-CN (f61-79) 5P, most likely arising from the parent peptide α_{s1}-CN (f1-79) 7P through cleavage at Met_{60}-Glu_{61}, was dephosphorylated and concurrently hydrolyzed into shorter peptides. Alkaline and/or

acid phosphatases acting on SerP residue dephosphorylated CPPs. N-terminal Ser was then exposed to aminopeptidase and released as a FAA. Bacterial CPase or exopeptidase such as cathepsin D/chymosin released α_{s1}-CN (f61-74) 4P and other derived CPPs through cleavage at Asn_{74}-$SerP_{75}$. In PR aged 30 months, α_{s1}-CN (f61-79) 5P (2,462.1 Da), α_{s1}-CN (f62-79) 5P (2,332.9 Da), α_{s1}-CN (f63-79) 5P (2,261.9 Da), α_{s1}-CN (f64-79) 5P (2,132.8 Da), α_{s1}-CN (f64-79) 4P (2,052.8 Da), α_{s1}-CN (f65-79) 4P (1,965.7 Da), α_{s1}-CN (f62-74) 4P (1,681.3 Da), α_{s1}-CN (f64-74) 4P (1,481.2 Da), α_{s1}-CN (f64-74) 3P (1,401.2 Da) and α_{s1}-CN (f65-74) 3P (1,314.1 Da) were the dominant CPPs (Figure 11d). Only 7 CPPs for PMC cheese and 11 CPPs for GR were derived from α_{s1}-CN (f59-79) 5P among which the most abundant were α_{s1}-CN (f61-79) 5P (2,462.1 Da) and α_{s1}-CN (f61-74) 4P (1810.5 Da) (Figure 11a and c and 13).
Considering the α_{s2}-CN peptide, CPPs derived from the N-terminal region of the α_{s2}-CN, *i.e.* α_{s2}-CN (f1-24) 4P. α_{s2}-CN (f1-24) derived CPPs were similar in number but significantly different in the four cheese varieties (Figure 11a-d). The dominant α_{s2}-CN derived CPPs were α_{s2}-CN (f1-24) 4P (3,132.9 Da) and α_{s2}-CN (f1-21) 4P (2,747.6 Da) in AA cheese (Figure 11b). The most abundant α_{s2}-CN derived CPPs were α_{s2}-CN (f1-18) 4P (2,355.1 Da) for PM and α_{s2}-CN (f6-18) 4P (1,751.4 Da), a shortened form of the primary CPP α_{s2}-CN (f1-18) 4P, for GR cheese (Figure 11a and c). α_{s2}-CN (f7-18) 4P (1,614.3 Da) and α_{s2}-CN (f8-18) 4P (1,515.1 Da) characteristically accumulated in PR cheese (Figure 5d). This means that a more extent proteolysis of casein fractions characterized the PR cheese. The CN hydrolysis became more evident when the chymosin retained in the cheese was largely inactivated by cooking the curd at high temperatures (~55 °C). The CN proteolysis by chymosin was covered by that of PL, which becames the principal proteolytic enzyme in the cheese. The presence and integrity of PL-mediated products of CN was a function of the milk, whether raw or pasteurized. Pasteurization reduced the milk PL activity only by ~15 percent, whereas PL activity increased during milk storage. Heat treatments modified the peptide profile by increasing the content of larger peptides and the shorter CPPs accumulated in long-ripened cheeses made from raw milk. Considering exclusively the CPPs with molecular mass between 3,000 and 3,500 kDa, the intensity of longer CPPs characterized the MALDI spectra of AA and GR cheese; these peptides were further transformed into a number of progressively lower molecular weight CPPs (Figure 11). In long ripened cheeses, a significant accumulation of shorter peptides containing the multi-phosphorylated motif -SerP-SerP-SerP-Glu-Glu-, more resistant to proteolytic enzymes, were observed. Moreover, a heterogeneous CPP pattern also differentiated the cheese samples within a given form because of the phosphatase gradient amongst peripheral and central parts of the GP cheese form ($3\ 10^5$ vs. $3\ 10^2$) (Pellegrino et al, 1997). This means that eating PR cheese increases the quota of the co-ingested mineral bound CPPs. Milk, ice cream and cheese have been observed to lower the incidence of dental caries in rats (Shaw et al., 1959) but the best results came out by eating cheese several times per week for the higher caries-protective effect (Papas et al., 1995).

3.13.2 β-CN derived CPP proteolysis in PR cheeses at different age of ripening

Most CPPs detected in milk, as discussed above, had C-terminal Lys, suggesting the PL activity. β-CN and α_{s2}-CN were the preferred substrates of PL, whereas α_{s1}-CN and κ-CN were resulted more resistant to enzyme digestion. In particular, β-CN, the CN fraction more affected by proteolysis, can be converted into pH 4.6-insoluble γ-CN and complementary soluble PP. The γ-CN detected by IEF analysis of cheese samples at 4- and 34-mo of ripening were considered good markers for GP ripening (Restani et al., 1996). Moreover, the concentration of β-CN rapidly decreased during the first 15 mo until reaching a plateau; it was still detectable in 22-mo-old samples (Gaiaschi et al., 2001). These results seems to conflict with previous results on CN proteolysis indicating that β-CN degradation was completed within one year of PR cheese ripening (Addeo et al., 1988) while they are consistent with subsequent findings according to which low levels of β-CN still remained intact in the 24-mo-old PR cheese (Addeo et al., 1995). Moreover, a trend inverse to that β-CN was observed for γ_1-CN and β-CN (f30-209), whose concentration increased until the 15-mo of ripening, and for γ_2-CN and γ_3-CN, whose production was greater between 15-mo and 22-mo (Gaiaschi et al., 2001). Previous results (data not published) have showed that the pH 4.6-insoluble N fraction represented approximately 70% (w/w) of defatted and freeze dried cheese for ripened 4- and 8-mo GP. After 14-38 mo, this fraction decreased to 50-55%, which indicated that CN fractions gave rise to an increased level of pH 4.6-soluble N compounds.
The analysis of HA-enriched CPPs of 5-, 15-, 26- and 35-mo-old PR samples has allowed to observe a progressive increase in the relative number of CPPs of mass ranged from 900 to 4,000 Da with aging. Approximately, 68% of β-CN derived CPPs occurring in the 5-mo-old PR cheese decreased to approximately 58%, 45% and 39% in the 15-, 26- and 35-mo-old PR cheeses, respectively. Moreover, MALDI spectra showed that the profiles of β-CN and α_{s1}-CN derived CPPs were very different after enrichment on HA, in the different samples (Figure 14). In the MALDI spectrum of 5-mo-old PR cheese, the majority of signals was associated to CPPs derived from β-CN (f1-28) 4P (3,479.4 Da) and β-CN (f29-57) 1P (3,570.5 Da)

(Figure 14, pink arrows). These MALDI signals lowly decreased in both number and intensity with increased ripening time (Figure 14, pink arrows).

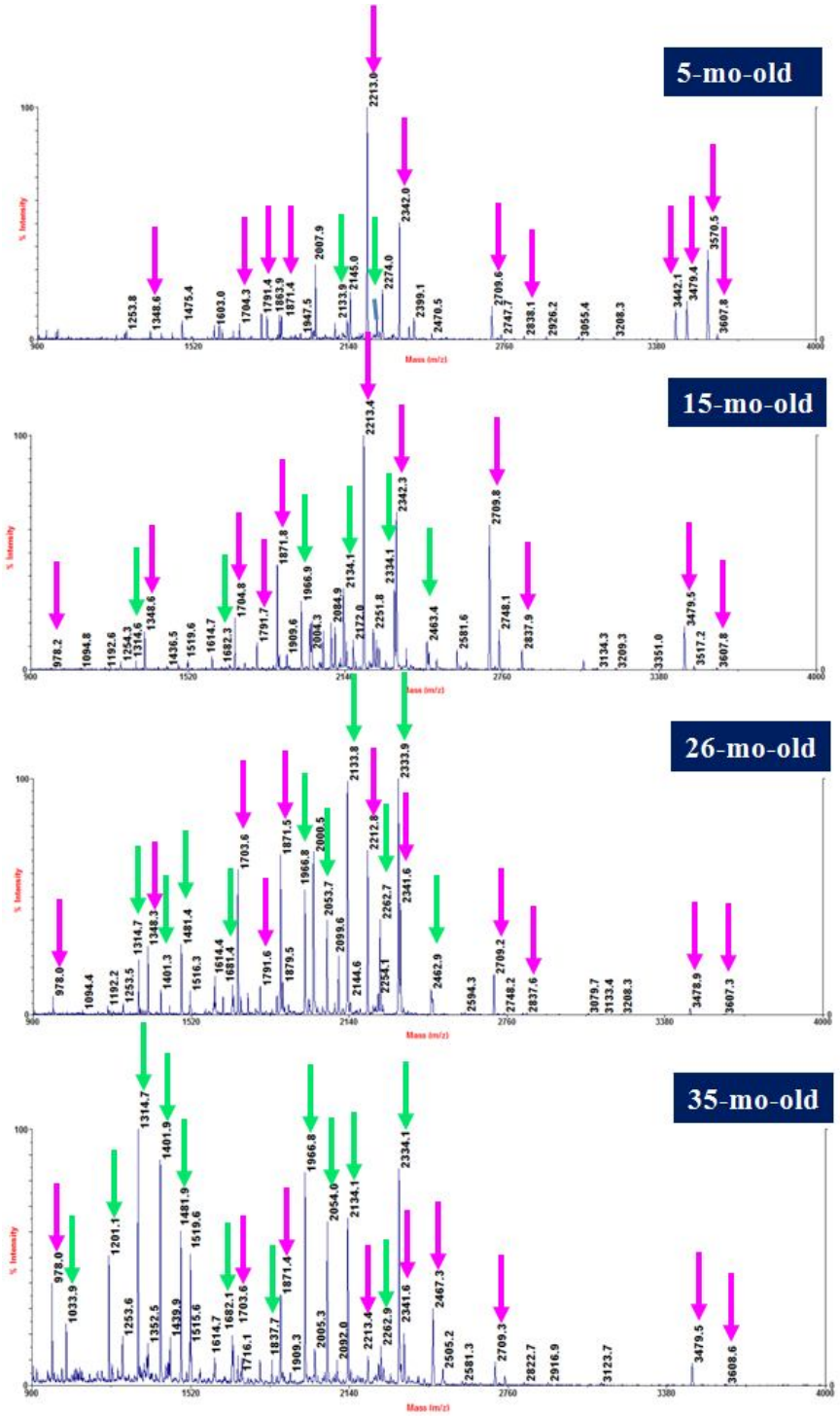

Figure 14. Comparison of different MALDI spectra recorded in the mass range of 0.9–4 kDa for cheese samples. Pink arrows indicate the most abundant signals of β-CN (f1-28) 4P derived CPPs and green arrows the most abundant signals of α_{s1}-CN (f61-79) 5P derived CPPs in PR cheese at 5-, 15-, 26- and 35-mo of ripening.

In 5- and 15-mo-old PR cheese samples, the degradation products of β-CN were more abundant than those derived from α_{s1}-CN whereas an inverse trend was observed in the 26- and 35-mo-old PR samples (Figure

14). These findings are consistent with previous results indicating that 80% of β-CN was hydrolyzed within the first year of ripening; afterwards the hydrolysis rate decreased. Moreover, a 15% of β-CN was further hydrolyzed within 30-mo of ripening, whereas 5% of the residual β-CN remained in the older GP cheeses (not shown data).
Eight PP, including two PP5 and two PP8 slow, raised from the two most common genetic variants, β-CNA^1 and A^2, were identified in the semi-hard Herrgard cheese. In addition to these peptides, novel CPPs such as β-CN (f29-96) 1P, (f30-97) 1P, (f29-93) 1P and (f30-93) 1P, in the two β-CN A^1 and A^2 variants, accumulated in the Herrgard cheese (Ardö et al., 2007). After enrichment of CPPs from pH 4.6-soluble fraction of 5-mo-old PR cheese, some CPPs were shorter than those found in the semi-hard Herrgard cheese, suggesting the enzymatic cleavage of the Ser_{57}-Leu_{58}, Phe_{52}-Ala_{53}, Asp_{43}-Glu_{44}, Gln_{40}-Thr_{41}, Gln_{39}-Gln_{40} peptide bonds (Figure 15), some of which were also detected in 6-mo-old Beaufort cheese (Dupas et al., 2009).

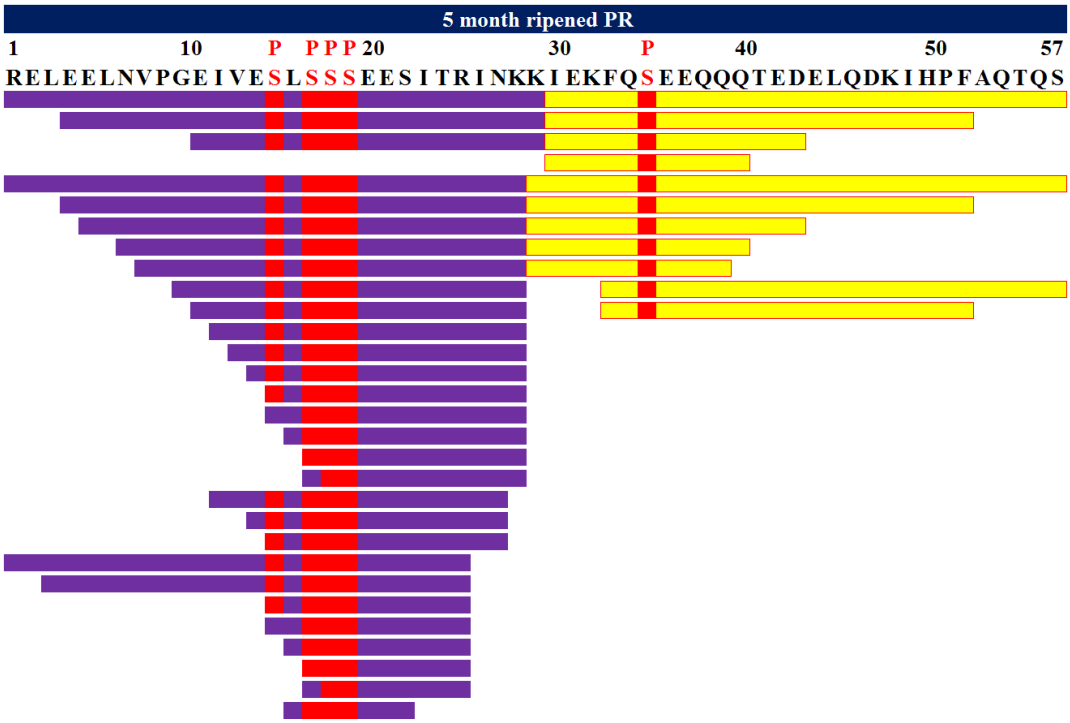

Figure 15. Amino acid sequence of the CPPs found in the 5-mo-old PR cheese aligned with that of β-CN (f1-57) 5P. The purple horizontal bar indicates CPPs derived from β-CN (f1-28)4P and the yellow bar indicates CPPs derived from β-CN (f29-57) 1P. The red shaded residue is a phosphoseryl residue.

The major CPPs, derived from β-CN (f29-57) 1P, were hydrolyzed in cheeses older than 5-mo.
The degradation mechanism of β-CN (f1-28) 4P in the different PR samples is similar to that found in GP (Ferranti et al., 1997) and involved the intervention of aminopeptidases to remove neutral amino acid from the N-terminus of oligopeptides as suggested by Roudot-Algaron (Roudot-Algaron et al., 1994). CPPs are shortened by the sequential removal of the phosphate group from the N-terminal SerP by phosphatases and the neutral Ser residue by aminopeptidases. The results obtained in the present study are not consistent with those from 6-mo-old Beaufort cheese that showed also CPPs with dephosphorylated residues in the internal positions of the peptide chain (Dupas et al, 2009). The dephosphorylation mechanism of Fiore Sardo CPPs was also different from that found, most likely due to the use of different rennet types (Pirisi et al., 2007). In PDO Fiore Sardo cheese, no apparent difference in susceptibility to dephosphorylation was found amongst the differently located SerP peptide residues. This resulted in the simultaneous occurrence of partly dephosphorylated peptides, either internally or externally. Moreover, β-CN (f16-22) 3P (978 Da) was the most abundant CPP in 38-mo-old ripened cheese (Ferranti et al., 1997), consistent with results from 24-mo-old PR cheese using the IMAC procedure (Lund & Ardö, 2004). Conversely, in MALDI spectrum of HA-enriched CPPs from 35-mo-old PR cheese, β-CN (f16-22) 3P was most intense signal among β-CN derived

CPPs but lower than that of CPPs derived from α_{s1}-CN (f59-79) 5P (Figure 14). A comparison of CPPs derived from β-CN (f1-28) 4P, isolated from the pH 4.6-soluble fractions of 5-, 15-, 26- and 35-mo-old PR cheese, is shown in Table 10.

Table 10. Comparison of CPPs derived from β-CN (f1-28) 4P detected in PR cheese at different ages.

β-CN (f1-28) 4P derived CPP				
Measured mass MH^+	**Ripened PR**			
	5 mo-old	**15 mo-old**	**26 mo-old**	**35 mo-old**
978.4	not detected (n.d.)	β-CN (f16-22) 3P		
1,065.3	n.d.	n.d.	n.d.	β-CN (f15-22) 3P
1,192.4	n.d.	β-CN (f16-24) 3P		
1,155.7	β-CN (f17-25) 2P			
1,235.6	β-CN (f17-25) 3P			
1,348.6	β-CN (f16-25) 3P			
1,435.7	β-CN(f15-25) 3P			
1,516.0	β-CN(f15-25) 4P			
1,857.2	β-CN (f12-25) 4P			
2,467.3	n.d.	n.d.	β-CN (f6-25) 4P	
2,838.3	β-CN (f3-25) 4P			
3,123.0	β-CN (f1-25) 4P			
1,575.0	β-CN (f16-27) 3P			
1,743.0	β-CN (f15-27) 4P			
2,084.8	β-CN (f12-27) 4P			
2,501.4	n.d.	β-CN (f7-27) 3P		
2,581.6	n.d.	β-CN (f7-27) 4P		
1,423.8	n.d.	β-CN (f18-28)2P	n.d.	β-CN (f18-28) 2P
1,591.2	β-CN (f17-28) 3P			
1,703.9	β-CN (f16-28) 3P			
1,791.7	β-CN (f15-28) 3P			
1,871.6	β-CN (f15-28) 4P			
1,999.7	β-CN (f14-28) 4P			
2,099.3	β-CN (f13-28) 4P			
2,213.1	β-CN (f12-28) 4P			
2,342.0	β-CN (f11-28) 4P			
2,399.2	β-CN (f10-28) 4P		n.d.	n.d.
2,594.0	β-CN (f8-28) 4P			n.d.
2,709.6	β-CN (f7-28) 4P			
2,951.4	β-CN (f5-28) 4P			n.d.
3,080.7	β-CN (f4-28) 4P			
3,479.5	β-CN (f1-28) 4P			
2,470.2	β-CN (f11-29) 4P			n.d.
3,208.7	β-CN (f4-29) 4P			n.d.
3,607.9	β-CN (f1-29) 4P	n.d.	n.d.	n.d.

The accumulation of β-CN (f16-22) 3P with PR cheese ripening time was parallel to the proteolysis of α_{s1}-CN that proceeded more quickly than that of β-CN. Moreover, β-CN (f15-22) 3P (1065.3 Da) was detected in only 35-mo-old PR cheese (Table 10). Only a few longer CPPs, including β-CN (f1-28) 4P, were still

detectable in 35-mo-old cheese as their degradation proceeded more quickly than their formation. However, because phosphorylation affects the proteolysis kinetics of cleavage sites proximal to the site of phosphorylation (Schlosser et al., 2001), multi-CPPs occurred with an increased frequency. Despite this, no specific marker of age has been found for PR cheese.

3.13.3 α_{s1}-CN-derived CPP proteolysis in PR cheeses at different age of ripening

The fragment α_{s1}-CN (f1-23) and its complementary fragment α_{s1}-I-CN (McSweeney et al., 1993) are mainly released from chymosin and then are further degraded by the cell wall proteinases of LAB (Fox & McSweeney, 1996). Chymosin plays an important role in the early stages of cheese making but it is rapidly denatured when the PR curd is heated to 55 °C. The concentration of α_{s1}-I-CN was very high during the first 4-mo of ripening and then gradually decreased (Gaiaschi et al., 2000). Moreover, α_{s1}-CN (f35-199) and α_{s1}-CN (f80-199) peptides released by PL action on the peptide bonds located between the Lys_{34}-Glu_{35} and Lys_{79}-His_{80} were found in GP and PR cheese as well as in other cheese varieties (Gaiaschi et al., 2000). The α_{s1}-CN (f35-199) concentration was similar in all ripening cheeses until 22-mo as a result of the balance between its formation and its breakdown to shorter peptides. The increase of α_{s1}-CN (f80-199) during ripening suggested a greater resistant to further proteolysis by endo and exopeptidases, even after 24 mo of ripening (Addeo et al., 1995; Gaiaschi et al., 2000). Cheese endoproteases were able to cleave α_{s1}-I-CN in three internal bonds such as the Phe_{32}, Met_{60} and Lys_{79} residues releasing peptides such as (f33-60) 3P and α_{s1}-CN (f61-79) 5P. Many CPPs derived from α_{s1}-CN (f61-79) 5P were captured by HA in PR cheeses of different ages. The percentages of α_{s1}-CN CPPs progressively increased from 12% to 18% in the 5- and 15-mo-old PR cheese until a plateau reached at 35% and 36% in the 26- and 35-mo-old PR cheeses, respectively. Thus, the most abundant MALDI signals were associated with α_{s1}-CN derived CPPs in the 26- and 35-mo-old PR samples (Figure 14, green arrows). Therefore, the degradation of α_{s1}-CN proceeded faster than that of β-CN, starting from 26-mo of ripening.
The sequences of some CPPs derived from β-CN and α_{s1}-CN were confirmed by LC-ESI-QTOF MS/MS as follows (Figure 16).

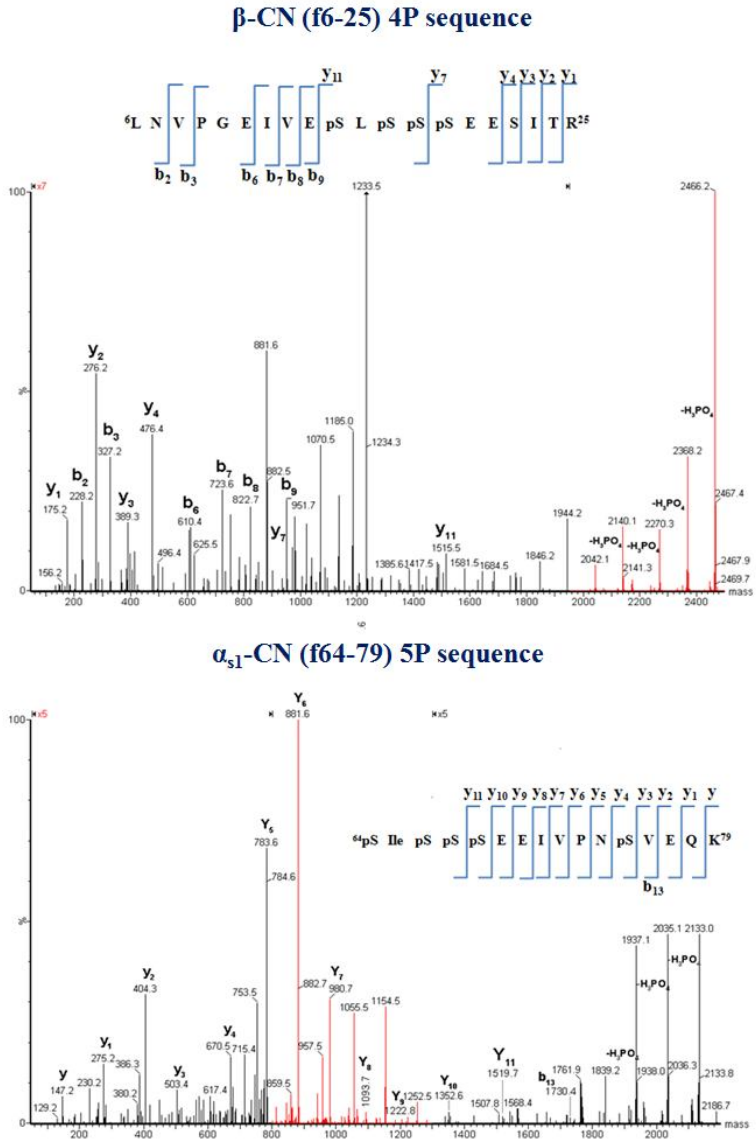

Figure 16. MS/MS analysis of the precursor ion at MH^+=2,466.9, corresponding to β-CN (f6-25) 4P, identified in PR cheese samples at 26- and 35-mo of ripening and of selected precursor molecular ion at MH^+= 2,133.3 Da, corresponding to α_{s1}-CN (64-79) 5P, identified in all the PR cheese samples. The mass loss of 98 Da corresponds to a phosphate group (H_3PO_4) so that this neutral loss value could be used to deduce the presence of a serine phosphate in the peptide chain. pS = phosphoserine residue.

The mass losses of 98 Da from the terminal Y ion series were detected by analysis LC-ESI-Q-TOF MS/MS analysis, indicating the presence of serine phosphate in the peptide chain (Figure 16).
The amino acid sequences of α_{s1}-CN CPPs are aligned to that of the parent peptide α_{s1}-CN (f59-79) 5P and compared in the different samples of PR cheese (Figure 17).

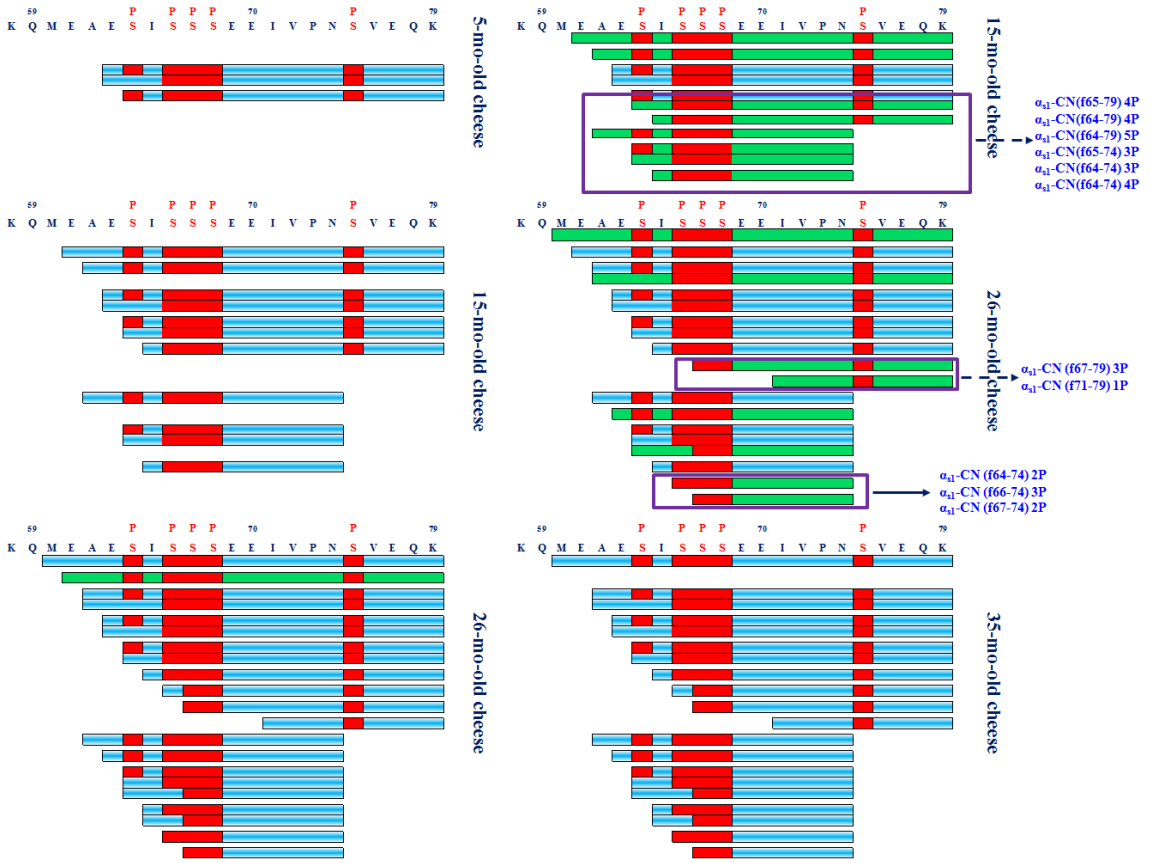

Figure 17. The amino acid sequences of the CPPs aligned with that of α_{s1}-CN (f59-79) 5P were compared in pairs, *i.e.* 5- and 15-, 15- and 26-, 26- and 35-mo-old PR cheese samples. The light blue horizontal bar indicates CPPs present in the samples and the green bar indicates those specific for PR cheese. The red shaded box indicates a phosphoseryl residue.

α_{s1}-CN (f61-79) 5P resulted differently hydrolyzed in 5-, 15- and 26-mo-old cheeses. Only three CPPs derived from α_{s1}-CN (f61-79) 5P were actually found in the 5-mo-old PR cheese while CPPs such as α_{s1}-CN (f65-79) 4P, α_{s1}-CN (f64-79) (5P and 4P), α_{s1}-CN (f65-74) 3P and α_{s1}-CN (f64-74) (4Pand 3P) were present in the 15-mo-old PR cheese (Figure 17). The formation of α_{s1}-CN (f67-79) 3P (1,686.6 Da), α_{s1}-CN (f71-79) 1P (1,094.2 Da), α_{s1}-CN (f64-74) 2P (1,321 Da), α_{s1}-CN (f66-74) 3P (1,201.1 Da) and α_{s1}-CN (f67-74) 2P (1,034.7 Da) in 26- and 35-mo-old cheeses may be ascribed to both proteolysis and dephosphorylation of the α_{s1}-CN (f61-79) 4P peptide (Figure 17). Our results showed that longer PL-mediated peptides were degraded into shorter CPPs. No significant difference in the proteolytic patterns of α_{s1}-CN (f59-79) 5P derived CPPs was observed between 26- and 35-mo-old PR when the accumulation of shorter CPPs plateaued. In particular, the signals of CPPs such as α_{s1}-CN (f65-74) 3P (1,314.7 Da) and α_{s1}-CN (f64-74) 3P (1,401.9 Da) were the most intense in the MALDI spectrum of 35-mo-old PR cheese, whereas α_{s1}-CN (f64-79) 5P (2,134.1 Da) and α_{s1}-CN (f62-79) 5P (2,334.1 Da) were the most intense in the 26-mo-old PR cheese (Figure 14). The amino acid sequences of accumulated CPPs were shorter for cheeses ripened for a long period of time, such as 35-mo-old PR cheese, at the expense of CPPs with longer amino acid sequences (Figure 14). This explains why the short-sequence CPPs such as α_{s1}-CN (f67-74) 2P (1,034.7 Da) and α_{s1}-CN (f66-74) 3P (1,201.1 Da) were well detected in 35-mo-old PR cheese. In contrast, same signals were found only by expanding the view of the m/z region in the MALDI spectrum of the 26-mo-old PR cheese (Figure 14).

The different PR cheese samples contained similar percentages of CPPs derived α_{s2}-CN (f1-24) 4P. For instance, the α_{s2}-CN (f8-18) 3P peptide, one of the shorter α_{s2}-CN CPPs, was found in all samples but variable rates of hydrolysis were observed for its conversion in shorter peptides.

Our procedure has allowed to confirm that α_{s2}-CN is more resistant to enzymes than α_{s1}-CN and β-CN, unlike what was previously reported for milk. Furthermore, according to data available in the literature, no significant hydrolytic difference was measured for the hydrolysis of hydrophobic 1-105 fragment (para-κ-CN) and water-soluble (glycomacropeptide, GMP) in the cheeses (Green & Foster 1974). This resistance to proteolysis reflects the relatively high level of secondary structure in κ-CN compared with the other CN

fractions (Swaisgood, 1992). In conclusion, the susceptibility of cheese CN fractions to hydrolysis proceeds at a decreasing rate according to the order β- > α_{s1}- > α_{s2}-CN > κ-CN.

3.14 Conclusions 3

Patterns of CPP proteolysis are resulted comparable in the different varieties of bovine cheese, which indicated that mechanisms of formation and degradation of CPP are similar regardless of the cheese variety. However, ingested cheese carries an amount of pH 4.6 soluble CPPs variable according to the cheese. The rate of proteolysis and dephosphorylation of cheese CN could be a function of the milk, whether raw or pasteurized. In the milk made from raw milk, longer CPP were further hydrolyzed in shorter CPP. Moreover, the presence of relatively few β-CN-derived CPPs of the various sizes and the low number of α_{s1}-CN derived CPPs in PMC could be the effect of the enzyme decline from the optimum level of enzyme activity to zero activity. α_{s1}-CN derived CPPs whose phosphorylated residues are hidden in the inner part of the sequence accumulated especially in long ripened PR. These CPPs failed to form in milk and began to form in very low amounts in young cheeses; conversely their amount increased with aging (Figure 14). Therefore, the different profile of CPPs could derive from the cheese variety and from the different length of ripening. The extent of proteolysis in a long ripened cheese depends on the enzymatic activity available as result of autolysis of starter or non-starter LAB and indigenous milk enzymes, especially PL. The latter has been recognized as the main endoprotease responsible for CN degradation of hard cheeses. In commercial milks, α_{s1}-, α_{s2}-, β- and κ-CN were preferentially hydrolyzed based on their affinity for PL according to the decreasing order: β- > α_{s2}- > α_{s1}- > κ-CN. This affinity is quite different in PR cheese, where α_{s1}-CN was hydrolyzed at higher amounts than α_{s2}-CN, according to this different order: β- > α_{s1}- > α_{s2}- > κ-CN. This is particularly evident in CPP profiling of the 5- to 35-mo-old cheeses using enrichment on HA. Many CPPs derived from β-CN (f29-57) 1P were detected in 5-mo-old PR cheese but they decreased or disappeared with aging (Figure 15). On the contrary, fewer α_{s1}- and α_{s2}-CN derived CPPs were detected in the younger PR cheese samples. This may indicate that β-CN is degraded within 15 mo of ripening faster than α_{s1}-CN and α_{s2}-CN, resulted more resistant to proteases. In contrast to α_{s2}-CN-derived CPPs, a higher number of α_{s1}-CN derived CPPs were frequently observed in 26- to 35-mo-old cheeses. Moreover, shorter length α_{s1}-CN and β-CN derived CPPs accumulated in the 35-mo-old cheese (Figure 14). Therefore, the MALDI profiles of CPPs from 5- or 15-mo-old PR cheeses are very distinct from those of 26- or 35-mo-old PR cheeses.
Notwithstanding the chromatographic procedure was ultimately unable to identify age-dependent signature CPPs in the pH 4.6-soluble fraction of PR cheese, it would be useful to distinguish between younger cheese (up to 15-mo) from that older than 26 mo. Perhaps separate profiles from the rind and core of younger and older cheeses would further help to find signature peptides as well as profiling the much more abundant non-CPPs could be useful to detect age-dependent signature peptides.

3.15 Refernces

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3.16 Topic 4: Milk speciation through the monitoring of proteotypic CPPs

Our strategy has combined direct MALDI-TOF-MS analysis and signature CPPs extracted from HA-CPP complexes to quantify species-specific casein peptides. Tryptic CPPs such as B β-CN (f1-25) 4P and WB β-CN (f1-28) 4P have been identified and then used as markers of bovine milk contaminating water buffalo milk. The method provides an alternative analytical means for detecting adulterated milks/cheeses with 0.5% LOD. The analysis of Mozzarella cheese real tests has allowed to detect the percentage of B milk fraudulently added to WB milk in the production of marketed cheese.

3.17 Materials and methods 4

3.17.1 Milk and cheese samples

Water buffalo and bovine milk samples were taken from herds located within the Campania region of Italy. Mixtures of WB milk containing 25%, 12.5%, 6.2%, 3.12%, 1.56% and 0.78% B milk (v/v) were prepared on a laboratory scale by weighing the exact amount of milk (~100 g final weight) to ±0.01 g for higher volumes and to ±0.001 g for lower volumes. Reference casein samples were obtained by isoelectric precipitation, according to the procedure described by Aschaffenburg & Drewry (Aschaffenburg & Drewry, 1959), from skimmed milk by centrifuging at 4,500 x g for 10 min. The protein concentration in the samples was preliminarily determined by the Biuret method according to Bradford (Bradford, 1976) using a protein assay dye reagent (Bio-Rad, Hercules, CA).
The extraction of CN from Mozzarella cheese was carried out according to the Official Journal of the European Communities (L 37/54, 7.2.2001). Dry mass of cheese (5 g) was suspensed in distilled water and homogenized for 1 min by an Ultra Turrax T50 (8,000 to 10,000 rpm). Suspension pH was adjusted to 4.6 with diluted acetic acid and centrifuged (3,000 x g for 5 min). The pellet was washed with distilled H_2O (40 ml) and dichloromethane (20 ml), homogenized again and centrifuged (3,000 x g for 5 min). After removal of the CN layer that lies between the aqueous and organic phases with a spatula, both phases were decanted off and the procedure was repeated until both extraction phases were colourless (two to three times). The protein residue homogenized with acetone (50 ml) was allowed to dry in the air or a stream of nitrogen and then finely to pulverize in a mortar.

3.17.2 Plasminolysis of milks in mixture and B and WB casein in ratio 1:1

Milk mixtures (1 ml) were added to an equal volume of 0.2 M NH_4HCO_3 (pH 8.0); the resulting solutions were shaken on a vortex for few seconds and then allowed to stand for 5 min. A 500 µL aliquot was transferred to a plastic culture tube (15 mm × 10 mm), 10 µl of 5 U ml^{-1} PL suspension was added (Boehringer; Mannheim, Germany) and the mixtures were incubated for 1 h in a water bath holder at 37 °C. An equal volume of 24% TCA (w/v) was added and the protein pellet was recovered by centrifugation for at 4,500 x g 10 min at room temperature. The precipitate was solubilised by the addition of 9 M urea (250 µL). B and WB casein (100 mg) in ratio 1:1 were dissolved in 2 ml of 50 mM AMBIC at pH 8.5 with 10 µl of PL suspension. After 4 h of incubation at 37 °C, the peptides were precipitated with TCA (12% final concentration), the supernatant was discarded and the pellet was washed (× 3 times, 1 ml) with dry acetone that had been kept at -20 °C. After each wash, the supernatant was discarded and the pellets were washed with diethyl ether (× 2.2 ml). Finally, the peptides were air-dried in a ventilated hood and kept at -20 °C until analysis. The PL-mediated peptides dissolved in a solution of 50% ACN and 0.1% TFA (1 mg/ml) from B and WB casein (1:1) were analysed by ESI-Q-TOF-MS and by MALDI-TOF before after enrichment as above described.

3.17.3 Semi-preparative isoelectric focusing of CN plasminolysate

Isoelectric focusing on ultra-thin-layer polyacrylamide isoelectric focusing gel (UTLIEF) was carried out according to the European reference method of analysis (Commission Regulation No 273/2008). The gel composition and pH gradient were the same for this apparatus and the PhastSystem apparatus (Moio et al.,

1998). After migration, the gel was stained using CBB according to the procedure of Neuhoff et al. (Neuhoff et al., 1988).

3.17.4 Peptide extraction from the gel bands and identification by MS

Gel bands were cut with a razor blade within 24 h from CBB-stained UTLIEF polyacrylamide gels. Excised spots were disengaged from the polyester backing and destained by the addition of 50% ACN (ACN/H_2O = 50/50, v/v) with 1 h of shaking. The coloured supernatant was discarded and the process was repeated until the supernatant was almost colourless. After washing twice in 100 µl of distilled water for 15 min, each gel piece was suspended in 0.25 mM AMBIC at pH 8.0 and stored at -20 °C overnight. An equal volume of ACN 0.1% TFA was then added and sonication was carried out for 1 h. The suspension was centrifuged at 12,000 x g for 1 min at 4 °C and the supernatant transferred to a new, clean vial. After SpeedVac drying to half of the initial volume, the sample was directly analyzed by LC-ESI-Q-TOF-MS as described above. The molecular mass distributions of peptides in different spot hydrolysates were searched against the WB β-CN sequence (Q9TSI0) using FindPept tools available at www.expasy.ch.

3.17.5 Calibration curve for B β-CN (f1-25) and WB β-CN (f1-28) peptides by using known quantities of WB and B CN in mixtures

WB casein containing 50%, 6.2%, 3.12%, 1.56% and 0.78% B CN (10 mg) and later CN isolated from Mozzarella cheese (10 mg) were adsorbed to microgranular HA (100 mg) previously equilibrated with the loading buffer (50 mM Tris-HCl, 0.2 M KCl, 4.5 M urea and 10 mM DTT, pH 8.0). After washing, the dried HA-bound CN/CPP trypsinolysis was performed *in situ* as described above. The HA-CPP complexes were deposited onto the MALDI plate, covered with the matrix solution (0.5 µL) containing 10 mg/mL DHB in H_2O/ACN/PA (49:50:1), which that promote co-crystallisation by air-drying at room temperature. The MALDI spectra were recorded as above.
The calibration curve was obtained by plotting the bovine β-CN (f1-25) 4P and water buffalo β-CN (f1-28) 4P ratio against the composition of adulterated WB milk.

3.18 Results and discussion 4

3.18.1 Analysis of β-CN plasminolysate in bovine and buffalo water species

The B and WB β-CN fractions are both very sensitive to hydrolysis by PL. The primary cleavage sites in B β-CN are Lys^{28}-Lys^{29}, Lys^{105}-His^{106} and Lys^{107}-Glu^{108}, cleavage of which leads to the formation of C-terminal polypeptides, β-CN (f29-209) (γ_1-CN), β-CN (f106-209) (γ_2-CN), β-CN (f108-209) (γ_3-CN), β-CN (f1-105/107) (PP 5), β-CN (f29-105/107) (PP 8-slow) and β-CN (f1-28) (PP 8-fast) as indicated in Figure 18. Additional cleavage sites include Lys_{113}-Tyr_{114} and Arg_{183}-Asp_{184}, that give rise to β-(f114-209) (γ_4-CN) and β-CN (f184-209) (γ_5-CN), respectively (O'Malley et al., 2000). β-CN (f69-209), called γ_4-CN and originated from the early hydrolysis of Lys_{68}-Ser_{69} bond of WB β-CN by PL, has no counterpart in B milk (Trieu-Cuot & Addeo, 1981; Somma et al., 2008; Di Luccia et al., 2009).

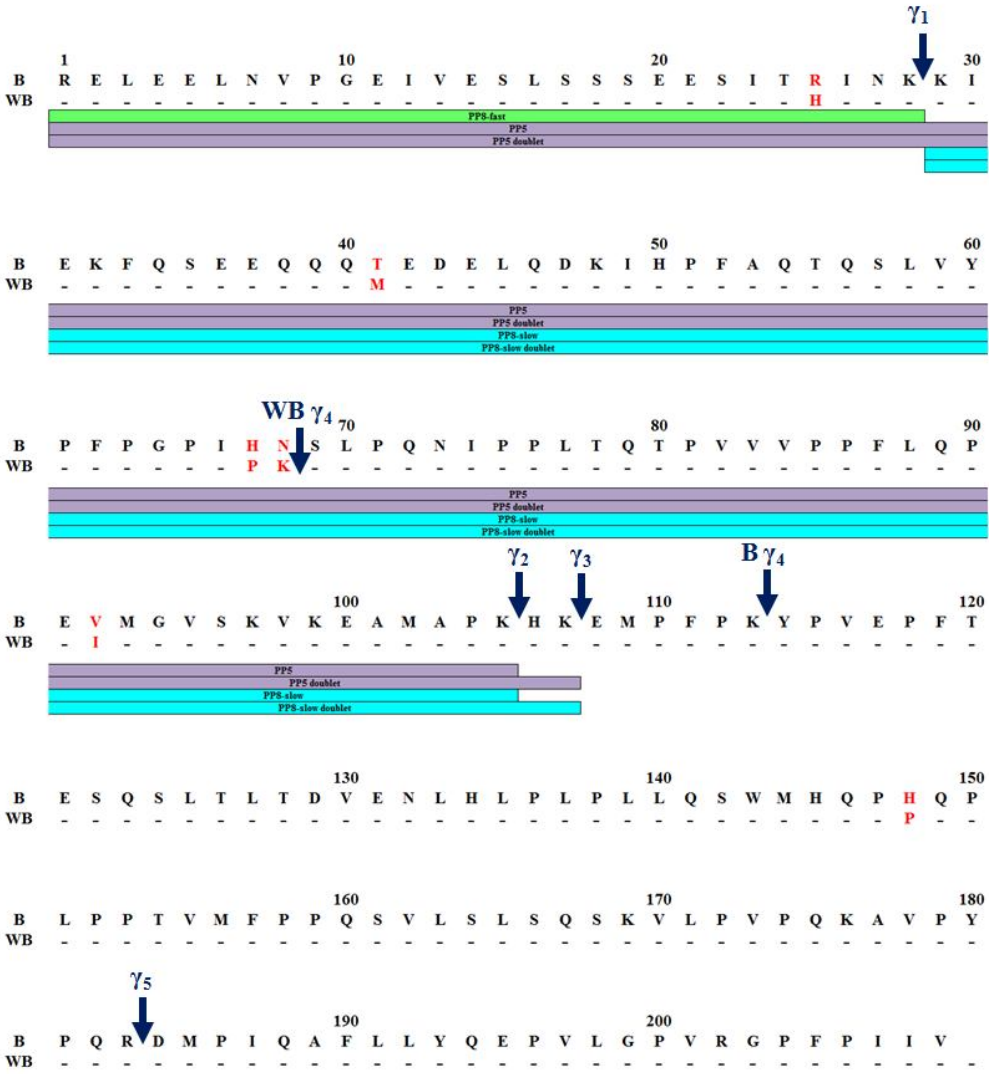

Figure 18. Amino acid sequence of bovine (B) and water buffalo (WB) β-CN. Arrows indicate bonds sensitive to plasmin. γ_1, γ_2, γ_3, B and WB γ_4 and γ_5 are γ-CN. The red letters are amino acid residues differentiating β-CN of two species. The green bar indicates PP8-fast; the purple bars indicate the two PP5, the blue ones the two PP8-slow.

In a recent work, a plasminolysate of B and WB casein was analyzed by ESI-Q-TOF-MS (Figure 19) (Addeo et al., under review).

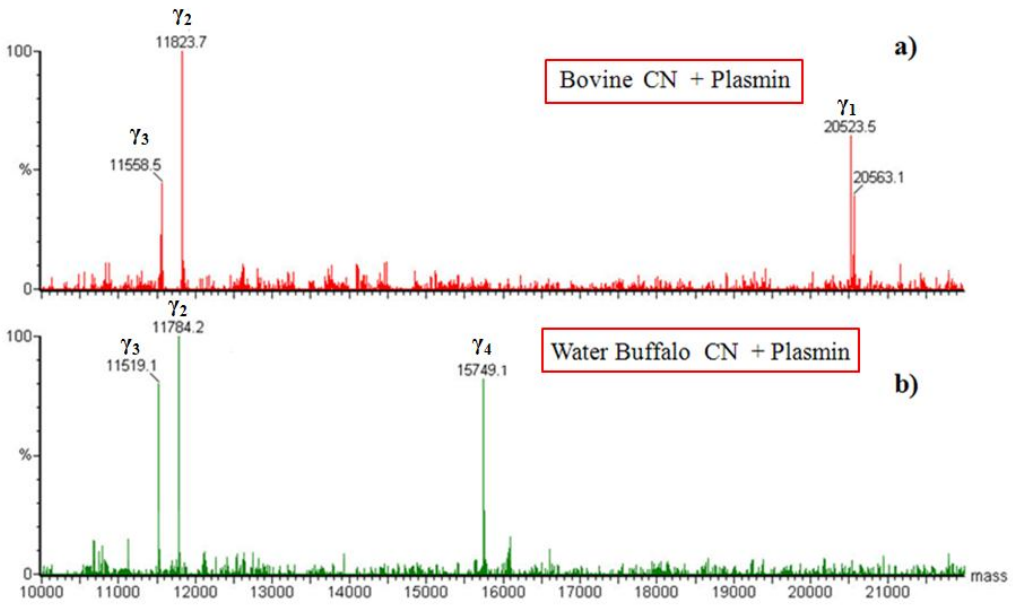

Figure 19. Partial view of the ESI-Q-TOF-MS spectra for the PL-mediated B (Panel a) and WB (Panel b) casein hydrolysate.

Two intense ion signals (γ_2-CN and γ_3-CN) were detcted in both species while γ_4-CN was exclusively observed in WB sample; the $Asn_{68} \rightarrow Lys_{68}$ substitution occurring in the sequence of WB β-CN added a further site of PL cleavage. The quantification of γ_4-CN by MALDI-TOF MS has been used for the detection of B CN in WB Mozzarella cheese (Angeletti et al., 1998). Therefore, γ_4-CN can be considered a molecular marker of the presence of water buffalo CN in any dairy product (Somma et al., 2008).
Different methods for the detection of foreign milk involve the use of CN, a more useful indicator than heat-sensitive whey proteins. The IEF-based method is the European reference that allows the distinct detection of B and WB γ_2- and γ_3-CN (Commission Regulation No 273/2008). PL hydrolysis of WB CN has been previously demonstrated to release a peptide comigrating with B γ_2-CN. The occurrence of this peptide may lead to false-positive detection of cow's milk for a genuine WB cheese if it is analyzed by applying a fast version of the European official method (Addeo et al., 2009).
Reference samples of B (lane 1) and WB CN (lane 8) and WB milk in mixture with different % B milk (lanes 2-7) were submitted to PL action. The separation by fast isoelectric focusing (FIEF) on mini-gel of PL-mediated peptides is shown in Figure 20.

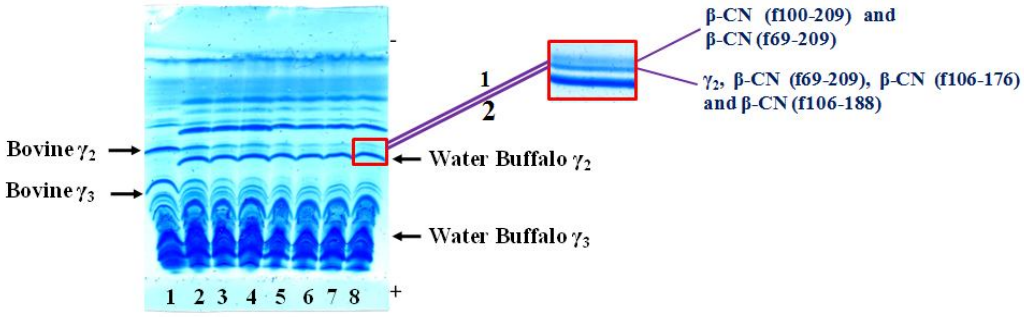

Figure 20. Isoelectric focusing on a PhastSystem apparatus of PL-mediated peptides of WB milk in mixture containing 25% (lane 2), 12.5% (lane 3), 6.2% (lane 4), 3.12% (lane 5), 1.56% (lane 6) and 0.78% (lane 7) B milk (v/v). Reference samples of bovine (lane 1) and water buffalo whole CN (lane 8) are analysed on the same gel. Staining has been carried out with CBB. The faint bands at similar pI of bovine γ_2-CN in pure water buffalo milk have been highlighted and the peptides extracted by gel bands have been identified by LC-ESI-Q-TOF-MS.

Two faint bands migrated at similar pI of B γ_2-CN in pure WB milk, leading to false-positive detection. Direct LC-ESI-Q-TOF-MS analysis of peptides extracted by two bands has allowed the identification of β-CN (f100-209) and β-CN (f69-209) in upper band and γ_2, β-CN (f69-209), β-CN (f106-176) and β-CN (f106-188) in the lower band (Figure 20). The additional peptides β-CN (f106-176) and β-CN (f106-188) derived from the internal cleavages of WB γ_2-CN. For this reason, an immunoblotting procedure based on the use of specific antipeptide antibody has been suggested as "gold standard" method in order to assurance WB milk purity and cheese genuineness (Addeo et al., 2009). Moreover, recently, the use of MALDI-TOF-MS has been suggested for confirmatory analysis. MALDI-TOF MS analysis of the tryptic digests of single-species defatted milk has allowed to detect the α_{s1}-CN (f8-22) proteotypic peptide characterized by different molecular masses in the several species (1,686.9 Da in WB, 1,759.1 Da in B and 1,718.9 in ovine milks) (Cuollo et al., 2010). α_{s1}-CN (f8-22) resulted an excellent signature peptides able to differentiate B, ovine and WB milks (Cuollo et al., 2010).
The MALDI analisys of PL-mediated peptides from a mixture of B and WB casein (1:1) showed different possible candidate proteotypic peptides before and after enrichment on HA (Figure 21).

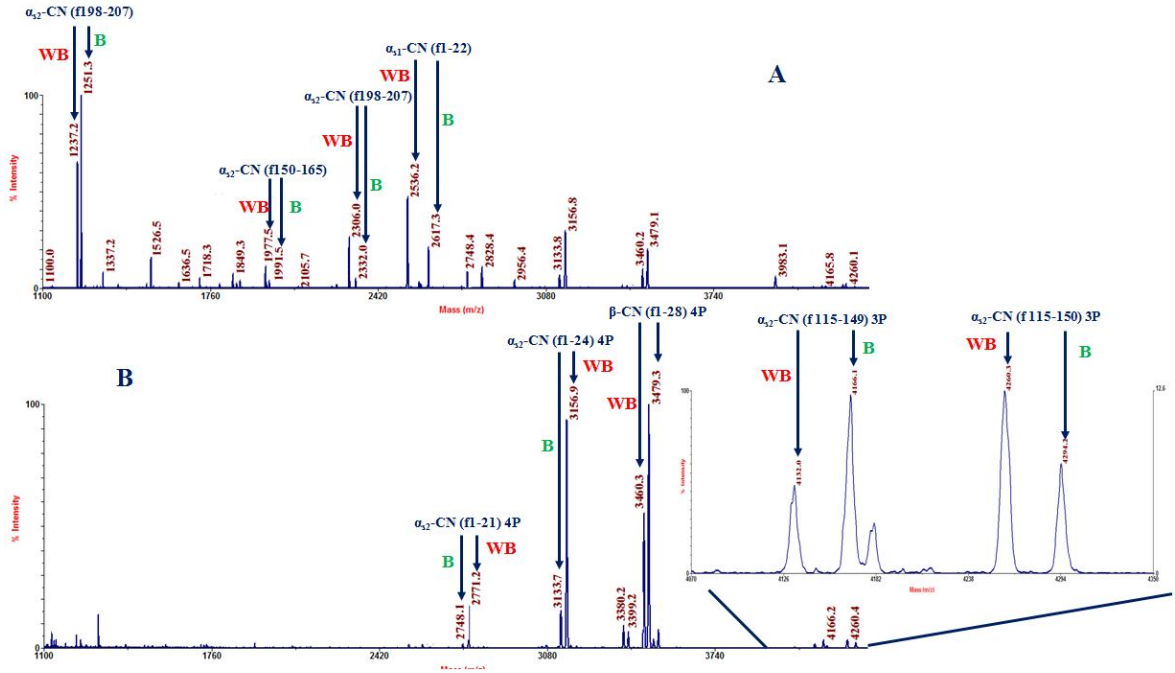

Figure 21. MALDI spectra of plasminosate containing water buffalo (WB) and bovine (B) CN in equal amounts before A) and after B) enrichment on HA. The identification of non-CPPs A) and CPPs B) couples from the two species is reported.

Proteotypic CPPs can be used as marker for B and WB milk only after enrichment for the great increase of their ionization (Figure 21B). The identification of CPPs released by PL and the relative sequence alignment is report in Figure 22.

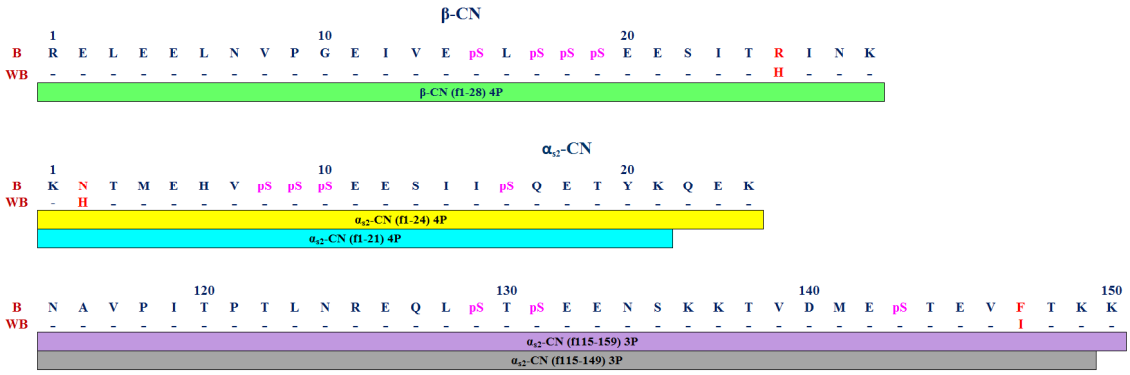

Figure 22. Sequence alignment of the CPPs detected in the plasminosate containing water buffalo (WB) and bovine (B) CN in equal amounts. The amino acid substitution of the possible proteotypic CPPs derived from β-CN and α_{s2}-CN are highlighted in red. pS = phosphoserine residue.

The Arg_{25} (B β-CN)→His_{25} (WB β-CN), Asn_2 (B α_{s2}-CN)→His_2 (WB α_{s2}-CN), Phe_{147} (B α_{s2}-CN)→Ile_{147} (WB α_{s2}-CN) amino acid substitutions in the analogue CPPs gave distinct mass MALDI signals allowing their use as signature peptides. The amino acid substitution appreciably perturbed the ionization efficiency of the analogue peptides. In particular, the signal intensity of B β-CN (f1-28) 4P was double than that of the WB analogue (Figure 21). Moreover, the PL action on β-CN released B and WB β-CN (f1-28) 4P but also a lower amount of B β-CN (f1-25) 4P. Notwithstanding the fact that Arg_{25}-X and Lys_{28}-X are evenly susceptible to PL, the latter is approximately tenfold more efficient with Lys-X as a substrate than with Arg-X bond (Weinstein & Doolittle, 1972). The MALDI spectra of the HA-tryptic CPPs and HA-PL CPPs derived from B (50%) and WB (50%) CN mixtures are shown in Figure 23.

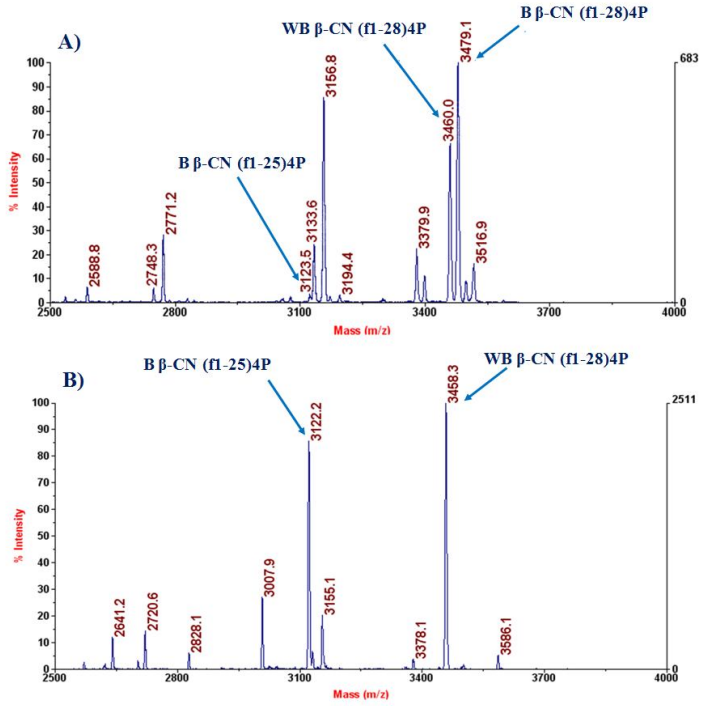

Figure 23. MALDI-TOF spectrum of a plasminosate A) and a tryptic hydrolysate B) after enrichment on HA from WB and bovine B casein in equal amounts. Ionization efficiency of B β-CN (f1-28) 4P (MH^+ 3479.1 Da) and WB β-CN (f1-28) 4P (MH^+ 3,460.0 Da) was not comparable A) while that of B β-CN (f1-25)4P (MH^+ 3,122.2 Da) and WB β-CN (f1-28) 4P (MH^+ 3,458.3 Da) was not appreciably affected by different length of two peptides.

WB and B β-CN (f1-28) 4P would occur in the CN plasminolysate of CN mixtures while trypsinolysis affords the formation of WB β-CN (f1-28) 4P and B β-CN (f1-25) 4P because of the amino acid substitution Arg_{25} (B)→His_{25} (WB) (Figure 23). The signals of the tryptic B β-CN (f1-25) and WB β-CN (f1-28) had almost the same amplitude, whereas those generated by PL were different (Figure 23). This difference can be explained by the formation in the B species of β-CN (f1-25) 4P simultaneously with lower amounts of β-CN (f1-28) 4P, the only peptide formed by the WB milk (Figure 23).

3.18.2 Standard curve of molecular mass vs. adulterant bovine milk

The analysis of triptic digests of B and WB mixtures is preferred to quantify the adulterant addition of B milk in WB milk. Consequently, for doubtful or low amounts of B milk, trypsin hydrolysates of milk or cheese CN were carried out. To this end, five samples of known composition were analyzed by MALDI-TOF-MS (Figure 24) in triplicate and spectra were obtained as outlined in the above described procedure.

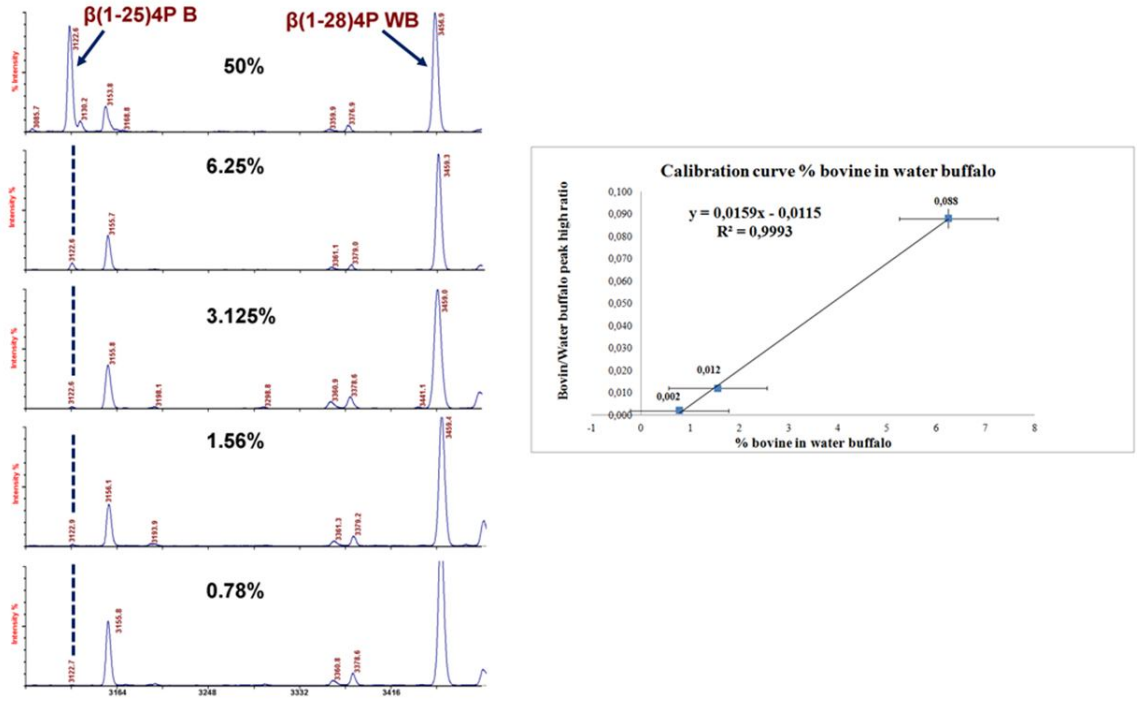

Figure 24. Identification of bovine β-CN (f1-25) 4P (MH^+ 3,122.6 Da) and water buffalo β-CN (f1-28) 4P, (MH+ 3,458.9 Da) peptides in tryspin digests containing different amounts of B CN (0.8-50%, w/w). Calibration curve were obtained by plotting the B β-CN (f1-25) 4P and WB β-CN (f1-28) 4P ratio against the composition of adulterated water buffalo milk.

A linear relationship was established between the ratio of the peak heights of WB β-CN (f1-28) 4P and the adulterant B β-CN (f1-25) 4P against percentage of B milk (y = 0.0159 – 0.0115, R^2 = 0.9993) (Figure 24). Using this calibration curve, the actual percentages of B milk in adulterated WB milk samples were calculated with a LOD of 0.5%. The detection of B β-CN (f1-25) 4P in tryptic digests of CN isolated from WB Mozzarella cheese have allowed to measure the % of B adulterant that resulted of ~2 % for the tested cheese samples. Therefore, the direct MALDI-TOF-MS analysis of the tryptic CN digests could be a promising alternative method to the official European electrophoretic method of analysis (Commission Regulation No 273/2008) as it provides better separation and an appropriate discrimination between real and false signature peptides.

3.19 Conclusions 4

Milk speciation through the monitoring whey proteins is not recommended because of their propensity for denaturation during heat and high-pressure processing. Heat-resistant CN is considered to be a better indicator for detecting WB milk adulteration. Two faint bands are resulted to migrate at similar pI of B γ_2-CN in pure WB milk; these bands contain some peptides responsible for false positive responses in B milk. This drawback limits the application of the European reference electrophoretic method of analysis for the authentication of WB milk/cheese. In order to prevent false positive responses, multiple proteotypic peptides could be detected for each parent CN. In this manner, the MS based methods become effective in discriminating pure or contaminated milk samples. The direct MALDI-TOF-MS and ESI-Q-TOF-MS analysis of the tryptic CN digests are promising alternative methods to the official European electrophoretic method of analysis providing both a better separation, similar sensitivity and specific detection of the proteotypic CPPs from adulterating bovine milk. In this regard, the analysis by ESI-Q-TOF-MS and MALDI-TOF-MS of the protein plasminolysate could provide second-generation methods for authenticating pure water buffalo milk/cheese. These higher-resolution and cost-effective methods provide quantitative detection of low amounts of B milk in WB milk and derived Mozzarella cheese.
Our method is based on the selective isolation of tryptic CPPs through the use of HA. The latter binds exclusively phosphorylated peptides, simplifying the MALDI spectra and the interpretation of the peptide profile. Moreover, a LOD of 0.5% has been achieved for tryptic B β-CN (f1-25) 4P for detecting B milk in adulterated water buffalo milk and derived mozzarella. The excellent specificity of proteotypic peptides opens the possibility to broaden the application to other species' milk or to any food protein as long as the protein sequence is known.

3.20 References

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3.21 GLOSSARY

AA	Asiago d'Allevo
ACN	Acetonitrile
ACP	Amorphous calcium phosphate
ALP	Alkaline phosphatase
AMBIC	Ammonium bicarbonate
B	Bovine
CM	Clinical mastitis
CN	Casein
CPase	Carboxypeptidase
CPPs	Casein phosphopeptides
DHB	2,5-dihydroxybenzoic acid
DTT	Dithiothreitol
EDT	Ethanedithiol
ESI-MS/MS	Electrospray ionization-tandem mass spectrometry
FA	Formic acid
GP	Grana Padano
GR	Gorgonzola
HA	Hydroxyapatite
HMM	High-molecular mass
HPLC	High performance liquid chromatography
ICAT	Isotope coded affinity tags
IEF	Isoelectric focusing
IMAC	Immobilized metal-ion affinity chromatography
IS	Internal standards
LAB	Lactic acid bacteria
LC-ESI-MS	Liquid chromatography-electrospray ionisation-mass spectrometry
LMM	Low-molecular mass
LOD	Limit of detection
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MOAC	Metal oxide affinity chromatography
Month	Mo
MS/MS or MS2	Tandem mass spectrometry
PA	Phosphoric acid
PL	Plasmin
PM	Pasteurized milk
PMC	Provolone del Monaco
PMNs	Polymorphonuclear neutrophils
PP	Proteose peptones
PR	Parmigiano Reggiano
PSD	Post-source decay
SC	Somatic cell
SCC	Somatic cell counts
SCM	Subclinical mastitis
TFA	Trifluoroacetic acid
Tris-HCl	Tris(hydroxymethyl) aminomethane hydrochloride
UHT	Ultra High Temperature
UTLIEF	Ultrathin-layer isoelectric focusing
WB	Water buffalo
α-La	α-lactalbumin
β-Lg	β-lactoglobulin

3.22 PUBLICATION LIST

Some of the research reported in this thesis have been published in the following refereed papers and book chapters.

3.22.1 Papers**:**

1. Caira, S., **Pinto, G.**, Balteanu V.A., L. Chianese & F. Addeo, 2013. A signature protein-based method to distinguish Mediterranean water buffalo and foreign breed milk. Food Chem. In press.

2. **Pinto, G.**, Caira, S., Cuollo, M., Fierro O., Nicolai M.A., L. Chianese & F. Addeo, 2012. Lactosylated casein phosphopeptides as specific indicators of heated milks. Anal. Bioanal. Chem. 402:1961-1972, ISSN 1618-2650.

3. **Pinto G**., Caira S., Cuollo M., Lilla S., O. Fierro & F. Addeo, 2010. Hydroxyapatite as a concentrating probe for phosphoproteomic analyses. J. Chromatogr. B Analyt. Technol. Biomed Life Sci 878:2669-2678, ISSN: 1570-0232.

4. Cuollo, M., Caira, S., Fierro, O., **Pinto G.**, G. Picariello & F. Addeo, 2010. Toward milk speciation through the monitoring of casein proteotypic peptides. Rapid Commun. Mass. Spectrom. 24:1687-1696, ISSN: 1097-0231.

3.22.2 Book chapters

1. Cuollo, M., Picariello, G., Caira, S., **Pinto, G.**, L. Chianese & F.Addeo, 2013. Current methods for assessing authenticity of cheese. In: Handbook of cheese in health: production, nutrition and medical sciences, Victor R. Preedy, Ronald Ross Watson and Vinood B. Patel (eds.), ISBN: 9789086862115. In press.

2. **Pinto, G.**, Caira, S., Cuollo, M., Lilla, S., L. Chianese & F. Addeo, 2012. Bioactive Casein Phosphopeptides in Dairy Products as Nutraceuticals for Functional Foods. In “Milk Protein” pp. 3-44. INTECH, Rijeka (HRV): Walter L. Hurley, ISBN: 9789535107439.

3. Caira, S., Pizzano, R., Picariello, G., **Pinto G**., Cuollo, M., L. Chianese & F. Addeo, 2012. Allergenity of Milk Proteins. In “Milk Protein” pp. 173-214. INTECH, Rijeka (HRV): Walter L. Hurley (ed.), ISBN: 9789535107439.

APPENDIX

Anal Bioanal Chem (2012) 402:1961–1972
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ORIGINAL PAPER

Lactosylated casein phosphopeptides as specific indicators of heated milks

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Abstract Casein phosphopeptides (CPP) were identified in small amounts in milks heated at various intensities by using matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry. CPP selectively concentrated on hydroxyapatite (HA) were regenerated using phosphoric acid mixed in the matrix. Unphosphorylated peptides not retained by HA were removed by buffer washing. This procedure enhanced the MALDI signals of CPP that are ordinarily suppressed by the co-occurrence of unphosphorylated peptides. CPP, belonging to the β-casein (CN) family, i.e., (f1-29) 4P, (f1-28) 4P, and (f1-27) 4P, and the α_{s2}-CN family, i.e., (f1-21) 4P and (f1-24) 4P, were observed in liquid and powder milk. The lactosylated counterparts were specific to intensely heated milks, but absent in raw and thermized/pasteurized milk. Most CPP with C-terminal lysines probably arose from the activity of plasmin; an enzyme most active in casein hydrolysis. A CPP analogue was used as the internal standard. The raw milk signature peptide β-CN (f1-28) 4P constituted ~4.3% of the total β-CN. Small amounts of lactosylated peptides, which varied with heat treatment intensity, were detected in the milk samples. The limit of detection of ultra-high-temperature milk adjunction in raw or pasteurized milk was ~10%.

Keywords Hydroxyapatite-bound phosphopeptides (CPP) MALDI analysis · Lactosylated CPP detection · Signature CPP for heated milks

Abbreviations

ACN	Acetonitrile
ALP	Alkaline phosphatase
AMBIC	Ammonium bicarbonate buffer
α-La	alpha-Lactalbumin
β-Lg	beta-Lactoglobulin
CN	Casein
CPP	Casein phosphopeptides
DHB	5-Dihydroxybenzoic acid
DTT	Dithiothreitol
HA	Hydroxyapatite
IMAC	Immobilized metal affinity chromatography
IS	Internal standard
LOD	Limit of detection
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
PA	Phosphoric acid
PP	Proteose peptones
PSD	Post-source decay
SA	Sinapinic acid
SCC	Somatic cell count
TFA	Trifluoroacetic acid
UHT	Ultra high temperature

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Introduction

The European Union Directives (92/46 CEE and 94/71 CEE) set specific rules for the production of heat-treated milk and milk-based products such as the use of specific temperature/time combinations for pasteurized and ultra high-temperature (UHT) milk. The use of low value ingredients such as UHT and milk powder in mixtures with raw or pasteurized milks is strictly prohibited. Nowadays, rapid methods are mainly based on the evaluation of furosine, a useful indicator of heat damage in processed milk. Studies of milk adulterations are also focused on lactosylation of milk proteins. Endogenous milk proteinases and those associated with somatic cells in high counts hydrolyze caseins of raw milk. Some heat-stable proteinases may continue to do it during storage of heat-treated milks. The number of somatic cells should not exceed 200,000 cells/mL bulk raw milk [1], although the European Union Directives (92/46 CEE and 94/71 CEE) set a limit of 400,000 cells/mL for SCC for drinking milk. Alternatively, an indirect means to monitor milk quality in relation to SCC would be to know one or more signature peptides. Milk contains colloidal and soluble casein subjected to hydrolysis by proteinases. Casein releases water-soluble peptides under the action of plasmin (EC 3.4.21.7) and lysosomal proteases, such as carboxylprotease and cathepsin D (EC3.4.23.5). While β-casein (CN) and α_{s2}-CN are the preferred substrates of plasmin, α_{s1}-CN [2] and κ-CN [3] are more resistant to enzymatic degradation. Therefore, one would expect to find β- and α_{s2}-CN-derived peptides in milk. Indeed, β-CN is the most hydrolyzed casein fraction partly converted into pH 4.6 insoluble γ-CN and complementary soluble proteose peptones (PP). PP include peptides such as β-CN (f1-105/107) (component PP5), (f1-28) (component PP8-fast), (f29-105/107) 1P (component PP8-slow) [4, 5], and others. Raw milk of good quality has 1–2 mg/mL PP, which can increase during storage. A significant PP5 accumulation has been reported for commercially packaged pasteurized milk [6]. Because as much as 30–40% plasmin survives UHT treatment [7], gelation could be caused for long-stored UHT milk [8].

While the role of proteases in casein proteolysis has been fully clarified, the subsequent lactosylation of water-soluble peptides remains to be clarified. Although determined for whey proteins and casein, lactosylation of milk peptides has not been deeply studied with the objective of distinguishing various types of milk [9]. For heat-treated liquid and powder milk, a reaction could occur between the ε-amino group of protein-bound lysine or amino-termini of peptides and the carbonyl group of lactose. In the initial step of the Maillard reaction, lactosylation occurs both during heating and, to a lesser extent, during milk powder storage [10]. The degree of lactosylation of β-CN increases steadily with temperature between 37 °C and 60 °C [11]. Lactose specifically binds to Lys-34 in α_{s1}-CN and to Lys-107 in β-CN under moderate heat treatment conditions (72–85 °C for 15–30 s). The number of binding sites increases to seven for α_{s1}-CN and five for β-CN after intensive treatment (142–145 °C for 2–5 min) [12]. The extent of lactosylation measured under the same conditions is 25% for β-lactoglobulins (β-Lg) and 35% for β-CN [11]. Especially in drinking milk, but also in milk powders, milk quality is greatly affected by the initial proteolysis of raw milk. Due to the specificity of plasmin, which acts preferentially on Lys-X bonds, milk heating induces lactosylation of the released peptide at basic C-terminal residues (mainly Lys or Arg). Even though the extent of lactosylation affects drinking milks differently, little is known about the impact of Maillard reactions on the formation of CPP markers.

Several milk peptides have been shown to derive from the proteolytic digestion of caseins [13]. Separation of signature peptides from other peptides may often be achieved by chemical methods. Thus, to reduce the complexity of the peptide fraction, the chromatography on hydroxyapatite (HA) has been recently introduced [14]. This method achieves the recovery of casein phosphopeptides (CPP) from complex peptide mixtures [14, 15] as an alternative to immobilized metal affinity chromatography (IMAC)-based methods [16, 17] and other affinity-based methods [18]. While unphosphorylated peptides are washed out, CPP were retained on microgranules of HA. This was useful for isolating CPP free of unphosphorylated peptides. A HA–CPP complex was then spotted onto a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) target plate for analysis [14]. Due to plethora of milk peptides, we focused on signature CPP released in definite number by milk proteinase(s). The efficient separation of the unphosphorylated peptides also enhanced the MALDI signals of CPP. The research results could demonstrate the nature and activity of milk proteinases and provide information about the proteolysis induced thereof. We were interested to detect heated milk and quantify peptides which undergo lactosylated once released by proteinases. By direct MALDI analysis, in situ immobilized CPP, both native and lactosylated, can be identified. Similarly, lactosylation sites can be localized on peptide sequence by post-source decay (PSD) fragmentation. The comparison between the potentially lactosylable and actually lactosylated sites could serve to differentiate processed milks according to heat intensity. The lactosylated CPP could serve to discriminate raw and pasteurized from UHT milk. In this work, we have realized a procedure for detecting UHT milk in amounts not lower than 10% spiking pasteurized milk. We suggest extending this approach to the analysis of any dairy products with suspected addition of UHT milk, milk protein, or milk powder.

Experimental

Chemicals

HA (Macro-Prep Ceramic Hydroxyapatite Type I) was supplied by Bio-Rad (Milan, Italy). Tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl), potassium chloride (KCl), urea, trifluoroacetic acid (TFA), acetonitrile (ACN) for HPLC, 85% orthophosphoric acid (PA), and AMBIC were from Carlo Erba (Milan, Italy). Dithiothreitol (DTT) was from AppliChem (Darmstadt, Germany). TPCK-treated trypsin from bovine pancreas was from Sigma (St. Louis, MO, USA). Sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB), and sodium acetate (AcNa) trihydrate were obtained from Fluka (St. Louis, MO, USA). Acetic acid was purchased from Baker Chemicals B.V. (Deventer, the Netherlands). Water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA). Pooled raw milk was from local dairy farms. The pasteurized UHT milks were purchased from a local store. Milk protein powder and milk powder were supplied by Sacco Industry (Milan, Italy).

Sample preparation

Pasteurized milk was added with UHT milk to a final concentration of 90% to 1%. Then, each sample, including isoelectric casein (sample I), milk protein powder (sample V), or milk powder (sample VI) in solution, raw milk (sample II), pasteurized milk (sample III), and UHT milk (sample IV), was treated with HA as described below. The chemical composition of milk samples as well as the time/temperature combinations are reported in Table 1.

HA-based phosphoprotein/CPP enrichment from various milk samples

HA (100 mg), previously equilibrated with the loading buffer (50 mM Tris–HCl, 0.2 M KCl, 4.5 M urea, and 10 mM DTT, pH 8.0), was put in contact with protein in solution (10 mg) or equivalent amounts of raw and skimmed milk. The HA-bound proteins were incubated for 15 min at room temperature and centrifuged for 5 min at 4,000×*g* per minute. The resin was successively washed with three different buffers: loading buffer (1 mL), 50 mM Tris–HCl at pH 8.0 (1 mL), and 20 mM Tris–HCl in 20% ACN (*v*/*v*) at pH 8.0 (1 mL). The resin was washed with Milli-Q water (1 mL) and freeze-dried with a SpeedVac concentrator system (Thermo Electron, Milford, MA).

In order to confirm the degree of lactosylation, the dried HA-bound CPP (1 mg) were dissolved in a 5% aqueous PA solution (120 μL) and desalted with a ZipTip C18 pipette tip before dephosphorylation with alkaline phosphatase (ALP), carried out according to the procedure previously described [14]. Assays were needed especially for CPP isolated from milk protein and milk powder. However, since HA is known to interact with phosphoprotein and CPP, the aqueous suspension of HA was trypsinized according to the procedure previously described [14]. The HA-bound tryptic CPP were then analyzed by MALDI.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MALDI-TOF mass spectra were recorded using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA) in positive linear mode. All spectra were acquired in the range of 10–30 kDa (for proteins) and 1–5 kDa (for peptides) with the following settings: an accelerating voltage of 25 kV (for proteins) or 20 kV (for peptides) and a grid voltage of 93% (for proteins) or 95% (for peptides) of the accelerating voltage, a guide wire of 0.15% (for proteins) or 0.05% (for peptides), and a delayed ion extraction time of 485 ns (for proteins) or 175 ns (for peptides). The laser power was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise ratio. All spectra were acquired with 200 shots in three replicates. Measured molecular masses, with a ±0.5-Da tolerance, matching the calculated masses, enabled the identification of ion signals. Different MALDI matrix solutions were freshly made for different types

Table 1 Protein and lactose content in heat-treated commercial milk samples

Sample		% protein	% lactose	Heating treatment	Time/temperature combinations
I	Isoelectric casein	100	0	No	No
II	Raw milk	3.2	4.9	No	No
III	Pasteurized milk	3.2	4.9	Pasteurization	15 s/71.7 °C
IV	UHT milk	3.1	4.9	Direct ultra high-temperature processing	2 s/135 °C
V	Milk protein powder	46	5	Membrane filtration[a] and spray drying	Air temperature at 200 °C
VI	Milk powder	36	56	Spray drying	Air temperature at 200 °C

[a] Concentrated commercial milk proteins were obtained by milk ultrafiltration on membrane

of molecules to be analyzed. For phosphoprotein analysis, 10 mg/mL of SA was dissolved in 50% ACN and 0.1% TFA in an ultrasonic bath. For CPP, 10 mg/mL of DHB was dissolved in 1 mL of H_2O/ACN/PA (49:50:1) using an ultrasonic bath. Prior to HA enrichment, the samples were analyzed by MALDI. Solid milk samples (1 mg/mL) were solubilized in H_2O/ACN (50:50) solution with 0.1% TFA. Liquid milk samples (10 μL) were diluted 1:100 with the above solution. Each protein solution (1 μL) was loaded in one well of a MALDI plate and covered with SA matrix (1 μL) for the preliminary analysis. After enrichment, ~1,000 HA-phosphoprotein microgranules were deposited onto the MALDI plate and covered with the SA matrix (1 μL) to promote analyte/matrix co-crystallization in the presence of 0.1% TFA. For CPP, 1% PA was included in the matrix solution to achieve maximum ionization efficiency by MALDI and minimize binding of CPP to HA [19].

PSD-MALDI-TOF experiments

PSD fragment ion spectra were acquired after isolation of the appropriate precursor ions using timed ion selection. In this manner, the spectra of lactosylated β-CN (f1-28) 4P (*m*/*z*3,803 Da) and its unphosphorylated counterpart (*m*/*z*3,483 Da) were acquired. Acquisition was performed with the following settings: accelerating voltage, 20 kV; grid voltage, 80% of the accelerating voltage; guide wire, 0.02; and delayed time, 100 ns. Each spectrum was the average of 300 shots in three replicates.

Synthesis of peptide analogues

The peptides were synthesized by solid-phase methods using the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy on a Pioneer peptide synthesizer (Synthesis System 9050 instrument; PE-Biosystems, Framingham, MA, USA). The level of the β-CN (1-28) 4P plasmin-derived fragment was chosen as the index for quantification of casein proteolysis. The method was applied to all of the milks to select a marker specific for intensely heated milks. The natural and the analogue-synthesized peptides (Table 2) were used to generate a calibration curve.

A constant concentration (10 μg/μL) of synthesized modified peptides was used to spike different solutions of synthesized natural peptides to create a calibration curve, with the natural/modified area ratio as a function of the concentration ratio of the corresponding peptides. The amount of β-CN (f1-28) 4P was obtained by spiking 10 μL raw milk with 10 μg/μL internal standard (IS) and calculating the quantity of this peptide according to the equation of the calibration plot.

Table 2 Amino acid sequence of synthesized natural and modified peptides

Synthetic natural peptide	[M+H]	Sequence
β-CN (f1-28) 4P	3,478.5 Da	RELEELNVPGEIVESpLSpSpSpEESITRINK
Synthetic modified peptide	[M+H]	Sequence
β-CN (1-25) 4P	3,196.3 Da	REWEELNVPGEIVESpLSpSpSpEESITR

Differentiating Trp residue is underlined. Sp indicates phosphorylated serine residue

Results and discussion

Strategy of HA-based enrichment of casein and CPP

Isolation of casein/CPP is one of the most difficult tasks because of the heterogeneity in the number of phosphate groups (P), varying from 1 to 13, attached to specific serine residues of the four casein fractions [20]. Phosphoproteins/peptides compete for the C sites on ceramic HA over a wide pH range [21], and both were eluted for MALDI analysis [15, 22]. Our procedure did not use elution because the HA-phosphocaseins/CPP complex was directly solubilized by spotting on the MALDI plate matrix mixed to TFA (for casein) or PA (for CPP) [14]. In addition to this, PA enhances the detection of phosphopeptide ions by MALDI-TOF [19]. The main objective of our work was to determine whether the HA-based procedure is suitable for characterizing signature peptides of heat-treated milk samples. Because the affinity of CPP for HA could decrease with increasing nonspecific binding of the unphosphorylated peptides, a preliminary study measured the bound and unbound proteins/peptides of raw, pasteurized, UHT, milk protein powder (casein+whey), and milk powder samples. Our results indicate that MALDI is useful in identifying lactosylation, one of the most common posttranslational protein modifications that occur during heating or spray drying. MALDI analysis provided molecular mass value of peptides and information on the presence/absence of signature peptides. Thus, severe heating induced changes of lactosylation in whey proteins of samples I to VI (Electronic supplementary material (ESM) Table S1). The signals of non-enzymatic lactosylation of β-Lg A and B and α-lactalbumin (α-La) B dominated the MALDI-TOF spectra of different samples (ESM Table S1 and Figs. S4 and S6). The molecular masses of β-Lg B (unmodified *m*/*z*18,280.4) and β-Lg A (unmodified *m*/*z*18,365.9) increased by 314.2 Da per lactose molecule or multiple integrals up to 6 (ESM Table S1). The lactosylation of β-Lg A and B compared with control raw milk was higher in powdered milk (sample VI, six lactose residues/molecule) than in protein milk powder (sample V, four lactose residues/molecule) and UHT milk (sample IV, one lactose residue/molecule). Unmodified β-Lg A and B

were still present in all samples except milk powder (ESM Table S1 and Fig. S6). α-La B was less lactosylated in the milk protein powder (sample V, three lactose residues/molecule) than in powdered milk (sample VI, four lactose residues/molecule). In UHT milk (sample IV), α-La B (unmodified *m/z* 1,4187.2) was lactosylated as β-Lg (one lactose residue/α-La molecule). This seems to contrast at least partially with previous findings on casein lactosylation which occurs at similar levels in UHT and in-bottle sterilized milk [12]. Indeed, the extent of lactosylated proteins in pasteurized milk does not differ from that measured in raw milk. In contrast, the different levels of lactosylation of proteins in the powder samples were probably due to the lower lactose content of ultrafiltered milk or whey. It seems that heating extensively enhanced lactosylation by exposing lysine residues on the surface of the proteins. However, Maillard reaction was incomplete even in the powdered milk preparations. In recent studies, Lys-47, Lys-138, and Lys-141 of β-Lg were lactosylated, while Lys-98, Lys-114, or Lys-122 of α-La were lactosylated in UHT milk [23]. In general, the degree of lactosylation increases steadily with temperature, progressively from pasteurization to spray drying passing through to UHT. However, the Amadori compound, the first intermediate in the Maillard reactions, is probably degraded by heat [24] via the Strecker degradation [25]. Assessment of the whey protein powder quality in infant formula on the basis of the number of lactose residues per protein molecule can detect five lactoses/α-La and ten lactoses/β-Lg [26]. In the solid state, all 15 lysine residues of β-Lg and the N-terminal leucine were lactosylated [27], in addition to Arg-124, for a total of 17 lactosylated amino acid residues [28].

While the effect of heat treatment on the whey protein denaturation has been well established by many decades of research, little is known about casein lactosylation. By applying our procedure, lactosylated casein would be detected in heated milk (ESM Fig. S4 and S6). All of the MALDI spectra for the milk samples actually contained the signals of (1) unmodified κ-CN or the mono-lactosylated component but milk powder (ESM Table S1 and Fig. S6); (2) multi-phosphorylated α_{s2}-CN after enrichment of isoelectric casein on HA; (3) lactosylated β-CN A^1 and A^2, which differed based on His^{67}(β-CN A^1) for Pro^{67}(β-CN A^2); and (4) diffusely lactosylated β-CN and α_{s1}-CN in milk protein (V) and milk powder (VI) samples due to the low resolution of MALDI (ESM Table S1 and Figs. S4 and S6). However, monomer κ-CN was missing in the milk powder sample because of its possible association with whey proteins. On the whole, HA procedure provides a system for the selective enrichment of caseins/CPP while whey proteins are washed away (ESM Table S1 and Fig. S8 and S9 and S10).

Identification of HA-bound CPP and unphosphorylated peptides

To follow the evolution of the peptide profile according to the heating intensity, the MALDI-TOF spectra were explored in the 10- to 14-kDa range. After enrichment on HA, raw (Fig. 1b and ESM Table S2) and pasteurized milk (ESM Fig. S11) had dominant signals of β-CN (f1-97) 5P (11,362.3 Da) and α_{s2}-CN(f53-150) 6P (11,860.4 Da). The impact of UHT treatment and of milk and milk protein drying seemed higher than in pasteurized milk (ESM Fig. S11).

The MALDI signals of unphosphorylated peptides significantly decreased, while those of CPP considerably increased. The results indicate that (1) a selective enrichment occurred as a result of displacement of CPP bound to HA by the addition of TFA; (2) the CPP enrichment reduced the sample complexity; and (3) low-abundance CPP in mixtures with unphosphorylated peptides can be detected directly

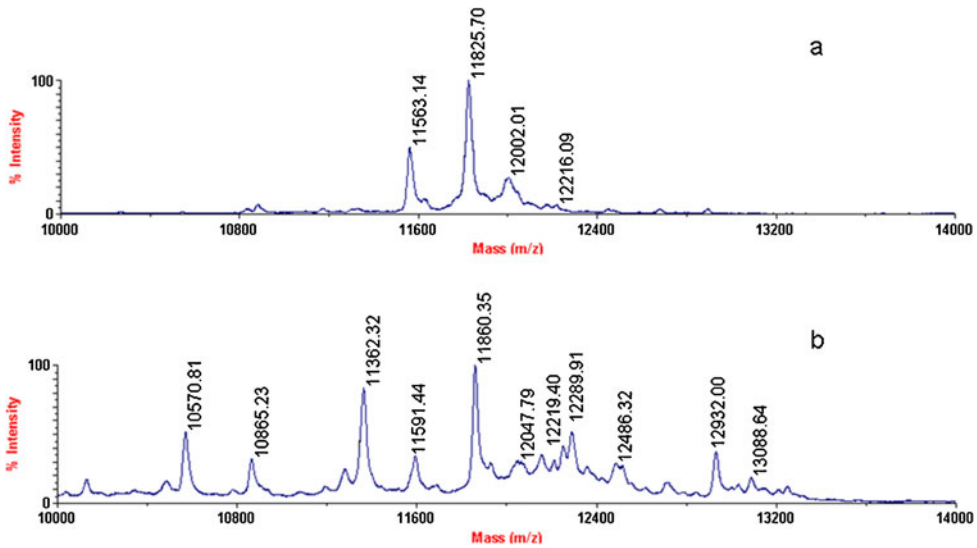

Fig. 1 MALDI-TOF spectra of raw milk peptides in the 10- to 14-kDa mass range before (**a**) and after enrichment (**b**) using HA as concentrating probe. Component identification is reported in ESM Table S2

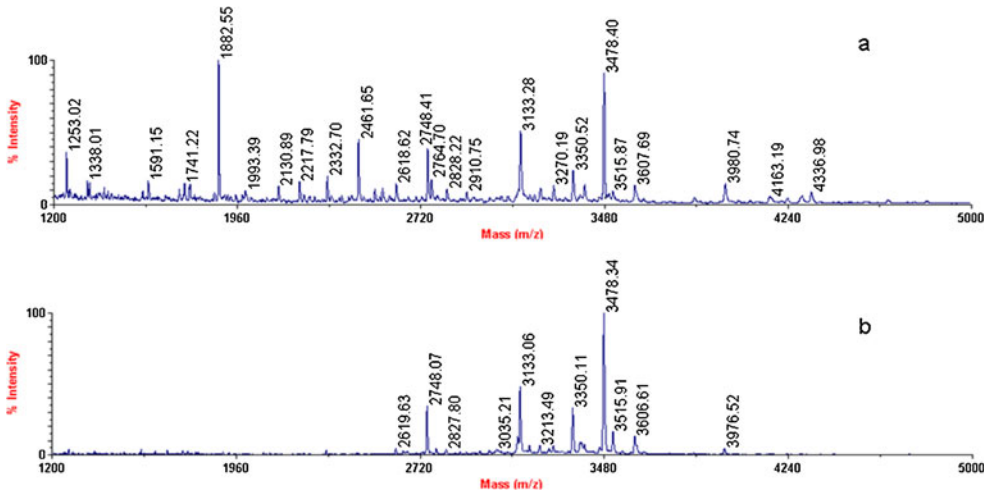

Fig. 2 MALDI-TOF spectra of raw milk peptides in the 1- to 5-kDa mass range before (**a**) and after enrichment (**b**) using HA as concentrating probe. Component identification is reported in ESM Table S3

"on-beads." This is confirmed by unphosphorylated γ_3- and γ_2-CN dominating the spectra of the raw milk (Fig. 1a) whose signals were lost during the repeated buffer washes, while β-CN (1-105) 5P and β-CN (1-107) 5P (complementary to γ_2-CN and γ_3-CN, respectively; Fig. 1b) were detected in HA-bound CPP. Taken together, these results demonstrate the outstanding enrichment capacity of HA for MS analysis.

MALDI-TOF in the 1.2- to 5-kDa range

Raw, pasteurized, UHT, milk protein, and milk powder samples were examined by MALDI-TOF in the 1.2- to 5-kDa range. As an example, the MALDI spectrum for raw milk is shown in Fig. 2a (ESM Table S3). Here was registered a substantial increase of peptides belonging to the β-CN, α_{s2}-CN and α_{s1}-CN family. This occurred probably because of the presence of active plasmin and somatic cell proteinases. After enrichment on HA, only phosphorylated peptides were detected, as shown in Fig. 2b (ESM Table S3).

Similarly, the major CPP were identified in the other commercial milk samples (ESM Fig. S12). In all the milk samples, both native and lactosylated forms of CPP were detected, except in raw and pasteurized milk. β-CN (f1-28) 4P, (f1-27) 4P, and α_{s2}-CN (f1-24) 4P were the main components of CPP (Fig. 3 and ESM Table S4). The native and lactosylated α_{s2}-CN (f1-21) 4P co-existed in milk powder. In this manner, we identified the lactosylated β-CN (f1-29) 4P as a signature peptide of milk proteins and powdered milk preparations because it was missing in other types of milk. It was missing in raw, pasteurized (71.7 °C for 15 s; Fig. 3a), and intensely pasteurized milk (121 °C for 2–4 s, spectrum not shown). α_{s1}-CN-derived CPP failed to form due to the internal location of the phosphorylation cluster. In contrast, they are released in long-ripened cheese [29].

To screen lactosylated CPP, in vitro dephosphorylation was carried out using ALP. The difference between the native and dephosphorylated peptide mass values allowed us to assign the phosphate groups correctly (one phosphate group=80 Da). The results of CPP dephosphorylation are shown in Table 3 (Fig. 4). Each peptide was a mono-lactosylated component. The degrees of phosphorylation and lactosylation of β-CN (f1-28) 4P were further confirmed by PSD MS experiments (Fig. 5).

The PSD spectrum in Fig. 5 exhibited a prominent neutral loss of 340 Da (one lactosyl group) from the precursor ion at *m/z*3,802.9 Da. Moreover, the PSD spectrum of lactosylated β-CN (f1-28) 4P yielded a neutral loss of 98 Da (H_3PO_4), consistent with a peptide containing four phosphoserine residues (Fig. 5). The PSD spectrum of lactosylated β-CN (f1-28) 0P confirmed the loss of one lactose group (*m/z*3,140.2 Da) from the peptide (Fig. 6).

In vitro trypsinolysis of HA-bound CPP or caseins from milk protein powder

Trypsinolysis of the HA-bound CPP in addition to the native β-CN (f1-25) 4P and β-CN (f1-25) 3P (3,123.0 and 3.043.6 Da) released mono-lactosylated counterparts (3,447.2 and 3,367.3 Da).

Notwithstanding the fact that Arg^{25}-X and Lys^{28}-X were evenly susceptible to trypsin, Lys^{28}-X was split by plasmin because of the strict specificity of enzyme towards lysine [30]. It is likely that lactosylation especially affects plasmin-mediated peptides [31]. Heating produced a consistent amount of lactosylated peptides. To observe this effect,

Fig. 3 The MALDI spectra show mass signals of β-CN derived lactosylated phosphopeptides (CPP) isolated by addition of hydroxyapatite (HA) microgranules to samples of pasteurized milk (**a**), UHT milk (**b**), milk protein powder (**c**), and milk powder (**d**). The inset magnifies the *m/z* values in 1- to 5-kDa range that are expected to show mass peaks corresponding to marker peptides. Monolactosylated CPP labelled with a *red circle* correspond to β-CN (f1-27) 4P (m/z 3675 Da), β-CN (f1-28) 4P (*m/z* 3803 Da) and β-CN (f1-29) 4P (*m/z* 3931 Da). As expected, none of these lactosylated CPP occurred in the spectrum of pasteurized milk (a). Identification of all the components is reported in ESM Table S4 ▶

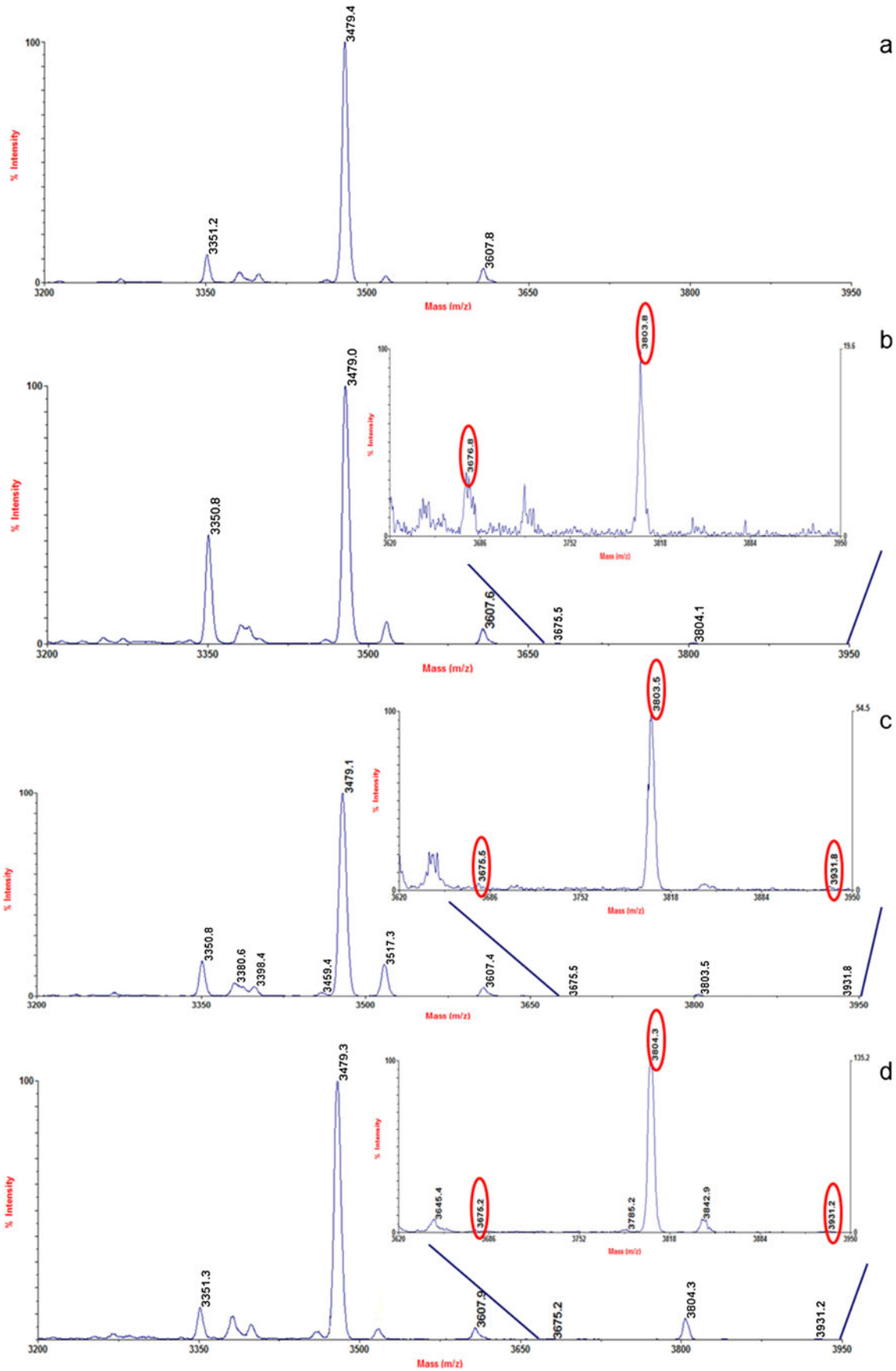
a
3479.4
3351.2
3607.8
% Intensity
Mass (m/z)
b
3479.0
3803.8
3676.8
3350.8
3607.6
3675.5
3804.1
c
3479.1
3803.5
3675.5
3931.8
3350.8
3380.6
3398.4
3459.4
3517.3
3607.4
d
3479.3
3804.3
3676.2
3931.2
3351.3
3607.9
3675.2

Table 3 MALDI identification of lactosylated CPP after in vitro dephosphorylation

Measured mass of dephosphorylated peptide (MH^+) (Da)	Theoretical mass (Da)	Identified peptide
3611.5	3,610.9	β-CN (f1-29) 0P+1Lactose
3483.5	3,482.7	β-CN (f1-28) 0P+1Lactose
3355.0	3,354.6	β-CN (f1-27) 0P+1Lactose

The spectra shown in Fig. 4 have been taken for milk powder sample

a shift of +324 Da was expected for peptides containing a reactive NH_2 group, yielding the so-called Amadori product. If Arg^{25} were lactosylated, Arg^{25}-X as well as Lys^{28}-X would be trypsin- and plasmin-resistant. Indeed, an amount of N-terminally mono-lactosylated β-CN (f1-28) 4P corresponding to ~3% total β-CN (f1-28) 4P was found in the milk protein powder sample. In contrast, a higher quantity of mono-lactosylated β-CN (f1-25) 4P, ~17% of the total β-CN, was released by trypsinolysis of HA-bound casein/CPP. This means that both lactosylated caseins and lactosylated CPP combine to determine the levels of signature peptides.

Such peptides were found in dried milks before digestion with trypsin.

Quantification of β-CN (f1-28) 4P in raw milk by MALDI-TOF-MS

Based on a set of naturally occurring peptides, β-CN (f1-28) 4P was chosen because it is a phosphorylated member of a plasmin-mediated CPP family that undergoes glycosylation during heat treatment. Specifically, for the absolute quantification of the mono-lactosylated β-CN (f1-28) 4P signature peptide, a synthetic 25-residue analogue that was identical to the N-terminal 25-mer peptide except for the amino acid substitution $Leu^3 \rightarrow Trp^3$ was used as IS. The natural proteotypic peptide was mixed with IS in known concentrations, and the MALDI intensity of the two co-occurring signals was measured (ESM Figs. S14 and S15). MALDI measurements performed on four binary solutions containing natural peptide/IS ratios of 0.22:1, 0.11:1, 0.055:1, and 0.0275:1 produced a line defined by the equation $y=6.2451x+0.3635$ ($R^2=0.9855$, mean of ten replicates; ESM Fig. S16). Using this approach, raw milk β-CN was found to have released ~4.3% of the β-CN (f1-28) 4P (ESM Fig. S17).

Specific detection of lactosylated milk CPP

Raw milk is rejected by dairy processing plants if the sample is found to be contaminated with UHT or other heat-treated milk. The HA-based method could ensure that UHT milk and sterilized milk are not used for the production of pasteurized milk. To demonstrate this, aliquots of pasteurized milk were artificially spiked with 90%, 70%, 50%, 30%, 10%, 5%, or 1% UHT milk. The limit of detection (LOD) was determined by MALDI searching for the lactosylated β-CN (f1-28) 4P, assumed as the signature peptide of UHT milk. The method did not discriminate UHT milk in amounts below 10% (Fig. 7).

Using improved MS equipment, in terms of mass resolution and sensitivity, we think that the LOD could be substantially lowered. Trypsinolysis of HA-bound CPP, enhancing the signal of lactosylated β-CN (f1-25) 4P, could further lower the LOD value.

Sequences of cleaved peptides

To verify the role played by proteolytic enzymes in casein hydrolysis, the amino acid sequence of CPP was investigated (Fig. 2b). The in vivo and in vitro plasmin-mediated peptides that arose from raw milk were compared (ESM Fig. S18 and Table S5).

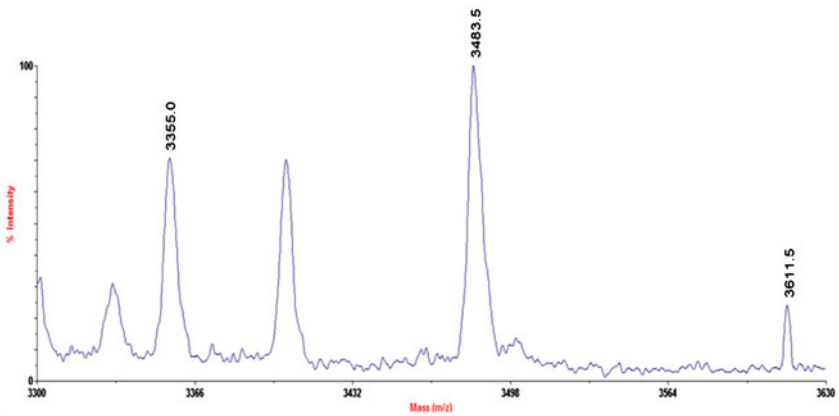

Fig. 4 An example of determination of phosphorylation stoichiometry of lactosylated CPP by combining alkaline phosphatase-based dephosphorylation with peptide identification by mass spectrometry. By comparing the MALDI spectra recorded for the phosphatase-treated to the untreated sample in Fig. 3, the candidate CPP were identified. Because of 320 Da mass shift (1 phosphate group=80 Da) each peptide, indicated with a *m/z* value in the MALDI spectra, had four phosphorylation sites

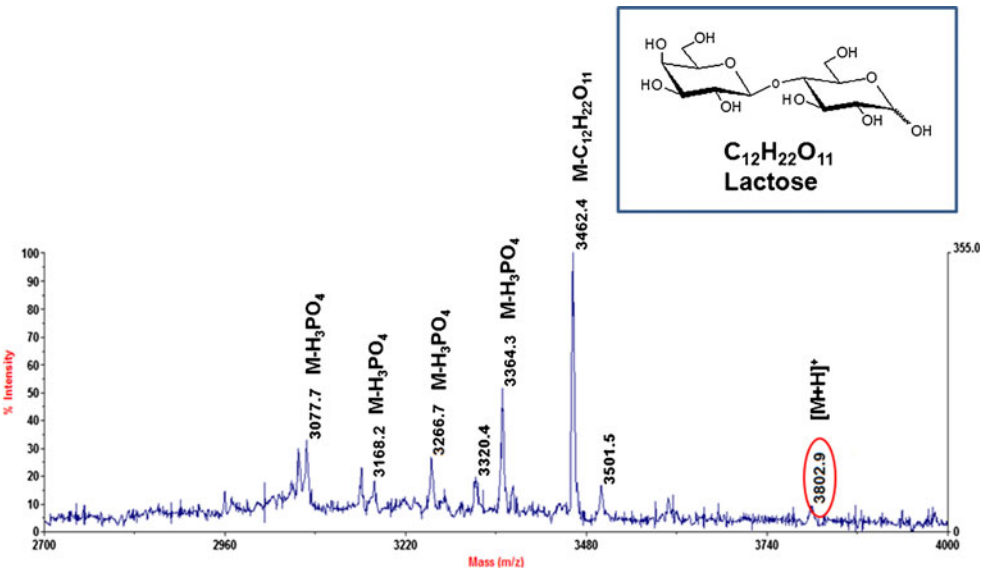

Fig. 5 Post-source decay (PSD)-MALDI spectrum of a HA-bound CPP occurring in the sample of milk powder. The *red circled* peak represents a peptide exhibiting a neutral loss of 340 Da (a lactosyl group) that was one of the most intense ions, and 392 Da for phosphoric acid (1P= 98 Da) from the precursor ion at m/z 3,802.9 Da. These structural informations allowed to assign four phosphates and one lactose group unequivocally to the peptide β-CN (f1-28) at *m/z* 3,803 Da that was present in the milk powder sample

The choice of plasmin as the proteolytic enzyme was dictated by the role played by this enzyme during milk storage. Because peptide bonds containing a C-terminal Lys or Arg can be hydrolyzed by plasmin, the majority of β- and α_{s2}-CN CPP reported in ESM Table S3 must have been released by plasmin. This agrees with the known activities of plasmin and other proteases associated with milk somatic cells. CPP differed only in the level of lactosylation, which was absent in raw milk but extensive in milk powder. Thus, lactosylated CPP may serve as suitable chemical markers of the intensity of heat treatment. When CPP levels are higher than normal, milk is of poor quality because it is rich in proteolytic enzymes. It seems that the level of lactosylation in milk is related to the intensity of the heating process.

Disadvantages and advantages of the HA procedure

Current techniques use gel electrophoresis and immunoblotting for separating and detecting specifically proteins. Antibodies developed against the Amadori compound containing lactose as a glycating agent were used as a surrogate for measuring furosine, distinguishing the lactosylated from non-lactosylated caseins [32]. In general, anti-peptide or monoclonal antibodies are specific reagents for measuring lactosylated peptides [32]. The drawbacks of polyclonal

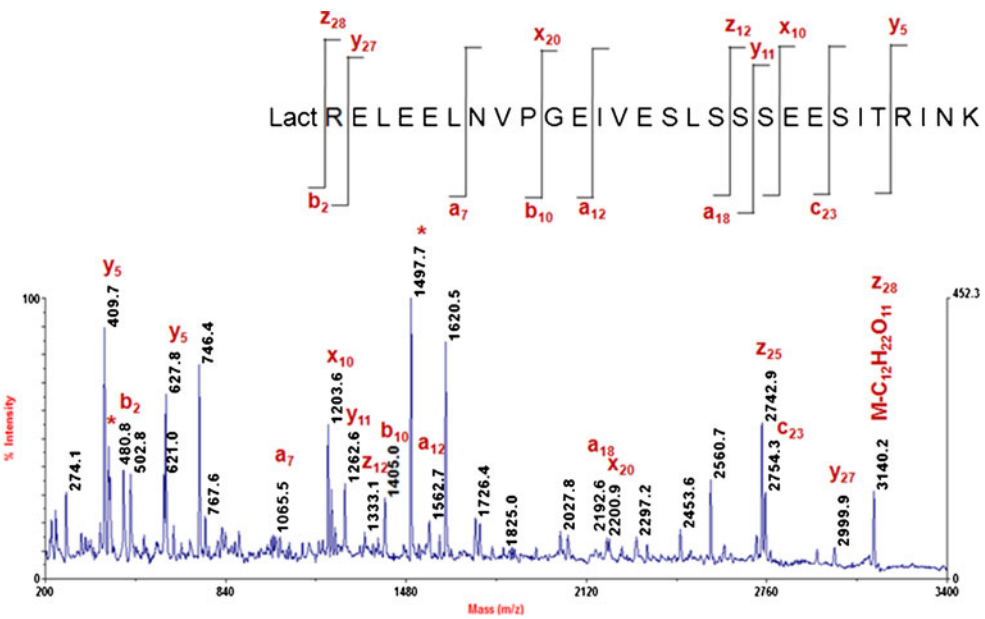

Fig. 6 PSD-MALDI-TOF-MS/MS spectrum of lactosylated β-CN (f1-28) 0P at *m/z*3,483 Da after dephosphorylation. The signal at *m/z*3,140.2 Da confirmed the loss of one lactose group

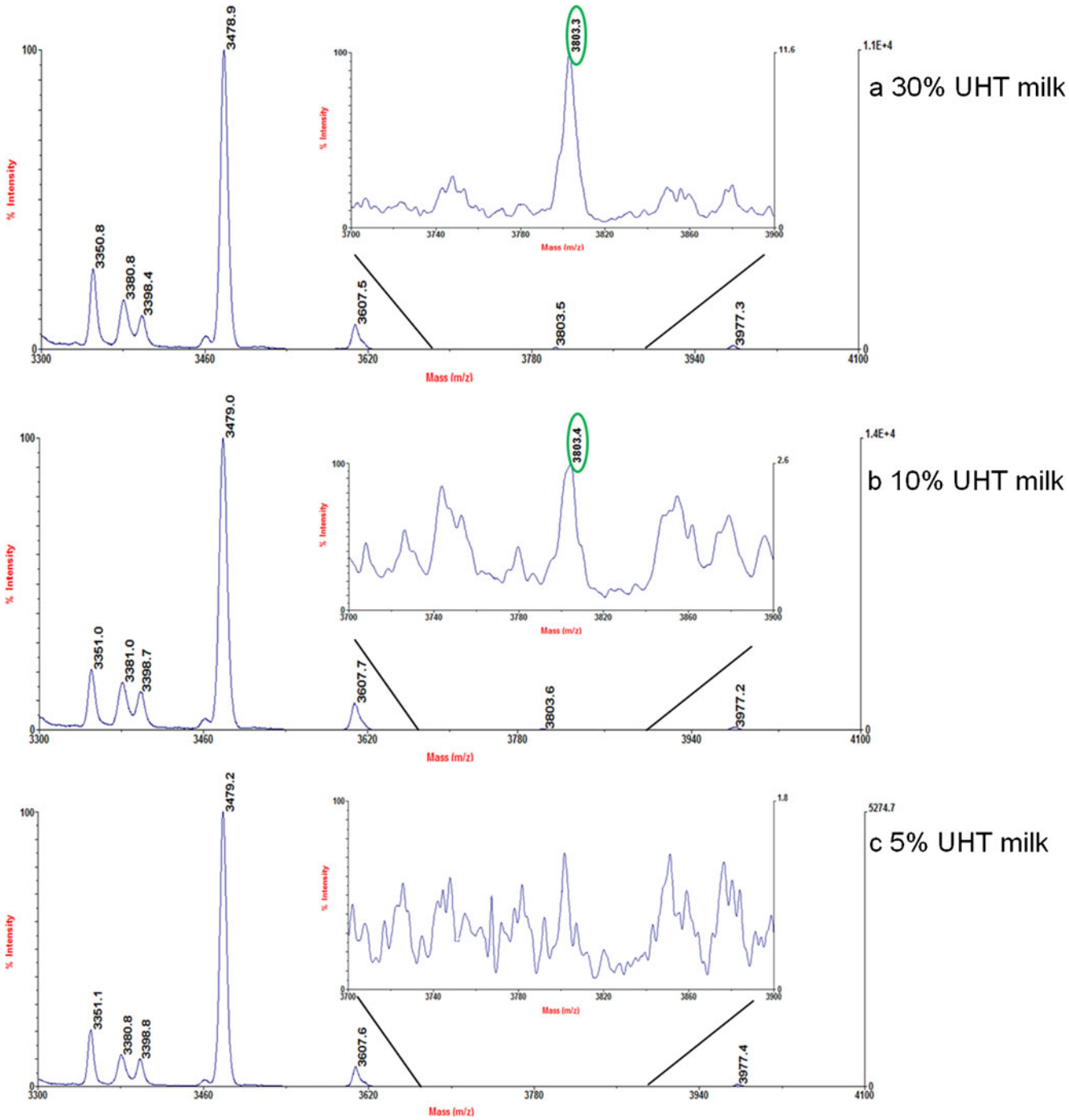

Fig. 7 Zoomed-in view of MALDI spectrum in the 1- to 5-kDa mass range of β-CN (f1-28) 4P signature lactosylated CPP from pasteurized milk spiked with 30% (**a**), 10% (**b**), and 5% (**c**) UHT milk. Lactosylated β-CN (f1-28) 4P mass signal was magnified and *circled in green*

anti-peptide antibodies are mainly (1) the amino acid sequences of the peptides would be known in advance to target their lactosylated proteins; (2) the antibodies might not recognize native proteins; and (3) a cross-reaction with other members of the protein families would not be excluded a priori. On other hand, concentration of low-abundance peptides/proteins by gel electrophoresis carries the risk that water-soluble peptides escape to the staining procedure with Coomassie Blue. Separation of proteins by 2DE coupled with identification of tryptic peptides through offline MALDI-TOF-MS is one of the most commonly used techniques in proteomic analysis [33]. Cumbersome 2D electrophoresis for protein separations requires extensive manual manipulation for mostly qualitative experiments. Online HPLC coupled with mass spectrometry

have been widely used for concentrating/identifying low-abundance peptides/proteins. However, one sample at a time is evaluated by HPLC, so samples are analyzed multiple times during a multi-sample sequence. In addition, low-abundance lactosylated CPP have to be concentrated before injection into the HPLC apparatus. The chromatographic techniques using IMAC and titanium dioxide (TiO_2) require both a preliminary purification [34, 35] and elution step [36, 37] for isolating phosphopeptides prior to MALDI analysis. The HA-based procedure has the advantage of capturing distinctly the plasmin-derived CPP and the counterparts that have undergone lactosylation under different heat treatments. The chemical changes induced by processing milk to heating should be confirmed by MS through identification of the amino acid sites modified by lactose. The advantages of using lactosylated signature peptides as analytical surrogates of the proteins are that (1) it is easier to separate and detect peptides than proteins, (2) structure of the lactosylated CPP does not alter during the analysis, (3) native CPP do not interfere with lactosylated counterparts, and (4) putative peptides suggested from databases can be easily recognized and eventually synthesized for external standards. Another advantage of the procedure is that different mass range acquiring analysis can be explored to detect co-existing HA-bound phosphoproteins and CPP in three separated range masses of the MALDI spectra. Once a lactosylated peptide is discriminative for the heat treatment type, the relative signature peptide could be detected and quantified within a restricted mass range.

Conclusion

The lactosylated phosphopeptides we detected were indicative of the casein quality. The ability to dynamically characterize lactosylation as a distinguished signature modification of caseins could help monitor the heat-induced modifications of casein micelles. CPP lactosylation can be regarded as a model for studying the accessibility of lactose to casein micelles. The major drawback encountered in concentrating CPP was resolved by capturing CPP on HA. In addition to this, the quality of MALDI signals of lactosylated CPP improved. These peculiarities make the new method able to detect also low-abundance signature peptides by MALDI.

The results of our experiments have demonstrated that (1) native and lactosylated CPP are equally bound to HA; (2) CPP have higher affinity for HA than unphosphorylated peptides co-purifying with them using other methods; (3) as expected, the extent of the casein lactosylation is a function of the heating intensity; and (4) mono-lactosylated β-CN (f1-28) 4P represents a distinct signature peptide that is detected distinctly from the native counterpart, making possible the detection of not less than 10% UHT milk spiking raw or pasteurized milk. However, the present procedure needs further signature peptides to distinguish thermized and pasteurized milks. Even with this limitation, the method could be applied in the design and control of any dairy product at the molecular level.

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Hydroxyapatite as a concentrating probe for phosphoproteomic analyses

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ABSTRACT

A novel method for the selective enrichment of casein phosphoproteins/phosphopeptides (CPP) from complex mixtures is reported herein. This method employs ceramic hydroxyapatite (HA) as a solid-phase adsorbent to efficiently capture phosphoproteins and CPP from complex media. Casein was chosen as the model phosphoprotein to test the protocol. CPP immobilized on HA microgranules formed a complex that was included in the matrix-assisted laser desorption/ionization mass spectrometry (MALDI) matrix before desorbing directly from the well plate. Casein fractions with different levels of phosphorylation were desorbed based upon the specific concentration of trifluoroacetic acid (TFA) included in the MALDI matrix. The HA-bound casein enzymolysis was performed in situ with trypsin to remove non-phosphorylated peptides and isolate the immobilized CPP. The latter were recovered by centrifugation, dried, and co-crystallized with a 1% phosphoric acid (PA) solution in the matrix that was appropriate for detecting CPP in MALDI-MS spectra. This approach for the selection of casein/CPP resulted in the identification of 32 CPP by MALDI-time of flight (TOF). The analytical process involved two steps requiring ~2 h, excluding the time required for the enzymatic reaction. The alkaline phosphatase (AP)-assisted de-phosphorylation of tryptic CPP allowed the phosphorylation level of peptides to be calculated concurrently with MALDI-TOF MS and liquid chromatography-electrospray ionization–mass spectrometry (LC–ESI–MS/MS). The effectiveness of the extraction procedure assayed on eggshell phosphoproteins resulted in the identification of 5 phosphoproteins and 14 derived phosphopeptides with a phosphoprotein global recovery of ~70% at least.

1. Introduction

Several reviews have described phosphoproteomics, which attempts to study protein phosphorylation on a large scale by elucidating the phosphorylation status of all proteins present in cells or organisms. One of the most significant phosphoproteomic studies described the "phosphokinome" of human cancer cell lines, identifying almost 1000 phosphorylation sites on 219 protein kinases [1]. Given their often substoichiometric concentration, phosphopeptides frequently escape detection because of a poor mass spectrometric (MS) precursor response and the low m/z values of MS2 fragment ions [2]. Moreover, proteins/peptides containing multiple phosphorylation sites are detected with low efficiency by MALDI-MS because of their attenuated or suppressed ionization [3,4]. Prior enrichment of phosphoproteins/phosphopeptides is one of the important methods to enhance the MS detection of phosphoanalyte signals. The objective of any separation and enrichment procedure from protein or peptide complex media is the quantitative recovery of phosphorylated peptides free of the non-phosphorylated components. To date, several strategies have been developed to fulfill this objective, most of which are based upon enrichment and/or down-scaling strategies. These strategies both reduce the amount of starting non-phosphorylated material and increase the intensity of the phosphoanalyte peaks in MS. Immobilized metal affinity chromatography (IMAC) [5–7], which chelates phosphoanalytes on metal ions (e.g., Zn^{+2}, Ga^{+3}, and Fe^{+3}) through metal-phosphate ion–pair interactions, has been suggested to be one of the best procedures for enriching phosphorylated proteins and peptides. Another method utilizes either titanium dioxide [8–11] (TiO_2) or zirconium dioxide (ZrO_2) [12–14] to capture phosphoanalytes through bidentate interactions [15], providing complementary information to IMAC [16]. Each affinity capture method has a particular specificity for the phosphoproteins/phosphopeptides that it can isolate and, consequently, isolates a different set of phosphopeptides. A comparative study of three affinity methods concluded that different, partially overlapping segments of the phosphoproteome were detected by each method and that, at present, no single method is sufficient for a comprehensive phosphoproteomic analysis [16]. In addition, the extent of phosphopeptide identification with any approach is highly dependent upon the sample and the laboratory performing the analysis [17]. The affinity techniques developed for the

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selective enrichment of phosphoproteins/phosphopeptides from complex samples gave reproducible but incomplete results [16] due to the poor binding of phosphopeptides present in low concentrations and the insufficient recovery of peptides with multiple phosphorylation sites [18]. IMAC also binds non-phosphorylated peptides because the metal ions also chelate protein carboxylate groups. To overcome this drawback, binding and washing are performed at low pH, at which carboxylic acid groups are not ionized, while elution is subsequently accomplished by raising the pH [19]. Esterification of acidic residues can also reduce the binding of non-phosphorylated peptides [20]. At the present time, which of these affinity isolation procedures would yield the best results is unclear. We developed a hydroxyapatite (HA)-based procedure for the chromatographic isolation of casein fractions according to the increasing phosphate content [21]. Since then, there have been a few reports on the use of HA for the enrichment of phosphoproteins/CPP [22,23]. Recent work has compared the efficiency of HA- and TiO_2-based methods, demonstrating that both showed an almost equal performance for CPP enrichment [22]. Basically, the HA-based protocol immobilizes CPP on HA microgranules after elution with a phosphate-containing buffer, which are then identified by off-line MALDI-MS [22]. To speed up this procedure for its routine application on a variety of samples, we investigated the possibility of using MALDI-TOF for direct analysis of the HA-bound phosphoproteins/CPP in a solution free of non-phosphorylated analytes. The efficiency of an analytical method is usually determined using model proteins. Whole casein was chosen as the reference protein for this study because it is formed by four protein families each having a known sequence and well documented phosphorylation sites [24]. Because a true standard for how these CPP are reported has already been established, there is no need to validate the assignment of peptides. Not all predicted masses, however, actually correspond to CPP. To reduce the misidentification rate, the assignment and sequence of peptides can be accomplished by MALDI-TOF post source decay and LC–MS/MS, which are both able to identify phosphorylated amino acids in the resultant mass spectra [25]. Because progress in mass spectrometry in the field of proteomics is outside the scope of this report, we mainly focus on the analysis of our hydroxyapatite-based method, which specifically captures both phosphoproteins and phosphopeptides as well as eliminates the sample loss that can occur with elution prior to MALDI analysis [22]. This hydroxyapatite (HA)-based method provides a very efficient means to specifically analyze microgranule-bound phosphoproteins and phosphopeptides.

2. Materials and methods

2.1. Materials and reagents

HA (Macro-Prep Ceramic Hydroxyapatite TYPE I) was purchased from Bio-Rad (Milan, Italy). Tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl), potassium chloride (KCl), urea, TFA, ACN for HPLC, 85% orthophosphoric acid (PA), and ammonium bicarbonate buffer (AMBIC) were purchased from Carlo Erba (Milan, Italy). TPCK-treated trypsin from bovine pancreas was purchased from Sigma (St. Louis, MO, USA). Sinapinic acid, sodium acetate trihydrate and 2,5-dihydroxybenzoic acid (DHB) were obtained from Fluka (St. Louis, MO, USA). Acetic acid was purchased from Baker Chemicals B.V. (Deventer, Netherlands). Alkaline phosphatase (Grade I, 4000 U) from calf intestine was supplied by Roche (Roche Diagnostics, GmbH, Mannheim, Germany). Dithiothreitol (DTT) was purchased from Applichem (Darmstadt, Germany). Water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Raw whole milk was collected from local dairy farms. The milk was skimmed by centrifugation at 4000 rpm at 4 °C for 30 min. Isoelectric casein was prepared by the addition of 10% (v/v) acetic acid to the resulting skim milk, followed by incubation for 30 min at 35 °C and the subsequent addition of 1 M NaOAc to produce a final pH of 4.6. After an additional 30-min incubation, the suspension was recentrifuged as above, the supernatant was discarded, the casein was washed twice with buffer diluted 1:4 and twice with Milli-Q water, and the sample was subsequently freeze-dried. The shell proteins from fresh commercial egg chicken were recovered according to the procedure previously described [26] from a local store. The calcified layer was finely grinded and demineralized in 10% acetic acid at 4 °C for 24 h with constant stirring. Insoluble material was removed by centrifugation and the supernatant was dialyzed against 5% acetic acid and lyophilized.

2.3. HA-based phosphoprotein/CPP enrichment

Lyophilized casein (10 mg) was dissolved in 100 μL of buffer containing 50 mM Tris–HCl, 0.2 M KCl, 4.5 M urea, and 10 mM DTT, pH 7.8. The casein solution was loaded onto 10 mg of HA that was previously packed with loading buffer. The HA-bound casein was incubated for 15 min at room temperature and centrifuged for 5 min at 4000 rpm. The resin was successively washed with three different buffers: 1 mL of loading buffer, 1 mL of 50 mM Tris–HCl, pH 7.8 (washing buffer), and 1 mL of buffer containing 20 mM Tris–HCl, pH 7.8 and 20% ACN (v/v). The resin was washed with 1 mL of Milli-Q water and freeze-dried with a SpeedVac concentrator system (Thermo Electron, Milford, MA, USA). For eggshell proteins, 5 mg/100 μl buffer were loaded onto 5 mg HA.

2.4. HA-based CPP enrichment

The HA-bound phosphoproteins/CPP enzymolysis was performed in situ with trypsin, which was added to the suspension at an enzyme/substrate ratio of 1:50 (w/w) in 50 mM Tris–HCl buffer, pH 7.8 containing 0.2 M KCl, 4.5 M urea, and 10 mM DTT. The reaction was carried out at 37 °C overnight and stopped by centrifuging the HA-CPP microgranules for 5 min at 4000 rpm. The microgranules were then washed as described above for the phosphoproteins. After washing with Milli-Q water, the microgranules were dried with a SpeedVac apparatus.

2.5. MALDI-TOF MS

MALDI-TOF mass spectra were recorded using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA). Mass spectra were acquired in positive linear mode due to the known instability of CPP in the reflector mode [27]. This situation is amplified by the multiply phosphorylated peptides generating dephosphorylated peptide in the metastable decomposition [28]. In addition, the co-presence in high number of phosphorylated peptides would complicate the reflector spectrum. Instead, interpretation of the linear spectrum is straightforward, since it contains only the $[M+H]^+$ ion for each species. Moreover, higher molecular weight peptides and phosphoproteins also show lower sensitivity in the reflector mode than in the linear mode due to longer flight path and action of reflector [29]. Finally, the LC–ESI–MS/MS experiments were carried out to confirm the assignment of CPP identity [30]. The spectra were acquired in the range of 15–30 kDa (for proteins) and 1–5 kDa (for peptides) with the following settings: an accelerating voltage of 25 kV (for proteins) or 20 kV (for peptides) and a grid voltage of 93% (for proteins) or 95% (for peptides) of the accelerating voltage, a guide wire of 0.15% (for proteins) or

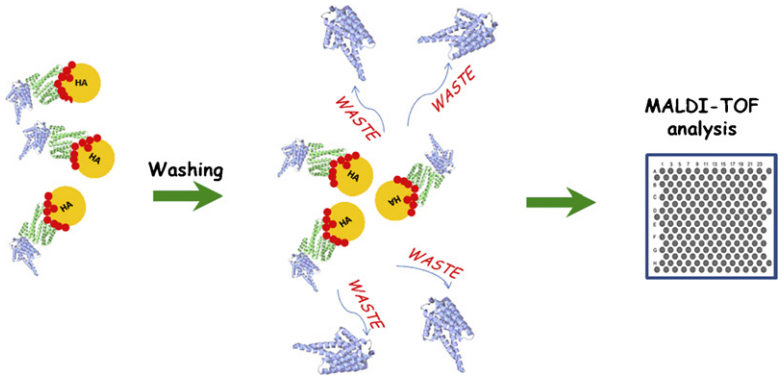

Fig. 1. Schematic representation of the HA-based technique for phosphoprotein enrichment. Phosphoproteins bind to HA through their phosphate groups, while the non-phosphorylated components are removed using washing buffers. The dried HA-phosphoprotein microgranules are then analyzed directly by MALDI-TOF after co-crystallization with sinapinic acid in an appropriate quantity of trifluoroacetic acid. ● = phosphate group.

0.05% (for peptides), and a delayed ion extraction time of 485 ns (for proteins) or 175 ns (for peptides). The laser power was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise ratio. All spectra were acquired with 200 shots in three replicates. Two different matrix solutions were freshly made each day from stocks of the required solvents and sonicated for 15 min in an ultrasonic water bath (Bransonic 220, Mazian Med Equip, Toronto, Canada) prior to use. The sample/wash (SW) method was based on the sample/matrix/wash (SMW) method described by Zhang et al. [31], with the exclusion of the wash step.

The HA-phosphoprotein microgranules ($n = \sim 1000$) were deposited onto the MALDI plate, covered with the matrix solution (10 mg/mL sinapinic acid (SA) in 50% ACN, 1 μL) to promote analyte/matrix co-crystallization and air-dried at room temperature in the presence of increasing TFA concentrations (0.01%, 0.05%, 0.1% and 0.5%). For HA-CPP, 1% PA was included in the matrix solution (10 mg/mL 2,5-dihydroxybenzoic acid (2,5-DHB) in 50% ACN) [32].

2.6. LC–ESI–MS/MS analysis

HA-CPP complexes (1 mg) were solubilized with 120 μL of 0.5% PA and analyzed by a CapLC nano-flow high-pressure pump system (Waters/Micromass, Manchester, UK) interfaced with a QTOF Micromass spectrometer (Waters/Micromass) operating in the positive ion mode. Chromatographic separations were performed on a reverse phase Atlantis dC18 capillary column (75 μm i.d.). The mobile phase was water (A) and ACN (B) with 0.1% FA. A linear gradient from 5% to 70% of B was applied to the pre-column and column over a 45-min period at a flow-rate of approximately 300 nL/min pre-column splitting using a pump operating at 5 μL min^{-1}. The source conditions were as follows: capillary voltage, 2.6 kV; cone voltage, 100 V; and RF1 lens, 40 V. Argon was used as the collision gas in the ESI-MS/MS experiments for peptide sequencing. Raw data were processed using MassLynx 4.0 ProteinLynx software.

2.7. Microscopic observation

The HA-CPP microgranules deposited onto a 96-well MALDI target were observed by stereomicroscopy before and after co-crystallization with the DHB matrix. The microscope (PBI International, Milan, Italy) was equipped with a binocular head illuminated with a 6-V, 20-W halogen lamp and a 100–230-V, 50/60-Hz power supply.

2.8. CPP de-phosphorylation

The HA-bound tryptic CPP complex (1 mg) was treated with 15 μL of a 5% aqueous PA solution to render soluble both the HA and phosphorylated components. The solution was then loaded on a Zip Tip C18 to obtain the salt-free phosphopeptide mixture and freeze-dried in a SpeedVac apparatus. The residue was then dissolved in a solution composed of 50 μL 0.4% AMBIC, pH 9 and 1 μL alkaline phosphatase (ALP) and incubated at 37 °C overnight. The de-phosphorylated CPP diluted 10 times with 0.1% H_2O/TFA (1 μL) was deposited in a MALDI well, covered with DHB matrix (10 mg/mL of the H_2O/ACN/PA = 49/50/1 solution) and allowed to crystallize. Spectra were acquired as indicated above.

2.9. Phosphoprotein workflow

An integrated strategy for the experimental analysis of the phosphoproteome combining MALDI-TOF analysis with the phosphoprotein enrichment steps is schematically illustrated in Fig. 1. The suspension of HA microgranules in the loading buffer was mixed with the protein solution to bind the target phosphorylated components to the adsorbent. The analytical method (Fig. 1) requires six consecutive steps: (1) enriching and separating phosphoproteins using HA microgranules (HA: protein, 1:1 [w/w]); (2) washing with the dilution/washing buffer to remove any non-phosphorylated proteins from the casein-HA microgranule; (3) drying the protein-HA microgranules; (4) loading the HA microgranules ($n = \sim 1000$) onto the multi-well MALDI target plate; (5) covering the microgranules with a sinapinic acid matrix (10 mg/mL in 50% ACN) to promote co-crystallization of the analyte/TFA (0.01–0.5%) mixture; and (6) in situ desorption of the phosphoproteins by direct MALDI-MS analysis (Fig. 2).

2.10. Phosphopeptide workflow

Whole casein (or eggshell proteins) was converted to tryptic digests for the selective enrichment of phosphorylated peptides using HA chromatography (Fig. 3). Preliminary experiments revealed that the highest CPP yield was obtained using a 1:1 HA-to-protein ratio (w/w), a 1:50 trypsin-to-protein ratio (w/w) and an 18-h digestion period. After addition of trypsin to the protein-HA microgranules, the supernatant, which contained non-phosphorylated tryptic peptides and trypsin, was discarded. The residue was sequentially washed with a loading/washing buffer/water solution, recovered by centrifugation and freeze-dried. The resultant dried phosphopeptide-HA microgranules, mixed with a DHB and 1% PA solution, were left to co-crystallize on a MALDI target plate and directly analyzed by MALDI-TOF. This version of the protocol could further speed up our previously developed procedure requiring phosphopeptide elution from HA before MS analysis [22]. Briefly, microgranules deposited on the target were covered by matrix solution containing 1% H_3PO_4, which

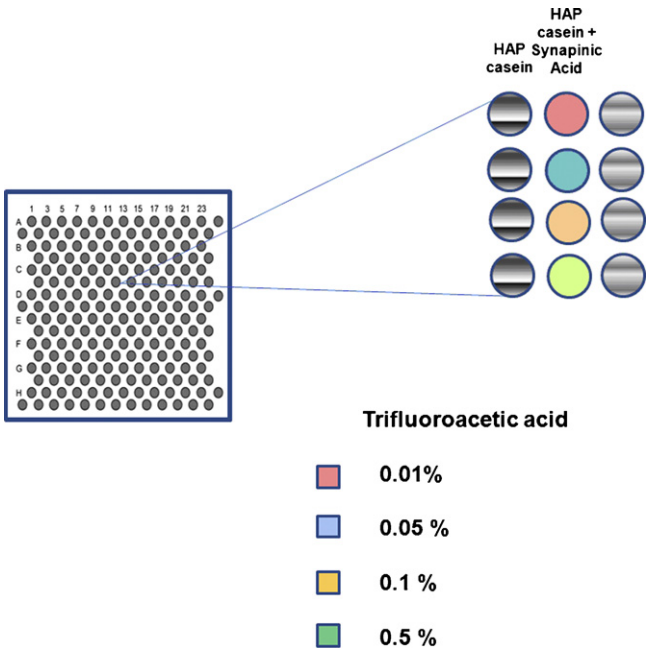

Fig. 2. Schematic representation of the co-crystallization of HA-bound casein with the sinapinic matrix on the MALDI target plate in the presence of increasing concentrations of TFA (0.01%, 0.05%, 0.1% and 0.5%).

dissolved the HA-captured CPP or eggshell phosphopeptides and making their analysis by MALDI possible.

3. Results

The identification of phosphoproteins by MALDI-TOF is not as straightforward as the identification of proteins due to their relatively low abundance, the variance in phosphorylation sites and the possible presence of phosphatases. Given that de-phosphorylation could occur even as the sample is processed, the results also depend on the specific activity of alkaline phosphatase. Therefore, phosphatase-free isoelectric casein was selected to study the specificity of interactions with HA. HA possesses pairs of positively charged C-sites and negatively charged P-sites [33] that are capable

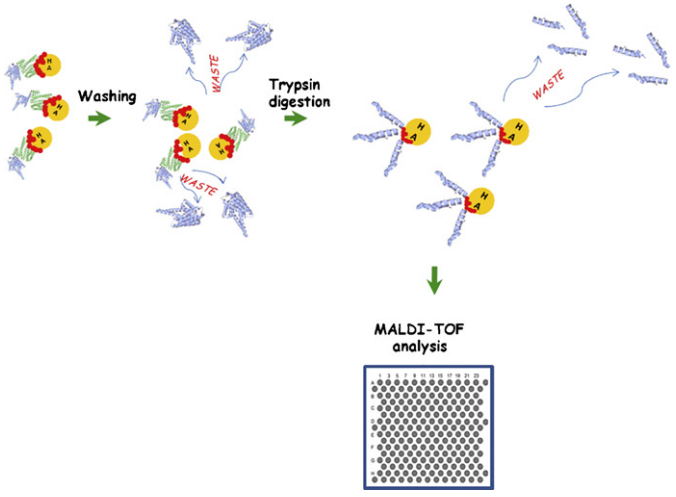

Fig. 3. Schematic representation of the HA-based CPP enrichment technique. HA-bound phosphoprotein enzymolysis is performed in situ with trypsin, and non-phosphorylated peptides are released in the flow-through. The dried HA-CPP microgranules included in the co-crystallization matrix are analyzed by MALDI-TOF directly on the MALDI plate, as indicated in Fig. 2.

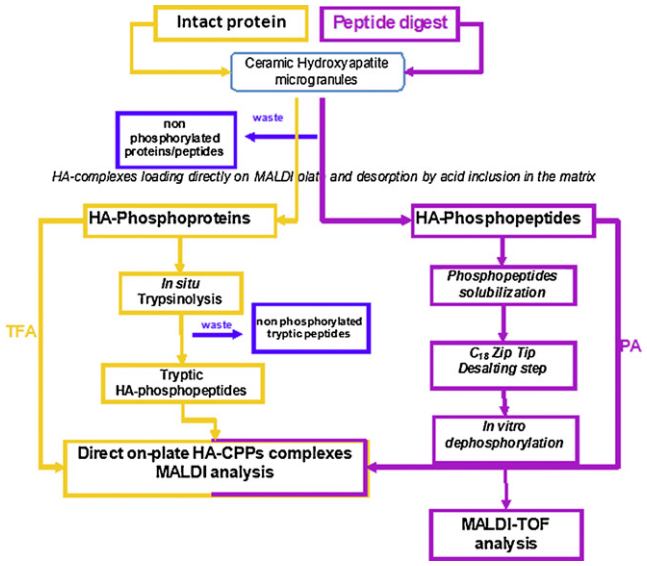

Fig. 4. Experimental scheme for the direct MALDI-MS analysis of phosphoproteins and CPP bound to HA microgranules. The samples were analyzed as follows. An aliquot of the solution containing casein and/or its derivative peptides was mixed with the HA microgranules to capture the phosphorylated components. Another aliquot containing HA-bound casein or tryptic CPP was spotted on the target, covered with the MALDI matrix solution containing exogenous acids (TFA for phosphoproteins, shown in yellow; PA for CPP, shown in violet) and the protein/peptide ions desorbed from the MALDI-TOF matrix. The HA-based protein/peptide enrichment procedure was tested using MALDI-TOF to discriminate the phosphoanalytes in two distinct mass ranges. The presence of phosphoanalytes in the mixture was verified through phosphate-specific reactions (e.g., sensitivity to alkaline phosphatase). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of interacting, under a wide range pH levels, with the side chain groups of proteins/peptides. These characteristics allow the HA surface to bind proteins with different p*I* [33]. In this study, HA was first tested for its ability to bind the four major casein families through their variable number of phosphate groups present in concentrations ranging from 1 to 13 moles/molecule [21]. The suspension of HA-bound casein complexes was then assayed for its susceptibility to trypsin in comparison to native casein in solution. The detailed procedure for the phosphoprotein/phosphopeptide enrichment and optimization is outlined in Fig. 4.

3.1. Analysis of phosphoproteins

Using the strategy depicted in Figs. 1 and 3, hydroxyapatite was tested for its ability to chelate casein and be used in the subsequent characterization of captured proteins using MALDI-TOF. Using 0.01% TFA, those casein components with a low number of phosphate groups were desorbed first from the on-bead HA microgranules. The resulting spectral signals were assigned to the corresponding κ-CN 1P variant A and β-CN (Fig. 5, panel a; Table 1), while those with multiple phosphorylation sites were desorbed by the addition of 10-fold concentrated TFA (Fig. 5, panel b). The results indicate that HA captured all casein fractions. Among the weakly HA-bound fractions, the caseins with a low number of phosphorylation sites were desorbed first. Almost exclusively κ-CN could be desorbed by the inclusion of 0.005% TFA in the MALDI matrix (Supplemental Figure S1). In addition, κ-CN had been previously found to elute first from a HA column using a buffer with a low phosphate concentration (5 mM) [21]. Based on the rationale that the casein can be fractionated according to the phosphate concentration of the eluent, a HA-based chromatographic method was developed [22] to separate low- and high-phosphorylated CPP. If this fractionation does not really apply to such CPP, however, the

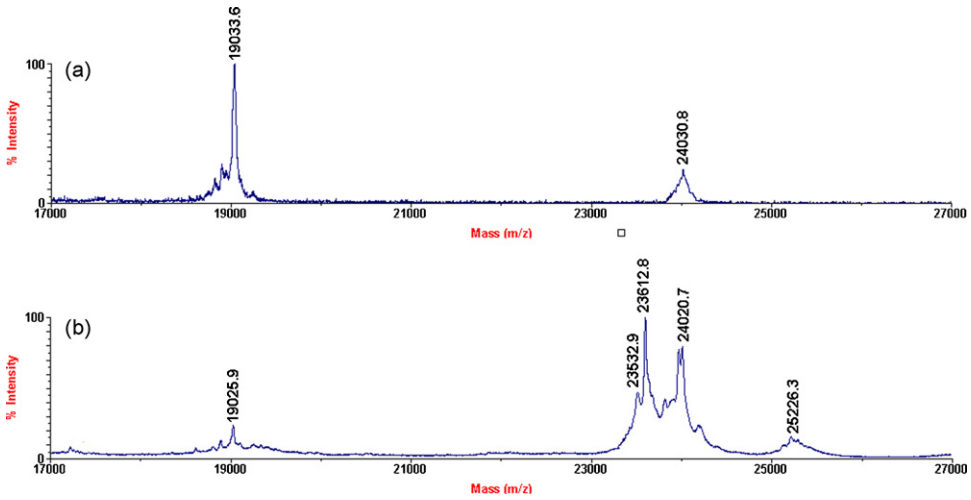

Fig. 5. MALDI-TOF analysis of phosphoproteins desorbed from HA microgranules by 0.01% TFA (a) or 0.1% TFA (b).

HA-bound peptides might all be eluted simultaneously [22]. Indeed, HA-bound proteins can all be recovered by an adsorbent collapse through TFA addition to the MALDI matrix. Because this procedure does not require any prior chromatographic separation of the phosphoproteins, it can be assumed to enable a single-step identification of the HA-bound phosphoproteins by comparing the measured mass to the theoretical mass (Table 1). More precisely, four coupled casein fractions, each with differing numbers of phosphate groups were identified (Table 1). β-CN A^1 and A^2 differed by the additional amino acid substitution His^{67} (β-CN A^1) → Pro^{67} (β-CN A^2). Notwithstanding the presence of β-CN in amounts three-fold higher than those of the heavily phosphorylated α_{s2}-CN, the ionization of the latter is not completely suppressed with respect to that of β-CN. The results demonstrate that MALDI-TOF MS is capable of identifying on-bead, immobilized casein fractions with single and multiple phosphorylation sites. Although casein is a well known model phosphoprotein, the method could present some limitations in more complex biological matrices. We tested the analytical power of our procedure by submitting the eggshell proteins to HA enrichment as shown in Fig. 10. Herein, the MALDI spectrum of the HA-bound eggshell phosphoproteins was more complex than that of casein showing several overlapping signals (Fig. 10). Undoubtedly, the procedure described herein has advantages over previous methods due to its simplicity, straightforwardness and reduced work-up. Using this method, analytical tests for the detection of multiple phosphoprotein markers can be developed.

3.2. Tryptic digestion and HA-based CPP enrichment

The casein digestion yielded the expected ratio of mono- to penta-phosphorylated CPP, irrespective of protein weight (1–10 mg), bound to the same quantity of HA (1 mg, Supplemental Figure S2). In complex mixtures, the signals of highly phosphorylated peptides are usually suppressed by those of non-phosphorylated peptides [4]. The analysis of the tryptic casein digest without any prior enrichment (Fig. 6, panel a) revealed a number of prominent signals that were assigned to non-phosphorylated tryptic peptides. Four signals represented the non-phosphorylated peptides α_{s1}-CN (f91–100; m/z 1267), α_{s1}-CN (f8–22; m/z 1759), α_{s1}-CN (f23–34; m/z 1383.7) and α_{s1}-CN (f133–151; m/z 2316.1). The high level of these peptides in the washes (Fig. 6, panel b) accounts for their scarce affinity for HA. This result confirms once again the low interaction of non-phosphorylated peptides and the attenuation of C-sites by the inclusion of 20% ACN in the HA loading/washing buffer [22]. In vitro, a solution of salts in milk concentrations containing β-casein (f1–25) 4P tryptic CPP caused the spontaneous formation of calcium phosphate nanoclusters [34]. In milk, nanometer-sized clusters of calcium phosphate are sequestered within the casein micelles that have a larger colloidal dimension [35]. Therefore, CPP would be expected to compete in a similar fashion as phosphoproteins with regard to the adsorption on HA C-sites. Indeed, the MALDI-TOF mass spectra confirmed that the phosphorylated peptides were selectively sequestered by the adsorbent (Fig. 7). Trials were conducted to determine the best methods for sample and matrix preparation. The microscopic images of HA-CPP on the MALDI well (Fig. 8, panel a) did not show signs of structural modification in detectable amounts with respect to HA (data not shown). Moreover, microgranule aggregation in the matrix solution was effectively prevented by employing dried HA-CPP. The matrix solvent rapidly evaporated, yielding crystals with a large surface area. As a consequence, the HA-CPP microgranules distributed within the DHB crystal matrix showed homogeneously sized needles (Fig. 8, panel b). HA-bound CPP complexes were checked by direct MALDI-TOF analysis of either microgranules internalized into the acidified matrix or in solution. The resultant spectra showed CPP peaks at the expected m/z with only a few changes in their heights (Supplemental Figure S3). Furthermore, ionization was optimized by the presence of Arg and Lys in C-terminal positions, which facilitated the identification of tryptic peptides by MALDI-TOF. The inclusion of 1% PA in the MALDI matrix solution improved the ionization efficiency for phosphorylated peptides in the mixture [32]. MALDI signals were improved by the use of 1% PA in this study compared to 0.1% TFA. Spectra were manually annotated, and the identification of tryptic CPP was confirmed by using the FindPept tools available at the

Table 1
List of the casein fractions identified in the MALDI-TOF spectra shown in Fig. 5.

Phosphoprotein	Theoretical mass (Da)	Measured mass (Da)
κ-CN 1 $P^{a,b}$	19,037.6	19,025.7
α_{s1}-CN 8P^a	23,614.9	23,612.8
α_{s1}-CN 7P^a	23,534.9	23,532.9
β-CN A^1 5 $P^{a,b}$	24,022.4	24,020.7
β-CN A^2 5P^a	23,983.4	23,980.3
β-CN A^1 4P^a	23,942.4	23,940.0
β-CN A^2 4P^a	23,903.4	23,900.9
α_{s2}-CN 12P^a	25,308.5	25,306.5
α_{s2}-CN 11P^a	25,228.5	25,226.3

[a,b]Signals occurring in the MALDI spectra from Fig. 5, panels a and b, respectively. A^1 and A^2, bovine β-casein A^1 and A^2 variants, respectively.

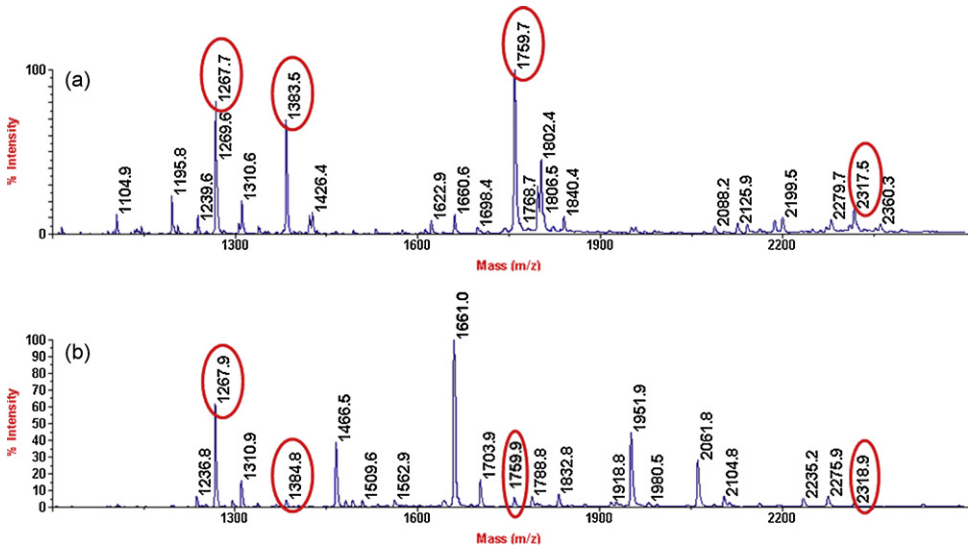

Fig. 6. Mass spectra of tryptic digests obtained after in situ hydrolysis of the HA-bound casein (a) and water soluble peptides from the last wash with 20 mM Tris–HCl, pH 7.8 containing 20% ACN (v/v) (b).

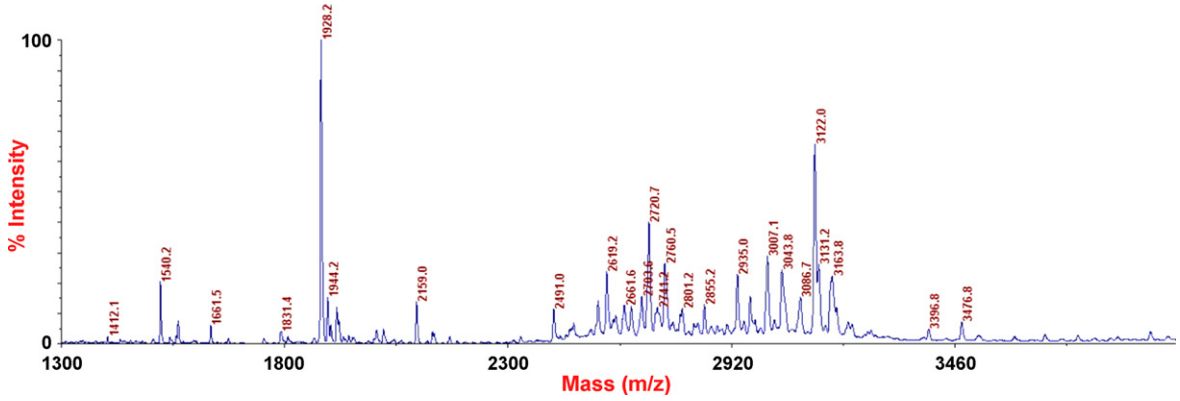

Fig. 7. Mass spectra of HA-bound CPP co-crystallized with the DHB matrix containing 1% PA. The components are identified in Table 2.

www.expasy.ch site (http://www.expasy.org/tools/findpept.html). As expected, the enriched CPP fraction contained a large number of peptides containing multiple phosphorylation sites (up to five phosphate groups/molecule, Fig. 9 and Table 2) and no non-phosphorylated peptides. The data shown in Table 2 emphasize the importance of the enrichment step for the identification of tryptic CPP. The MALDI-TOF spectra (Fig. 9) definitely confirmed the identity of casein-derived tryptic CPP complexes on the basis of the expected masses. Alternatively, alkaline phosphatase action reduced the number of candidate peptides, leading to the characteristic 80-Da mass shift that corresponds to $-HPO_3$ group loss or multiple integers. Thus, a simplified MALDI spectrum containing exclusively non-phosphorylated peptides was obtained (Fig. 9, panel b). The number of P groups was calculated by the *n* (80-Da) mass shift. The masses of native peptides were missing after the alkaline phosphatase treatment (Fig. 9, panel a), and the tryptic CPP annotated with respect to those expected (in parentheses) (Table 2) that were raised from β-, α_{s1}-, and α_{s2}-CN were 6 (2), 7 (3), and

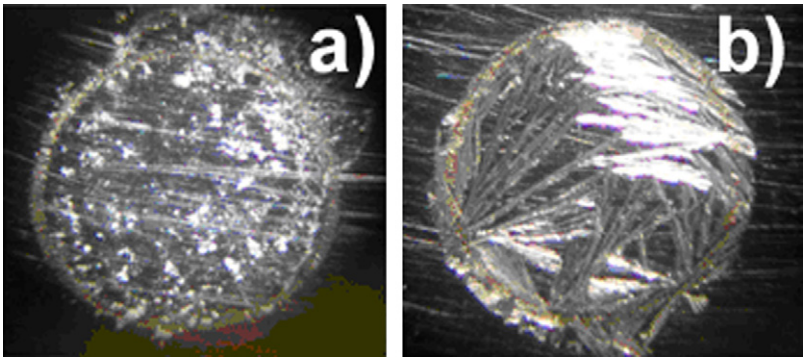

Fig. 8. Stereoscopic microscope images of HA-CPP complexes on the MALDI plate before (a) and after co-crystallization (b) of the HA-CPP sample with the DHB matrix in PA solution.

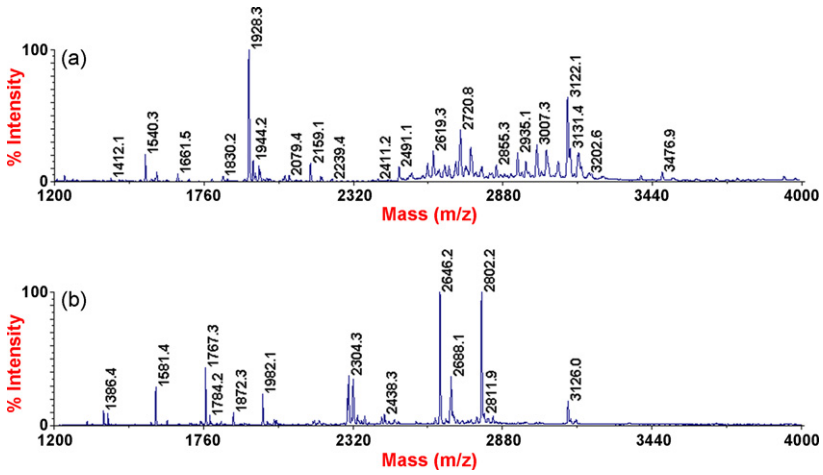

Fig. 9. Mass spectra of HA-bound CPP (a) co-crystallized with the MALDI DHB matrix containing 1% PA or (b) fully de-phosphorylated using alkaline phosphatase.

10 (5), respectively. Neither GMP nor other tryptic κ-CN peptides were detected in the casein digest. The peptide modification (e.g., partial de-phosphorylation, oxidization and N-terminal residue pyroglutamination) that occurred in the tryptic digests could explain the increased number of CPP complexes that are derived from the same phosphoprotein. For egg phosphoproteins in situ trypsinolysis allowed to obtain HA-bound phosphorylated tryptic peptides as shown in MALDI spectra (Fig. 11, Table 3). Fourteen HA-bound tryptic phosphopeptides derived from 5 phosphoproteins were identified and some were partially dephosphorylated as apparent from the characteristic 80-Da mass difference. The number of peptides is partially consistent with the long list of tryptic eggshell phosphopeptides recently reported [26]. Identification of ovocleidin-116- and osteopontin-derived phosphopeptides confirmed the co-presence of eggshell phosphoproteins with multiple phosphorylation consensus sequences. The function of these modifications is unknown although the phosphorylation sites are preserved in the multiple phosphorylated proteins [26].

Table 2
List of the HA-bound tryptic native and de-phosphorylated CPP complexes identified in the MALDI spectra shown in Fig. 9.

Molecular mass (Da) HA-bound CPP		De-phosphorylated CPP		Number of phosphate groups	CPP identity
Expected	Measured	Expected	Measured		
1410.1	1411.1	1251.3	1252.3	2	α_{s2} (f126–136)
1466.1	1467.1	1385.6	1386.4	1	α_{s2} (f138–149)
1539.4	1540.0	1378.6	1379.4	2	α_{s2} (f126–137)[a]
1660.7	1661.1.	1580.7	1581.4	1	α_{s1} (f106–119)
1846.1	1847.0.	1766.7	1767.4	1	α_{s1} (f43–58)
1927.8	1928.0.	1766.7	1767.4	2	α_{s1} (f43–58)
1943.8	1944.2	1782.7	1783.4	2	α_{s1} (f43–58) + M (O)
1952.0	1952.0	1872.1	1872.3	1	α_{s1} (f104–119)
2060.1	2062.1	1981.8	1982.2	1	β (f33–48)
2158.8	2159.1	1998.8	1998.8	2	α_{s2} (f42–59)[a]
2238.8	2239.4	1998.8	1998.8	3	α_{s2} (f42–59)[a]
2410.8	2411.3	2170.9	2170.9	3	α_{s2} (f2–20)
2432.4	2432.1	2352.1	2352.0	1	β (f30–48)[a]
2490.8	2491.2	2170.9	2170.9	4	α_{s2} (f2–20)
2539.0	2539.1	2299.0	2299.2	3	α_{s2} (f2–21)
2598.0	2598.0	2438.1	2438.3	2	α_{s1} (f37–58)[a]
2618.1	2619.0	2299.0	2299.2	4	α_{s2} (f2–21)
2678.0	2678.0	2438.1	2438.3	3	α_{s1} (f37–58)[a]
2703.1	2703.1	2304.0	2304.3	5	α_{s1} (f59–79) pyroQ
2720.1	2720.1	2321.0	2321.3	5	α_{s1} (f59–79)
2746.1	2747.1	2427.1	2427.7	4	α_{s2} (f1–21)[a]
2759.6	2760.5	2439.2	2439.5	4	α_{s1} (f62–83)[a]
2855.0	2855.0	2695.2	2695.8	2	α_{s1} (f35–58)[a]
2886.0	2886.0	2646.3	2646.2	3	β (f2–25)
2935.0	2935.0	2695.2	2695.8	3	α_{s1} (f35–58)[a]
2966.0	2966.0	2646.3	2646.2	4	β (f2–25)
3007.1	3007.0	2688.2	2688.2	4	α_{s2} (f46–70)
3088.0	3087.0	2688.2	2688.2	5	α_{s2} (f46–70)
3042.0	3043.1	2802.4	2802.2	3	β (f1–25)[a]
3122.0	3122.0	2802.4	2802.2	4	β (f1–25)[a]
3132.0	3131.4	2812.3	2812.5	4	α_{s2} (f1–24)[a]
3448.3	3445.9	3128.4	3126.4	4	α_{s2} (f1–27)[a]
3477.1	3476.9	3157.6	3157.0	4	β (f1–28)[a]
3605.1	3605.0	3285.7	3285.4	4	β (f1–29)[a]

[a] Peptide with tryptic cleavage sites that were missed.
M, methionine oxidation.

Table 3
List of the HA-bound tryptic phosphopeptide complexes from eggshell phosphoproteins identified in the MALDI spectra shown in Fig. 9.

Theoretical mass	Measured mass M[H+] (Da)	Identification	Phosphate number
1806.9	1807.4	Ovocleidin-17 (f47–62)	1P
2275.2	2276.8	Ovocleidin-17 (f53–74)	2P
2089.1	2090.1	Ovalbumin (f340–359)	1P
1826.7	1827.0	Ovocleidin-116 (f704–719)	1P
1905.6	1906.6	Ovocleidin-116 (f704–719)	2P
1986.6	1986.7	Ovocleidin-116 (f704–719)	3P
2148.3	2148.6	Ovocleidin-116 (f389–407)	1P
3137.1	3138.7	Ovocleidin-116 (f690–719)	2P
3217.1	3218.7	Ovocleidin-116 (f690–719)	3P
3297.1	3298.6	Ovocleidin-116 (f690–719)	4P
1887.9	1888.0	Osteopontin (4–19)	1P
1966.8	1967.5	Osteopontin (4–19)	2P
1957.8	1958.7	Osteopontin (8–22)	3P
2147.0	2148.6	Ovocalyxin-32 (221–241)	1P

These authors have identified 39 phosphoproteins including low abundance components containing at least 155 different phosphorylation sites with a ratio of pS/pT/pY of approximately 90:10:1. We were able to identify only 5 high abundance phosphoproteins and 14 derived phosphopeptides each containing only the phosphorylation site pS. In-depth study using fresh material and use of alternative trypsin enzymes able to access hindered sites to trypsin could afford the enrichment of more low abundance peptides.

3.3. Localization of phosphate residues in CPP

The determination of phosphorylated site(s) could be important for the establishment of the functional consequences of the phosphorylation. In many cases, peptide flexibility depends on the phosphorylated sequence (e.g., participation of the phosphorylated loop in a protein-protein interaction or reposition of loops after de-phosphorylation). Fragmentation of the MS1 precursor ions by LC–ESI–MS/MS for the selective detection of CPP ordinarily exhibits a strong neutral loss, although not for all phosphopeptides, when collisionally activated in positive mode [36]. For all chromatographic peaks, the fragmentation provided the sequence information that was used to ultimately identify the phosphorylated peptide. Supplemental Figures S4–S9). The α_{s2}-CN (f138–149) 1P peptide, which contains the residues Thr^{138} and Ser^{143} as candidates for phosphorylation, only had Ser^{143} phosphorylated (Supplemental Figure S4). Moreover, the α_{s2}-CN (f126–137) 2P peptide, which contains Ser^{129}, Thr^{130} and Ser^{131} residues, had $PSer^{129}$ and $PSer^{131}$, but not Thr^{130}, phosphorylated (Supplemental Figure S5). This result was confirmed by the presence of $PSer^{129}$, $PSer^{131}$ and non-phosphorylated Thr^{130} in the peptide α_{s2}-CN(f126-136) 2P, which was longer by one residue. Further examples of peptide sequences with a lower number of phosphorylated Ser than was expected were α_{s1}-CN (f43–58) 1P and 2P, in which the candidate residues were Ser^{46} and Thr^{49}, with the phosphorylation of both resulting in the 2P form or phosphorylation of only Thr^{49} resulting in the 1P form (Supplemental Figures S7 and S8). The co-presence of α_{s1}-CN(f35–58) 2P and 3P, α_{s1}-CN (f37–58) 2P and 3P, and β-CN (f1–25) 4P and 3P peptides (Supplemental Figure S9) provided confirmatory evidence of partially phosphorylated parent casein fractions. Given these data, the application of tandem MS/MS represents a major improvement in peptide identification with respect to probability-based Databank searches by resolving the problem posed by the presence of multiple phosphorylation sites for proper peptide characterization.

Whether the MALDI or LC–MS/MS technique was applied to large-scale phosphoproteomics, a high number of phosphorylated peptides immobilized on HA microgranules could be detected.

3.4. Quantification and recovery of CPP

In order to quantify the casein-derived tryptic CPP using HA beads, two synthetic peptides, a natural and the modified counterpart that differed by single amino acid substitution, were used as internal standards (IS) (Table 4). The bovine α_{s1}-CN (f43–58) 2P, the most abundant CPP in the phosphopeptide mixture, was chosen to evaluate the recovery of CPP. The $Ile^{44} \rightarrow Gly^{44}$ amino acid substitution in the peptide analogues were introduced to give rise to a distinct mass MALDI signal, without appreciable perturbation of the ionization efficiency. The 0.9/1.0 quasi-equimolar peptide mixture yielded a very similar MALDI ionization efficiency (Supplemental Figure S10). In this manner, the natural peptide analogues were able to function appropriately as IS. A constant concentration (10 μg/μl) of synthetic modified peptide was used to spike solutions containing different concentration of synthetic natural peptide (Supplemental Figure S11). Using synthetic natural/modified area ratio, obtained by integration via software, a calibration curve was built up as a function of their concentration ratio (Supplemental Figure S12). The combined data yielded a linear calibration curve, $y = 1.1147x + 0.1001$ ($R^2 = 0.967$) (mean of 10 replicates) for α_{s1}-CN (f43–58) 2P. Standard deviations for the measurements are indicated by error bars (Supplemental Figure S12). For quantification purposes, IS (1 μg) was added to HA-CPP complex (1 mg) and solubilized with a 0.5% aqueous PA solution. The amount of α_{s1}-CN (f43–58) 2P derived from whole casein (0.05 mg) was found to be 920 ng (Supplemental Figure S13). Therefore, the use of defatted milk tryptic digests in amount as low as 0.1–100 μl/mg dried HA allowed to detect 0.027–27 ng α_{s1}-CN (f43–58) 2P. This means ~70% CPP recovery from either milk or casein (Figs. 10 and 11).

4. Discussion

Using the procedure described above, HA-bound phosphorylated peptides did not contain any non-phosphorylated casein-derived γ_2- and γ_3-CN peptides. The new approach worked

Table 4
List of synthetic native and modified peptides used as internal standard. The amino acid residues differentiating synthetic analogue from natural tryptic CPP are underlined.

α_{s1}-CN peptide	Synthetic native peptide [M+H]+	Native sequence
(f43–58) 2P	1926.7	D<u>I</u>GS(P)ES(P)TEDQAMEDIK
α_{s1}-CN peptide	Synthetic modified peptide [M+H]+	Modified sequence
(f43–58) 2P	1870.6	D<u>G</u>GS(P)ES(P)TEDQAMEDIK

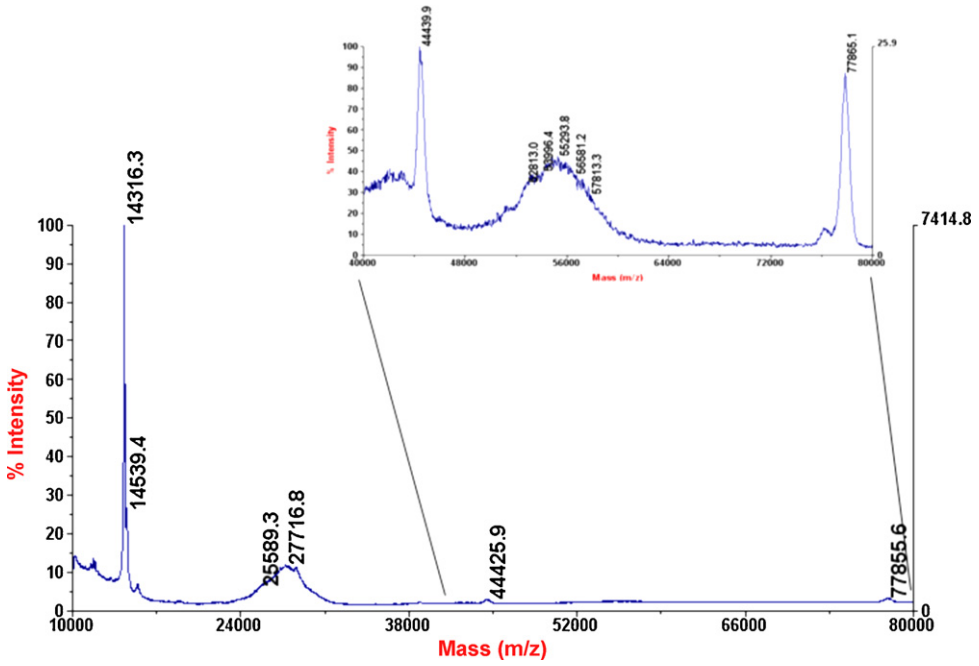

Fig. 10. MALDI-TOF analysis of eggshell phosphoproteins desorbed from HA microgranules.

well with egg proteins as MALDI spectra did not contain non-phosphorylated peptides in appreciable amounts. Therefore, the proposed procedure and our application can accommodate a wide variety of biological matrices.

Various strategies for the enrichment of phosphopeptides based on different chemical strategies have improved the overall recovery of low-abundance proteins. Amongst the strategies for selective enrichment of phosphoproteins/phosphopeptides, the IMAC (Fe^{3+})-based techniques have been claimed to allow recovery up to 90% of phosphoproteins [37]. These methods could be coupled to other fractionation steps to improve the overall recovery of low-abundance proteins. Our method is more selective, more cost-effective and faster than any IMAC-based method as it is based on one-step procedure inclusive of enrichment on HA and MALDI analysis. Using a phosphorylated peptide analogues as internal standard, α_{s1}-casein was recovered up to 70% working either on milk or derived casein.

In addition, when HA was deliberately overloaded with phosphoanalytes to saturate the adsorbent, the number of identified CPP complexes did not vary, demonstrating a lack of preferentiality amongst CPP absorption (Supplemental Figure S2). The role of exogenous acid addition is important because its omission resulted in a complete lack of phosphoprotein signals in the MALDI matrix spectra. To achieve the goal of detecting weakly phosphorylated species, very low TFA concentrations have to be used. In contrast, more tightly bound proteins could be successively eluted by increasing concentrations of TFA. As in the classical method for casein fractionation, the phosphorylated members of this protein family were fractionated into sub-mixture pools by controlling the desorption process on the MALDI plate. Basically, the in situ fractional desorption of casein is controlled by the level of phosphorylation, which also governs the affinity of CPP for HA. Trypsinolysis released CPP according to the reactivity of the lysine residues of the individual caseins that were either HA-bound or in solution in the expected number. Only κ-CN CPP was missing in the HA-bound CPP because hydrolysis of κ-CN by trypsin can be insignificant [38]. Our results demonstrated that CPP could be enriched for MS analysis within only a few minutes, showcasing the outstanding enrichment capacity of HA. Two different mass range acquiring analysis could be performed to demonstrate that the co-existing bound phosphoproteins and CPP can be identified in two separated zones of the MALDI spectra. Conversely, enzyme-mediated CPP have been isolated from cheese and characterized by MS [39,40], but the isolation procedures used in those studies were long, tedious, cumbersome and not suitable for the analysis of a large number samples. Our procedure has the advantage of capturing phosphoproteins on HA and identifying the in situ immobilized CPP by direct MALDI analysis with easy sample manipulation. This procedure circumvents the leakage of CPP by in situ desorption on the MALDI plate. Controlling the desorption process to the equilibrium point at which there are no phosphoproteins/CPP still binding HA, the procedure can be used for quantitative purposes either for CPP or non-phosphorylated peptides concentrated to a known volume, provided that internal standards are available. In summary, for the first time, HA-immobilized phosphoproteins/CPP are directly analyzed on-bead by MALDI-TOF MS without requiring a prior elution of the phosphorylated components. This procedure demonstrates several possibilities: (1) detecting phosphorylated proteins/peptides even in complex mixtures, (2) determining phosphorylated and de-phosphorylated sites, and (3) attaining information about both weakly and heavily phosphorylated peptides. All of our results indicate that this

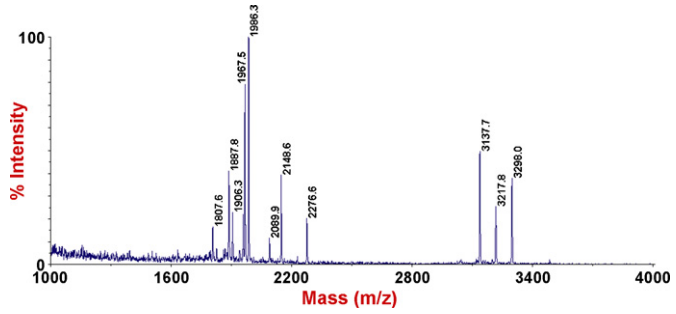

Fig. 11. Mass spectra of tryptic digests obtained after in situ hydrolysis of the HA-bound eggshell proteins. The non-phosphorylated peptides were eluted by washing buffer and the HA-bound CPP identified by FindPept tools and literature data [35].

method will allow proteins/peptides with different level of phosphorylation to be accurately measured. The time required for the HA-based procedure, from the application of the soluble protein mixture to the readout of the MALDI spectra, is less than 2 h (excluding the trypsinolysis step). The development of such a specific strategy opens up the possibility for quantitative applications in phosphoproteomics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.07.024.

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RCM

Toward milk speciation through the monitoring of casein proteotypic peptides

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The possibility of detecting extraneous milk in singles species cheese-milk has been explored. A mass spectrometry (MS)-based procedure has been developed to detect 'signature peptides', corresponding to the predefined subset of 'proteotypic peptides', as matchless analytical surrogates of the parent caseins. Tryptic digests of skimmed milk samples from four species were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. Amongst the candidate signature peptides that are able to differentiate milks from the four species, the α_{s1}-casein (CN) f8-22 peptide was selected as a convenient marker for bovine, ovine and water buffalo milk while the f4-22 peptide was selected as a marker for the two caprine α_{s1}-CN A and B variants, which differ by a Pro^{16} (B)->Leu^{16} (A) substitution. MALDI analysis of the digest allowed the detection of α_{s1}-CN f8-22 and caprine α_{s1}-CN f4-22. The accurate evaluation of caprine milk in a quaternary mixture required the development of a liquid chromatography/electrospray ionization (LC/ESI)-MS procedure. Five synthetic signature peptide analogues, which differed from their natural counterparts by a single amino acid substitution, were used as internal standards to quantify the α_{s1}-CN, which was chosen as a reference milk protein, from the different species. The limits of detection were 0.5% (1% for caprine) for either the MALDI or the LC/ESI-MS method. The isotopic-label-free quantification of isoform- or variant-specific signature peptides has disclosed a convenient approach for targeting proteins in complex mixtures. Copyright © 2010 John Wiley & Sons, Ltd.

The use of non-bovine cheese milks contaminated with cheaper all year long available bovine milk constitutes a violation of the law regulating mono-species cheese, which must be free of any foreign milk(s). In order to satisfy the growing demand of consumers within European Union or abroad, fraudulent substitution with bovine milk in the manufacturing of water buffalo mozzarella cheese is drastically increasing, especially during the summer peak lean period when milk shortages occur. To give an idea about the proportion for the adulterations of traditional Protected Designation of Origin (PDO) *Mozzarella di Bufala Campana*, according to official data from the Italian Ministry of Agricultural, Food and Forestry Policies, nearly 10% of sampled commercial cheese could be irregular for bovine milk content ranging up to 30%.

The exclusive use of sheep's milk is mandatory in the manufacturing of several traditional worldwide diffused high-grade ovine European PDO cheeses. Typical examples are Feta cheese from Greece, Roquefort from France, Idiazabal from Spain, and Pecorino Romano, Fiore Sardo, Pecorino Sardo, Pecorino Toscano and Canestrato Pugliese from Italy. Addition of cheaper cow's milk to goat's milk is another common practice in cheese-making.[1] The availability of caprine and ovine milk is also subjected to seasonal fluctuations. European legislation imposes to adequately label the combined use of sheep's and goat's milk in cheeses, which implies the loss of the PDO qualification. To protect consumers from fraudulent practices, authenticity of the cheese needs to be validated. Thus, an analytical methodology is required to verify the absence of any contaminating milk in the target milk/cheese. Intensive research has focused on the discovery of appropriate markers and relative methods for detecting adulterating milks/cheeses. For this purpose, quick immunological tests, chromatographic techniques, and electrophoretic methods have been proposed. The determination of IgG for the rapid detection of raw, pasteurized and sterile bovine milk has been recommended for immediate decision-making (~20 min).[2] An indirect, competitive enzyme-linked immunosorbent assay (ELISA) has also been developed for the detection of bovine milk in that of different species using anti-bovine IgG antibodies.[3,4] No test kit is presently available for the simultaneous detection of the four animal species in a milk sample. Bovine milk adulterating water buffalo milk has been recognized by high-performance liquid chromatography (HPLC) separation using the bovine marker β-lactoglobulin A.[5] Similar procedures have been developed for detecting bovine milk in binary[6] or tertiary milk mixtures by capillary electropho-

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resis.[7] Currently, cheese made from non-bovine milks is considered adulterated when the error in the evaluation of the extraneous milk does not exceed 1%.[8] Fast detection of bovine α-lactalbumin and β-lactoglobulin in water buffalo and ovine milk using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently been reported.[9] Heating milk over a wide range of temperatures/times denatures whey proteins while the stability of caseins is not greatly affected. For this reason, caseins rather than heat-denatured whey proteins are better targets for the detection. Proteome studies have not been widely developed as analytical tools in solving problems relevant to milk adulteration. For complex milk mixtures especially, such methods may not provide a satisfactory response in identifying all the components. Presently, the European Union reference method is based on the detection of bovine γ_2- and γ_3-CN by gel isoelectric focusing (IEF).[8] A version using anti-peptide antibodies has improved the bovine level detection to 0.2%[10] and simplified the IEF profile, by showing almost exclusively the γ_2-CN band.[11,12] A single electrophoretic test, which can specifically discriminate between ovine and caprine milks, is not available. Mass spectrometry has been increasingly used to resolve highly complex protein mixtures. On the other hand, any unfavorable analytical response requires confirmation by a subsequent technique or method. The European Union has increasingly encouraged the diffusion of mass spectrometry especially as confirmative technique to attest cheese labeling. Angeletti *et al*.[13] demonstrated the efficiency of MALDI-TOF MS for the detection of bovine caseins in water buffalo mozzarella cheese, through the relative quantification of a 'X component' exclusive of buffalo milk, later identified as the buffalo β-CN f69-209.[14] Here, a MALDI-TOF-based method for the simultaneous detection of casein-derived peptides in ternary (or quaternary) milk mixtures is presented. To date, although a MALDI-TOF MS analysis is generally applicable to the detection of any of the peptides selected as markers, an on-line liquid chromatography/electrospray ionization (LC/ESI-MS) procedure was developed to profile quaternary milk mixtures. This strategy combines milk protein trypsinolysis, MS detection of species-specific casein peptides and quantification using synthetic peptide analogues as internal standards.

EXPERIMENTAL

Materials

Milk samples were collected from local herds. All chemicals were of the highest purity commercially available and were used without further purification. Ammonium bicarbonate, trifluoroacetic acid (TFA), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (4-CHCA), and TPCK-treated trypsin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). HPLC-grade H_2O and CH_3CN were provided by Carlo Erba (Milan, Italy).

Sample preparation

Milk samples were manually expressed into sterile polystyrene containers, immediately frozen, and stored at −20°C until use, to prevent undesired proteolysis. Ovine/caprine milk, containing 0 and 1% cow's milk (CRM 599), was utilized as an external reference material and was obtained from the Institute for Reference Materials and Measurements (Geel, Belgium). Skim milk was obtained by centrifugation at 3000 *g* for 20 min at 2°C (Labofuge 400R, Heraeus Instruments, Hanau, Germany). After the fat had been removed with a spatula, isoelectric casein was prepared by precipitation, according to Aschaffenburg and Drewry.[15] Briefly, 10 mL of skimmed milk was diluted threefold with distilled water, acidified with 1 mL 10% (v/v) acetic acid and adjusted to pH 4.6 with 1 M sodium acetate buffer. Samples were kept for 30 min at 37°C and then centrifuged (4000 *g*, 5°C, 10 min). The casein precipitate was washed twice with 150 mM sodium acetate buffer, pH 4.6, washed twice with cold acetone and finally with diethyl ether. The air-dried isoelectric casein was then stored at −20°C until analysis. Quaternary mixtures of the four raw milks (water buffalo, caprine, ovine and bovine milk) were obtained by mixing the same volume of milk. To determine the limits of detection, water buffalo milk was added to 0.5, 1.0, and 5.0% of each foreign milk sample. The ternary mixtures of milk were obtained similarly, except that caprine milk was omitted. Isoelectric casein from the milk mixtures was obtained as described above. Mixtures of trypsinized milk proteins were also prepared by mixing together trypsinized mono-species milks.

Tryptic digestion of casein

Dry isoelectric casein (10 mg) was dissolved in 4 g/L ammonium bicarbonate at pH 8.5. TPCK-treated or sequencing-grade modified trypsin, dissolved in the same buffer, was added to the casein solution at an enzyme/substrate (E/S) ratio of 1:50 (w/w) and incubated for 4 h at 37°C. Six samples of casein tryptic digests were prepared by varying the reaction time and the trypsin-to-casein ratio (w/w). The enzymatic reaction was monitored at 1, 2, 3, and 4 h at an E/S ratio of 1:150, 1:100, 1:50, 1:40, 1:30, and 1:20. Samples were freeze-dried and then re-dissolved in 0.1% TFA at a 2 mg/mL concentration.

Reversed-phase (RP)-HPLC/ESI-MS analysis

RP-HPLC-ESI/MS analysis was carried out using an HP1100 modular chromatographer (Agilent Technology, Palo Alto, CA, USA) coupled on-line to a single-quadrupole MSD (Agilent) apparatus. Aliquots (15 μL) of the casein tryptic digests were diluted to 100 μL with 0.1% TFA and loaded onto a Vydac reversed-phase C_{18} column (250 mm, 2.1 mm i.d.). Peptide mixtures were separated by applying a linear gradient from 5% to 60% of solvent B (0.1% TFA in acetonitrile, v/v) over 60 min at a constant flow rate of 0.2 mL/min; solvent A was 0.1% TFA in water (v/v). Column effluent was monitored by UV detection at 220 nm. Mass spectra were acquired with a scan range from *m/z* 400–2300 at a scan cycle of 4.90 s per scan with a 0.1 s inter-scan delay. The source temperature was 180°C. Spectra were acquired in the positive ion mode, while the capillary voltage and the cone voltage were set to 3.6 kV and 40 V, respectively. N_2 was used as both the drying and nebulizing gas at a flow rate of 10 L/min. LC/MS patterns were elaborated using the LC/MSD

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ChemStation Data Analysis Software A.08.03 supplied with the instrument, which allows integration and calculation of the area of the ion extracted peaks.

MALDI-TOF MS

MALDI-TOF MS analyses were performed on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with an N_2 laser ($\lambda = 337$ nm). For the analysis of peptides resulting from the hydrolysis of proteins, 4-CHCA was used as the matrix, which was prepared by dissolving 10 mg of 4-CHCA in 1 mL of aqueous 50% (v/v) acetonitrile containing 0.1% (v/v) TFA. The instrument operated with an accelerating voltage of 20 kV. External mass calibration was performed with the signal of the matrix dimer at $[M+H]^+ = 379.05$ and with the monoisotopic masses of peptide standards, including angiotensin I ($[M+H]^+ = 1296.68$), ovine α_{s1}-casein 1-23 peptide ($[M+H]^+ = 2764.55$) and bovine insulin ($[M+H]^+ = 5730.61$), thereby achieving an accuracy in the measurement of the peptide mass of better than 75 ppm. For the analysis of peptides, the mass spectra were acquired in the positive reflector ion mode using delayed extraction (DE) technology. In the reflector ion mode, resolution at the full width/half maximum of the peak was normally $\geq$5000. Higher mass ranges were explored operating in the linear positive ion mode, using SA (10 mg/mL in 50% acetonitrile/0.1% TFA) as the matrix. Raw data were elaborated using the software program Data Explorer version 4.0 (Applied Biosystems).

Peptide recognition

Signals in the mass spectra were assigned to the corresponding tryptic peptides according to the expected molecular mass from the known casein sequences, taking into account enzyme specificity, using several bioinformatics tools, such as the GPMAW 7.0 software (Lighthouse Data, Odense, Denmark) or other on-line resources.

Synthesis of peptide analogues

All peptides were synthesized by solid-phase methodology using the Fmoc (9-fluorenylmethyloxycarbonyl)/*t*-butyl strategy on a Pioneer peptide synthesizer (Synthesis System 9050 instrument; PE-Biosystems, Framingham, MA, USA). For quantification of bovine, water buffalo, sheep, and goat α_{s1}-CN, the synthetic peptides shown in Table 1 were used as internal standards. The identity of peptides purified by RP-HPLC was assessed by MALDI-TOF MS. The convergent indications of retention time and molecular mass helped to localize the signature peptide of goat α_{s1}-CN variant A within the LC/MS profile, while the corresponding area of the extracted ion was used for quantification.

RESULTS AND DISCUSSION

In proteomic experiments, the mass spectrometric analysis of proteolytic digests rarely provides complete protein coverage. Peptides of interest, predicted on the basis of cleavage enzyme specificity, often escape detection whereas other fragments originating from diverse protein regions exhibit an intrinsically higher detectability. Although information about protein isoforms or variants can be lost, the detection of proteotypic or 'signature' peptides is sufficient to establish protein parentage, whether or not the protein sequence has previously been reported. Monitoring a signature peptide as an analytical surrogate for a specific protein has the advantage, in general, of not requiring the proteolytic digest to be fractionated prior to MS, as peptides are detected at higher specificity and sensitivity than their parent proteins. One of the challenges in the application of this process involves the selection of a proteotypic set of peptides that best represents the target proteins. Therefore, verification of the authenticity of a single-species milk sample would necessitate the *in silico* prediction and the empirical monitoring of proteotypic peptides by the tryptic digestion of bovine, caprine, ovine and water buffalo casein.

Four species casein trypsinolysis

A preliminary study was conducted to define the most convenient operating conditions for the casein trypsinolysis. After a 240 min incubation at a trypsin-to-casein ratio of 1:40, casein was completely hydrolyzed. The equivalent effect, without a significant change in the MALDI mass spectrum of the hydrolysate, was obtained using a trypsin-to-casein ratio of 1:20 for 60 min. Longer incubation may yield non-specific cleavages by trypsin that are incompatible with an analytical procedure that requires the shortest time-to-result possible. To overcome these shortcomings, we compared the performance of TPCK-treated and sequencing-grade modified trypsin during casein digestion and probed for the formation of autolysis products. As expected, proteomic grade trypsin minimized autolysis and enabled higher enzyme-to-substrate ratios (e.g., 1:20 to 1:150), thus enhancing digestion efficiency. The exposure to higher sequencing-grade trypsin concentrations that yielded the highest level of peptides during hydrolysis involved a time period for incubation of at least about 1 hour at an enzyme-to-protein ratio of 1:20 (w/w).

Table 1. Analytical data from the synthesized peptides used as internal standards. The amino acid residues differentiating synthetic analogues from natural tryptic peptides are underlined

α_{s1}-CN peptide	Native peptide $[M+H]^+$	Sequence	Modified sequence	Synthetic peptide $[M+H]^+$
Bovine 8-22	1759.95	HQGLPQEVLNENLLR	HQGGPQEVLNENLLR	1703.88 (Gly^{11})
Water buffalo 8-22	1687.92	HQGLPQGVLNENLLR	HQGGPQGVLNENLLR	1631.86 (Gly^{11})
Ovine Var. C 8-22	1718.92	HQGLSPEVLNENLLR	HQGGSPEVLNENLLR	1662.86 (Gly^{11})
Caprine 4-22 Var. B	2164.13	HPINHQGLSPEVPNENLLR	HPINHQGGSPEVPNENLLR	2108.06 (Gly^{11})
Caprine 4-22 Var. A	2180.16	HPINHQGLSPEVLNENLLR	HPINHQGLSPEALNENLLR	2152.16 (Ala^{15})

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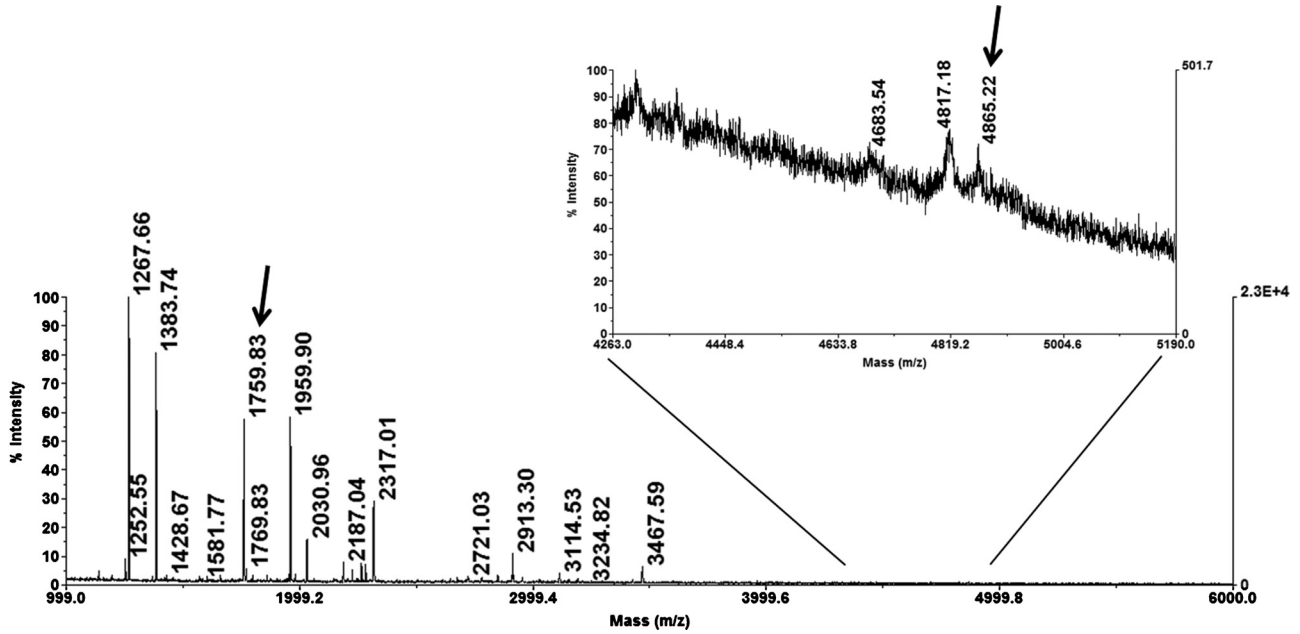

Figure 1. MALDI-TOF MS analysis of the tryptic digests of bovine casein. The peptide ion signals for α_{s1}-CN f8-22 (MH^+ 1759.83) and β-CN f53-97 Var. A^1 (MH^+ 4865.22, inset) are indicated by arrows.

The direct MALDI mass spectrum of the tryptic digest of bovine casein (Fig. 1) uncovered several signals, indicated by arrows on the peak tops, which were selected as possible signature peptides for bovine casein. MALDI MS screening was reiterated for caseins from the other three species, thus defining a restricted set of intense and unique signature peptides (Table 2). Due to the enhanced ion production of the Arg-terminating tryptic peptides,[16] the spectrum appeared dominated by the α_{s1}-CN-derived peptides, even though α_{s1}-CN and β-CN occur approximately at the same level of abundance in whole casein. β-CN contains three Arg residues, among which Arg^1 is in part released as a free amino acid by tryptic cleavage or integrated, together with Arg^{25}, in a hardly detectable fourfold phosphorylated peptide. The Arg-containing peptide ion signal of β-CN f191-202 at *m/z* 1383.74, generated by a chymotrypsin-like cleavage, is among the most prominent signals. On the other hand α_{s1}-CN contains six Arg residues, five of which (except Arg^1) generate peptides of optimal molecular size to be detected by both MALDI or ESI-MS.

In separate experiments, defatted milk was directly incubated with trypsin for various periods of time by adjusting the enzyme-to-casein ratio. None of the spectra for defatted milk tryptic digests contained whey protein-derived signals at a significant intensity to interfere with the α_{s1}-CN signature peptides (Supplementary Fig. S1, see Supporting Information). This may be mainly attributed to either the proportion of whey proteins to casein (~20:80) or the lower susceptibility of non-casein proteins to proteases. Once the origins of the prominent signals from the signature peptides had been confirmed, the most convenient operating conditions for mass spectrometric milk speciation using direct

Table 2. List of measured and expected masses of eligible signature tryptic peptides from the four casein families in the bovine, water buffalo, ovine and caprine species. The most widespread genetic variants of caseins have been considered. Peptides in bold are prominent in the MALDI spectra of the tryptic digests

Molecular mass						
Measured Mass (MH^+)	Expected Mass (MH^+)	Peptide	Bovine	Water buffalo	Sheep	Goat
1384.92	1384.72	α_{s1}-CN(23-34)	**1384.92**	**1384.92**	-	-
1306.74	1306.71	α_{s1}-CN(23-34)	**-**	**-**	**1306.74**	**1306.74**
1759.13	1758.94	α_{s1}-CN (8-22)	**1759.13**	**1686.92**	**1718.92**	**-**
2164.36	2164.12	α_{s1}-CN (4-22)	**-**	**-**	**-**	**2164.36 Var. B**
2181.14	2180.15	α_{s1}-CN (4-22)	**-**	**-**	**-**	**2181.14 Var. A**
2062.12	2060.84	β(33-48) P*	**2062.12**	**2092.14**	**2062.12**	**2062.12**
2710.62	2709.40	α_{s2}-CN (92-113)	2710.62	2710.62	-	-
2693.42	2693.37	α_{s2}-CN (93-114)	-	-	2710.62	**2693.42**
2866.72	2865.50	α_{s2}-CN (92-114)	2866.72	2866.72	-	-
2849.52	2849.47	α_{s2}-CN (93-115)	-	-	2866.72	2849.52
3113.72	3112.70	β-CN (69-97)	3113.72	**3126.72**	3152.82	3152.82
4839.32	4838.63	β-CN (53-97)	**4864.62** A^1 4824.72 A^2	4852.82	4839.32	4839.32

* P: phosphorylation.

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trypsinolysis of defatted milk were a time period for incubation of at least about 1 h at 37°C at an enzyme-to-casein ratio 1:20.

Selection of signature peptides

Figure 2 reports the four-species sequence alignments of the most frequent genetic variants of α_{s1}-CN and β-CN. To select convenient proteotypic peptides, α_{s1}-CN and β-CN have been preferentially considered due to their prevalence in milk. Table 2 reports the list of eligible proteotypic peptides, based on sequence alignments of the four casein families from the four species. Here the expected signature and detected peptides for milks from different species are compared. While trypsin releases the α_{s1}-CN f8-22 from bovine, ovine and buffalo milk, the $Lys^4 \rightarrow Asn^4$ substitution in caprine α_{s1}-CN results in the production of the longer tryptic peptide f4-22. Furthermore, the caprine α_{s1}-CN f4-22 peptide could occur under two distinct molecular masses, i.e., $[M+H]^+$ 2164.13 (variant B) or 2180.16 (variant A) (Fig. 3). Therefore, bulk caprine milk is typified by at least two signature tryptic peptides, i.e., α_{s1}-CN f4-22 Pro^{16}(A) and Leu^{16}(B), because of the high frequencies of the parent proteins.[15,16] The measured mass of α_{s1}-CN f4-22 A and B was consistent with that expected. Thus, the α_{s1}-CN f4-22 and f8-22 peptides were the most convenient signature peptides for assessing milk adulteration because (i) they have

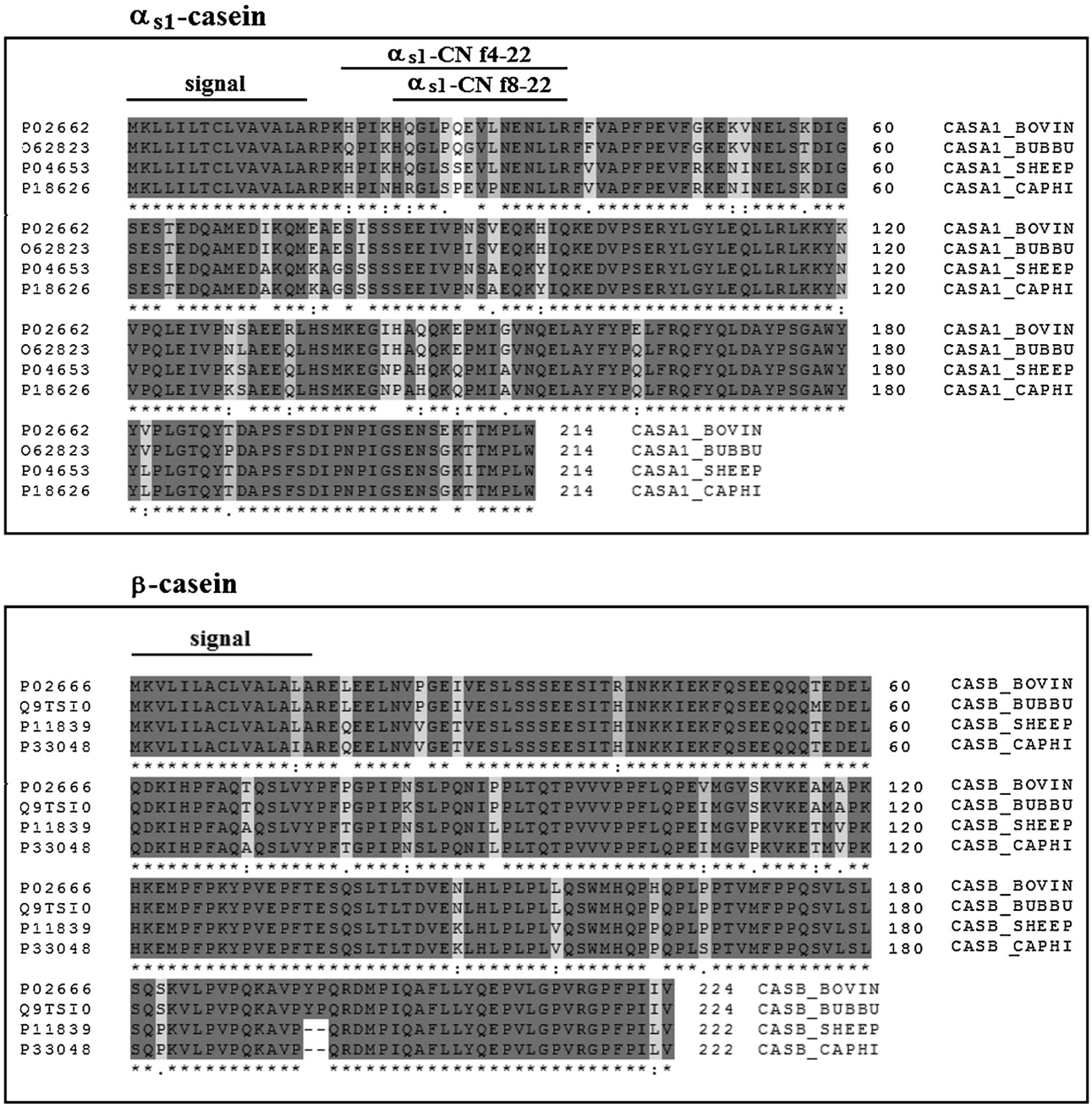

Figure 2. Sequence alignment of the four-species α_{s1}- and β-casein (ClustalW[25]). Proteotypic α_{s1}-casein peptides are to be localized into the shadowed region. According to the Uniprot database, alignment of primary structure takes into consideration the most frequent α_{s1}- and β-casein variants. The high degree of homology among the casein sequences restricts the choice of the signature peptides to a few options.

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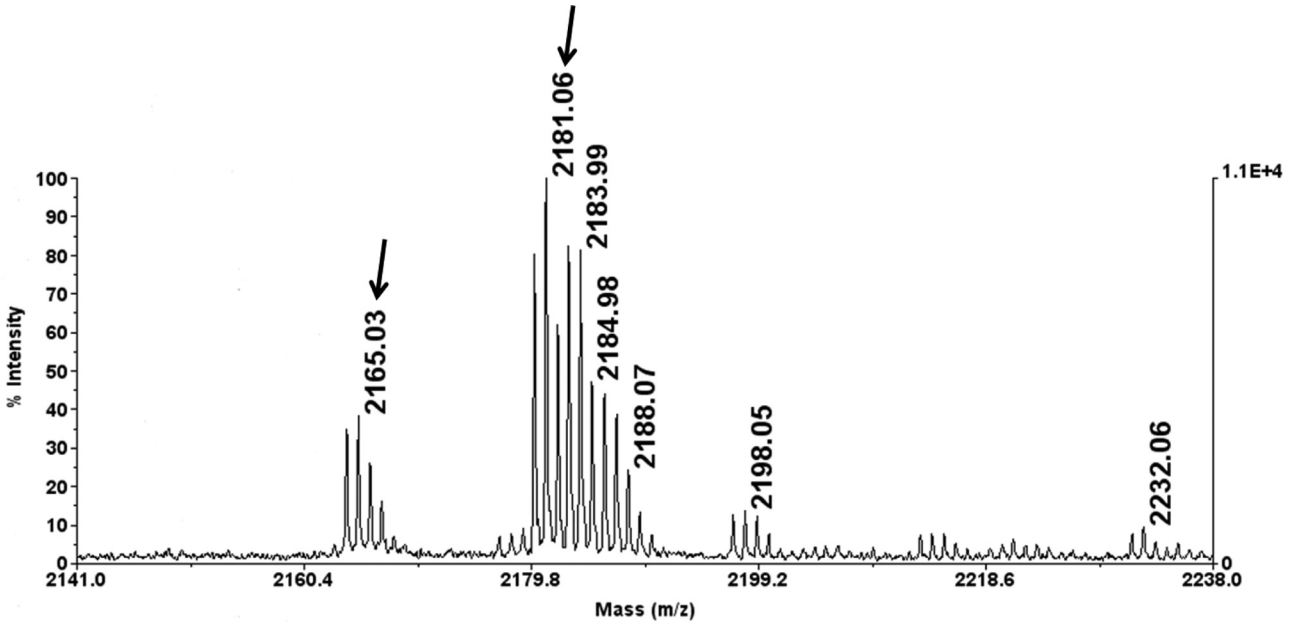

Figure 3. Caprine α_{s1}-CN f4-22 occurred under two distinct molecular masses, i.e., $[MH]^+$ 2164.13 (variant B) and 2180.16 (variant A), in the reflector ion mode MALDI-TOF spectrum for the tryptic digest of defatted goat milk. The isotopic signal cluster of α_{s1}-CN f4-22 Var. A partially overlaps that of β-CN f114-132 ($[MH]^+$ 2183.07).

molecular mass values that are different enough to distinguish casein from the four species; (ii) they are derived from the major α_{s1}-CN fraction; (iii) they have strong ionization efficiency values, which were empirically determined; (iv) their relatively small size makes it suitable to obtain low-cost synthetic analogue peptides; (v) they lack, apart from rare exceptions, post-translational modifications, which would complicate quantification; and (vi) they have very few neighboring basic amino acids on either side of the peptide (e.g., KRXX or XXRK), which might yield trypsin-missed cleavages. MALDI spectra for the tryptic digests of the four single-species milks are shown in Figs. 4(a)–4(d). Compared with one another, a uniform degree of digestion, regardless of the milk species, was detected.

Because most of the caseins occur in different genetic variants, bulk tank milks, composed of a mixture of a number of individual milks, would give tryptic peptide patterns that were more complex than the individual samples carrying one or two genetic variants. As an example, a gel isoelectric focusing analysis for the bulk milk sample of our experiments was found to contain exclusively ovine α_{s1}-CN C (result not shown), although five variants (A to E) have been described for the ovine α_{s1}-CN locus.[17] However, it must be considered that some alleles have low frequencies and are undetectable in bulk milk.[18] Allele A, which differs from the C counterpart by the $Pro^{13}\rightarrow Ser^{13}$ substitution, does not exceed 0.6%.[17] A search for the signals from the predicted tryptic peptides of the A variant was also conducted within the MALDI spectra taking into account the two possible forms of the phosphorylated or non-phosphorylated α_{s1}-CN f8-22 peptide (*m/z* 1788.86 and 1708.90, respectively). Only the variant C-derived peptide signal at *m/z* 1718.92 was detected in the bulk milk sample. This finding is consistent with a missing phosphorylation site on the α_{s1}-CN f8-22 peptide derived from the C variant, justified by the unfavorable $S^{12}PE^{14}$ consensus sequence for casein kinase recognition.[19,20]

Clearly, the presence of additional protein variants would necessitate the consideration of other possible proteotypic peptides.

Quantification and limit of detection of signature peptides in bulk milks by MALDI-TOF MS

Comparisons of the MALDI-TOF spectra allowed the discrimination of signature peptides according to their milk species. As an initial test, spectra were acquired for tryptic digests of the four-species milks mixed in equal volumes (Fig. 5(a)). Then, quantitative evaluations were carried out by MALDI-TOF MS for the four milks in mixtures, comparing the concentrations of the proteotypic peptides and their synthetically modified counterparts, which were being used as internal standards (IS) (Fig. 5(b)). The $Leu^{11}\rightarrow Gly^{11}$ amino acid substitution in the peptide analogues was introduced to give rise to a distinct mass MALDI signal, with respect to the natural peptides, without appreciably perturbing the ionization efficiency of the peptide mixtures. This represents the ultimate proof that the analogues of the natural peptides function appropriately as IS. Afterwards, for quantification purposes, fixed amounts of IS were added to the tryptic digests of milk samples and the individual peak heights compared to that of the respective native tryptic proteotypic peptides. Because of the non-predictable peptide-dependent MALDI MS ion production, the relative or absolute concentration of proteotypic peptides cannot be directly inferred from the signal intensity.[21] The incorporation of IS into the MALDI matrix/analyte mixture provided a means to achieve a quantitative estimation of the proteotypic peptides. In contrast to other approaches, in which whole sets of tryptic peptides are used as IS to determine the relative changes of protein

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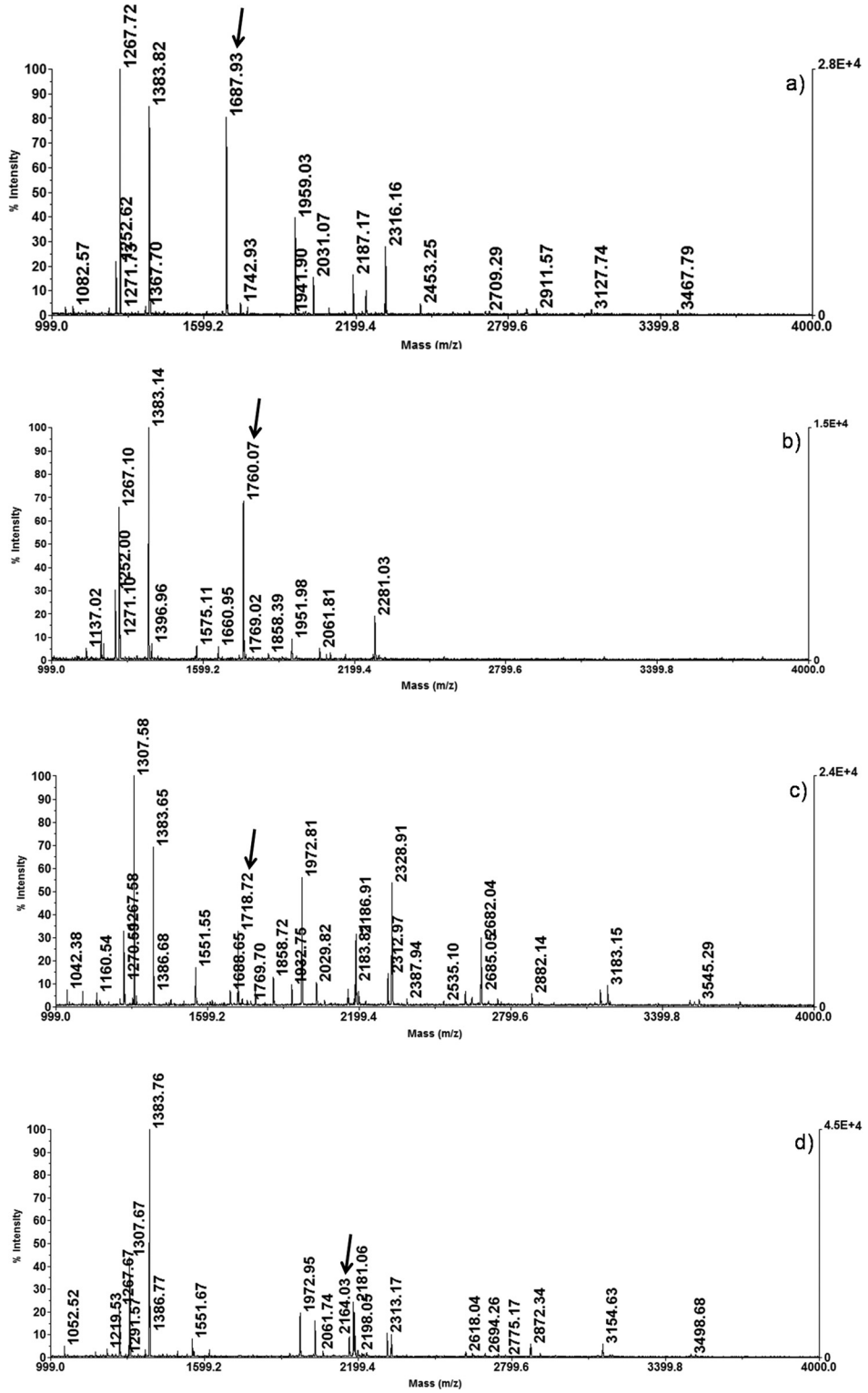

Figure 4. MALDI-TOF MS identification of the signature peptides in the tryptic digests of single-species defatted milk. α_{s1}-CN f8-22 in (a) water buffalo, (b) bovine and (c) ovine milk digests, and α_{s1}-CN f4-22 Var. A in caprine (d) milk digests are indicated by arrows.

amounts,[21] our strategy did not require complicated algorithms for normalization. It must be noted that a preliminary examination confirmed that equimolar amounts of each synthetic peptide reproducing natural sequence and its modified counterpart (Table 1) gave rise to two nearly identical MALDI intensity peaks (Supplementary Figs. S2–S5, see Supporting Information). This means that if IS peptides are added to a milk sample, at a value that corresponds to 1% adulteration, the intensity of the proteotypic tryptic peptide signal from any of the four species could be compared in terms of quantity to the adulterated milk (lower, higher or equal to 1%). The latter represents the maximum error allowed according to the European Union Official Method of analysis for assessing adulteration. To determine the limit of detection

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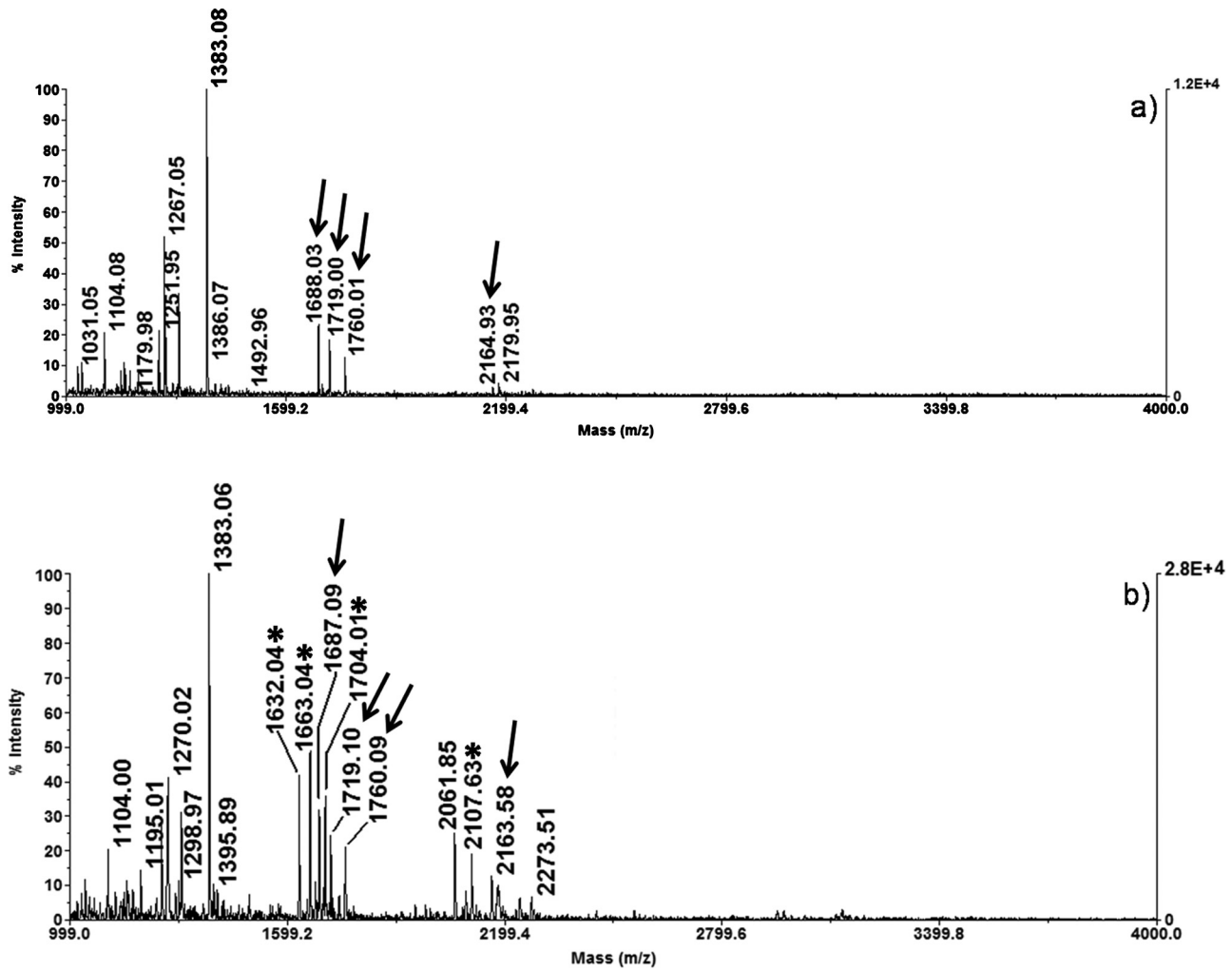

Figure 5. MALDI-TOF MS spectrum of tryptic digests from an equi-volume quaternary milk mixture (a); spiked with synthetic modified analogues that were used as internal standards (b). Natural proteotypic peptides are indicated by arrows, while asterisks label the synthetic analogue counterparts containing the $Leu^{11} \rightarrow Gly^{11}$ amino acid substitution.

(LOD) of the analytical procedure, assays were performed involving the spiking of water buffalo milk with known scalar amounts of extraneous milks, i.e., 5, 1, or 0.5% simultaneously. This range of additions reproduces the actual adulterated milk lots that are frequently delivered to cheese plants. We verified that the progress of the tryptic digestion was independent of the concentration of adulterating milk within the explored 0.5–5% range. MALDI-TOF MS was able to detect bovine and ovine milk, distinct from their analogue water buffalo counterpart, until the 0.5% adulteration threshold (Supplementary Fig. S6, see Supporting Information). However, the LOD for caprine milk was 1% (Supplementary Fig. S7, see Supporting Information). This is likely attributable to (i) the casein composition of caprine milk, which has a lower α_{s1}-CN content compared to that of the other species and (ii) the splitting of the signature tryptic α_{s1}-CN f4-22 peptide into two signals (α_{s1}-CN f4-22 A and B variants) because of the amino acid substitution Pro^{16}(A)$\rightarrow Leu^{16}$ (B), each with high, though different, frequencies within the caprine breeds of different countries.[22,23] The rare caprine α_{s1}-CN C variant is restricted to hardly any breeds.[24] The derived tryptic peptide containing the amino acid His^{8}(C)$\rightarrow Ile^{8}$ (B) substitution was not found in the bulk milk used in this study. As this finding can undermine the evaluation issue, the reliability of the MALDI-TOF method was assessed by characterizing systematically varied composition samples. To this end, the natural tryptic α_{s1}-CN peptide, which forms in quantities that correspond to the degree of adulteration in the main dispersing milk, was evaluated using the four synthetic peptide analogues as IS. The areas of the MALDI MS signals, obtained by integration via software, were used to build up a calibration curve as a function of the signature peptide concentration. The ratio of the peak height of each member of a species of interest to that of the corresponding IS was plotted as a function of the percentage of water buffalo milk adulteration. The combined data yielded calibration curves with equations $y = 3.9605x$ ($R^2 = 0.9996$) and $y = 4x$ ($R^2 = 0.9999$) for bovine and the other two milks (Supplementary Figs. S8–S11, see Supporting Information), respectively, showing a nearly optimal linearity. The absolute amount of total α_{s1}-CN for each contaminating species was calculated by measuring the peptide signal that was generated by the parent protein, using the peptide signature as reference.

The calibration curve (mean of 10 replicates) was used to measure the amount of peptide (expressed in ng), yielding reproducible results for the α_{s1}-CN content over at least one order of magnitude, between 0.02 (or below) and 0.2 ng/μL.

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Standard deviations for the measurements are indicated by error bars (Supplementary Figs. S8–S11, see Supporting Information). The lowest concentration of detectable peptide was 0.0065 ng or 5 fmol, at a signal-to-noise (S/N) ratio of approximately 20:1 (in linear mode) and 10:1 (in reflector mode). The weight of α_{s1}-CN f8-22 from the extraneous species, evaluated according to the relevant calibration curve, was directly converted into adulteration percentage. Consider, for example, an experiment where the adulteration of sheep milk with bovine milk has been measured in very low percentages (Supplementary Fig. S12, see Supporting Information).

The described procedure allows the estimation of individual α_{s1}-CN peptides in milk mixtures. This was not exactly equivalent to determining the percentage of the contaminating milks because the α_{s1}-CN peptides contributing to bulk milks are either species- or sample-dependent. Therefore, for the calculation it was necessary to assume a fixed α_{s1}-CN-to-milk ratio or to use a standard mixture of ovine/goat and bovine milk (1% batch) as a reference sample (CRM 599, BCR) (Supplementary Fig. S12, see Supporting Information). However, neither the α_{s1}-CN nor total casein contents of single milks in reference samples are specified. The availability of such standard samples (or others) minimally biases the quantification while the systematic errors of the measurement are eliminated after correcting for the spectral background with non-adulterated reference materials (0% batch). The significant difference between caprine and other signature peptides give clearly identifiable and unique signals even in the tryptic digests of defatted milk. However, we found that the isotopic cluster of the caprine signature peptide at $[M+H]^+$ 2180.16 (variant A) partially overlaps the caprine β-CN f114-132 peptide ($[M+H]^+$ 2183.07). Moreover, unmodified trypsin generated 50-69 peptide at $[M+H]^+$ 2163.05 by autolysis, which interfered with the signature peptide α_{s1}-CN f4-22 variant B signal at $[M+H]^+$ 2164.13. Thus, because of the many interfering components, the MALDI-TOF-based procedure could overestimate the level of caprine milk in adulterated milks.

LC/ESI-MS-based strategy for quantification of goat α_{s1}-CN

To overcome this drawback, an LC/ESI-MS-based protocol was developed to separate the caprine α_{s1}-CN f4-22 variant A and B peptides. Trypsinized four-species milk was spiked with diverse quantities of the caprine α_{s1}-CN f4-22 variant A analogue (MH^+ 2152.16, see Table 1). By LC/ESI-MS analysis the mixtures gave rise to a total ion current (TIC) pattern from which the ions of the natural α_{s1}-CN f4-22 variant A and the relative IS were easily localized by ion extraction (Supplementary Fig. S13, see Supporting Information). Retention times for the target peptides were determined earlier by separate injections of the respective synthetic peptides. The convergent indications of chromatographic retention time and molecular mass of the components eliminated the possible interfering peptides. Interestingly, the evaluation of goat milk in complex mixtures was then unaffected by the typology of commercial trypsin. The peaks of the extracted ions were integrated and the area ratios were plotted against the IS concentration (Supplementary Figs. S14–S18, see Supporting Information). Moreover, the area ratios of the natural peptide α_{s1}-CN f4-22 variant A and the IS ions, for equal volumes of four-species milks, were plotted against the IS concentrations (Supplementary Figs. S14–S18, see Supporting Information).

In both cases, a usable linear relationship was found in the investigated concentration range, as already demonstrated by the relative calibration curves. In an analogous way, a linear response was also found for the natural peptide α_{s1}-CN f4-22 variant B, as shown in Supplementary Figs. S19 and S20 (see Supporting Information). In this case, the amino acid substitution $Pro^{16}\rightarrow Leu^{16}$ did not affect the ionization efficiency of the peptides. Therefore, the use of a single synthetic proteotypic peptide analogue allowed for the evaluation of both caprine α_{s1}-CN variants. The results clearly indicate that LC/ESI-MS, when used to test very small volumes of goat's milk, has a LOD as low as 1% (Supplementary Fig. S18, see Supporting Information). The MALDI-based procedure, simultaneously monitoring multiple peptides, has allowed the detection of four signature peptides (one for each species). Using LC/ESI-MS, five proteotypic peptides were distinctly detected, of which two were derived from caprine α_{s1}-CN variants. In principle, a single LC/ESI-MS analysis of a tryptic digest of a milk sample spiked with the entire set of synthetic analogues would allow the one-step milk speciation also encompassing quantitative determination. The practicability of such a strategy will be the focus of further investigations. In any case, the MALDI-based screening offers a series of evident advantages, such as the quickness of the analysis, the minimal sample handling, the immediateness of the data interpretation, all combined with a specificity and robustness of the analytical response comparable to that from LC/MS. For forensic purposes, both the MS-based methods (i.e., MALDI and LC/ESI-MS) provided precise and reliable quantitative results.

In effect, the presence of extraneous caseins in milks that are suspected of adulteration could be confirmed by a longer list of candidate proteotypic peptides (Table 2). In other words, to prevent false positive responses, multiple peptides could be detected for each parent casein. The possibility of quantifying samples based on additional proteotypic peptides also permits the minimization of the magnitude of fluctuations depending on several experimental factors, including changes in ionization efficiency over time and matrix effects leading to ion suppression or enhancement from co-eluting components, that cannot be accurately controlled in MS-based analyses. In this manner, the MS-based procedures in the above outlined versions become effective in discriminating pure or contaminated milk samples. The excellent specificity of proteotypic peptides opens the possibility of universally applying MS-based methods to any protein of any food as long as the protein sequence is known. Therefore, provided that opportune IS are available, each of the selected proteotypic peptides can potentially serve as a probe for protein abundance within a complex sample. The sensitivity and specificity of the MS-based detection of signature peptides could be further enhanced by the selected reaction monitoring technique.[21]

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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Chapter 1

Bioactive Casein Phosphopeptides in Dairy Products as Nutraceuticals for Functional Foods

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Additional information is available at the end of the chapter

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1. Introduction

Nutraceuticals, a term combining the words "nutrition" and "pharmaceutical", is *a food or food product that provides medical or health benefits including the prevention and treatment of diseases*. A functional food essentially provides a health benefit beyond the basic nutrition, whereas nutraceutical is used to describe an isolated or concentrated molecular extract of bioactive compounds. Milk is a unique food providing a variety of essential nutrients necessary to properly fuel the body. Inactive food proteins can release encrypted bioactive peptides *in vivo* or *in vitro* by digestive enzymatic hydrolysis. Bioseparation protocols offer unique possibilities for a number of application areas, *e.g.,* hydrolyzate-based nutraceutical ingredients for functional foods, dietary supplements and medical foods.

Many ingredients are included in the wide range of nutraceuticals, such as essential amino acids, conjugated fatty acids, vitamins, minerals and polyphenols. They have already been patented and incorporated in functional foods and nutritional beverages. Such components are believed to improve overall health and well-being, reducing the risk of specific diseases or minimizing the effects of other health concerns. However, milk is devoid of flavonoids, the most common group of vegetable polyphenolic compounds, which act as antioxidants and free radical scavengers. In contrast, the ingestion of soy and green tea extract may reduce the risk of developing prostate cancer and may protect against various other types of cancer [1-2]. An interesting patented invention has made available an extended-release form of polyphenols and riboflavin (vitamin B2) coated with methylcellulose [3]. Coating slows down the release of polyphenols in the nutraceutical preparation [3]. Possible applications of coating technology could be extended to all of the bioactive peptides susceptible to digestive enzymes. For example, glutathione can be maintained in human blood at normal levels by supplying it as dry-filled capsules [4]. Nutrients and bioactive compounds may be

microencapsulated by using mixtures of proteins or peptides and oils. Encapsulation of ω-3 *fatty acids* (FA) enhances the stability and bioavailability of bioactive food ingredients [5]. By these means, new transparent bioavailable beverages containing ω-3 rich oils, phospholipids and minerals in an oxidatively stable food system were created [6]. Iron or calcium casein phosphopeptides (CPP) were embedded in the chitosan lactate fiber as a protective agent against oil oxidation [6]. A recent patent relates to a nutraceutical composition consisting of a sweetener admixture for food or drink comprising calcium lactate, calcium acetate, vitamin D3 and sucralose (for fortified zero calorie formulation) or sugar (white/brown; for fortified sugar preparation) [7]. Milk proteins playing a physiological role include proteins such as β-lactoglobulin, α-lactalbumin, immunoglobulins, lactoferrin, heat-stable proteose peptones, serum albumin and various acid soluble phosphoglycoproteins. Casein (CN), representing 80% of total milk proteins, consists of four α_{s1}-, α_{s2}-, β-, and κ-CN families in the approximate ratio 38:11:38:13. Research performed in recent years has shown that caseins and whey proteins are rich in encrypted biologically active peptides such as exorphins (casomorphins), CPP and immunopeptides [8]. The peptides are released by enzymes in the form of mature bioactive components or the precursors thereof [9]. They are 3- to 20-residue long peptides released during *in vivo* gastrointestinal digestion. Historically, the opioid peptides were discovered as the result of a systematic search for exogenous substances, namely (i) first discovered in 1979, opioid agonist peptides derived from milk proteins were characterized in 1986; (ii) in 1982, angiotensin-converting enzyme (ACE)-inhibitory peptides were found to be antihypertensive peptides; (iii) then, fibrinogen-like sequences with antithrombotic activity were found; (iv) phagocytic activity and lymphocyte proliferation of numerous immunomodulating peptides were observed; (v) CPP facilitating the absorption of minerals, especially calcium, magnesium and iron were found; and (vi) antimicrobial peptides were discovered [10]. There are many milk peptides that possess multifunctional activities, *i.e.*, they can play two or more hormone-like roles. Bioactive peptides grouped according to their function in human well-being are shown in Figure 1.

Nutraceutical products comprising short bioactive peptides showing *in vitro* or *in vivo* antimicrobial, ACE-inhibitory activity and/or antihypertensive and/or antioxidant activity are being considered for possible use by the pharmaceutical industry. The CN hydrolyzates could serve as food preservatives to reinforce the body's natural defenses or as pharmaceutical products for facilitating the control of blood and/or bacterial infections [12]. Much research has been devoted to increasing mineral transport by phosphorylated groups of peptides [13]. CPP in commercial hydrolysed casein (Tatua Cooperative Dairy Co. Ltd, New Zealand and Arla Foods Ingredients and Sweden) seem to help the absorption of chelated calcium, iron, copper, zinc and manganese in the intestine (Table 1). Thus, CPP-bound amorphous calcium phosphate (ACP) displayed anticariogenic effect when added to dentifrices or oral care products by localizing calcium and phosphate ions at the tooth surface. Similarly, it has been claimed that a chewing gum or other confectionery product containing a combination of CPP-ACP and sodium bicarbonate as active ingredients can provide dental health benefits [14]. In experiments on humans, synthetic CPP-ACP nanocomplexes incorporated in mouth rinses and sugar-free chewing gums have been proven to be potential anticariogenic agents [15] (Table 1).

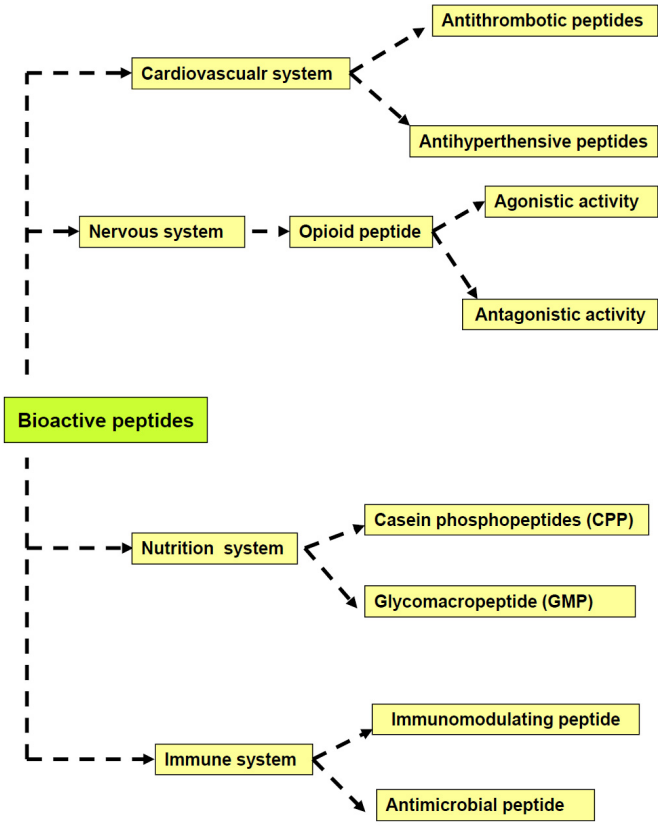

Figure 1. Main bioactivity of peptides formed by the enzymatic digestion of milk proteins (source: [11]).

Brand name	Product type	Claimed functional bioactive peptides	Health/function claims	Manufacturer
HCP102/HCP 105	Hydrolysate ingredient	CPP	Helps mineral absorption	Tatua (New Zealand)
Capolac	Hydrolysate ingredient	CPP	Helps mineral absorption	Arla Foods Ingredients (Sweden)
Recaldent	Chewing gum	ACP-CPP	Anticariogenic	Cadbury Adams (USA)
Recaldent	Toothpaste	ACP-CPP	Anticariogenic	GC Tooth Mousse (USA)

ACP= amorphous calcium phosphate; CPP= Casein phosphopeptides

Table 1. Commercial dairy products and ingredients with health or function claims based on CPP content (source: modified from [16]).

2. Bioactive peptides released *in vitro* by the hydrolysis of milk proteins

Peptides with various bioactivities can be produced according to two different methods: i) *in vitro* fermentation of milk inoculated with starter cultures and ii) *in vitro* digestion of milk proteins by one or more proteolytic enzymes.

(i) The proteolytic system of lactococci is able to degrade milk proteins using cell-wall-bound proteinases by releasing di-, tri-, and/or oligo-peptides and amino acids supporting the growth of bacteria. In addition, lactococcal peptidases released into the curd/cheese consequently to autolysis can further degrade the internalized peptides to amino acids [17]. The higher the exopeptidase activity in cheeses, the greater the age of the cheese [18]. Consistently, yogurt and other cultured dairy drinks have some of the highest counts of cells that actually survived and thus possessed the lowest number of peptides derived from the aminopeptidase activity. In a comprehensive review of literature, no enzyme with carboxypeptidase (CPase) activity has been reported for either lactococci or other LAB [19-20]. The bacterial peptidases have different and partly overlapping specificities (Figure 2).

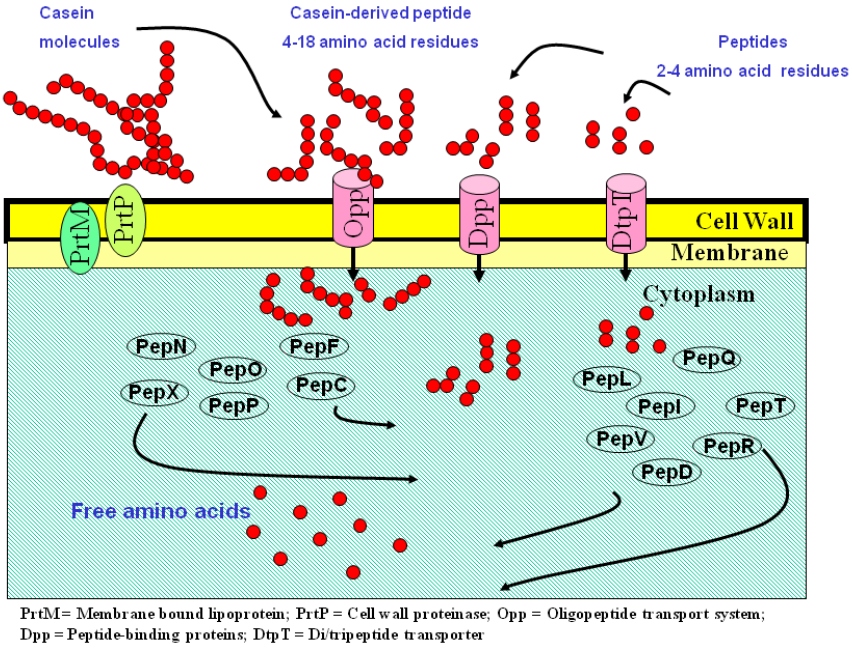

Figure 2. A simplified model presenting proteolysis, transport, peptidolysis and regulation of the proteolytic system of *Lactococcus lactis* on casein breakdown [20-22]. Intracellular peptidases PepO and PepF are endopeptidases, PepN/PepC/PepP are general aminopeptidases, PepX is X-prolyldipeptidyl aminopeptidase, PepT is tripeptidase, PepQ is prolidase, PepR is prolinase, PepI is proline iminopeptidase, and PepD and PepV are dipeptidases D and V. The role of PepN, PepC, PepI, PepP and PepA and PepO, PepF, PepX, PepQ, PepR, PepV, PepT and peptidolytic cycles are depicted schematically (various alternative routes of breakdown are possible for most peptides).

Although the intracellular endopeptidases PepN and PepC are unable to hydrolyze casein molecules, the X-prolyl dipeptidyl aminopeptidases (PepX) are active on oligopeptides hydrolyzing the internal bonds of casein-derived peptides. Taken together, these enzymes are able to remove the N-terminal residues from peptides, with the specificity primarily depending on the nature of the N-terminal amino acid [20-21]. Di- and tripeptides generated

by endopeptidases, general aminopeptidases and PepX are next subjected to additional cleavage by the tripeptidase, PepT, and dipeptidases, PepV and PepD (Figure 2). Other peptidases with more specific substrate specificities include PepA, which liberates N-terminal acidic residues from 3- to 9-residue long peptides; PepP, which prefers tripeptides carrying proline in the middle position; PepR and PepI, which act on dipeptides containing proline in the penultimate position; PepQ, which cleaves dipeptides carrying proline in the second position; and PepS, which shows preference for peptides containing two to five residues with Arg or aromatic amino acid residues in the N-terminal position [20-21,23] (Figure 2). This proteolytic system is able to support LAB growth to high cell densities (10^9–10^{10} cfu/mL) in milk containing only small amounts of hydrolytic products that are transportable into the cells for assimilation. *Lb. helveticus* and *Lb. delbrueckii* ssp. *bulgaricus* possess cell wall proteinase activity stronger than that of lactococci. The number of intracellular proteins released by *St. thermophilus* is greater than that by *Lb. helveticus* [24]. Proteins released in cheese from a starter-based thermophilic LAB, such as *Lb. helveticus*, *Lb. delbruecki* subsp. *lactis* and *Streptococcus salivarius* subsp. *thermophilus* and *Propionibacterium freudenreichii*, have been identified using 2D-PAGE and mass spectrometry (MS) analysis [24]. Similarly, bioactive peptides have been determined using High Performance Liquid Chromatography (HPLC) and offline Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry-Time-Of-Flight (MALDI-MS-TOF) [25]. These peptides were all generated from CN and released upon proteolysis depending on the bacterial strain [26]. In this manner, proteinases play a primary role, as they are able to generate specific bioactive peptides. Recombinant human α_{s1}-casein digested by trypsin gave rise to several ACE-inhibitory peptides and calcium-binding CPP. These peptides did not form in cheese whey, although they can be formed from CN during fermentation using various commercial dairy starters [27]. The combination of the LAB bacteria and proteolytic enzymes could serve to increase the range of bioactive peptides.

(ii) *In vitro hydrolysis* of CN by pepsin and trypsin could produce many bioactive peptides. Pepsin, an endopeptidase with broad specificity, preferentially cleaves hydrophobic, preferably aromatic, residues. Trypsin specifically hydrolyses peptide bonds just after a lysine or an arginine residue of β-casein A^1 and A^2 variant (11 and 4, respectively); κ-casein A and B (9 and 5); α_{s2}-CN A (24 and 6); or αs1-CN B (14 and 6). In this manner, tryptic hydrolysates of CN contain uneven peptides of up to 8159 Da and also free amino *acids* [28]. We have demonstrated that CN hydrolysate by pepsin (P) and trypsin (T) in succession does not contain peptides with molecular mass greater than 2431 Da. β-CN resulted extensively hydrolyzed into a high number of oligopeptides by using proteases with well defined cleavage specificity. Controlled partial hydrolysis by proteinases could lead to the formation of partially degraded proteins critical for obtaining new functional products. The choice of digestion enzymes needs to be evaluated carefully because influences the hydrolysate final composition. A high number of peptides with antimicrobial, anti-hypertensive and opiod-like activity has been identified (Table 2), some of which exactly matched those described in the literature for potential bioactivity (Table 2). The potent opioid β-CM7 peptide retained part of its original opioid activity-

like food hormone when progressively shortened. The synthetic β-casomorphin derivatives have been shown to be highly specific and potent β-type opioid receptor ligands [29].

We also performed sequential milk protein (powder sample) digestions using various endoproteases facilitating a consistent partial hydrolysis. The high degree of specificity in terms of cleaving peptide bonds exhibited by a cocktail of enzymes (P, T and P432 from Biocatalysts, U.K) yielded a limited number of CPP. After fractionation on an HA column,

Identity of bioactive peptides	Bioactivity	References	PT peptides
β-CN (f60-70); (f59-61); (f59-64); (f60-68)	Opiod	[29-30]	(f58-68); (f59-63); (f59-62)
β-CN (f74-76)	ACE-Inhibitory	[31-33]	(f69-80); (f71-80); (f75-80)
β-CN (f80-90); (f84-86)			(f80-88); (f81-89); (f81-88); (f81-92); (f81-93); (f81-94)
β-CN (f108-113)			(f108-103)
β-CN (f140-143)			(f139-142)
β-CN (f177-183)			(f177-183); (f177-184); (f179-182)
β-CN (f169-174)			(f169-176); (f172-176)
β-CN (f193-198)			(f193-199)
β-CN (f193-202)			(f193-202)
β-CN (f1-25)4P	Mineral carrier, immunomodulatory, cytomodulatory	[34-38]	(f12-17)1P; (f12-25)3P; (f12-25)4P; (f15-25)2P; (f15-25)3P; (f19-25)1P; (f19-25)2P
β-CN (f29-41)1P	Mineral carrier	[13]	(f33-42)1P; (f33-43)1P; (f33-44)1P
β-CN (f84-86)	ACE-Inhibitory	[31-33]	(f80-87); (f81-88); (f81-89); (f81-92); (f81-93); (f81-94)
α_{s1}-CN (f23-34)			(f24-32)
αs_1-CN (f25-27)			(f25-31)
α_{s1}-CN (f90-96); (f90-95); (f91-95)	Opiod (agonist)	[39-41]	(f92-95); (f91-95); (f90-95)
α_{s1}-CN (f142-147)	ACE-Inhibitory	[31-33]	(f142-145); (f143-146); (f143-150); (f144-149)
α_{s1}-CN (f157-164)			(f155-164)
α_{s1}-CN (f194-199)			(f194-199); (f197-199); (f193-199)
α_{s1}-CN	Mineral carrier	[13]	(f110-119)1P
α_{s1}-CN			(f41-55)1P; (f68-79)2P
α_{s2}-CN (f174-179)	ACE-Inhibitory	[31-33]	(f174-179)
α_{s1}-CN (f189-193)			(f189-193)
α_{s2}-CN	Mineral carrier	[13]	(f138-146)1P
α_{s2}-CN			(f124-137)2P; (f126-136)2P; (f126-137)2P
κ-CN (f33-38)	Opiod (antagonist)		(f58-65)1P

PT= Pepsin and Trypsin action; P= Phosphate group.

Table 2. Identity of bioactive peptides found in the PT digest of milk protein powder sample. The bioactivity of the peptide from which they derive and the references are reported.

both non-CPP and CPP were identified as shown in Table 3. Different oxidation rates of Met residues in the same protein resulted in formation of the peptides with different molecular mass. In most cases, a single Met-containing peptide and its oxidized counterpart were identified. However, in many cases, when proteins contained several consecutive endoprotease sensitive bonds, different long peptides containing the same Met-oxidation site(s) were identified. The data in Table 3 suggest that the cocktail enzymes containing amino- and CPases, in addition to P and T, progressively reduce the size of peptides without altering the degree of phosphorylation. Evaluation of protein/peptide quality can take advantage of the tandem MS for the detection of native, partly oxidized and partly dephosphorylated peptides.

By this means, phosphorylated peptides (2 and 3P/mole) of a precursor of lactophorin (LP) (28 kDa milk glycoprotein), proteose-peptone component 3, and glycosylation-dependent adhesion molecule 1 were detected. In addition, three low molecular weight non-CPP derived from LP were detected.

Native milk proteins used as the substrate for digestion by enzymes did not form CPP. This suggests that denatured LP and other whey proteins could have the tendency to form low molecular-mass peptide aggregates characterized by a poor solubility. For this reason, the use of milk protein and/or any milk substrate powder must be discouraged to eliminate phosphates and salts from the substrate. The enzymatic hydrolysis of the casein implies the use of endoproteases. However, the protein hydrolysate with alcalase is used in infant formulae, dietetic foods, nutraceuticals, ice creams, dressings, fermented products, yogurts, and personal care products. CPP released by alcalase are truncated with respect to those released by trypsin. The identified peptides can be categorized into two groups, one containing multiphosphorylated peptides and the other tri-, di- and mono CPP. Each group contained a number of variously long peptides due to the broad specificity of alcalase cleaving peptide bonds mainly on the carboxyl side of Glu, Met, Leu, Tyr, Lys, and Gln. The exoproteases responsible for the hydrolysis are inactivated by heating for ~10 min to ~85 °C. The *in vitro* sequential use of pepsin, pancreatic proteases and extracts of human intestinal brush border membranes, mimicking the respective gastric, duodenal and jejuneal *in vivo* digestion of CN, exhibited significant bioactive effects. A limited number of CN and whey protein peptides survived the *in vitro* simulated gastro-intestinal digestion. The anionic character seems to confer a marked resistance to multi-phosphorylated CPP hydrolysis by endoprotease. Ten out of 19 CPP contained SerP available for binding minerals, and four of these peptides, α_{s1}-CN (f57-90)5P, α_{s1}-CN (f56-90)5P, α_{s1}-CN (f55-76)5P, β-CN (f1-52)5P, were reported for the first time in the CN digests [42]. Only β-CN (f1-25)4P, 3P and 2P survived the simulated gastrointestinal digest of CN [43].

The ingress of foreign material in general, such as CPP, across the mucosal brush-border into the enterocyte is conditioned by the efficient dephosphorylation of peptides by alkaline phosphatase. This aspect deserves more in-depth investigation.

Parent protein	Molecular mass (Da)		Start		End	Peptide sequence	Peptide modifications
	Expected	Calculated					
α_{s1}-CN	1222.5	1222.5	110	-	119	(L)EIVPN**S**AEER(L)	1P
	1517.8	1517.6	68	-	79	(S)**S**EEIVPN**S**VEQK(H)	2P
	1525.7	1525.5	41	-	53	(L)SKDIG**S**E**S**TEDQA(M)	2P
	1586.6	1586.5	43	-	55	(K)DIG**S**E**S**TEDQAME(D)	2P; Oxidation (Met)
	1672.7	1672.6	41	-	54	(L)SKDIG**S**E**S**TEDQAM(E)	2P; Oxidation (Met)
	1785.8	1785.6	41	-	55	(L)SKDIG**S**E**S**TEDQAME(D)	2P
	1801.7	1801.6	41	-	55	(L)SKDIG**S**E**S**TEDQAME(D)	2P; Oxidation (Met)
	1963.9	1963.8	39	-	55	(N)ELSKDIGSE**S**TEDQAME(D)	1P; Oxidation (Met)
	1989.9	1989.7	37	-	52	(K)VNEL**S**KDIG**S**E**S**TEDQ(A)	3P
	2060.7	2060.7	37	-	53	(K)VNEL**S**KDIG**S**E**S**TEDQA(M)	3P
α_{s2}-CN	900.4	900.3	58	-	65	(S)**S**EESAEVA(T)	P
	937.4	937.3	141	-	147	(D)ME**S**TEVF(T)	1P; Oxidation (Met)
	1067.4	1067.3	57	-	65	(S)**S S**EESAEVA(T)	2P
	1089.5	1089.4	138	-	146	(K)TVDME**S**TEV(F)	1P
	1105.4	1105.4	138	-	146	(K)TVDME**S**TEV(F)	1P; Oxidation (Met)
	1252.6	1252.5	138	-	147	(K)TVDME**S**TEVF(T)	1P; Oxidation (Met)
	1410.6	1410.5	126	-	136	(R)EQL**S**T**S**EENSK(K)	2P
	1538.7	1538.6	126	-	137	(R)EQL**S**T**S**EENSKK(T)	2P
	1623.7	1623.6	1	-	13	KNTMEHVS**S S**EES(I)	2P
	1639.7	1639.5	1	-	13	KNTMEHVS**S S**EES(I)	2P; Oxidation (Met)
	1680.8	1680.6	124	-	136	(L)NREQL**S**T**S**EENSK(K)	2P
	1719.7	1719.5	1	-	13	KNTMEHV**S S S**EES (I)	3P; Oxidation (Met)
	1808.9	1808.7	124	-	137	(L)NREQL**S**T**S**EENSKK(T)	2P
β-CN	639.3	639.3	12	-	16	(E)IVE**S**L(S)	1P
	900.5	900.4	19	-	25	(S)**S**EESITR(I)	1P
	1067.4	1067.4	18	-	25	(S)**S S**EESITR(I)	2P
	1354.6	1354.5	15	-	25	(E)SLS**S S**EESITR(I)	2P
	1434.6	1434.5	15	-	25	(E)SL**S S S**EESITR(I)	3P
	1447.7	1447.5	33	-	43	(K)FQ**S**EEQQQTED(E)	1P
	1576.7	1576.6	33	-	44	(K)FQ**S**EEQQQTEDE(L)	1P
	1689.8	1689.6	33	-	45	(K)FQ**S**EEQQQTEDEL(Q)	1P
	1775.8	1775.7	12	-	25	(E)IVESL**S S S**EESITR(I)	3P
	1855.6	1855.6	12	-	25	(E)IVE**S**L**S S S**EESITR(I)	4P
κ-CN	968.4	968.4	145	-	152	(A)TLED**S**PEV(I)	1P
	1734.7	1734.7	147	-	161	(L)ED**S**PEVIESPPEINT(V)	1P
Lactophorin	1226.5	1226.51	34	-	43	(L)SKEPSI**S**RED(L)	1P
	1306.6	1306.5	34	-	43	(L)SKEP**S**I**S**RED(L)	2P
	1419.6	1419.56	34	-	44	(L)SKEP**S**I**S**REDL(I)	2P
	1499.7	1499.5	34	-	44	(L)**S**KEP**S**I**S**REDL(I)	3P

Phosphoserine residues are coloured red.

Table 3. Native and partly Met-oxidized CPP isolated from a three enzyme (Pepsin, trypsin and P432) milk protein hydrolyzate.

3. *In vivo* digestion of casein, formation of CPP and their physiological importance

Among the biologically active peptides, CPP characterized by SerP and/or ThrP residues account for ~30% of monoesters of hydroxyl amino acids. They mainly occur in the Ser/Thr-Xaa-SerP/Glu/Asp sequence consensus, where Xaa is any amino acid residue but Pro. The three-phosphorylated motif -SerP-SerP-SerP-Glu-Glu- occurs in α_{s1}-CN (f66-70), α_{s2}-CN (f8-12), α_{s2}-CN (f56-60), and β-CN (f17-21). According to the current CN nomenclature, bovine α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN possess 8-9, 11-13, 4-5, and 1-2 phosphate (P) residues, respectively, and the P number could change according to the casein variant [44]. For example, β-CN D has one SerP residue less than the A counterpart due to the substitution $Lys^{18} \rightarrow Ser^{18}$.

The *in vivo* digestion of milk proteins takes place mainly in the stomach under the action of pepsins, gastric digestive proteinases that are able to digest ~20% proteins. Afterwards, the pepsin digests pass to the duodenum where peptides are further hydrolyzed by pancreatic enzymes. The digestion is completed by membrane proteases and a variety of peptidases embedded in the brush border of the small intestine and released by the intestinal microflora. These peptidases release an amino acid residue or a dipeptide from the N- and C-terminal side of oligopeptides [45-47]. Phosphatase(s) located in the brush border of the apical membrane of enterocytes, act(s) in removing phosphate groups, thus promoting partial or full peptide dephosphorylation of peptides in different body districts. The phosphorylated sequence is responsible, at the intestinal pH, for binding Ca^{++}, Zn^{++}, and Mg^{++} and for the *in vivo* resistance of the complex to gastrointestinal proteases [48]. Fe complexed to β-CN (f15-25)4P was scarcely hydrolyzed throughout the digestion, suggesting that the coordination of iron ions to CPP inhibits the action of both phosphatase and peptidases [49]. Brush border enzyme alkaline phosphatase activity could improve the absorption of Fe complexed CPP by releasing Fe from peptides. Moreover, Fe complexed to β-CN CPP was absorbed more than Fe complexed to α_{s1}-CN CPP [50]. The differences in protein composition between cow and breast milk could explain some of the differences in the Fe bioavailability of the latter [50]. Iron deficiency, a major worldwide nutritional problem, can be reduced by CPP. Fe complexed CPP prevents the formation of poorly absorbed high molecular weight ferric hydroxides. Zinc absorption can also be enhanced by the formation of Zn complexed to CCP, in particular to β-CN (f1-25)4P [51]. Some portion of the mineral complexed CPP formed in the small intestine was resistant to the digestive and enteric bacteria enzymes and found in the feces of rats fed a casein-based diet [52]. Although literature data regarding intestinal CPP absorption are conflicting, the peptides seem to interact directly with the plasma membrane. One possible mode of CPP action on the transmembrane flux of calcium is that CPP might insert themselves into the plasma membrane and form their own calcium selective channels or act as calcium-carrier peptides rapidly internalized via endocytosis or other processes and eventually provide ionized calcium in the cytosol [53]. Cellular uptake studies of fluorine-18 labeled CPP in human colorectal adenocarcinoma cell line (HT-29) and human head and neck squamous cell carcinoma line (FaDu) cells at 37 °C and 48 °C showed a poor cell penetration because of the poor transport of the phosphopeptides through the cell membrane [54]. The results from *in vivo* studies are still too controversial, as there are many factors affecting Ca availability, such

as the various co-present dietary compounds in the intestinal lumen [55]. Despite the vigor of the saturable active transport process by the duodenum, most of the absorption of ingested calcium occurs in the ileum (88% of calcium), jejunum (4%) and duodenum (8%) [56]. An important factor determining the contribution of the ileum to overall calcium absorption is the relatively long transit time of calcium in the segments of the small intestine, accounting for approximately 102 min in the ileum and 6 min in the duodenum [57]. The higher absorption of calcium occurred when inorganic P was added to the Ca-CPP preparation. CPP exhibit a potent ability to form soluble complexes with Ca^{2+} and other trace elements, preventing the formation of Ca-phosphate precipitate in the intestine. CPP could limit the inhibitory effect of phosphate on Ca availability and increase Ca transport across the distal small intestine [55]. All components of the diet reaching the ileum make calcium soluble or keep it in solution within the ileum. Several molecules, particularly CPP, stimulate the passive diffusion of minerals. CPP have been for the first time detected in human ileostomy fluid, confirming their ability to survive gastrointestinal passage into the human distal ileum [58]. CPP released during milk digestion appeared to be stable for up to 8 h in ileostomy contents [58]. The *in vivo* formation of bovine CPP was demonstrated in the small intestinal fluid of minipigs after ingestion of a diet containing casein [59] and in the stomach and duodenum after ingestion of milk or yogurt [60]. The *in vivo* survival of CPP to the prolonged intestinal passage in the distal small intestine is a prerequisite for their function as bioactive substances [58]. CPP are protected from degradation in the gut by the milk matrix, provided that they are ingested as milk constituents and not as isolated CPP. Whole casein or individual casein fractions are used as raw materials to obtain CPP as dietary supplements. Ca could be bound to either SerP or Glu residues [61], suggesting that CPP may enhance the solubility of calcium in the intestinal lumen, thereby increasing the mineral availability for absorption in the small intestine [62,13]. Chemically synthesized CPP, *i.e.*, β-CN (f1-25)4P and αs1-CN (f59-79)5P, carrying the characteristic cluster Ser(P)-Ser(P)-Ser(P)-Glu-Glu, increase the intracellular calcium uptake by the human cultured HT-29 tumor cells [63], Caco-2 cells [64] and osteoblasts [65]. A more pronounced effect has been observed for β-CN-derived peptides than for α_{s1}-CN-counterparts. It has been suggested that CPP promote calcium binding, which would depend on the structural conformation conferred by the two phosphorylated 'acidic motif' and the N-terminal sequence of β-CN [63].

Dental caries are initiated via the demineralization of tooth hard tissue by organic acids directly from the diet or produced from fermentable carbohydrate by dental plaque cariogenic bacteria. CPP can help to replace the minerals that were previously lost consequently to caries [66-67]. Hence, there is a great interest in developing CPP as nutraceutical ingredients for the formulation of functional foods.

4. CPP enrichment by different techniques

CPP preferably comprise components released by four different casein families, each having a molecular weight greater than 500 Da. Multiply and singly, tryptic CPP can be simultaneously detected using MALDI-TOF, and the location of phosphate groups by a combination of tandem mass spectrometry and computer-assisted database search programs, such as SEQUEST (Trademark, University of Washington, Seattle Wash) [68-69].

Nano-electrospray MS/MS has been used for phosphopeptide sequencing and exact determination of phosphorylation sites [70]. However, mass spectrometric analysis of proteolytic digests of proteins rarely provides full coverage of the phosphorylated sequence, with parts of the sequence often going undetected. In addition, protein phosphorylation is often sub-stoichiometric, such that ionization of CPP present in lower abundance in complex hydrolysates is ordinarily suppressed by strongly ionizable non-phosphorylated peptides. The MALDI-MS desorption/ionization efficiency for phosphopeptides was reported to be an order of magnitude lower than that recorded for the non-phosphorylated counterpart, and ionization became more difficult as the number of phosphate groups increased [71]. Direct analysis of phosphopeptides utilizes two orthogonal MS scanning techniques, both based on the production of phosphopeptide-specific marker ions at m/z 63 and/or 79 in the negative ion mode. These scanning methods combined with the liquid chromatography (LC)-electrospray mass spectrometry (ESI) and nano-electrospray MS/MS allow the selective detection and identification of phosphopeptides even in complex proteolytic digests. Thus, even when the signal of the phosphopeptide is indistinguishable from the background, as in the conventional MS scan, low-abundant and low-stoichiometric phosphorylated peptides can be selectively determined in the presence of a large excess of non-phosphorylated peptides. This strategy is particularly well suited to phosphoproteins that are phosphorylated to varying degrees of stoichiometry at multiple sites [72]. However, the identification and characterization of phosphoproteins would be greatly improved using selective enrichment of CPP prior to MS analysis. An ancient technique for phosphoprotein enrichment consisted of the precipitation of phosphopeptides as insoluble barium salts and recovery by centrifugation, as according to the Manson & Annan method [73]. High-throughput phosphoproteome technologies currently rely on combining pre-separation of proteins, most commonly by high-resolution two-dimensional polyacrylamide gel, in-gel tryptic cleavage of proteins, and subsequent MALDI-TOF or ESI-MS/MS mass spectrometry analysis of peptides [74]. The high resolving power of 2-DE with the sensitive MS requires extensive manual manipulation of samples. Alternative methods are based on chemical derivatization. For example, for β-elimination, a strong base such as NaOH or $Ba(OH)_2$ is used to cleave the phosphoester bonds of phosphoserine and phosphothreonine and form dehydroalanine or dehydroaminobutyric acid, respectively, each able to react with different nucleophiles, such as ethanedithiol (EDT) or dithiothreitol (DTT). This procedure provides a considerably simpler method to enrich CPP. By using cross-linking reagents with affinity tags, such as biotin, interfering non-cross-linked peptides are eliminated, and CPP are highly enriched [75]. Although the chemical derivatization methods are highly selective, they are not widely applied in phosphoproteome studies due to sample loss by the multiple reaction steps and unavoidable side reactions [76]. Immobilized metal-ion affinity chromatography (IMAC, with Fe^{3+}, Ga^{3+}, Ni^{2+} and Zr^{4+} metal ions) and metal oxide affinity chromatography (MOAC, with TiO_2, ZrO_2, Al_2O_3 and Nb_2O_5) have been widely used for the quantitative binding of CPP on resin or adsorbent. Iminodiacetic acid (IDA, a tridentate metal-chelator) or nitrilotriacetic acid (NTA, a quadradentate metal chelator) are often used as IMAC functional matrices reacting with multivalent metal ions to form chelated ions with positive charges useful for the purification of phosphopeptides. Usually Fe^{3+}, Ga^{3+} and Al^{3+} are bound to a chelating support prior to fractionating the complex mixture of peptides

before MS analysis [77-78]. Ga^{3+} showed selectivity for CPP higher than Fe^{3+} and Al^{3+} [77,79]. Phosphopeptides bound to IMAC resin are successively recovered in the column effluent by increasing either pH or the phosphate concentration in the buffer [80]. The negatively charged CPP selectively interact with TiO_2 microspheres via bidentate binding at the dioxide surface [81-83]. TiO_2-MOAC showed higher specificity than immobilized gallium (Ga^{3+}), immobilized iron (Fe^{3+}), or zirconium dioxide (ZrO_2) affinity chromatography for phosphopeptide enrichment. The main problem associated with the chelating resins is the metal-ion leaching, which leads to CPP loss during the enrichment procedure. The selectivity of these methods was somewhat compromised by the detection of several acidic non-CPP that were also retained by the TiO_2 column [84]. To overcome this drawback, the carboxyl groups are methyl esterified which eliminates the non-specific adsorption of acidic peptides on IMAC [85]. Considerable efforts have been expended to remove acidic non-CPP by washing the resin with 2,5-dihydroxybenzoic acid (DHB) [86] or phthalic acid [87]. It has been found that aliphatic hydroxyl acid modified metal oxide works more efficiently and more specifically than aromatic modifiers such as DHB and phthalic acid in titania and zirconia MOC [88]. However, all affinity techniques developed for the current enrichment strategies of CPP gave reproducible but incomplete results due to *poor binding* of low concentrations of CPP and the insufficient *recovery* of multiple phosphorylated peptides [89]. Recently, a specific hydroxyapatite (HA)-based enrichment procedure has been developed for complex mixtures of phosphoprotein/CPP [90]. Salt such as calcium phosphate, also occurring in bone and tooth tissue in the HA form, with the formula $[Ca_{10}(PO_4)_6(OH)_2]$, has been previously used to enrich bone proteins [91]. The phosphate groups of phosphoproteins interact with crystalline lattice Ca^{2+} [92] more strongly than do the carboxyl groups [93]. Moreover, increasing protein phosphorylation leads to tighter binding of the proteins/CPP to HA [92]. One might conclude that the affinity of the multi-phosphorylated proteins/peptides for HA is significantly higher than that of the same components with lower phosphorylation. Essentially, the HA-based protocol immobilizes on HA microgranules proteins/peptides through their phosphate groups, while the non phosphorylated components are washed out using various buffers. In a previous article, CPP immobilized on HA were progressively eluted, increasing phosphate in the elution buffer, and then identified by off-line MALDI-MS [94]. This procedure was accelerated, and loss during elution was minimized by spotting HA-CN/CPP microgranules onto a MALDI target and analyzing the peptides directly by MALDI-TOF [90]. This method was useful for measuring the phosphorylation level of phosphoproteins/CPP quickly, with less than 2 h elapsing from the fractionation of the protein/CPP to the readout of the MALDI spectra (excluding the trypsinolysis step).

The more important advantages of the procedure are the possibility of (1) detecting phosphorylated proteins/peptides even in complex mixtures, (2) determining phosphorylated sites and those dephosphorylated by phosphatase, (3) attaining information regarding weakly and heavily phosphorylated peptides and (4) adding the HA-CPP complex directly to food, which is enabled by the use of an edible resin such HA [90]. Moreover, use of available commercial CPP preparations by the food industry is difficult for three primary reasons: i) the matrix bound to CPP is often not edible and such products can be hazardous; ii) the

preparation of CPP is a long and a laborious procedure that requires cumbersome and expensive manipulation; and iii) CPP have an unpleasant taste even in modest amounts, which disadvantageously limits their direct utilization as a human food ingredient. A novel HA-based method for food grade CPP preparation has been performed on tryptic digests of casein. HA captured all CPP free of non-CPP [90]. There were approximately 32 HA bound CPP, and all non-CPP peptides were eluted [90]. HA-based enrichment procedure has been successfully applied to phosphopeptide recovery from complex biological fluids such as human serum thus providing a great source of potential biomarkers of disease. Four primary phosphopeptides derived from fibrinogen were enriched from human serum (Figure 3a-b, Table 4). A similar set of phosphorylated peptides was previously obtained using a modified IMAC strategy coupled to iterative mass spectrometry-based scanning techniques [95], using the titanium ion-immobilized mesoporous silica particles and MALDI-TOF [96] and cerium ion-chelated magnetic silica microspheres [97].

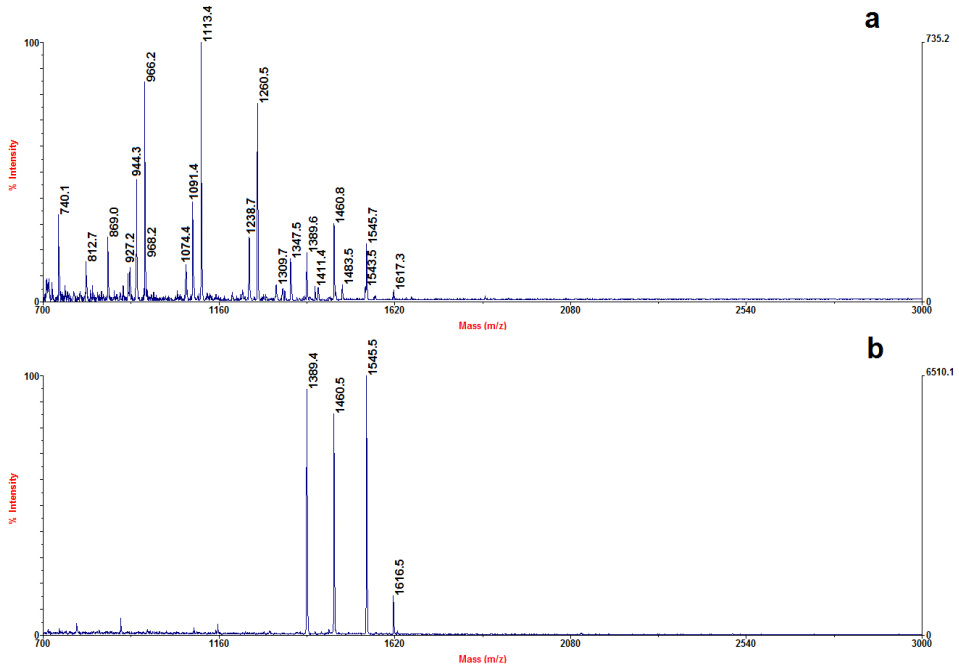

Figure 3. MALDI-MS-TOF spectra for the human serum before (a) and after (b) enrichment by HA (insert is the zoomed between 700 and 3000 Da).

Fibrinopeptide A (FPA) (f1-16)1P, (SerP^{3}), a 16-residue long peptide (1615 Da) (Table 4), is the segment anchored on the thrombin surface [98]. The other three phosphopeptides, (f1-

15)1P, (f2-15)1P and (f2-16)1P (Table 4), are hydrolytic products of FPA. The serum level of fibrinogen and its hydrolytic products may reflect the expression and activation of enzymes including kinase, phosphatase, and protease [99]. An altered ratio of FPA (f2-15) and FPA (f1-16) is detected in patients affected by hepatocellular carcinoma; the D^{2}[pS]GEGDFLAEGGGV^{15} peptide is upregulated, and the A^{1}D[pS]GEGDFLAEGGGVR^{16} peptide is down-regulated greatly. The other two peptides, A^{1}D[pS]GEGDFLAEGGGV^{15} and D^{2}[pS]GEGDFLAEGGGVR^{16}, varied only slightly between the two groups [96]. The proportions of fibrinogen and their phosphorylation products offer new opportunities for basic research in exploring new frontiers in bio-marker discovery.

Molecular Mass (Da)		Fibrinogen α-chain sequence	Phosphorylation sites
Theoretical	Measured MH^{+}		
1388.5	1389.4	D^{2}**S**GEGDFLAEGGGV^{15}	1
1459.5	1460.5	A^{1}D**S**GEGDFLAEGGGV^{15}	1
1544.6	1545.5	D^{2}**S**GEGDFLAEGGGVR^{16}	1
1615.6	1616.5	A^{1}D**S**GEGDFLAEGGGVR^{16}	1

Phosphoserine residues are coloured red.

Table 4. Identification of phosphorylated fibrinogen fragments from human serum immobilized on HA.

4.1. CPP in commercial milk as specific indicators of heated milks

Because of the lower value, the addition of UHT and milk powder to raw or pasteurized milk is prohibited (EU Directives 92/46 CEE and 94/71 CEE) for cheese milk. The intensity of heat treatment was found to correlate with the furosine content. Glycated proteins and peptides formed during the initial stages of the Maillard reaction are indirectly evaluated through the furosine content [100-101]. The Amadori compound formed upon the reaction of lysine residue with a lactose molecule will prevent the digestive enzymes from reaching the binding sites. Native and lactosylated forms of β-CN (f1-28)4P, (f1-27)4P and α_{s2}-CN (f1-24)4P, although typical of UHT milk and milk powder, are missing in raw, pasteurized milk (71.7 °C for 15 s) and intensely pasteurized milk. The lactosylated peptides that varied with heat treatment characterize UHT milk added in amounts not lower than 10% to raw and pasteurized milk [102]. Milk delactosed with microbial β-galactosidase did not suppress the Maillard reaction; indeed, the furosine concentration increased to 35-400 mg/100 g of protein [103]. As expected, a lactose-reduced UHT milk had β-CN (f1-28)4P glycated mainly by its monosaccharides (Figure 4a).

Therefore, the nonenzymatically glycated CPP derived from the reaction of one molecule of glucose or galactose with a lysine residue (m/z 3641) can be considered to be the signature peptide of lactose-reduced milk (Figure 4).

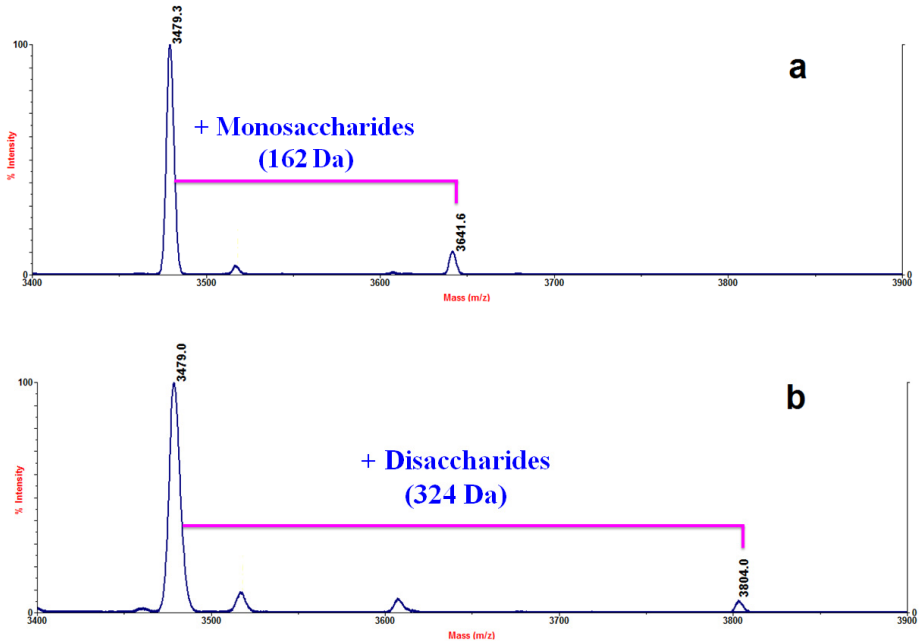

Figure 4. Enlarged view of the MALDI spectrum of β-CN (f1-28)4P (MH^+ = 3479 Da) signature glycosylated CPP in lactose-reduced UHT milk (a) and lactosylated CPP in UHT milk (b). The mass differences corresponded to lactose and glucose/galactose residues.

4.2. CPP in yogurt

Yogurt is a fermented milk defined as the "food produced by culturing one or more of the optional dairy ingredients (cream, milk, partially skimmed milk, and skim milk) with a characteristic bacteria culture that contains the lactic acid-producing bacteria, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*". A heat treatment of 90 °C for 10 min is considered optimal to obtain a good quality yogurt [104], and the addition of milk powder increases the content of furosine to more than 300 mg/100 g protein [105]. In yogurt, enzymes could give rise to the liberation of a particularly high number of bioactive peptides, among them CPP, which could be partly due to LAB proteolytic activity. Comparison of CPP in raw, pasteurized and intensely heated milks has previously shown that there is a plethora of milk peptides among which a few were glycosylated CPP [102]. Yogurt is prepared from intensely heated milk instead of low-pasteurized drinking milk. The CPP of two preparations were enriched on HA and analyzed by MALDI-TOF (Table 5). The proportion of CPP with molecular masses between 2.5 and 4 kDa was significantly higher in yogurt than in pasteurized milk as shown in Table 5. Only four fragments of CPP-derived peptides produced during yogurt preparation occur in pasteurized milk (Table 5).

Molecular Mass (Da)		CPP sequence	Yogurt	Pasteurized milk
Measured MH^+	Theoretical		Relative Intensity	
α_{s1}-CN derived CPP				
1327.5	1326.4	α_{s1}-CN (f44-54)2P	0.3	n.d.
5009.1	5008.7	α_{s1}-CN (f39-79)6P	0.6	n.d.
5089.3	5088.7	α_{s1}-CN (f39-79)7P	1.2	n.d.
6959.0	6958.7	α_{s1}-CN (f35-90)8P	0.6	n.d.
7087.1	7086.9	α_{s1}-CN (f34-90)8P	0.2	n.d.
α_{s2}-CN derived CPP				
1493.6	1492.7	α_{s2}-CN (f139-150)1P	1.2	n.d.
1617.5	1616.5	α_{s2}-CN (f1-12)3P	2.0	n.d.
1931.7	1930.8	α_{s2}-CN (f1-15)3P	8.6	n.d.
2007.9	2006.7	α_{s2}-CN (f7-21)4P	0.4	n.d.
2356.2	2355.1	α_{s2}-CN (f1-18)4P	20.8	n.d.
2666.7	2665.4	α_{s2}-CN (f51-72)4P	2.3	n.d.
2748.6	2747.5	α_{s2}-CN (f1-21)4P*	3.0	26.9
2876.8	2875.7	α_{s2}-CN (f1-22)4P	1.8	n.d.
3005.2	3004.8	α_{s2}-CN (f2-24)4P	0.4	n.d.
3134.3	3133.0	α_{s2}-CN (f1-24)4P*	24.2	46.0
3382.0	3381.1	α_{s2}-CN (f49-76)4P	8.4	n.d.
3461.9	3461.1	α_{s2}-CN (f49-76)5P	1.4	n.d.
3666.0	3665.9	α_{s2}-CN (f16-45)2P	0.9	n.d.
4166.4	4165.3	α_{s2}-CN (f115-149)3P*	0.6	n.d.
4294.7	4293.5	α_{s2}-CN (f115-150)3P*	0.6	n.d.
β-CN derived CPP				
960.5	959.9	β-CN (f30-36)1P	0.8	n.d.
1462.5	1461.4	β-CN (f17-27)3P	1.8	n.d.
1511.6	1510.4	β-CN (f17-28)2P	0.6	n.d.
1515.5	1514.4	β-CN (f15-25)4P	1.3	n.d.
1591.4	1590.4	β-CN (f17-28)3P	19.5	n.d.
1628.9	1628.3	β-CN (f15-26)4P	0.7	n.d.
1645.4	1644.3	β-CN (f14-25)4P	1.1	n.d.
1743.8	1742.5	β-CN (f15-27)4P	16.0	n.d.
1791.4	1790.6	β-CN (f15-28)3P	7.9	n.d.
1871.6	1870.6	β-CN (f15-28)4P	100.0	n.d.
1999.8	1998.7	β-CN (f14-28)4P	0.4	n.d.
2240.4	2239.0	β-CN (f8-25)4P	1.6	n.d.
2709.4	2708.5	β-CN (f7-28)4P	4.1	n.d.
2967.4	2966.7	β-CN (f1-24)4P	0.2	n.d.
3080.5	3079.9	β-CN (f4-28)4P	0.6	n.d.
3123.2	3122.9	β-CN (f1-25)4P*	n.d.	5.8
3351.6	3350.2	β-CN (f1-27)4P	3.2	14.8
3479.9	3478.4	β-CN (f1-28)4P*	70.1	100.0
3607.8	3606.6	β-CN (f1-29)4P*	13.2	6.0
3803.8	3802.4	β-CN (f1-28)4P + 1 lactose	0.7	n.d.
3849.3	3848.8	β-CN (f1-31)4P	0.1	n.d.
3978.2	3977.0	β-CN (f1-32)4P	n.d.	0.5
κ-CN derived CPP				
6229.8	6228.8	κ-CN (f106-163)1P	0.3	n.d.
6788.5	6787.5	κ-CN (f106-169)1P	0.1	n.d.

n.d. not detected; * CPP detected *in vitro* because it was liberated by plasmin in raw milk and enriched on HA.

Table 5. List of HA-enriched CPP identified in commercial samples of yogurt and pasteurized milk. Relative intensity of each peak is reported.

For example, β-CN (f1-28)4P, a peptide representing 100% intensity (assumed as base peak) of the signals in MALDI spectra, was reduced by approximately 30%; this loss was associated with the transformation of pasteurized milk into yogurt. One can deduce that the original peptide undergoes degradation even considering the higher number of formed CN peptides. In yogurt, β-CN (f1-28)4P, the most common CPP in pasteurized milk, was hydrolyzed into the peptide β-CN (f15-28)4P, which thus becomes the most abundant CPP.

β-CN (f1-29)4P, β-CN (f1-28)4P, β-CN (f1-25)4P, β-CN (f1-24)4P, α_{s2}-CN (f1-24)4P, α_{s2}-CN (f1-21)4P, resulting from the CN hydrolysis by plasmin and enriched on HA, were also found as C-terminally shortened peptides. During fermentation and storage, α_{s2}-CN (f115-150)3P and α_{s2}-CN (f115-149)3P derived by plasmin action did not react further to produce shorter peptides, most likely because of the absence of proteolytic enzymes. In the yogurt fraction recovered by centrifugation, only five multi-phosphorylated α_{s1}-CN and two low-phosphorylated κ-CN, κ-CN (f106-163)1P and κ-CN (f106-169)1P, were identified. Few CPP were less phosphorylated than the native peptides due to the presence of milk phosphatase, which was denatured in all pasteurized cheese-milks. The presence of lactosylated β-CN (f1-28)4P CPP was indicative of yogurt made with high-heat treated or milk fortified with milk protein powder [102]. Proteolysis of milk proteins in model yogurt systems has shown a similar set of primary CPP (Table 5). Therefore, the question is raised how CPP, derived from the enzymatic hydrolysis of yogurt CN are digested and absorbed in adult humans. For this reason, it is important to know the gastrointestinal resistance of CPP if used as a functional ingredient for fruit beverages. In the various stages of human digestion, a large quantity of CPP is produced in the stomach by partial hydrolysis of CN through pepsin action and in the small intestine by trypsin; these peptides are successively refined by endoproteases/exopeptidases. Although analysis of the intestinal contents of milk and yogurt ingestion has revealed the presence of CPP [60], their further resistance to gastrointestinal enzymes is poorly documented. Fragment β-CN (f1-24)4P has been previously identified in the lumen contents of rats after 60 min of digestion as a β-CN (f1-25)4P derived peptide [106]. Moreover, after yogurt ingestion, β-CN (f1-32)4P CPP was released in the human stomach [60] and β-CN (f1-31)4P was found in a yogurt sample. The fragment β-CN (f1-28)4P constitutes a clear example of the multi-functionality of milk-derived peptides because some regions in the primary structure of caseins contain overlapping peptide sequences that exert different biological effects, in this case both mineral binding and immunostimulatory action [107]. Even with the difference in the peptide pattern, it is evident that CPP binding iron (or other metal ions) remains soluble in the digestive tract, where they escape further enzyme digestion [106]. The authors have studied in depth the simulated digestion of CPP from peptide precursors. These studies greatly benefit from the knowledge of enzyme specificities and degradation mechanisms. CPase and chymotryptic activity of pancreatin exhibits broad specificity, cleaving bonds on the carboxyl side of several amino acid residues of CPP. The latter, which are in the mass range 960-7087 Da (Table 5), are good candidates for intestinal absorption and for playing a possible physiological role in mineral bioavailability. However, there are conflicting results on the lack of α_{s1}-CN (f43-52)2P and α_{s2}-CN (f1-19)4P identified by other authors after CN hydrolysis with pancreatin, an enzyme used during the intestinal step of simulated

physiological digestion [108]. Generally, the physiological effects of CPP may not always be extended to precursor peptides, although they are structurally similar. There are not enough data concerning the effects of CPP addition to probiotic acid fermented milks. However, probiotic bacteria such as *Lactobacillus acidophilus* and *Bifidobacterium* spp., selected because of their beneficial action, which they may manifest on the health of the consumer, grow slowly in milk because of the lack of the proteolytic activity [109]. For this reason, and also to reduce the fermentation time, probiotic yogurt is manufactured by yogurt bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) with the addition of probiotic culture. In parallel, non-digestible food ingredients, *i.e.*, "prebiotics", resist digestion in the small intestine and reach the colon, where they act as a growth factor for *Bifidobacterium* species and are metabolized into short chain fatty acids by a limited number of the microorganisms also comprising the colonic microflora. Prebiotics are principally oligosaccharides (fructo-oligosaccharides, inulins, isomalto-oligosaccharides, lactilol, lactosucrose, lactulose, pyrodextrins, soy oligosaccharides, transgalacto-oligosaccharides, and xylo-oligosaccharides) that stimulate bifidobacteria growth. In probiotic yogurt containing inulin as a prebiotic, the number of bioactive peptides increased, which means that elevation in the proteolytic activity has a synergistic effect with probiotic counts of yogurt cultures. Therefore, the most proteolytic strains of *St. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, spp. enhance the growth of *Lb. acidophilus* and *Bifidobacterium*. In our studies, the overall opiate activity of the bio-yogurt preparation containing *Lb. acidophilus* and *Bifidobacterium* spp. and inulin as a prebiotic was approximately twice that typical of traditional yogurt. In addition, the above yogurt preparation contained a variety of opioid agonistic and antagonistic, immunomodulation, anti-thrombotic, ACE-inhibitor, and anti-microbial activities. Traditional and probiotic yogurt both possess a characteristic soluble fraction composed by peptides exhibiting biological activity, amongst others. This fraction was found to include CPP, β-casomorphins and antithrombotic peptide precursors that did not differ greatly from one another. In a study comparing the proteolytic, amino-, di-, tri- and endopeptidase activity of nine strains of *St. thermophilus*, six strains of *Lb. delbrueckii*, fourteen strains of *Lb. acidophilus* and thirteen strains of *Bifidobacterium* spp., aminopeptidase activity was detected for all bacterial strains – traditional yogurt strains and probiotic bifidobacteria - both at the extracellular and intracellular levels. High dipeptidase activity was demonstrated by all bacterial strains for *Lb. delbrueckii* ssp. *bulgaricus*, *Lb. acidophilus*, and *Bifidobacterium* spp., whereas *St. thermophilus* had greater dipeptidase activity at the extracellular level.

4.3. CPP in a few cheese varieties

Whole milk contains a variety of endogenous plasmin-mediated CN peptides. In addition, CPP were released following cell lysis and release of intracellular LAB enzymes. This phenomenon was observed especially at the end of ripening in long-ripened cheeses, such as Comté [110], Grana Padano [111], Parmigiano-Reggiano and semi-hard Herrgard cheese [112]. In Grana Padano cheese, 45 CPP were identified, of which 24 originated from β-casein, 16 from α_{s1}-casein and 5 from α_{s2}-casein. These CPP formally derive from three parent peptides, namely β-CN (f7-28)4P, α_{s1}-CN (f61-79)4P and α_{s2}-CN (f7-21)4P [111]. By

comparing CPP of Grana Padano and Herrgard cheese, it was clear that CPP were all progressively shortened and dephosphorylated during ripening. CPP were very resistant to enzymatic degradation, especially when SerP residue was at the N-terminal end. The number of CPP identified according to the different procedures was comparable for Grana Padano [111] and Herrgard cheese [112]. In both the cheeses, CPP were progressively shortened and dephosphorylated during ripening, with both cheeses constituting heterogeneous mixtures of peptides phosphorylated at various sites sharing N- and C-terminally truncated CPP. However, some peptides proved very resistant to enzymatic degradation, especially when SerP residue was present at the N-terminal end of CPP [111]. The only SerP residue located at the N-terminus of CPP was subjected to dephosphorylation, exposing the dephosphorylated residue to aminopeptidase action. A heterogeneous CPP pattern also differentiated the cheese samples within a given form because of the phosphatase gradient amongst peripheral and central parts of the Grana Padano cheese form ($3\ 10^5$ *vs.* $3\ 10^2$). This is due to heat sensitivity in the temperature range 57 and 62 °C and the acid pH at which these enzymes are denatured. These data explain the discrepancy in the amount of serine, which varied by as much as 50% of SerP from the periphery towards the center of the cheese form [113]. In contrast, the CPP fraction of Herrgard cheese was more uniform, with the two cheese varieties sharing active plasmin and amino-peptidases from lactic acid bacteria. Because milk pasteurization denatures alkaline phosphatase while it activates plasmin [114], proteolysis in the above cheese is plasmin-dependent. It is therefore likely that CPP of pasteurized milk cheeses are intrinsically more stable than raw milk cheeses [111]. CPP in artisanal PDO ovine Fiore Sardo cheese have been previously reported [115]. Patterns of CPP similar to that observed for bovine cheese indicated that mechanisms of formation and degradation of CPP were similar regardless of the milk species and cheese variety. The dephosphorylation mechanism in Fiore Sardo was different from that found in Grana Padano cheese, most likely because of the use of different rennet types. In PDO Fiore Sardo cheese, no apparent difference in susceptibility to dephosphorylation was found amongst the differently located SerP peptide residues. This resulted in the simultaneous occurrence of partly dephosphorylated peptides, either internally or externally. CPP enrichment by HA, for example of pH 4.6 soluble fractions of hard Parmigiano Reggiano (PR) (30-mo-old), semi-hard, *pasta filata* Provolone del Monaco (PM) (6-mo-old), semi-cooked Asiago d'Allevo (AA) (3-mo-old) and mold-ripened cheese Gorgonzola (GR) (2-mo-old) cheese, has allowed the identification of CPP in high number (Figure 5) which may explain the broad-specificity of the cheese enzymes involved in CN proteolysis. Some CPP were derived from the Lys-X or Arg-X cleavage by plasmin primarily located in the *N*-terminal region of caseins, such as β-CN (f1-28)4P (Lys^{28}-Lys^{29}) or β-CN (f1-29)4P (Lys^{29}-Ile^{30}), α_{s1}-CN (f61-79)5P (Lys^{79}-His^{80}) and α_{s2}-CN (f1-24)4P (Lys^{24}-Asn^{25}). The native plasmin-derived CPP were then further hydrolyzed by cheese aminopeptidases and CPase into shorter peptides.

It is likely that ingested cheese carries a concentrated pH 4.6 soluble CPP fraction and a variable number of CPP according to the cheese variety. Above all, the presence and integrity of plasmin-mediated products of CN is a function of the milk, whether raw or pasteurized. Pasteurization reduces the milk plasmin activity only by ~15 percent, whereas plasmin activity increases during milk storage. UHT does not inactivate the plasmin in milk,

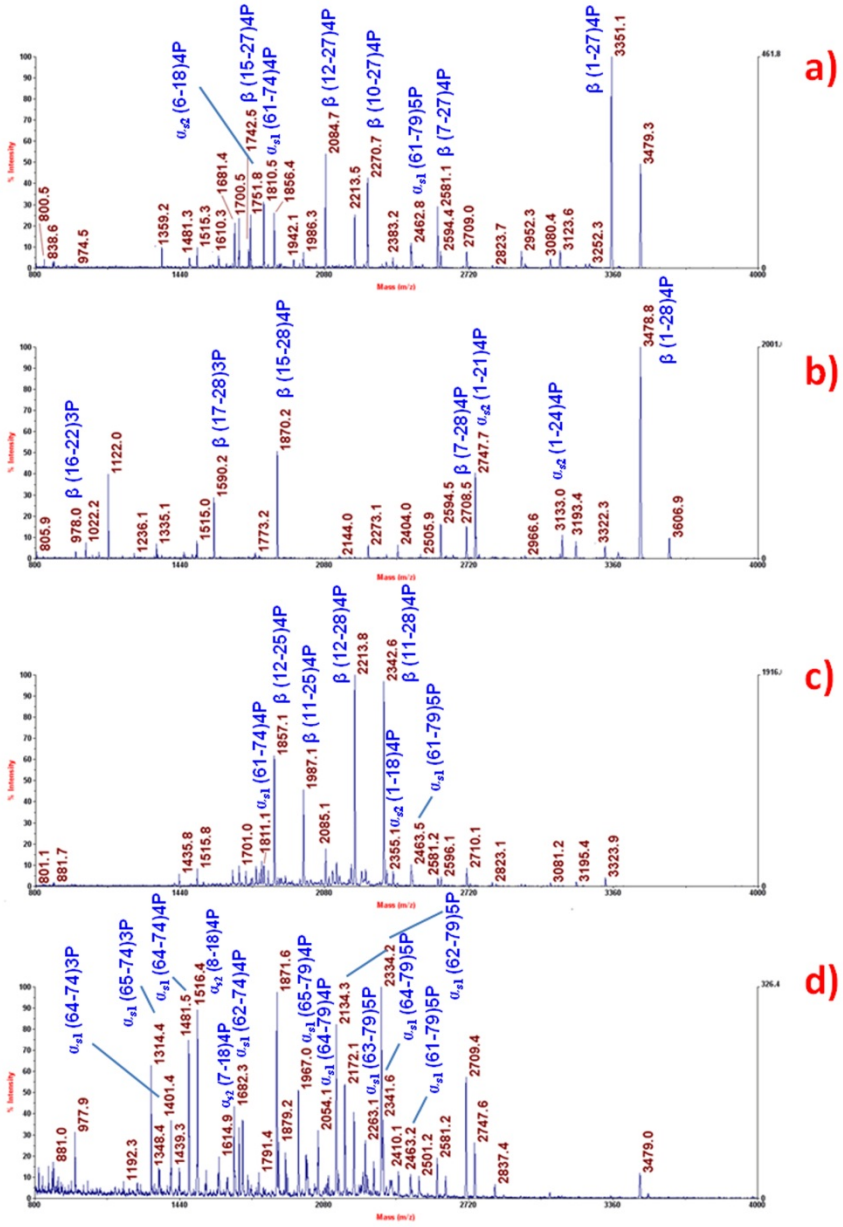

Figure 5. The MALDI spectra of CPP isolated by the addition of HA to pH 4.6 soluble fractions of Gorgonzola (a), Asiago (b), Provolone del Monaco (c), and Parmigiano Reggiano (d) cheeses. The inset magnifies the m/z values in the lower molecular mass peptide range 0.8-4 kDa.

and proteolytic activity will continue to damage milk. Heat treatments modify the peptide profile by increasing the content of larger peptides. The CN breakdown occurring during the ripening of PR cheese proceeded more slowly in PM cheese.. This means that eating PR cheese increases the quota of the co-ingested mineral bound CPP. In contrast, GR cheese show a different CPP level such as that of β-CN (f1-27)4P (3350.2 Da), resulting the most abundant CPP, when compared with hard cheeses (Figure 5a and 6); β-CN (f1-27)4P was further hydrolyzed into the shorter β-CN (f7-27)4P (2580.4 Da), β-CN (f10-27)4P (2270.0 Da), β- CN (f12-27)4P (2083.8 Da) and β-CN (f15-27)4 (1742.4 Da). The most abundant CPP in all three cheeses derived from the peptide β-CN (f1-28)4P, but long-ripened PR cheese was dissimilar from the other cheeses in its content of α_{s1}-CN (f62-79)5P (2332.9 Da) (Figure 5d and 6).

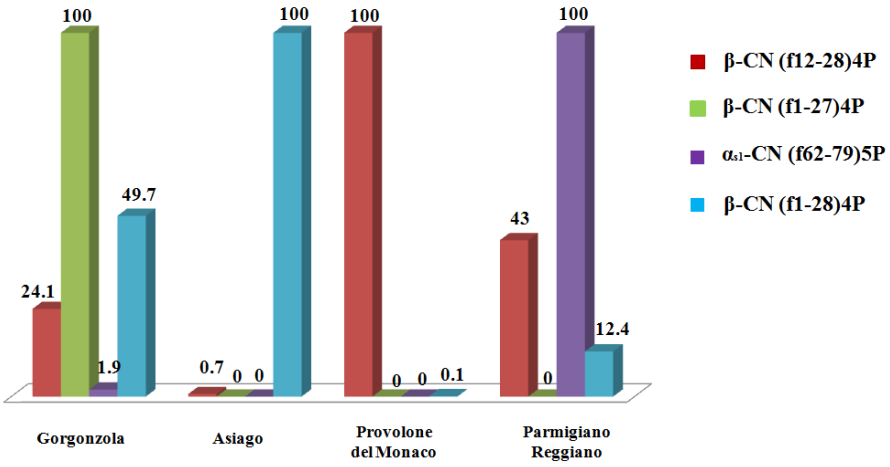

Figure 6. Histogram representation of CPP at 100% relative intensity and their performance in four cheeses.

β-CN (f1-28)4P (3478.4 Da) in AA cheese was the most abundant signal of the MALDI spectra and it was partially hydrolyzed into the shorter peptides β-CN (f7-28)4P (2708.5 Da), β-CN (f15-28)4P (1869.7 Da) and β-CN (f17-28)3P (1589.6 Da) (Figure 5b and 6). Considering exclusively the CPP molecular mass in the 3-3.5 kDa range of AA and GR cheese, the intensity of a high number of peptides transformed into a number of progressively lower molecular weight CPP, with accompanying liberation of peptides (Figure 5). The most common group of CPP occurred in the mass range of 1.7-2.9, reaching the maximum intensity for β-CN (f12-28)4P (2212.0 Da) in PM and α_{s1}-CN (f62-79)5P (2332.9 Da) in PR cheese (Figure 5c-d and 6). The presence of β-CN (f16-22)3P (977.7 Da) was discovered in both AA and PR cheeses and was not detected in the PM and GR cheeses (Figure 5). Our results show that longer plasmin-mediated peptides degraded into shorter CPP. These peptides became more evident when the chymosin retained in the cheese was largely inactivated by cooking the curd at high temperatures (~55 °C). The hydrolysis of CN by

chymosin was covered by that of plasmin, which became the principal proteolytic enzyme in the cheese. This phenomenon is particularly evident in PM raw milk cheese for which the plasmin-mediated β-CN (f1-28)4P peptide, representing ~0.1% of the CPP, was almost completely hydrolyzed into the shorter peptides β-CN (f11-28)4P (2341.1 Da), β-CN (f12-28)4P (2212.0 Da), β-CN (f11-25)4P (1985.7 Da) and β-CN (f12-25)4P (1856.6 Da) (Figure 5c). When comparing the PR and PM cheese, the former had a high extent of β-CN (f1-28)4P as judged by the higher levels of the peptide. This demonstrates that CPP of PR cheese are progressively transformed into a number of lower-molecular-weight peptides. In contrast, the quasi-total absence in the PM cheese of β-CN (f1-28)4P and relatively few of the various sizes β-CN-derived CPP (Figure 6) could be the effect of the enzyme decline from the optimum level of activity to zero enzyme activity.

α_{s1}-CN CPP originated for the greater parts from the internal regions of the amino acid sequence, namely αs1-CN (f61-79)5P and αs1-CN (f33-60)3P.

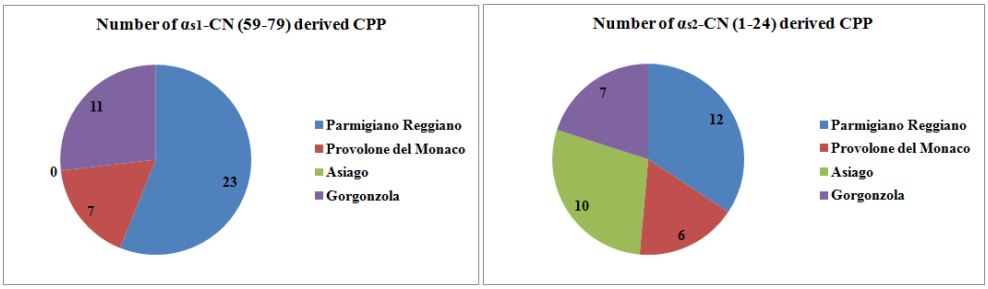

Figure 7. The number of CPP derived from α_{s1}-CN (f59-79)5P and α_{s2}-CN (f1-24)4P in Parmigiano Reggiano, Provolone del Monaco, Asiago and Gorgonzola cheeses.

In PR cheese, 23 casein-derived CPP were found to derive from the internal region of α_{s1}-CN, *i.e.*, α_{s1}-CN (f59-79)5P, whereas they were not detected in AA (Figure 7).

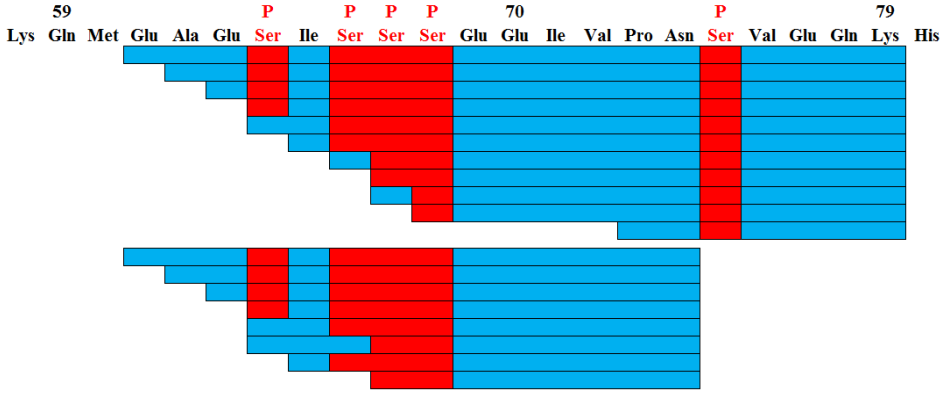

Figure 8. Amino acid sequence of the α_{s1}-CN (f59-79) 4P peptide and the CPP identified in PR cheese. Phosphoserine residues are indicated by red boxes.

The profile of the CPP depicts the mechanisms of both the proteolysis and dephosphorylation in a long ripened cheese. Peptide α_{s1}-CN (f61-79)5P, most likely arising from the parent peptide α_{s1}-CN (f1-79)7P through cleavage at Met^{60}-Glu^{61}, was dephosphorylated and concurrently hydrolyzed into shorter peptides. Alkaline and/or acid phosphatases acting on SerP residue dephosphorylated CPP. N-terminal Ser was then exposed to aminopeptidase and released as a free amino acid. Bacterial CPase or exopeptidase such as cathepsin D/chymosin release α_{s1}-CN (f61-74)4P and other derived CPP through cleavage at Asn^{74}-$SerP^{75}$ (Figure 8). In PR aged 30 months, α_{s1}-CN (f61-79)5P (2462.1 Da), α_{s1}-CN (f62-79)5P (2332.9 Da), α_{s1}-CN (f63-79)5P (2261.9 Da), α_{s1}-CN (f64-79)5P (2132.8 Da), α_{s1}-CN (f64-79)4P (2052.8 Da), α_{s1}-CN (f65-79)4P (1965.7 Da), α_{s1}-CN (f62-74)4P (1681.3 Da), α_{s1}-CN (f64-74)4P (1481.2 Da), α_{s1}-CN (f64-74)3P (1401.2 Da) and α_{s1}-CN (f65-74)3P (1314.1 Da) were the dominant CPP (Figure 5d and 8). Indeed, only 7 CPP for PM cheese and 11 CPP for GR were derived from α_{s1}-CN (f59-79)5P, namely α_{s1}-CN (f61-79)5P (2462.1 Da) and α_{s1}-CN (f61-74)4P (1810.5 Da) (Figure 5a and c and 7). Considering the α_{s2}-CN peptide, the α_{s2}-CN (f1-24)4P CPP were similar in number but significantly different in the case of the four cheese varieties (Figure 6). The dominant α_{s2}-CN-derived CPP were α_{s2}-CN (f1-24)4P (3132.9 Da) and α_{s2}-CN (f1-21)4P (2747.6 Da) in AA cheese (Figure 5b). The most abundant α_{s2}-CN-derived CPP were α_{s2}-CN (f1-18)4P (2355.1 Da) for PM and α_{s2}-CN (f6-18)4P (1751.4 Da), a shortened form of the primary CPP α_{s2}-CN (f1-18)4P, for GR cheese (Figure 5a and c). α_{s2}-CN (f7-18)4P (1614.3 Da) and α_{s2}-CN (f8-18)4P (1515.1 Da) characteristically accumulated in PR cheese (Figure 5d). Similar and discrete phosphorylated CPP derived species for α_{s2}-CN (f1-24) (3P and 4P) and β-CN (f1-28) (3P and 4P) occurred in all cheeses. For the other casein fractions, primary CPP are fully phosphorylated, such as α_{s1}-CN (f61-79)5P, whereas the derived peptides show a level of phosphorylation less than native form as observed in PM and GR. A higher dephosphorylation level characterized the CPP profile of PR cheese (Figure 8). The different profile of CPP could derive from the different length of ripening and from the cheese variety.

CPP in ovine cheeses

Cheeses that contain CPP are also manufactured from ovine milk. Proteolytic enzymes in Pecorino cheese originate from chymosin, pepsin and other clotting preparations such as paste rennet. These enzymatic activities are complemented by those secreted by the vegetative spores of *Penicillium roqueforti* during the maturation of blue-veined cheeses. The CPP patterns of Pecorino and Roquefort cheese have been characterized and main components identified. The sequence alignment of the CPP released throughout the hydrolysis of the β-CN (f1-28)4P in Pecorino and Roquefort cheeses are compared in Figure 9.

In PR cheese of different ages, the released CPP were progressively degraded at C- and N-terminal ends. CPases work from the C-terminal end and aminopeptidases from the N-terminal end, both removing the terminal amino acid residues incrementally. LAB does not produce CPases; thus, the ability to liberate the carboxyterminal amino acid and peptides is typical of the mold. The N-terminal amino acid seemed to be released faster than the C-

terminal residues because of the lower activity of CPases. There are negligible differences in the CPP level of different cheese lots primarily because of the action of the enzymes from *P. roqueforti* after its sporulation in blue-veined cheese. More long-chain CPP β-CN, such as β-CN (f1-28)5P-4P, β-CN (f1-27)5P-4P and β-CN (f1-24)5P-4P, were detected in Pecorino cheese, whereas β-CN (f7-28)5P-4P and β-CN (f7-27)5P-4P resulted from the longer CPP in Roquefort cheese (Figure 9). In these cheese varieties, both α_{s1}- and β-CN have been described as completely hydrolyzed at the end of ripening. This contradicts other findings indicating ~50% CN hydrolysis. Plasmin, NSLAB, and Lactobacilli contaminating flora proteinases are mainly responsible for extensive proteolysis in Parmigiano-Reggiano cheese, which is ripened for ~24 months at ~18-20 °C [116]. Here, chymosin is denatured by the high cooking temperature used during the manufacture of cheese. Molds develop at approximately 2 to 5 weeks of ripening, concurrently degrading CN into peptides of various sizes [117]. A similar mechanism for β-CN-derived CPP was found in Grana Padano cheese. Ser was proteolytically cleaved by aminopeptidases, and SerP hindered cleavage by the latter and continued its action after dephosphorylatation of SerP.

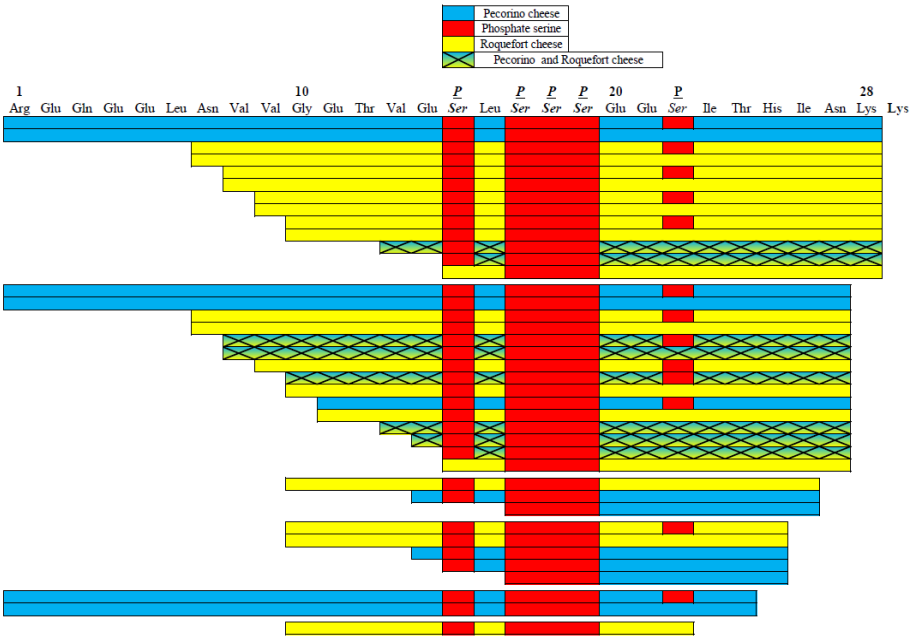

Figure 9. Amino acid sequence of the β-CN (f1-28)4P peptide and the CPP identified in Pecorino (blue line) and Roquefort (yellow line) cheeses. CPP common to the two cheeses are indicated by crosses, and phosphoserine residues are indicated by red boxes.

5. Anticariogenic effect of CPP in yogurt and cheese

Due to proteolytic activity, a great number of CPP are formed in raw milk cheese. In contrast, the enzymatic digestion of proteins to peptides can be reduced by milk

pasteurization. Notwithstanding this, yogurt remains a consistent source of bioavailable CPP even if milk is heated to high temperatures (90 °C, 30 min) to create an inoculation medium in which the bacteria can grow and produce lactic acid. LAB provide plenty of CPP in the form of soluble complexes with Ca^{2+} that are effective in avoiding the life-threatening calcium phosphate precipitation, enhancing the intestinal absorption of minerals and retention in the human body [34,118]. The mineral-binding power of CPP also depends on the number of binding sites and their relative accessibility [119]. Dairy products such as cheese and yogurt are both rich in multi-phosphorylated peptides capable of interacting with colloidal calcium phosphate to manifest anticariogenic properties in human and animal [120-121,13]. The mechanism of anticariogenicity might be due to a direct chemical effect of casein and calcium phosphate components [122]. Tooth enamel is a polymeric substance consisting of crystalline calcium phosphate, embedded in a protein matrix. Thus, CPP can significantly enhance localization of ACP at the tooth surface, inhibiting enamel demineralization and promoting remineralization. In the development of teeth and bone, CPP act as hydroxyapatite nucleator and control the growth of the crystals, resulting in unique crystal morphology. A new calcium phosphate remineralization technology has been recently developed based on the complex CPP-ACP [Recaldent™ CASRN691364-49-5] [66]. This preparation is claimed to stabilize calcium and phosphate ions in high concentrations by binding ACP to pellicle and plaque of the tooth surface. Moreover, CPP-ACP inhibited the adhesion of *Streptococcus mutans* to the tooth surface producing a copious reservoir of bioavailable calcium ions [67]. Cheese and yogurt CPP have the ability to stabilize calcium phosphate in solution, forming small CPP-ACP nanocomplexes. The calcium-binding ability of CPP has been applied by clinical dentists to show that CPP stabilize high concentrations of calcium, phosphate, and fluoride ions on the tooth surface by binding them to pellicle and plaque [66,123]. In dental plaque, CPP-ACP binds onto the surface components of the intercellular plaque matrix. Incorporation of CPP-ACP into the plaque will increase the calcium and phosphate content by forming a stable supersaturated solution of calcium phosphate. Thus, the availability of calcium in plaque provides a natural anticaries protective effect, either by suppression of demineralization promoted by fermentative acids in mouth, through an increased remineralization by binding calcium ions to teeth enamel, or possibly a combination of both. An inverse relation between plaque calcium and caries incidence has been evidenced [124-125]. Reynolds (1997) [126] has demonstrated that CPP-ACP can actually remineralize subsurface lesions in human enamel, and this is indeed the basis of one claim of his patent [127]. The diffusion of available CPP-ACP in the mouth is controlled by two main factors: i) the molecular weight of the diffusing species, the square of which is inversely proportional to the diffusion coefficient; and ii) the binding characteristics of the diffusing species, which dictate how much CPP-ACP is free to diffuse at a given time [128]. At neutral pH, calcium diffusion is limited by the quantity of bound calcium, reducing the effective diffusion coefficient (De) and creating a measurable restricted effective diffusion coefficient (rDe), where $rDe = De/(R + 1)$ and R is the ratio of bound to free calcium. A large number of potential binding sites for calcium can have significant effects on the calcium diffusion coefficient; this effect is maintained at

low pH, although overall diffusion is slightly faster [129-130]. Conversely, one may infer that ACP may also bind to dental plaque and tooth enamel, thus having beneficial effects on teeth remineralization [67]. In addition, SerP residues of the CCP-HA complex are exposed to intestinal alkaline phosphatase, which favors metal ion bioavailability by releasing inorganic phosphate. Milk, ice cream, and cheese have been observed to lower the incidence of dental caries in rats [131]. Elderly people that eat cheese several times per week had a lower incidence of root surface caries development [132]. CPP of yogurt have been observed to have an inhibitory effect on demineralization and are able to promote remineralization of dental enamel [133]. Moreover, anticariogenic activity has also been reported for egg phosphopeptides (viz. phosvitin and phosphophorin) [134-135]. The heating process affects the bioavailability of CPP; for example, milk sterilization can induce dephosphorylation of phosphoseryl residues and dehydroalanine residue formation [136].

6. CPP as nutraceutical ingredients in functional foods

6.1. Examples of commercial CPP preparation

Some commercial products have been developed containing moderately hydrolyzed milk proteins as the sole protein source. Hypoallergenic formulas are based on partially or extensively hydrolyzed proteins. Both formulae are better tolerated by small premature infants than native cow milk protein. Ultrafiltered micellar casein and microfiltered whey protein concentrate are known to slow down the digestive process. Typical composition data of commercial phosphopeptide preparations (m/m) include 91.3% (TN x 6.47) or 94.8 (dry basis) protein, 16.0% CPP, and 6.0% free amino acids. Compared with the expected CPP composition, the commercial preparation contained ~5% undigested protein, ~30% peptides in the intermediate molecular mass 5000-20000 Da range, and ~48% of molecular mass in the 500-5000 Da range (Tatua, New Zealand). The preparation is generally very complex and dependent on the procedure used to perform casein hydrolysis. Therefore, commercial products can be considered to be an enriched-CCP preparation containing 16% CPP without specification of the peptide size and phosphorylation degree. Preliminary analysis performed by MALDI-TOF analysis indicated that the signal in the mass spectra originated exclusively from the β-CN digestion. The intensity of non-CPP such as β-CN (f191-209), β-CN (f184-209), β-CN (f177-209) and β-CN (f170-209) was sufficiently high to obscure other CN peptides (MALDI spectrum not shown). As reported above, a number of available techniques allow the separation of CPP and non-CPP. To reduce the large dynamic range of non-CPP, HA was used for CPP enrichment [90,94,102]. CPP included multiply phosphorylated peptides (up to 4 phosphorylation residues). CPP β-CN (f1-28)4P and 3P, β-CN (f1-25)4P, 3P and 2P and β-CN (f2-25)4P, 3P and 2P were found and may be indicative of the progressive CPP dephosphorylation (MALDI spectrum not shown). Interestingly, in addition to β-CN, the commercial CPP (Tatua, NZ) also displayed α_{s1}- or α_{s2}-CN-derived CPP, invisible or weakly visible before the sample was treated with HA (Table 6).

Finally, a method for reducing the complexity of peptide mixtures was the separation of non-CPP and CPP by trapping CPP on HA under neutral conditions. This could be the principle for an industrially based production of CPP.

Parent Protein	Molecular Mass (Da)		Start		End	Peptide Sequence	Peptide Modifications
	Measured	Theoretical					
α_{s1}-CN	1138.4	1138.4	115	-	123	(N)SAEERLHSM(K)	1P
	1526.7	1526.7	35	-	47	(K)EKVNELSKDIGSE(S)	1P
	1831.9	1831.8	75	-	89	(N)SVEQKHIQKEDVPSE(R)	1P
	1859.9	1859.9	75	-	89	(N)SVEKHIQKEDVPSER(Y)*	1P
	1926.5	1926.7	43	-	58	(K)DIGSESTEDQAMEDIK(Q)	2P
	1950.9	1950.9	104	-	119	(K)YKVPQLEIVPNSAEER(L)	1P
	1988.0	1987.9	75	-	90	(N)SVEQKHIQKEDVPSER(Y)	1P
	2054.7	2054.7	43	-	59	(K)DIGSESTEDQAMEDIKQ(M)	2P
	2185.8	2185.8	43	-	60	(K)DIGSESTEDQAMEDIKQM(E)	2P
	2400.9	2400.9	41	-	60	(L)SKDIGSESTEDQAMEDIKQM(E)	2P
	2597.0	2597.0	37	-	58	(K)VNELSKDIGSESTEDQAMEDIK(Q)	2P
	2856.1	2856.1	37	-	60	(K)VNELSKDIGSESTEDQAMEDIKQM(E)	2P
	3113.2	3113.3	35	-	60	(K)EKVNELSKDIGSESTEDQAMEDIKQM(E)	2P
	3193.2	3193.3	35	-	60	(K)EKVNELSKDIGSESTEDQAMEDIKQM(E)	3P
	3699.6	3699.5	61	-	90	(M)EAESISSSEEIVPNSVEQKHIQKEDVPSER(Y)	4P
	3779.6	3779.5	61	-	90	(M)EAESISSSEEIVPNSVEQKHIQKEDVPSER(Y)	5P
	3974.5	3974.6	59	-	90	(K)QMEAESISSSEEIVPNSVEQKHIQKEDVPSER(Y)	4P; Oxidation (Met)
	4479.0	4479.0	23	-	60	(R)FFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM(E)	2P
α_{s2}-CN	1432.6	1432.6	135	-	146	(N)SKKTVDMESTEV(F)	1P
	1538.6	1538.6	126	-	137	(R)EQLSTSEENSKK(T)	2P
	1578.6	1578.6	123	-	134	(T)LNREQLSTSEEN(S)	2P
	1694.7	1694.7	125	-	137	(N)REQLSTSEENSKK(T)	2P
	1793.7	1793.7	123	-	136	(T)LNREQLSTSEENSK(K)	2P
	1921.8	1921.8	123	-	137	(T)LNREQLSTSEENSKK(T)	2P
	2715.3	2715.3	115	-	137	(R)NAVPITPTLNREQLSTSEENSKK(T)	2P
	3051.3	3051.2	1	-	24	KNTMEHVSSSEESIISQETYKQEK(N)	3P
	3131.2	3131.2	1	-	24	KNTMEHVSSSEESIISQETYKQEK(N)	4P
	3786.6	3786.6	115	-	146	(R)NAVPITPTLNREQLSTSEENSKKTVDMESTEV(F)	3P
	4034.6	4034.8	115	-	148	(R)NAVPITPTLNREQLSTSEENSKKTVDMESTEVFT(K)	3P
β-CN	1600.7	1600.7	29	-	40	(K)KIEKFQSEEQQQ(T)	1P
	1785.7	1785.7	35	-	48	(Q)SEEQQQTEDELQDK(I)	1P
	1945.8	1945.8	29	-	43	(K)KIEKFQSEEQQQTED(E)	1P
	2559.2	2559.1	29	-	48	(K)KIEKFQSEEQQQTEDELQDK(I)	1P
	2805.2	2805.2	1	-	24	RELEELNVPGEIVESLSSSEESIT(R)	2P
	2885.2	2885.2	1	-	24	RELEELNVPGEIVESLSSSEESIT(R)	3P
	2906.3	2906.3	29	-	51	(K)KIEKFQSEEQQQTEDELQDKIHP(F)	1P
	2961.3	2961.3	1	-	25	RELEELNVPGEIVESLSSSEESITR(I)	2P
	2965.3	2965.2	1	-	24	RELEELNVPGEIVESLSSSEESIT(R)	4P
	3041.2	3041.3	1	-	25	RELEELNVPGEIVESLSSSEESITR(I)	3P
	3053.4	3053.4	29	-	52	(K)KIEKFQSEEQQQTEDELQDKIHPF(A)	1P
	3121.1	3121.3	1	-	25	RELEELNVPGEIVESLSSSEESITR(I)	4P
	3396.5	3396.5	1	-	28	RELEELNVPGEIVESLSSSEESITRINK(K)	3P
	3476.5	3476.5	1	-	28	RELEELNVPGEIVESLSSSEESITRINK(K)	4P

* indicates a CPP containing the alternative non-allelic deletion of Gln[78] [137]; Phosphoserine residues are coloured red.

Table 6. CPP identified in a commercial CPP preparation (Tatua Co-operative Dairy Company Ltd) by tandem MS sequencing.

6.2. Examples of industrial methods for CPP preparation

Starting from CN, the overall preparation process gave 16% CPP (Figure 10), less than the theoretical yield of 23% (Y_{theor} =22.8%), which means approximately 20% yield on the basis of weight, a yield higher than that obtained by other researchers [138-140]. In their production

experiments, the authors obtained a CPP preparation as high as 18.8% degree of hydrolysis (DH).

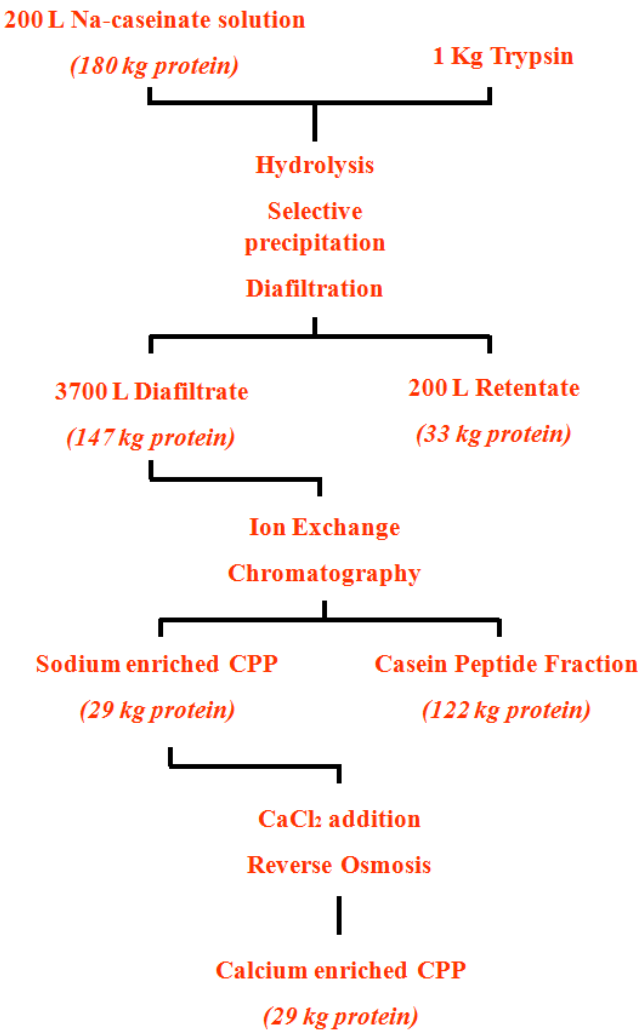

Figure 10. Schematic representation of the process-scale isolation of tryptic CPP from caseinate, showing the protein flow through the process (source: [141]).

The example of 2000 L Na-caseinate solution containing 180 kg protein and 1 kg trypsin yielded 29 kg of calcium-enriched CPP, corresponding to a yield of ~16% (w/w) [141]. A variety of raw materials such as acid casein, sodium caseinate, and calcium caseinate, as well as skimmed raw milk, milk concentrate by ultrafiltration, pasteurized and UHT milk near the limit date for consumption, may be used as the substrate for CPP production. The

optimal parameters for the hydrolysis with trypsin were 37 °C and pH between 7.5 and 8.5. The casein hydrolysate solution was roughly fractioned by ultrafiltration with appropriate membranes to obtain soluble CPP both in the "permeate" and "retentate". Lower-molecular peptides/phosphopeptides occurred in the "permeate" and larger in the "retentate". The diafiltrate containing the tryptic casein digest was loaded on the ion exchange resin, and the non-CPP flowed through and were recovered for further use, *e.g.*, as substrate for bacterial culture. Bound CPP, free of non-CPP, are eluted using sodium hydroxide, *e.g.*, 0.2 M, and conductivity and absorbance monitored at 280 nm. The eluate containing CPP is collected and then concentrated, typically by reverse osmosis. Peptides were pasteurized (85 °C, 15 s) and spray dried, yielding sodium enriched CPP (Na-CPP). In other cases, to obtain calcium-enriched CPP, the concentrate is added with $CaCl_2$ in excess, diafiltered, and then CPP solution concentrated by reverse osmosis until the filtrate conductivity was negligible (less than 3 mS cm^{-1}). The concentrate is spray dried, and the product is labeled as calcium-enriched CPP (Ca-CPP).

6.3. Traditional and new processes for the use of CPP in alimentary products

A Ca^{2+}/ethanol selective precipitation procedure was used to produce a CPP and non-CPP concurrently from an alcalase digest of whole casein in which the traditional and new processes for CPP production were reported [142]. CN is trypsinized, and the pH of the solution is adjusted to 4.6 to separate the non-peptide material. The CPP-Ca^{2+} aggregation was induced by ethanol addition in the supernatant and recovered as precipitate for freeze drying. In the novel process, the step of non-peptide material removal was omitted, and non-CPP (CNPP) was recovered as supernatant for use in alimentary products. For casein, the use of alcalase, a cheap enzyme suitable for industrial application, for hydrolysis was suggested [142]. The CN hydrolysates were separated into the two types of peptides using combined treatment with $CaCl_2$ and ethanol. CPP and non-CPP comprised components with molecular weight lower than 2509 Da and 2254 Da, respectively, as determined using size exclusion HPLC. A DH of 20% for the CN hydrolysate was achieved. At the end, the recovery of CPP reached 24%. The phosphorus component of CPP was 3.08%, and nitrogen recovery was approximately 76% [142]. CPP generally had an improved solubility and transparency even under acid conditions and could be used as ingredient for beverages such as sport drinks, soft drinks, health drinks, fermented products, vitamin concentrates, fruit or fruit fractions.

6.4. Patented methods for CPP production as ingredients for alimentary products

A method for the preparation of selected anticariogenic CPP comprised the steps of complete digestion of CN as soluble monovalent cation salt with a proteolytic enzyme: the addition of a mineral acid to the solution to adjust the pH to approximately 4.7; the removal of any produced precipitate; the addition of $CaCl_2$ to a concentration of approximately 1.0% (w/v) to cause the aggregation of CPP; the separation of the aggregated CPP from the solution through a filter with a molecular weight exclusion limit within the range 10000 to

20000 while passing the bulk of the remaining CPP in solution; the diafiltration of the separated CPP with water through a filter; the concentration of the solution; and the drying the retentate [143]. Peptides not included in the aggregation were removed by ultrafiltration/diafiltration. By this means, anticariogenic CPP at purity greater than 90% were obtained [143]. CPP including calcium, magnesium or both salts (or zinc, ferric or other salts) are produced by submitting CN to proteolytic enzyme hydrolysis, ultrafiltering the resulting hydrolyzate to produce a permeate containing CPP, adding a bivalent cation salt to the peptides to form CPP aggregates, and separating by ultrafiltration the CPP aggregates and non-CPP [144]. When CPP salts need to be converted to free phosphopeptides, they can be restored by acidification with HCl; the solution is then diafiltered extensively through a 1000 molecular weight cut-off membrane to remove excess calcium chloride.

6.5. Example of CPP applications as nutraceuticals in functional foods

The CPP-salts complex can be added to different foods. A stable acidic beverage or other alimentary products can be obtained by digesting casein with trypsin, precipitating the insoluble components at acid pH, adjusting the pH of the obtained supernatant to approximately 6.0, then adding calcium chloride and ethanol to recover an acid-soluble calcium complex of CPP and the reaction product and adding them to a soft drink [145]. The acid-soluble calcium complex of CPP enhances calcium absorption from food because calcium may be absorbed by the body in the form of soluble calcium. Acid-soluble CPP is a mixture of α_{s1}-, α_{s2}- and β-CPP, forming essentially no turbidity in solution at pH of 3.0 or less, and having purity greater than 90%, molecular weights between approximately 2500 and 4600 Da, and the ability to solubilize at least 100 ppm calcium at a concentration of 0.5 mg/ml CPP. The acid-soluble CPP produced *in vitro* also have a solubilizing capability on iron. It is widely believed that iron must be solubilized for absorption through the small intestine. Accordingly, health may also be enhanced by the absorption of the soluble iron in the drink by the human body. Similarly, magnesium may be solubilized in a drink or edible product of this type [145]. Therefore, in addition to drinks, preparation of CPP with high negative charge could be used as additive for healthy foods or for dietetic or pharmaceutical compositions, as they are capable of increasing the *in vivo* absorption of calcium or other ions [146]. Another interesting invention relates to processes and the compositions that are useful to remineralize the teeth of mammals, particularly humans, and impart acid resistance thereto. These compositions included a gum base or carrier, sweetening agents, CPP-ACP preparation and food-grade acids [147]. Because many chewing gum and confectionery products usually contain acids, many consumers enjoying chewing gum and confectionery products ingest acids causing demineralization of the tooth surface. CPP-calcium phosphate complexes are known to have anticariogenic teeth strengthening effects that could be used address the problem of dissolution or demineralization of tooth enamel and the resultant formation of dental caries. Exogenous CPP-ACP preparations have also been added to milk in 2.0-5.0 g⁄L amounts to remineralize enamel subsurface lesions, which actually increased with respect to the control [148].

These inventions create functional foods with undoubted beneficial effects on human health, possibly promoting recalcification of bones, protecting the tooth enamel from decay and other possible health benefits.

7. Conclusion

Proteins are no longer considered merely nutritional components because they possess encrypted peptides with possible biological properties [149-153]. The cited literature has highlighted that bioactive peptides may be released *in vitro* or *in vivo* by digestive and bacterial enzymes starting from casein or generally inactive precursors [151,154-155]. We have examined the case of casein-derived phosphopeptides that can be applied as dietary supplements in "functional foods" and produced on an industrial scale. With this, it has been demonstrated that, each time a health-enhancing nutraceutical is required for a functional food, an appropriate enzymatic hydrolysis of casein needs to be designed [156-157]. In addition to the anticariogenic activity, the most important function displayed by CPP is that soluble and encrypted casein phosphopeptides can arrive without modification of the phosphorylated sequence to the brush border membrane. It is postulated that short-sequence peptides reach their putative receptors in many tissues without modification. Whether they enter blood circulation or whether their action is restricted to a peripheral circle are current questions that await response. In conclusion, this review has considered milk and cheese CPP for specific food ingredients. The contribution of *in vitro* casein digests by gastric proteases to potential biologically active substances in the intestine must be simultaneously considered. Scientists are currently involved in investigations to define the *in vivo* fate of all of the bioactive peptides. The *in vitro* studies have allowed the scientists to compare the predicted and the experimental sequence of CPP. This step is preliminary to the clinical investigations designed to determine the bioactivity of the milk hydrolysates. These findings open an industrial perspective that will permit the unrestricted use of CPP in healthy promoting food application.

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Chapter 7

Allergenicity of Milk Proteins

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Additional information is available at the end of the chapter

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1. Introduction

Adverse reactions to food are currently classified into toxic and non-toxic reactions. There is a normal range of concentrations of naturally occurring toxic compounds, which can easily increase during food processing. For example, thermal processing can cause the unintended and undesirable formation of toxic compounds, such as acrylamide in fried potato chips and furan in sterilized canned vegetables, together with losses of certain nutrients. The incidence of non-toxic reactions depends on individual susceptibility to a specific food or food ingredient, although these reactions are often dose-dependent. The non-toxic types may be divided further into immune- and non immune-mediated reactions. The term "hypersensitivity" is used for immune-mediated reactions, and the term 'intolerance' is used for non immune-mediated reactions (Figure 1). Immune-mediated reactions may be IgE-mediated (i.e., allergy or type I hypersensitivity) or non-IgE-mediated, whereas food intolerance may be enzymatic, pharmacologic or undefined. The incidence of immune-mediated adverse reactions to foods has increased in recent decades. In healthy subjects, orally ingested dietary proteins induce antigen-specific systemic hyporesponsiveness, termed oral tolerance. This phenomenon is well described in animal models, although the mechanisms remain unknown. Abrogation of oral tolerance or failure to induce oral tolerance may result in the development of food hypersensitivity. Immune reactions that cause tissue damage may be mediated by four reaction types that were defined by Coombs and Gell [1] (Figure 2). Type I, or anaphylactic hypersensitivity, is mediated by the reaction of an antigen with specific IgE antibodies that are strongly bound through their Fc receptor (CD23, IgεR) to the surface of the mast cell. Crosslinking of Igε receptors by divalent hapten leads immediately to the release of mediators, for example, some cytokines [primarily interleukin 4 (IL4)] and histamine, which are both activities of eosinophil chemotactic factor (ECF) and neutrophil chemotactic factor (NCF) (Figure 2A). This type of hypersensitivity occurs within minutes of antigen exposure. Some reaction mediators produce local skin,

gastrointestinal and respiratory tract manifestations, and a systemic allergic reaction to an allergen that is associated with a dangerously low blood pressure. Moreover, some of the mediators exhibit chemoattractant activity and induce the infiltration of neutrophils, eosinophils, macrophages, lymphocytes and basophils within 6-12 h after challenge. The localized late-phase inflammatory response may also be mediated partly by cytokines that are released from mast cells. In type II or antibody-dependent cytotoxic hypersensitivity, antibodies recognize antigens on the surface of specific cells or tissues. Once activated, the complement system can initiate a variety of responses that can lyse and destroy cells. Phagocytic and cytotoxic K cells, which have receptors for the Fc-part of IgG or an activation component of complement, i.e., C3b, may also destroy cells (Figure 2B). In type III, or immune complex-mediated hypersensitivity, the soluble antigen can activate the complement and deposited phagocytes. Leucocytes may release tissue-damaging mediators and activate phagocytes, culminating in tissue damage. The complex can also induce thrombin-mediated platelet aggregation and release a vasoactive amine (Figure 2C). Type IV reaction, or delayed-type hypersensitivity, arises more than 24 h after an encounter with the antigen. Type IV reactions are mediated by antigen-sensitized CD4+ T cells (T helper cells) that release inflammatory mediators [e.g., IL2 and interferon-y (lFN-y)], attract phagocytes to site of infection, activate an inflammation response and lyse invading cells (Figure 2D). Because lytic enzymes are secreted from the phagocytic cells into the surrounding tissue, localized tissue destruction can progress.

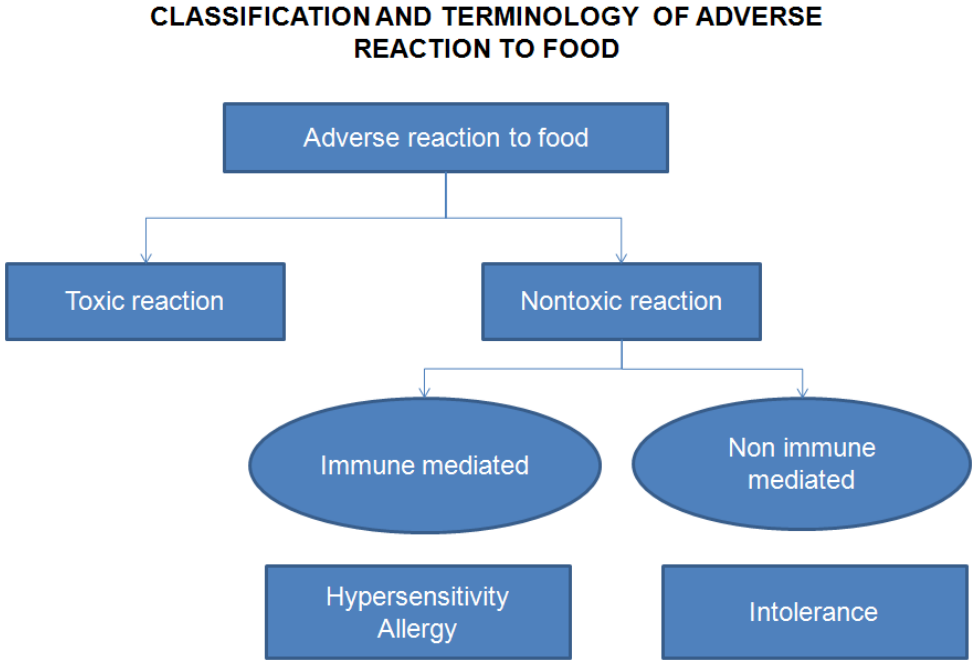


Figure 1.

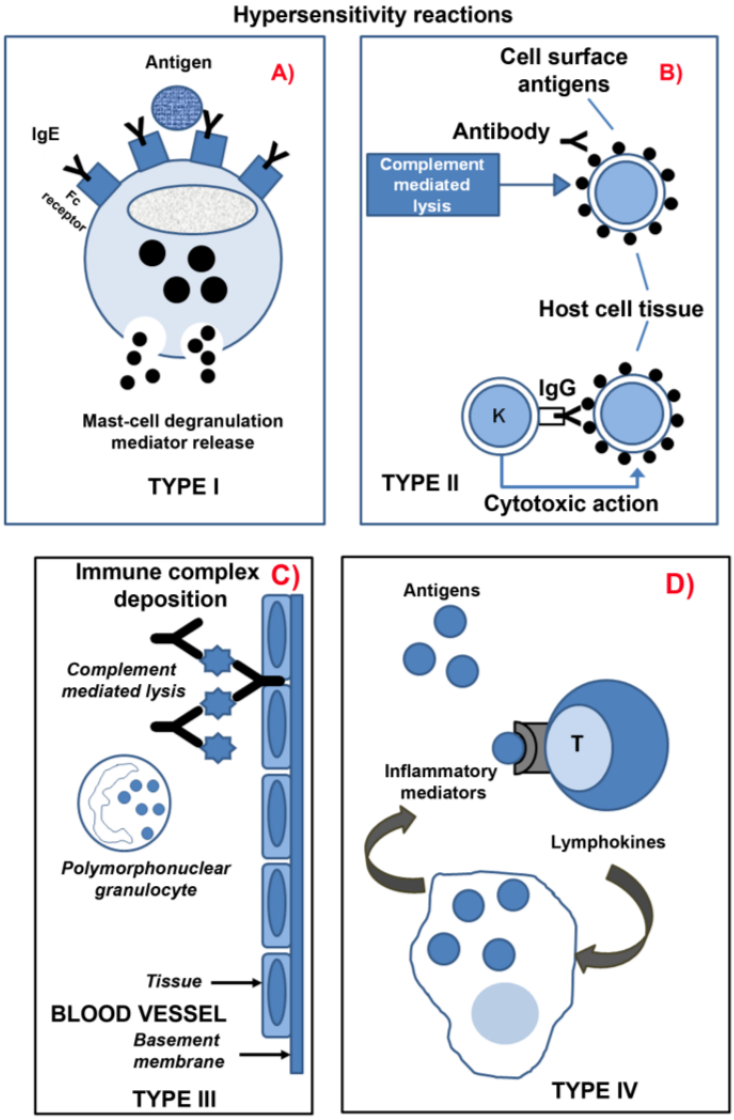


Figure 2. Types of Allergy Mechanisms according to Coombs and Gell [1].

The mechanisms underlying of allergic sensitization to food include genetic susceptibility, aberrant barrier functions of the skin epithelium and gut mucosa and dysregulation of immune functions. Despite a wide range of clinical manifestations, there are at least two common prerequisites for the development of a general food allergy (FA). First, intraluminal antigens must penetrate the mucosal barrier of the intestine. Second, the absorbed antigens must cause harmful immune responses [2].

2. Adverse reaction to milk

Non-toxic adverse reactions to milk are primarily caused by either lactose intolerance or milk allergy.

2.1. Lactose intolerance

Milk intolerance is due to the inherited lack of the specific enzyme, β-galactosidase that is required to hydrolyze lactose. For lactose malabsorption, the most common therapeutic approach excludes lactose-containing milk from the diet. To make yogurt edible, exogenous β-galactosidases that hydrolyze yogurt lactose or probiotics for their bacterial lactase activity are added. However, further studies are required to clarify the role probiotics play in lactose intolerance therapy, which includes considering their well-known beneficial effects on intestinal function, gas metabolism and motility [3]. A prolonged contact time between β-galactosidase and lactose delays the gastrointestinal transit time and chronic lactose ingestion to improve colonic adaptation. It is known that high concentrations of β-galactosidase are physiologically present in neonates, but a genetically programmed and irreversible decline of the activity occurs after weaning [4], which results in primary lactose malabsorption. The secondary hypolactasia because of intestinal mucosa brush border damage that increases the gastrointestinal transit time is a transient and reversible condition [5]. Bloating, flatulence, abdominal pain, the passage of loose and watery stools, excessive flatus and diarrhea are gastrointestinal symptoms of lactose intolerance [6]. However, lactose occurring in the colonic lumen does not necessarily produce gastrointestinal symptoms because of the variable amount of residual intestinal mucosal lactase activity that possibly digests lactose. The availability of recombinant β-galactosidase as an exogenous lactase has resolved problems concerning bacteria that release β-galactosidase during gastric passage. By this means, lactose is partially or fully degraded to glucose and galactose and is therefore easily eliminated by simple dietary adjustments that mediate the effects of lactose intolerance [7]. An accurate diagnosis of lactose intolerance can significantly reduce patient anxiety and avoid unnecessary examinations and treatments [8].

2.2. Milk allergy

Cow's milk allergy (CMA) is a complex disorder that implies an immunologically mediated hypersensitivity reaction with varying mechanisms and clinical presentations. The type I reactions appear to be the most common immune reaction to milk. However, the dominance of IgE reactions (Type I) may be an artifact as the reaction is easy to diagnose because of an immediate IgE measurement, whereas other reaction types are more difficult to diagnose. Non-IgE-mediated hypersensitivity has been increasingly diagnosed, and it is likely that several mechanisms operate in an individual patient. In clinical work, hypersensitivities are classified as IgE- and non-IgE-mediated or as immediate and late reactions based on the appearance of the first types of symptoms [9]. Cow’s milk is a member of the “Big-8” food allergens that include egg, soy, wheat, peanuts, tree nuts, fish and shellfish in terms of prevalence [9]. The incidence of CMA varies with age. CMA is prevalent in early childhood

with reported incidences between 2 and 6% [10-12] and decreases into adulthood to an incidence of 0.1–0.5% [13-14]. It has been suggested that infants have milk allergies because milk is usually the first source of foreign antigens that they ingest in large quantities, and the infant intestinal system is insufficiently developed to digest and immunologically react to milk proteins. When milk is eliminated, the inflammation response is controlled. After several years, oral tolerance is developed, and milk can again be tolerated [15]. Most milk-allergic children are considered symptom-free by 3 years of age, but several studies have indicated that older children may also have immune reactions to milk. Children whose CMA has been diagnosed at an age older than 3 years do not tend to outgrow the problem. In adults, CMA is less common than lactose intolerance [16-17], even though it has been reported that approximately 1% of the adult population has milk-specific IgE antibodies. However, studies on CMA in adults are scarce. Little is known about the clinical symptoms, eliciting doses, and allergens involved. It has established that CMA in adults is rare but serious [18]. In a study by Stöger et al. [19], the main target organs in adult CMA were the skin and the respiratory tract. Gastrointestinal (mild to moderate) and cardiovascular (severe) symptoms were observed less often in adults compared with children. Milk allergies and hypersensitivity may be more common now than they were several decades ago. Further, the prevalence of atopic diseases has also increased in recent decades. Because genetic diversity has not changed over a short period, environmental factors are believed to have influenced the phenotype. Such factors may include increased air pollution, such as passive smoking, and dietary factors, such as the duration of breastfeeding, amount of antioxidants and the type of dietary fats (favoring saturated fat and n-6 fatty acids). Another approach is the hygiene hypothesis, which states that early exposure to microbial antigens may reduce the risk of having allergies [20]. For as long as milk allergy and hypersensitivity have been experienced, and still may be, these conditions may be misdiagnosed as a disease other than an allergy/hypersensitivity, particularly if symptoms are delayed. Classical IgE-mediated milk allergies with objectively recorded skin reactions may have been diagnosed easily, whereas hypersensitivity with subjective gastrointestinal reactions may have been diagnosed as lactose intolerance, irritable bowel syndrome or some other intestinal disorder. In adults, viral infection, antibiotic treatment or stress may alter intestinal integrity so that its balance is disturbed and the number of protecting agents, such as microflora and mucosal immunity, are altered [21]. This process may result in milk hypersensitivity. Over one-third of women with IgE-mediated reactions to milk proteins have been reported to exhibit their first symptoms of hypersensitivity during or shortly after pregnancy, and one-quarter reported the first symptoms during a period of severe emotional stress [22].

3. Milk allergens

No single major allergen has been identified in cow's milk according to either challenge tests or laboratory procedures; [23]. Indeed, clinical challenge tests demonstrate that most CMA patients react to several protein fractions of cow's milk and each allergenic protein may have several epitopes, which are widely spread along the molecules. The cow milk proteins prevalently implicated in allergic responses in children are the whey proteins α-

Lactalbumin (α-La)(Bos d 4) and β-Lactoglobulin (β-Lg) (Bos d 5), in addition to the casein (CN) fraction (Bos d 8) [24-26]. In adults, the predominant allergen is CN, whereas sensitization to whey proteins is rare. Biochemical characteristic of allergenic cow milk proteins are reported in Table 1. Currently, milk allergen analyses are generally based on immunoassay methods, such as enzyme-linked immunosorbent assay (ELISA) [27]. Commercial test kits are available for the determination of CN, β-Lg, or total milk proteins (CN and β-Lg) [28]. Interlaboratory studies were performed to evaluate the reliability and reproducibility of these kits [29]. Antibody cross-reactivity has been reported for some milk proteins [30]. Confirmatory tests are required to corroborate ELISA detection results and improve the detection specificity of undeclared milk allergens. For the last several decades, mass spectrometry has become the dominant technology for the identification of peptides and proteins. The primary current approaches used for protein identification are top-down [31-33] and bottom-up [34] sequencing. Top-down sequencing involves introducing the intact protein into the gas phase. The protein is identified by measuring either the protein molecular weight or its fragmentation pattern using various techniques [35]. The bottom-up approach is more common. The sample is usually digested with an enzyme, such as trypsin, followed by accurate sequence analysis by tandem mass spectrometry (MS/MS) of the proteolytic fragments. For protein identification, an algorithm is used for database searching based on amino acid sequence assignments.

Cow's Milk Proteins (100%)	Protein	Allergen Name	Allergenicity	Total Protein %	MW (kDa)	pI	Amino Acid Residues	Calcium sensitivity	Phosphate groups
Caseins (80%)	α_{s1}-Casein	Bos d 8	Major	32	26.6	4.9 - 5.0	199	+++	8-9
	α_{s2}-Casein	"	"	10	25.2	5.2 - 5.4	207	++++	10-13
	β-Casein	"	"	28	24,0	5.1 - 5.4		++	4-5
	γ1-Casein	"	"	Traces	20,5	5.5	181	+	1
	γ2-Casein	"	"	Traces	11,9	6.4	104		
	γ3-Casein	"	"	Traces	11.5	5.8	102		
	κ-Casein	"	"	10	19	5.4 - 5.6	169		
Whey proteins (20%)	α-Lactalbumin	Bos d 4	Major	5	14.2	4.8	123		
	β-Lactoglobulin	Bos d 5	Major	10	18.3	5.3	162		
	Immunoglobulins	Bos d 7	–	3	150	–	–	–	1-3
	BSA	Bos d 6	–	1	66.3	4.9 - 5.1	582		
	Lactoferrin	–	–	Traces	80	8.7	703		

Table 1. Chemical characteristics of cow's milk proteins and their inclusion in the official list of allergens

3.1. Casein

The CN fraction is composed of four proteins α_{s1}-, α_{s2}-, β-, and κ-CN, in approximate proportions of 40%, 10%, 40%, and 10%, respectively, and αs1-CN is a major allergen according to IgE and T cell recognition data [36-39]. The specificity of the IgE response to the different purified CNs has been analyzed on 58 sera from patients allergic to whole-CN [40].

Multi-sensitization was observed, which most likely corresponded to a co-sensitization to the different CN components after the disruption of the CN micelles. The CN fractions cross-link to aggregates termed nanoclusters, which combine into micelles. In the new state of aggregation, there is a hydrophobic central part and peripheral hydrophilic one that include phosphorylation sites. Although the primary structure of CNs is known, the micelle structure remains relatively unknown. αs2-, αs1-, and β-CN bind calcium to form a core that is covered with a κ-CN layer. The κ-CN latter protects CN micelles from precipitation in the presence of milk constitutive calcium ions. During the last five decades, different models for the structure of the bovine CN micelle have been proposed. Walstra has summarized the common structural elements into a "ball-shaped model" [41]. According to Walstra, a well-accepted model considers the CN micelle as follows: (i) it is roughly spherical; although it does not have a smooth surface; (ii) it is built of smaller units, termed sub-micelles, which mainly contain CN and have a mixed composition; (iii) sub-micelles vary in composition and consist of two main types-one primarily consists of α_s and β-CNs and the other primarily consists of α_s- and κ-CNs; (iv) the sub-micelles can be linked together by small calcium phosphate clusters bridging them; (v) the sub-micelles aggregate until they have formed a micelle in which those with κ-CN are outside; and (vi) consequently, molecular chains of the C-terminal end of κ-CN protrude from the micelle surface to form a "hairy" layer that prevents any further aggregation of sub-micelles by steric and electrostatic repulsion. The hairy layer is also held responsible for the stability of the micelles against flocculation. Destabilization of CN micelles can be made by treating milk with milk clotting enzymes, which limitedly degrade κ-CN affording to CN micelle fusion and formation of a para-κ-CN aggregate. Finally, a variable proportion, up to 5% total, consists of a heterogeneous group of CNs termed γ-CNs (γ-CN) that result from the limited proteolysis of β-CN by plasmin, the native milk protease. Plasmin disperses in low amounts from blood to milk during secretion to generate CN fragments whose structure are shown in Figure 7 with peptides labeled according to the current nomenclature [42]. CNs are highly sensitive to proteolysis and do not maintain a unique folded conformation [43], which has led them to being termed rheomorphic. Because the folded structure is limited, heating does not generally change the structure and hence its IgE binding [44]. α_{s1}-CN, α_{s2}-CN, and β-CN can chelate Ca^{2+}, Zn^{2+}, and Fe^{3+}, respectively. The four CNs share little sequential homology, but, despite this lack of homology, simultaneous sensitization is often observed.

3.1.1. α_{s1}-CN

α_{s1}-CN consists of major and minor polypeptides that include the same amino acid sequence but have different degrees of phosphorylation [45]. Allergenic epitopes were identified by Spuergin et al. [46] in α_{s1}-CN regions 19–30, 93–98 and 141–150 as immunodominant epitopes. Some sequential IgE-binding regions were recognized at AA 17–36, 39–48, 69–78, 93–102, 109–120, 123–132, 139–154,159–174, and 173–194 using sera from nine older children (> 9 years old), and the epitopes AA 69–78 and 175–192 were recognized by 60% and 80% of sera, respectively, from older children [47]. A later study by Elsayed et al. [48] has

demonstrated that the N- and C-terminal peptides, AA 16–35 and 136–155, respectively, have the highest human IgE-binding affinity, and AA 1–18 and 181–199 exhibited high binding to rabbit IgG. cDNA coding for α_{s1}-CN from a bovine mammary gland cDNA library with allergic patients' IgE Abs has been isolated. IgE epitopes of α_{s1}-CN were determined with recombinant fragments and synthetic peptides that spanned the α_{s1}-CN sequence using microarray components and sera from 66 cow's milk-sensitized patients. The allergenic activity of recombinant α_{s1}-CN and the α_{s1}-CN-derived peptides exhibited IgE reactivity, but mainly the intact recombinant α_{s1}-CN induced strong basophil degranulation. The results indicate that α_{s1}-CN contains several sequential IgE epitopes, but the isolated peptides were less potent than the complete allergen in initiating effector cell degranulation. These results suggest that primarily intact α_{s1}-CN or larger IgE-reactive portions thereof are responsible for IgE-mediated symptoms of FA [49].

3.1.2. α_{s2}-CN

α_{s2}-CN is the most hydrophilic of the four CNs because of an anionic group cluster. α_{s2}-CN consists of two major and several minor components that exhibit varying levels of phosphorylation. α_{s2}-CN contains two cysteines and forms disulfide-linked dimers. Using 99 synthetic decapeptides, 10 regions binding IgE from the sera were identified as allergenic, i.e., 31–44, 43–56, 83–100, 93–108, 105–114, 117–128, 143–158, 157–172, 165–188, and 191–200 [50]. Studies on the presence of α_{s2}-CN epitopes 87–96 and 159–168 with weak binding to 145–154 and 171–180 with originate from individuals with persistent cow's milk allergy. Patients with transient allergies exhibited only weak binding to α_{s2}-CN peptides [51].

3.1.3. β-CN

β-CN is the most hydrophobic component of total CN. Sequence variants are known because of both partial proteolysis and variant genes [42]. β-CN is less phosphorylated than α_{s1}-CN and α_{s2}-CN, with five potential phosphorylated sites located in the N-terminal region. Plasmin cleaves β-CN into γ_1-, γ_2-, and γ_3-CN. Synthetic decapeptides were used to estimate the β-CN region that binds IgE of patients, and peptides 1–16, 45–54, 55–70, 83–92, 107–120, 135–144, 149–164, 167–184, and 185–208 were described as typical for patients with persistent CMA. Sera from eight young patients exhibited a simpler IgE pattern because the sera were bound to peptides 1–16, 45–54, 83–92, 107–120, and 135–144 with weak binding to residues 57-66 and the C-terminal region [52].

3.1.4. κ-CN

κ-CN consists of a major carbonate-free component and a mini-bonded polymer that ranges from dimers to octamers. κ-CN plays an important role in the stability and coagulation properties of milk [89]. κ-CN is most likely more structured than α_s- and β-CN and contains specific disulfide bonds [53-54]. The β-CN can rearrange on heating [55]. κ-CN is sensitive to proteolysis and is hydrolyzed by chymosin to produce *para*-κ-CN and caseinomacropeptide in the cheese-making process. Allergenic potential sequences remain in cooked cheeses. κ-

CN is essential to the stability of CN micelles [55]. Diagnosing patients with persistent cow's milk allergies with the use of 80 overlapping synthetic decapeptides helped to identify some regions that bind to IgE from sera, specifically sequences 15–24, 37–46, 55–80, 83–92, and 105–116 [37].

3.2. Whey proteins

Whey proteins contain two major allergens, β-Lg and α-La, and minor constituents, e.g., lactoferrin, bovine serum albumin (BSA), and immunoglobulins. In this fraction, proteolytic fragments of CN and fat globule membrane proteins can occur.

3.2.1. β-Lg

β-Lg (Bos d 5) is the major whey protein in ruminant milks, comprising 50% of the total whey protein. β-Lg is found in the milk of other mammals but is missing from the milk from rodents, lagomorphs and humans. Notably, 13-76% of patients are found to react with β-Lg. β-Lg has a molecular weight of approximately 18 kDa [37] and belongs to the lipocalin superfamily. The β-Lg (Bos d 5) allergen is capable of binding lipids, including retinol, β-carotene, saturated and unsaturated fatty acids, and aliphatic hydrocarbons, and transporting hydrophobic molecules, which is an important function [56-57]. Under physiological conditions, β-Lg is an equilibrium mixture of monomeric and dimeric forms. The proportion of monomers increases after heating to 70°C. β-Lg contains five cysteine residues, of which four are engaged in intra-chain disulfide bridges. Because of the single unpaired cysteine, β-Lg predominantly exists as a stable dimer that tends to dissociate into monomers at a pH between 2 and 3 [58]. β-Lg is present in several variants, i.e., A, B, and C, that are found in the Jersey breed. β-Lg is sensitive to thermal processes. Ehn et al. [59] reported that heating β-Lg to 74°C and 90°C reduced IgE binding significantly. Heating to 90 °C reduced IgE binding more extensively. Chen et al. [60] reported that nearly 90% loss and denaturation of β-Lg are observed in processed milk and that high heat is the major cause of protein aggregation. Circular dichroism demonstrated no significant conformational changes at temperatures below 70°C for as long as 480 s. The rapid changes of β-Lg occurred between 80°C and 95°C. Fifty percent of the maximal changes could be reached within 15 s. Guyomarc'h et al. [61] reported that large micellar aggregates, 4 × 106 Da, are formed upon heating milk that contained 3:1 ratios of β-Lg and α-La together with κ-CN and α_{s2}-CN. Proteolysis and use of monoclonal antibodies proved that β-Lg possesses many allergenic epitopes spread over the β-Lg structure [62]. Major human IgE epitopes for β-Lg amino acid fragments are composed of residues 41–60, 102–124, and 149–162; intermediate 1–8, 25–40, and 92–100; and minor 9–14, 84–91, 125–135, and 78–83 [63]. Similar IgE epitope regions (21–40, 40–60, 107–117, and 148–168) were reported for a rat model of β-Lg allergy [64,65].

3.2.2. α-La

α-La (Bos d 4) is a homologue of C-type lysozymes. It is a member of glycohydrolyase family 22 and Pfam family and weighs 14,186 Da in the mature form and between 15,840 to

16,690 Da for the glycosylated forms. α-La is stabilized by binding to calcium. Polverino de Laureto et al. [66] reported that α-La is cleaved by pepsin at pH 2 in the region of residues 34–57, which produces large fragments. Veprintsev et al. [67] reported that differential scanning calorimetry of α-La at pH 8.1 exhibited transitions at 20°C–30°C with calcium chelator ethylene glycol tetra-acetic acid and near 70°C with the addition of calcium. McGuffey et al. [68] investigated the heating effects of purified α-La and demonstrated that the extent of irreversible aggregation varies at temperatures between 67°C and 95°C. When milk is heated to 95°C, α-La denatures more slowly than β-Lg. The folded α-La structure is destabilized at low pHs with the formation of a molten globule[69]. The stability to denaturation is also strongly lowered by the reduction of disulfides [70]. Disulfide exchange can occur during thermal denaturation, which leads to the formation of aggregates [71]. Although evolved from a lysozyme [72], the function of α-La is to form a complex with galactosyltransferase, which alters the substrate specificity and increase the lactose synthase rate in milk. The galactosyltransferase and α-La is termed lactose synthase [73]. α-La alone does not have any catalytic activity as a lysozyme or a synthase. Several other properties of α-La and possible additional functions have been described [74], which include binding of several ligands and antimicrobial activity, both as a complete molecule [75] or as peptides [76]. The cytotoxic effects against mammalian cells have also been investigated [77]. The major component of α-La is unglycosylated. However, a mass spectrum of α-La contains at least 15 distinct peaks [78], and a minor glycosylated form (approximately 10%) results from asparagine 45 glycosylation. A study concerning the allergenic properties of α-La demonstrated that in 60% of the study patients, allergic sera were specific for intact α-La with only 40% binding to peptides obtained after tryptic hydrolysis. Residue 17–58 was the most frequently recognized in the sequence 59–93, 99–108, and 109–123 [79]. The linear epitopes were identified by using sera of patients suffering from persistent allergies and IgE to cow's milk levels > 100 kU(A)/L. Serum IgE bound most strongly to peptides 1–16, 13–26, 47–58, and 93–102 [80].

3.2.3. Minor allergens

Bovine serum albumin (BSA) (Bos d 6), which is a heat-labile protein, is a major allergen in beef but a minor allergen in milk [81-84]. Accordingly, beef allergic individuals are at risk of being allergic to cow's milk and vice versa. BSA allergies account for 0-88% of sensitization events, whereas clinical symptoms occur in up to 20% of patients. BSA is one of the proteins most frequently involved in binding with circulating IgE [85-86]. Bovine immunoglobulins (Bos d 7) may be also responsible for clinical symptoms in CMA.

4. Post-translational modifications

In evaluating the allergenic potential of a protein, post-translational modifications of amino acid residues should be considered in addition to sequential and conformational IgE binding domains. Notably, such modifications may either generate additional IgE epitopes or induce changes in protein folding that affect IgE-protein interactions. Accordingly, recombinant allergens do not generally have the IgE-binding capacity of their natural

counterparts, most likely because of a deficiency in the post-translational events [87]. Regarding milk proteins, selective phosphorylation of serine residues in all of the four CNs, O-glycosylation of threonine residues in κ-CN and N-glycosylation of asparagine residues in α-La have been long described.

4.1. Phosphorylation

The removal of phosphate groups from CNs significantly reduces the CN-binding capacity of IgE from patients who suffer from milk allergies, which indicates that at least part of anti-CN IgE is directed against CN domains that comprise a major phosphorylation site [88, 89]. It has been suggested that currently observed co- and cross-sensitization to the different CNs that are encoded by different genes and display few amino acid sequence homologies can be caused by the occurrence of common highly conserved major sites of phosphorylation, i.e., the Ca^{2+} binding CN sequence SerP-SerP-SerP-Glu-Glu that corresponds to α_{s1}-CN 66-70, β-CN 17-21 and α_{s2}-CN 8-12 and 56-60 [87]. Most likely, sensitization to milk is caused by a large release of phosphopeptides that are resistant to further degradation by digestive enzymes [90] during intestinal proteolysis of milk proteins. However, serine phosphorylation poorly affects the overall antigenic potential of individual CNs. Notably, antisera raised against native β- and α_{s2}-CNs can recognize their targets after dephosphorylation or deletion of a major phosphorylation site [87]. Furthermore, polyclonal antisera that are produced in rabbits using a bovine β-CN 1-28 phosphorylated peptide as an antigen have been utilized to detect all of the tryptic phosphopeptides that originate exclusively from the 1-28 region of β-CN, regardless of the content of the phosphorylated Ser residues, and none of those generated by the other bovine CN fractions [91]. β-CN from human milk contains the phosphopeptide cluster ^{5}Glu-Ser-Leu-SerP-SerP-SerP-Glu-Glu^{12}, also found in the bovine β-CN sequence 14-21; however, a lower level of phosphorylation has been generally observed. For example, according to the phosphopeptide analysis of human milk that is reported in Table 2 and Figure 3, the 2092.8 Da component, which corresponds to β-CN(f2-18)2P, caused the third peak in intensity order in combination with the fully phosphorylated components. This lower phosphorylation level is lacking in its bovine counterpart. The overall higher degree of phosphorylation of bovine CNs can play a role in sensitizing humans to bovine milk.

4.2. Glycosylation

The effect of glycosylation on the allergenic potential of milk proteins has been long disregarded despite efforts to identify the domains responsible for the allergenicity of milk proteins, mostly based on an epitope mapping approach. Notably, the role of carbohydrate epitopes in initiating an allergic reaction is still unclear [92]. Potential glycosylation sites have been identified in major milk proteins, i.e., N^{45} and N^{71} of mature α-La [93] and T^{131}, T^{133}, T^{135}, T^{136}, T^{142}, and S^{141} of mature κ-CN [94]. Approximately 10% of α-La has been found glycosylated at N^{45}, giving rise to at least 14 distinct peaks by electrospray-ionization mass spectrometry analysis [95]. However, these glycosylated forms were not included among the IgE epitopes in a study because they were not detected by matrix-assisted laser

desorption/ionization time-of-flight (MALDI-TOF) analysis of α-La; notwithstanding in the same study, IgE reactivity of sera from patients allergic to α-La were proven to be sensitive to periodic acid treatment [93]. As reported in Table 3, the genetic variant A of water buffalo α-La that carries an $N^{45} \rightarrow D^{45}$ substitution cannot be glycosylated. To assess the effect of glycosylation on the allergenicity of α-La, it might be used as substrate for IgE reactivity testing of sera from patients sensitized to α-La.

Molecular mass (Da)		CPP identification
Theoretical	Measured MH^+	
Human β-Casein		
2488.1	2489.1	β-CN (f1–18)5P
2408.1	2409.1	β-CN (f1–18)4P
2328.2	2329.1	β-CN (f1–18)3P
2248.2	2249.3	β-CN (f1–18)2P
2168.2	2169.2	β-CN (f1–18)1P
2252.0	2252.7	β-CN (f2–18)4P
2172.0	2173.0	β-CN (f2–18)3P
2092.0	2093.0	β-CN (f2–18)2P
2012.0	2013.0	β-CN (f2–18)1P
3100.8	3101.0	β-CN (f1–23)5P
3020.8	3021.8	β-CN (f1–23)4P
2940.9	2941.0	β-CN (f1–23)3P
2860.9	2861.9	β-CN (f1–23)2P
2780.9	2781.9	β-CN (1–23)1P
Human α_{s1}-Casein		
3077.0	3078.9	α_{s1}-CN (f15–38)3P
3068.1	3069.1	α_{s1}-CN (f12–36)2P
2488.5	2489.5	α_{s1}-CN(f8–27)2P
2408.5	2409.5	α_{s1}-CN (f8–27)2P
2118.8	2119.5	α_{s1}-CN (f68–83)5P

Table 2. Identification of human milk soluble TCA 12% peptide fractions enriched on hydroxyapatite after MALDI-TOF analysis by FindPept (http://www.expasy.org/tools/findpept.html) software.

Despite some indications that the allergenic character has been identified in the glycosidic moiety of native κ-CN [96], at present this issue remains to be settled. Glycated forms of κ-CN account for approximately 40% of the κ-CN that normally occurs in bovine milk, but glycans are not randomly distributed among potential glycosylation sites. The hierarchy of glycan addition proceeds according to the order T^{131}, T^{142}, T^{133}, whereas the other sites remain latent until these sites are occupied [97]. κ-CN is cleaved by chymosin during the primary stage of cheese making at the peptide bond F^{105}-M^{106}. The C-terminal 106-169 fragment, known as glycopeptide because all of the glucides originally present in κ-CN are

retained, is released and lost in the whey. Therefore, cheese is devoid of any glycosylated major component. Potential allergenicity of κ-CN glycoforms has been suggested by analyzing the IgE binding capacity of an individual human serum from an adult atopic patient who had outgrown a cow milk allergy in early childhood. Bovine κ-CN has been selectively recognized by IgE immunostaining of an electrophoretic profile of milk proteins. No additional IgE-reactive proteins other than bovine κ-CN have been found in either bovine cheese, regardless of the cheese making technology and time ripening, or in ewe, goat and water buffalo milk. Moreover, chemical removal of glucide chains from bovine κ-CN has not impaired IgE binding, thus proving a primary involvement of the glycoside moiety of the protein in IgE recognition. According to the specificity displayed by IgE, N-acetylneuraminic acid as a terminal unit of a tetrasaccharide chain has been argued to be an IgE epitope [98].

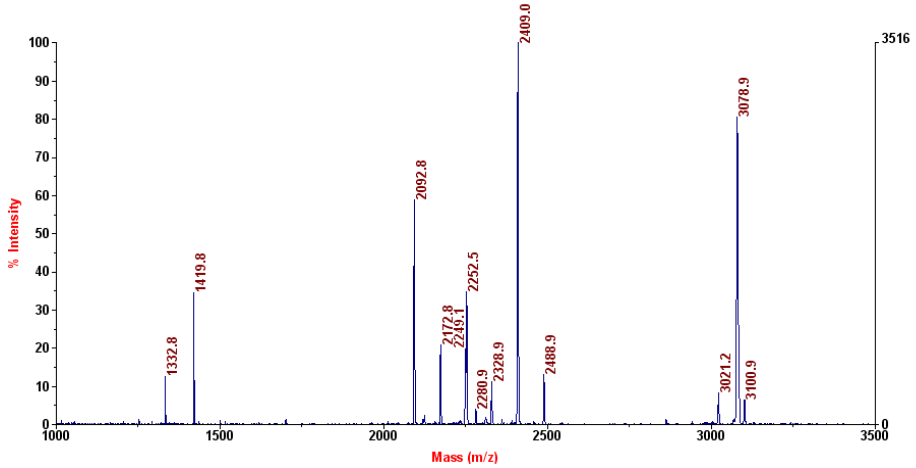


Figure 3. Mass spectrum of human milk soluble TCA 12% fraction enriched on hydroxyapatite by MALDI-TOF.

Site	10	17	45
Species			
Bovine A	Gln	Gly	Asn
Bovine B	Arg	Gly	Asn
Water buffalo A	Arg	Asp	Asp
Water buffalo B	Arg	Asp	Asn
Caprine	Gln	Asp	Asn
Ovine	Gln	Asp	Asn

Table 3. Position and amino acid differences among the genetic variant of α-La from four animal species.

5. "Allergenomics"

The application of proteomic methodologies for the analysis of food allergens has been termed "allergenomics" [99]. For "type I" FA, IgE-binding indicates that the target carries the risk to be an allergen. MS-based proteomic methods have identified many proteins and allergens. MALDI [100] and electrospray ionization (ESI) [101] and MS/MS sequencing are the techniques most widely used to produce high-quality spectra of post-translationally modified peptides [102-104] or intact proteins (see [105]). The characterization of glycosylated allergens has been partly overcome by specific enrichment using lectin or hydrophilic resins (HILIC) prior to MS analysis [106,107]. Native and de-glycosylated peptides are analyzed by MALDI or ESI-MS. Because of the difficulty of profiling oligosaccharides released by glycoprotein [108-110], glycan profiles are obtained after permethylation of the oligosaccharide chains according to the procedure of Das et al. [111]. By this means, glycosylated site(s) are identified together with the peptide backbone. Although widespread, several studies are dedicated to milk protein analysis for the detection of allergenic proteins or peptides in dairy products. Thus is determined by the concentration of allergenic compounds in food products that are often secondarily masked by dominant non-allergenic proteins. Among the various methods currently used to detect allergens in food products, immunochemical techniques that rely on antibody-binding properties have been developed. Commercially based kits are used for rapid screening, and enzyme-linked immunosorbent assays (ELISA) provide evaluations. Limits of detection (LOD) attained by ELISA tests are in the range of 1–5 ppm. Because the epitopes to be detected and their possible cross-reactivity with matrix components are unknown, detection reliability strongly depends on various factors that include the thermal changes of whey proteins, which are of primary importance. Furthermore, in several foods, linear epitopes can be released by parent protein hydrolysis, whereas retain their allergenic potential. As cited above**,** MS measurements can be finalized to evaluate the molecular mass of proteins and derived-peptides (MS1), determine the amino acid sequence and identify post-translational modifications (MS/MS or MS^n). Two-dimensional electrophoresis (2DE) separates proteins according to the pI (i.e., first dimension, isoelectric focusing, IEF) and subsequently molecular weight (SDS-PAGE) in an orthogonal dimension. By this means, the separation of thousands of proteins has been achieved using highly specific stains that visualize specific protein classes, i.e., phosphoproteins, or nonspecific stains that simultaneously target total proteins without particular functional groups. Protein spots are localized, excised from the gel and subjected to an in-gel tryptic digestion. Mass spectrometry analysis either by MALDI reflectron TOF or microcapillary liquid chromatography MS-MS detects the proteins based on the expected masses of peptides available in databases and other plant proteins in pollen diffusates. More directly, tandem mass spectrometry is used to identify the peptide sequence and search for allergens in databases. More elegantly, allergens are localized on the gels after a one- or two-dimensional electrophoretic separation followed by a nitrocellulose transfer of the proteins (i.e., western blotting), which is stained with sera from allergic patients as a source of specific IgE. Combined with the analysis by mass spectrometry of electrophoretically separated allergens, immunoblotting is useful for the rapid determination of allergen

identities. Allergen–IgE complexes are also detected using conjugated anti-human IgE as a secondary antibody. Once localized in a 2DE map, the allergen can be monitored using allergen specific antibodies [112,113]. Immune-reactive allergenic protein(s) are identified along the immunoblots by comparison with a reference electrophoretic map. All of the major milk proteins are allergen candidates because sera of allergic patients contain various percentages of immune-reactive proteins that are recognized by IgE [114]. The order of milk protein allergenicity is as follows: α_{s2}-CN>α_{s1}-CN>β-CN>κ-CN>β-Lg> serum albumin > IgG-heavy chain>Lf. This list contains allergenic proteins that have been identified by experiments on several MS platforms [115]. Thus far, it is quite difficult to find a separation method that can accommodate the diversity of proteins equally. Therefore, modern separation techniques have been performed off-line or by online ion-exchange/reversed phase liquid chromatography prior to MS analysis. ESI-MS is currently the interface most frequently used to perform an LC separation of intact proteins. The protein identification is most commonly achieved after a proteolytic digestion and molecular weight determination of the LC-separated peptides. "Shotgun" proteomics is the most effective LC/MS-based strategy because a trypsin-digested protein sample generates thousands of peptides that are subsequently separated by LC prior to MS/MS sequencing [103,116]. Proteins with at least one matching peptide are candidates to occur in the sample. However, with CNs, it is difficult to determine which fractions are present in the sample if they share the same set of phosphopeptides or have only one constituent peptide detected. LC-Q/TOF MS/MS has been used to detect wine CN as a fining agent. Two peptides were identified from α_{s1}-CN and four peptides from the tryptic digestion of α- and β-CNs [117]. A similar strategy could be applied to monitoring allergens in processed milk products. Signature peptides could be identified as CN or whey protein allergens by submitting protein concentrates to trypsinolysis. In this manner, information on the molecular weight of the intact allergen is lost, but cross-reactive immunogenic peptides can be discriminated. Because of the higher sensitivity of MS in the detection of peptides, MS expands the dynamic range of the protein species detected. MS is a method for discovering "hidden" or traces of allergens. Proteomics has become pivotal to the development of modern structural immunology and to the understanding of interacting systems that are involved in immune responses, regardless of FA status.

5.1. Allergen quantification methods

Difference gel electrophoresis (DIGE) is utilized to compare multiple proteins in samples migrating in parallel in the same chamber. The proteins are labeled with three distinct fluorescent dyes on the same 2D gel and differentially visualized via fluorescence at different wavelengths. This methodology enables the detection of a differential presence of proteins and small differences in protein abundance. Allergens can be quantified by LC–MS. An accurate evaluation of the protein/peptide requires a suitable standard. In the direct quantification of intact proteins, the intensity of multi-charged analyte ions is compared with that of an internal or external standard. For example, quantification of cow's milk allergens in fruit juice samples [118] and whey drink [119] was performed by simultaneously monitoring several multiple-charged ions of whey protein components.

With a similar approach, internal standard β-Lg was used to quantify non-bovine β-Lg in different milk-derived products [120]. The use of "bottom-up" methods, such as SILAC, ICAT, and iTRAQ, for quantitative analysis in proteomics has progressively increased [121,122]. Quantification of allergens in complex samples requires simple and precise methods of analysis, such as selected reaction monitoring (SRM) [123]. SRM is presently considered the gold standard for absolute quantification, whereas multiple reaction monitoring (MRM) can monitor the masses of selected signature peptides. For an allergen evaluation, internal reference peptides are required for food product monitoring by LC–SRM MS [124]. Hydrolyzed protein samples are spiked with known amounts of synthetic peptides and monitored by LC–MS in the SRM mode. Absolute amounts of peptide(s) are determined by the ratio of the ion intensities of natural and synthetic peptides (Figure 4).

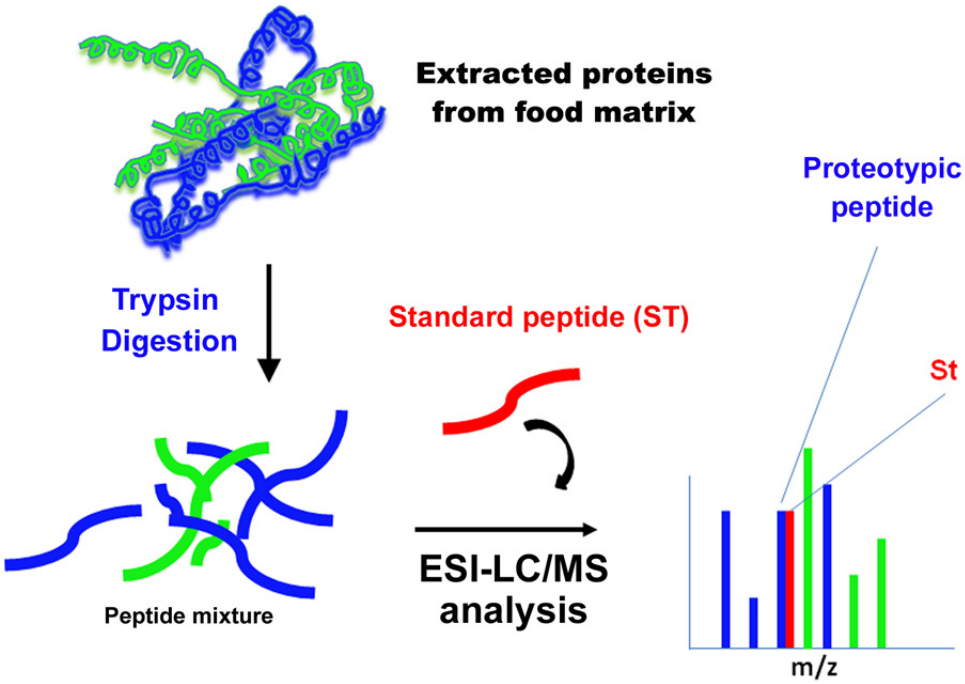


Figure 4. Mass Spectrometry procedure analysis for protein absolute quantification. Proteotypic peptides are selected with a preliminary fullscan. For quantitative analysis whole protein extracts are trypsinized and the peptide mixture is spiked with external standard peptide. Proteotypic peptides (blue colored) selected as analytical probes of the target protein(s), are quantified by comparing the ionic intensities.

This strategy has demonstrated its validity for using signature peptides as analytical surrogates to measure allergens in crude protein extracts. One advantage of the SRM procedure is the possibility of one-step monitoring of a variety of allergens. Recently LC–triple quadrupole MS operating in an MRM mode has been effectively demonstrated to simultaneously detect allergens from seven different potentially allergenic matrices, such as

milk, eggs, soy, hazelnuts, peanuts, walnuts and almonds. The detection limits were in the 10-1000 μg/g range. However, prior knowledge of allergens was required to monitor the most suitable allergenic peptides [125]. Based on the above-specified considerations, allergen evaluation requires the following: (i) allergen extraction from the food; (ii) enzyme proteolysis, usually trypsinolysis; and (iii) identification of signature peptides that are characteristic of food proteins or food ingredients. The signature peptides should be determined experimentally by prior LC/MS analysis of food-derived digested protein extracts.

5.2. Standardization of allergen preparations

Although the search for clinically relevant allergens has progressed, the characterization of allergens still requires studies on milk proteins as starting material. Pure native and recombinant allergens are needed as reference materials to calibrate methods among different laboratories. Recently, a panel of 46 food plant and animal allergens [126,127,128] has been made available within an EU-funded research project. In a recent 2DE application, calibration has been utilized for microbial complex protein systems using data obtained by MS [129]. Developing more allergen standards could be realized in the near future. Moreover, the search for allergenic sequence stretches would comprise only those immunodominant produced during digestion that can to translocate the gut barrier and reach the mucosal immune system. Among the digestion/adsorption models of food protein stability, pepsin digestion has been included in the Food and Agriculture Organization/World Health Organization to assess food safety [130]. A model study has established milk-derived peptide candidate-mediated resistance to proteases to display allergic effects. The survival of milk protein epitopes [131,114] requires structure determination. To this end, a simulated digestion of bovine milk proteins in vitro that includes the sequential use of pepsin, pancreatic proteases, and extracts of human intestinal brush border membranes, has allowed the identification of produced peptides by MS. The presence of characteristic β-Lg resistant peptides could implicate β-Lg in the case of a cow's milk allergy [132]. The identification by MS of peptides arising from simulated digestion is complicated by a lack of enzyme specificity. Currently, there is no treatment to fully resolve or provide long-term remission from FA allergies. The research for therapy is mainly focused on the introduction of anti-IgE antibodies and specific oral tolerance induction. Immunotherapy appears to be an attractive approach; however, the risk of anaphylaxis should be considered. To this purpose, engineered proteins have been designed, i.e., anaphylaxis-initiating epitopes have been removed within these proteins, while preserving the tolerance-inducing epitopes [133-135]. It appears clear that to successfully pursue similar strategies, the precise identification of epitopes is necessary. It is expected that such approaches will be extended to an increasing number of food sources, whereby MS will play a key role for characterizing novel protein entities. The accurate characterization of the offending sequences could also be the starting point for developing less allergenic food products through the use of enzymatic, microbiological and technological processes to effectively remove allergens [136–139].

6. Dairy research versus CMA

6.1. Milk and dairy products from mammals different from cow

According to the current clinical approach to FA and intolerance based on an elimination diet, the treatment of choice is complete avoidance of cow milk. Although of moderate importance in an adult diet, cow milk elimination has a significant nutritional significance in the infant diet, especially during early childhood. Milk from other mammals has been suggested as a possible alternative to cow milk. At first, goat milk had been proposed as a hypoallergenic infant food or cow milk substitute in human diet, but much of this thesi has no credible scientific evidence. Despite the immunological cross-reactivity between cow and goat milk proteins, due to the close biochemical similarity associated with the same phylogenetic origin [140], it has been estimated that from 40 to 100% of patients allergic to cow milk proteins can tolerate goat milk intake [141]. However, clinical and immunochemical studies aimed at evaluating goat milk safety for cow milk allergic subjects have demonstrated that goat milk cannot be a substitute for cow milk without risk of anaphylactic reactions [142,143]. It has been suggested that evidence for goat milk tolerance in clinical trials can be due, at least in part, to a higher number of genetic polymorphisms in goat CNs, especially for α_{s1}-CN [144]. Null or reduced expression of α_{s1}-CN in individual goats; consequently, the overall α_{s1}-CN content in goat bulk milk is lower than that found in cow bulk milk. According to this general finding, and taking into account that little β-Lg persists in cheese, fresh cheese produced from raw milk has been suggested to be a promising hypoallergenic protein source [145]. Unexpectedly, water buffalo milk yogurt has successfully been employed as an alternative food for children with cow milk allergies [146] despite homologous proteins from cow and water buffalo milk [147]. In contrast, several studies have reported the existence of allergies to goat and sheep milk [148-151] and cheese [152,153] in patients with tolerance to cow milk proteins. Overall, this type of allergy is less common and occurs later than that initiated by cow milk proteins, which is likely because goat and sheep dairy products are not usually included in an infant diet. Moreover, IgE epitopes have been widely recognized in the CN components of goat and sheep milk. Differences in the degree of CN phosphorylation, on average lower in goat and sheep milk than in cow milk, rather than differences in IgE epitope sequences, may be involved in initiating selective allergies to goat and sheep milk, as observed in recent cases. In addition to the four ruminant species of dairy interest (i.e., cow, water buffalo, sheep and goat), other monogastric mammals produce milk for human consumption, such as mares and donkeys. Mare milk, which is more similar to human than cow milk, has been proven to be an acceptable substitute of cow milk for children with severe IgE-mediated cow milk allergy; although the evidence of its tolerability by a supervised oral challenge test is recommended [154]. However, mare milk availability is limited, and its collection is difficult. Donkey milk provides nutritional adequacy and excellent palatability similar to that of mare milk but is more readily available. The composition of donkey milk is more similar to human milk than cow milk because of the higher lactose content (6.5 vs. 5 g/100 mL), lower protein content (1.2 vs. 3.2 g/100 mL), lower CN/whey protein ratio (approximately 1 vs. 4) and a higher non-protein nitrogen fraction level (0.29 vs. 0.18%) [155]. These features have prompted clinicians to propose donkey milk as a valuable breast milk substitute. Additionally, donkey

milk intake has demonstrated positive effects in the diet therapy of patients allergic to cow milk proteins [156]. Although the mechanism of this tolerance is unclear, the reduced allergenic properties of donkey milk can be related to the structural differences compared with bovine milk. Because of scientific and clinical interest in donkey milk, characterization of the whey protein fraction [157], caseome [158], and the minor protein components [159] of donkey milk have been recently provided. Presently, milk from mammals with a geographically restricted distribution area, such as reindeer living in Northern Europe, has been utilized to overcome immunological cross-reactivity among proteins from mammals other than cows. In particular, β-Lg from reindeer milk, although belonging to the lipocalin family and similar to its homologous bovine protein, lacks several IgE epitopes of bovine β-Lg that are involved in CMA [160]. Recently, camel milk, mainly available in the Gulf area and Mauritania, is of growing interest to both nutritionists and pediatricians because of its high nutritive value and unique electrophoretic protein patterns, which strongly suggest a different immunological reactivity of camel milk proteins with respect to the bovine counterparts [161].

6.2. Gut microflora

It has been suggested that gut flora may be involved in the etiology of atopic diseases. It has been demonstrated that the gut microflora differs in children with high or low rates of allergy. Commensal gut flora play a role in inducing an oral tolerance, and the importance of the intestinal microbiota in developing food allergies is essential at early ages when the mucosal barrier and immune system are still immature. Probiotics interact with the mucosal immune system by the same pathways as commensal bacteria. A recent study has demonstrated that probiotic bacteria induced in vivo increased plasma levels of IL-10 and total IgA in children with allergic predisposition. Many clinical studies have reported significant benefits by probiotics supplementation in FA prevention and management. However, not everyone agrees on the effectiveness of probiotics supplementation. The differences are most likely related to the selected populations and probiotic strains used. The hygiene hypothesis proposes that disturbances in the gastrointestinal microbiota are associated with increased prevalence of allergic and autoimmune diseases [162]. Changes in the establishment of gut microbiota have been observed in Western infants [163,164]. This is most likely because of improved hygiene and cleanliness in Western countries and excessive use of antibiotics, which causes a reduced bacterial stimulus. Several clinical studies have reported differences in the composition of bacterial communities in the feces of children with and without allergic diseases. Many of those studies have highlighted the involvement of *Bifidobacterium* and *Bacteroides* in the protection against the development of atopy [165-168], but this observation remains a matter of debate [162]. Moreover, the mechanisms underlying such protective effects remain elusive. There is increasing evidence that T-regulatory cells derived from the thymus or induced in the periphery including the gut mucosa [169,170] are key players of immune regulation [171-173]. Using a single strain mouse model and defined bacterial communities and conventional mice, it has been recently demonstrated that the gut microbiota plays a protective role against allergen sensitization and allergic response in a mouse model of FA [174]. The difference between healthy and

allergic children may be in their microflora. At 3 weeks of age, infants in whom atopy developed then had more Clostridia and fewer *bifidobacteria* in their feces compared with infants who remained healthy. Moreover, fecal *bifidobacteria* microflorae were different between healthy and allergic children; the healthy infants' microflora was mainly *Bifidobacterium bifidum*, whereas the microflora was mainly *Bifidobacteria adolescentis* in the allergic infants. It can be hypothesized that individual species, rather than an entire genus, can affect the manifestation of allergy. In a recent study, the microflorae of milk-hypersensitive and control adults before and after a 4-week supplementation with probiotic bacteria (*Lactobacillus rhamnosus* GG, ATCC 53103) have been studied. The anaerobic microflora before supplementation was comparable between the healthy and hypersensitive subjects, whereas the response after supplementation was different. The number of *bifidobacteria* in the healthy subjects increased significantly after supplementation. However, this did not occur with the supplementation in milk-hypersensitive subjects; this may be because of altered intestinal integrity. However, other studies have suggested a beneficial effect of probiotic bacteria in milk-hypersensitive subjects. In one study, symptoms of hypersensitivity abated along with an elimination diet in 28% of the patients in 4 years. It can be hypothesized that with milk elimination and long-lasting probiotic treatment, the intestinal severity of IgE-mediated hypersensitivity reactions may increase the intestinal microflora or even eliminate them.

6.3. Reduction of allergenicity of milk proteins by hydrolysis

The main objective of the milk industry is to supply products while preserving both the nutritive value and safety against developing allergies. Nutritional value is preserved by exposing liquid or powdered milk to low heat treatments to reduce heat susceptible amino acid side chain modifications and preserve the integrity of triacylglycerols, native vitamins and other milk components. As noted above, infants can develop milk allergies because of increased gut permeability to large molecules, in addition to other causes [175]. This result is supported by measurements of unmodified proteins or partially modified proteins in the sera of infants and adults [176]. Milk proteins have a molecular mass between 14 and 80 kDa. To reduce allergenicity, milk proteins can be submitted to different hydrolysis procedures. Attempts to classify products by protein hydrolysis include “extensive” or “high degree” hydrolysis and “partial” or “low degree” hydrolysis. The rationale of such a classification is the spectrum of peptide molecular weights or the ratio of α amino acids to total nitrogen. For quality assurance, *in vitro* product characterization requires size measurements of the peptides that are generated by protein hydrolysis and then an in vivo allergenicity determination. The in vivo step would include evaluating immunogenic or allergenic effects in a recipient infant. The European Union regulates that infant formulas contain immunoreactive proteins in quantities lower than 1% of nitrogen compounds [177] to reduce allergenicity knowing that only pure amino acids are strictly non-allergenic. This criterion could be encountered by milk proteins that have undergone extensive hydrolysis partially to cleave amino acids [178-180]. In contrast, formulas with moderately reduced allergenicity (partially hydrolyzed) are not recommended for the treatment of allergies because of the high amounts of residual allergens [181]. The low quantity of native proteins

or residual high molecular mass peptides may produce adverse effects in highly sensitive patients. Therefore, a milk hydrolysate can be considered safe and non-allergenic if the nitrogen fraction does not contain unmodified milk proteins or high molecular mass peptides [178-180]. In the latter case, the product could be classified in the "low degree" protein hydrolysate category. However, the antigenic properties of protein hydrolysates may not be dependent on the molecular size of the peptide components alone [182]. By comparing protein structures with known allergens and allergen epitopes, protein allergenicity has been predicted [183]. Although this is true for crystallized proteins, such as α-La, β-Lg and Lf, this procedure cannot be applied to uncrystallized CNs. Because infants who are diagnosed for milk protein allergies must ingest foods that exclude the causal protein, including those ingested by the mother and filtered in breast milk, extensively hydrolyzed milk formulas are used for the development of appropriate dietary and management strategies. Preclinical testing of infant formulas is necessary to characterize the molecular properties and residual antigenicity of proteins [184-186]. Stringent criteria specify that extensively hydrolyzed CN with a molecular weight below 5000 Da should be reduced by at least 99.99%. There is a need for accurate diagnostic methods to confirm the amount of extensively hydrolyzed CN. Milk for allergic infants would consist of extensively hydrolyzed CN and whey proteins of which at least 99.99% of the hydrolysis products have molecular weights below 5000 Da. The crucial criterion is for the level of allergens to be sufficiently low as to cause no significant reaction, even in infants who are highly allergic to cow's milk. There have been no reports of adverse reactions because of whey [187, 188] and CN hydrolysates [189-191]. Therefore, caution must be maintained that milk formulas destined to infants with milk allergies contain correctly hydrolyzed proteins. This generic indication requires that molecular properties and residual antigenicity of proteins would be characterized [184-186]. In vitro incubation of milk proteins with pepsin, trypsin, and chymotrypsin causes the cleavage of numerous peptides of various sizes. Bacterial, fungal and plant proteases may also act as hydrolyzing agents. Various enzyme combinations, such as alcalase, pancreatin and enzymes from fungal sources, have been utilized to produce protein hydrolysates. Commercial hypoallergenic products are currently characterized by an average degree of hydrolysis (DH) of the protein components. The DH 19 milk protein value is calculated from the increase of the number of primary amino groups compared to that of native proteins. In practice, the DH value could vary from 1 to 100% in the case of total hydrolysis of the proteins. In the case of partly hydrolyzed proteins, intact and partly hydrolyzed proteins are visible bands along an electrophoretic pattern of the products. Two commercial formula preparations with DH values of 6.3 and 1.3% contained some intact β-Lg and peptides with an Mr between 6000 and 8000 Da [192]. Using gel permeation chromatography, quantitative results on peptides with an Mr larger than 10 kDa were obtained [192]. Regardless of the technique used, descriptive information was obtained on either the molecular mass or the origin of the peptides. As a result, consumption of infant formulas by allergic patients cannot be attributed to one specific protein or high molecular mass peptide. To suppress or reduce the antigenicity of peptides, natural enzyme cleaving

of many or most of the peptide bonds is required. In this manner, epitopes that determine the antigenicity of the protein molecules are destroyed. This result proves that evaluation of the adequacy of infant formula composition in preventing or delaying antigenicity is not based solely on DH or Mr determination. In highly sensitized infants with IgE-mediated cow's milk allergies, life-threatening anaphylactic shock usually develops shortly after the consumption of claimed hypoallergenic milk products in which a number of epitopes would have survived in the highly proteolytic environment. The possibility of using well-characterized monoclonal antibodies in ELISA tests can be used for assessing the origin of immunoreactive bovine milk proteins. Because the clinical significance of residual antigenicity requires prior molecular approaches, hypoallergenic products may first be screened for peptide identification in hydrolyzed milk products. Although protein hydrolysates can provide a positive effect, they can contain undefined peptide components, which are undesirable for pharmaceutical production purposes. In many cases, hydrolysates are produced by methods that are not well-controlled. Other complications arise from the raw starting material and differences in processing that lead to lot-to-lot hydrolysate composition variability. For these reasons, constant chemically defined products are needed. The data presented here represent the initial steps that have been taken to identify peptides treated with pepsin (P) and trypsin (T) and were used in succession to hydrolyze commercial milk protein powder (PT hydrolysate). To mimic commercial milk hydrolysates, the protein powder was treated with enzymes after a thermal shock deactivation treatment. Subsequently, the peptides are identified. RP-HPLC fractionation was used to aid with the peptide separation. In Figure 5, the hydrolysate was analyzed to demonstrate a correlation between proteolytic enzymes and the presence of peptides. Commercial milk protein hydrolysates may contain trace amounts of allergenic proteins whose molecular weights were determined by MALDI-TOF analysis. Among the number of peptides present in the hydrolyzed sample, the proteins/peptides exhibited molecular masses less than or equal to 2431 Da (Figure 6). This means that the CNs and whey proteins were digested by pepsin and trypsin into peptides with masses less than 3000 Da. This type of hydrolysate is not expected to elicit allergic reactions in already sensitized allergic patients (neither anaphylactic shock nor positive passive cutaneous anaphylaxis), as verified in experimental animals [193]. LC-ESI-MS/MS analysis was performed on the hydrolysate to identify peptides occurring herein. No sequence peptides with 3 or 4 residues were detected because the MALDI signals were acquired at a mass gate of m/z 400. Because some short peptides were in the hydrolysate, milk proteins were hydrolyzed by P and T into oligopeptides with different biological activities. In Figure 7, the amino acid sequence of the four bovine CN fractions and β-Lg are reported with a subscript that indicates the number of amino acid residues in MS/MS-identified fragment.

The proteins in the milk powder sample, which contained modified amino acid residues that may indicate the quality of the protein in milk powder, were not examined within the present work.

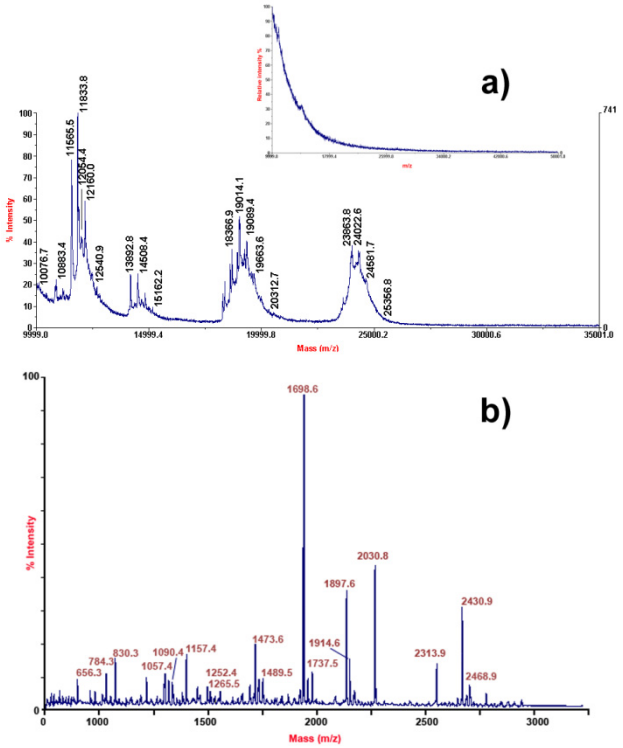


Figure 5. MALDI spectra of a sample of milk powder before and after sequential hydrolysis with pepsin and trypsin. A search for residual intact proteins and high molecular derived peptides (a) and measurements of molecular mass value of oligopeptides in the mixture (b). No peptide at a molecular mass higher than 3430.97 Da was observed in the MALDI spectrum.

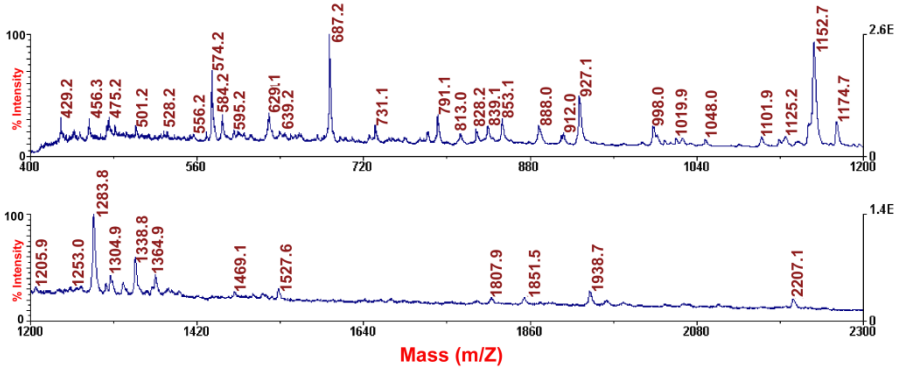
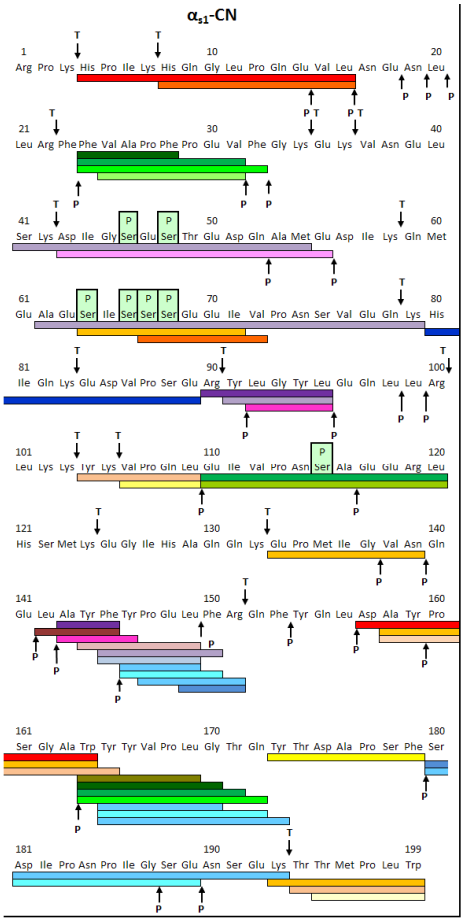
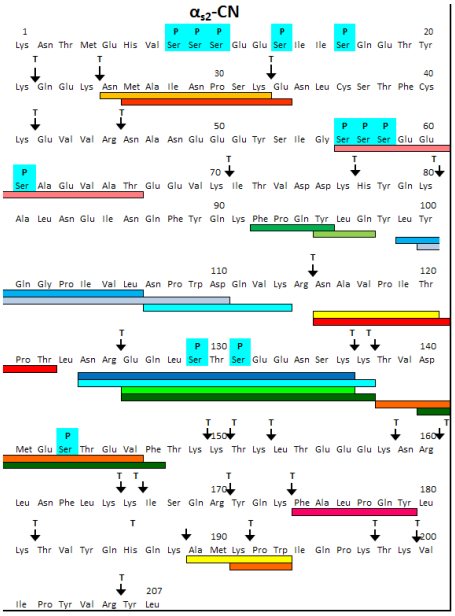
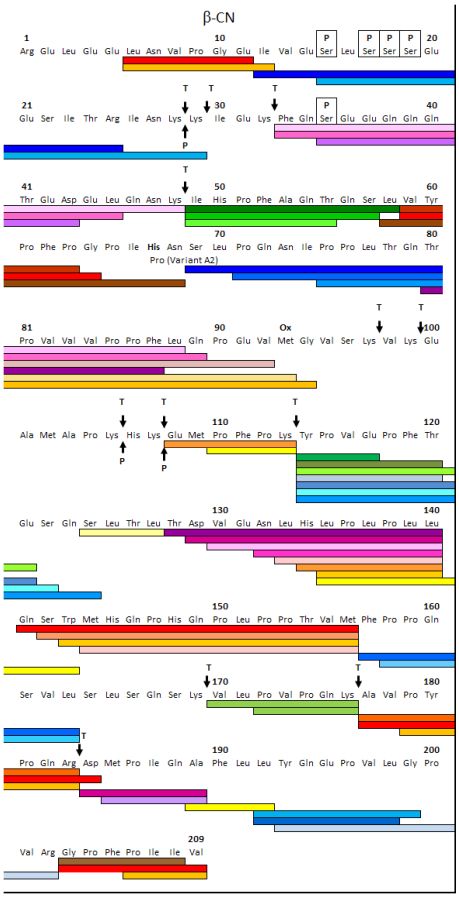


Figure 6. MALDI analysis of a milk sample after sequential hydrolysis with pepsin and trypsin. A molecular mass value corresponds to that of oligopeptides in mixture. No peptide at a molecular mass higher than 2207.1 Da was observed in the MALDI spectrum.


α_{s1}-CN
1 Arg Pro Lys His Pro Ile Lys His Gln Gly Leu Pro Gln Glu Val Leu Asn Glu Asn Leu 20
21 Leu Arg Phe Phe Val Ala Pro Phe Pro Glu Val Phe Gly Lys Glu Lys Val Asn Glu Leu 40
41 Ser Lys Asp Ile Gly Ser Glu Ser Thr Glu Asp Gln Ala Met Glu Asp Ile Lys Gln Met 60
61 Glu Ala Glu Ser Ile Ser Ser Ser Glu Glu Ile Val Pro Asn Ser Val Glu Gln Lys His 80
81 Ile Gln Lys Glu Asp Val Pro Ser Glu Arg Tyr Leu Gly Tyr Leu Glu Gln Leu Leu Arg 100
101 Leu Lys Lys Tyr Lys Val Pro Gln Leu Glu Ile Val Pro Asn Ser Ala Glu Glu Arg Leu 120
121 His Ser Met Lys Glu Gly Ile His Ala Gln Gln Lys Glu Pro Met Ile Gly Val Asn Gln 140
141 Glu Leu Ala Tyr Phe Tyr Pro Glu Leu Phe Arg Gln Phe Tyr Gln Leu Asp Ala Tyr Pro 160
161 Ser Gly Ala Trp Tyr Tyr Val Pro Leu Gly Thr Gln Tyr Thr Asp Ala Pro Ser Phe Ser 180
181 Asp Ile Pro Asn Pro Ile Gly Ser Glu Asn Ser Glu Lys Thr Thr Met Pro Leu Trp 199


α_{s2}-CN
1 Lys Asn Thr Met Glu His Val Ser Ser Ser Glu Glu Ser Ile Ile Ser Gln Glu Thr Tyr 20
Lys Gln Glu Lys Asn Met Ala Ile Asn Pro Ser Lys Glu Asn Leu Cys Ser Thr Phe Cys 40
Lys Glu Val Val Arg Asn Ala Asn Glu Glu Glu Tyr Ser Ile Gly Ser Ser Ser Glu Glu 60
Ser Ala Glu Val Ala Thr Glu Glu Val Lys Ile Thr Val Asp Asp Lys His Tyr Gln Lys 80
Ala Leu Asn Glu Ile Asn Gln Phe Tyr Gln Lys Phe Pro Gln Tyr Leu Gln Tyr Leu Tyr 100
Gln Gly Pro Ile Val Leu Asn Pro Trp Asp Gln Val Lys Arg Asn Ala Val Pro Ile Thr 120
Pro Thr Leu Asn Arg Glu Gln Leu Ser Thr Ser Glu Glu Asn Ser Lys Lys Thr Val Asp 140
Met Glu Ser Thr Glu Val Phe Thr Lys Lys Thr Lys Leu Thr Glu Glu Glu Lys Asn Arg 160
Leu Asn Phe Leu Lys Lys Ile Ser Gln Arg Tyr Gln Lys Phe Ala Leu Pro Gln Tyr Leu 180
Lys Thr Val Tyr Gln His Gln Lys Ala Met Lys Pro Trp Ile Gln Pro Lys Thr Lys Val 200
Ile Pro Tyr Val Arg Tyr Leu 207


β-CN
1 10 20
Arg Glu Leu Glu Glu Leu Asn Val Pro Gly Glu Ile Val Glu Ser Leu Ser Ser Ser Glu
21 30 40
Glu Ser Ile Thr Arg Ile Asn Lys Lys Ile Glu Lys Phe Gln Ser Glu Glu Gln Gln Gln
41 50 60
Thr Glu Asp Glu Leu Gln Asn Lys Ile His Pro Phe Ala Gln Thr Gln Ser Leu Val Tyr
70 80
Pro Phe Pro Gly Pro Ile His Asn Ser Leu Pro Gln Asn Ile Pro Pro Leu Thr Gln Thr
Pro (Variant A2)
81 90 Ox 100
Pro Val Val Val Pro Pro Phe Leu Gln Pro Glu Val Met Gly Val Ser Lys Val Lys Glu
110 120
Ala Met Ala Pro Lys His Lys Glu Met Pro Phe Pro Lys Tyr Pro Val Glu Pro Phe Thr
130 140
Glu Ser Gln Ser Leu Thr Leu Thr Asp Val Glu Asn Leu His Leu Pro Leu Pro Leu Leu
150 160
Gln Ser Trp Met His Gln Pro His Gln Pro Leu Pro Pro Thr Val Met Phe Pro Pro Gln
170 180
Ser Val Leu Ser Leu Ser Gln Ser Lys Val Leu Pro Val Pro Gln Lys Ala Val Pro Tyr
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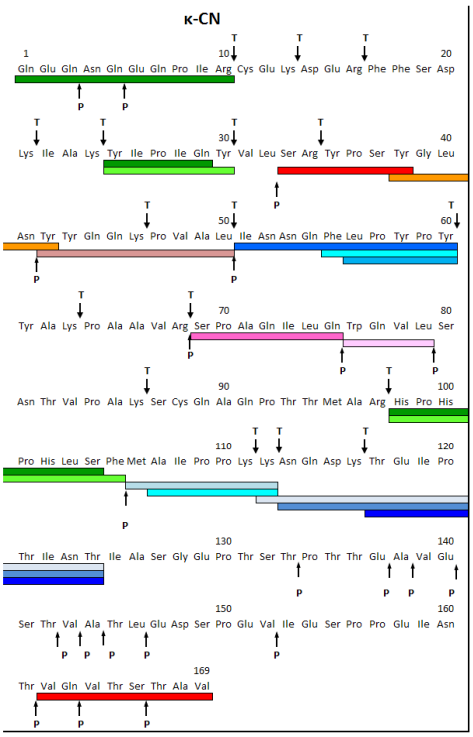
209
Val Arg Gly Pro Phe Pro Ile Ile Val


κ-CN
1 10 20
Gln Glu Gln Asn Gln Glu Gln Pro Ile Arg Cys Glu Lys Asp Glu Arg Phe Phe Ser Asp
30 40
Lys Ile Ala Lys Tyr Ile Pro Ile Gln Tyr Val Leu Ser Arg Tyr Pro Ser Tyr Gly Leu
50 60
Asn Tyr Tyr Gln Gln Lys Pro Val Ala Leu Ile Asn Asn Gln Phe Leu Pro Tyr Pro Tyr
70 80
Tyr Ala Lys Pro Ala Ala Val Arg Ser Pro Ala Gln Ile Leu Gln Trp Gln Val Leu Ser
90 100
Asn Thr Val Pro Ala Lys Ser Cys Gln Ala Gln Pro Thr Thr Met Ala Arg His Pro His
110 120
Pro His Leu Ser Phe Met Ala Ile Pro Pro Lys Lys Asn Gln Asp Lys Thr Glu Ile Pro
130 140
Thr Ile Asn Thr Ile Ala Ser Gly Glu Pro Thr Ser Thr Pro Thr Thr Glu Ala Val Glu
150 160
Ser Thr Val Ala Thr Leu Glu Asp Ser Pro Glu Val Ile Glu Ser Pro Pro Glu Ile Asn
169
Thr Val Gln Val Thr Ser Thr Ala Val

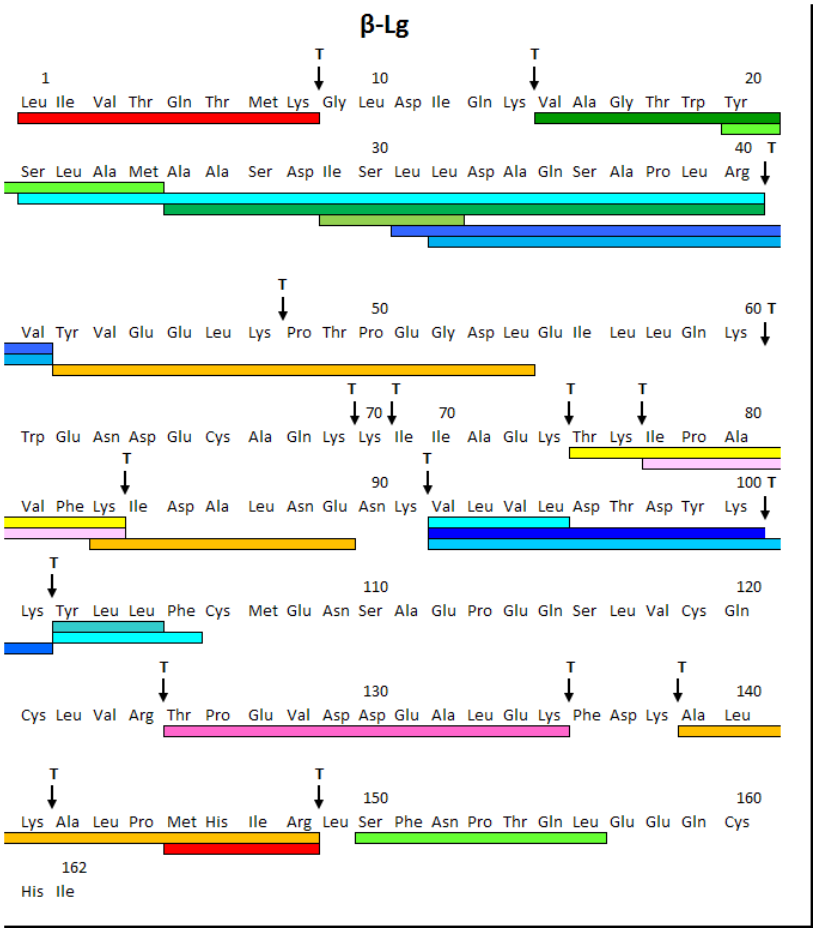


Figure 7. Primary structure of bovine β-, α_{s1}-, α_{s2}-, κ-CN and β-Lg together with identified peptides in commercial milk protein powder sample hydrolysed by sequential action of pepsin and trypsin. Protein/enzyme ratio 100/1 (w/w). Incubation with each enzyme was carried out for 16h at appropriate pH. Ends of horizontal bars indicate first and last amino acid residue of peptides isolated within this work. Arrows indicate casein peptide bonds broken by pepsin (P) and trypsin (T) as reported by Pelissier [194].

7. Oral immunotherapy as future perspective in CMA management

The primary treatment for managing food allergies is eliminating the offending food from the diet. In the case of milk, it is extremely difficult to achieve complete elimination because milk can be masked in any number of foods, which may lead to unwanted severe reactions. The natural course of a cow's milk protein allergy is the acquisition of tolerance spontaneously through an elimination diet, and 85% of patients overcome CMA by the time they are 4-5 years old [195-198]. In recent years, a number of studies have been published

regarding desensitization or oral tolerance to food antigens, particularly to cow's milk. Major advances in understanding the immunological processes involved in the development of CMA have revealed a considerable number of allergenic epitopes and the heterogeneity of allergic responses. Importantly, an elimination diet of dairy foods has negative consequences in terms of inadequate calcium and vitamin intake. In the literature, several conflicting studies have reported on possible desensitizing therapies in the treatment of FA allergies. The possibility to obtain an oral desensitization is now gaining acceptance widely, even if the mechanism is still unclear. Oral tolerance or desensitization is the active non-response of the immune system to an antigen through sublingual, oral administration. Tolerance or the long-term loss of allergic reactivity follows a desensitization treatment. In the literature, several studies have reported on possible physiopathogenetic mechanisms of oral desensitization, but the exact mechanism is still unknown. That tolerance may be involved in the mechanism of desensitization is still uncertain [199-201]. A growing understanding of the molecular and cellular mechanisms of oral tolerance is reinforcing advances in potential therapies for food allergies and is pivotal to eventually curing allergies in sensitized individuals. Oral desensitization should be taken into consideration in the management of food-allergic patients even if the physiopathogenetic mechanisms are still unexplained. Moreover, this treatment should be considered, particularly for children, because elimination from the diet of some foods (e.g., milk and eggs) for these patients could cause psychological and/or nutritional problems. Oral immunotherapy (oral desensitization) may be a promising treatment strategy for cow's milk allergy in children and valuable for other foods, such as eggs or peanuts. Although the mechanisms of IgE-mediated allergies are fairly well understood, the immunology and variety of non-IgE-mediated reactions remains largely unknown. A better understanding of these allergy mechanisms is a prerequisite to the development of improved diagnostics, which in turn will facilitate an improved understanding of the epidemiology of CMA, particularly for non-IgE-mediated reactions.

8. Conclusions

A better understanding will also aid the development of hypoallergenic dairy products, especially for adults with CMA for whom there is currently a dearth of suitable low-allergenic dairy products. Some of the risk factors for developing CMA have been identified; a familiar history of atopy is one of the main determinants. However, the mechanisms of allergic sensitization and the precise interactions between genetics and various environmental factors that lead to CMA remain unclear. The first few months of life, during which the immune system is still maturing, appear to be a critical risk period for allergic sensitization. For at-risk infants with at least one atopic parent, breastfeeding during this period is currently the best identified preventative strategy; the use of hydrolyzed formulas is recommended for babies who cannot be breastfed. The use of immunomodulatory dietary adjuvants, such as probiotics, is an emerging approach with considerable promise for primary prevention. For CMA sufferers, the avoidance of dietary milk proteins remains the only effective management strategy but carries with it nutritional implications, particularly for adequate vitamin and calcium intake as well as protein and energy where unorthodox

alternative diets are implemented. Increasing knowledge of the molecular and cellular mechanisms of oral tolerance reinforces the advances in potential FA therapies and is pivotal to eventually curing allergies in sensitized individuals. Unraveling the links between innate and adaptive immunity and characterizing the roles of dendritic cells and T cells in directing immune responses and homeostasis to environmental antigens are likely to remain a focus of fundamental FA research in the coming years.

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