EXTRACTION AND IMMOBILIZATION OF VEGETABLE ASPARTIC PROTEASES FOR CHEESE MAKING

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Ora dunque rimangono queste tre cose:
la fede, la speranza e la carità.
Ma la più grande di tutte è la carità.
1 Corinzi 13:13

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SUMMARY

The worldwide increase in cheese production and consumption, together with the reduced supply and consequent price increase of calf rennet, has led to the search of new milk coagulant agents. Much research interest has been thus directed towards discovering milk clotting enzymes which would satisfactory replace calf rennet in cheese manufacture. Microbial rennet produced by genetically engineered bacteria has proven to be a suitable substitute even though the consumer constraints on the use of animal rennet either for religion reasons (Judaism and Islam) or diet habits (vegetarianism) or consumer concerns regarding genetically engineered foods (Germany, Netherlands and France forbid the use of recombinant calf rennet) have led to a growing interest toward vegetable coagulants. Therefore, several plant sources of milk-clotting enzymes have been investigated.

Unfortunately, most of the "plant rennets" were found unsuitable because they produced extremely bitter dairy products due to a strong proteolysis. However, an exception to this general rule is represented by the extract of flowers of the *Cynara* genus. In particular, flowers *of Cynara scolymus* and *Cynara cardunculus* contain two different aspartic proteases named cynarase and cardosin, respectively. The latter enzyme was successfully used for cheese manufacture in several areas of Portugal and Spain. Regarding the cynarase, its use is limited since the flowers of globe artichoke (*Cynara scolymus*) represent an edible part before the blooming. In fact, in Mediterranean countries, the globe artichoke plays an important economic role, so that Italy is the first producer with ~475 kt. However, the artichoke processing industry generates large amounts of agricultural solid wastes of which ~60% is represented by the leaves. Therefore, I found of great interest to investigate the presence of aspartic proteases, possessing milk clotting properties, in the *Cynara scolymus* leaves as well as in the flowers of *Carduus defloratus,* as unproductive and/or intensely exploited agricultural lands.

The obtained results showed the presence of clotting enzyme both in the leaves of *Cynara scolymus* and in the flowers of *Carduus defloratus.* In the first case, the extraction of proteins from plant tissue resulted difficult due to the presence of interfering compounds such as cell wall cellulose, lipids and other polysaccharides. To optimize protein extraction an enzymatic pretreatment with a mixture of carbohydrases was successfully carried out. However, the yield was always very low and, therefore, this protocol would be hardly suitable for an industrial application.

For these reasons, in the second part of the work, I have studied the possibility to immobilize the enzyme extracted from the flowers of *Carduus defloratus* by using a commercial rennet from *Mucor meihei* as model enzyme. The immobilization of the enzyme extracted from both sources was successfully carried out by using an epoxy support and, in particular, the results showed that the *Carduus defloratus* enzyme exhibited higher stability than the commercial rennet one.

All these results suggest the possibility to use an immobilized "vegetable rennet", extracted from the flowers of *Carduus defloratus,* for the production of novel dairy products.

RIASSUNTO

1. Introduzione

Le proteasi svolgono un ruolo di primo piano in vari settori delle biotecnologie e sono al primo posto nel mercato mondiale degli enzimi utilizzati in vari settori produttivi e nei processi di trasformazione. Soprattutto nell'industria alimentare le proteasi hanno una vasta gamma di applicazioni nelle diverse fasi di produzione degli alimenti. Una delle prime applicazioni biotecnologiche è nel settore caseario dove il caglio viene utilizzato nel processo di produzione dei formaggi. Il principale enzima proteolitico responsabile della caseificazione risulta essere la “chimosina”, presente nel caglio naturale ottenuto dall’abomaso di giovani vitelli. Negli ultimi dieci anni l'aumento a livello mondiale della produzione di formaggi e la riduzione della disponibilità del caglio da vitello hanno portato ad un crescente interesse per lo ricerca di nuove proteasi con capacità coagulanti il latte. Un ulteriore stimolo alla ricerca di possibili sostituti del caglio animale deriva dal sempre maggiore frequente rifiuto ad alimentarsi con formaggi ottenuti con l'uso di caglio animale dettato da motivi religiosi, da scelte di regimi alimentari speciali. come la dieta vegana, e dal timore dell’utilizzo di alimenti derivanti da, o contenenti, organismi geneticamente modificati. Più di recente, anche l'incidenza di malattie contagiose come l’encefalopatia spongiforme bovina (BSA) ha ridotto sia l'offerta che la domanda di caglio bovino.

Recenti pubblicazioni nel settore della coagulazione del latte mediante l’utilizzo di enzimi ottenuti da fonti vegetali ha evidenziato un crescente interesse per l’industria lattiero-casearia. Anche se sono state individuate diverse proteasi vegetali ad attività coagulante il latte estratte da *Carica papaya*, *Ananas comosus*, *Ficus acacia*, *Lactuca sativa*, *Cucumis melo*, la maggior parte di tali enzimi risulta essere inadeguato per la produzione di formaggi a causa della loro eccessiva attività proteolitica che abbassa la resa finale del prodotto e produce sapori amari o comunque sgradevoli. Un'eccezione è rappresentata dagli estratti acquosi dei fiori del genere *Cynara*. I fiori di *Cynara cardunculus,* in particolare, sono stati utilizzati per anni nella produzione tradizionale di alcuni formaggi portoghesi (Serra e Serpa) e spagnoli (Los Pedroches e Serena) che, pur prodotti su scala artigianale in fattoria o piccolo caseificio, rappresentano un importante contributo socio-economico per il settore caseario a livello locale e regionale di questi paesi. Secondo diversi autori la loro capacità coagulante è dovuta alla presenza in tali vegetali di specifiche aspartico proteasi (EC 3.4.23). Tra queste, quelle maggiormente caratterizzate sono la “cardosina A” e la “cardosina B”, presenti nei fiori di *Cynara cardunculus*. Come la chimosina, la cardosina A scinde il legame peptidico tra Phe105-Met106 della k-caseina (k-CN) bovina mentre, la cardosina B è simile alla pepsina, in termini di specificità e di attività. Questi enzimi sono caratterizzati da elevata attività coagulante e proteolitica e producono formaggi caratterizzati da una consistenza morbida e cremosa con un aroma leggermente piccante.

La caratterizzazione di caglio vegetale prodotto dai fiori del genere *Cynara* è stata effettuata principalmente sulle specie *Cynara cardunculus,* mentre la conoscenza sulle proprietà delle proteasi dai fiori di carciofo (*Cynara scolymus*) è estremamente limitata e di scarso interesse in quanto il carciofo viene utilizzato come alimento prima della fioritura. Per questo motivo alcuni autori hanno suggerito di ricercare la presenza di possibili proteasi in estratti grezzi di altre parti della pianta (foglie, radici e pappus).

L'Italia è il primo produttore di carciofi al mondo con una produzione nel 2011 di 474.550 tonnellate. Durante la loro raccolta e trasformazione, circa il 60% della biomassa viene scartata e, di questa, la maggior parte è rappresentata dalle foglie (FAO, 2011). Finora, questi scarti sono stati presi in considerazione, per quanto riguarda una loro possibile utilizzazione, come mangimi per animali o per la produzione di fibre. Per questi motivi, all'inizio del mio lavoro sperimentale, mi sono proposta di utilizzare questi sottoprodotti come possibili materie prime da utilizzare per la produzione di enzimi per la cagliatura del latte, al fine di dare un valore aggiunto ai rifiuti provenienti dai carciofi.
Un'altra strategia di ricerca di aspartato proteasi è stata quella di individuarla in specie selvatiche coltivabili in suoli marginali. Tra queste, alcune specie largamente studiate anche per la produzione di biomassa sono quelle del genere *Carduus*. In particolare, ho preso in considerazione il *Carduus defloratus L. subsp. Carlinifolius,* detto anche cardo alpino, che si adatta bene ai terreni aridi e marginali e presenta capolini floreali grandi e abbondanti.
Pertanto, l'obiettivo del mio lavoro sperimentale è stato quello di valutare la presenza ed il possibile utilizzo di proteasi con attività coagulante estratte dalle foglie di *Cynara scolymus* e dai fiori di *Carduus defloratus L. subsp. Carlinifolius,* e di procedere alla loro eventuale immobilizzazione per la produzione di nuovi prodotti caseari.

2. Risultati

Estrazione di attività coagulante dalle foglie di carciofo (*Cynara scolymus)*
Essendo generalmente la struttura cellulare dei tessuti vegetali alquanto complessa, di conseguenza anche l'estrazione delle proteine dai tessuti vegetali presenta quasi sempre una certa complessità operativa. Per tali ragioni, al fine di ottimizzare la resa dell'estrazione proteica, sono stati presi in considerazione protocolli di estrazione effettuata a differenti pH mediante omogeneizzazione con Ultraturrax. Inoltre, l'utilizzo di enzimi pectinolitici e cellulosolitici spesso rappresentano un utile strumento nel trattamento di questo tipo di matrici. Si è pensato, quindi, di trattare preventivamente le foglie di carciofo con una miscela di questi enzimi (*Viscozyme*) e solo successivamente omogeneizzate. In questo modo la parete cellulare poteva essere degradata e la successiva fase di estrazione essere facilitata. Pertanto sono stati esaminati gli effetti di differenti quantità di *Viscozyme* a diversi tempi di incubazione per verificare le migliori condizioni sperimentali. Così è stato possibile stabilire, pertanto, che una concentrazione di *Viscozyme* al 6%, in un tempo di incubazione pari a 20 minuti, era sufficiente per ottenere la massima resa di estrazione. I risultati ottenuti confermarono così che il pretrattamento enzimatico effettivamente promuoveva una ottima estrazione proteica attraverso l'idrolisi della parete cellulare e la riduzione delle interazioni tra polisaccaridi e le proteine, determinando il conseguente rilascio di queste ultime in forma solubile. Inoltre i dati ottenuti hanno indicato che anche il pH influiva in maniera significativa sulla resa di estrazione, essendo pH 5 il valore di pH a cui era possibile ottenere il massimo di estrazione. Al fine di valutare la presenza nelle foglie di proteasi con attività coagulante, veniva successivamente effettuato un saggio qualitativo mediante caseogramma, un metodo che prevede l'esecuzione di un'elettroforesi in gel di poliacrilammide in condizioni native a pH alcalino e poi un incubazione di quest'ultimo a 37°C su un gel di agarosio e latte a pH 5, pH ottimale per la coagulazione. E' da sottolineare che, in questo caso, solo l'estratto proteico ottenuto

a pH 5 mostrava attività coagulante. Questo risultato era probabilmente dovuto al fatto che il pH influenzava le interazioni elettrostatiche tra le singole molecole proteiche e/o tra i polisaccaridi e le proteine andando a modificare la conformazione dell'enzima e, di conseguenza, la sua attività. Inoltre, l'estratto non trattato con il *Viscozyme* presentava una banda di coagulazione con una bassa mobilità, mentre quello preincubato con il *Viscozyme* presentava una specie con attività coagulante ad alta mobilità. Questo risultato probabilmente era dovuto alla capacità degli enzimi presenti nella miscela del *Viscozyme* di promuovere efficacemente la liberazione dalla matrice polisaccaridica dell'attività enzimatica coagulante. Successivamente, l'estratto proteico è stato parzialmente purificato mediante frazionamento in solfato d'ammonio, che ha indicato la precipitazione dell'attività coagulante nella frazione 20-50% del sale.
Questa prima tappa di purificazione ha messo in evidenza che la resa di estrazione del campione non trattato con *Viscozyme* era molto bassa (circa 1%) rispetto a quella osservabile quando il campione veniva trattato con la miscela di enzimi pectinolitici e cellulosolitici (circa 60%): nel primo caso l'attività totale si traduceva in 0,086 U rispetto alle 64 U dell'estratto trattato con *Viscozyme*. Per tale ragione la successiva fase di purificazione, mediante cromatografia a scambio anionico, è stata effettuata solo sull'estratto ottenuto mediante trattamento con *Viscozyme*. L'attività coagulante veniva eluita dalla colonna cromatografica ad una concentrazione di circa 0.3 M di NaCl contenuto nella soluzione tampone eluente. Il peso molecolare della specie proteica avente attività coagulante (37-50 KDa) è stato determinato esaminando il suo profilo elettroforetico su gel di poliacrilammide, in condizioni denaturanti ed in presenza di SDS, che mostrava solo due bande proteiche. Per comprendere la natura enzimatica di questo enzima è stato eseguito anche un saggio con la "pepstatina", inibitore specifico per le aspartato proteasi. I risultati ottenuti hanno confermato che l'enzima estratto apparteneva a tale "famiglia" di proteasi.

<u>Estrazione di attività coagulante dai fiori di cardo *(Carduus defloratus)*</u>
Un altro scopo del mio lavoro sperimentale è stato quello di verificare la presenza di enzimi con attività coagulante del latte anche nei fiori di *Carduus defloratus,* una specie botanica molto simile a quella del genere *Cynara.* A tale fine è stata eseguita un'estrazione delle proteine dai fiori freschi del cardo a pH 5 che ha mostrato un recupero di circa 0,96 mg/gr. Successivamente l'estratto proteico è stato sottoposto ad una parziale purificazione mediante precipitazione con solfato d'ammonio per rimuovere diversi contaminanti, tra i quali i composti fenolici. Le frazioni quindi sono state analizzate sia in SDS-PAGE e sia per la loro attività coagulante mediante caseogramma. Anche in questo caso è stato possibile osservare la presenza di attività coagulante solo nel precipitato ottenuto nella frazione 20-50% di solfato di ammonio. L'analisi elettroforetica ha messo in evidenza che in questa frazione erano presenti solo tre bande proteiche aventi peso molecolare compreso tra 15 e 37 KDa. E' molto probabile che l'attività coagulante osservata sia legata alla specie molecolare di 37 KDa, peso molecolare caratteristico dell'enzima "cardosina" estratta dai fiori di *Cynara cardunculus*. Analizzando i dati ottenuti analizzando gli estratti proteici dei fiori di cardo con quelli ottenuti dalle foglie di carciofo è stato possibile constatare che il contenuto in proteine degli estratti di carciofo (76.81mg/g) era molto inferiore a quello dei fiori di cardo (463mg/g). Inoltre nel primo caso era possibile ottenere 0.133 U/mg di attività coagulante contro le 4.17 U/mg riscontrate negli estratti proteici di cardo.

Anche nel caso di *Carduus defloratus* è stato possibile determinare mediante saggio di inibizione con pepstatina che l'enzima estratto apparteneva alla famiglia delle aspartato proteasi.

Immobilizzazione di enzimi con attività coagulante

Negli ultimi tempi gli enzimi sono oggetto di sempre maggiore attenzione da parte dell'industria per le potenzialità di un loro utilizzo nei processi produttivi. Tuttavia uno dei maggiori problemi nell'uso degli enzimi a livello industriale è la bassa stabilità delle macromolecole proteiche. Uno dei settori in cui l'uso degli enzimi è in costante crescita è l'industria alimentare e, in particolare, l'industria lattiero-casearia. E' noto che gli enzimi con attività coagulante il latte, estratti dalle piante, hanno un'attività proteolitica molto accentuata e portano, quindi, alla produzione di formaggi con caratteristiche organolettiche poco accettabili dal consumatore e con basse rese. Pertanto, un ulteriore obiettivo del lavoro di tesi è stato quello di procedere all'immobilizzazione di un enzima di origine vegetale avente attività coagulante, al fine di poter controllare il processo enzimatico e di addivenire ad un possibile riuso del catalizzatore biologico con conseguente auspicabile abbattimento dei costi.
Pertanto, in una prima fase di messa a punto della metodologia, mi sono concentrata nello studio di immobilizzazione di un enzima commerciale, avente attività coagulante, estratto da *Mucor meihei.* Una prima tappa sperimentale è stata quella di verificare il pH funzionale di questo enzima mediante il saggio con la k-caseina, ed i risultati ottenuti hanno messo in evidenza che la migliore attività catalitica era osservabile a pH 7.0.
Per l'immobilizzazione sono stati utilizzate come supporto sfere di materiale poliacrilico aventi gruppi epossidici capaci di formare legami covalenti con l'enzima. Come suggerito dalla casa produttrice di queste sfere, per il processo di immobilizzazione 400 µL di enzima, contenenti 11.2 U, sono stati incubati a 4°C per 16 h con 100 mg di sfere. Successivamente, dopo aver rimosso il sovranatante, le sfere sono state lavate in apposito tampone e saggiate in presenza di k-caseina determinando il tempo di coagulazione della proteina substrato.
Al fine di poter valutare il pH ottimale di immobilizzazione si è proceduto ad effettuare diversi tentativi a pH 5, 6 e 7. I risultati ottenuti hanno indicato che la migliore resa di immobilizzazione (circa il 90% di proteine presenti nell'estratto) e di attività coagulante era osservabile a pH 6.0. La probabile causa della perdita di attività osservata immobilizzando l'enzima a valori di pH superiori o inferiori potrebbe essere dovuta ad ulteriori reazioni tra i gruppi epossidici e diversi gruppi nucleofili presenti sulla proteina enzimatica. Infatti, a pH acido i gruppi carbossilici delle proteine reagiscono con i gruppi epossidici presenti sulle sfere formando dei legami esteri, mentre a pH neutro o alcalino reagiscono sia i gruppi tiolici che amminici. E' probabile, pertanto, che queste diverse condizioni di pH influiscano sul numero di legami covalenti che si instaurano tra l'enzima e le sfere, modificando la capacità dell'enzima di interagire con le proteine substrato.
Poiché una delle più importanti caratteristiche degli enzimi immobilizzati è quella di possedere proprietà di stabilità, sono stati eseguiti esperimenti per valutare questo parametro. A tale scopo l'enzima immobilizzato è stato conservato a 4°C a pH 7 (valore di massima attività catalitica). I risultati ottenuti hanno mostrato che l'enzima immobilizzato in tali condizioni perdeva circa l'80% della sua attività coagulante dopo 48 h. La causa di tale instabilità è stata individuata nella alta concentrazione di enzima legato alle sfere che determinava un alto livello di autoproteolisi.

Successivamente ho proceduto all'immobilizzazione dell'enzima estratto dai fiori di *Carduus defloratus,* per una possibile applicazione industriale di tale enzima. Anche in questo caso, al fine di valutare l'effetto del pH sull'attività coagulante dell'enzima è stato eseguito un saggio preliminare di attività usando come substrato la k-caseina. In questo caso il valore di pH ottimale è risultato essere pari a 5.0. Successivi esperimenti, volti a determinare il pH ottimale di immobilizzazione seguendo lo stesso protocollo applicato per il *Mucor meihei,* hanno indicato che la migliore resa di immobilizzazione si otteneva a pH 5.0 (82.5%). Per quanto riguarda l'attività coagulante dell'enzima immobilizzato, essa è risultata assente quando l'immobilizzazione veniva effettuata a pH 6, mentre l'enzima immobilizzato a pH 7 conservava circa il 79% dell'attività coagulante. In questo caso si può supporre che questi risultati siano dovuti alla reattività dei gruppi presenti sulla superficie dell'enzima e che una minore resa nell'immobilizzazione possa essere attribuita ad un maggior numero di legami covalenti multipli, condizione che determinava una maggiore stabilità dell'enzima. Infatti esperimenti volti a valutare la stabilità dell'enzima immobilizzato hanno mostrato che l'enzima estratto dai fiori di *Carduus defloratus* esibiva una stabilità maggiore se paragonato a quello estratto da *Mucor meihei.* Infatti, dopo 72 h l'enzima vegetale non mostrava alcuna perdita di attività.
Dall'analisi complessiva dei dati ottenuti in questo lavoro di tesi è possibile affermare che sia nelle foglie di carciofo che nei fiori di cardo è presente una significativa attività enzimatica coagulante il latte, dovuta alla presenza di una proteina catalitica appartenente alla famiglia delle "aspartato proteasi". Essendo le rese di estrazione dell'enzima da cardo (*Carduus defloratus)* maggiormente vantaggiose, si è proceduto alla immobilizzazione di tale proteasi attraverso una metodologia messa a punto immobilizzando l'enzima coagulante il latte estratto da *Mucor meihei*.
I risultati degli esperimenti di estrazione, purificazione e immobilizzazione riportati, seppure meritevoli di ulteriori studi volti al miglioramento della resa e stabilità dell'enzima oggetto di studio, rappresentano una valida base per una possibile futura realizzazione ed applicazione a livello industriale di un bioreattore enzimatico di origine vegetale per la coagulazione "in line" del latte nella produzione lattiero-casearia.

1. Introduction

1.1 Enzyme global market situation

The statistical analyses revealed that the global market for industrial enzymes was rather immune from the economic difficulties. In fact, demand for industrial enzymes in mature economies such as US, Western Europe, Japan and Canada was relatively stable during the recent years. The worldwide market of industrial enzymes was estimated to reach $ 3.3 billion in 2010 and is forecast to reach US $ 3.74 billion by 2015 (Boutros *et al.,* 2012). Key factors promoting market growth include new enzyme technologies improving cost efficiency and productivity, and the growing interest among consumers in substituting petrochemical products and harsh chemicals for other bio-organic compounds like enzymes. Other factors propelling market growth is the surging demand from textile manufacturers, animal feed producers, detergent manufacturers, pharmaceutical and cosmetic companies (Sarrouh *et al.,* 2012).
Enzymes are a very well established product in biotechnology (Norus, 2006; Sarrouh *et al.,* 2012) and a recent survey on world sales of enzymes ascribes 31% for food enzymes, 6% for feed enzymes and the remaining for technical enzymes. For the food and beverage enzymes segment the world sales have been estimated about $ 1 billion in 2010. In particular, milk and dairy had the highest sales within this segment (Sarrouh *et al.,* 2012).
Further published reports on enzymes market highlighted the fact that proteases constitute the largest product segment in the global industrial enzymes market, representing about the 60% of the total industrial enzyme market (Figure 1.1) with an important place in food, pharmaceutical and detergent industries, as well as in the preparation of leather, textile and wool (Doran, 2002; Gupta *et al.*, 2002; Abidi *et al.*, Chandel *et al.,* 2007; 2008; González-Rábade *et al.,* 2011).

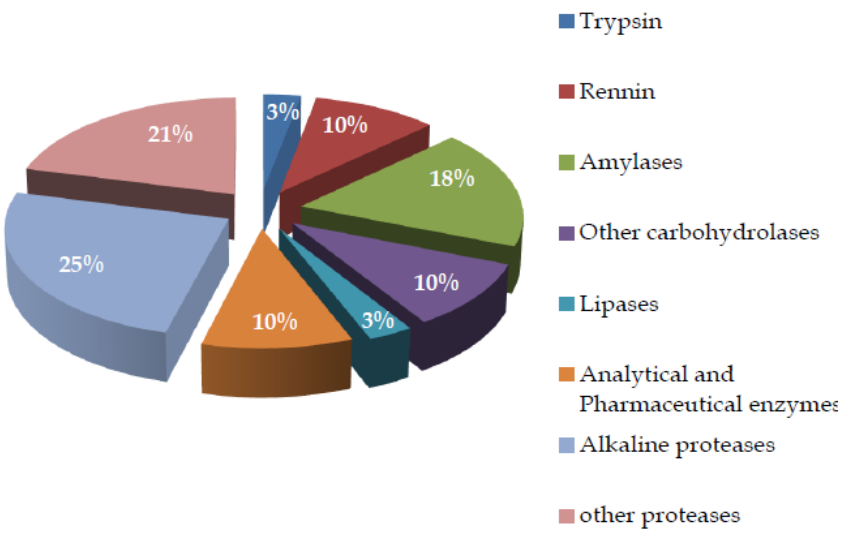

Figure 1.1- Market share of industrial enzymes (Chandel *et al.,* 2007).

1.2 Proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases, also called peptidases, are classified as hydrolases (group 3) and within the subgroup 4, i.e. those which are able to

hydrolyse peptide bonds. On the basis of the site of action on protein substrates, proteases can also be subdivided into endopeptidases or exopeptidases. The first cleave peptide bonds within and distant from the ends of a polypeptide chain, whereas the second catalyse the hydrolysis of the peptide bond near N- or C-terminal ends of the substrates (Rao *et al.,* 1998).
On the other hand, following the catalytic mechanism and the presence of amino acid residues at the active site, the proteases can be grouped as aspartic-, cysteine-, glutamic-, metallo-, asparagine-, serine-, threonine proteases, and others with mixed or unknown catalytic mechanism. Based on the optimal pH, they are referred to as neutral, acidic and alkaline proteases (Jaswal *et al.,* 2008; Vishalakshi *et al.,* 2009). Classification and nomenclature of proteolytic enzymes, as well as a detailed description of individual proteases, is available in the MEROPS database (Rawlings *et al.,* 2012).
Proteases occur naturally in all organisms and are involved in many physiological and industrial processes. They play a role in the production of nutrients for cell growth and proliferation, protein degradation, and as regulatory components for different physiological functions. Blood coagulation, inflammation, pharmacologically active peptide synthesis from precursor proteins, activation of zymogens, secretory protein transportation and release of hormones are some of essential and important protease functions. Moreover, proteases have different industrial applications, mainly in detergent and food industry. In fact, they are used in leather treatment and new generation pharmaceutical production. Proteases are also used for fundamental scientific researches such as structure-function relationships, peptide synthesis and protein sequencing (Rao *et al.,* 1998).
The use of proteases in food industry dates back to antiquity. They have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates and meat tenderization. In particular, the major application of proteases in the dairy industry is the manufacture of cheese (Cheong *et al.,* 1993).
Although also cysteine and serine proteases possess the ability to clot milk under proper conditions, aspartic proteases are those mainly utilized in the milk coagulation (Shah *et al.,* 2014).

1.2.1 Aspartic proteases

Aspartic proteases (EC 3.4.23), also known as acidic proteases or aspartyl proteases, are a subfamily of endopeptidases having two aspartic acid residues that are critical for their catalytic activity (i.e. Asp32 and Asp215, pepsin numbering) within their active site. They have been grouped into three families, namely pepsin (A1), retropepsin (A2) and enzymes from pararetroviruses (A3). Structurally, most aspartic proteases belong to A1 pepsin family. Like other pepsin-like enzymes, aspartic proteases are synthesized as pro-enzymes (zymogens). After cleavage of the signal peptide, the pro-enzyme is secreted and autocatalytically activated (Davies, 1990). These enzymes have molecular weights in a range of 30–45 kDa and contain one or two conserved aspartic acid residues at the active site (Sumantha *et al.,* 2006). Aspartic proteases are optimally active at acidic pH (pH 3–5). They are acidic in nature, having isoelectric points in the range from 3 to 4.5 and are inhibited by pepstatin A (Rao *et al.,* 1998). Aspartic proteases were the first type of enzymes known, the first protease type described, the second protein crystallized, and also the target for numerous early investigations by the pioneers of modern enzymology and protein chemistry (Szecsi, 1992). They have been extensively studied and

characterized and are widely distributed among vertebrates, plant, yeast, nematodes, parasites, fungi and viruses (Simoes *et al.,* 2004).
Because of their high activity and stability in acidic environments, aspartic proteases play an important role in the food processing industry. These include milk clotting during cheese manufacturing (Claverie-Martin and Vega-Hernandez, 2007), production of seasonings products (Kanlayakrit and Maweang, 2006), degradation of turbidity complexes in fruit juices and alcoholic liquors (Sumantha *et al.* 2006), production of "renneted casein" and "casein hydrolysates" which are used as fat emulsifier and texture modifier agents in food and pharmaceutical formulations (Phelan *et al*. 2009). In addition, increased fiber hydrolysis from feedstock available from agricultural processing operations (Abbas and Bao, 2009), meat tenderization (Ashie *et al*., 2002) and peptide synthesis (Kumar and Bhalla, 2005; Filippova and Lysogorskaia, 2003) are further industrial applications of aspartic proteases.

1.2.2 Milk clotting aspartic proteases

Milk coagulation is a crucial step in cheese making and, thus, it has been widely studied. Bovine chymosin (EC 3.4.23.4), also called rennin, obtained from the abomasum of unweaned calves is generally utilized in milk coagulation. This enzyme, characterized by Foltmann in 1996, exhibits a high ratio of milk clotting/proteolytic activity in contrast with the bovine "rennet" obtained from adult animals which is rich in pepsin and, consequently, has poorer technological properties (Nielsen and Foltmann, 1995).
Bovine chymosin produces a very specific cleavage between Phe105 and Met106 of κ-casein, destabilizing the casein micelle structure (Turhan and Mutlu, 1998) (Figure 1.2). Therefore, cleavage specificity as opposed to extended proteolysis defines a good milk coagulant.

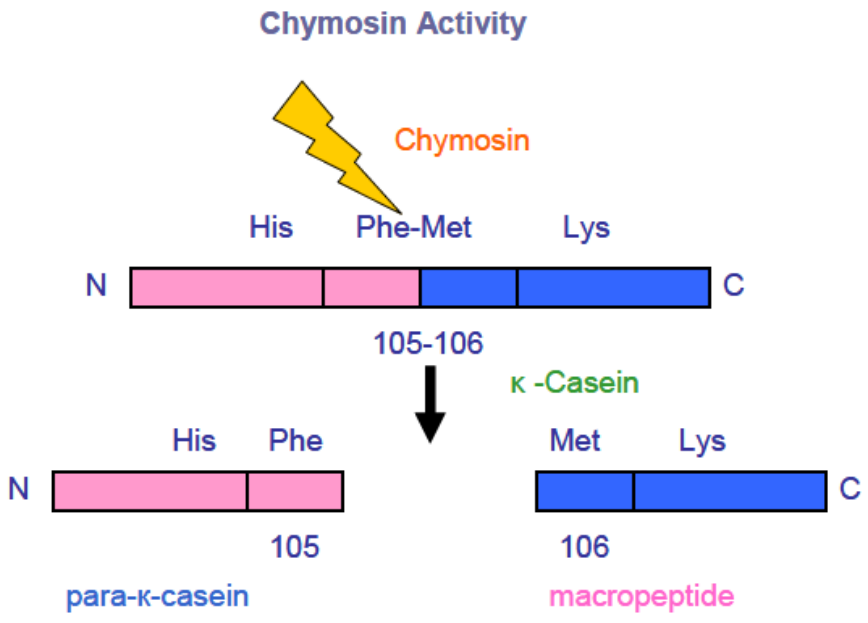

Figure 1.2- Chymosin cleaves the k- casein's peptide bond between Phe105 and Met106.

Enzymatic milk coagulation is a two phase process, any variation in the chemical environment affecting the two stages of reaction separately. In the first phase the enzyme hydrolyzes the κ-casein molecule, splitting the protein in two fragments: the (hydrophobic) para-κ-casein and the (hydrophilic) macropeptide (GMP), the latter being able to diffuse into the milk whey. The second phase consists in the coagulation of the casein micelles that have been destabilized by proteolytic attack (Claverie-Martin and Vega-Hernandez, 2007). This phase is sensitive to temperature, pH, and Ca^{2+} concentration. These reactions are shown schematically in Figure 1.3.

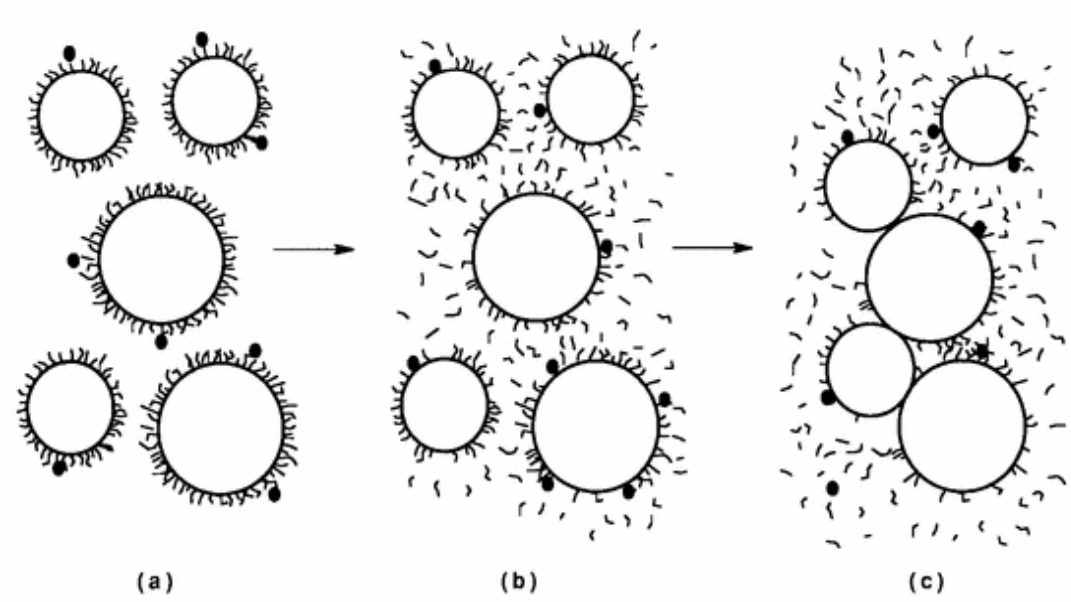

Figure 1.3- Schematic diagram of the attack by chymosin (shown as small filler circles) on casein micelles. Three different points in the reaction are illustrated. In (a) the k-casein coat of the micelles is intact, and chymosin has just been added; (b) some time later, much of the k-casein has been hydrolysed and a proportion of the GMP is in solution, but sufficient amount of k-casein remains to prevent aggregation; (c) at a later time, nearly all of the k-casein has been hydrolysed and the micelles started to aggregate (Dalgleish, 1999).

1.2.3 Plant proteases as milk clotting enzyme for cheese making

Plant extracts have been used as milk coagulants in the manufacture of dairy products since ancient times. The majority of plant proteases belongs to the A1 family, being the members of this enzyme family active at acidic pH, inhibited by pepstatin and possessing two aspartic acid residues responsible for the catalytic activity (Siomoes *et al.,* 2004). The physiological role of these enzymes is not so far elucidated. Most probably they may be involved in the hydrolysis of both storage and extracellular proteins (Schaller 2004).

Plant coagulants include enzymes from *Cynara cardunculus* (Louro Martins *et al.,* 1996; Sousa and Malcata, 2002), *Cynara scolymus* (Sidrach *et al.,* 2005), seven *Papilionoideae* species -*Eriosema shirense*, *E. ellipticum*, *E. pauciflorum*, *E. gossweilleri*, *E. psoraleoides*, *Adenolichosanchietae* and *Droogmansia megalantha* (Lopes *et al.,* 1998)-, *Solanum dobium fresen* (Ahmed *et al.,* 2009), *Centaurea calcitrapa* (Reis *et al.,* 2000; Pintado *et al.,* 2001), *Calotropis procera* (Sanni, *et al.,* 1999), *Helianthus annuus* (Park *et al.,* 2000), *Lactuca sativa* (Lo Piero *et al.,* 2002), *Ficus carica* (fig), *Carica papaya* (papaia), *Ananas sativa* (pineapple) and *Ricinus communis* (castor oil) (Garg and Johri, 1994). Almost all the enzymes used as milk

coagulants belong to aspartic proteases, but enzymes from other families, such as cysteine and serine proteases, have also been used.
However, the excessive proteolytic activity of most vegetable coagulants has limited their use in cheese manufacturing due to lower cheese yield and defects in flavor and texture (Lo Piero *et al.,* 2002). An exception is represented by the aqueous extracts of *Cynara* genus flowers. In particular, the flowers from *Cynara cardunculus* have been used for years in the manufacture of several traditional Portuguese (Serra and Serpa) and Spanish (Los Pedroches and Serena) dairy products (Chazarra *et al.,* 2007). These cheeses are normally produced on an artisanal scale, in farm-houses or small dairies. However, they give an important socio-economical contribution to the dairy sector at local and regional areas of each country. According to several authors the ability of such "vegetable rennet" to clot is due to the presence of aspartic proteases (EC 3.4.23).
The most characterized plant aspartic proteases are cardosin A and cardosin B. Like chymosin, cardosin A cleaves bovine k-casein (k-CN) between Phe105 and Met106, while cardosin B is similar to pepsin in terms of specificity and activity (Padros *et al.,* 2007). These enzymes are characterized by high clotting and strong proteolytic activity producing cheeses with a creamy soft texture and genuine and slightly piquant aroma (Fernandez-Salguero *et al.,* 2002).
The studies on plant rennet from flowers of genus *Cynara* have been focused mainly on the species *C. cardunculus* since the knowledge on the properties of proteases extracted from the flowers of globe artichoke (*C. scolymus*) is limited because this vegetable is extensively used as food before the blooming.
Moreover, the search for new potential milk-clotting enzymes from plants is in continuous progress to make them industrially useful following the increasing global demand for diversified and high quality cheese production (Hashim *et al.,* 2011). Generally, these enzymes are extracted from natural sources by aqueous maceration of various plant organs such, as flowers, seeds, roots and leaves, following several different ways of preparing the plant material extracts. The dried whole or crushed thistle flowers are soaked in water at room temperature for different times. Then, the filtrate is collected and the crude extract is used as coagulant (Roseiro *et al.,* 2003).
An alternative method of extraction is dried flower grinding with crude kitchen salt, laying the paste on a cotton cloth (which acts as a strainer) and solubilizing the enzymes by percolation with warm milk (Sousa and Malcata 2002). Precipitation with ammonium sulfate could be an effective way to produce substantial amounts of active proteases from *C. cardunculus* flowers (Barros *et al.,* 2001).

1.3 Overview of enzyme immobilization

Enzymes, being specific and efficient biocatalysts able to work without need of extreme temperature, high pressures or corrosive conditions, became increasingly important in a wide range of industrial sectors to replace classical catalysts (Liang *et al.,* 2000). Nowadays, one of the most important driving forces in science is to develop sustainable technologies for the manufacture of chemical compounds. Beyond the mild reaction conditions, shorter synthetic routes, biodegradability of the catalyst and use of environmentally friendly solvents, biocatalytic processes are also characterized by chemo-, regio-, and stereo-selectivity, resulting in low amount of general wastes. Conversely, some enzyme features are not adequate for an industrial application: difficult recovery for reutilization, unsatisfactory thermal, pH,

and stability properties, possible inhibition by substrates and products, low affinity toward non natural substrates.
Therefore, enzyme properties need to be significantly improved before their use as industrial catalysts (Guisan *et al.,* 2006). Under this perspective, enzyme immobilization is one of the most important engineering strategies and may be defined as any technique that is able to allow the reuse or continuous use of the biocatalysts. The potential advantages associated with the immobilization of the enzymes can be resumed as follows (End et al., 2004):

- Higher stability with respect to temperature, pH and catalyst poisoning.
- Repeated use.
- Higher resistance to stress and contamination.
- Easy separation from reaction media.
- Increased reaction rate due to high catalyst concentration.

Further important characteristics of immobilized enzymes are the simplicity and the cost effectiveness of the methodology used (Guisan, 2006). Up to date, an universally applicable method for the immobilization of all enzymes or for all the possible applications of a certain enzyme does not exist. The choice of a certain procedure, support, or enzyme strongly depends on the chosen process and must be evaluated from case to case, as well as the development of a procedure of immobilization can be sometimes laborious and expensive (End *et al.,* 2004).
Based on the nature of interaction that leads to immobilization, the methods are classified as physical or chemical (Peter *et al.,* 2005; Seville, 2011) and as irreversible and reversible (Guisan, 2006). According to another largely used classification system, three types of immobilization can be distinguished: binding to a carrier, entrapment (encapsulation) and cross-linking (Chibata *et al.,* 1987; Sheldon *et al.,* 2007 a) (Figure 1.4).

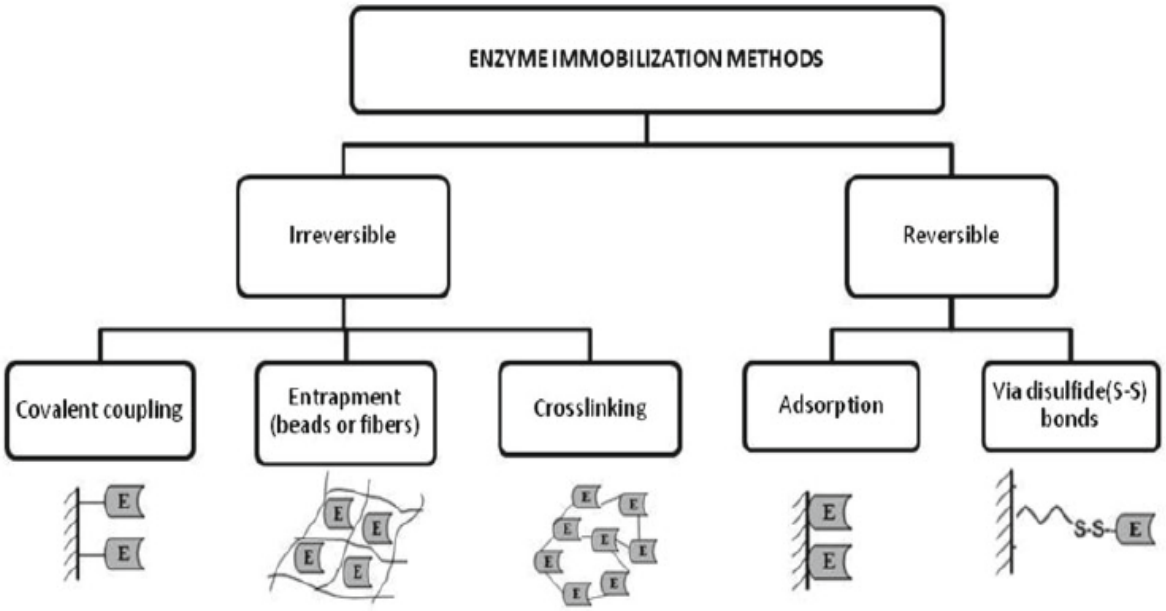

Figure 1.4- Schematic representation of the main different methods of enzyme (E) immobilization (Brena et al., 2013).

Protease immobilization for milk coagulation has recently receiving a renewed interest for several new potential applications. In fact, the use of immobilized proteases would permit the "renneting" of milk as a continuous process. In addition, it should be possible to recover and reuse the enzyme for coagulation of numerous batches of milk. The following Table 1.1 shows the different supports and the main immobilization methods so far used for various proteases used for milk coagulation (Garg et al., 1993).

Table 1.1- Immobilized proteases for milk coagulation.

Enzyme	Support	Immobilization method	Reactor	Reference
Chymotrypsin and rennin	Agarose	Covalent	Packed bed and stirred tank	Green & Crutchfield (1969)
Rennin	DEAE-cellulose	Adsorption	Packed bed and stirred tank	Green & Crutchfield (1969)
Pepsin and trypsin	EMA resin	Covalent	—	Goldstein (1973)
Pepsin	Porous glass	Covalent	Packed bed	Ferrier *et al.* (1972)
Renin, *R. miehei* protease, pepsin	Porous glass	Covalent	Fluidized bed	Cheryan *et al.* (1975a, b, 1976)
Pepsin	Alumina	Adsorption	Fluidized bed	Taylor *et al.* (1977)
Pepsin	BSA coated porous glass	Glutaraldehyde	Fluidized bed	Lee *et al.* (1977)
Chymosin	Polymer; wax	Entrapment	—	Shindo & Arima (1979); Shindo *et al.* (1980)
Rennet	Sepharose-4B	Covalent	Packed bed	Angelo & Shahani (1983)
Pepsin	Duolite amine resin	Glutaraldehyde	—	Ronald *et al.* (1984)
Mucor sp. L_{42} protease	Succinicamido-propylated glass	Covalent	Packed bed	Park *et al.* (1986a, b)
Calf rennet, pepsin and *R. miehei* rennet	Agarose	Covalent	Packed bed and stirred reactor	Mashaly *et al.* (1988); Wahba *et al.* (1989)
Rennilase-L	Alkylamide sand	Covalent	—	Anprung *et al.* (1989)

1.3.1 Epoxy support

Epoxy groups are very stable at neutral pH values even in wet conditions and, hence, such commercial supports can be stored for long periods of time and can be prepared quite far from the place where the enzyme has to be immobilized (Mateo *et al.,* 2000). Epoxy groups are able to react with different nucleophilic groups on the protein surface (i.e., amino, hydroxy, or thiol moieties) to form extremely strong linkages (secondary amino bonds, ether bonds, thioether bonds) with minimal chemical modification of the protein (i.e., p*K* values of the new secondary amino groups are very similar to those of the pre-existing primary amino ones). At the end of the immobilization process, epoxy groups can be easily blocked by reaction with various thiol or amino compounds under mild conditions, preventing further uncontrolled reactions between the support and the enzyme that could decrease enzyme stability (Mateo *et al.,* 2003).

Weak nucleofilic agents mainly reacted with epoxy groups according to the following mechanism (Figure 1.5):

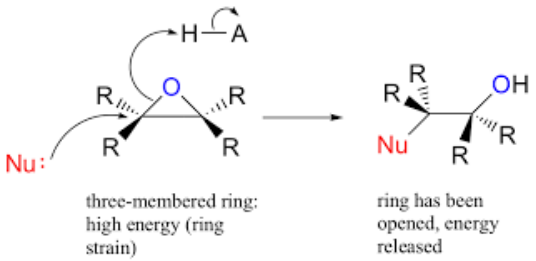

Figure 1.5- Reaction between a nucleofilic group (Nu:) and an epoxy group.

1.4 Aim of the thesis

The preliminary objective of the research thesis was to investigate the presence of milk clotting activities in the protein extracts of artichoke leaves (*Cynara scolymus*) to identify a new vegetable source of milk-clotting proteases assigning an added value to wastes of artichoke processing industry. Even though an aspartic protease having coagulating activity was isolated from artichoke leaves, the presence in the plant extracts of interfering compounds and the resulting very low enzyme yield led to consider the developed process hardly suitable for a possible industrial application. Therefore, in the second part of the work the possibility to extract milk-clotting enzyme(s) occurring in the flowers of another plant of the Asteraceae family -the *Carduus defloratus subsp. carlinifolius* (Lam.), commonly known as alpine thistle- was investigated. In the final part of the work, the immobilization of the enzyme extracted from alpine thistle flowers, as well as of the commercial rennet from *Mucor meihei* , was carried out.
The research activities were the following:

- Extraction, purification and characterization of milk clotting proteases from *Cynara scolymus* leaves
- Extraction, purification and characterization of milk clotting proteases *Carduus defloratus* flowers.
- Immobilization of commercial rennet from *Mucor meihei*
- Immobilization of milk clotting proteases from *Carduus defloratus* flowers

1.5 References

- **Abbas, C.A. Bao, W.L.** (2009). Increased Fiber hydrolysis by protease addition. U.S. Patent 0098638 A1.
- **Abidi, F., Llimam, F., Nejib, M.M.** (2008). Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. *Process Biochem.,* 43, 1202-1208.
- **Aguilar, C.F., Cronin, N.B., Badasso, M., Dreyer, T., Newman, M.P., Cooper, J.B., Hoover, D.J., Wood, S.P., Johnson, M.S. Blundell, T.** (1997). The three-dimensional structure at 2.4Å resolution of glycosylated proteinase A from the lysosome-like vacuole of *Saccharomyces cerevisiae*. *J. Mol. Biol.,* 267, 899-915.
- **Ahmed., I.A.M., Morishima, I., Babiker, E.E., Mori, N. (**2009). Characterization of partially purified milk clotting enzyme from *Solanum dubium* Fresen seeds. *Food Chem.,* 116, 395-400.
- **Ashie, I.N.A., Sorensen, T.L. Nielsen, P.M.** (2002). Effects of papain and a microbial enzyme on meat proteins and beef tenderness. *J. Food Sci.,* 67, 2138-2142.
- **Barros, R.M., Ferreira, C.A., Silva, S.V., Malcata, F.X.** (2001). Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate. *Enzyme Microb. Technol.,* 29, 541–547.
- **Brena, B., Gonzalez-Pombo, P., Batista-Viera,F.** (2013). Immobilization of enzyme: a literature survey. Methods Mol., Biol., 1015, 15-31.

- **Chandel, A.K., Rudravaram, R., Rao, L.V., Ravindra, P., Narasu, M.L.,** (2007). Industrial enzyme in bio-industrial sector development,. An Indian perspective. J. of Commercial Biotechnology., 13, 238-291.
- **Chazarra, S., Sidrach, L., López-Molina, D., Rodríguez-López, J.N.** (2007). Characterization of the milk-clotting properties of extracts from artichoke (Cynara scolymus, L) flowers. *Int. Dairy J.* 17, 1393-1400.
- **Cheong, C., Chun, S.S., Kim, Y.H.** (1993). Production and properties of an alkaline protease from Pseudomonas sp. SJ-320. *Korean Bidern. Journal.,* 26, 479-484.
- **Claverie-Martin, F., Vega-Hernandez, M.C.** (2007). Aspartic proteases in cheese making. In: Polaina, J. and Maccabe, A.P. (eds). Industrial Enzymes. Springer, Netherlands, 207-219.
- **Dalgeish D.G**. (1999). Enzymatic coagulation of milk. In: Fox P.F. (ed). Cheese: chemistry, physics,and microbiology (general aspects) vol.1, 2 nd ed. Aspen Publishers, Inc., Maryland (USA).
- **Davies, D.R.** (1990). The structure and function of aspartic proteases. *Annu. Rev. Biophys. Biomol. Struct.,* 19, 189-215.
- **Doran, P.M.** (2002). Properties and applications of hairy-root cultures. In: Oksman-Caldenty, K.M. and Barz, W.H. (eds). Plant biotechnology and transgenic plants. New York, Mercel Dekker Inc., 143-162.
- **Fernandez-Salguero, J., Sanjuan, E.** (1999). Influence of vegetable and animal rennet on proteolysis during ripening in ewe's milk cheese. *Food Chem.,* 64, 177-183.
- **Filippova, I.Y., Lysogorskaia, E.N.** (2003). Modified proteinases in peptide synthesis in organic media. *Russ. J. Bioorganic. Chem*., 29, 544-550.
- **Garg, S.K., Johri, B.N.** (1993). Immobilization of milk-clotting proteases (Rew.). *World J. Microb. Biot.,* 9, 139-144.
- **Gonzalez-Rabade, N., Badillo-Corona, J.A., Aranda-Barradas, J.S., Oliver-Salvador, M.C.** (2011). Production of plant proteases *in vivo* and *in vitro* - A review. *Biotech. Adv.,* 29, 983-996.
- **Gupta, R., Beg, Q.K., Lorenz, P.** (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol.,* 59, 15-32.
- **Hashim, M.M., Mingsheng, D., Iqbal, M.F., Xiaohong, C.** (2011). Ginger rhizome as a potential source of milk coagulating cysteine protease. *Phytochem*., 72:458-464.
- **Jacob, M., Jaros, D., Rohm, H.** (2011). Recent advances in milk clotting enzymes. *Int. J. Dairy Technol.,* 64,14-33.
- **Jaswal, R.K., Kocher, G.S., Virk, M.S.** (2008). Production of alkaline protease by Bacillus circulans using agricultural residues: A statistical approach. *Indian J. of Biotech.,* 7, 356-360.
- **Kanlayakrit, W., Maweang, M.** (2006). Production of seasoning “Mirin” from Thai rice by fermentation. *Kasetsart J.,* 40, 39-46.
- **Kumar, D., Bhalla, T.C.,** (2005). Microbial proteases in peptide synthesis: approaches and applications. *Appl. Microbiol. Biotechnol.,* 68, 726-736.
- **Liang, J.F., Li, Y.T., Yang, V.C.** (2000). Biomedical application of immobilized enzyme. *J. Pharm. Sci.,* 89, 970-990.
- **Lo Piero, A.R., Puglisi, I., Petrone, G.** (2002). Characterization of “lettucine”, a serine-like protease from Lactuca sativa leaves, as a novel enzyme for milk clotting. *J. Agric. Food Chem.,* 50, 2439-2443.

- **Lopes, A., Teixerira, G., Liberato, M.C., Pais, M.S., Clemente, A.** (1998). New vegetal sources for milk clotting enzymes. *J. Mol. Catal. B-Enzym.,* 5, 63-68.
- **Martins, A.P.L., de Vasconcelos, M.M.P., De Sousa, R.B.** (1996). Thistle (*Cynara cardunculus* L) flower as a coagulant agent for cheese making. Short characterization. *Lait.,* 76, 473-477.
- **Mateo, C., Abian, O., Fernandez-Lafuente, R., Guisan, J.M.** (2000). Increase in conformational stability of enzymes immobilized on epoxy activated supports by favoring additional multipoint covalent attachment. *Enzyme Microb. Technol.* 26, 509-515.
- **Mateo, C., Fernandez-Lorente, G., Abian, O., Fernandez-Lafuente, R., Guisa, J.M.** (2000). Multifunctional Epoxy Supports: A New Tool To Improve the Covalent Immobilization of Proteins. The Promotion of Physical Adsorptions of Proteins on the Supports before Their Covalent Linkage. *Biomacromolecules*, 1, 739-745.
- **Mateo, C., Torres, R., Fernandez-Lorente, G., Ortiz, C., Fuentes, M., Hidalgo, A., Lopez-Gallego, F., Abian, O., Palomo, J.M., Betancor, L., Pessela, B.C.C., Guisan, J.M**. (2003).Epoxy-amino groups: a new tool for improved immobilization of proteins by the epoxy method. *Biomacromolecules,* 4, 772-777.
- **Nielsen, P.K., Foltmann, H.** (1995). Purification and Characterization of Porcine Pepsinogen B and Pepsin. *Arch. Biochem. Biophys.,* 322, 417-422.
- **Norus, J.** (2006). Building sustainable competitive advantage from knowledge in the region: the industrial enzymes industry. *Eur. Plan. Stud.,* 14, 681-696.
- **Phelan, M., Aherne, A., FitzGerald, R.J., O'Brien, N.M.** (2009). Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *Int. Dairy J.,* 19, 643-654.
- **Prados, F., Pino, A., Fernandez-Salguero, J.** (2007). Effect of a powdered vegetable coagulant from cardoon Cynara cardunculus in the accelerated ripening of Manchego cheese. *Int. J. Food Sci. Technol.,* 42, 556-561.
- **Rao, M.B., Tanksale, A.M., Ghatge, M.S., Deshpande, V.V.** (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.,* 62, 597-635.
- **Rawlings, N.D., Barrett, A.J., Bateman, A.M.** (2012). The database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.,* 40, 343-350.
- **Reis, P.M., Lourenco, P.L., Domingos, A., Clemente, A.F., Pais M.S., Malcata, F.X.** (2000). Applicability of extracts from *Centaureae calcitrapa* in ripening of bovine cheese. *Int. Dairy J.,* 10, 775-780.
- **Roseiro, L.B., Barbosa, M., Ames, J.M., Wilbey, R.A.** (2003). Cheesemaking with vegetable coagulants—the use of Cynara L. for the production of ovine cheeses. *Int. J. Dairy Technol.,* 56, 76-85.
- **Sarrouh, B., Santos, T.M., Miyoshi, A., Dias, R., Azevedo, V.** (2012). Up-To-Date Insight on Industrial Enzymes Applications and Global Market. *J. Bioprocess Biotechniq.,* S4:002. doi: 10.4172/2155-9821.S4-002.
- **Schaller, A.** (2004). A cut above the rest: the regulatory function of plant proteases. *Planta*, 220,183-197.
- **-Shah, M.A., Mir, S.A., Paray, M.A.** (2014). Plant proteases as milk clotting enzymes in cheesemaking: a review. *Dairy Sci. Technol.,* 94, 5-16.
- **Sidrach, L., Garcıa-Canovas, F., Tudela, J., Rodrıguez-Lopez, J. N.** (2005). Purification of cynarases from artichoke (*Cynara scolymus* L.): Enzymatic properties of cynarase A. *Phytochemistry,* 66, 41-49.
- **Simoes, I., Faro, C.** (2004). Structure and function of plant aspartic proteinases. *Eur. J. Biochem.,* 271, 2067-2075.

- **Sousa, M.J., Malcata, F.X.** (2002). Advances in the role of a plant coagulant (Cynara cardunculus) in vitro and during ripening of cheese from several milk species. *Lait,* 82, 151–170**.**
- **Sumantha, A., Larroche, C., Pandey, A.** (2006). Microbiology and Industrial Biotechnology of Food-Grade Proteases: A Perspective. *Food Technol. Biotechnol.,* 4, 21-220.
- **Szecsi, P.B.** (1992). The aspartic proteases. Scand J Clin Lab Invest 210, 5-22.
- **Tamer Ml, Mavituna F** (1997). Protease from freely suspended and immobilized Mirabilis jalapa. *Process Biochem.,* 32, 195-200.
- **Vishalakshi, N., Dayanand, A.** (2009). Production of alkaline protease from *Streptomyces gulbargensis* and its application in removal of blood stain. *Indian J. of Biotech.,* 81, 280-285.
- **Yegin, S., Fernandez-Lahore, M., Gama-Salgado, A.J., Guvenc, U., Goksungur, Y., Tari, C.** (2011). Aspartic proteinases from Mucor spp. in cheese manufacturing. *Appl. Microbiol. Biotechnol.,* 89, 949-60.
- **Yin, L.J., Hsu, T.H., Jiang, S.T.** (2013). Characterization of acidic protease from Aspergillus niger BCRC 32720. *J. Agric. Food Chem.,* 61, 662-6.

2. Results

2.1 Extraction and characterization of milk clotting proteases from artichoke (*Cynara scolymus*, L.) leaves and alpine thistle (*Carduus defloratus* subsp. *carlinifolius*, Lam.) flowers

2.1.1 Introduction

Calf rennet has been the first milk-clotting enzyme and still is the most widely used in cheese making procedures. The coagulating properties of calf rennet derive from the presence of chymosin (EC 3.4.23.4), an aspartic protease which is considered to be the best coagulating agent due to its high specificity for cleaving the Phe105-Met106 bond of k-casein (Ahmed *et al.*, 2009).
The worldwide increase in cheese production and consumption, together with the increase of calf rennet price, led to the search for new coagulant agents (Guiama *et al.*, 2010). Much research interest has been directed towards discovering a milk-clotting enzyme which would satisfactorily replace calf rennet in cheese manufacture. Microbial rennet produced by genetically engineered bacteria has proven to be a suitable substitute but, the consumer constraints for religion (i.e., Judaism and Islam) or diet (vegetarianism) reasons, as well as for consumer concerns regarding genetically engineered foods (i.e., Germany, Netherlands and France forbid the use of recombinant calf rennet), have led to a growing interest toward vegetable coagulants (Egito *et al.,* 2007. Ahmed *et al.,* 2009).
Plant sources for milk-clotting enzymes have been identified in *Ananas comosus* (Cattaneo *et al.*, 1994), Carica papaya (Cabezas *et al.*, 1981), Ficus carica (El-Shibiny *et al.*, 1973), Calm viscera (Gupta *et al*., 1977) and Cynara sp. (Heimgartner *et al.*, 1990; Sidrach *et al.*, 2005). Unfortunately, most of the plant rennets were found unsuitable because they produce extremely bitter cheese because of their strong proteolytic activity (Ahmed *et al.*, 2009). An exception to this general rule is represented by extracts of *Cynara* genus flowers. In particular, flowers *of Cynara scolymus* and *Cynara cardunculus* contain two aspartic proteases named cynarase and cardosin, respectively. The latter is used successfully as source for the manufacture of cheese in several areas of Portugal and Spain (Verissimo *et al*., 1995; Roseiro *et al*., 2003; Llorente *et al.,* 2004; Sidrach *et al.,* 2005). Regarding cynarase, the use of this enzyme is limited because flowers of globe artichoke (*Cynara scolymus*) is extensively consumed as food before blooming. In fact, in Mediterranean countries, globe artichokes (*Cynara scolymus* L.) play an important economic role, and Italy is the first producer with ~475 kt. However, the artichoke processing industry generates large amounts of agricultural solid wastes of which ~60% is represented by their leaves (Faostat, 2011).
For this reason I found of interest to investigate on the presence of aspartic proteases in the discarded parts of artichoke, such as leaves, pappus and roots. The preliminary aim of my thesis work was, thus, to investigate the presence of clotting activities in the protein extracts of artichoke leaves to identify a new vegetable source of milk-clotting proteases assigning an added value to wastes of artichoke processing industry.
Following the difficulties encountered during the isolation of the coagulating activities from *Cynara scolymus* leaves, an additional source of vegetable rennet, obtainable at low cost, of the Asteraceae family (*Carduus defloratus subsp. carlinifolius* (Lam.) flowers, commonly known as alpine thistle) was taken into consideration. The alpine thistle is a perennial herbaceous plant belonging to the “wild flora” and is widely

distributed throughout the Italian territory both at mid and high latitudes. Its typical habitat is the meadow, but also the cloudy forest, walls, rocks and screes are possible environments, being the preferred substrate both siliceous or calcareous with alkaline pH and basic nutritional value. Moreover, the ability of growing on dry soil makes the alpine thistle suitable to be used as wildflower in recovery of marginal soils such as unproductive and/or intensely exploited agricultural lands. As wildflower, *Carduus defloratus carlinifolius* is easy to find and it is a species very close in terms of systematic classification to *Cynara cardunculus* and *Cynara scolymus*.

2.1.2 Materials and methods

2.1.2.1 Materials

Cynara scolymus, L. subsp. *scolymus* leaves were obtained from plant grown in Salerno province (Campania, Italy). Flowers of *Carduus defloratus* L. *subsp. carlinifolius,* Lam. harvested in Gallicchio (PZ), a municipality of National Park "Appennino Lucano Val d'Agri Lagonegrese" (Basilicata, Italy). The electrophoresis reagents and non fat dry milk were purchased from BIO-RAD (Milano). Rennet from *Mucor miehei* (10 U/μg) was purchased in a local market. Viscozyme, a multi-enzyme mixture containing a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase, were purchased from Novozyme (Bagsvaerd, Denmark). All chemicals and reagents of analytic grade were purchased from Sigma-Aldrich (Milano, Italy).

2.1.2.2 Methods

2.1.2.2.1 Protein extraction

Crude protein extracts were obtained by using Ultra-Turrax (IKA-WERKE, Germany) homogenizer under different conditions.

Fresh artichoke leaves (50 g) were homogenized with 150 mL of the following three different buffers: 0.1M sodium citrate/pH 3, 0.1M sodium acetate/pH 5 and 0.1M Tris-HCl/pH 7. For enzymatic protein extraction, artichoke leaves were enzymatically pretreated with a commercial mix of carbohydrases (Viscozyme L, Sigma-Aldrich, Milano) as follows: artichoke leaves were incubated in a sorbitol solution (13% w/v) at pH 5 (grams of fresh leaves/buffer volume, 1:5 w/v) under continuous stirring in a water bath at 37°C for 5 min. Afterwards, different amounts of Viscozyme (3, 6, 12 or 24%, v/w of fresh leaves) were added to the leaf suspension and the enzymatic hydrolysis was carried out in an orbital incubator (Künher, Switzerland) at 200 rpm and 37°C for different times (5, 10, 20 and 40 min).

For thistle flowers protein extraction, 80 gr of fresh flowers were homogenized in 240 mL of 0.1M sodium acetate/pH 5 with an Ultra-Turrax (IKA-WERKE, Germany according to modified method of Chen *et al.* (2003).

In all cases, homogenates were centrifuged at 30,000xg for 30 min at 4°C. The supernatants were collected and filtered through Whatman n. 1 filter papers. Protein concentration was determined using the Bradford assay with bovine serum albumin as a standard.

2.1.2.2.2 SDS-PAGE analysis

SDS–PAGE was performed in a vertical gel apparatus (Mini-Protean, Bio-Rad) by using 12% (w/v) polyacrylamide gels as described by Laemmli (1970).
Samples containing different amounts of proteins were applied to the gel and electrophoresed at 100 V for 45 min. Proteins were stained with 0.2% (w/v) Coomassie brilliant blue in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 45 min.

2.1.2.2.3 Two-dimensional electrophoresis (2DE)

Protein extracts from leaves (130 µg) were separated by 2DE. The first dimensional step was performed using an Ettan IPGphor II system (GE healthcare). Immobilized pH 3-10 linear gradient strips (11 cm, BioRad) were rehydrated overnight with 130 µg of reduced protein extracts previously suspended in 200 µL of IPG strip rehydration buffer (8 M urea; 2% CHAPS; 20 mM DDT; 2% IPG buffer; 0.002% bromophenol blue). The proteins were focused up to 11,000 Vh at a maximum voltage of 6000 V at 20°C. IPG strips were soaked in the equilibration solution (6 M urea, 2% w/v SDS, 30% w/v glycerol, 50 mM Tris-HCl, pH 8.8, 0.002% w/v bromophenol blue) for 15 min with 1% DTT (w/v) and 15 min with 2.5% iodoacetamide (w/v) at room temperature. The second dimensional electrophoresis was performed as described above on 12% polyacrylamide gel.

2.1.2.2.4 Purification steps

Ammonium sulphate precipitation- Crude extracts were brought to different ammonium sulphate saturations (0-20, 20-50, 50-80%) by slowly adding the appropriate amount of solid ammonium sulphate during continuous stirring at 4°C. The precipitates were collected by centrifugation at 30,000xg for 30 minutes at 4°C and redissolved in one-fifth of the starting volume of sodium acetate buffer (0.1 M pH 5). Finally, the obtained fractions were dialyzed 3 times for 6 hours, against 100 volumes of the same buffer.
Anion exchange chromatography- Anion exchange chromatography was performed with HPLC System Gold 126 NM (Beckmann Coulter). Before loading the sample, the column (Shodex, QA-825) was equilibrated with 20 mM diethylamine/pH 9. The chromatographic separation was performed by the following experimental conditions: the unbound proteins were eluted in 20 mM diethylamine/pH 9, whereas the bound proteins were eluted with a linear gradient of NaCl (0-0.5M) in 15 min; flow rate was adjusted at 1 mL/min and the chromatogram was visualized by reading absorbance at 220 and 280 nm using an UV/VIS detector (Beckman). The fractions were collected and then analyzed for enzyme activity and protein concentration.

2.1.2.2.5 Milk-clotting activity

Qualitative evaluation- The clotting activity was qualitatively evaluated according to the method called “caseogram” described by Foltman *et al.* (1985). In this method, the sample was allowed to migrate in a polyacrylamide gel in non-denaturing conditions at 20 mA for 50 min in Tris-HCl buffer pH 8.8. After this step, the acrylamide gel was equilibrated with 0.15 M sodium acetate, pH 5, for 30 min and then placed on the top of a previously fresh prepared 1% (w/v) agarose gel, containing 1% (w/v) skim-milk powder in 0.1 M sodium acetate pH 5.3. The so assembled system was then incubated at 37°C in order to allow the appearance of the clotting signal in correspondence of the electrophoretic bands.
Quantitative determination- For quantitative measurements of the enzymatic clotting activity a spectrophotometric assay was performed according with Douillard

et al. (1970). The enzyme was incubated at 25°C in 1 mL 0.1 M sodium acetate, pH 5, buffer containing 0.1% k-casein as substrate in a disposable spectrophotometer cuvette (pathlength 1 cm). The progress of the reaction was displayed by following the increase of absorbance at 550 nm as function of time (min) until the kinetic sigmoid curves reached the plateau. The recorded kinetic curves were processed with SigmaPlot software in order to evaluate the clotting time which mathematically corresponds to the abscissa value of the first minimum of the second derivative obtained from sigmoid curve. The clotting activity was calculated as the inverse of the clotting time (min^{-1}). One unit (U) is defined as the amount of enzyme able to produce 0.2 min^{-1} of clotting activity under the above described conditions.

2.1.2.2.6 Inhibition studies

In order to assign clotting activity to aspartic protease activity, extracts were assayed in the presence of pepstatin A, a specific inhibitor of aspartic protease. Crude extract (0.05 U of enzyme) were pre-incubated with 1 µM pepstatin A, previously dissolved in assay buffer, for 30 min at 25°C. Same amount of enzyme in the absence of the inhibitor was run as control. The reaction started by the addition of the preincubated extracts to the assay mixtures containing 0.1% k-casein as above described for quantitative determination of clotting activity. Inhibition rate was assessed by comparison the sigmoid curves obtained either in the presence or absence of pepstatin A.

2.1.2.2.7 Statistical analysis

Microsoft Excel and SigmaPlot software were used for data processing and validation.

2.1.3 Results and Discussion

2.1.3.1 *Cynara scolymus*

The extraction of proteins from plant tissue is often difficult due to the presence of interfering compounds such as rigid cellulose cell wall, storage of polysaccharides, lipids and other contaminants. In this study we tried to optimize protocols for protein extraction by using Ultra-turrax homogenizer system at different pH values (3, 5 and 7). The effectiveness of the used protocol was evaluated by comparing the protein yields. The experimental results demonstrated that the amount of proteins in the homogenate supernatants was significantly influenced by the pH value of the extraction buffer, showing a highest protein yield in samples homogenized at pH 5 (Figure 2.1-A). In fact, working at pH 5, it was possible to obtain up to 12 mg of protein per gram of fresh leaves, about twice the amount of protein obtained at other pH values. In addition, each protein extract was tested through caseogram experiments to assess the presence of clotting activity. The clotting signal was clearly detected only in the sample homogenized at pH 5 (Figure 2.1-B, lane 2), while no clotting activity was found in the other two samples extracted at pH 3 and pH 7 (lanes 1 and 3). It is worth noting that, for caseogram experiments, protein samples were subjected to gel electrophoresis in buffer at pH 8.8 and then were balanced to pH 5 which is also the reported optimum pH for clotting activity (Sidrach *et al.,* 2005; Ahmed *et al*., 2009).

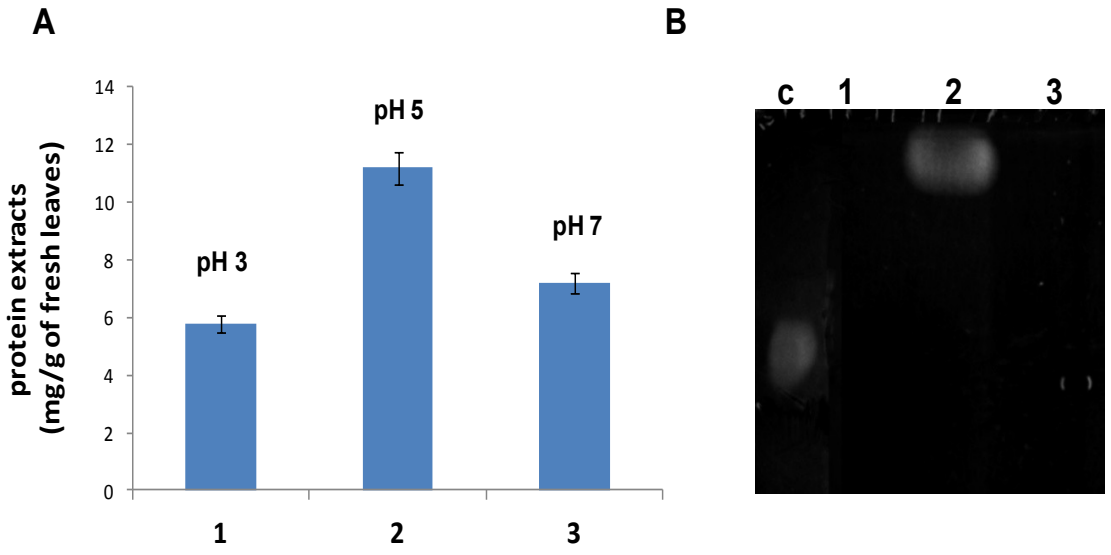

Figure 2.1 – Influence of extraction pH on protein content and milk-clotting activity of supernatant of artichoke leaf homogenates. Protein content of homogenate supernatants, obtained at different pHs, was estimated with Bradford assay (A) and the corresponding milk-clotting activity was evaluated in caseogram experiment (B) performed with 20 µg of each protein extract. Commercial rennet from *Mucor miehei* (0.1 µg) was used as positive control (lane c).

Although the extraction at pH 5 allowed to obtain a significant amount of protein for laboratory analyses, the yields resulted quite low for a large-scale production. With the aim to increase protein yields for industrial applications, we modified the extraction protocol by treating the artichoke leaves with a mixture of carbohydrases (Viscozyme L). Enzymes contained in Viscozyme promote the hydrolysis of the cell wall matrix facilitating its breakdown and, consequently, protein release (Ansharullah *et al.,*1997; Wang *et al.,* 1999). However, several factors, such as carbohydrase concentration, incubation time and temperature influence the effectiveness of the enzymatic pretreatment, and their effects may be either independent or interactive.
For this reason we have tested different amounts of Viscozyme (3, 6, 12, or 24%, v/w of fresh leaves) for different times (5, 10, 20 and 40 min). As expected, both enzyme concentration and incubation time significantly affected the yield of protein extraction. In particular, we found that the treatment with 6% Viscozyme for 20 min gave rise to the optimal experimental conditions. In fact, it was determined that the extension of the incubation time did not correspond to any significant increase in extracted proteins. Therefore, under these conditions, the total protein content of the crude extract was found to be about two times higher than that of the untreated samples (Table 2.1). The increase in proteins extraction was certainly due to the action of the Viscozyme that, hydrolyzing the cell wall polysaccharide network, reduced the protein-polysaccharide interactions and allowed the release of more intracellular constituents (Tang *et al*., 2003). The SDS-PAGE profiles of the supernatants of homogenates derived from Viscozyme-untreated (lane 1) and –treated (lane 2) artichoke leaves are reported in the panel A of Figure 2.2. As shown, protein bands occurring in the Viscozyme-treated sample exhibited higher electrophoretic mobility.

Table 2.1 - Purification of soluble clotting activity occurring in the homogenate supernatants derived from artichoke leaves pretreated without (-) and with (+) carbohydrolytic enzymes (Viscozyme)

	Proteins (mg)		Total activity (U)		Specific activity (U/mg)		Yield (%)		Purification (fold)	
	-	+	-	+	-	+	-	+	-	+
Homogenate supernatant	463	797	9.26	106.11	0.020	0.133	100	100	1	1
20-50 % $(NH_4)_2SO_4$	81.4	121.5	0.086	64.32	0.001	0.529	0.93	60.6	0.05	3.9
Anion exchange Chromatography	n.a.	6.5	n.a.	4.09	n.a.	0.63	n.a.	3.85	n.a.	4.7

n.a.*:* not analyzed.

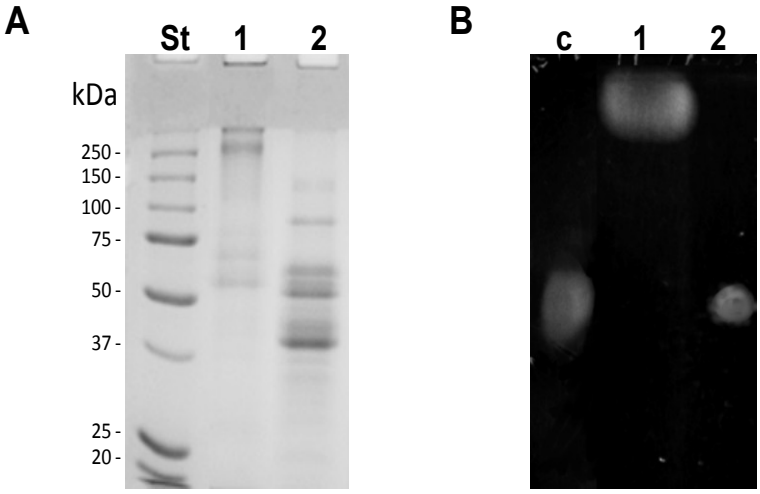

Figure 2.2 – Effect of pretreatment with Viscozyme on protein content and milk-clotting activity of supernatant of artichoke leaf homogenates. Homogenate supernatants (20 µL) obtained without (lane 1) and with (lane 2) leaf pretreatment with Viscozyme were analysed by SDS-PAGE 12% (A) and caseogram (B). In the latter, 0.1 µg commercial rennet from *Mucor miehei* were used as positive control (lane c). St, molecular weight standards (Biorad).

These results could be easily explained by considering that the long chain polysaccharides occurring in the cell wall were solubilized in the homogenate supernatant obtained from Viscozyme-untreates establishing chemical-physical interactions with proteins that remained entrapped in a high molecular weight complexes. Some of these macromolecular complexes were unable to enter into the gel, whereas some others migrated only in the upper part of the polyacrylamide gel (panel A of Figure 2.2, lane 1). On the contrary, the Viscozyme-catalyzed polysaccharide hydrolysis by hindering polysaccharide-protein assembly allowed the proteins to freely migrate according to their single molecular weights (panel A of Figure 2.2, lane 2). The effects of leaf Viscozyme pretreatment were also observed in caseogram in which the clotting signal appeared linked to a protein with high

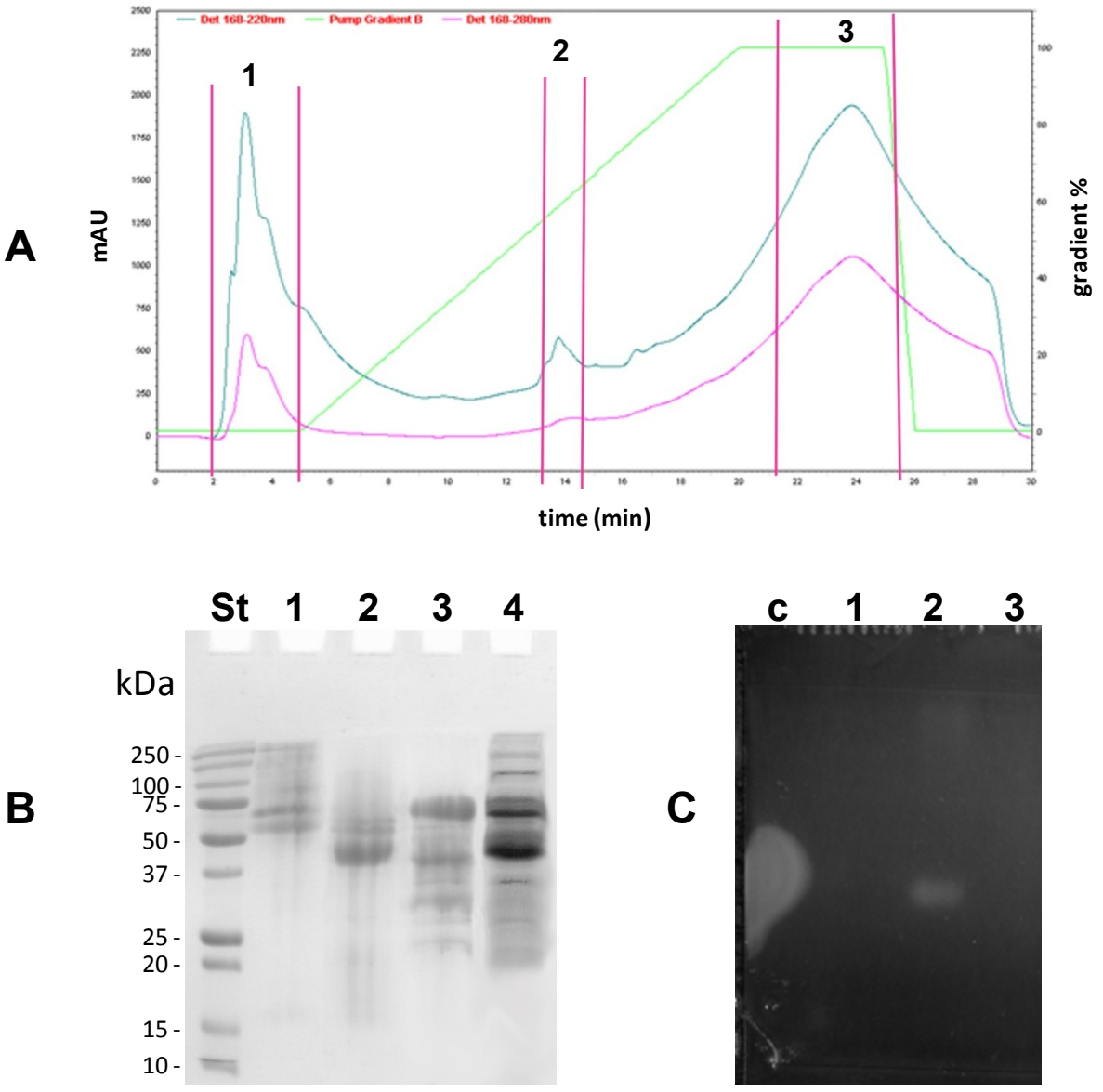

Figure 2.3 –Anion exchange chromatographic pattern (A) of 20-50% ammonium sulfate precipitate, and relative SDS-PAGE (B) and caseogram (C) of the eluted fractions
20-50% ammonium sulfate precipitated proteins (1 mg), derived from the homogenate supernatant of Viscozyme-treated artichoke leaves, were applied to an anion-exchange column equilibrated at pH 9.0 and eluted with a 0-0.5M NaCl gradient (A). The numbered peaks were manually collected and analyzed by SDS-PAGE (20 µL) (B) and caseogram (C). Numbers on lanes correspond to peak numbers; 20 µg of 20-50% fraction were analysed as control (lane 4). St, molecular weight standards (Biorad). Commercial rennet from *Mucor miehei* (0.1 µg) was used as positive control (lane c) in the caseogram.

electrophoretic mobility (panel B of Figure 2.2, lane 2) compared to the Viscozyme-untreated sample (lane 1), suggesting that the protein responsible for the clotting activity was one of the protein entrapped in the macromolecular polysaccharide-protein complexes.
Afterwards, a partial purification of homogenate supernatant of artichoke leaves of the protein endowed with clotting activity was carried out subjecting both Viscozyme-untreated and -treated samples to fractional precipitation by ammonium sulfate (0-20%; 20-50%; 50-80%) addition. The three protein fractions, collected at different ammonium sulfate saturations, were then dialyzed and assayed for clotting activity. The obtained results indicated that only the 20-50% ammonium sulfate fraction was found to be active by examining both Viscozyme-untreated and treated samples. However, the recovery of enzyme activity for Viscozyme-untreated samples resulted extremely low (0.93 %), while for the Viscozyme-treated samples a yield of about 61% was obtained (Table 2.1). Therefore, protein purification was continued by using Viscozyme-treated sample.
A further attempt of purification was performed by subjecting the 20-50% ammonium sulfate fraction to ion-exchange chromatography. In particular, on the basis of 2D analysis that revealed the presence of different protein species mainly distributed around an isoelectric point of pH 5.0 and in a range of pH 7.5-9.0 it was decided to use an anionic column equilibrated at pH 9.0. The elution pattern recorded during the chromatographic separation is reported in the panel A of Figure 2.3. The three main peaks were collected and used for both electrophoretic and caseogram analysis (panel B and C of Figure 2.3, respectively). The latter highlighted that the clotting activity was present only in the peak number 2 (panel C of Figure 2.3, lane 2) which was eluted around 0.3 M NaCl. SDS-PAGE analysis of this chromatographic fraction revealed three bands with molecular weight between 70 and 37 kDa with a major band (corresponding to the functional protein) having a molecular weight of around 45 kDa (panel B of Figure 2.3, lane 2).
However, by considering the summary of the purification data reported in Table 2.1, this step seemed to be useful by comparing the high amount of contaminant proteins eliminated, even though it involves an unsatisfactory loss of the clotting activity probably due to a very low enzyme stability. Therefore, it was reasonable to continue the characterization studies of the protein endowed with clotting activity by using the 20-50% ammonium sulfate fraction. Since several authors state that plant proteases from *Cynara scolymus* flowers belong to the family of aspartic proteases (Sidrach *et al*., 2005; Chazarra *et al*., 2007; Oliveira *et al*., 2010), a spectrophotometric assays to follow the clotting activity of 20-50% ammonium sulfate precipitate pre-incubated in the absence or presence of pestatin A, a specific inhibitor of aspartic proteases, was carried out. The obtained results show that the clotting activity of the sample was completely inhibited when pepstatin A occurred in the reaction mixture (Figure 2.4, blue line), thus indicating that also the leaves of *Cynara scolymus* contain a protein with aspartic protease activity. Same results were obtained when protease from *Mucor miehei*, a known aspartic protease used as control, was assayed in the presence of pepstatin A (Figure 2.4, green line).
In conclusion, by taking into consideration the marked enzyme instability of the aspartic protease isolated from *Cynara scolymus* leaves, an additional possible source, obtainable at low cost, of vegetable rennet of the Asteraceae family (*Carduus defloratus subsp. carlinifolius* (Lam.) flowers, commonly known as alpine thistle) was investigated.

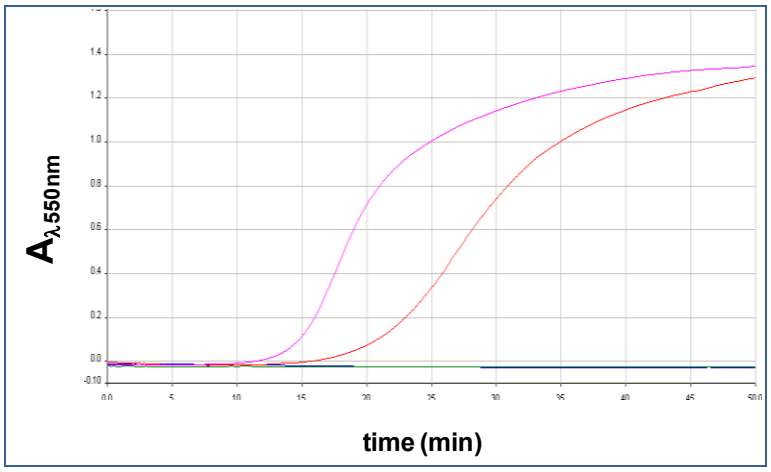

Figure 2.4 – Spectrophotometric clotting activity assays performed in the absence or presence of pepstatin A. Kinetic determination of clotting activity was performed at 25°C by using k-casein as substrate and 20-50% ammonium sulfate precipitate derived from homogenate supernatant of Viscozyme-treated artichoke leaves (0.05 U) preincubated without (red) and with (blue) pepstatin A (1 μM). Protease (0.05 U) from *Mucor miehei* preincubated without (violet) and with (green) pepstatin A (1 μM) was used as control. Further details are available under Methods.

2.1.3.2 *Carduus defloratus* subsp. *carlinifolius*, Lam.

Therefore, the other aim of this study was to explore the presence of rennet-like proteases in alpine thistle (*Carduus defloratus carlinifolius*) flowers, a "wildflower" systematically very close to *Cynara* species. For this purpose, crude protein extracts were obtained from fresh flowers after homogenization in acetate buffer at pH 5.0, with a recovery of about 0.96 mg of protein/g of fresh flowers (Table 2.2).

Table 2.2 - Purification of soluble clotting activity from alpine thistle flowers (80 g)

	Proteins (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Homogenate supernatant	76.81	320.24	4.17	100	1
20-50 % $(NH_4)_2SO_4$	25.59	196.78	7.69	61.44	1.8

Flower extracts were subjected to fractional precipitation by adding ammonium sulfate (0-20%; 20-50%; 50-80%) with the aim to partially purify the enzymatic activity and to remove some low molecular weight contaminants, such as phenolic compounds (Barros *et al.*, 2001; Vairo-Cavalli *et al.*, 2005). Protein fractions, collected at different ammonium sulfate saturations, were analyzed by SDS-PAGE and assayed for clotting activity (Figure 2.5). Lane 1 in the panel A of Figure 2.5 shows the electrophoretic profile of the proteins contained in the crude extract of

alpine thistle flower. These proteins were successfully fractionated with different percentages of saturation of ammonium sulfate (panel A of Figure 2.5, lanes 2-4). Moreover, the parallel caseogram, reported in panel B of Figure 2.5, demonstrated a marked clotting activity both in the crude extract (lane 1) and in the 20-50% ammonium sulfate fraction (lane 3). Since the electrophoretic profile of the latter fraction shows only two major bands between 37 and 25 kDa an one at 15 kDa, it is possible to assume that, mostly probably, the enzymatic clotting activity could be attributed to a protein with a molecular weight of about 35 kDa. This hypothesis is supported by the data in the literature concerning the protein "cardosin" and "cynarase" isolated from *Cynara cardunculus* and *Cynara scolymus* flowers, two aspartic proteases with similar molecular weight and endowed with milk–clotting activity (Verissimo *et al*., 1995; Silva *et al*., 2003).

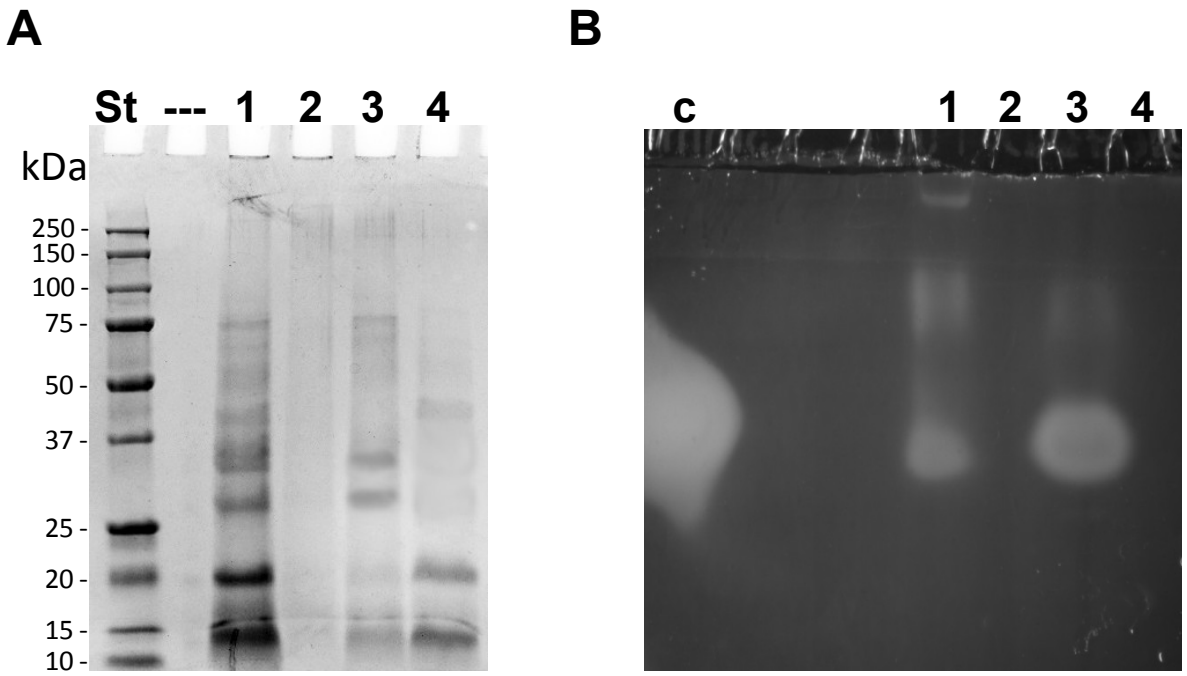

Figure 2.5 - SDS-PAGE (A) and caseogram (B) of ammonium sulfate fractions obtained from crude extract of alpine thistle flowers.
Crude extract (9.2 µg, lanes 1) and ammonium sulfate 0-20% (lanes 2), 20-50% (lanes 3) and 50-80% (lanes 4) fractions (20 µl each) were analyzed by 12 % SDS-PAGE (A) and caseogram (B). St, molecular weight standards (Biorad). Commercial rennet from *Mucor miehei* (0.1 µg) was used as positive control (lane c).

By analyzing the purification reports of the enzyme isolated from artichoke leaves (Table 2.1) and the ones of the enzyme isolated from alpine thistle flowers (Table 2.2), it is possible to conclude that even though the protein amount extracted/g of alpine thistle fresh flowers is lower (from 10- to 15- fold) if compared to that obtained from the artichoke leaves, however, the specific enzyme activity values were much higher (from 30- to 200-fold) when alpine thistle extract was assayed. Moreover, further purification step by ammonium sulfate fractionation allowed to obtain a sample (20-50% ammonium sulfate) with a double enzyme specific activity value, by

removing almost 70% of initial proteins and with a satisfactory recovery of the total enzymatic activity (more than 60%).
As already described for the artichoke leaf protease, also for the characterization of the alpine thistle enzyme, clotting assays were conducted in the presence of pepstatin A, a specific aspartic protease inhibitor. The obtained results showed a complete inhibition of clotting activity contained both in the alpine thistle flower crude extract and in the 20-50% ammonium sulfate derived fraction (Figure 2.6), thus suggesting that, also in this case, we were dealing with an aspartic protease.

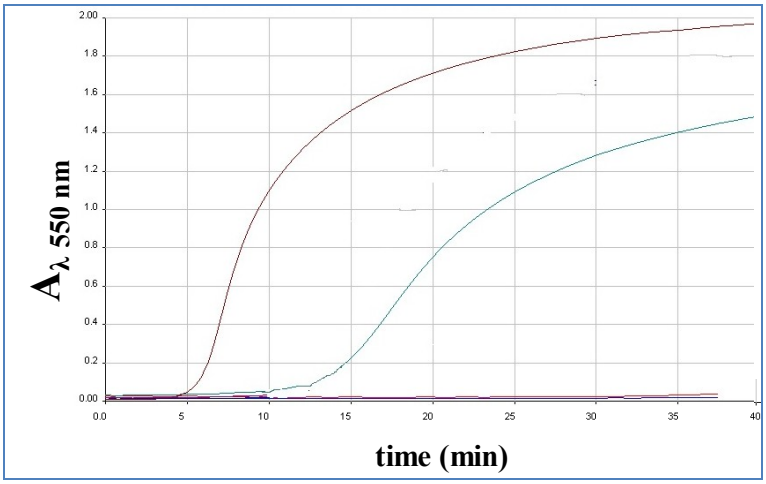

Figure 2.6 – Spectrophotometric clotting activity assays performed in the absence or presence of pepstatin A. Kinetic determination of clotting activity was performed at 25°C by using k-casein as substrate and 20-50% ammonium sulfate precipitate derived from homogenate supernatant of alpine thistle flowers (0.05 U) preincubated without (brown) and with (blue) pepstatin A (1 μM). Protease (0.05 U) from *Mucor miehei* preincubated without (green) and with (red) pepstatin A (1 μM) was used as control. Further details are available under Methods.

2.1.4 Conclusions

Numerous attempts have been made to replace calf rennet because of limited supply and increasingly high prices. Therefore, there is a renewed interest to identify milk-clotting enzymes from novel plant sources. In this work we investigated on the presence of “vegetable rennet” in *Cynara scolymus* leaves and in *Carduus defloratus* flowers. The obtained results showed that both plants contain aspartic proteases with milk-clotting activity. The enzyme occurring in the artichoke leaf extracts was found to exhibit a low specific activity and to be markedly unstable. Conversely, *Carduus defloratus* flowers revealed an advantageous plant source in terms of enzyme high specific activity and stability. It is possible to conclude that the extract of *Carduus defloratus* flowers may represent a promising candidate for further studies on clotting enzyme immobilization for possible inline milk coagulation in cheese making.

2.1.5 References

- **Ahmed, I.A.M., Morishima, I., Babiker, E.E., Mori, N.** (2009). Characterisation of partially purified milk-clotting enzyme from Solanum dubium Fresen seeds. *Food Chem.,* 116, 395-400.
- **Ansharullah, A., Hourigan, J.A., Chesterman, C.F.** (1997). Application of carbohydrases in extracting protein from rice bran. *J. Sci. Food Agric.,* 74, 141-146.
- **Barros, R., Ferreira, C.A., Silva, S.V., Malcata, F.X.** (2001). Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate enzyme. *Microb. Technol.,* 29, 541-547.
- **Cabezas, L., Esteban, M.A., Marcos, A.** (1981). Agar gel diffusion of *Cynara humilis* L. proteinases and other enzymes. *Alimentaria,* 128, 17-22.
- **Cattaneo, T.M.P., Nigro, F., Messina, G., Giangiacomo M.** (1994). Effect of an enzymatic complex from pineapple pulp on the primary clotting phase. *Milchwissenschaft,* 49, 269-272.
- **Chazarra, S., Sidrach, L., Lopez-Molina, D., & Rodrıguez-Lopez, J. N.** (2007). Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus* L.) flowers. *Int. Dairy J.,* 17, 1393-1400.
- **Chen, S., Zhao, J., Agboola, S.,** (2003). Isolation and partial characterization of rennet-like proteases from Australian cardoon (*Cynara cardunculus* L.). *J. Agric. Food Chem.,* 51, 3127-3134.
- **Egito, A.S., Girardet, J.M., Laguna, L.E., Poirson, C., Molle, D., Miclo, L., Humbert, G., Gaillard, J.L.** (2007). Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine k casein. *Int. Dairy J.,* 17, 816-825.
- **El-Shibiny, S., Abd El-Salam, H.M., Rifaat, I.D., Fahmi, H.A.** (1973). Studies on milk clotting enzyme from plant source. II Separation of milk clotting enzymes from latex of *Ficus carica* var. Soltani. *J. Food Sci. Tech.,* 5, 30-34.
- **FAO Statistical Database**, 2011: http://www.faostat.org/.
- **Faro, C.J., Moir, A.J.G., Pires, E.V.** (1992). Specificity of a milk clotting enzyme extracted from the thistle Cynara cardunculus L.: action on oxidized insulin and k-casein. *Biotechnol. Lett.,* 14, 841-846.
- **Foltman, B., Szecsi, P.B., Tarasova, N.I.** (1985). Detection of proteases by clotting of casein after gel electrophoresis. *Anal. Biochem.,* 146, 353-360.
- **Guiama, V.D., Libouga, D.G., Ngah, E., Mbofung, C.M.** (2010). Milk clotting activity of berriers extracts from nine Solanum plants. *Afr J Biotechnol.,* 9, 3911-3918.
- **Gupta, B.C., Eskin, M.A.N.** (1977). Potential use of vegetable rennet in the production of cheese. *Food Tech.,* 31, 62-65.
- **Heimgartner, U., Pietrazak, M., Greetsen, R., Brodelius, P., Figueredo, M.A., C. Figueiredo, C.** (1990). Purification and partial characterization of milk clotting protease's from flowers of *Cynara cardunculus. Phytochemistry*, 29, 1405-1410.
- **Laemmli, U.K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227, 680-685.
- **Llorente, B:E., Brutti, C.B., Caffini, N.O.** (2004). Purification and characterization of milk clotting aspartic proteinase from globe artichoke (*Cynara scolymus* L.). *J. Agric. Food Chem.,* 52, 8182-8189.

- **Lourenço, P.M., de Castro, S., Martins, T.M., Clemente, A., Domingos, A.** (2002). Growth and proteolytic activity of hair roots from *Centaurea calcitrapa*: effect of nitrogen and sucrose. *Enz. Microb. Technol.,* 31, 242-249.
- **Oliveira, A., Pereira, C., Soares da Costa, D., Teixeira, J., Fidalgo, F., Pereira, S.** (2010). Characterization of aspartic proteinases in C. cardunculus L. callus tissue for its prospective transformation. *Plant Scie.,* 178, 140-146.
- **Roseiro, L.B., Barbosa, M., Ames, J.M., Wilbey, A.** (2003). Cheesemaking with vegetable coagulants—The use of Cynara L. for the production of ovine milk cheese. *Int. J. of Dairy Tech.,* 56, 76-85.
- **Sidrach, L., Garcıa-Canovas, F., Tudela, J., Rodrıguez-Lopez, J.N.** (2005). Purification of cynarases from artichoke (*Cynara scolymus* L.): Enzymatic properties of cynarase A. *Phytochemistry,* 66, 41-49.
- **Silva, S.V., Allmere, T., Malcata, F.X., Andrén, A.** (2003). Comparative studies on the gelling properties of cardosins extracted from *Cynara cardunculus* and chymosin on cow skim milk. *Int. Dairy J.,* 13, 559-564.
- **Tang, S., Hettiarachchy, N.S., Eswaranandam, S., Crandall., P. (2003).** Protein Extraction from Heat-stabilized Defatted Rice Bran: II. The Role of Amylase, Celluclast, and Viscozyme. *J. of Food Scie.,* 68, 471-475.
- **Vairo-Cavalli, S., Claver, S., Priolo, N.** (2005). Extraction and partial characterization of a coagulant preparation from Silybum marianum flowers. Its action on bovine caseinate. *J. of dairy,* 72, 271-275.
- **Verissimo, P., Esteves, C., Faro, C., Pires, E.** (1995). The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnol. Lett.,* 17, 621-626.
- **Wang, M., Hettiarachchy, N.S., Qi, M., Burks, W., Siebenmorgen, T.** (1999). Preparation and functional properties of rice bran protein isolate. *J. Agri. and Food Chem.,* 47, 411-416.

2.2 Immobilization of aspartic proteases with milk-clotting activity from *Mucor miehei* and *Carduus defloratus L. subsp. carlinifolius*

2.2.1 Introduction

Enzymes are becoming increasingly important in industrial processes due to their ability to work at milder pH, temperature and pressure conditions, limited by-product formation, high activity, and great selectivity. The growth of interest for possible industrial enzyme application is mainly driven by a general need of environmentally friendly technologies and sustainable production methods (Krajewska, 2004; Iyer *et al*., 2008). Despite all the advantages, the successful implementation of enzymes is not always straightforward, because a lot of issues arise during process standardization. One of the major problems during the technological transfer of an enzyme-catalyzed procedure is the frequent either lack or decrease of enzyme activity due to extreme temperature and pH, stress conditions, or presence of specific salts or surfactants (Polizzi *et al*., 2007; Iyer *et al*., 2008). Since the recovery yield and reusability of free enzymes as industrial catalysts are quite limited, attention has been directed to immobilized enzymes, which offers advantages over soluble native or modified ones (Tischer *et al*., 1999). In fact, insolubility of immobilized enzymes allows: (i) continuity in the enzymatic processes, (ii) controlled product formation, (iii) rapid stop of the reaction, (iv) easy enzyme separation from reaction mixture and its recycle, (v) improved enzyme stability, and (vi) great variety of engineering designs (Cao *et al*., 2003). Immobilized enzymes are defined as *"enzymes physically confined or localized in a certain defined region of space with retention of their activities, which can be used repeatedly and continuously"* (Makkar, 1982). Various types of immobilization techniques are available. However, the selection of carriers is highly dependent on the purpose, type of biocatalyst, reaction and reactor. Methods of enzyme immobilization can be classified into four main categories which are adsorption, covalent linkage, encapsulation and entrapment (Mateo *et al*., 2007; Brady *et al*., 2009).

Immobilized enzymes are used mostly in food technology, pharmaceutical industry and biotechnology. Traditionally, cheese manufacturing is a batch process and current practice is to use milk-clotting enzymes in a soluble form. The worldwide increase in cheese production and consumption, together with the reduced supply and consequent price increase of calf rennet, has led to try to immobilize milk-clotting activities to allow enzyme reutilization in the cheese manufacturing procedures (Garg *et al*., 1993; Shah *et al.,* 1995).

The aim of this work was to immobilize two aspartic proteases having milk clotting activity. One was commercially available from the fungal organism *Mucor miehei*, and is the enzyme widely used in cheese-making; the other was extracted from *Carduus defloratus subsp. Carlinifolius* flowers, a new source of vegetable rennet as reported in the paragraph 2.1.3.2. Therefore, the objective was to improve the stability and to reuse these enzymes following their immobilization on an inert support. Moreover, as far as vegetable proteases are concerned, it is well known that "plant rennets" are generally characterized by strong proteolytic activity leading to the bitter cheese production. The immobilization of these enzymes could allow to overcome this drawback since the immobilized enzyme is removed after the reaction and is not incorporated into the cheese curd matrix. In addition, it is also possible to control the enzyme proteolytic activity during the entire process and cheese ripening.

For the immobilization of both enzymes a polyacrylic support, containing polar epoxy groups, was used as it is economic and technically friendly in both laboratory and industrial scale. Epoxy supports are able to react with different nucleophilic groups occurring on the protein surface (i.e., amino-, hydroxyl-, or thiol- moieties) to form covalent linkages (Mateo *et al*., 2000). The process parameters affecting the immobilization system were studied to evaluate its potential as a possible candidate for future industrial applications.

2.2.2 Materials and Methods

2.2.2.1 Materials

Rennet from *Mucor miehei* (10 U/μg) was purchased in a local market. Flowers of *Carduus defloratus* L. *subsp. carlinifolius,* Lam. were harvested in Gallicchio (PZ), a municipality of National Park "Appennino Lucano Val d'Agri Lagonegrese" (Basilicata, Italy). The Immobead IB-150P beads (particle size 150-500 μm) were obtained from ChiralVision (Netherlands). Comassie Protein Reagent was purchased from Thermo Scientific (USA). All chemicals and reagents of analytic grade were purchased from Sigma-Aldrich (Milan, Italy).

2.2.2.2 Methods

2.2.2.2.1 Enzyme purification

Fresh thistle flowers were homogenized in 0.1 M sodium acetate, pH 5 (1:3 w/v) with an Ultra-Turrax (IKA-WERKE, Germany) according to modified method of Chen *et al*. (2003). The homogenate was centrifuged at 30,000xg for 30 min at 4°C and the supernatant was collected and filtered through Whatman n. 1 filter paper. Then, the crude extract was brought to two different ammonium sulphate saturations (0-20% and 20-50%) by slowly adding the appropriate amount of solid ammonium sulphate during continuous stirring at 4°C. The precipitates were collected by centrifugation at 30.000xg for 30 minutes at 4°C. The 20-50% sediment was preserved and redissolved in 0.1 M sodium acetate buffer, pH 5 (10% of the starting volume). The sample was then dialyzed 3 times for 6 hours against 100 volumes of the same buffer. Protein concentration and enzyme specific activity were finally determined.

2.2.2.2.2 Immobilization procedure

An aliquot of either rennet from *Mucor miehei* or dialyzed 20-50% ammonium sulfate thistle flower sample were added to 100 mg of dry Immobead IB-150P beads, in the optimum ratio of 11.2 U and 16 U, suspended in 0.4 mL final volume of 0.1 M phosphate buffer at different pH (5.0, 6.0 or 7.0). The beads-enzyme mixture was shacked for 3 hours at 4°C on the rotary mixer to allow the reaction. After that, the vials were let stand overnight at 4°C in order to promote the natural decantation of the beads. Finally, the supernatants, containing the unbound enzyme molecules, were removed from the sedimented beads and used to determine both protein content and clotting activity. The sedimented beads were washed four times with 1 mL of the different buffers used for immobilization process as follow: first washing: 1 min, by gently mixing; second and third washing: 15 min on the rotary mixer; fourth washing: 5 min on the rotary mixer. After each washing the beads were left to settle at 4 °C and then recovered and resuspended for subsequent washing, while the

supernatants were removed and analyzed for protein content and clotting activity as well as the collected beads after the last washing.

2.2.2.2.3 Determination of protein concentration

Protein amount was determined by using Coomassie protein assay kit through Bradford assay in microtiter plate using BSA as protein standard.

2.2.2.2.4 Clotting activity assay

For quantitative measurements of the enzymatic clotting activity a spectrophotometric assay was performed, according with Douillard *et al*., (1970). The enzymatic source (containing either free or linked enzyme) was incubated at 25°C in 1 mL of 0.1 M phosphate buffer at different pH (5-8) containing 0.1% k-casein as substrate in a disposable spectrophotometer cuvette (pathlength 1 cm). Suitable controls at the respective pH values were also run including the buffered substrate without enzyme. Reaction rates were measured by following the increase of absorbance at 550 nm as function of time (min) until the kinetic sigmoid curves reached the plateau (Infinite 200 PRO spectrophotometer, Tecan, Switzerland). The recorded kinetic curves were processed with SigmaPlot software in order to evaluate the clotting time which mathematically corresponded to the abscissa value of the first minimum of the second derivative obtained from sigmoid curve. The clotting activity was calculated as the inverse of the clotting time (min^{-1}). One enzyme unit (U) was defined as the amount of enzyme able to produce 0.2 min^{-1} of clotting activity in the above described conditions. In all experiments the highest measured enzymatic activity was taken as reference (100% of clotting activity). Each result represented the average values of three independent estimations.

2.2.2.2.5 Determination of immobilized activity

The amount of enzyme that was immobilized on the beads was determined by measuring the total residual enzyme activity that remains in the enzyme solution after immobilization (Sur) and into the four washes (W1, W2, W3 and W4) and by subtracting this activity from the total starting activity. More in detail, the following parameters were determined:

Immobilized activity = starting activity – activity in the supernatant

Yield (%)= (immobilized activity/starting activity)*100

Efficiency (%)= (observed activity/immobilized activity)*100

Activity recovery (%)= (observed activity/starting activity)*100

2.2.2.2.6 Reusability of the immobilized enzymes

The reusability of the immobilized enzymes was monitored for 17 days by incubating 180 mg of enzyme containing wet beads (corresponding to 100 mg of starting dry beads), at 4°C in 0.4 mL of 0.1 M phosphate buffer, pH 7. At 1, 2, 3, 6 and 17 days the buffer was discarded and the beads were subjected to the clotting activity assay as above described (2.2.2.2.4 section). After each assay the beads were washed with 1.0 mL of 0.1 M phosphate buffer, pH 7, and incubated at 4°C resuspended in 0.4 mL of the same buffer until the subsequent assay. Each result represented the average values of three independent estimations.

2.2.2.2.7 Statistical analysis

Microsoft Excel and SigmaPlot software were used for data processing and validation.

2.2.3 Results and Discussion

2.2.3.1 *Mucor miehei* protease immobilization

A commercial protease from *Mucor miehei* rennet was used as model enzyme to investigate the immobilization process on polyacrylic support containing polar epoxy groups. The degree of immobilization and the retained activity are important parameters in the development of an industrial process that uses immobilized enzymes. The effect of different pH values, reaction times and enzyme concentrations are some of the key points to optimize an immobilization protocol.

With the aim to understand the stability of the enzyme under the immobilization experimental conditions, we have assayed the functionality of the free enzyme at different pH values (5-8) before and after storage for 16 h (predicted immobilization time) at 4°C in 0.1 M phosphate buffer at different pH values (5-8). The obtained data revealed that the optimum pH for the functionality of the aspartic protease from *Mucor miehei* was about 7, value at which the highest enzymatic activity was recorded both before and after enzyme storage for 16 h (Figure 2.7). In fact, we found that at pH 8 and pH 6 the enzyme exhibited 70.5 and 92.8% of its clotting activity, respectively, whereas at pH 5 a marked loss of enzyme activity was observed (more than 40%). It should be noted that when the enzyme was stored at different pH values and then assayed at its optimal pH (pH 7.0) no significant change in its activity was detectable.

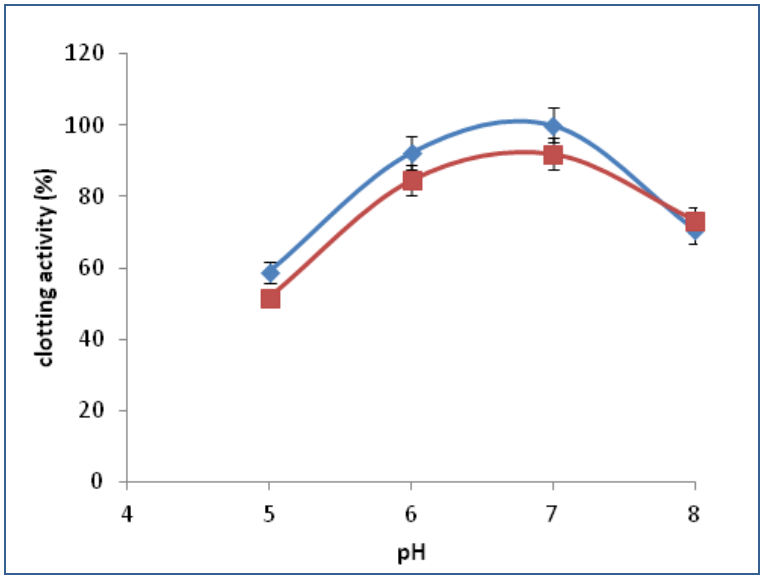

Figure 2.7 – Effect of pH on aspartic protease from *Mucor miehei*. Free enzyme was assayed for clotting activity at specific pH values before (blue curve) and after (red curve) storage for 16 h at 4°C at the corresponding pH. Further details are available in 2.2.2.2.4 section.

These data are in agreement with the ones recently reported by Celebi *et al*. (2014), showing a reduction of clotting activity of *Mucor miehei* rennet decreasing the reaction pH from 7 to 5.

It should be highlighted that the storage of the free enzyme at the different pH values, and for the time predicted for its immobilization (16 h at 4°C), did not produce any reduction in its clotting activity, this result representing a promising assumption for the subsequent scheduled experiments.
Once established, with the above described preliminary study, the pH range of functionality of *Mucor miehei* protease, we proceeded with the immobilization of the enzyme by mixing the free enzyme with dry Immobead IB-150P beads (experimental details are reported in 2.2.2.2.2 section). Enzyme immobilization, as known, is influenced by different factors. One of the most important is the pH during the immobilization process. Therefore, the first part of the work has been addressed to the determination of the pH at which the highest amount of immobilized protein, as well as highest enzyme activity, was obtained. Immobilization experiments were carried out at three different pHs (5.0, 6.0 and 7.0) and both proteins and clotting activity were calculated at each pH value in both reaction mixture supernatants and washing solutions applied to remove the unbound proteins. As it is possible to observe, neither protein nor clotting activity were detectable after the fourth washing (Table 2.3). In addition, the data reported in the Table indicate that the maximal protein-beads binding occurred at pH 7.

Table 2.3 – Protein content and enzyme clotting activity (ECA) occurring in the supernatant of *Mucor miehei* rennet immobilization reaction mixtures (Sur) performed at different pHs and in the subsequent four washing solutions of the beads (W1, W2, W3 and W4). N.D., not detectable.

	Sur		W1		W2		W3		W4	
	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)
pH 5	0.0310	0.280	0.0420	0.360	0.0394	0.325	0.0115	0.091	N.D.	N.D.
pH 6	0.0230	0.210	0.0450	0.410	0.0450	0.391	0.0018	0.038	N.D.	N.D.
pH 7	0.0278	0.298	0.0285	0.305	0.0255	0.285	0.0093	0.103	N.D.	N.D.

Conversely, no difference was observed among the yields of the enzyme clotting activity immobilized ("expected") at the three different pH values (Table 2.4). However, the "observed" immobilized clotting activity, measured as both efficiency and recovery of the procedure, resulted significantly higher when protein immobilization was carried out at pH 6.
To understand whether the immobilized enzyme exhibited a different pH optimum with respect to the unbound enzyme, the clotting activity was assayed at 25°C in a pH range between 5.0–8.0. In fact, it is well known that immobilization may alter enzyme optimal pH value (Yang et al. 2004; Godjevargova et al., 2000), this effect being the consequence of the involvement of reactive amino acid residues of the protein in the covalent binding to the immobilization support.
The obtained results (data not shown) indicated a curve of enzymatic activity very similar to the one obtained by using free *Mucor miehei* rennet (Figure 2.7, blue curve). Similar behavior was described for other immobilized enzymes such as α-amylase (He *et al*., 2000; Rauf *et al*., 2006).

Table 2.4 – Yield, efficiency and enzyme clotting activity (ECA) recovery after rennet *Mucor miehei* immobilization at different pHs.

	ECA (U)			Yield (%)	Efficiency (%)	Recovery (%)
	Unbound	Immobilized				
		expected	observed			
pH 5	1.06 ± 0.32	10.14 ± 0.22	6.56 ± 0.11	90.6	64.6	58.6
pH 6	1.05 ± 0.43	10.15 ± 0.29	9.88 ± 0.06	90.6	97.3	88.2
pH 7	0.99 ± 0.31	10.20± 0.22	8.89 ± 0.18	91.1	87.1	79.4

One of the most important characteristics of the immobilized enzymes are their stability during storage for their possible reutilization. A loss of functionality of covalently immobilized enzymes is due to the reactions occurring during their immobilization at different pHs. In fact it is known that epoxy group react with different nucleophilic groups present on the protein surface under different pH conditions. At slightly acidic pH the carboxylic groups reacted with the epoxy group to form ester bond (Mateo *et al.,* 2007). Conversely, at neutral or slightly alkaline conditions, epoxy group reacted manly with the thiol groups and then with the amino groups (Cerdobbel *et al.,* 2010b). This different reactivity at different pHs may affect the number of covalent bonds between protein and beads consequently increasing the rigidity and reducing the catalytic activity of the enzyme (Mateo *et al.,* 2003).
The reusability of *Mucor miehei* rennet immobilized at different pHs was monitored for 6 days by storing at 4°C 180 mg of enzyme containing wet beads (corresponding to 100 mg of starting dry beads) in 0.4 mL of 0.1 M phosphate buffer at pH 7, to reduce the multipoint covalent attachment of protein nucleophilic reactive residues. In fact, it has been reported that the covalent reaction between proteins and epoxy support is extremely slow at neutral pH (Mateo *et al*., 2000; Torres et al., 2003).

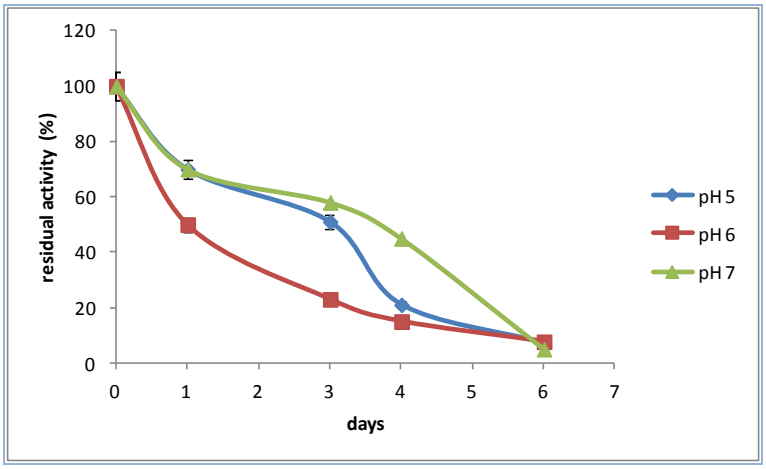

Figure 2.8 - Stability of immobilized *Mucor miehei* rennet during storage. Enzyme immobilized at different pH (5, 6 and 7) was incubated at 4°C and pH 7 for 6 days and monitored for its activity by following spectrophotometrically k-casein (1%) clotting. Further details are available in 2.2.2.2.5 section.

Figure 2.8 shows that, independently on the pH used for its immobilization, the enzyme exhibited low stability during storage at 4°C and pH 7.0. In particular, we observed a 65% loss of clotting activity after two days of storage of the enzyme previously immobilized at pH 6, while a loss of only 35% was observed by assaying the enzyme immobilized at pH 5 or 7 at the same time of storage. Moreover, when the enzyme was immobilized at pH 7 only 50% of its activity was lost after four days of storage, whereas the enzyme immobilized at both pH 5 and 6 lost almost 80% of their activity. Finally, all the immobilized enzymes resulted completely inactive after six days of storage. All these findings suggest a low stability of the immobilized *Mucor miehei* rennet under the experimental conditions used, thus suggesting that further experiments are needed to obtain a prolonged reusability of such enzyme.

2.2.3.2 *Carduus defloratus* protease immobilization

Partially purified *Carduus defloratus* protease was prepared as previously described under 2.2.2.2.1 paragraph. For the immobilization of this enzyme we followed the same experimental procedure previously developed for the *Mucor miehei* rennet. Thus, we performed a preliminary study to evaluate both enzyme optimal pH and the best stability conditions during storage at different pH values (5, 6 and 7) for 16 h at 4°C. We found that alpine thistle flower enzyme showed the highest level of activity at pH 5, value that was used as reference (100% of clotting activity). Figure 2.9 shows that the clotting activity decreased by increasing the pH, reaching a value of almost 40% of the control at pH 7 (blue curve). In parallel, the curve obtained with samples pre-incubated at the corresponding pH showed a very similar trend by exhibiting about 90% of the activity detected with control samples (Figure 2.10, red curve). It should be noted that when the enzyme was stored at different pH values and then assayed at its optimal pH (pH 5.0) no significant change in its activity was detectable.

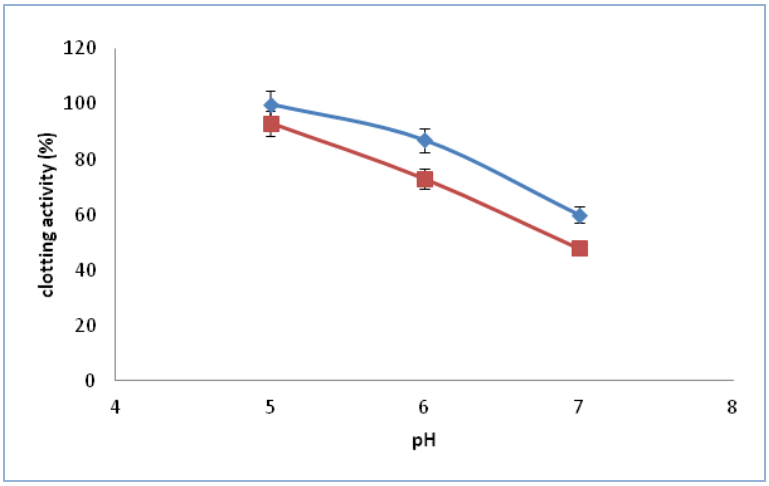

Figure 2.9 - **Effect of pH on aspartic protease from *Carduus defloratus* flowers.** Free enzyme was assayed for clotting activity at specific pH values before (blue curve) and after (red curve) storage for 16 h at 4°C at the corresponding pH. Further details are available in 2.2.2.2.4 section.

Also the immobilization of the protease from *Carduus defloratus* was carried out by using polyacrylic beads containing polar epoxide groups. Free enzyme was incubated at three different pH values (5.0, 6.0 and 7.0) with dry Immobead IB-150P

beads, as described in 2.2.2.2.2 section, and both protein content and clotting activity were evaluated at each pH value in both reaction mixture supernatants and washing solutions applied to remove the unbound proteins. Table 2.5 shows that neither protein nor clotting activity were detectable after the fourth washing. In addition, the data reported in the Table 2.5 indicate that the maximal protein-beads binding (80%) occurred at pH 5, the same value at which the highest yields (82.5%) of the enzyme clotting activity immobilized ("expected") was observed (Table 2.6).

Table 2.5 – Protein content and enzyme clotting activity (ECA) occurring in the supernatant of *Carduus defloratus* flower immobilization reaction mixtures (Sur) performed at different pHs and in the subsequent four washing solutions of the beads (W1, W2, W3 and W4). N.D., not detectable.

	Sur		W1		W2		W3		W4	
	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)	Protein (mg)	ECA (U)
pH 5	0.160	0.12	0.105	0.81	0.045	0.65	0.015	0.09	N.D.	N.D.
pH 6	0.192	2.02	0.150	1.82	0.195	1.52	0.042	0.22	N.D.	N.D.
pH 7	0.256	2.86	0.038	0.42	0.032	0.32	0.013	0.15	N.D.	N.D.

However, the "observed" immobilized clotting activity, measured as both efficiency and recovery of the procedure, resulted significantly higher when protein immobilization was carried out at pH 7 (79.1% and 53.8%, respectively). It is worthy to note that almost all the enzyme molecules immobilized at pH 6 resulted completely inactive, thus suggesting that at this pH value the binding of the enzyme to the beads dramatically compromise its activity.

Table 2.6 – Yield, efficiency and enzyme clotting activity (ECA) recovery after *Carduus defloratus* flower protease immobilization at different pHs.

	ECA (U)			Yield (%)	Efficiency (%)	Recovery (%)
	Unbound	Immobilized				
		expected	observed			
pH 5	2.8 ± 0.9	13.2 ± 1.2	7.66 ± 0.42	82.5	58.0	47.8
pH 6	5.6 ± 1.1	10.4 ± 1.8	0.02 ± 0.01	65.0	0.2	0.1
pH 7	5.1 ± 1.0	10.9 ± 1.5	8.62 ± 0.92	68.1	79.1	53.8

The reusability of *Carduus defloratus* flower enzyme, immobilized at two different pH values (5 and 7) was monitored for 17 days by storing at 4°C 180 mg of enzyme containing wet beads (equivalent to 100 mg of starting dry beads) in 0.4 mL of 0.1 M phosphate buffer at pH 5, corresponding to the optimal pH of the enzyme. Figure 2.10 shows that the enzyme exhibited low stability during storage at pH 5. In particular, we observed a 70% loss of clotting activity after only three days of storage

of the enzyme previously immobilized at pH 5, while no loss of activity was observed by assaying the enzyme immobilized at pH 7 at the same time of storage. Moreover, when the enzyme was immobilized at pH 7 a loss of 70% of its activity was observed only after 17 days of storage. All these findings clearly suggest a markedly higher stability and potential reusability of the enzyme extracted from alpine thistle flowers with respect to the *Mucor miehei* rennet, stimulating further experiments for the application of such enzyme in a possible inline milk coagulation and cheese making.

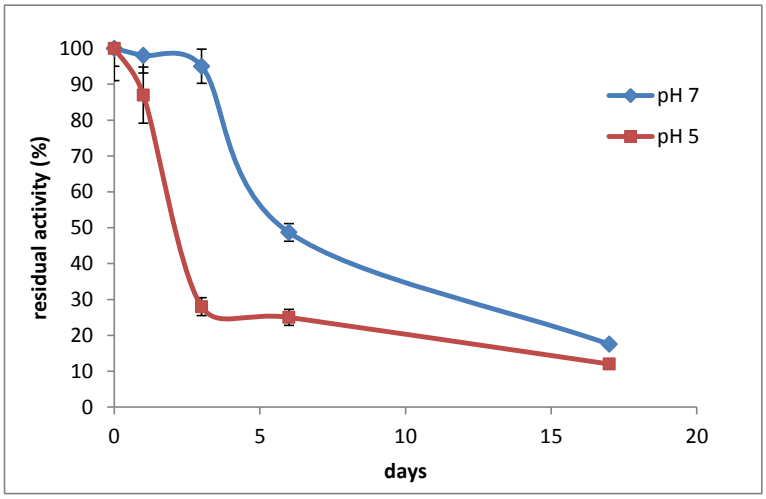

Figure 2.10 - Stability of immobilized protease from *Carduus defloratus* flowers during storage. Enzyme immobilized at different pH (5 and 7) was incubated at 4°C and pH 5 for 17 days and monitored for its activity by following spectrophotometrically k-casein (1%) clotting. Further details are available in 2.2.2.2.5 section.

2.2.4 Conclusions

Since the recovery yield and reusability of free enzymes as industrial catalysts are quite limited, there is an increasing attention toward enzyme immobilization. In this scenario protease immobilization useful for milk coagulation has receiving a renewed interest. In particular, immobilized proteases would allow to curdle the milk as a continuous process by recovering and reusing the enzymes for further coagulations. Moreover, the use of immobilized milk-clotting proteases could ensure curds only in the presamic step with respect to the batch process allowing the control of the proteolityc activity since proteases are not incorporated into the cheese.
In this work the aspartic protease from *Mucor miehei* and by *Carduus defloratus* flowers were immobilized on Immobead IB-150P beads containing reactive polar epoxy groups. Comparing the obtained results, we can conclude that *Mucor miehei* protease was highly efficiently immobilized. However, the observed low stability of the immobilized enzyme under the conditions used led to suggest that further experiments are needed to try to obtain a prolonged reusability of such enzyme. Conversely, showing the reported immobilization procedure of *Carduus defloratus* protease high efficiency as well as good yield, and exhibiting the immobilized enzyme satisfactory stability and reusability, such protease may represent a promising vegetable rennet for future industrial applications in cheese production.

2.2.5 References

- **Brady, D., Jordaan, J.** (2009). Advances in enzyme immobilization. *Biotechnol. Lett.,* 31, 1639-1650.
- **Cao, L., Langen, L.V., Sheldon, R.A.** (2003). Immobilized enzymes: carrier-bound or carrier free? *Curr. Opin. Biotechnol.,* 14, 387-394.
- **Celebi, M., Ozdemir, Z.O., Eroglu, E., Guney, I.** (2014). Statistically Defining Optimal Condition of Coagulation Time of Skim Milk. *J.Chem.Soc.Pak.,* 36, 1.
- **Cerdobbel, A., Desmet, T., De Winter, K., Maertens, J., Soetaert, W.** (2010b). Increasing the thermostability of sucrose phosphorylase by multipoint covalent immobilization. *J. Biotechnol.,* 150, 125-130.
- **Chen, S., Zhao, J., Agboola, S.,** (2003). Isolation and partial characterization of rennet-like proteases from Australian cardoon (*Cynara cardunculus* L.). *J. Agric. Food Chem.,* 51, 3127–3134.
- **Douillard, R., Ribadeau-Dumas, B.** (1970). Determination avec la casein –k de l'activete proteolytique de la pressure de la pepsine de porc et des pepsins bovines. *Bull*. *Soc. Chim. Belg.,* 52, 1429-1445.
- **Garg, S.K., Johri, B.N.** (1993). Immobilization of milk-clotting protease. *World J. Microbiol. Biotechnol.,* 9, 139-144.
- **Godjevargova, T., Konsulov, V., Dimov, A., Vasileva, N.** (2000). Behavior of glucose oxidase immobilized on ultrafiltration membranes obtained by copolymerizing acrylonitrile and N-vinylimidazol. *J. Membrane Sci.,* 172, 279-285.
- **He, D., Cai, Y., Nie, L., Yao, S.** (2000). α-Amylase immobilized on bulk acoustic-wave sensor by UV-curing coating. *Biochem. Eng. J.,* 6, 7.
- **Iyer, P.V., Ananthanarayan, L.** (2008). Enzyme stability and stabilization–aqueous and non-aqueous environment. *Process. Biochem.,* 43, 1019-1032.
- **Krajewska, B.** (2004). Application of chitin-and chitosan-based materials for **enzyme** immobilizations: A review *Enzyme Microb. Technol.,* 35, 126-139.
- **Makkar, H.P.S.** (1982). Immobilization of β-D-galactosidase from Lactobacillus bulgaricus on polyacrylamide in the presence of protective agents and properties of the immobilized enzyme. *J. of Bioscience,* 4, 153-157.
- **Mateo, C., Fernandez-Lorente, G., Abian, O., Fernandez-Lafuente, R., Guisan, J.M.** (2000). *Biomacromolecules* 1, 739-745.
- **Mateo, C., Torres, R., Fernandez-Lorente, G., Ortiz, C., Fuentes, M., Hidalgo, A., Lopez-Gallego, F., Abian, O., Palomo, J.M., Betancor, L., Pessela, B.C.C., Guisan, J.M**. (2003).Epoxy-amino groups: a new tool for improved immobilization of proteins by the epoxy method. *Biomacromolecules,* 4, 772-777.
- **Mateo, C., Palomo, J.M., Fernandez-Lorente, G., Guisan, J.M., Fernandez-Lafuente, R.** (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques *Enzyme Microb. Technol.,* 40,1451-1463.
- **Polizzi, K.M., Bommarius, A. S., Broering J.M., Chaparro-Riggers J.F.** (2007). Stability of biocatalysts. *Curr. Opin. Chem. Biol.*, **11**, 220-225.
- **Rauf, S. Ihsan, A. Akhtar, K. Ghauri, M.A. Rahman, M. Anwar, M.A. Khalid. A.M.** (2006). Glucose oxidase immobilization on a novel cellulose acetate–polymethylmethacrylate membrane. *J. of Biotech.,* 121, 351-360.
- **Shah, B., Kumar, S.R., Devi, S.** (1995). Immobilized proteolytic enzymes on resinous materials and their use in milk clotting. *Process Biochem.,* 30, 63-68.
- **Tischer, W., Kasche, V.** (1999). Immobilized enzymes: crystals or carriers?. *Trends Biotechnol.,* 17, 326-335.

- **Yang, Y.M., Wang, J.W., Tan. R.X.,** (2004). Immobilization of glucose oxidase on chitosan-SiO2 gel. *Enzyme Microb. Technol.,* 34, 126-131.
- **Torres, R., Mateo, C., Fernandez-Lorente, G., Ortiz, C., Fuentes, M., Palomo, J. M. Guisan, J. M. Fernandez-Lafuente R**. (2003). A Novel Heterofunctional Epoxy-Amino Sepabeads for a New Enzyme Immobilization Protocol: Immobilization-Stabilization o *â*-Galactosidase from *Aspergillus orza. Biotechnol. Prog.* 19, 1056-1060.

3. Conclusions

Milk enzymatic coagulation is the main step for producing cheese and represents the oldest application of enzymes as biotechnological tool in food sector. Chymosin, often called “rennet”, is the main enzyme employed in this process. In the last decade, the limited supply of natural rennet has stimulated the research to find out alternative sources of milk-clotting enzymes able to satisfactorily replace calf rennet. In this scenario various proteases have been isolated from several vegetable sources and studied for their potential application as milk-clotting enzymes.

In this thesis I report the presence of aspartic proteases in two different vegetable sources, such as artichoke (*Cynara scolymus*) leaves and alpine thistle (*Carduus defloratus sub. Carlinifolius*) flowers, in the course of studies aimed to identify novel milk-clotting enzymes. In particular, I have chosen the mentioned vegetable sources because the leaves represent a well known waste of the artichoke working process and the alpine thistle flowers a perennial herbaceous plant, very close in terms of systematic classification to *Cynara cardunculus* and *Cynara scolymus*, able to grow up on dry soils and, thus, suitable to be used in recovery of unproductive and/or intensely exploited agricultural lands.

The first phase of my experimental work was focused to optimize protein extraction from *Cynara scolymus* leaves. In fact, artichoke leaves result a quite difficult vegetable tissue during both homogenization procedures and transfer of cell content into a soluble extract because of leaf wooden structure and of the occurrence of numerous interfering molecules. An enzymatic pretreatment of the vegetable tissue with a mixture of carbohydrases was successfully carried out obtaining an improved extraction yield. In fact, the resulting homogenate supernatant showed a significant milk-clotting activity, of about 45 kDa, that was further purified by ammonium sulphate precipitation (20-50%) and anionic exchange chromatography. In addition, inhibition studies performed in the presence of pepstatin A demonstrated that the observed clotting activity corresponded to an aspartic protease.

However, since the enzyme occurring in the artichoke leaf extracts was found to exhibit low specific activity and very low stability, an additional source of vegetable rennet obtainable at low cost, extracted from *Carduus defloratus* flowers*,* was taken into consideration. The obtained results showed that also this vegetable source contained an aspartic protease endowed with milk-clotting activity but, conversely, *Carduus defloratus* flowers revealed a more advantageous milk-clotting enzyme source in terms of biocatalyst yield and stability. Therefore, such enzyme proved to be a promising candidate worthy of further studies of both purification and immobilization.

Hence, the second step of my research was focused on the immobilization of the aspartic protease extracted from *Carduus defloratus* flowers on a polyacrylic support activated with polar epoxy groups, having previously tuned such methodology to immobilize a commercially available aspartic protease widely used in cheese-making and present in the fungal organism *Mucor miehei*. The aim of these studies was not only to obtain a stable and reusable plant derived milk-clotting enzyme but also to improve the flavor of the cheese produced by vegetable rennet. In fact, it is well known that the plant milk-clotting enzymes are generally characterized by strong proteolytic activity leading to undesirable bitter cheeses. Enzyme immobilization could allow to overcome this drawback by enzyme removal after the reaction avoiding its presence into the cheese curd matrix and the ripening of the latter. In addition, it might represent a promising methodology for possible inline milk coagulation in cheese making. The obtained findings let to conclude that even though *Mucor miehei* protease was highly efficiently immobilized, the observed enzyme low

stability under the conditions used suggest that further experiments are needed to try to obtain its prolonged reusability. Conversely, since the setup immobilization procedure of *Carduus defloratus* protease showed high efficiency as well as good yield, producing a satisfactorily stable and reusable enzyme, such immobilized protease may represent a promising candidate as vegetable rennet for future industrial applications.

Appendix

Comunications

Esposito M., Giosafatto C.V.L., Mariniello L., Sorrentino A., Rossi-Marquez G., Di Pierro P. *Cynara scolymus* leaves as a potential source for vegetable rennet useful for cheese making. ItalianForum on Industrial Biotechnology and Bioeconomy (IFIB). Naples, Italy. October 2013.

Esposito M., Giosafatto C.V.L., Mariniello L., Porta R., Rossi-Marquez G., Di Pierro P. Proteolytic and clotting activity in *Cynara scolymus* leaves: new horizons for the cheese manufacture. 57th National Meeting of the Italian Society of Biochemistry and Molecular Biology. Ferrara, Italy. September, 2013.

Papers

Rossi Márquez G., Di Pierro P., Esposito M., Mariniello L., Porta R. (2014). Application of transglutaminase-crosslinked pectin/whey protein films as water barrier coatings in fried and baked foods. Food Bioprocess Technology. 7:447-455. DOI 10.1007/s11947-012-1045-9 .

Mariniello L., Porta R., Sorrentino A., Giosafatto C.V.L., Rossi Marquez G., Esposito M., Di Pierro P. (2013). Transglutaminase-mediated macromolecular assembly: production of conjugates for food and pharmaceutical applications. MiniReview: Amino Acids. DOI 10.1007/s00726-013-1561-6.

Porta R., Rossi-Marquez G., Mariniello L., Sorrentino A., Giosafatto C.V.L., Esposito M. and Di Pierro P. (2013). Edible Coating as Packaging Strategy to Extend the Shelf-life of Fresh-Cut Fruits and Vegetables. Editorial Journal of Biotechnology and Biomaterials. 3:4. DOI: 0.4172/2155-952X.1000e124.

Porta R., Mariniello L., Di Pierro P., Sorrentino A., Giosafatto C.V.L., Rossi Marquez G., Esposito M. (2012). Water barrier edible coatings of fried foods. Editorial. Journal of Biotechnology and Biomaterials. 2:7. DOI: 10.4172/2155-952X.1000e116.

Rossi Márquez G., Di Pierro P., Esposito M., Mariniello L., Porta R. (2012). Transglutaminase-crosslinked whey protein/pectin coating acts as an effective water vapour barrier in fried and baked food. Biopolpack, ISBN 88-901491-4-0.

Rossi-Marquez G., Porta R., Coppola B., Mariniello L., Esposito M. and Di Pierro P. Effects of hydrocolloid coatings containing transglutaminase on the shelf-life of different vegetable foods. Italian Congress of Food Science and Technology, Milan, Italy. May 2013.

Experiences in foreign laboratories

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