# NOVEL EDIBLE COATINGS TO IMPROVE QUALITY AND SHELF-LIFE OF FOODS PRODUCED BY PALESTINIAN INDUSTRIES

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*“To my family”*

# CONTENTS

**RIASSUNTO**

L'attuale allarmante scenario dell'inquinamento dovuto alla produzione di materiale plastico è esemplificato dal dato che più di 700 mila buste di plastica e 400 mila bottiglie di plastica vengono consumate nel mondo ogni minuto. La conseguenza di tale iper-consumo di materiale plastico è che circa 35 milioni di tonnellate di rifiuti di materiale non biodegradabile vengono prodotte annualmente in tutto il mondo, di cui solo il 7% del totale viene oggi riciclato. Inoltre, la maggior parte delle materie plastiche di origine petrolifera che viene a contatto con gli alimenti risulta pericolosa per la salute umana, potendo causare gravi danni al nostro sistema endocrino. Le soluzioni, finora ipotizzate, a questo enorme problema ambientale sono individuate nella auspicabile (i) accelerazione della biodegradazione degli attuali materiali plastici, (ii) sintesi di nuovi materiali plastici di origine petrolifera che siano biodegradabili, (iii) produzione di materiali biodegradabili sostitutivi delle tradizionali plastiche in quanto derivati da fonti naturali ("bioplastiche"). Diverse bioplastiche edibili sono già attualmente in commercio ed utilizzate per rivestire o proteggere alimenti altamente deperibili e per conservare le loro proprietà nutrizionali e organolettiche, avendo la capacità di estendere la shelf-life dei singoli prodotti e di ridurre gli effetti negativi causati dalla loro lavorazione, come il cambiamento di colore, la diminuzione della loro consistenza e lo sviluppo di odori e sapori non graditi. Le biomacromolecole proteiche e polisaccaridiche rappresentano due dei principali biopolimeri oggetto di studio finora utilizzati per preparare film biodegradabili/edibili. Le bioplastiche derivate da tali biomacromolecole presentano però ancora diversi svantaggi rispetto ai polimeri sintetici a causa delle loro limitate e non comparabili proprietà meccaniche e/o di barriera. Il presente lavoro è stato, pertanto, svolto per tentare di preparare e caratterizzare nuovi materiali idrocolloidali in grado di essere una valida alternativa alle plastiche tradizionali utilizzate per il packaging di prodotti alimentari, realizzando bioplastiche a base di un concentrato di proteine estratte da semi della “veccia amara” (*Vicia ervilia*) (BVPC), o a base di chitosano (CH), un polimero derivato dal secondo polisaccaride più abbondante in natura dopo la cellulosa, la chitina.

Per ottenere film innovativi con caratteristiche chimico-fisiche e proprietà superiori a quelle finora descritte per altre bioplastiche, è stata studiata l'efficacia (i) dell'aggiunta di nuovi composti con attività plastificanti (le poliammine alifatiche), a soluzioni formanti film costituite sia da BVPC che da CH, (ii) di procedure di miscelazione (“blending”) del BVPC con il polisaccaride pectina (PEC) e di reticolazione proteica mediante l’uso dell'enzima transglutaminasi (TGasi). I risultati ottenuti hanno suggerito come l'uso quale additivo di spermidina (SPD), o di una combinazione della poliammina con un plastificante primario come il glicerolo (GLY), possa aprire nuove vie di preparazione di biomateriali idrocolloidali edibili dotati di proprietà meccaniche e/o di barriera specifiche per il rivestimento/protezione di selezionati prodotti alimentari. I risultati ottenuti indicano, infatti, che la SPD non solo è in grado di agire di per sè da plastificante, interagendo con le proteine sia con legami di idrogeno che con legami ionici (come dimostrato dall'analisi FT-IR), ma anche di facilitare la riduzione, provocata dal GLY, delle forze intermolecolari tra le catene proteiche, migliorando conseguentemente la flessibilità e l'estensibilità del biomateriale prodotto. Pertanto, la SPD si è dimostrata essere non solo un efficace plastificante primario, ma anche un valido plastificante secondario a causa della sua capacità di migliorare le prestazioni in tal senso del GLY. Inoltre, la miscelazione del BVPC con la PEC, in presenza o assenza di TGasi, ha rappresentato un ulteriore

efficace approccio metodologico per ottenere una nuova bioplastica edibile. Infatti, l'aggiunta di tale polisaccaride alla soluzione proteica ha marcatamente diminuito la permeabilità ai gas dei film a base di BVPC, mentre i legami isopeptidici ottenuti grazie all'azione catalitica della TGasi hanno determinato un ulteriore miglioramento delle loro proprietà barriera. Questi risultati, supportati da analisi morfologiche mediante SEM, suggeriscono che le caratteristiche funzionali osservate erano determinate dalla struttura più compatta dei film proteici ottenuta a seguito della reticolazione enzimatica prodottasi in presenza di PEC.

Il secondo tipo di bioplastiche oggetto della tesi è rappresentato dai biomateriali a base di CH. Poiché i rifiuti di chitina, principalmente derivati dalla lavorazione dei crostacei, rappresentano un serio problema di inquinamento ambientale, attenzione è stata anche rivolta a migliorare le proprietà meccaniche e di barriera di biomateriali ottenuti con il CH, biopolimero derivante dalla deacetilazione della chitina, sfruttando l'effetto plastificante della SPD in assenza o presenza di GLY. I risultati ottenuti hanno dimostrato che i film di CH contenenti SPD sono più estensibili, esibendo un allungamento a rottura superiore a quello osservato nei film plastificati con GLY, a causa di interazioni idrofobiche e legami di H, evidenziati mediante analisi FT-IR, che si instaurano tra la poliammina e le catene di CH. Inoltre, è stato dimostrato che la presenza concomitante di appropriate concentrazioni di SPD e GLY migliora la plasticità del nuovo biomateriale prodotto, conferendo ad esso la capacità di poter essere anche termosaldato. Infine, tutti i film a base di CH preparati hanno mostratp una chiara attività antimicrobica, rivelandosi quindi candidati credibili per potenziali applicazioni come rivestimenti e/o involucri adatti per la conservazione degli alimenti.

Un'applicazione preliminare dei nuovi biomateriali ottenuti, sia quelli a base proteica che polisaccaridica, è stata realizzata rivestendo ed analizzando a diversi tempi campioni di formaggio fresco, preparato e conservato sia sotto sale che in assenza di sale secondo la metodologia tipica del formaggio palestinese di Nablus ("Nabulsi cheese"). Infatti, molti fenomeni indesiderati -quali il cambiamento del colore, la produzione di aromi sgradevoli, lo sviluppo di sapore amaro e la consistenza granulare- sono noti avvenire nei giorni seguenti la produzione di tale prodotto caseario, come anche nel corso di una prolungata conservazione in grandi contenitori in presenza di un'alta concentrazione salina. I risultati ottenuti hanno dimostrato che il packaging del "Nabulsi cheese" mediante i biomateriali prodotti (principalmente quelli preparati con BVPC) aveva gli stessi effetti positivi di quelli ottenuti con la conservazione sotto sale o mediante packaging con pellicole non biodegradabili a base di LDPE.

Pertanto, una eventuale produzione industriale di "Nabulsi cheese" non salato, ma semplicemente rivestito e protetto con pellicole costituite dai materiali biodegradabili oggetto della presente tesi, consentirebbe non solo un incremento della durata di conservazione del prodotto caseario fresco, ma anche di incrementare significativamente la prevedibile richiesta da parte dei consumatori di un formaggio fresco non salato, che sarebbe percepito come alimento maggiormente salutare e pronto per un suo possibile utilizzo anche nel settore dell'industria dolciaria.

**ABBREVIATIONS**

AFM, atomic force microscopy
BV, bitter vetch
BVPC, bitter vetch protein concentrate
CH, chitosan
RH, relative humidity
FT-IR, fourier-transform infrared
ATR, attenuated total reflectance
EB, elongation at break
FFS, film forming solution
GLY, glycerol
HDPE, high density polyethylene
LDPE, low density polyethylene
PEC, pectin
SEM, scanning electron microscopy
SNC, salted Nabulsi cheese
PA, polyamine
SPD, spermidine
TA, titratable acidity
TGase, transglutaminase
mTGase, microbial transglutaminase
TS, tensile strength
TSA, tryptic soy agar
TSB, tryptic soy broth
UNC, unsalted Nabulsi cheese
UW, unwrapped
YM, Young's module
W, wrapped
WV, water vapor

**SUMMARY**

The current alarming scenario of plastic pollution shows that more than 700 thousand plastic shopping bags and 400 thousand plastic bottles are consumed worldwide every minute. Consequently, about 35 millions tons of plastic wastes are annually produced in the entire world but only 7% of them are recycled. Moreover, when in contact with foods, many petrol-derived plastics can be harmful for human health, causing severe damages to the endocrin system. Possible solutions to these huge environmental and health problems may be (i) the biodegradation of the plastic materials, (ii) the synthesis of oil-derived biodegradable materials, (iii) the synthesis of substituting biodegradable materials derived from natural sources called "bioplastics". Several edible bioplastics are currently used to coat or wrap highly perishable foodstuff to protect their nutritional and organoleptic properties by extending food shelf-life and reducing the negative effects caused by food processing, such as enzymatic browning, texture breakdown and off-flavors development.

Protein and polysaccharides represent two of the main biopolymers used to prepare biodegradable/edible films, even though they still present several disadvantages due to the limited mechanical and/or barrier properties of the derived bioplastics. The present work was carried out to prepare and characterize new hydrocolloid materials made with either a concentrate of proteins extracted from bitter vetch (*Vicia ervilia*) seeds (BVPC) or from chitosan (CH), a polymer derived from chitin, the second most abundant polysaccharide occurring in nature.

To achieve innovative films with improved features we investigated the effectiveness of the addition of new compounds possessing plasticizing activities to BVPC and CH film forming solutions, such as the aliphatic polyamines, as well as the procedures of both blending of BVPC with the polysaccharide pectin (PEC) and protein crosslinking by the enzyme transglutaminase of microbial origin (mTGase). Our findings suggested that the use of spermidine (SPD) or of a combination of the polyamine with a primary plasticizer such as glycerol (GLY), as additives of BVPC-based films, may open new possibilities to generate hydrocolloid edible biomaterials endowed with improved mechanical and/or barrier properties specifically suitable for the coating/wrapping of different food products. The obtained results indicates that SPD is not only able to act as a plasticizer itself, by interacting with proteins by both hydrogen and ionic bonds as demonstrated by FT-IR analysis, but that it also facilitates GLY-dependent reduction of the intermolecular forces along the protein chains, consequently improving film flexibility and extensibility. Thus, SPD was demonstrated to be not only a primary, but also as a secondary plasticizer because of its ability to enhance GLY plasticizing performance.

Moreover, the blending of BVPC with PEC in the presence or absence of mTGase is the other way to achieve further innovative edible bioplastics. In fact, PEC addition markedly decreased the gas permeability of BVPC films and mTGase-catalyzed protein crosslinks determined a further enhancement of their barrier properties. These findings, supported by SEM morphological analyses, suggest that the improved functional features depend on film more compact structure due to crosslinked proteins grafted with PEC.

Since chitin waste, mainly produced from seafood processing (crustacean shells), still represents a major environmental issue, the attention was also addressed to improve the mechanical and barrier properties of CH-based bioplastics by assaying SPD, with and without GLY, as new plasticizer. Our findings

demonstrated that SPD containing CH films were always more extensible, exhibiting an elongation at break higher than that observed with glycerol-plasticized films, as a consequence of the occurrence of both hydrophobic and H-bonding interactions of SPD with CH chains observed in FT-IR spectra. Furthermore, the concurrent presence of appropriate concentrations of SPD and GLY enhanced the plasticity of the new biomaterial, conferring to it the ability to be also heat-sealed. Finally, all the prepared CH films exhibited a clear antimicrobial activity, thus representing credible candidates as food preservative coatings and/or wrappings.

A preliminary application of the new obtained biomaterials was realized by wrapping salted and unsalted Nabulsi cheese samples. In fact, many undesirable changes such as discoloration, off-flavor production, slime and gas formation, bitterness and textural problems may occur with Nabulsi cheese, a typical Palestinian fresh dairy product produced by traditional methods, during its storage in large cans, also in spite of high brine concentration. The obtained results demonstrate that the wrapping of the unsalted Nabulsi cheese by hydrocolloid films (mostly BVPC-based ones) has the same effects of LDPE wrapping, as well as of the salting treatment, in preventing the lowering of pH and the increase of titratable acidity occurring during the storage of the unwrapped dairy product. A possible industrial production of unsalted Nabulsi cheese wrapped with the reported edible film would present the advantages to increase its shelf-life, avoid any postprocess contamination, and enhance the following possible demand for an unsalted, healthy and ready-to-eat cheese, potentially to be used also in sweet pastry.

# 1. INTRODUCTION

## 1.1 Plastics and bioplastics

Packaging is one of the most important processes to maintain food quality for storage, transportation and end-use. The main function of packaging is to achieve preservation and the safe delivery of food products until consumption. Therefore, an effective packaging contributes to extend the shelf-life and to maintain the quality and the safety of the food products (Han, 2005). The world annual production of plastic shopping bags overcomes 5 million tons and 60 thousand of them are used every five seconds, more than 100 billions of them being consumed only in Europe each year. In addition, 2 millions of plastic bottles are consumed worldwide every five minutes. Currently, more than 35 millions tons of wastes deriving from different plastic products are produced each year in the world and only 7% of them are recycled, the remaining waste being deposited in the landfills or dispersed in the oceans. Most of the packaging available in the market is developed from synthetic traditional polymers such as polyethylene, polypropylene, or polystyrene causing health hazards due to migration of toxic additives into the consumables (Ansorena et al., 2018).

Global production of plastics has increased twenty-fold since the 1960s, reaching 322 million tons in 2015, and it is expected to double again over the next 20 years (Fig. 1). In a business-as-usual scenario, the ocean is expected to contain 1 ton of plastic for every 3 tons of fish by 2025 and, by 2050, more plastics than fish (by weight) (World Economic Forum, 2016). Plastics disposal in either ground or water threatens soil fertility and marine life, whereas plastics burning releases poisonous chemicals in the air. Nowadays, people are more aware about the harmful effects of the presence in the environment of wastes derived from plastic materials.

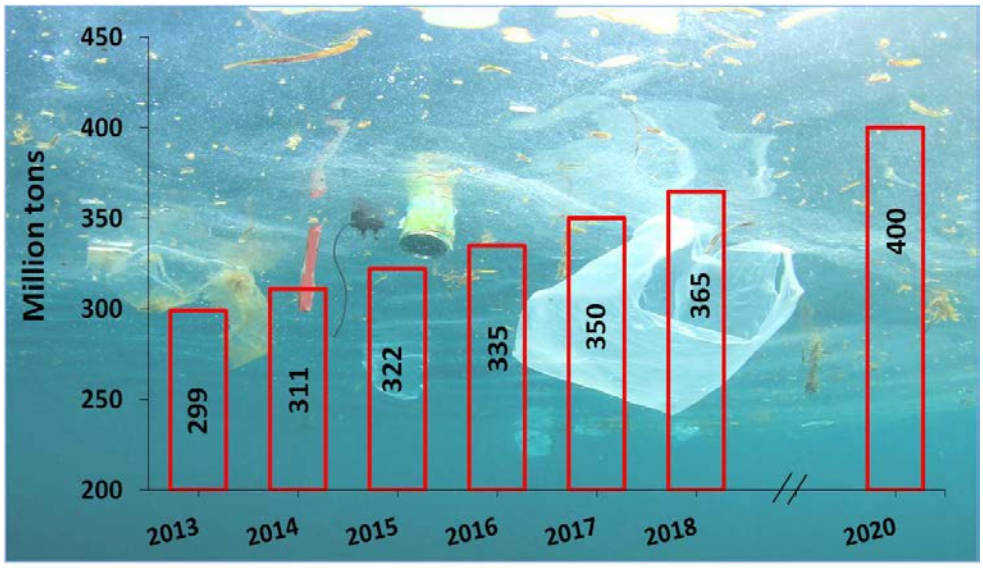


***Fig. 1. Global plastics production.***

Plastics can degrade by a variety of mechanisms such as chemical, thermal, photoxidation and biodegradation, all of which take an extremely long time depending on the molecular weight of the different polymers (some types of plastics could take up to 1000 years to degrade) (Pramila and Ramesh, 2011). Microorganisms can also

play a role in this process, since over 90 genera of bacteria, fungi and actinomycetes have been found to possess a slow ability to degrade plastics (Mahdiyah and Mukti, 2013). Conversely, Bombelli et al. (2017) recently reported the fast bio-degradation of polyethylene by larvae of the wax moth *Galleria mellonella* producing ethylene glycol.

A possible solution to reduce the consumption of the traditional plastics of petrochemical origin, and consequently the plastic waste disposal, is their replacement with biodegradable materials (generally called “bioplastics”), as it is reported in the following recent publication.

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# Plastic Pollution and the Challenge of Bioplastics

Opinion

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## Opinion

The world annual production of plastic shopping bags (PBS) overcomes 5 million tons and 60 thousand PBS are used every five seconds, more than 100 billions of them are being consumed only in Europe each year. In addition, 2 millions of plastic bottles are consumed worldwide every five minutes. Currently, more than 35 millions tons of wastes deriving from different plastic products are produced each year in the world and only 7% of them are recycled, the remaining waste being deposited in the landfills or dispersed in the oceans. Plastics disposal in either ground or water threatens soil fertility and marine life, whereas plastics burning releases poisonous chemicals in the air. Nowadays, people are more aware about the harmful effects of the presence in the environment of wastes derived from plastic materials. A possible solution to reduce the consumption of the traditional plastics of petrochemical origin and, consequently, plastic waste disposal, is their replacement with biodegradable materials (generally called “bioplastics”). Bioplastics seem an attractive eco-friendly alternative since they can be easily degraded by the enzymes present in different microorganisms. The main biopolymers used so far to prepare these innovative biomaterials are some aliphatic polyesters (e.g. polylactic acid and polyhydroxyalkanoates), various polypeptides (e.g. soy and whey proteins, collagen, gelatin) and numerous polysaccharides (e.g. cellulose, starch, chitin, pectins) obtained from plant or animal feedstocks. It is possible to produce bioplastics by starting from bio-based monomers (e.g. lactic acid), obtained by fermentation or conventional chemistry and polymerizing them in a second step. Further ways are both the polymer biosynthesis directly in microorganisms or in genetically modified crops (e.g. polyhydroxyalkanoates) and the utilization of natural biopolymers (e.g. polysaccharides and/or proteins) recovered from organic waste. However, different bioplastics are prepared for different applications. Cellulose is the most used biopolymer in the food industry, where edible coatings are produced to improve food quality and can be consumed together with the packaged products. Novel edible films with different morphological, mechanical and permeability properties and containing various antimicrobial and/or antioxidant additives are increasingly issued from the scientific literature in order to improve the shelf-life of different foods. Conversely, starch is currently the most used biopolymer outside food industry and thermoplastic starch, often blended with caprolactones and other biodegradable esters, constitutes today about 50% of the total bioplastics market. Packaging is the largest field of application for bioplastics covering almost 40% of the produced biodegradable articles (consumer goods, 22%; transport sector, 14%; construction and building sector, 13%). However, although global bioplastics market has been reported (Allied Market Research) to reach more than 30 billion dollars by 2020, the biodegradable materials are still considered “potential” rather than “commercial” materials. In fact, they replaced until now only 1% of the 300 million tons of the total plastics produced annually, their limits being poor mechanical and barrier properties. But, as demand is rising and more sophisticated products and applications are emerging, the market of bioplastics is rapidly changing. According to the latest data (European Bioplastics), global production capacity is predicted to grow from around 4.2 million tons in 2016 to approximately 6.1 million tons in 2021. The most important factors able to influence such a positive trend might be

(i) An enhanced consumer awareness to increase the demand of bioplastics, as well as new laws facilitating the companies to produce them

(ii) Improved interactions between academia, industry and government institutions and, mostly

(iii) The production of new biomaterials with improved and tailored characteristics. In this respect the preparation of innovative biodegradable films reinforced by nanoparticles (e.g. $TiO_2$, FeO, $SiO_2$, mesoporous silica, carbon nanotubes) and/or by enzyme-catalyzed (peroxidase, tyrosinase, transglutaminase) crosslinks, may represent a promising approach to meet the challenge to stop plastics pollution.

Bioplastics seem an attractive eco-friendly alternative since they can be easily degraded by the enzymes normally present in different microorganisms. The global market for bioplastics is predicted to grow continuously over the next years. In fact, according to the latest market data compiled by European Bioplastics in collaboration with the nova-Institute, global production capacities of bioplastics should grow from around 2 million tons in 2017 to approximately 2.5 million tons by 2022 (Fig. 2) (European Bioplastic, nova-Institute, 2017).

The main polymers used so far to prepare these innovative biomaterials are some aliphatic polyesters (e.g. polylactic acid and polyhydroxyalkanoates), various polypeptides (e.g. soy and whey proteins, collagen, gelatin) and numerous polysaccharides (e.g. cellulose, starch, chitin, pectins) obtained from plant or animal feedstocks. It is possible to produce bioplastics by starting from bio-based monomers (e.g. lactic acid), obtained by fermentation or conventional chemistry and polymerizing them in a second step. Further ways are both polymer biosynthesis directly in microorganisms or in genetically modified crops (e.g. polyhydroxyalkanoates) and the utilization of natural biopolymers (e.g. polysaccharides or proteins) recovered from organic wastes. However, different bioplastics are prepared for different applications.


***Fig. 2. Global production capacities of bioplastics.***

Bioplastics made of polysaccharides or proteins, while possess good gas barrier features towards oxygen and carbon dioxide, usually exhibit poor mechanical properties and, due to their hydrophilic nature, are too much water sensitive to be applied to the majority of foods having high or intermediate moisture (Krochta and DeMulder-Johnston 1997; Nisperos-Carriedo, 1994). These disadvantages can be resolved by the preparation of blended films, as protein-polysaccharide films and/or by adding lipids or other components like nanoparticles able to reinforce film network (Porta et al., 2011b; Rhim, 2007; Rostamzad et al., 2016). An additional strategy to improve the characteristics of protein-based films is to create a crosslinked structure of its network either chemically or enzymatically. Among the enzymes able to create protein crosslinkings, transglutaminase (EC 2.3.2.13; TGase) is certainly the most efficient, being able to catalyze isopeptide bonds between reactive Gln and Lys residues existing in the polypeptide sequence. Mahmoud and Savello (1992; 1993) were the first to have utilized TGase as crosslinker to produce milk whey protein

homo- and hetero-polymers containing films. Afterwards, different authors proposed further proteins and methodologies to produce TGase-crosslinked biomaterials with specific properties suitable for coating specific food products, generally utilizing TGase of microbial origin (mTGase) for its several advantages (broad substrate specificity and optimal pH, calcium-independence, commercial availability) (Cui et al., 2017; Elango et al., 2017; Mariniello and Porta, 2003; Porta et al., 2011b, 2011c; Rossi Marquez et al., 2014, 2017). However, this kind of food coating needs, of course, that the used proteins are able to act as acyl donor and/or acceptor substrates of the enzyme and, thus, most of the studies in this direction were mainly focused on few specific proteins able to easily form crosslinked polymers in the presence of mTGase.

### 1.2 Edible films: coating and wrapping

Edible coatings and wrapping are generally used to cover or protect the food surface(s). The difference between coating and wrapping is that the coating is created directly on food surface itself, whereas in the second case the film represents a stand-alone wrapping material (Pavlath and Orts, 2009). Films may take, thus, the form of pouches, capsules, casings, or bags (Sánchez-Ortega et al., 2014). Edible films may prevent food moisture losses and quality changes and/or help in control exchange of various gases as well as they may prevent the quality loss of various components of food products and/or ensure their surface resistance (Akhtara et al., 2015). Edible packaging can be carriers for flavourings, antimicrobial agents or antioxidants (Šuput et al., 2015) and are degraded by microorganisms in composting processes to produce natural breakdown compounds. They can also ensure sterility of the food surface, representing an useful tool for providing food safety preventing pathogens contamination by direct contact of the package with its surface.

According to Dhanapal et al. (2012), dipping is the most common method to apply coatings to fruits and vegetables when the coating solution is highly viscous (Fig. 3a). Dipping is carried out by introducing the product, for a time between 5 and 30 s, in a coating solution under controlled conditions of density and surface tension. However, when the coating solution is not highly viscous, a spraying procedure can be used (Fig. 3b). The food product is introduced into the coating system and it is sprayed by controlling the final drop size of the solution, which depends on the thickness of the spray gun, nozzle temperature, air and liquid flow rates, humidity of incoming air and polymer solution, drying time and temperature. Finally, the brushing method is used in some products, such as fresh beans and strawberries, when the reduction of the moisture loss is an issue (Fig. 3c). Thin coatings onto the surface of the product are obtained in all cases and they could act as semi-permeable membranes reducing gas transfer rates and creating new packaging materials to extend food shelf-life (Valdés et al., 2015). Substantial advances have been made over the last two decades in the field of biodegradable polymers, mostly derived from renewable natural resources, to produce bioplastics with features similar to those typical of oil-based materials (Pathak et al*.,* 2014). In particular, protein-based edible films and coatings have attracted an increasing interest in recent years since they might be used to improve the shelf-life of different food products (Zink et al*.,* 2016). These biomaterials are generally first evaluated for their mechanical and barrier properties as a function of different types and concentrations of plasticizers, generally small and non-volatile organic additives used to increase film extensibility and reduce its cristallinity, brittleness and water vapour (WV) permeability.

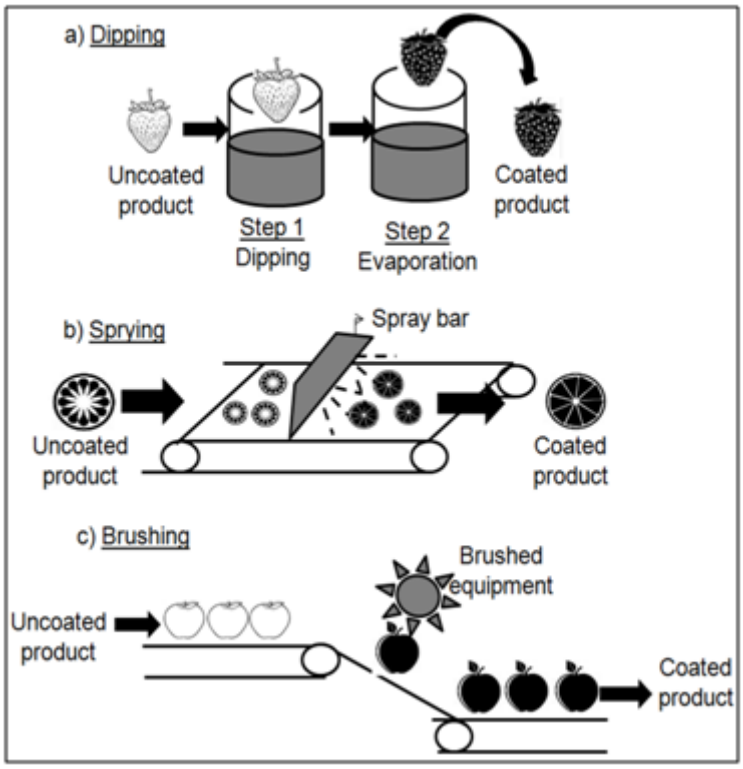


***Fig. 3. Main coating methods used in food industry (Dhanapal et al., 2012).***

### 1.3 Plasticizers

Numerous additives, called plasticizers, have long been known for their effectiveness in enhancing the flexibility of synthetic plastics (Mekonnen et al., 2013) and new types of plasticizers specifically suitable to bio-based materials are being developed. For technical and economic reasons, these additives are a large and increasingly significant components of the polymer industry (Rahman and Brazel, 2004). Generally, plasticizers are small, relatively non-volatile, organic molecules that are added to polymers to reduce brittleness, impart flexibility, and improve toughness reducing crystallinity, glass transition and melting temperatures. (De Groote et al*.,* 2002). Plasticization reduces the relative number of polymer–polymer contacts, thereby decreasing the rigidity of the three-dimensional structure and allowing deformation of material without rupture (Varughese and Tripathy, 1993). Consequently, plasticizers improve processability, flexibility, durability and, in some cases, reduce the cost of production (Snejdrova and Dittrich, 2012; Ljungberg and Wesslen, 2003). Therefore, as the bioplastic industries is continuously growing, the demand for new kinds of plasticizers endowed with specific characteristics and performances compatible with the single bioplastics is parallel growing (Vieira et al., 2011; Mekonnen et al., 2013).

Water is the main solvent in the natural biopolymer technology able to reduce the glass transition temperature and to increase the free volume of biomaterials and, thus, water is considered the most powerful ‘‘natural’’ plasticizer of hydrocolloid-based films (Cheng et al., 2006; Karbowiak et al., 2006). Recently, many studies have focused on the use of plasticizers of different chemical nature, such as glycerol (GLY) (Cheng et al., 2006: Cao et al., 2009; Porta et al,. 2015; Sanyang et al., 2016; Santana at al., 2018; Fernandez-Bats et al., 2018; Fitri et al., 2018), ethylene glycol, diethylene glycol, triethylene glycol, tetraethylene glycol, polyethylene glycol (Suyatma et al., 2005; Cao et al., 2009), sorbitol (Cao et al., 2009; Sanyang et al.,

2016; Escamilla-García et al., 2017), fatty acids (Pommet et al., 2003; Jongjareonrak et al., 2006), and several monosaccharides (glucose, mannose, fructose, sucrose) (Veiga-Santos et al., 2007; Galdeano et al., 2009).

### 1.4 Bitter vetch (*Vicia ervilia*) protein concentrate

Bitter vetch (BV, *Vicia ervilia*) is an annual Vicia genus cultivated for forage and seed yield (Fig. 4). In particular, BV seeds, containing up to 25% of protein, are an abundant, inexpensive and renewable source of both protein and energy (Sadeghi et al., 2009).

***Fig. 4. Bitter vetch and derived seeds.***

Thus, BV proteins might represent an affordable alternative protein source to produce edible films for both pharmaceutical and food applications. In particular, Arabestani et al. (2013; 2016), recently described edible films obtained from a BV protein concentrate (BVPC), showing promising both barrier and mechanical properties, and the ability to give rise to both edible films and biodegradable containers. Moreover, Porta et al. (2015) determined the properties of BVPC films reinforced by mTGase-catalyzed protein crosslinking. The surface of films prepared in the presence of enzyme appeared more compact and smoother and the film cross sections showed the disappearance of the discontinuous zones observed in the control films and, on the contrary, a very homogeneous structure. mTGase-crosslinked films exhibited also markedly decreased oxygen (700-fold) and carbon dioxide (50-fold) permeability, compared to controls, as well as significantly different mechanical properties being increased film resistance and stiffness. More recently, Fernandez-Bats et al. (2018) added mesoporous silica nanoparticles and its (3-aminopropyl)triethoxysilane derivative to improve the mechanical as well as the gas and WV barrier properties of the BVPC-based films. Finally, the demonstrated antimicrobial and antifungal activities of the obtained biomaterials, increased by nisin addition to the film forming solutions (FFSs), suggest the possibility of their potential application as bio-preservative active packaging to improve the shelf-life of different food products.

### 1.5 Chitosan

Chitosan (CH) is a linear polysaccharide of randomly distributed β-(1–4)-linked D glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (Fig. 5), derived by deacetylation at high temperatures of chitin, the second most abundant natural polysaccharide after cellulose (Siripatrawan, 2016; Muxika et al., 2017).

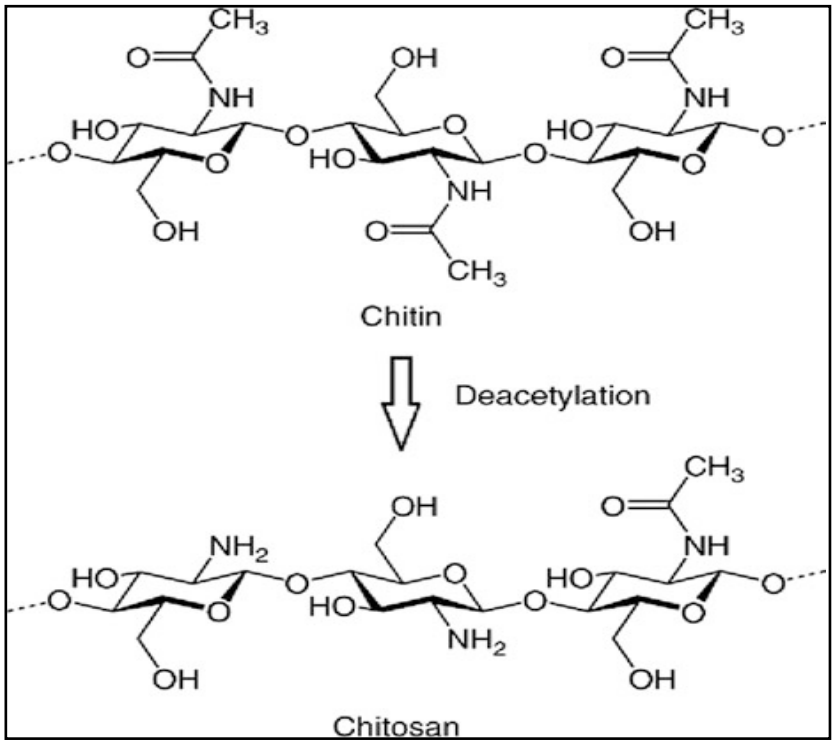


***Fig. 5. Structure of chitin and chitosan (Siripatrawan, 2016).***

Although various factors (e.g. chitin source, alkali concentration, deacetylation procedure) may affect some of its properties, CH (pKa, 6.3) is easily dissolved in acidic solutions, i.e. when its free amino groups are fully protonated (Babu et al., 2013; Kaur and Dhillon, 2014; Rinaudo, 2006; Yeul and Rayalu, 2013; Van den Broek et al., 2015). The degree of both deacetylation (ranging from 40 to 98%) and polymerization determines CH molecular weight (generally ranging from $5x10^4$ to $2x10^6$ Da) and its consequent possible utilization (Aljawish et al., 2015). The unique physicochemical and biological features of CH make it worthy in regard to various biomedical, pharmaceutical and agricultural applications and, because of its broad antibacterial and antifungal properties, CH edible films have been promoted as promising biomaterials also for food coating and protection (Kong et al., 2010). In fact, although CH-based films exhibit weak mechanical properties, as well as unsatisfying WV barrier features, they remain the most up-and-coming ones among the various hydrocolloid bioplastics so far proposed, because they are biodegradable, biocompatible, non-toxic and obtainable in large quantities from waste products of seafood industries (crustacean shells) (Elsabee and Abdou, 2013; Mayachiew and Devahastin, 2008; Van der Broek et al., 2015). In addition, it is worthy to note that CH has been considered as a GRAS (Generally Recognized As Safe) food additive for both consumers and the environment (FDA, 2012).

Several advantages have been demonstrated when different food products were CH-coated. CH was shown to be able to form a semi-permeable layer on the surface of various fruits and vegetables, and to delay the rate of respiration and their ripening by reducing food moisture and weight loss (Alvarez et al., 2013; Chofer et al., 2012; Eum et al., 2009; Gol et al., 2013; Sun et al., 2014). Moreover, CH films have also been used as carriers releasing different bioactive agents like essential oils, as well as antimicrobials and/or antioxidants (Acevedo-Fani et al., 2015; Avila-Sosa et al., 2012; Zivanovic et al., 2005), and to protect fish, red meat, poultry and their

processed products with the aim to decrease color changes, lipid oxidation, growth of pathogenic and spoilage bacteria and to extend product shelf-life (Chamanara et al., 2013; Gómez-Estaca et al., 2010; Samelis, 2006; Yingyuad et al., 2006). Many different attempts have been made to improve mechanical, barrier and functionality properties of CH films by their blending with other biopolymers and/or plasticizers (Baron et al., 2017; Di Pierro et al., 2006, 2007; Escamilla-García et al., 2017). For instance, CH films plasticized with sorbitol show a lower WV permeability than those plasticized with GLY at similar concentrations, due to the lower ability of sorbitol to bind water (Siripatrawan, 2016).

### 1.6 Nabulsi cheese

Nabulsi cheese (Fig. 6) is one of the traditional white brined cheese known and widely consumed in various countries of the Middle East, particularly Palestine and Jordan, also used to produce a typical dessert called *Knafeh*. This cheese is classified as unripened, semi-hard dairy product with moisture content ranging from 45% to 55% and salt content around 9% (Ayyash and Shah, 2011). The white colour of Nabulsi cheese is an important factor for consumer acceptance and directly related to product quality. Colour or appearance of the cheese is related to its physical structure and chemical nature (Rudan et al., 1998).

***Fig. 6. Nabulsi cheese.***

Nabulsi cheese is produced mainly from sheep and goat milk, but also form cow milk, and its most favorable characteristics can be described as follows:

1- shape is rectangular and the dimensions are generally 8×4×1.5 cm.
2- color is normally white, yellowish color occurring only when cow milk is used.
3- texture analysis indicates a semi-hard cheese, with no gas holes, becoming soft and elastic when heated.
4- flavor is characteristic for ewe and goat milk and it is influenced by the particular aroma of *Prunus mahaleb* and *Pistacia letiscus* extracts added to the boiling brine.

The traditional method used for Nabulsi cheese preservation is to boil the salted cheese in brine (18-20%), and it can be stored in 20% brine solution for 1 year after moulded curd pieces have been boiled in brine solution for 10 min (Abd El-Salam and Alichanidis, 2004). The cheese is generally stored in cans of various sizes, without refrigeration, containing its boiling brine. Boiling procedure of Nabulsi cheese is quite similar to milk pasteurization (Yamani et al., 1987). Most Mediterranean cheeses contain high salt content -such as Feta (5.0%), Halloumi (3.0-5.0%) and Akawi (5.0%) cheeses- (Ayyash and Shah, 2011). Therefore, following the traditional

preservation method the final Nabulsi dairy products will contain high amounts of salts which can result in negative effects for the consumers suffering of high blood pressure and other disease related to high sodium intake. Moreover, many undesirable changes such as discoloration, off-flavor production, slime and gas formation, as well as bitterness and textural problems, may occur to the Nabulsi cheese produced by traditional methods during storage in large cans in spite of high brine concentration (Mazaherh et al., 2009; Yamani, 1997). This explains why the dairy industries are recently increasing the production of Nabulsi cheese obtained from pasteurized cow milk and containing either lower salt amount on the surface or even no salt.

In conclusion, an innovative packaging, made of edible coating and/or wrapping material derived from cheap or waste biological sources, could represent a strategical way to protect Nabulsi cheese from the moment of its production until its final retail destination and consumption.

## 1.7 General objectives of the experimental work

The main objectives of the present thesis were: (i) to produce new hydrocolloid edible films, prepared from low price vegetal sources, such as BVPC, or from a waste-derived biological source, such as the polysaccharide CH; (ii) to identify innovative plasticizing additives, to be used alone or in combination with GLY, to improve the mechanical and barrier characteristics of BVPC and CH films; (iii) to study the stability of the new FFSs formulated by analyzing their zeta-potential and particle size; (iv) to examine the physicochemical and biological properties of the obtained biodegradable/edible films in comparison with those o different commercial materials; (v) to test the ability of the obtained biomaterials to improve Nabulsi cheese shelf-life by its coating and/or wrapping.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Bitter vetch protein concentrate

BV seeds were obtained from a local market in Gallicchio (Potenza, Italy) and the contained proteins were extracted as previously described (Arabestani et al., 2013) with some modifications (Fig.7). The seeds were grinded at speed of 1300 r.p.m. for 5 minutes and the flour was solubilized in distilled water at pH 11 by stirring at medium speed for 1 h at room temperature. After centrifugation at 3200x g for 10 min, the supernatant was collected and the pH was adjusted to 5.4 by 1 N HCl addition to form a precipitate which was then separated by centrifugation at 3200x g for 10 min. Finally, the pellet was poured and uniformally distributed on a plastic plate and dried at 37°C and 25% relative humidity. The dry protein concentrate was finally grinded and its protein content (77%) determined by the Kjeldahl's method (Kjeldahl, 1883).

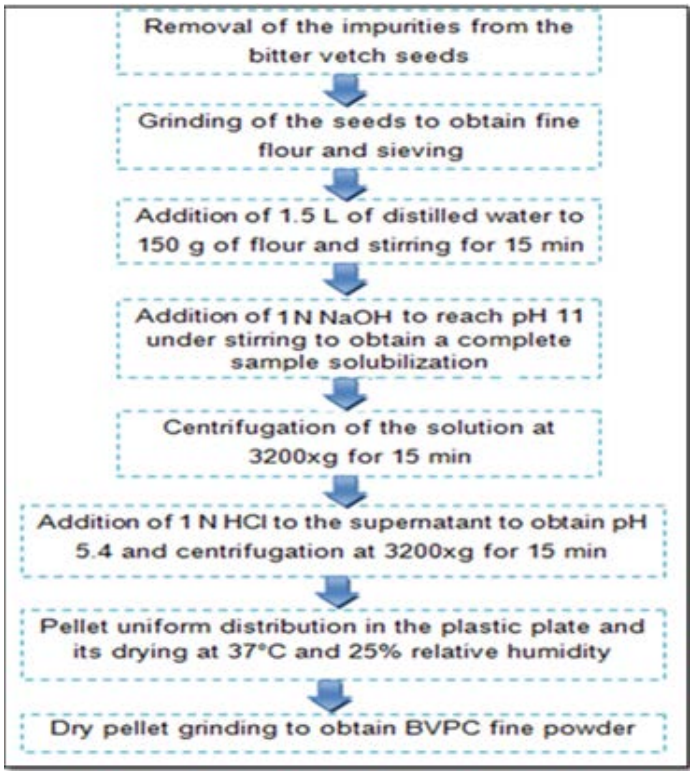


***Fig. 7. Summary of BVPC preparation.***

#### 2.1.2 Chitosan

CH (mean molar mass of $3.7x10^4$ g/mol) with a degree of 9% N-acetylation, was a gift from prof. R.A.A. Muzzarelli (University of Ancona, Italy). The mean molar mass of CH was determined by a viscometric method, as previously described (Costa et al., 2015), by dissolving 0.2 g of CH in 10 mL of 0.1 M acetic acid, containing 0.2 M sodium chloride, and obtaining five different dilutions of the original solution. The degree of N- acetylation was determined by the first derivative ultraviolet spectrophotometric method, as described by Muzzarelli and Rocchetti (1985), based on recording of the first derivative of the CH UV spectra at 202 nm by using a standard curve obtained by varying N-acetylglucosamine concentrations.

### 2.1.3 Other materials

Microbial TGase (mTGase) from *Streptoverticillium* sp. (Activa WM; specific activity 90 units/g) was supplied by Prodotti Gianni SpA (Milano, Italy). The enzyme solution was prepared by dissolving the commercial preparation in distilled water at a concentration of 100 mg/mL and the mixture was centrifuged at 10,000×g for 2 min to remove the precipitate.

Citrus peel low-methylated (7%) pectin (PEC) (Aglupectin USP) was from Silva Extracts srl (Gorle, BG, Italy).

Viscofan NDX edible casings were from Naturin Viscofan GmbH (Tajonar-Navarra, Spain); Mater-Bi (S 301).

MaterBi, as well as high density (HDPE) and low density (LDPE) polyethylene materials, were from local market shopping bags, Naples, Italy.

Spermidine (SPD) was from Sigma Chemical Company (St. Louis, MO, USA).

GLY (about 87%) was from the Merck Chemical Company (Darmstadt, Germany).

All other chemicals and solvents used in this study were analytical grade commercial products.

## 2.2 Film forming solution preparation and characterization

To prepare the different BV protein FFSs, BVPC was dispersed in distilled water (2 g/100 mL) and the pH value was adjusted to pH 12.0 by using 0.1 N NaOH under constant stirring until the powder was completely solubilized. Aliquots of BVPC solution were then added with different concentrations of polyamines (PAs) and incubated for 30 min either at 25 or 80°C to obtain FFSs containing both native and denatured BV proteins. Two additional aliquots of BVPC solution were brought at pH 8.0 and 11.0, respectively, by 0.1 N HCl addition and then incubated in the presence of PAs for 30 min either at 25 or 80 °C. Where indicated, different concentrations of GLY were added to the obtained FFSs at the end of incubation.

CH stock solution (2 %) was prepared by dissolving the polysaccharide in 0.1 N HCl at room temperature under overnight constant stirring at 700 rpm (Di Pierro et al., 2006). CH FFSs were obtained at pH 4.5 by using CH (0.1-0.6 %) mixed or not with different concentrations of SPD (2-10 mM) and/or GLY (2-40 mM).

Zeta potential and Z-average values of the different BVPC and CH FFSs were determined by a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) equipped with an automatic titrator unit (MPT-2) to study the effect of different pH or any additives on the zeta potential and Z-average of FFSs. The device was equipped with a helium-neon laser of 4 mW output power operating at the fixed wavelength of 633 nm (wavelength of laser red emission). The instrument software programmer calculated the zeta potential through the electrophoretic mobility by applying a voltage of 200 mV using the Henry equation. The zeta potential and Z-average were studied in triplicates immediately after their preparation to prevent possible alterations in molecular interaction during storage (Schmid et al., 2015).

In the following two articles an insight into zeta potential measurements in biopolymer film preparation is reported, as well as stabilization studies of the polysaccharide FFSs by nanoparticle Z-average and zeta potential monitoring is described.

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# Insight into Zeta Potential Measurements in Biopolymer Film Preparation

**Mohammed Sabbah, Marilena Esposito, Prospero Di Pierro, C. Valeria L. Giosafatto, Loredana Mariniello and Raffaele Porta***

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Micro- and nano-particle charge is one of the main factors determining the physical stability of both emulsions and suspensions and can be quantified by measuring their so called *"zeta potential"*. When all the particles have a large either negative or positive zeta potential value, they will repel each other and, as a consequence, the suspension becomes stable. By contrast, whether the zeta potential is close to 0 mV, the tendency for flocculation increases. Zeta potential is, however, a feature of the particle in its environment and not of the particle itself. In fact, its net charge in solution affects the ion distribution surrounding the particle, thus resulting in an increase in the concentration of counter-ions. The region over which this influence extends is called *"electrical double layer"* (EDL) and EDL splits into two regions (Figure 1). In the first, called "*stern layer*", the ions are of opposite charge with respect to the particles and, being strongly bound to them, move with them. The second layer, conversely, is a "*diffuse layer*" where the ions are less strongly attached and, inside it, there is a boundary line between the ions moving with the particles and the not moving ones. This region, called *"slipping plane"*, is known as the surface of hydrodynamic shear and the potential existing in the slipping plane is called zeta potential [1].

Electrophoresis is the most widely used technique for measuring zeta potential. By directly analysing the electrophoretic mobility of a particle, the zeta potential may then be determined using the *"Henry equation"*:

$$U_E = \frac{2\varepsilon z f(Ka)}{3\eta}$$

where $U_E$ is the electrophoretic mobility, $\varepsilon$ is the dielectric constant, $z$ is the zeta potential, $\eta$ is the viscosity and $f(Ka)$ is Henry's function. For measuring zeta potential in aqueous solutions of moderate electrolyte concentration, a Henry's function value of 1.5 is used (*Smoluchowski approximation*) whereas, if zeta potential is measured in a non-polar solvent, $f(Ka)$ is set to 1.0 (*Huckel approximation*).

Zeta potential is attracting an increasing interest for the characterization of electrochemical surface properties of both micro- and nano-particles being a key parameter for a number of applications, including characterization of biomedical polymers, electrokinetic transport of particles or blood cells, biocompatibility tests for pharmaceuticals and medical devices, membrane separation, protein purification, mineral processing, water treatment, characterization of clothing material properties in the textile industry [2,3].

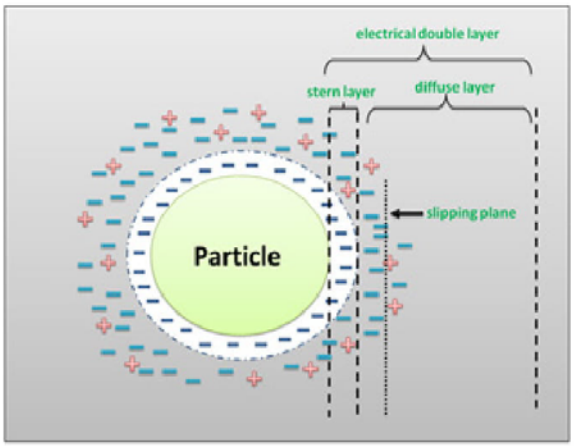


**Figure 1:** Micro- or nano-particle electrical double layer.

The use of biodegradable and/or edible films was proposed in the late sixties, originally to extend the shelf life of various fresh, frozen and manufactured food items and to improve their quality [4]. Many bio-macromolecules including proteins and carbohydrates have been thus far used, blended or not, as edible films and coatings [4-7]. Protein- and polysaccharide-based films show good tensile features, whereas lipid-based films have proved to be good water vapour barriers [8-10]. The formation of biopolymer supra-molecular structures induced by electrostatic interactions is related to the nature of raw materials, as well as to other factors such as concentration of the components, their mix ratio, pH, temperature and ionic strength [11-13]. In addition, Porta et al. [14,15] studied the effect of transglutaminase (TGase), an enzyme able to form protein inter- and/or intra-molecular crosslinks, on the mechanical and barrier properties of protein-based films showing that the enzyme is a very useful tool to produce innovative bio-plastics from renewable biomass sources. Giosafatto et al. [16] characterized citrus pectin (PEC) edible films containing TGase-modified phaseolin, and reported that the mechanical, as well as barrier properties to $CO_2$, $O_2$ and water vapor of the latter were comparable to the ones of commercial plastics. More recently, Porta et al. [17] investigated the microstructure and some features of bitter vetch (*Vicia ervilia*, BV) seed protein films reinforced by microbial TGase. BV, an annual grain legume crop thus far widely cultivated only for forage because of its high nutritional value, shows several favourable characteristics, such as having high yields and being a cheap and abundant protein source [18,19]. Arabestani et al. [20] found that the very low gas permeability, in addition to good mechanical properties, of BV protein films prepared in the presence of the enzyme conferred to these new bio-materials potential practical applications not only as edible coatings in food packaging but also as biodegradable containers.

On the other hand, polysaccharides as starch, alginate, cellulose, chitosan, carrageenan, PEC, or their derivatives, impart hardness, crispness, compactness, thickening quality, viscosity, adhesiveness, and gel forming ability to a variety of films. These films, because of the make up of the polymer chains, exhibit excellent gas permeability

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properties, resulting in desirable modified atmospheres that enhance the shelf life of the products without creating anaerobic conditions [21]. Additionally, polysaccharide films and coatings can be used to extend the shelf life of muscle foods by preventing dehydration, oxidative rancidity and surface browning, even though their hydrophilic nature makes them poor barriers for water vapour [22].

Among the different polysaccharides, PEC appears to be suitable for low moisture foods [21]. PEC, mainly extracted from citrus peel and apple pomace, is a heterogeneous group of acidic macromolecules well known for their long and safe use in the food industry as a thickening and stabilizer agents. Because of its gelling characteristics, bio-adhesivity, biocompatibility and non-toxicity properties, PEC is also a promising biopolymer as a drug delivery vehicle. In fact, it has been used in mucoadhesive systems to increase the retention time of the dosage form in the gastrointestinal tract, thus enhancing drug absorption after its oral administration [23,24]. The ability of PEC to adhere to mucous membranes seems to be dependent on the different type of polysaccharide employed [25,26]. In particular, the degree of esterification or amidation of the galacturonic acid residues inside the macromolecule is often used to characterize PEC and describes the different properties of the various PEC preparations [27].

All the above literature indicates that the variations of the film physical properties are closely related to polyelectrolyte nature of the biopolymer component(s) and to their capacity to influence the microstructural network by their ionisable groups. The charge on the polymer chains is related to pH and ionic strength of the solution and could affect the polyelectrolyte aggregation with formation of nano-complexes. Therefore, a careful analysis of this relationships needs experimental details that may be provided by zeta potential measurements carried out on the film forming (FF) mixtures used to prepare the differently tailored biodegradable/edible films. Moreover, the analysis of the various factors able to influence the zeta potential value of each FF solution/suspension could be useful to render "stable" the latter. It is worthy to note that the mixing of the different components of a specific FF solution/suspension -as well as the pH, the ionic strength, the polyelectrolyte ratio, or even the method of adding of each component and the speed of mixing- may markedly influence the zeta potential and the size of the obtained micro- or nano-complexes.

To this aim, the zeta potential of FF solutions of pure polysaccharide (PEC) and protein (phaseolin), as well as of a protein mixture (BV seed protein concentrate, BVPC), was measured at different pH values by titration from pH 8.0 to pH 2.0 using the Zetasizer nano-ZSP (Malvern, Worcestershire, UK). The results reported in Table 1 showed that PEC negative zeta potential progressively decreased from -37.50 mV, recorded at pH 8.0, to -6.84 mV recorded at pH 2.0, indicating that PEC FF solution starts to move from a stable to a more unstable form at about pH 5.0. Nguyen et al. [28] found that the adsorption of PEC onto positive liposomes yielded a reproducible increase in particle size and a shift of the zeta potential from positive to negative side, whereas PEC adsorption onto negative liposomes did not render any significant changes probably due to electrostatic repulsion. Furthermore, zeta potential is commonly used also to investigate protein solution/suspension stability [29]. Yin et al. [30] recently studied the surface charge and conformational properties of phaseolin, the major globulin occurring in red kidney beans (*Phaseolus vulgaris L*) [31], and reported that the zeta potential of phaseolin, measured in the absence of NaCl, increased from -44.0 mV at pH 9.4 to 27.9 mV at pH 3.0, the protein isoelectric point occurring at pH 4.2. In addition, zeta potential decrease was observed by increasing NaCl concentration from 0 to 200 mM at pH below the isoelectric point. Table 1 summarizes the results of our recent experiments concerning the changes in the zeta potential values of phaseolin when the protein was dissolved in 125 mM NaCl at 25°C as a function of pH. Also in this case the zeta potential was observed to increase (from -13.80 mV at pH 8.0 to 3.48 mV at pH 2.0). Finally, Table 1 reports also the changes in the zeta potential values measured with the mixture of proteins extracted from BV seeds. The detected zeta potential of BVPC was found to increase from -17.00 mV at pH 8.0 to 23.50 mV at pH 2.0, indicating that the electrostatic repulsion pattern may be gradually modified as a result of the gradual deprotonation of carboxyl groups and protonation of the amino groups of each BV protein composing the mixture. Further preliminary experiments (unpublished data) allowed to correlate film mechanical properties with the increase of the negative zeta potential of BV protein/PEC nano-complexes occurring in the FF solution. Our findings are in agreement with previous studies [32,33] explaining the observed increase of flexibility of both polysaccharide/essential oil and whey protein/gelatin composite films by the influence of the nano-emulsion/solution electrical charge. In this respect, it was suggested that the repulsive forces among macromolecules of the same charge could increase the distance between the polymer chains and, consequently, determine a plasticizing effect in the case of charged polymeric film structures.

| | Zeta potential (mV) | | |
|---|---|---|---|
| ***pH*** | ***PEC*** | ***Phaseolin*** | ***BVPC*** |
| 8 | -37.50 ± 1.06 | -13.80 ± 0.21 | -17.00 ± 0.01 |
| 7 | -38.50 ± 1.13 | -13.02 ± 0.70 | -15.80 ± 1.20 |
| 6 | -37.60 ± 1.13 | -11.20 ± 1.27 | -17.90 ± 0.56 |
| 5 | -30.90 ± 1.82 | -6.39 ± 0.64 | -9.30 ± 0.03 |
| 4 | -27.30 ± 1.48 | -6.13 ± 0.06 | 9.33 ± 0.07 |
| 3 | -10.55 ± 1.41 | 0.44 ± 0.13 | 13.50 ± 0.21 |
| 2 | -6.84 ± 1.48 | 3.48 ± 0.62 | 23.50 ± 0.21 |

**Table 1:** Zeta potential of PEC, phaseolin and BVPC measured at different pHs.

In conclusion, zeta potential measurement is proposed as an useful tool for assessing biopolymer interactions in each specific FF solution/suspension, before casting, during the preparation of bio-based edible films. When zeta potential value results either less negative than -10mV or lower than 10mV, the particle solution/suspension is extremely unstable and, in turn, the physical properties of the derived edible film would be hard to tailor for specific applications.

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Editorial OMICS International

# Stabilization of Charged Polysaccharide Film Forming Solution by Sodium Chloride: Nanoparticle Z-Average and Zeta-Potential Monitoring

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Different natural biopolymers are becoming the issue of an expanding number of studies reporting their potential applications in food, pharmaceutical and cosmetic technologies, as well as in tissues engineering [1-4]. In this respect, the utilization of charged polysaccharides like chitosan (CH) or pectin (PEC) appears to be one of the most interesting way in manufacturing of biodegradable new materials [4].

CH is a cationic biopolymer under pH 6.0 and derives from the N-deacetylation of chitin, the natural polysaccharide responsible for the hardness of the crustacean shells [5]. CH is a copolymer consisting of β-(1–4)-2-acetamido-D-glucose and β-(1–4)-2-amino-D-glucose units with the latter usually exceeding 60%. Due to its non toxic, biodegradable and biocompatible properties, CH is extensively used in the biomedical area as wound healing material and for sustained release of drugs [6]. Moreover, CH-based edible films and coatings allow extending and preserving shelf life of different foods [7-11].

PEC is, conversely, an anionic polysaccharide at pH higher than 4.6, widely occurs in fruits and vegetables and is generally used in food and pharmaceutical industries as gelling and thickening agent [12]. Mainly extracted from citrus peel and apple pomace, PEC represents a heterogeneous group of acidic biomacromolecules and, because of its gelling characteristics, as well as bioadhesivity, biocompatibility and non toxicity properties, it is also a promising biopolymer able to act as a drug delivering vehicle. In fact, PEC has been successfully used in mucoadhesive systems to increase the retention time of the dosage form in the gastrointestinal tract, thus enhancing drug absorption after oral administration [12,13]. Furthermore, the degree of esterification or amidation of the galacturonic acid residues inside the macromolecule is often used to characterize the different PECs and defines the different properties of the various polysaccharide preparations [14].

Dinamic light scattering technique is a particularly useful tool for exploring homo- or hetero-association of biomacromolecules like proteins and polysaccharides, and allows to study the influence on it of different experimental factors, such as the change in pH or ionic strength of the solution [15]. Micro- and nano-particle charge is one of the main factors determining the physical stability of both emulsions and suspensions, as well as solutions, and such physical stability can be monitored by measuring particle "zeta-potential" [16]. When all the particles have a high either negative or positive zeta-potential value (> ± 30 mV), they will repel each other and, as a consequence, the emulsions, suspensions or solutions become more stable. By contrast, whether the zeta-potential is in the range between 0 and ±10 mV, the tendency to flocculation dramatically increases [17]. Zeta-potential cannot be measured directly but it can be calculated by using theoretical models and experimental determination of the motion of dispersed particles. The charge intensity of the polysaccharide occurring in the film forming solution (FFS), as well as the hydrodynamic diameter of its particles (Z-average), may provide crucial information on how the biopolymer will behave during the process of polyelectrolyte multilayer construction, being also useful both in the choice of the biomaterial to be used and in the establishment of the more suitable experimental conditions. Among these, FFS pH value and ionic strength are critical parameters governing the polymer film formation.

In the present editorial we report the results of our studies devoted to determine the zeta-potential and the Z-average of FFSs prepared with two different charged polysaccharides, CH and PEC, in the presence of different sodium chloride concentrations, in order to obtain useful information on FFS stability for consequent possible multilayer nanostructure preparation.

2 g of CH, obtained as previously described [18], were dissolved in 100 mL of 0.1 N HCl and the solution, after overnight stirring, was diluted to 1 mg/mL and its pH adjusted to pH 3.5 by using 0.1 N HCl. In addition, 1.0 g of citrus peel low-methylated (7%) PEC (Aglupectin USP from Silva Extracts srl, Gorle, BG, Italy) was dissolved in 100 mL of deionized water and the solution was stirred until PEC was completely solubilized. Then, the solution was diluted to 1 mg/mL and the pH adjusted to pH 7.0 by using 1.0 N HCl. FFS zeta-potential, particle size and conductivity were determined by a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) with automatic titrator unit (MPT-2). The two different FFSs, containing either CH or PEC, were automatically added with different amounts of sodium chloride (0-0.24%, w/v) under constant stirring at 25°C. The device was equipped with a helium-neon laser of 4 mW output power operating at the fixed wavelength of 633 nm (wavelength of laser red emission). The instrument software programme calculated the zeta-potential through the electrophoretic mobility by applying a voltage of 200 mV using the Henry equation. All the results are reported as mean ± standard deviation.

Different NaCl concentrations were added to the CH and PEC FFSs to evaluate the influence of the increased ionic strength and electrical conductivity (EC) on both Z-average and zeta-potential of the polysaccharide particles. Our findings indicate that CH FFS showed highly positive values (+68 mV) of zeta-potential, which dramatically decreased to around (+47 mV) in the presence of very low NaCl concentration (0.02 %) (panel A of Figure 1) and at an almost unvaried FFS EC (around 0.5 ms/cm) (panel A of Figure 2). The increases in FFS saline concentrations until 0.24%, with concomitant enhancement of EC to 4.5 ms/cm, did not determine significant further decreases in the CH FFS zeta-potential.

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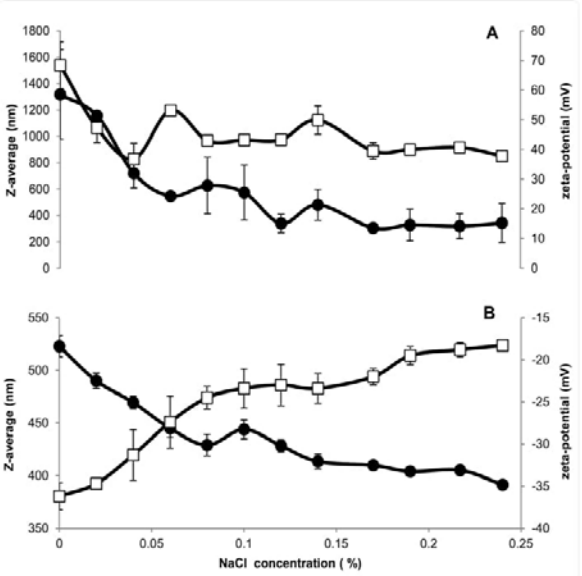


**Figure 1:** Influence of different concentrations of NaCl on CH (panel A) and PEC (panel B) Z-average (●) and zeta-potential (□).

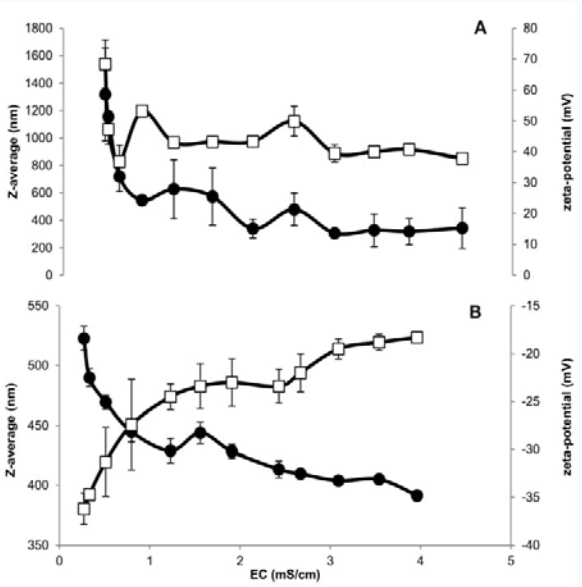


**Figure 2:** Influence of different conductivity on CH (panel A) and PEC (panel B) Z-average () and zeta-potential (□).

Conversely, PEC FFS exhibited a negative zeta-potential value in the absence of NaCl (-36 mV), which progressively decreased until to -23 mV with the increase of NaCl concentration up to 0.1% (EC, 1.5-2.0 ms/cm) (panel B of Figures 1 and 2). Further enhancements in NaCl concentration until 0.24% (EC, 4.0 ms/cm) led to a zeta-potential value, however, never lower than -18 mV. It is worthy to note that these results are in agreement with previous data reported by McConaughy et al. [19] showing that the increase of galacturonate solution ionic strength significantly lowered polysaccharide zeta-potential value.

Ionic strength increase was shown to influence also Z-average of both CH and PEC nanoparticles. In fact, CH Z-average of 1350 nm resulted halved at 0.04% NaCl concentration (EC, 0.6 ms/cm), further decreasing to 300-350 nm in the range of NaCl between 0.12-0.24% (EC, 2-4.5 ms/cm) (panel A of Figures 1 and 2). In contrast, PEC particle Z-average value of 523 nm progressively decreased with the increase of NaCl concentration, reaching the minimum Z-average (~400 nm) in the range of 0.15-0.24% NaCl (EC, 2.5-4.0 ms/cm) (panel B of Figures 1 and 2).

In conclusion, the reported data suggest that a concentration of NaCl in FFS higher than 0.04% (EC>0.6 ms/cm) should be used to stabilize CH nanoparticles, whereas a concentration of NaCl close to 0.06% (EC ~0.8 ms/cm) seems appropriate for PEC nanoparticle stabilization. In fact, we obtained under these experimental conditions the best compromise with a monitored both highly positive (> +40 mV for CH) or negative (~ -27 mV for PEC) zeta-potential and the lower polysaccharide Z-average (<400 nm for CH and ~450 nm for PEC, respectively). More in general, our findings outline the existence of a relationship between the properties of the charged polysaccharides and the ionic strength and conductivity of their environment, thus providing insight on how it is possible to monitor biopolymer FFS stability to obtain multilayered films in view of their potential application in food, pharmaceutical and cosmetic industries.

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## 2.3 Film preparation and characterization

### 2.3.1 Casting

Fig. 8 illustrates the procedure of film preparation, starting by the different FFSs obtained as described in 2.2. Each FFS (50 mL) was casted onto a polystyrene Petri dish (8 cm diameter) and dried at 25 °C and 45% RH for 48 h.

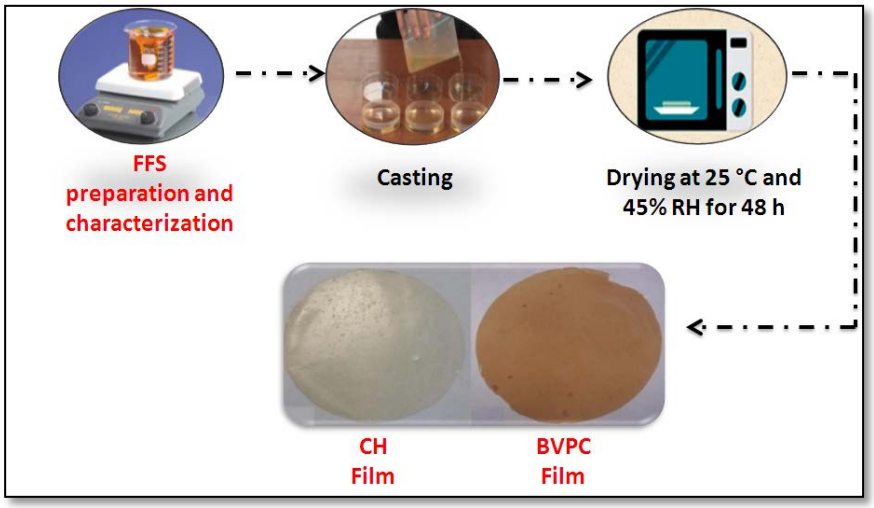


***Fig. 8. Film preparation procedure.***

The obtained film was then peeled from the casting surface and stored at 25 °C and 50% RH into a dessicator over a saturated solution of $Mg(NO_3)_2.6H_2O$ before being tested. Finally, each film was characterized for morphological, physicochemical and antimicrobial properties and for its ability to be heat-sealed and to wrap cheese products.

### 2.3.2 Morphology

Film surface and cross section ultrastructure were analyzed by using a Scanning Electron Microscope (SEM). Films were cut using scissors, mounted on stub and sputter-coated with platinum-palladium (Denton Vacuum Desk V), before observation with Supra 40 ZEISS (EHT= 5.00 kV, detector inlens).

Film surface morphology was studied using an Atomic Force Microscope (AFM) (Bruker, model Nanos). A sharpened $Si_3N_4$ cantilever, with a spring constant of 0.2 N/m and a V-shaped tip 450 µm long, was positioned over each sample and images (42 × 42 µm) under ambient conditions were obtained.

### 2.3.3 Physicochemical analysis

#### 2.3.3.1 Thickness measurements

Film thickness was measured with a micrometer model HO62 (Metrocontrol Srl, Casoria (Naples, Italy) at five random positions over the film area. Values are mean ± standard deviation (SD) of five replicates.

#### 2.3.3.2 Mechanical properties

Film tensile strength (TS), elongation at break (EB) and Young's module (YM) were measured by using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). Film samples were cut, using a

sharp scissors, into 10 mm wide and 40 mm length strips equilibrated for 2 h at 50% ± 5% RH and 23 ± 2 °C in an environmental chamber. Five specimens of each film type were tested (1 KN load and 1 mm/5 min speed), as previously reported (ASTM D882-97, 1997).

#### 2.3.3.3 Barrier properties

Film permeabilities to $O_2$ (ASTM D3985-05, 2010) and $CO_2$ (ASTM F2476-13, 2013) and WV (ASTM F1249-13, 2013) were determined in triplicate for each film by using a TotalPerm apparatus (Extrasolution s.r.l., Pisa, Italy). Aluminium masks were used to reduce film test area to 5 $cm^2$, whereas the testing was performed at 25 °C under 50% RH.

#### 2.3.3.4 FT-IR spectroscopy

The analysis of film structural links was performed using FT-IR measurements on a Bruker model ALPHA spectrometer, equipped with attenuated total reflectance (ATR) accessory. Spectra were obtained by averaging 24 scans over the spectral range of 400 to 4000 $cm^{-1}$. Data analysis of each film was performed with Origin Pro 8.6 program (Origin Lab, Northampton, MA, U.S.A.).

#### 2.3.3.5 Heat sealability

The heat sealability of the different biomaterials was examined by using an automatic heat sealer (MAGIC VAC® AXOLUTE Mod: P0608ED, Italy) equipped with a vacuum pump (60 cm/Hg -0.80 bar /11.6 PSI). All the samples were cut into strips of 5x2.5 cm, and one strip was placed onto another strip of the same sample (Fig. 9).

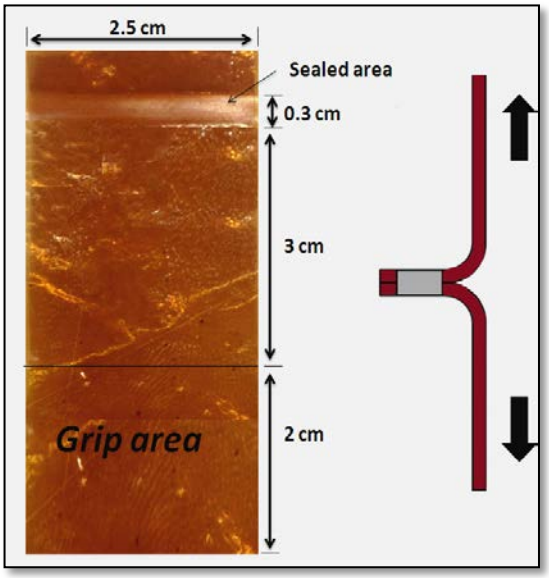


***Fig. 9. Visualization of the film sealing procedure.***

All the samples were previously conditioned at 25 °C and 50 ± 5% RH for 48 h and few drops of distilled water were dispersed onto the sealing areas (0.3 cm) before inserting the two strips into the heat sealer. Sealing temperature, dwell time and pressure were automatically assessed and the resulting welds were analyzed according to ASTM E88-07a (ASTM, 2007) with an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). The seal strength (N/m) was calculated by dividing the maximum peak force to the film width.

### 2.3.4 Antimicrobial activity

*Salmonella typhimurium* ATCC 14028 was grown in tryptic soy broth (TSB, Becton Dickinson Difco, Franklin Lakes, NJ) and on tryptic soy agar (TSA; Oxoid Ltd., Hampshire, UK). In all the experiments, bacteria were inoculated and grown overnight in TSB at 37 °C and, the day after, were transferred into a fresh TSB tube and grown to mid-logarithmic phase. Bacterial cells were then diluted in TSB to approximately $2x10^7$ CFU/mL and inoculated by surface streaking into TSA plates using a swab. 1 $cm^2$ CH film strip was placed onto the center of the inoculated plate and pressed to ensure full contact with the agar surface. Plates were incubated at 37 °C for 24 h and the presence or absence of bacterial growth under the film was evaluated.

## 2.4 Film applications to dairy products

### 2.4.1 Nabulsi cheese preparation

Unsalted (UNC) and salted (SNC) Nabulsi cheeses were prepared from fresh cow milk, obtained from local market (Napoli, Italy), as previously described by Al-Dabbas et al., (2014) with the following modifications: 5 L of fresh milk were tempered to 35 °C and rennet was added; after 60 min the curd was cut and settled for 15 min, and then transferred into cheesecloth that was inside perforated square stainless steel frame, 20x20x2 cm; the curd was then pressed with 25 Kg weight for 2 h and the cheese cut into small blocks (2x2x2 cm) by using a stainless steel cheese slicer. Dry salt (2.5%, w/w of cheese) was dispersed onto the surface of the half of the cheese blocks to prepare the SNC samples.

### 2.4.2 Cheese wrapping

Films prepared both from BVPC FFS containing 42 mM GLY and from CH FFS containing 25 mM GLY were used to wrap both UNC and SNC, after their equilibration for 2 days at 50±5% RH and 23±2 °C in an environmental chamber. The two types of film (7.5×9.5 cm) were heat-sealed by three sides giving rise to an open bag in which each cheese block was placed. Then, vacuum was applied to discard all air inside the bag and also the fourth side of the films was heat-sealed by using a professional vacuum packing machine (MAGIC VAC® AXOLUTE Mod: P0608ED, Italy) equipped with a vacuum pump (60 cm/Hg -0.80 bar /11.6 PSI). All the wrapped cheese samples were finally stored at 4 °C for different times. LDPE was used as wrapping control films.

### 2.4.3 pH variation

The pH of unwrapped and wrapped cheeses was measured according to AOAC procedures (AOAC, 1990). Cheese samples (10 g) were homogenized, at the maximum speed for 5 min, in 100 mL of distilled water by an Ultra-Turrax T8 homogenizer (IKA-WerkeGmbH, Staufen, Germany). After stirring for 15 min, the homogenates were centrifuged at 2500xg for 10 min and the obtained supernatants were filtered through both cotton lint and paper filter. Measurement of pH was carried out using a digital pH meter, model 211 (Hanna instruments, PBI International).

### 2.4.4 Titratable acidity

Titratable acidity (TA) of unwrapped and wrapped cheeses was measured according to Di Pierro et al. (2011). Cheese samples (10 g) were added to 50 mL of distilled water at 40 °C and homogenized for 5 min at the maximum speed with an

Ultra-Turrax T8 homogenizer. After centrifugation at 3000x g for 10 min, the supernatants were filtered. Then, 5 drops of phenolphthalein (1% in ethanol) were added to 25 mL of supernatant and TA was determined by addition of 0.1 N NaOH until the solution became pink. TA, expressed as milliequivalent/100 g, was calculated as follow:

$$TA = \frac{a \times b \times 100}{c}$$

where a and b correspond to the concentration and the volume of titrant solution, respectively, and c refers to the weight (g) of the analyzed sample.

#### 2.4.5 Cheese weight loss

Cheeses were individually weighed on an automatic analytical balance (ORMA s.r.l. BCA310 S. Milano) with a precision of ± 0.0001 g, at the beginning and during the storage period, and the percentage weight loss from the initial cheese weight was calculated by the following equation:

$$\text{weight loss (\%)} = \frac{W1 - W2}{W1} \times 100$$

where W1 is the cheese initial weight and W2 is the cheese weight after storage. Three experiments performed with each cheese sample were detected.

### 2.5 Statistical analysis

JMP software 10.0 (SAS Institute, Cary, NC, USA), was used for all statistical analyses. The data were subjected to analysis of variance, and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $p<0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1 Plasticizing effects of polyamines in protein-based films

Plasticizers generally improve the processability of the different biomaterials. Therefore, as the bioplastic industry is continuously growing, the demand for new kinds of plasticizers endowed with specific characteristics and performances compatible with each single bioplastic is growing in parallel. GLY is commonly used as plasticizer in different hydrocolloid films and coatings, since it increases the free space inside the film matrix following hydrogen bond formation between the polymer chains. This interaction generally increases film permeability and decreases film mechanical properties. BVPC films in the absence of high concentrations of a plasticizer like GLY are brittle, difficult to manipulate, and, consequently, impossible to study. Moreover, the addition of high amounts of GLY were shown unable to give rise to handleable films even when BV proteins were previously denatured by heat treatment at 80 °C for 30 min. The attention was, thus, focused on the possible plasticizing effects of positively charged small mol. weight compounds, such as the aliphatic polyamines, already shown to be able to plasticize PEC-based films (Esposito et al., 2016). To this aim, BVPC was used to obtain protein-based SPD plasticized films both in the absence and presence of GLY.

In a first published article (Int. J. Mol. Sci., 18, 1026, 2017) it has been demonstrated the specific ability of SPD to act as an effective cationic plasticizer also for protein-based films. The presence of appropriate SPD amounts allowed to obtain easily manipulable films at low GLY concentrations and even in the absence of GLY. Moreover, in a second published article (Int. J. Mol. Sci., 18, 2658, 2017) it was demonstrated the SPD positive influence on the morphological, mechanical, and barrier properties of films prepared from both native and heat-denatured BV proteins at different concentrations of GLY and pH values.

One of the main technical challenges in food processing and storage today is the development of tailor-made coating materials with appropriate characteristics according to the specific requirements of the various fresh or processed foodstuffs. Our findings suggest that the use of SPD or of a combination of the polyamine with a primary plasticizer such as GLY as additives of protein-based films, may open new possibilities to generate hydrocolloid edible films endowed with different mechanical and barrier properties specifically suitable for the coating of different food products. Lower film EB and higher YM values were detected in the presence of the polyamine without or with small amounts of GLY. The obtained results suggest that SPD not only acts as a plasticizer itself by ionically interacting with proteins, but that it also facilitates GLY-dependent reduction of the intermolecular forces along the protein chains, consequently improving the film flexibility and extensibility. Thus, SPD may be considered not only as a primary, but also as a secondary plasticizer because of its ability to enhance GLY plasticizing performance. Moreover, SPD reduce the BVPC film thickness from 108 µm with GLY alone to 82 µm this is due to the ionic interaction that occur between SPD and protein (Fig. 10).

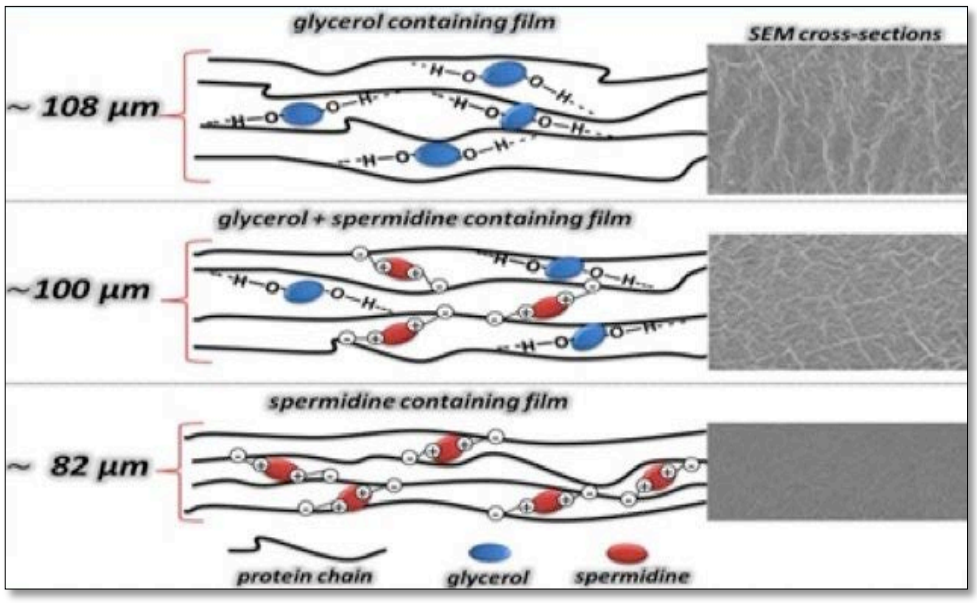


***Fig. 10. Proposed model of the effects of GLY or SPD alone, and of GLY together with SPD, on the BVPC film thickness (SEM images of the cross-sections of each film are reported).***

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# Plasticizing Effects of Polyamines in Protein-Based Films

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**Abstract:** Zeta potential and nanoparticle size were determined on film forming solutions of native and heat-denatured proteins of bitter vetch as a function of pH and of different concentrations of the polyamines spermidine and spermine, both in the absence and presence of the plasticizer glycerol. Our results showed that both polyamines decreased the negative zeta potential of all samples under pH 8.0 as a consequence of their ionic interaction with proteins. At the same time, they enhanced the dimension of nanoparticles under pH 8.0 as a result of macromolecular aggregations. By using native protein solutions, handleable films were obtained only from samples containing either a minimum of 33 mM glycerol or 4 mM spermidine, or both compounds together at lower glycerol concentrations. However, 2 mM spermidine was sufficient to obtain handleable film by using heat-treated samples without glycerol. Conversely, brittle materials were obtained by spermine alone, thus indicating that only spermidine was able to act as an ionic plasticizer. Lastly, both polyamines, mainly spermine, were found able to act as "glycerol-like" plasticizers at concentrations higher than 5 mM under experimental conditions at which their amino groups are undissociated. Our findings open new perspectives in obtaining protein-based films by using aliphatic polycations as components.

**Keywords:** polyamines; spermidine; spermine; edible film; bitter vetch; plasticizer

## 1. Introduction

The interest in protein-based films and coatings has increased considerably over recent years due to their advantages with respect to the conventional petroleum derived materials and to other biodegradable materials made of polysaccharides and/or lipids [1,2]. Protein films generally exhibit better gas barrier and mechanical properties than polysaccharide films but both generally possess poor water vapor barrier characteristics as a consequence of their hydrophilic nature [3–7].

One of the main additives of all bio-based edible films is the plasticizer, which is generally a small molecule such as glycerol (GLY) or sorbitol. The plasticizer is able to improve film extensibility and flexibility by decreasing the attractive intermolecular forces and increasing both free volume and chain mobility [8]. Our recent studies demonstrated the effectiveness of the aliphatic diamine putrescine, as well as of the polyamine (PA) spermidine (SPD), as alternative plasticizers for pectin edible films [9]. The PAs SPD and spermine (SPM) are reported to be essential components of all living cells [10–12] because they are involved in cellular growth and proliferation [13,14], the differentiation of immune cells and the regulation of inflammatory reactions [15,16]. Normal levels of PAs are maintained not

only by endogenous and intestinal microorganism biosynthesis, but also by their exogenous supply through the diet [13,17,18] which provides the largest amount. Moreover, since the biosynthesis of PAs was shown to decrease with age, their content in the diet seems to be important in maintaining the full functionality of the different tissues in the elderly [19]. On the other hand, the very low toxicity of PAs, attested by an acute oral toxicity of 0.6 g/kg in rats [20] and by an $LD_{50}$ value higher than 2 g/kg in mice [21], indicated their possible addition to film forming solutions (FFSs) in obtaining safe edible biobased materials.

Therefore, attention was focused on the possible effects of both SPD and SPM not only on the functionality of the polysaccharide-based films but also as plasticizers of the protein-based films, in comparison with a well-known and largely used plasticizer such as GLY. To this aim, protein concentrate, obtained from bitter vetch (*Vicia ervilia*; BV) seeds and recently proposed as a promising source for both edible films and biodegradable containers [22–24], was used as raw material of protein-based film forming solution. BV is an ancient grain legume crop, originated in the Mediterranean region, which can be found today in many countries around the world. This annual Vicia genus shows several favorable features, such as having high yields and being a cheap protein source resistant to drought and insects. Not only for these reasons, but also because of its high nutritional value, capacity of nitrogen fixation and ability to grow in poor soils, BV is widely cultivated for forage [25].

Here we present data showing the specific ability of SPD to act as an effective cationic plasticizer for protein-based edible films, both in the absence and presence of GLY.

## 2. Results and Discussion

Since the ability of both diamine putrescine and triamine SPD to act as plasticizers of polysaccharide-based films has been recently demonstrated [9], we were stimulated to investigate the ability of the PAs to play the same role in protein-based films. Therefore, a protein mixture, extracted from BV seeds previously characterized for their chemical composition and capacity to prepare biodegradable films and containers, was selected as biomaterial source [24]. The obtained BV protein concentrate (BVPC) was always used either after its heat-treatment at 80 °C for 30 min, to obtain denatured proteins, or after its mild incubation at 25 °C to preserve native protein structures. First of all, the changes of zeta potential and Z-average of the nanoparticles occurring in the BVPC FFSs were investigated at different pH values and different SPD and SPM concentrations, both in the absence and presence of GLY.

As shown in Figure 1, high GLY concentrations (42 mM = 50% *w*/*w* protein) alone were found to significantly increase the negative zeta potential of all BVPC FFSs over pH 6.0 in the absence of PAs, probably as a consequence of the intermolecular hydrogen bonds formed by GLY with the electronegative atoms occurring in numerous reactive groups of BV proteins. Conversely, 5 mM SPD, present in BVPC FFSs during their heat-treatment both at 25 and 80 °C, always markedly decreased the negative zeta potential between pH 6.0 and 8.0, as a result of ionic interactions between the positively charged SPD amino groups and the BV negatively charged proteins in such pH range. Same results were obtained when 42 mM GLY was added to the FFSs after heat treatments, thus indicating that GLY did not significantly influence the SPD ionic interaction with BV proteins. As shown in Figure 2, the decreased negative zeta potential measured at pH 8.0 was dependent on SPD amount at all the GLY concentrations used and, as reported in Table 1, the SPM effect on zeta potential was even more pronounced than the SPD one, as well as not influenced by the presence of GLY.

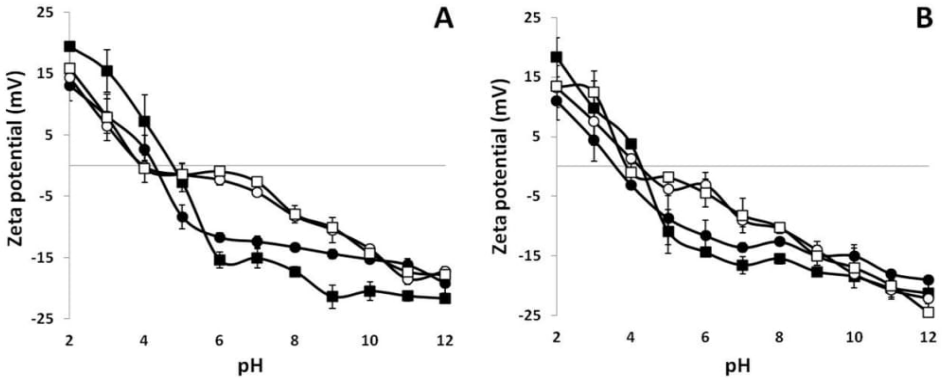


**Figure 1.** Effect of 5 mM spermidine (SPD) on zeta potential of 25 °C-treated (**A**) and 80 °C-treated (**B**) bitter vetch protein concentrate (BVPC) film forming solutions (FFSs) measured at different pH values in the absence or presence of 42 mM glycerol (GLY). The different FFSs contained only BVPC (●), BVPC + GLY (■), BVPC + SPD (○), BVPC + SPD + GLY (□). The results are expressed as mean ± standard deviation. Further experimental details are given in the text.

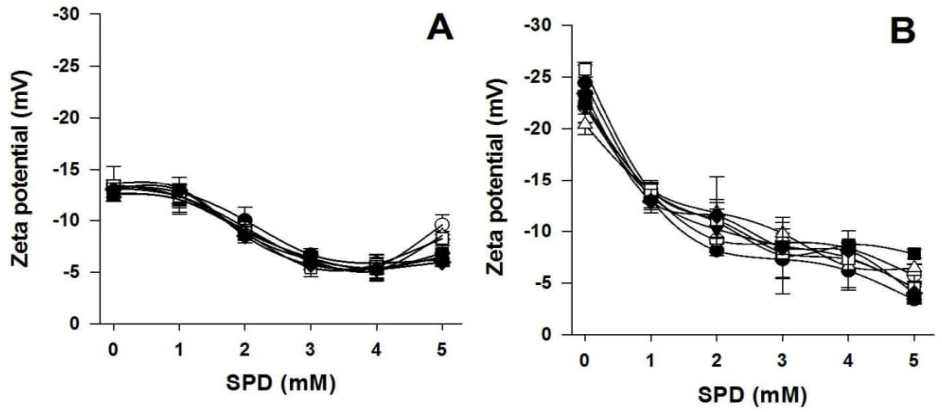


**Figure 2.** Effect of different SPD concentrations on 25 °C-treated (**A**) and 80 °C-treated (**B**) BVPC FFS zeta potential measured at pH 8.0 in the absence or presence of GLY. The different FFSs were prepared in the absence (●) or presence of 4 (○), 8 (▼), 17 (△), 25 (■), 33 (□) and 42 mM (♦) GLY. The results are expressed as mean ± standard deviation. Further experimental details are given in the text.

Further experiments were carried out to determine the Z-average of nanoparticles occurring in the FFSs when BVPC was pretreated for 30 min either at 25 or 80 °C. The results reported in Tables 2 and 3 indicate that, in both cases, the size of BV protein particles were always <1000 d.nm (and not significantly different) in the range of pH over 8.0, also in the presence of either SPD or SPM, their amino groups being uncharged and unable to ionically interact with BV proteins. Conversely, when PAs became positively charged, i.e., under pH 8.0, the protein mean particle size was found to increase over 1000 d.nm, as a result of PA/protein ionic bindings. Superimposable data were obtained both in the absence and presence of GLY. Therefore, these results confirm the ionic interaction of aliphatic PA amino groups with the negative charges occurring on the amino acid lateral chains of BV proteins under pH 8.0, as well as they indicate that the presence of GLY does not significantly influence PA/BV protein binding. In addition, when only GLY was present in the sample, a BV protein mean particle size >1000 d.nm was detected under pH 6.0, as a consequence of the formation of macromolecular aggregates in proximity of protein isoelectric points. In fact, GLY is a small

molecule able to interact only with protein proton acceptors by intermolecular hydrogen bonds and BV aggregates were obtained under pH 6.0 also in the absence of GLY.

**Table 1.** Effect of different concentrations of polyamines (PAs) on 25 °C-treated (**A**) or 80 °C-treated (**B**) BVPC FFSs zeta potential measured at either pH 8.0 or 11.0 in the absence and presence of 42 mM GLY *.

| | Addition | Zeta Potential (mV) | | | |
|---|---|---|---|---|---|
| | | pH 8.0 | | pH 11.0 | |
| | | −GLY | +GLY | −GLY | +GLY |
| **A** | none | −13.30 ± 0.68 | −13.00 ± 0.69 | −23.5 ± 1.01 | −20.5 ± 1.56 |
| | +2 mM SPD | −10.00 ± 1.25 § | −8.48 ± 0.45 § | −22.2 ± 1.79 | −21.8 ± 0.52 |
| | +3 mM SPD | −6.68 ± 0.55 § | −5.26 ± 0.86 § | −23.5 ± 2.42 | −23.6 ± 1.34 |
| | +5 mM SPD | −3.92 ± 0.41 § | −3.57 ± 0.64 § | −22.1 ± 0.85 | −22.3 ± 1.22 |
| | +2 mM SPM | −5.09 ± 1.16 § | −6.13 ± 0.27 § | −21.1 ± 1.83 | −22.9 ± 2.76 |
| | +3 mM SPM | −3.40 ± 0.77 § | −4.00 ± 0.60 § | −23.6 ± 0.84 | −24.1 ± 2.97 |
| | +5 mM SPM | −1.80 ± 0.81 § | −2.20 ± 0.92 § | −20.5 ± 1.20 | −21.4 ± 1.70 |
| **B** | none | −14.40 ± 1.72 | −13.40 ± 1.21 | −22.0 ± 1.92 | −21.9 ± 1.08 |
| | +2 mM SPD | −8.13 ± 0.21 § | −11.50 ± 3.86 | −24.9 ± 3.07 | −25.9 ± 3.93 |
| | +3 mM SPD | −7.27 ± 1.83 § | −8.42 ± 0.65 § | −25.6 ± 2.98 | −24.5 ± 1.40 |
| | +5 mM SPD | −3.40 ± 0.35 § | −4.01 ± 0.93 § | −21.4 ± 2.40 | −22.2 ± 2.01 |
| | +2 mM SPM | −4.69 ± 1.09 § | −7.35 ± 0.72 § | −22.7 ± 2.29 | −26.8 ± 0.70 |
| | +3 mM SPM | −2.34 ± 0.61 § | −2.43 ± 0.74 § | −21.6 ± 0.77 | −21.2 ± 2.22 |
| | +5 mM SPM | −1.83 ± 0.49 § | −1.62 ± 0.65 § | −20.1 ± 1.50 | −20.7 ± 2.40 |

* The results are expressed as mean ± standard deviation. § Significantly different values as compared to the ones obtained under the same experimental conditions in the absence of PAs. Further experimental details are given in the text.

**Table 2.** Effect of 5 mM polyamines (PAs) on Z-average of the nanoparticles contained in the 25 °C-treated BVPC FFS measured at different pH values both in the absence and presence of 42 mM GLY *.

| pH | Z-Average (d.nm) | | | | | |
|---|---|---|---|---|---|---|
| | None | +GLY | +SPD | +SPD/GLY | +SPM | +SPM/GLY |
| 12 | 254.3 ± 14.4 | 272.5 ± 21.7 | 273.8 ± 2.0 | 280.4 ± 4.3 | 262.9 ± 9.3 | 258.5 ± 13.1 |
| 11 | 250.1 ± 13.6 | 283.9 ± 16.2 | 272.7 ± 14.0 | 282.3 ± 17.7 | 263.0 ± 2.3 | 346.8 ± 19.2 |
| 10 | 237.5 ± 14.0 | 270.4 ± 6.3 | 298.3 ± 12.0 | 312.7 ± 12.5 | 243.0 ± 4.8 | 336.1 ± 6.9 |
| 9 | 310.1 ± 23.8 | 292.5 ± 5.7 | 319.9 ± 11.4 | 471.6 ± 65.2 | 305.7 ± 38.6 | 423.4 ± 44.8 |
| 8 | 304.3 ± 44.0 | 289.4 ± 9.6 | 477.3 ± 100.4 | 424.9 ± 55.3 | 501.8 ± 5.6 | 816.7 ± 59.5 |
| 7 | 344.2 ± 30.2 | 358.0 ± 8.4 | >1000 § | >1000 § | >1000 § | >1000 § |
| 6 | 330.5 ± 16.3 | 335.7 ± 43.2 | >1000 § | >1000 § | >1000 § | >1000 § |
| 5 | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § |
| 4 | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § |
| 3 | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § |

* The results are expressed as mean ± standard deviation. § Significantly different values as compared to the ones obtained under the same experimental conditions at higher pH. Further experimental details are given in the text.

**Table 3.** Effect of 5 mM polyamines (PAs) on Z-average of the nanoparticles contained in the 80 °C-treated BVPC FFS measured at different pH values both in the absence and presence of 42 mM GLY *.

| pH | Z-Average (d.nm) | | | | | |
|---|---|---|---|---|---|---|
| | None | +GLY | +SPD | +SPD/GLY | +SPM | +SPM/GLY |
| 12 | 228.9 ± 6.8 | 229.9 ± 5.4 | 242.5 ± 9.0 | 227.7 ± 5.4 | 320.0 ± 10.6 | 303.3 ± 35.5 |
| 11 | 234.5 ± 6.2 | 232.9 ± 1.3 | 229.6 ± 3.0 | 233.4 ± 3.0 | 370.8 ± 66.4 | 426.3 ± 21.6 |
| 10 | 225.4 ± 10.0 | 213.2 ± 3.3 | 210.6 ± 6.1 | 217.8 ± 3.9 | 329.0 ± 7.6 | 394.6 ± 77.2 |
| 9 | 206.6 ± 1.7 | 197.0 ± 1.4 | 187.2 ± 6.3 | 189.6 ± 10.2 | 370.7 ± 4.9 | 374.6 ± 17.0 |
| 8 | 187.9 ± 0.4 | 188.8 ± 1.3 | 227.7 ± 7.0 | 173.4 ± 3.6 | 560.6 ± 11.3 | 680.9 ± 90.0 |
| 7 | 195.4 ± 0.7 | 187.7 ± 8.7 | >1000 § | >1000 § | >1000 § | >1000 § |
| 6 | 179.4 ± 0.9 | 185.4 ± 6.0 | >1000 § | >1000 § | >1000 § | >1000 § |
| 5 | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § |
| 4 | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § |
| 3 | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § | >1000 § |

* The results are expressed as mean ± standard deviation. § Significantly different values as compared to the ones obtained under the same experimental conditions at higher pH. Further experimental details are given in the text.

Figures 3 and 4 showed the results of the experiments carried out by casting the different FFSs prepared in the presence of either SPD or SPM, respectively, at pH 8.0 (i.e., when PAs became positively charged and the mean size of the particles occurring in the FFSs was <1000 d.nm) with the aim to determine the best conditions for obtaining handleable (i.e., not brittle nor sticky and, thus, easily to manipulate) and characterizable films. Panel A of both Figures shows that handleable yellowish films were formed in the presence of GLY alone at a minimal concentration of 33 mM (40%, *w*/*w* protein) by using not denatured BV proteins. Indeed, lower GLY concentrations gave rise to very brittle materials impossible to analyze unless SPD (Figure 3A), and less effectively SPM (Figure 4A) were present in the FFSs. But the most significant result was that handleable films were also obtained by adding SPD alone at a minimal concentration of 4 mM (Figure 3A), thus indicating that such PA is able to completely substitute GLY as a plasticizer not only to obtain polysaccharide–based [9] but also native protein-based films. Conversely, when denatured BV proteins were used, a different picture was recorded (Figure 3B), since quite sticky films were obtained by using GLY alone at all concentrations, whereas 2 mM SPD alone was sufficient to obtain handleable films both in the absence and presence of different GLY amounts. In addition, both panels of Figure 4 clearly indicate that SPM was absolutely unable to replace GLY as plasticizer by using both native and heat-denatured BV proteins, even though its presence reduces the amount of GLY needed to obtain handleable films.

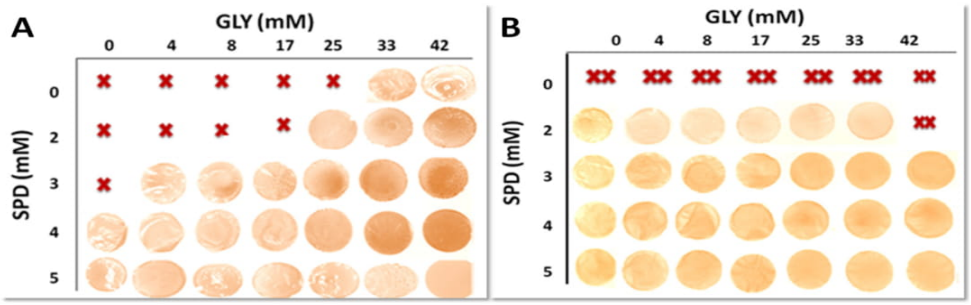


**Figure 3.** Films obtained by casting BVPC FFSs previously incubated for 30 min, either at 25 °C (**A**) or 80 °C (**B**), at pH 8.0 and in the presence of different concentrations of SPD and/or GLY. Not handleable–either brittle (X) or sticky (XX)-and handleable (photo) films are indicated. Further experimental details are given in the text.

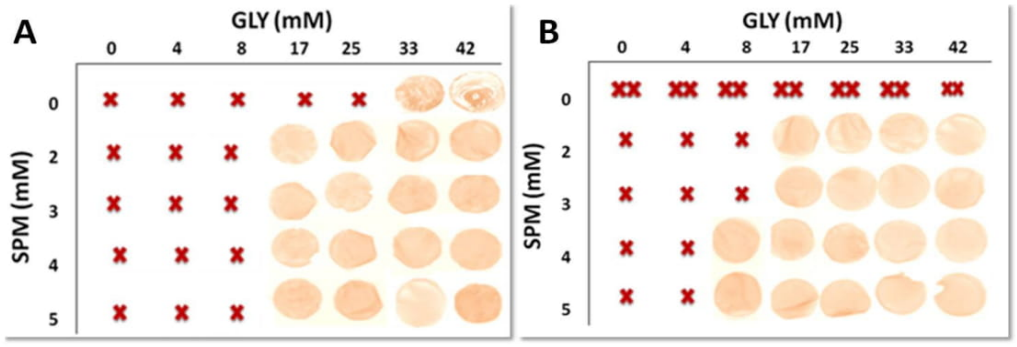


**Figure 4.** Films obtained by casting BVPC FFSs previously incubated for 30 min, either at 25 °C (**A**) or 80 °C (**B**), at pH 8.0 and in the presence of different concentrations of SPM and/or GLY. Not handleable –either brittle (X) or sticky (XX)- and handleable (photo) films are indicated. Further experimental details are given in the text.

In order to confirm the different interactions of SPD and GLY with BV proteins and investigate thermal behavior of the different films, differential scanning calorimetry (DSC) experiments were carried out. Preliminary DSC analyses indicated that both melting and glass transition temperature values of BV protein films prepared in the presence of 5 mM SPD were significantly higher (148 ± 2 °C and 70 ± 10 °C, respectively) than those measured with the films prepared in the presence of 42 mM GLY (130 ± 3 °C and 42 ± 7 °C, respectively), thus suggesting a more rigid structure determined by the ionic PA binding.

Finally, Figure 5 compares native and denatured BV protein films obtained at pH 8.0 and pH 11.0 with different amounts of either SPD or SPM and in the absence or presence of a high concentration of GLY (42 mM). Figure 5B indicates that GLY allowed the formation of handleable films only at pH 11.0, by using heat-denatured BV proteins both in the absence and presence of PAs at all concentrations used. Conversely, the showed results indicate that GLY always allows to produce handleable films at both pH values, in the absence or presence of PAs, when native BV proteins were used, with the only exception of the sample containing 5 mM SPM which, at pH 11.0, gave rise to a very sticky material.

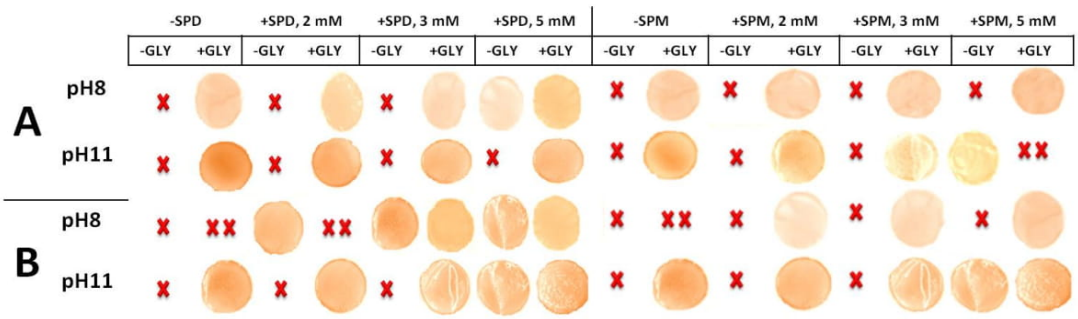


**Figure 5.** Films obtained by casting 25 °C-treated (**A**) and 80 °C-treated (**B**) BVPC FFSs previously incubated at either pH 8.0 or 11.0 in the presence of different concentrations of either SPD or SPM and/or 42 mM GLY. Brittle (X), sticky (XX) or handleable (photos) films are indicated. Further experimental details are given in the text.

On the other hand, whereas lower concentrations of each PA alone resulted in the inability to produce handleable films at pH 11.0, as expected being uncharged over pH 8.0, surprisingly 5 mM of both PAs (SPD with only heat-denatured proteins) produced handleable films at pH 11.0 also in the absence of GLY (Figure 5A,B). These findings could be explained with the formation of hydrogen

bonds between BV proteins and PA undissociated $-NH_2$ groups, in a similar way to those formed by GLY –OH groups. Following this hypothesis both PAs, mainly SPM, could be considered also as "GLY-like plasticizers" when used at high concentrations (>5 mM) and at pH values that do not allow the dissociation of their amino groups. This assumption seems to be confirmed by the measured values of the thickness of films prepared at pH 11.0 in the presence of 5 mM PAs (83.60 ± 1.63 µm for SPD and 92.83 ± 2.31 µm for SPM containing heat-denatured protein films, respectively; 94.33 ± 2.54 µm for SPM containing native protein film) which were in the same order of magnitude as those detected for the GLY-containing films (95.10 ± 2.23 µm and 85.50 ± 2.08 µm for native and heat-denatured protein films, respectively) at pH 11.0. In contrast, as shown in Table 4, the measured thickness values of SPD-containing films prepared at pH 8.0 were always significantly lower in the absence of GLY, increasing with the increase of the concentration of GLY, thus suggesting that the ionic interaction of SPD with BV proteins affected a more compact film network, as compared to the one originating from the GLY-induced hydrogen bonds. A possible model of such a hypothesis is illustrated in Figure 6.

**Table 4.** Thickness (µm) of films obtained by casting 25°C-treated (**A**) and 80°C-treated (**B**) BVPC FFSs previously incubated at pH 8.0 in the presence of different concentrations of SPD and/or GLY *.

| Components | | | | A | B |
|---|---|---|---|---|---|
| GLY, | 42 mM | | | 100.20 ± 1.44 | 74.66 ± 1.50 |
| SPD, | 2 mM | | | 58.12 ± 2.50 § | 56.16 ± 2.87 § |
| | 3 mM | | | 59.43 ± 1.81 § | 57.66 ± 1.63 § |
| | 4 mM | | | 67.66 ± 1.93 § | 59.85 ± 2.51 § |
| | 5 mM | | | 79.75 ± 2.68 § | 61.33 ± 1.75 § |
| GLY, | 25 mM + | SPD, | 2 mM + | 79.50 ± 3.56 § | 74.33 ± 2.65 |
| GLY, | 33 mM | | | 87.83 ± 3.25 § | 76.66 ± 2.25 |
| GLY, | 4 mM + | SPD, | 3 mM + | 57.50 ± 2.08 § | 56.16 ± 1.72 § |
| GLY, | 33 mM | | | 105.67 ± 2.84 | 75.83 ± 3.06 |

* The results are expressed as mean ± standard deviation. § Significantly different values as compared to the ones obtained under the same experimental conditions in the presence of 42 mM GLY. Further experimental details are given in the text.

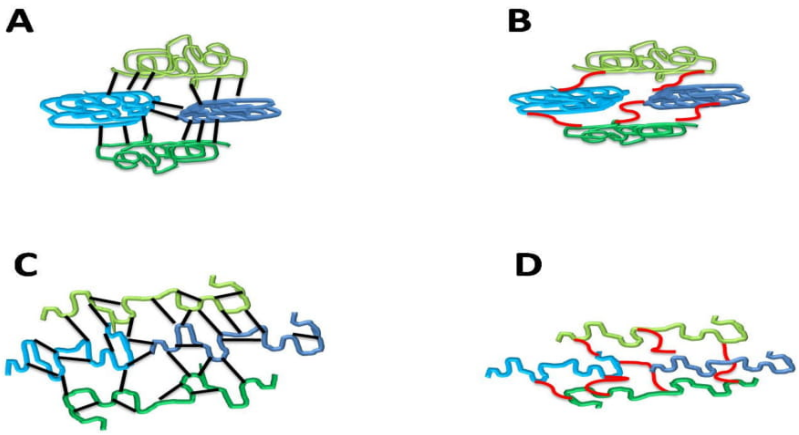


**Figure 6.** Proposed model of hydrogen bond GLY (black bar in **A**,**C**) and ionic SPD (red bar in **B**,**D**) interactions with BV proteins at pH 8.0 following FFS treatment for 30 min at either 25 °C (**A**,**B**, native proteins) or 80 °C (**C**,**D**, denatured proteins). The different kind of binding of GLY (hydrogen) and SPD (ionic) to BV proteins is proposed to be responsible for the different film thickness (minor thickness = major compactness for PA-containing film).

## 3. Materials and Methods

### *3.1. Materials*

Bitter vetch seeds were purchased from a local market in Gallicchio (PZ), Italy. GLY (about 87%) was supplied from the Merck Chemical Company (Darmstadt, Germany), whereas SPD and SPM were from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals and solvents used in this study were analytical grade commercial products.

### *3.2. BV Protein Concentrate Preparation*

Proteins contained in BV seeds were extracted as previously described [22] with some modifications. The seeds were grinded by using a rotary mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at speed of 1300 r.p.m. for 5 min and the flour was dispersed in distilled water (1:10, *w*/*v*), brought at pH 11.0 with 0.1 N NaOH and stirred at medium speed for 1 h at room temperature. After centrifugation at 3200× *g* for 10 min, the supernatant was collected and the pH adjusted at 5.4 by 0.1 N HCl addition to form a precipitate which was then separated by a new centrifugation at 3200× *g* for 10 min. Finally, the pellet was poured and uniformly distributed on a plastic plate and dried at 37 °C and 25% relative humidity. The obtained BVPC was finally grinded and its protein content (77%) determined by the Kjeldahl's method [26], using a nitrogen conversion factor of 6.25.

To prepare the different FFSs, BVPC was dispersed in distilled water (2 g/100 mL) and the pH value was adjusted to pH 12.0 by using NaOH 0.1 N under constant stirring until the powder was completely solubilized. Aliquots of BVPC solution were then added with different concentrations of PAs and incubated for 30 min either at 25 or 80 °C to obtain FFSs containing both native and denatured BV protein samples. Two additional aliquots of BVPC solution were brought at pH 8.0 and 11.0, respectively, by 0.1 N HCl and then incubated in the presence of PAs for 30 min either at 25 or 80 °C. Where indicated, different concentrations of GLY were added to the obtained FFSs at the end of incubation.

### *3.3. FFS Zeta Potential and Mean Hydrodynamic Diameter Determination*

Zeta potential and mean hydrodynamic diameter (Z-average) of the BVPC FFSs prepared at pH 12.0, containing or not high concentrations of PAs and/or GLY, were titrated automatically from pH 12.0 to pH 2.0 as previously described [5], by measuring the dynamic light scattering with a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) using a He–Ne laser (wavelength of 633 nm) and a detector angle of 173°. The effect of different concentrations of PAs and GLY on both zeta potential and Z-average of FFSs were also studied with BVPC FFSs at pH 8.0 and 11.0 immediately after their preparation to prevent possible alterations in molecular interaction during storage [27].

### *3.4. BVPC Film Preparation*

The same volumes (50 mL) of all the different FFSs, containing or not PAs and/or GLY, were poured onto 8 cm diameter polystyrene Petri dishes (7.8 mg BV proteins/cm$^2$) and finally allowed to dry in an environmental chamber at 25 °C and 45% RH for 48 h. The handleable dried films were peeled intact from the casting surface and their thickness was immediately afterwards measured with a micrometer (Electronic digital micrometer, DC-516, sensitivity 0.001 mm) at different positions for each film sample to prevent possible film structural changes [27].

### *3.5. Differential Scanning Calorimetry Analysis*

Thermal features of BV protein films were investigated by DSC (Perkin Elmer DSC-7) in which film samples of 6.0 mg were equilibrated at 25 °C for 2 min and heated to 240 °C at a rate of 20 °C/min.

### *3.6. Statistical Analysis*

The data were subjected to statistical analyses by JMP software 5.0 (SAS Institute, Cary, NC, USA) and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $p < 0.05$.

## 4. Conclusions

Zeta-potential and Z-average characterization of native and heat-denatured bitter vetch protein particles occurring in film forming solutions in the presence of the polyamines spermidine and spermine demonstrated that spermidine is able, specifically, to act as a cationic plasticizer in obtaining protein-based films. Conversely, spermine was found to only lower the minimal amount of glycerol needed to act as plasticizer by interacting with proteins through hydrogen bonds. All the spermidine containing films exhibited a significantly reduced thickness compared to the glycerol-plasticized ones, suggesting the formation of more compact protein networks due to ionic interactions between the positively charged polyamine and the protein negative charges. In addition, both polyamines, mainly spermine, showed to be able to act as "glycerol-like" plasticizers when their amino groups are undissociated.

The present findings open new perspectives in the preparation of a variety of protein-based edible films by using polyamines as plasticizers.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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# Tuning the Functional Properties of Bitter Vetch (*Vicia ervilia*) Protein Films Grafted with Spermidine

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**Abstract:** Bitter vetch protein films containing positively charged spermidine, alone or with low amounts of glycerol, showed high tensile strength that progressively decreased by increasing the plasticizer concentration. Accordingly, lower film elongation at break and higher Young's module values were detected in the presence of the polyamine without or with small amounts of glycerol. These data suggest that spermidine not only acts as a plasticizer itself by ionically interacting with proteins, but that it also facilitates glycerol-dependent reduction of the intermolecular forces along the protein chains, consequently improving the film flexibility and extensibility. Thus, spermidine may be considered not only as a primary, but also as a secondary plasticizer because of its ability to enhance glycerol plasticizing performance. Such double behavior of the polyamine was confirmed by the film permeability tests, since spermidine increased the barrier properties to gases and water vapor, while glycerol emphasized this effect at low concentrations but led to its marked reversal at high concentrations. Film microscopic images also substantiated these findings, showing more compact, cohesive, and homogeneous matrices in all spermidine-containing films.

**Keywords:** edible film; food coating; plasticizer; spermidine; glycerol; *Vicia ervilia*

## 1. Introduction

Substantial advances have been made over the last two decades in the field of biodegradable polymers, mostly derived from renewable natural resources, to produce bioplastics with features similar to those typical of oil-based materials [1]. In particular, protein-based edible films and coatings have attracted an increasing interest in recent years since they might be used to protect pharmaceuticals or improve the shelf life of different food products [2]. These biomaterials are generally first evaluated for their mechanical and barrier properties as a function of different types and concentrations of plasticizers, generally small and nonvolatile organic additives used to increase film extensibility and reduce its cristallinity, brittleness, and water vapor permeability. Plasticizers normally act by decreasing the intermolecular forces along the polymer chains, thus reducing the relative number of polymer–polymer contacts and producing a decrease in the cohesion and tensile strength, thereby increasing the flexibility of the film by allowing its deformation without rupture [3–5]. Consequently, plasticizers generally improve the processability of the different biomaterials. Therefore, as the bioplastic industry is continuously growing, the demand for new kinds of plasticizers endowed with specific characteristics and performances compatible with each single bioplastic is growing in parallel [4,5].

In the attempt to refine the mechanical and barrier properties of hydrocolloid biopolymers, the effect of the aliphatic polyamines spermidine (SPD) and spermine on both polysaccharide- and

protein-based films was recently investigated [6,7]. Polyamines are low-molecular-weight polycations, widely distributed in nature and able to mimic the action of divalent ions like $Ca^{2+}$ both in vitro and in vivo [8]. Although the role of polyamines has been associated with aging, various metabolic disorders, cell growth, and cancer, their precise biochemical function is one of the remaining mysteries of molecular cell biology [9]. The very low toxicity of SPD, attested by an acute oral toxicity of 600 mg/kg in rats [10] and by an LD50 value higher than 2000 mg/kg in mice [11], allows its possible addition to edible film-forming solutions (FFSs) to obtain safe food coatings. Thus, SPD effect was tested by including the polyamine in FFSs containing a protein concentrate obtained from bitter vetch (*Vicia ervilia*; BV) seeds and different amounts of glycerol (GLY), added as plasticizer [7]. BV, an annual legume species of the *Vicia* genus, is widely cultivated only for livestock feed in temperate regions of Europe, western and central Asia, north Africa, and Americas [12–15]. BV seeds, containing up to 25% of protein, were demonstrated to be a potentially useful source for food packaging applications, being not only an abundant protein source but also an inexpensive one for edible film production [15–18]. It was recently observed [7] that films made with a BV protein concentrate (BVPC) in the absence of high concentrations of GLY are brittle, difficult to manipulate, and, consequently, impossible to study. Moreover, the addition of high amounts of GLY were shown unable to give rise to handleable films even when BV proteins were previously denatured by heat treatment at 80 °C for 30 min. Conversely, the presence of appropriate SPD amounts allowed to obtain easily manipulable films at low GLY concentrations and even in the absence of GLY. Therefore, a plasticizing-like effect and a facilitating action on GLY plasticizing effect has been hypothesized for this aliphatic polycation [7]. In this paper, we report SPD influence on the morphological, mechanical, and barrier properties of films prepared from both native and heat-denatured BV proteins at different concentrations of GLY and pH values.

## 2. Results and Discussion

Since the addition of millimolar concentrations of SPD to BVPC FFSs allows the formation of handleable films even in the absence of GLY [7], we were first stimulated to determine the mechanical properties of these GLY-unplasticized films, as well of those prepared in the presence of different amounts of SPD (2–5 mM, corresponding to about 4–9% $w/w$ BV protein) and increasing GLY concentrations (4–42 mM GLY, corresponding to about 5–50% $w/w$ BV protein). To investigate the influence of SPD and GLY on both native and denatured protein-based films, a BVPC pretreated for 30 min at either 25 or 80 °C was used as a film protein source. Furthermore, to study the influence of the positively charged amino groups of SPD in structuring the film protein network and the consequent film mechanical properties, tensile strength (TS), elongation at break (EB), and Young's module (YM) values detected for films prepared at pH 8.0 (i.e., under SPD amino group p*K*a values) were compared to those detected for films obtained at pH 11.0 (i.e., over SPD amino group p*K*a values) [19].

The data reported in Table 1 indicate that all films made at pH 8.0 with both native and heat-denatured BV proteins and containing SPD alone or with low amounts of GLY showed higher TS, with a maximum value of 12.00 ± 1.21 MPa observed when 3 mM SPD was used in the heat-denatured BVPC FFSs. In addition, the film TS appeared to be progressively lower in all samples containing SPD and increasing GLY concentrations, reaching a very low value (less than 1.0 MPa) at 42 mM GLY. It is worth noting that these latter values are significantly lower than those detected by analyzing films prepared in the presence of GLY alone at high concentrations, thus confirming a positive influence of SPD on GLY plasticizing action.

**Table 1.** Effect of different concentrations of spermidine (SPD) and glycerol (GLY) on the tensile strength (TS) (MPa) of native (A) and heat-denatured (B) bitter vetch (BV) protein films obtained at pH 8.0 *.

| | Addition | GLY, mM | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **0** | **4** | **8** | **17** | **25** | **33** | **42** |
| A | none | ND | ND | ND | ND | ND | 1.42 ± 0.72 | 1.60 ± 0.40 |
| | +2 mM SPD | ND | ND | ND | ND | 1.28 ± 0.65 | 1.02 ± 0.26 [a] | 1.42 ± 0.07 [a] |
| | +3 mM SPD | ND | 3.49 ± 0.40 | 1.33 ± 0.24 | 1.47 ± 0.14 | 1.03 ± 0.42 | 0.58 ± 0.07 [a] | 0.39 ± 0.05 [a] |
| | +4 mM SPD | 3.45 ± 0.41 | 8.38 ± 1.90 [b] | 6.24 ± 0.80 [b] | 2.03 ± 0.20 [b] | 1.32 ± 0.32 [b] | 0.61 ± 0.04 [a,b] | 0.58 ± 0.02 [a,b] |
| | +5 mM SPD | 3.03 ± 1.61 | 3.44 ± 1.58 | 5.30 ± 1.33 | 2.91 ± 0.36 | 1.66 ± 0.43 [b] | 0.62 ± 0.05 [a,b] | 0.66 ± 0.08 [a,b] |
| B | none | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 |
| | +2 mM SPD | 7.14 ± 0.24 | 7.78 ± 1.04 | 4.43 ± 0.80 [b] | 2.34 ± 0.40 [b] | 2.34 ± 0.40 [b] | 1.18 ± 0.34 [b] | ND1 |
| | +3 mM SPD | 12.00 ± 1.21 | 7.65 ± 0.67 [b] | 4.95 ± 1.24 [b] | 2.66 ± 0.95 [b] | 1.76 ± 0.72 [b] | 1.42 ± 0.24 [b] | 1.17 ± 0.42 [b] |
| | +4 mM SPD | 7.96 ± 1.33 | 6.72 ± 1.96 | 6.79 ± 2.15 | 3.08 ± 1.39 [b] | 1.60 ± 0.36 [b] | 0.89 ± 0.30 [b] | 0.95 ± 0.38 [b] |
| | +5 mM SPD | 5.71 ± 1.76 | 4.02 ± 1.33 | 3.23 ± 0.70 | 1.25 ± 0.58 [b] | 1.05 ± 0.22 [b] | 0.96 ± 0.11 [b] | 0.86 ± 0.40 [b] |

* Not detectable values because of the film brittleness (ND) or stickiness (ND1). Significantly different values as compared to the ones obtained under the same experimental conditions without SPD ([a]) or GLY ([b]) (*t*-test, $p < 0.05$); *F*-test was positive ($p < 0.05$) for variations of TS with SPD and GLY concentrations. Further experimental details are given in the text.

Consequential and predictable effects were recorded when the EB of the same films was measured (Table 2). In fact, the lowest EB values were detected when analyzing the films prepared from both native and heat-denatured BVPC in the presence of SPD without or with low concentrations of GLY. Also in this case, EB was observed to rise in parallel with the increase of GLY amount contained in the FFSs where SPD was also present, reaching the maximum values (EB > 80%) at the highest GLY concentration (42 mM). Also such values resulted significantly different (much higher) than those detected when the films were prepared in the presence of high GLY concentrations (33 and 42 mM) but in the absence of SPD.

**Table 2.** Effect of different concentrations of SPD and GLY on the elongation at break (EB, %) of native (A) and heat- denatured (B) BV protein films obtained at pH 8.0 *.

| | Addition | GLY, mM | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **0** | **4** | **8** | **17** | **25** | **33** | **42** |
| A | none | ND | ND | ND | ND | ND | 15.21 ± 2.18 | 35.08 ± 3.43 |
| | +2 mM SPD | ND | ND | ND | ND | 40.61 ± 2.41 | 54.69 ± 5.45 [a] | 60.16 ± 4.52 [a] |
| | +3 mM SPD | ND | 24.62 ± 4.79 | 47.94 ± 4.56 | 50.28 ± 2.85 | 59.36 ± 6.25 | 64.41 ± 4.12 [a] | 72.31 ± 8.34 [a] |
| | +4 mM SPD | 0.89 ± 0.05 | 8.44 ± 2.67 [b] | 31.15 ± 3.85 [b] | 55.82 ± 4.67 [b] | 64.55 ± 5.48 [b] | 67.40 ± 6.64 [a,b] | 71.78 ± 2.05 [a,b] |
| | +5 mM SPD | 1.05 ± 0.15 | 4.56 ± 0.87 [b] | 9.28 ± 4.38 [b] | 22.78 ± 3.69 [b] | 60.23 ± 7.20 [b] | 72.58 ± 5.10 [a,b] | 86.26 ± 3.04 [a,b] |
| B | none | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 |
| | +2 mM SPD | 1.00 ± 0.01 | 5.35 ± 1.73 [b] | 13.11 ± 3.25 [b] | 32.91 ± 3.34 [b] | 35.85 ± 1.59 [b] | 59.02 ± 4.48 [b] | ND1 |
| | +3 mM SPD | 3.80 ± 0.80 | 15.17 ± 2.19 [b] | 24.66 ± 2.81 [b] | 33.26 ± 4.93 [b] | 37.02 ± 2.84 [b] | 51.57 ± 4.56 [b] | 80.88 ± 4.92 [b] |
| | +4 mM SPD | 6.44 ± 1.54 | 14.60 ± 2.48 [b] | 25.50 ± 2.59 [b] | 34.64 ± 3.24 [b] | 40.66 ± 5.74 [b] | 42.05 ± 3.68 [b] | 44.52 ± 4.89 [b] |
| | +5 mM SPD | 9.94 ± 1.66 | 18.01 ± 3.52 [b] | 32.38 ± 3.44 [b] | 34.51 ± 4.76 [b] | 46.66 ± 4.58 [b] | 20.36 ± 3.21 [b] | 21.45 ± 2.71 [b] |

* Not detectable values because of the film brittleness (ND) or stickiness (ND1). Significantly different values as compared to the ones obtained under the same experimental conditions without SPD ([a]) or GLY ([b]) (*t*-test, $p < 0.05$); *F*-test was positive ($p < 0.05$) for variations of EB with SPD and GLY concentrations. Further experimental details are given in the text.

The same behavior was recorded when measuring the YM of these films, with very high YM values detected for films containing SPD alone (more than 400 MPa) or with low GLY amounts. Also, the YM of SPD-containing films was observed to progressively decrease to very low values (less than 10 MPa) with increasing GLY concentrations up to 42 mM (Table 3). Also in this case, the YM of films containing both SPD and high amounts of GLY resulted much lower than the YM values observed for films prepared in the presence of high concentrations of GLY but in the absence of SPD.

All these data support our hypothesis on the ability of SPD not only to act as a plasticizer itself by ionically interacting at pH 8.0 with the negative charges occurring onto BV proteins, but also to facilitate in this way GLY action in reducing the intermolecular forces along the protein chains,

and consequently to further improve film flexibility and extensibility. This assumption was confirmed by the data of the mechanical properties of the films prepared in the presence of SPD at pH 11.0 (i.e., when its amino groups are uncharged being over their respective p*K*a values) in comparison with those obtained for the films prepared at pH 8.0 (i.e., when SPD is fully protonated). In fact, Figure 1 clearly indicates that films derived from native BV proteins have different TS, EB, and YM when they are prepared at pH 8.0 or 11.0 in the presence of 3 mM SPD and low GLY concentrations. In particular, the films obtained at pH 8.0 exhibited lower TS and YM and higher EB compared to those prepared at pH 11.0, thus indicating that the different kind of interaction between the polyamine and the folded protein chains is responsible for the different effects on the film mechanical properties. These differences have been proved not to be significant when heat-denatured BV protein films were tested, probably because the unfolded biopolymer chains are influenced by the uncharged SPD in the same way as by the protonated polyamine (Figure 2). In this case, polyamine–protein hydrogen bonds and hydrophobic interactions may be hypothesized.

**Table 3.** Effect of different concentrations of SPD and GLY on the Young's module (YM, MPa) of native (A) and heat-denatured (B) BV protein films obtained at pH 8.0 *.

| | Addition | GLY, mM | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **0** | **4** | **8** | **17** | **25** | **33** | **42** |
| A | none | ND | ND | ND | ND | ND | 80.0 ± 1.4 | 30.7 ± 0.6 |
| | +2 mM SPD | ND | ND | ND | ND | 25.4 ± 1.6 | 18.4 ± 3.6 $^{a}$ | 9.7 ± 1.5 $^{a}$ |
| | +3 mM SPD | ND | 154.9 ± 16.0 | 125.2 ± 13.6 | 23.8 ± 2.5 | 12.4 ± 2.4 | 14.3 ± 2.3 $^{a}$ | 4.3 ± 0.5 $^{a}$ |
| | +4 mM SPD | 425.3 ± 25.8 | 381.9 ± 17.1 | 121.9 ± 12.4 $^{b}$ | 42.2 ± 5.6 $^{b}$ | 16.1 ± 3.3 $^{b}$ | 13.1 ± 3.8 $^{a,b}$ | 3.5 ± 0.2 $^{a,b}$ |
| | +5 mM SPD | 397.8 ± 20.0 | 320.1 ± 27.1 $^{b}$ | 185.7 ± 19.3 $^{b}$ | 59.8 ± 4.0 $^{b}$ | 10.0 ± 1.3 $^{b}$ | 6.9 ± 0.5 $^{a,b}$ | 2.9 ± 0.5 $^{a,b}$ |
| B | none | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 |
| | +2 mM SPD | 804.8 ± 50.5 | 325.0 ± 17.0 $^{b}$ | 184.5 ± 4.3 $^{b}$ | 63.1 ± 4.5 $^{b}$ | 26.3 ± 4.2 $^{b}$ | 11.8 ± 1.1 $^{b}$ | ND1 |
| | +3 mM SPD | 454.0 ± 54.2 | 310.5 ± 25.1 $^{b}$ | 143.5 ± 5.4 $^{b}$ | 48.0 ± 1.6 $^{b}$ | 23.7 ± 4.0 $^{b}$ | 22.1 ± 3.8 $^{b}$ | 7.1 ± 1.3 $^{b}$ |
| | +4 mM SPD | 356.7 ± 20.5 | 196.4 ± 15.5 $^{b}$ | 150.2 ± 9.5 $^{b}$ | 46.5 ± 6.5 $^{b}$ | 22.5 ± 3.5 $^{b}$ | 21.9 ± 5.0 $^{b}$ | 17.7 ± 5.3 $^{b}$ |
| | +5 mM SPD | 226.6 ± 6.6 | 80.7 ± 3.7 $^{b}$ | 82.1 ± 3.4 $^{b}$ | 38.4 ± 4.2 $^{b}$ | 21.7 ± 2.1 $^{b}$ | 23.3 ± 6.0 $^{b}$ | 16.1 ± 5.8 $^{b}$ |

* Not detectable values because of the film brittleness (ND) or stickiness (ND1). Significantly different values as compared to the ones obtained under the same experimental conditions without SPD ($^{a}$) or GLY ($^{b}$) (*t*-test, $p < 0.05$); *F*-test was positive ($p < 0.05$) for variations of YM with SPD and GLY concentrations. Further experimental details are given in the text.

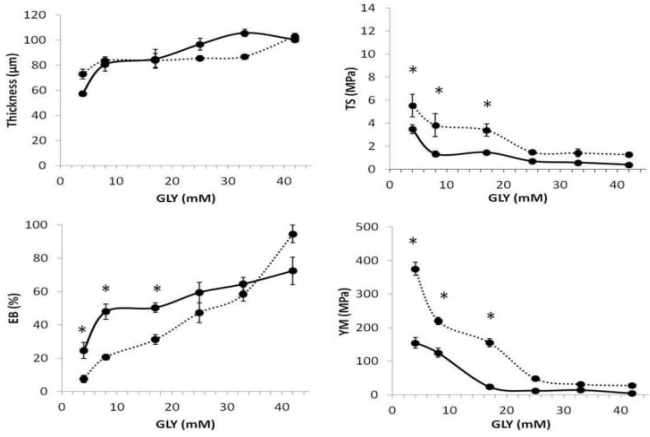


**Figure 1.** Effect of different GLY concentrations on the mechanical properties of films prepared with BV proteins treated at 25 °C, in the presence of 3 mM SPD at either pH 8.0 (solid line) or pH 11.0 (dotted line). Brittle and unhandleable films were obtained by casting FFSs prepared in the absence of GLY. The results are expressed as mean ± standard deviation (* values significantly different at $p < 0.05$). Further experimental details are given in the text.

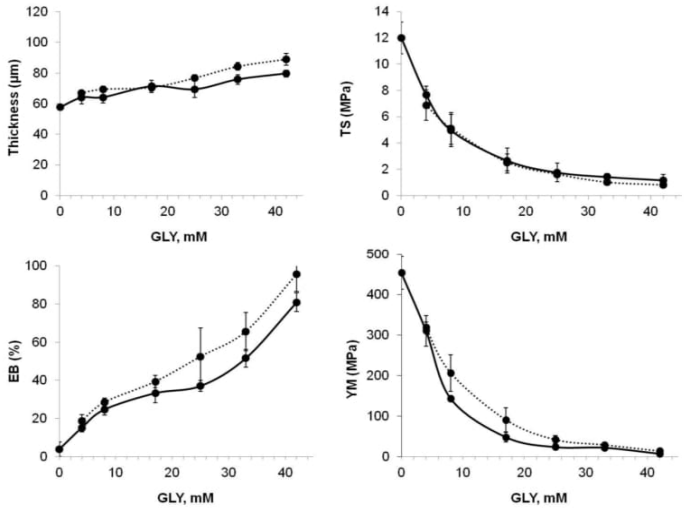


**Figure 2.** Effect of different GLY concentrations on the mechanical properties of films prepared by BV proteins treated at 80 °C, in the presence of 3 mM SPD at either pH 8.0 (solid line) or pH 11.0 (dotted line). Brittle and unhandleable films were obtained by casting FFSs prepared in the absence of GLY at pH 11.0. The results are expressed as mean ± standard deviation. Further experimental details are given in the text.

Lastly, a further demonstration that SPD was able to enhance the plasticizing performance of GLY was given by the significantly lower EB values (52.56 ± 3.90 vs. 94.45 ± 5.26 for native BV protein films; 51.17 ± 4.80 vs. 95.56 ± 7.62 for denatured BV protein films) and higher YM values (34.11 ± 1.66 vs. 27.66 ± 1.16 for native BV protein films; 68.78 ± 3.60 vs. 13.70 ± 2.70 for denatured BV protein films) detected for films prepared at pH 11.0 in the presence of 42 mM GLY and in the absence of SPD, compared to those containing 42 mM GLY and 3 mM SPD.

Since a primary plasticizer is generally defined as a molecule that, when added to a material, makes it softer, more flexible, and easier to be processed, our findings lead to consider SPD as a possible primary plasticizer of protein-based films. In fact, the addition of millimolar concentrations of SPD allowed BV proteins to give rise to handleable materials as a result of an increase of their elongation and softeness. However, SPD can also be considered as a secondary plasticizer, namely, an "extender", because of its ability to enhance the plasticizing performance even of a well-known primary plasticizer such as glycerol [20]. Such double behavior of the polyamine as both a primary and a secondary plasticizer seems to be confirmed also by the analysis of the permeability properties of BVPC films obtained in the presence of different concentrations of SPD and GLY. In fact, the data reported in Tables 4 and 5 showed that the addition of increasing SPD concentrations into FFSs caused in the derived films an increase in the barrier properties to gases ($CO_2$ and $O_2$) as well as to water vapor (WV) and that the concomitant presence of low GLY concentrations emphasized this effect. Further increases of GLY concentration into the films, however, led to a marked reversal of the positive barrier effect, thus indicating that an excessive film plasticization promoted film permeability to both gases and WV. It is worthy to note that the same effect was observed by testing films prepared with both native and heat-denatured BV proteins.

**Table 4.** Effect of different GLY and SPD concentrations on gas permeability ($cm^3$ mm $m^{-2}$ $day^{-1}$ $kPa^{-1}$) of native (A) and heat-denatured (B) BV protein films obtained at pH 8.0 *.

| | Addition | $CO_2$ Permeability | | | | $O_2$ Permeability | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | GLY, mM | | | | | | | |
| | | 0 | 8 | 25 | 42 | 0 | 8 | 25 | 42 |
| A | none | ND | ND | ND | 3.025 ± 0.210 | ND | ND | ND | 1.910 ± 0.013 |
| | +3 mM SPD | ND | 4.520 ± 0.685 | 0.990 ± 0.264 | 1.750 ± 0.163 [a] | ND | 8.107 ± 0.579 | 0.037 ± 0.010 | 0.059 ± 0.008 [a] |
| | +4 mM SPD | 7.592 ± 0.597 | 0.236 ± 0.062 [b] | 0.538 ± 0.084 [b] | 1.768 ± 0.313 [a,b] | 1.780 ± 0.104 | 0.017 ± 0.002 [b] | 0.048 ± 0.001 [b] | 0.078 ± 0.009 [a,b] |
| | +5 mM SPD | 0.982 ± 0.208 | 1.240 ± 0.140 | 2.613 ± 0.559 [b] | 2.396 ± 0.259 [a,b] | 0.293 ± 0.091 | 0.011 ± 0.008 [b] | 0.086 ± 0.006 [b] | 0.063 ± 0.001 [a,b] |
| B | none | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 | ND1 |
| | +3 mM SPD | 15.910 ± 2.030 | 0.380 ± 0.031 [b] | 0.567 ± 0.040 [b] | 0.630 ± 0.020 [b] | 3.559 ± 0.421 | 0.018 ± 0.002 [b] | 0.047 ± 0.003 [b] | 0.065 ± 0.004 [b] |
| | +4 mM SPD | 1.250 ± 0.241 | 0.463 ± 0.093 [b] | 0.389 ± 0.012 [b] | 2.321 ± 0.540 [b] | 2.130 ± 0.240 | 0.026 ± 0.004 [b] | 0.042 ± 0.001 [b] | 0.057 ± 0.003 [b] |
| | +5 mM SPD | 0.322 ± 0.007 | 0.664 ± 0.035 [b] | 0.823 ± 0.064 [b] | 2.319 ± 0.106 [b] | 1.671 ± 0.113 | 0.031 ± 0.002 [b] | 0.052 ± 0.012 [b] | 0.090 ± 0.001 [b] |

* Not detectable values because of film brittleness (ND) or stickiness (ND1). Significantly different values as compared to the ones obtained under the same experimental conditions without SPD ([a]) or GLY ([b]) (*t*-test, $p < 0.05$); *F*-test was positive ($p < 0.05$) for variations of permeability with SPD and GLY concentrations. Further experimental details are given in the text.

**Table 5.** Effect of different GLY and SPD concentrations on water vapor (WV) permeability ($cm^3$ mm $m^{-2}$ $day^{-1}$ $kPa^{-1}$) of native (A) and heat-denatured (B) BV protein films obtained at pH 8.0 *.

| Addition | | WV Permeability | | | |
|---|---|---|---|---|---|
| | | GLY, mM | | | |
| | | 0 | 8 | 25 | 42 |
| A | none | ND | ND | ND | 0.045 ± 0.007 |
| | +3 mM SPD | ND | 0.023 ± 0.001 | 0.102 ± 0.030 | 0.240 ± 0.019 [a] |
| | +4 mM SPD | 0.418 ± 0.010 | 0.039 ± 0.003 [b] | 0.116 ± 0.014 [b] | 0.228 ± 0.007 [a,b] |
| | +5 mM SPD | 0.308 ± 0.162 | 0.073 ± 0.002 [b] | 0.190 ± 0.061 [b] | 0.193 ± 0.001 [a,b] |
| B | none | ND1 | ND1 | ND1 | ND1 |
| | +3 mM SPD | 0.550 ± 0.045 | 0.022 ± 0.002 [b] | 0.067 ± 0.003 [b] | 0.088 ± 0.001 [b] |
| | +4 mM SPD | 0.367 ± 0.042 | 0.050 ± 0.002 [b] | 0.085 ± 0.001 [b] | 0.169 ± 0.008 [b] |
| | +5 mM SPD | 0.028 ± 0.004 | 0.036 ± 0.004 | 0.070 ± 0.001 [b] | 0.237 ± 0.001 [b] |

* Not detectable values because of film brittleness (ND) or stickiness (ND1). Significantly different values as compared to the ones obtained under the same experimental conditions without SPD ([a]) or GLY ([b]) (*t*-test, $p < 0.05$); *F*-test was positive ($p < 0.05$) for variations of permeability with SPD and GLY concentrations. Further experimental details are given in the text.

Figure 3 shows the surface and cross-sectional morphology of films derived from FFSs containing native (A–C) or heat denatured BVPC (D,E), mixed with either GLY (A), SPD alone (B,D), or with both plasticizers (C,E). According to the SEM images, BVPC films containing GLY and SPD together presented a relatively smoother and more uniform and continuous appearance with respect to films containing only GLY, which appeared less cohesive and exhibited an evident heterogeneity. Moreover, also the surfaces and the cross sections of the SPD-containing films prepared in the absence of GLY showed features typical of more compact matrices, likely accountable for the lower permeability detected with all films containing the polyamine. These results are in agreement with the marked reduction of thickness of SPD containing films, prepared with both native and denatured BVPC (Table 6).

**Table 6.** Effect of 5 mM SPD and, or 42 mM GLY on the thickness of native (A) and heat-denatured (B) BV protein films obtained at pH 8.0 *.

| | Addition | Thickness, μm |
|---|---|---|
| **A** | +GLY | 107.93 ± 1.84 [b] |
| | +SPD | 82.60 ± 1.08 [a,b] |
| | +GLY + SPD | 100.40 ± 1.76 [a,b] |
| **B** | +GLY | ND |
| | +SPD | 76.62 ± 1.01 [b] |
| | +GLY + SPD | 87.23 ± 1.13 [b] |

* Not detectable value because of film stickiness (ND). The values obtained in the presence of SPD (panel A) were significantly different from those obtained under the same experimental conditions in its absence ([a]) (*t*-test, $p < 0.05$); *F*-test was positive ($p < 0.05$) for variations of thickness with SPD and GLY ([b]). Further experimental details are given in the text.

Finally, as far as a possible application of SPD/GLY-plasticized films is concerned, we report in Table 7 the mechanical and barrier properties of various hydrocolloid (polysaccharide- and protein-based) films previously described in the literature, as well as those of some commercial bioplastic (Viscofan NDX and Mater-Bi S-301) and plastic (HD-PE 02) materials analyzed in the present study. The comparison of the features of the SPD-containing BVPC films described here with those of the other hydrocolloid materials indicated similar TS values when BVPC films were prepared in the absence of GLY, whereas higher EB characterized the BVPC films when also GLY was present. Conversely, the barrier effects toward both gases and WV seemed to be similar in all films. More in

particular, it is worthy to note that the BVPC films prepared in the presence of low concentrations of both SPD and GLY showed mechanical and barrier characteristics comparable with those of Viscofan NDX [21], a widely commercialized, collagen-derived edible film largely used in casings for fresh or processed sausages, or dry-cured snacks. Therefore, plant-derived casings made with BVPC and low amounts of both SPD and GLY deserve to be produced and tested as possible alternatives to the casings of animal origin.

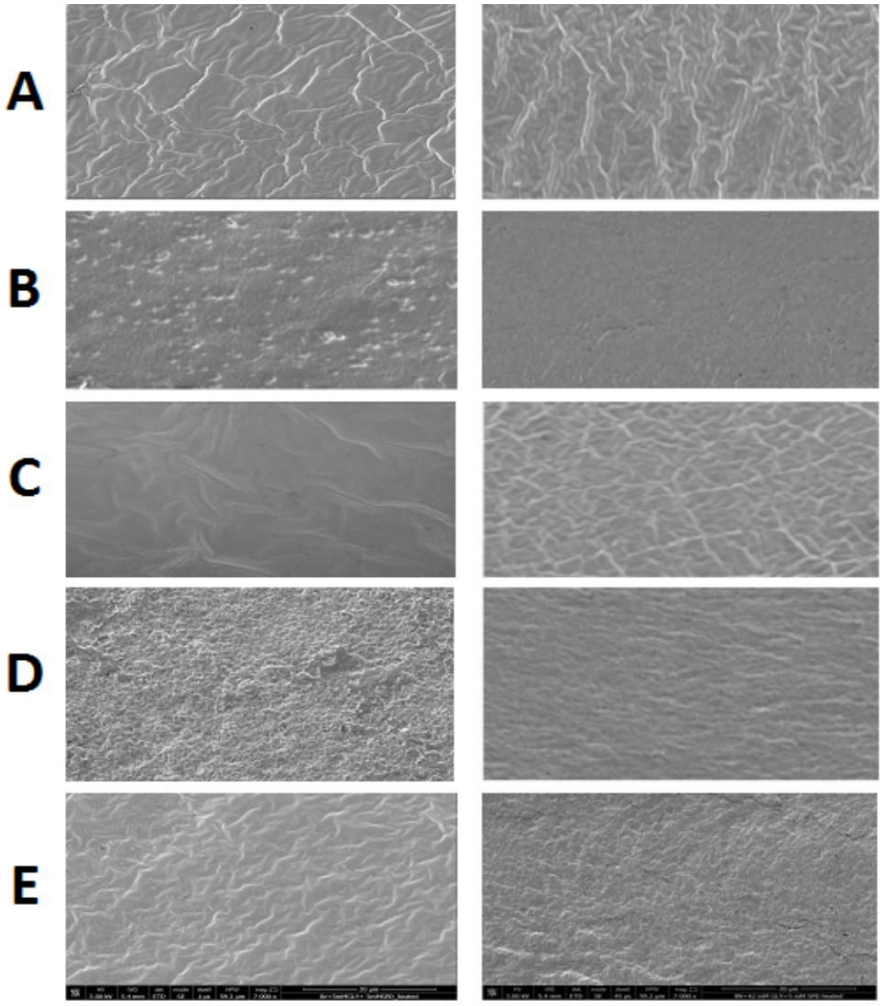


**Figure 3.** SEM micrographs at 7000× magnification of the film surfaces (left) and cross sections (right) obtained from native (**A**–**C**) and heat-denatured (**D**,**E**) BVPC in the presence of 5 mM SPD with (**C**,**E**) or without (**B**,**D**) 42 mM GLY, as well as in the absence of both SPD and GLY (**A**). The sample prepared with heat-denatured BVPC in the absence of SPD was not analyzed because not handleable for its stickiness. The images shown were chosen as the most representative of each sample. Further experimental details are given in the text.

**Table 7.** Thickness, mechanical, and barrier properties of some commercial materials and of different protein- and polysaccharide-based films described in the literature.

| Film | [Reference] | Thickness (μm) | TS (MPa) | EB (%) | YM (MPa) | Permeability ($cm^3$ mm $m^{-2}$ $day^{-1}$ $kpa^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | $CO_2$ | $O_2$ | WV |
| **Protein-Based** [a] | | | | | | | | |
| SP + 30% GLY | [22] | 98 ± 7 | 3.8 ± 0.4 | 6.5 ± 0.6 | ND | ND | 0.0012 | 0.43 ± 0.04 |
| SP + 40% SOR | [22] | 106 ± 7 | 4.0 ± 0.2 | 7.2 ± 1.1 | ND | ND | 0.0011 | 0.02 ± 0.01 |
| WG + 20% GLY | [23] | 53 | 2.6 ± 1.1 | 22.0 ± 10.0 | 2.6 ± 1.1 | ND | ND | 0.54 |
| WP + 40% GLY | [24] | 120 ± 8 | 0.83 ± 0.01 | 51.0 ± 1.0 | 43.0 ± 1.5 | 1.02 ± 0.01 | 0.20 | 8.25 ± 0.31 |
| **Polysaccharide-Based** [b] | | | | | | | | |
| CHI + 25% GLY | [25] | 55 ± 1 | 15.1 ± 0.5 | 22.1 ± 2.1 | ND | 0.02 ± 0.01 | 0.025 | 0.0005 |
| PEC + 30% GLY | [6] | 90 ± 1.2 | 11.0 ± 1.0 | 6.9 ± 0.2 | ND | 0.02 ± 0.01 | ND | 9.8 ± 0.4 |
| PEC + 8 mM SPD | [6] | 70 ± 0.5 | 5.6 ± 0.3 | 8.3 ± 0.6 | ND | 0.08 ± 0.01 | ND | 20.3 ± 0.3 |
| PEC + GLY + SPD | [6] | 101 ± 1.3 | 2.7 ± 0.3 | 28.1 ± 1.2 | ND | 1.02 ± 0.03 | ND | 10.2 ± 0.2 |
| **Commercial** [c] | | | | | | | | |
| Viscofan (NDX) | [PS] | 30.0 ± 0.4 | 36.6 ± 8.1 | 13.1 ± 2.9 | 356 ± 29 | 3.71 ± 0.16 | 0.03 ± 0.01 | 0.08 ± 0.01 |
| Mater-Bi (S-301) | [PS] | 16.0 ± 1.3 | 18.4 ± 2.7 | 317.9 ± 35.9 | 42.9 ± 0.8 | 5.23 ± 0.01 | 0.74 ± 0.01 | 0.04 ± 0.01 |
| HD-PE 02 | [PS] | 36.2 ± 1.7 | 13.1 ± 1.4 | 501.9 ± 43.3 | 75.2 ± 2.7 | 10.89 ± 1.48 | 3.21 ± 0.59 | 0.0002 |

[a] SP, soy protein; SOR, sorbitol; WG, wheat gluten; WP, whey protein. [b] CHI, chitosan; PEC, pectin; ND, not detected. [c] The results were obtained in the present study [PS] and are expressed as means ± standard deviation, either of six specimens for the determination of thickness and mechanical properties or of three specimens analyzed in permeability tests. Further experimental details are described under Materials and Methods.

## 3. Materials and Methods

### *3.1. Materials*

Bitter vetch seeds were obtained from a local market in Gallicchio (PZ), Italy. Viscofan NDX edible casings were from Naturin Viscofan GmbH (Tajonar-Navarra, Spain); Mater-Bi (S 301) and high density polyethylene (HD-PET) materials were from local market shopping bags, Naples, Italy. SPD was from Sigma Chemical Company (St. Louis, MO, USA), GLY (about 87%) was from the Merck Chemical Company (Darmstadt, Germany), and all other chemicals and solvents used in this study were analytical grade commercial products.

### *3.2. BVPC Film Preparation*

BVPC, the derived FFSs, and films containing or not different concentrations of SPD and, or GLY, were prepared as previously described [7]. The flour obtained from BV seeds grinded in a rotary mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at a speed of 1300 r.p.m. for 5 min was dispersed in distilled water (1:10, *w*/*v*), brought to pH 11.0 with 0.1 N NaOH, and stirred at medium speed for 1 h at room temperature. The suspension was centrifuged at 3200× *g* for 10 min and the pH of the collected supernatant was adjusted to 5.4 by 0.1 N HCl addition. The obtained precipitate was then separated by a new centrifugation at 3200× *g* for 10 min, poured, uniformly distributed on a plastic plate, and dried at 37 °C and 25% relative humidity (RH). The obtained BVPC was finally grinded and dispersed in distilled water (2 g/100 mL), and the pH value was adjusted to pH 12.0 by using 0.1 N NaOH under constant stirring until the powder was completely solubilized. Aliquots of BVPC solution were brought to pH 8.0 and 11.0, respectively, by 0.1 N HCl and then incubated in the presence of different concentrations of SPD (2–5 mM, corresponding to about 4–9% *w*/*w* BV protein) for 30 min, either at 25 or 80 °C to obtain FFSs containing both native and denatured BV protein samples. Where indicated, increasing concentrations of GLY (4–42 mM GLY, corresponding to about 5–50% *w*/*w* BV protein) were added to the obtained FFSs at the end of incubation. All the different FFSs (50 mL), containing or not SPD and, or GLY, were poured onto 8-cm-diameter polystyrene Petri dishes (7.8 mg protein/cm$^2$) and allowed to dry in an environmental chamber at 25 °C and 45% RH for 48 h. Finally, the dried films were peeled from the casting surface and stored at 25 °C and 50% RH. Film sample strips (10 mm wide and 50 mm long), obtained by using a sharp razor blade, were conditioned in an environmental chamber at 25 °C and 50% RH for 2 h by placing them into a dessicator over a saturated solution of $Mg(NO_3)_2 \cdot 6H_2O$ before being tested.

### *3.3. BVPC Film Properties*

Film thickness was measured in six different points with a micrometer (Electronic digital micrometer, DC-516, sensitivity 0.001 mm) and film TS at break, EB, and YM were determined in five specimens for each sample (1 KN load and 1 mm/5 min speed) as previously reported [26], by using an Instron universal testing instrument model No. 5543A (Instron Engineering Corp., Norwood, MA, USA).

The measurements of film permeability to $O_2$ [27], $CO_2$ [28] and water vapor (WV) [29] were determined in triplicate for each sample at 25 °C under 50% RH by using a TotalPerm apparatus (ExtraSolution s.r.l., Pisa, Italy).

Film surface and cross section morphology were observed using a Scanning Electron Microscope (Nova NanoSem 450-FEI) (SEM). For film surface analysis, the samples were placed on an aluminum stub by using a graphite double-sided adhesive tape, whereas for film cross sections the samples were fractured in liquid nitrogen and rested vertically on the sides of a rectangular aluminum piece fixed on stubs using a double-sided adhesive tape. A thin coat of gold and palladium was sputtered at a current of 20 mA for 120 s. The sputter-coated samples were then introduced into the specimen chamber and the images were acquired at an accelerating voltage of 3 kV, through the Everhart Thornley Detector. Micrographs for sample surfaces and cross sections were obtained at 7000× magnifications.

### *3.4. Statistical Analysis*

JMP software 10.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. The data were subjected to the analysis of variance (*F*-test) to evaluate the effect of SPD and GLY on film mechanical and barrier properties (significance at $p < 0.05$), whereas the means of the obtained data were analyzed using the Tukey-Kramer HSD (*t*-test) for pair comparison between control and treated samples (significance at $p < 0.05$).

## 4. Conclusions

Our findings suggest that the use of SPD or of a combination of the polyamine with a primary plasticizer such as GLY as additives of protein-based films, may open new possibilities to generate hydrocolloid edible films endowed with different mechanical and barrier properties specifically suitable for the coating of different food products. In fact, one of the main technical challenges in food processing and storage today is the development of tailor-made coating materials with appropriate characteristics according to the specific requirements of the various fresh or processed foodstuffs: meat, fish, dairy products, fruit, vegetables, as well as ready-to-eat meals [30]. In fact, although desirable mechanical and permeability properties remain the main factors to consider when selecting packaging materials, these features are particularly strategical for any edible coating in the processing and end use of food products, representing the main parameters to ensure food integrity against mechanical damage, microbial spoilage, and duration of the guarantee term.

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**Author Contributions:** Mohammed Sabbah, Prospero Di Pierro, and Raffaele Porta designed the experiments and wrote the paper; Mohammed Sabbah performed all the experiments except the SEM analyses carried out by Valentina Roviello. All authors have given approval to the final version of the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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### 3.1.1 FT-IR analysis of BVPC films

The broad band ranging between 3100 and 3500 $cm^{-1}$, corresponding to the stretching of O–H and N–H bonds (Martins et al., 2010), showed an increase of intensity of the signals only when the films containing GLY were analysed (Fig. 11). Moreover, the peaks detected between 2929 $cm^{-1}$ and 2877 $cm^{-1}$, attributed to C–H stretching vibration with amino group, significantly changed in the films containing GLY and/or SPD. The obtained data suggest a C–H stretching vibration with hydroxyl group in all obtained films. Fig. 12 shows that the peak observed at 1746 $cm^{-1}$, corresponding to N-H band of primary amines and detected in all film samples, markedly increased only in the film containing SPD alone. In fact, when also GLY occurred, the intensity of such peak dramatically decreased, probably as a consequence of SPD-GLY interaction. The intensity of the bands ranging between 1632 $cm^{-1}$ and 1519 $cm^{-1}$ , corresponding to amide-I and amide-II, were observed to be reduced in the films plasticized with SPD, probably due to the ionic interaction of the polyamine with BV proteins (Fig. 12). Similar findings have been reported by Ahmed Ouameur et al. (2004). In fact these Authors, reporting the reduction of the intensity of the amide-I and amide-II bands of human serum albumin in the presence of polyamines, explained this result suggesting a polycation–protein interaction.

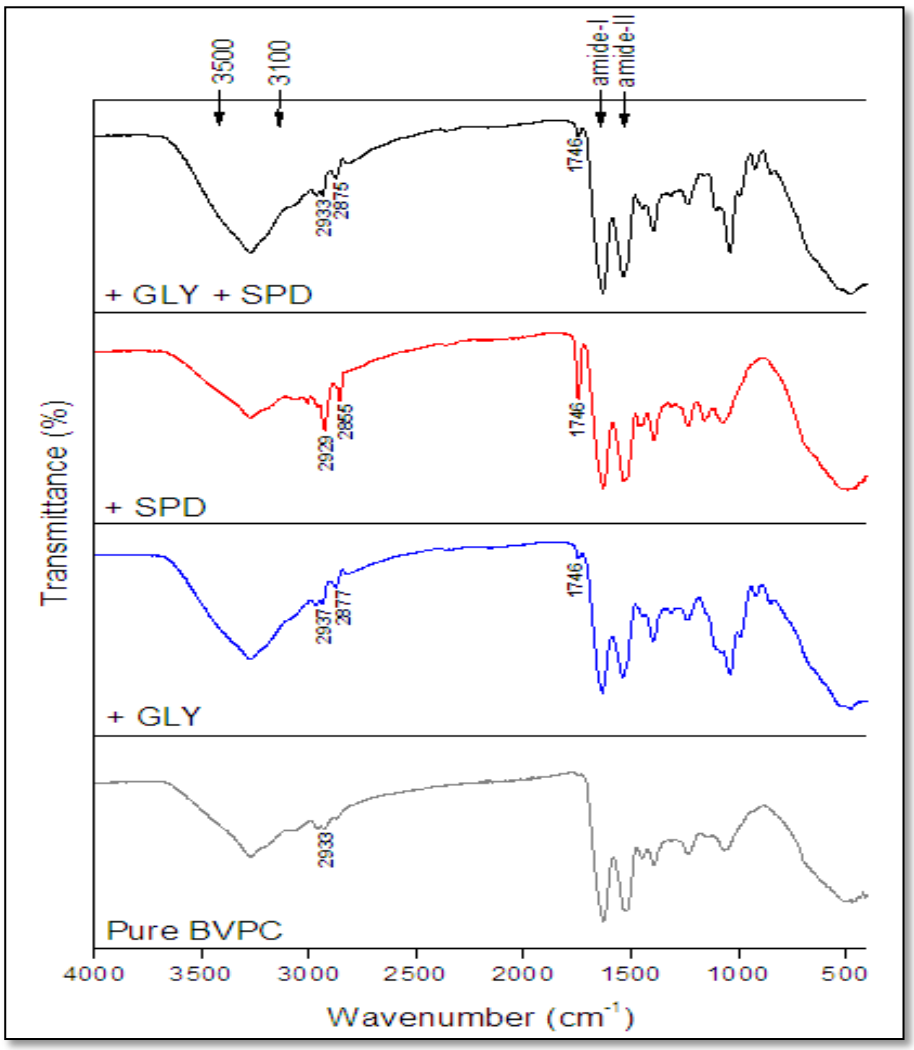


***Fig. 11. FT-IR spectra of pure BVPC powder and BVPC films prepared at pH 8 in the presence or absence of 42 mM GLY and/or 5mM SPD.***

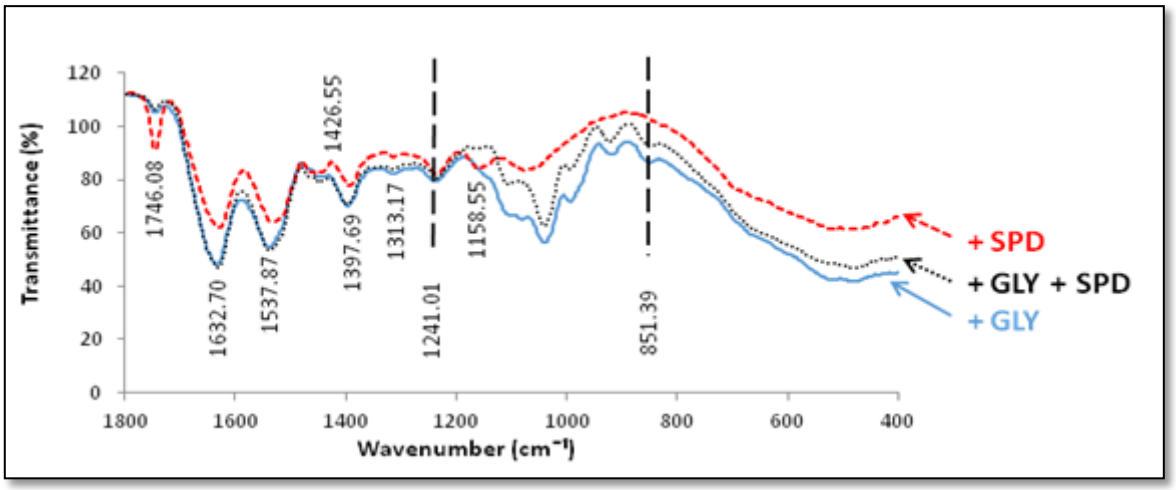


***Fig. 12. FT-IR spectra of BVPC films prepared at pH 8 in the presence or absence of 42 mM GLY and/or 5 mM SPD at wavenumber (1800–400 cm$^{-1}$).***

Moreover, no significant differences were detected in the peaks occurring in the range from 1537-1313 cm$^{-1}$, corresponding to the symmetrical stretching of the C=O and C=N bonds. Conversely, whereas several bands located between 1241-851 cm$^{-1}$ disappeared when SPD was used to plasticize BVPC films, a new band at 1158 cm$^{-1}$, corresponding to the tertiary amino C–N and probably due to ionic SPD-protein interaction, was clearly evident in the SPD-containing film spectrum (Fig. 12). More in particular, the bands located at 960–850 cm$^{-1}$ are known to be specifically related to the occurrence of O–H hydrogen bonds (Coates, 2000). Finally, the marked decrease of intensity in the spectrum region 1241-851 cm$^{-1}$, observed in the presence of both plasticizers with respect to the spectra of films plasticized by GLY alone, should be due to both ionic interaction between SPD and protein and hydrogen bonds between GLY and protein (Fig. 12).

### 3.2 Blended films of bitter vetch (*Vicia ervilia*) proteins and pectin: properties and effect of transglutaminase

As film components, polysaccharides are able to control gas transmission, whereas proteins often provide mechanical stability. Therefore, these biomacromolecules can be utilized either individually or, frequently, they are mixed to produce blended hydrocolloid films. In fact, combined use of compatible proteins and polysaccharides may allow preparing films showing improved mechanical or permeability properties with respect the ones obtained from proteins or polysaccharides alone. In addition, since chemical and/or enzymatic modifications of polysaccharides and proteins could provide further promising ways to improve film properties, we prepared BVPC/PEC blended films in the absence or presence of the enzyme TGase isolated from *Streptoverticillium sp*.. The microbial molecular form of the enzyme (mTGase), catalysing the production of protein inter- and/or intra-molecular ε-(γ-glutamyl) lysine isopeptide bonds, is able to transform soluble substrate proteins into high molecular weight polymers also inside the edible films (Porta et al., 2011a). Therefore, we expected that the addition of PEC, as well as of PEC and mTGase, into BVPC FFSs could determine significant changes in film mechanical and/or permeability features. In the following article the morphological

and the main functional characteristics of these new blended BVPC/PEC films were described.

The blended hydrocolloid film obtained by using BVPC in the presence of PEC and mTGase revealed a promising way to obtain possible edible food coatings as well as effective drug delivery systems. In fact, unsatisfactory tensile properties and poor barrier ability toward the main environmental gases are considered thus far the major limitations to a wider use of biodegradable/edible films, being undesirable characteristics to maintain the quality of numerous foods and drugs. The improved mechanical features exhibited by BVPC/PEC films prepared in the presence of mTGase, together with their high barrier activity to both $CO_2$ and $O_2$, represent a clear advancement in the search for effective substitutes of traditional plastics. Furthermore, the cross-sectional SEM analysis confirmed that BVPC/PEC films containing mTGase possess a more compact and homogeneous microstructure, with evident continuous zones, in comparison with the reticular structure of control samples of BVPC/PEC obtained in absence of mTGase. Therefore, these findings suggest these new hydrocolloid materials as potential interesting bioplastics for specific applications in both food and pharmaceutical industries.

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# Blend films of pectin and bitter vetch (*Vicia ervilia*) proteins: Properties and effect of transglutaminase

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ABSTRACT

Hydrocolloid solutions were prepared by blending pectins and *Vicia ervilia* seed proteins at complexation pH and edible films were obtained by casting the solutions both in the absence and presence of microbial transglutaminase. Protein/pectin films exhibited a tensile strength double than the one observed with films containing only proteins, with an increase of about 3-fold observed in the presence of enzyme. Also the elongation at break resulted higher in the films containing transglutaminase, leading to conclude that films are more extensible mostly when both pectins and enzyme occur in the film forming solutions. A direct correlation between the improved film mechanical properties and the negative increase of zeta-potential of the originating film forming solutions was recorded. Conversely, gas permeability of protein/pectin films markedly decreased and transglutaminase addition determined a further enhancement of their barrier properties. These findings, supported by morphological analyses, suggest that the improved film functional features depend on their more compact structure due to crosslinked bitter vetch proteins grafted with pectin.

*Industrial relevance:* The innovative packaging is becoming an important focus as food industries increasingly endeavor to reduce the environmental impact of their products. Biodegradable and/or edible materials made from renewable sources are interesting alternatives to produce ecofriendly food coatings being able to substitute petrochemical films and to reduce plastic wastes. Development of blended polysaccharide/protein-based biomaterials is an attractive option in the attempt of tuning biodegradable films endowed with tailored properties. In this study an improvement of bitter vetch protein edible films by pectin grafting and transglutaminase treatment is investigated.



## 1. Introduction

Advanced researches on edible films and coatings made from renewable resources have been carried out over the past twenty years due to the increasing demand for environmentally-friendly replacements for petroleum-based polymeric materials. Different edible films have been proposed to provide fortification of natural layers of numerous food products, as well as to prevent food moisture and flavour losses or to selectively control the exchange of gases such as oxygen and carbon dioxide (Embuscado & Huber, 2009). As film components, lipids are generally used to reduce water transmission, polysaccharides are able to control gas transmission, whereas proteins often provide mechanical stability. All these biomolecules can be utilized either individually or, more frequently, they are mixed to produce blended hydrocolloid films (Bifani et al., 2007; Lin & Zhao, 2007; Bourtoom, 2008; Vargas et al., 2008; Porta, Mariniello, Di Pierro, Sorrentino & Giosafatto, 2011b; Giosafatto et al., 2014; Song & Zheng, 2014). In particular, protein/polysaccharide complexation may produce soluble complexes with desired functional characteristics to be components for ecomaterials. In fact, combined use of compatible proteins and polysaccharides may allow preparing films showing improved mechanical or permeability properties with respect the ones obtained from proteins or polysaccharides alone (Song & Zheng, 2014). Therefore, numerous new protein/polysaccharide-based biomaterials have been recently developed and characterized, several of them obtained from abundant natural sources. Since some of the latter, like milk whey and chitin, have traditionally been regarded only as waste products (Di Pierro et al., 2006; Mariniello et al., 2007; Di Pierro, Sorrentino, Mariniello, Giosafatto & Porta, 2011; Rossi Marquez et al., 2014), their utilization to produce useful bioplastics seems a really promising way for a possible recycling.

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In previous articles, bitter vetch (*Vicia ervilia*) protein concentrate (BVPC) edible films with promising both barrier and mechanical properties were described (Arabestani, Kadivar, Shahedi, Goli & Porta, 2013; Porta et al., 2015; Arabestani et al., 2016). Bitter vetch, an annual grain legume crop thus far widely cultivated only for forage because of its high nutritional value, shows several favourable characteristics, such as having high yields and being a cheap and abundant protein source (Lopez Bellido, 1994; Sadeghi, Mohammadi, Ibrahim & Gruber, 2009). The obtained BVPC, containing >86% (db) proteins, was recently analyzed for protein identification and the occurrence of convicilin, legumin A2, legumin B, alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and allergen Len c 1.0101 was detected (Arabestani et al., 2016). Therefore, in the attempt to refine BVPC film performance, new possible formulations by adding pectins (PEC) to the protein film forming solution (FFS) were selected. PEC are a heterogeneous group of acidic polysaccharides, occurring in numerous fruit and vegetables, generally obtained from citrus peel and apple pomace. They are composed of β-1,4-linked D-galacturonic acid residues wherein the uronic acid carboxyls are either fully or partially methyl esterified. PEC provide gel forming ability, hardness and adhesiveness to a variety of films even though their hydrophilic nature makes them poor barriers for water vapour (Nisperos-Carriedo, 1994). In addition, since chemical and/or enzymatic modifications of polysaccharides and proteins could provide further promising ways to improve film properties, we prepared BVPC/PEC blended films also in the presence of the enzyme transglutaminase (EC 2.3.2.13) isolated from *Streptoverticillium* sp. (mTGase) (Pasternack et al., 1998; Kieliszek & Misiewicz, 2014). Transglutaminases, enzymes able to produce protein inter- and/or intra-molecular ε-(γ-glutamyl)lysine isopeptide bonds, are able to catalyze the conversion of soluble substrate proteins into high molecular weight polymers also inside the edible films (Porta, Di Pierro, Sorrentino & Mariniello, 2011a). Therefore, we expected that the occurrence of PEC, as well as of PEC and mTGase, in the BVPC FFS could determine significant changes in film mechanical and/or permeability features. In the present study we analyze the morphological and the main functional characteristics of these new blended BVPC/PEC films.

## 2. Materials and methods

### 2.1. Materials

Bitter vetch seeds were obtained from a local market in Isfahan (Iran), citrus peel low-methylated (7%) PEC (Aglupectin USP) was from Silva Extracts srl (Gorle, BG, Italy), whereas mTGase (Activa WM), derived from the culture of *Streptoverticillium* sp., was supplied by Prodotti Gianni SpA (Milano, Italy). Sodium hydroxide, hydrochloric acid (37%) and glycerol (about 87%) were purchased from the Merck Chemical Company (Darmstadt, Germany). All other chemicals and solvents used in this study were analytical grade commercial products.

### 2.2. BVPC and mTGase preparation

Bitter vetch proteins were extracted from the seeds as previously described (Arabestani et al., 2013) by their solubilization at pH 11 and, after stirring for 1 h at room temperature, by centrifugation at 3200 ×*g* for 10 min. The pH of the supernatant was then adjusted to 5.4 by 0.1 N HCl and the obtained precipitate, separated by centrifugation at 3200 ×*g* for 10 min, was finally dissolved at pH 7.0. The solution was dried at 50 °C in a vacuum oven, and the dry protein concentrate ground in a coffee grinder. The protein content of both bitter vetch seeds and BVPC was determined by the Kjeldahl's method.

mTGase solution was prepared by dissolving the commercial product, containing 1% of enzyme and 99% of maltodextrins, in distilled water (180 mg $mL^{-1}$). The mixture was centrifuged at 10,000 ×*g* for 2 min and the estimation of enzymatic activity occurring in the supernatant was carried out by a colorimetric hydroxamate assay (Kieliszek & Misiewicz, 2014). Specific activity of the enzyme preparation was detected to be 92 U/g.

### 2.3. PEC preparation

PEC (1.0 g) were dissolved in 100 mL of distilled water. The solution was stirred until PEC were completely solubilized. Then, the pH of the solution was adjusted to pH 5.0 by using HCl 3 N.

### 2.4. Turbidity titration

Bitter vetch protein/PEC complexation was followed by turbidity titration of acidic and basic protein groups (Di Pierro et al., 2013) The titrations were performed at 22 °C on all the film-forming solutions containing PEC at different BVPC/PEC ratios of 1:1, 5:1, and 40:1 (w/w), respectively, and on a solution containing only BVPC at a protein concentration of 45 mg/mL. The initial pH was adjusted to 10.0 ± 0.05 with 0.1 N NaOH, and the solutions were titrated with 0.1 N HCl to reach pH 3.5, corresponding to a pH value lower than PEC pKa (4.6). The pH was noted when the value was stable for at least 1 min. The reproducibility of two repeated titrations was ±0.05 pH units. The solution turbidity was determined using a UV/visible spectrophotometer at 600 nm with distilled water as blank reference. Complexation pH (pHc) was recorded as the pH value corresponding to a change in slope of each curve.

### 2.5. Film preparation

5 g of BVPC powder were dispersed in 100 mL of distilled water, under constant stirring, and 0.1 N NaOH was added to adjust the pH to 12 for a complete protein solubilization. Then, the pH was adjusted to pH 6.5 by 0.1 N HCl and 50% (w/w protein) glycerol was added. The obtained BVPC FFS was incubated at 25 °C for 1 h in the absence or presence of mTGase (20 U/g BVPC) for zeta-potential and particle size analyses. BVPC FFS was cast on polystyrene Petri dishes (7.5 mg proteins/$cm^2$) for 48 h at 25 °C and 45% RH. Dried films were peeled intact from the casting surface and conditioned at 25 °C and 53% RH for 2 h, by placing them in a dessicator over a saturated solution of $Mg(NO_3)_2 \cdot 6H_2O$, before being tested.

Two additional kinds of FFS were prepared: BVPC FFS containing PEC (40:1, w/w) was incubated at 25 °C for 1 h in the absence (BVPC/PEC) or presence (BVPC/PEC/mTGase) of mTGase (20 U/g BVPC) and then both analyzed for zeta-potential and particle size before their casting for 48 h at 25 °C and 45% RH. Dried films were peeled intact from the casting surface and conditioned as the controls. Film thickness was measured with a micrometer (Electronic digital micrometer, DC-516, sensitivity 0.001 mm) at different positions for each film sample. At least five measurements were taken on each film sample and the thickness mean values were considered in the different tests.

### 2.6. Zeta-potential and particle size measurements

Zeta-potential, particle size and polydispersity index of the different BVPC FFSs, containing or not PEC and mTGase, were analyzed using the Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK). Three independent zeta-potential measurements at pH 6.5 were carried out on each sample of FFS (1.0 mL) introduced in the measurement vessel. Temperature was set at 25 °C, applied voltage was 200 mV and duration of each analysis was approximately 10 min. The software calculated mean diameter of particles, determined at pH 6.5 with the Zetasizer Nano-ZSP by using dynamic light scattering, includes also the particle double layer (hydrodynamic diameter called Z-Average.), whereas the polydispersity index represents the relative variance in the particle size distribution. The device uses a helium-neon laser of 4 mW output power operating at the fixed wavelength of 633 nm (wavelength of laser red emission). All the results were reported as mean ± standard deviation.

### 2.7. Mechanical properties

Film tensile strength, elongation at break and Young's modulus were determined by using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). Film samples strips (10–11 mm wide and 50 mm long), obtained by using a sharp razor blade, were equilibrated for 2 h at 50% RH and 25 °C in an environmental chamber, and ten samples of each film type were tested. Tensile properties were measured according to the ASTM D882-97 (1997) using Test Method A, the static weighing, constant rate-of-grip separation test. The initial grip separation was 40 mm, and the crosshead speed was 10 mm/min in tension mode.

### 2.8. Gas and water vapor permeabilities

The permeability of the films to oxygen and carbon dioxide was determined using a modified ASTM D3985-81 (1981) with a MultiPerm apparatus (ExtraSolution s.r.l., Pisa, Italy). Duplicate samples of each film were conditioned in an environmental chamber for 2 h at 50% RH before measurement. Aluminum masks were used to reduce the film test area to 5 $cm^2$, whereas the testing was performed at 25 °C under 50% RH.

Water vapour permeability (WVP) was evaluated by a gravimetric test according to ASTM E96-93 (1993) by introducing 7 g of silica gel in each Fisher/Payne permeability cup (Carlo Erba, Italy). Film samples (6 cm diameter) were put on top of the cup and sealed by means of a top ring kept in place by three tight clamps, each film area exposed to vapour transmission being 10 $cm^2$. Silica gel containing cups were weighed and then placed in a ventilated desiccator containing an electronic hydrometer in the presence of a saturated KCl solution, which provided a constant water activity of 0.8434 at 25 °C. The desiccator was finally stored in a Heareus thermostated incubator at 25.0 ± 0.1 °C. Cups were weighed at scheduled times, and the amount of water vapour transmission rate through the film was estimated by the linear portion of the diagram obtained by plotting the weight increment of the cup as a function of time. It was assumed that the steady state was reached once the regression analysis made by using the last four data points resulted in $r^2 \geq 0.998$. The WVP was calculated from the equation

$$WVP = X/(A\Delta p)\, dm/dt$$

where $dm/dt$ is the slope of the cup weight versus time curve once steady state was reached ($X$ is the film thickness, $A$ is the film exposed area, and $\Delta p$ is the water vapour pressure across the film). By assuming that the vapour pressure inside the cup, due to the presence of silica gel, is equal to zero, $\Delta p$ becomes equal to the vapour pressure inside the desiccator and was calculated by multiplying water activity and the saturated vapour pressure ($P_0$) at 25 °C ($P_0 = 3.167$ kPa).

### 2.9. Swelling experiments

Disks of freshly prepared films were cut out with a punch, bisected with a razor and immersed in 0.1 M Tris HCl buffer, pH 6.5. The length of the straight edge was measured with a traveling microscope to 10 μm (PTI Liss, Hampshire, England). The polymer volume in the swollen state is a measure of fluid imbibed and retained by the film. Six independent determinations for each swelling experiment have been carried out.

### 2.10. Scanning electron and atomic force microscopy

Film cross section was observed using a Scanning Electron Microscope (SEM, Phillips XL30, the Netherlands). Films, fractured in liquid nitrogen, were rested vertically on the sides of a rectangular aluminum piece and fixed on stubs using double sided adhesive tape, then coated with gold (300 s, 20 mA), and finally observed at a magnification of 500× and 4000×. An acceleration potential of 20 kV was used during micrograph.

Film surface morphology was studied using an Atomic Force Microscope (AFM) (Bruker, model Nanos). A sharpened $Si_3N_4$ cantilever, with a spring constant of 0.2 N/m and a V-shaped tip 450 μm long, was positioned over each sample and images (42 × 42 μm) under ambient conditions were obtained.

### 2.11. Statistical analysis

JMP software 5.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. The data were subjected to analysis of variance, and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $P < 0.05$.

## 3. Results and discussion

### 3.1. Preparation of BVPC films in the presence of PEC and mTGase

The detected protein content value from ground bitter vetch seeds (flour) was about 28% (db), whereas the deriving BVPC used as protein source for the preparation of the different films contained about 85% (db) proteins. First of all the possibility of using PEC, a well known gel producing polysaccharide, in the manufacture of films blended with bitter vetch proteins was investigated. In addition, the obtained BVPC/PEC blended films were also prepared in the presence of mTGase, as PEC was previously shown to do not negatively affect enzyme functionality (Mariniello et al., 2003). In fact, the aim of our experiments was attempting to confer improved functional properties to the edible films recently obtained by using only bitter vetch proteins (Porta et al., 2015) in the absence or presence of mTGase.

To produce BVPC/PEC films the behavior of PEC in the presence of bitter vetch proteins, at both different BVPC/PEC ratios (w/w) and different pH values, was preliminarily investigated to determine the best experimental conditions for obtaining soluble protein/polysaccharide complexes. In fact, it is well known that, according to the model proposed by Weinbreck, de Vries, Schrooyen & de Kruif (2003), proteins and PEC occur in free molecular forms when they are dissolved in water at pH values higher than their pHc, whereas at pHc they interact by forming soluble complexes that generally aggregate and then precipitate with further pH decreases.

Fig. 1 illustrates the turbidity titration curves of different BVPC/PEC film-forming solutions prepared at various BVPC/PEC ratios. The titration curves obtained with all the BVPC/PEC ratios assayed became almost superimposable by decreasing the pH from 6.0 to pH 4.0. Moreover, although the titration curves corresponding to a BVPC/PEC ratio of 1:1, 5:1 and 40:1 (w/w) all changed their slopes at pH 6.5 (pHc), BVPC/PEC ratio of 40:1 resulted that suitable to obtain soluble protein/polysaccharide complexes at lowest PEC concentrations. Therefore, BVPC/PEC FFSs at pH 6.5 (i.e. when soluble bitter vetch protein/PEC complexes occur), both in the absence and presence of mTGase, were prepared and preliminarily analyzed for their zeta-potential, particle size and polydispersity index. Table 1 shows that the presence of PEC significantly increases the negative zeta-potential of the blended BVPC/PEC FFS and that this value was further enhanced in the presence of mTGase. Conversely, we observed an enhancement of the size of BV protein/PEC nano-complexes compared to BVPC sample which resulted decreased by the concomitant presence of mTGase. It is worthy to note that the same effect of TGase on both zeta-potential and particle size was detected also when BVPC FFS was prepared in the absence of PEC. Moreover, Table 1 shows that the addition of the enzyme determined a reduction in the Z-average, as well as an increased homogeneity of the particle size distribution as indicated by polydispersity index lower values. Since the TGase effect was observed both in the absence and presence of PEC, it could be hypotisized that BV protein crosslinking promoted an increased compactness in the macromolecular

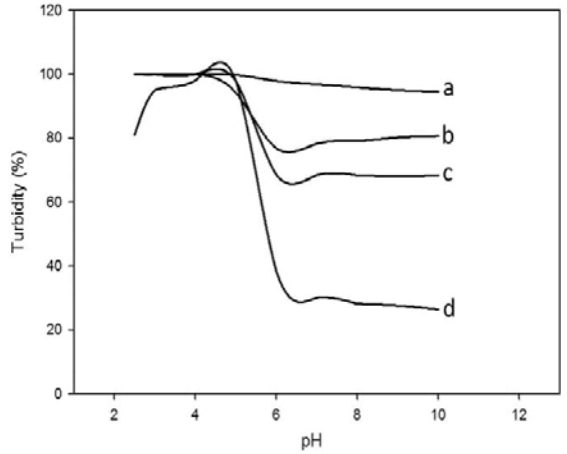


**Fig. 1.** Turbidometric titration curves of BVPC/PEC FFSs prepared with different protein/polysaccharide ratios (w/w). BVPC/PEC ratio (w/w) of 1:1 (a), 5:1 (b) and 40:1 (c); control curve (d) corresponds to BVPC FFS prepared in the absence of PEC. Further experimental details are given in the text.

nanocomplexes containing or not the polysaccharide. Therefore, we found of interest to examine the mechanical and barrier properties of the derived films with the aim to investigate a possible correlation of FFS physicochemical patterns with the functional features of the different films obtained.

### 3.2. Film mechanical properties

The results reported in Table 2 indicate that the blended BVPC/PEC films showed tensile strength values significantly higher than the ones of the corresponding films obtained from solutions prepared without PEC. In fact, the addition of PEC was observed to almost double BVPC film tensile strength, whereas the concomitant presence of mTGase in the BVPC/PEC FFS determined a much higher effect increasing about 3-fold the tensile strength value of the films prepared with only bitter vetch proteins. These findings, following the observed zeta-potential rising trend of the corresponding FFSs (Table 1), could be explained by considering the formation of a BVPC/PEC ionic supramolecular network that is even crosslinked in the presence of enzyme (Mariniello et al., 2003; Ashby & Jones, 2005). In fact, mTGase-catalyzed BV protein covalent bonds might be responsible for the improved film resistance with respect to the films prepared without enzyme both in the absence (Porta et al., 2015) and presence of PEC.

Furthermore, Table 2 shows that also the elongation at break resulted higher in the BVPC/PEC films containing mTGase with respect to both films prepared in the absence of the enzyme with (1.6-fold) or without (2-fold) PEC. Conversely, it was previously observed that the enzyme decreased the elongation at break of BVPC films prepared in the absence of PEC (Porta et al., 2015). These findings, thus, lead to conclude that mTGase containing films become more extensible only when also PEC occur in the FFS, suggesting that also this phenomenon should be correlated to the increase of zeta-potential of the originating FFSs. These findings are in agreement with previous studies (Jiang, Li, Chai & Leng, 2010; Acevedo-Fani et al., 2015) explaining the observed increase of flexibility of both polysaccharide/essential oil and whey protein/gelatin composite films by the influence of the nano-emulsion or solution electrical charge. In this respect, it was suggested that the repulsive forces among macromolecules of the same charge could increase the distance between the nano-complexes and, consequently, determine a plasticizing effect in the case of charged polymeric film structures.

**Table 1**
Zeta-potential, Z-average and polydispersity index of BVPC and BVPC/PEC FFSs prepared in the absence and presence of mTGase.[1]

| Sample | Zeta-potential (mV) | Z-average (nm) | Polydispersity index |
|---|---|---|---|
| BVPC | $-25.3 \pm 0.64^{a}$ | $286.4 \pm 21.2^{a}$ | $0.751 \pm 0.157^{a}$ |
| BVPC + mTGase | $-27.4 \pm 0.53^{b}$ | $181.9 \pm 1.7^{b}$ | $0.489 \pm 0.027^{b}$ |
| BVPC/PEC | $-35.6 \pm 0.56^{c}$ | $476.5 \pm 34.4^{c}$ | $0.731 \pm 0.107^{a}$ |
| BVPC/PEC + mTGase | $-37.8 \pm 0.50^{d}$ | $269.5 \pm 4.0^{d}$ | $0.471 \pm 0.016^{c}$ |

Different letters in the columns indicate significant differences at $P < 0.05$.
[1] Experimental details are given in the text.

**Table 2**
Mechanical properties of BVPC films prepared in the presence or absence of PEC and mTGase.[1]

| Film | Tensile strength (MPa) | Elongation at break (%) | Young's module (MPa) |
|---|---|---|---|
| BVPC (control) | $1.52 \pm 0.15^{a}$ | $30.15 \pm 2.32^{a}$ | $79.01 \pm 2.15^{a}$ |
| BVPC/PEC | $2.90 \pm 0.25^{b}$ | $41.17 \pm 4.76^{b}$ | $97.15 \pm 5.10^{b}$ |
| BVPC/PEC + mTGase | $4.29 \pm 0.47^{c}$ | $64.08 \pm 1.98^{c}$ | $60.26 \pm 2.60^{c}$ |

Different letters in the columns indicate significant differences at $P < 0.01$.
[1] Experimental details are given in the text.

Table 2 also shows that BVPC/PEC/mTGase films exhibited the lowest Young's module value, about 40% lower than that observed for BVPC/PEC films prepared in the absence of enzyme and 23% lower than the one of the films obtained with BVPC alone. Therefore, all these data clearly indicate an increase of the film stiffness determined by the mTGase-catalyzed BV protein cross-links. The observed highest tensile strength and deformability, together a low Young's modulus, lead to define the BVPC/PEC blended films obtained in the presence of mTGase as typical elastomers having a marked mechanical resistance (Dangaran, Tomasula & Qi, 2009.

### 3.3. Film permeability

To evaluate the possible influence of mTGase on film barrier properties, the permeabilities to the main environmental gases of BVPC/PEC blended films obtained at pHc, in the presence or absence of the enzyme, were analyzed. Table 3 shows that the permeability to both $CO_2$ and $O_2$ of BVPC films containing 40:1 (w/w) PEC was markedly lower (>20- and 300-fold, respectively) compared to the control values obtained with BVPC films. Moreover, our findings indicated that mTGase addition to the BVPC/PEC FFSs determined a further increase (almost 3- and 2-fold toward $CO_2$ and $O_2$, respectively) in the film barrier properties probably associated with a more compact film structure determined by the enzymatic cross-linking. In fact, it is well known that the film network structure, as well as the specific processing conditions, markedly affects film barrier properties to both $CO_2$ and $O_2$. Several studies have previously shown that cross-linking of polymers contained in various biomaterials are able to determine a decrease in film permeability (Miller & Krochta, 1997). Conversely, WVP values determined with BVPC/PEC films prepared both in the

**Table 3**
Gas and water vapor permeabilities of BVPC/PEC films prepared in the presence or absence of mTGase.[1]

| Film | Thickness | $CO_2$ | $O_2$ | WVP |
|---|---|---|---|---|
| | μm | $cm^3\ mm\ m^{-2}\ d^{-1}\ kPa^{-1}$ | | |
| BVPC (control) | $80.01 \pm 0.02^{a}$ | $21.14 \pm 2.10^{a}$ | $22.520 \pm 1.820^{a}$ | $15.01 \pm 1.78^{a2}$ |
| BVPC/PEC | $84.98 \pm 0.03^{b}$ | $0.91 \pm 0.05^{b}$ | $0.076 \pm 0.004^{b}$ | $16.01 \pm 1.65^{a}$ |
| BVPC/PEC + mTGase | $114.01 \pm 0.02^{c}$ | $0.34 \pm 0.02^{c}$ | $0.036 \pm 0.002^{c}$ | $20.15 \pm 1.78^{a}$ |

Different letters in the columns indicate significant differences at $P < 0.01$.
[1] Experimental details are given in the text.
[2] Porta et al. (2015).

absence (16.01 ± 1.65 $cm^3$ mm $m^{-2}$ $d^{-1}$ $kPa^{-1}$) and presence (20.15 ± 1.78 $cm^3$ mm $m^{-2}$ $d^{-1}$ $kPa^{-1}$) of mTGase resulted not significantly different from the ones previously reported for BVPC films (Porta et al., 2015). This result can be explained by considering that the barrier effect to water vapour depends mainly on the film hydrophilicity that is not significantly affected by the protein crosslink formation inside the network. However, swelling experiments performed at pH 6.5 showed low but significant differences among BVPC films prepared in the presence or absence of both PEC and mTGase. In fact, the swelling index of BVPC films containing PEC was found lower than the one measured for the BVPC film alone (V/Vo = 0.035 ± 0.003 vs 0.045 ± 0.002) because of the strong ionic protein/PEC interactions occurring at pHc. Moreover, when mTGase was added to the FFS, film swelling index was further reduced to a value of 0.026 ± 0.002, thus confirming a free volume decrease determined by the enzyme-catalyzed cross-links produced in the film network.

### 3.4. Morphological characteristics of BVPC/PEC films

Finally, on the basis of the different functional properties of the blended BVPC/PEC films, observed when mTGase was added to the FFS, film microstructure was investigated by carrying out both AFM and SEM experiments. Fig. 2 shows the three-dimensional (left) and topographic (right) AFM images of the surface of BVPC/PEC films prepared in the absence (panel A) or presence (panel B) of mTGase. A RMS roughness value (Rq) of 66.4 nm was calculated for the films prepared without the enzyme, whereas a Rq value of 214.1 nm was determined for those obtained in its presence, thus indicating a higher surface roughness of the films obtained from solutions containing mTGase. Moreover, cross-sectional SEM analysis of the same samples (Fig. 3) showed that BVPC/PEC films containing mTGase (panel B) possess a more compact and homogeneous microstructure, with evident continuous zones, in comparison with the reticular structure of control samples (panel A). The knitting morphology of BVPC/PEC films could explain also their more marked water uptake ability observed during swelling experiments.

## 4. Conclusion

The development of biopolymer-based films, mechanically resistant and exhibiting good barrier properties, are receiving an increasing attention for their possible practical application as alternatives to oil-sourced polymers. Due to their low cost and high yield, bitter vetch proteins present undoubtful advantages for developing new edible ecomaterials. The blended hydrocolloid film obtained by using BVPC in the presence of PEC and mTGase is a promising way to obtain possible edible food coatings as well as effective drug delivery systems. In fact, unsatisfactory tensile properties and poor barrier ability toward the main environmental gases are considered thus far the major limitations to a wider use of biodegradable/edible films, being undesirable characteristics to maintain the quality of numerous foods and drugs. Therefore, the improved mechanical features exhibited by BVPC/PEC films prepared in the presence of mTGase, together with their high barrier activity to both $CO_2$ and $O_2$, confer to these new hydrocolloid materials potential interesting applications in both food and pharmaceutical industries.

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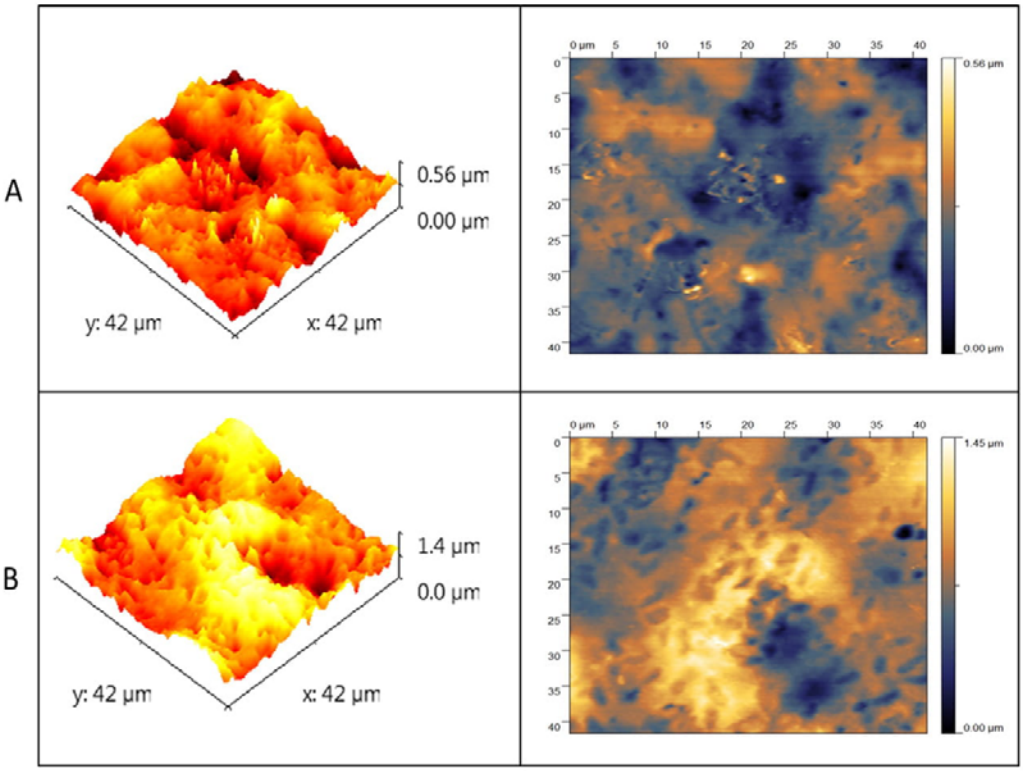


**Fig. 2.** Three-dimensional (left) and topographic (right) AFM images of BVPC/PEC films obtained in the absence (panel A) or presence (panel B) of mTGase. The images shown were chosen as the most representative from each sample. Experimental details are given in the text.

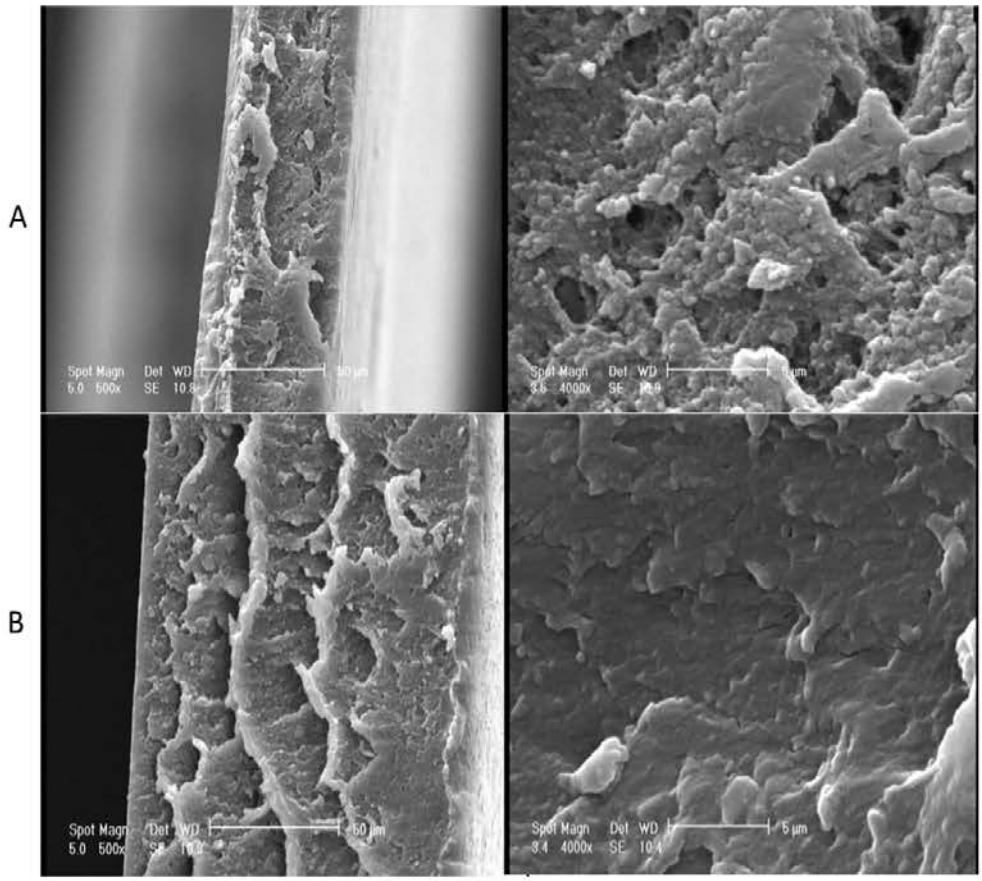


**Fig. 3.** SEM micrographs at 500× magnification (left) and at 4000× magnification (right) of BVPC/PEC films obtained in the absence (panel A) or presence (panel B) of mTGase. The images shown were chosen as the most representative from each sample. Experimental details are given in the text.

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### 3.3 Development and properties of new chitosan-based films plasticized with spermidine and/or glycerol

Although CH-based films exhibit weak mechanical properties, as well as unsatisfying WV barrier features, they remain the most up-and-coming ones among the various hydrocolloid bioplastics so far proposed, because they are biodegradable, biocompatible, non-toxic and obtainable in large quantities from waste products of seafood industries (crustacean shells). Recent studies have shown that aliphatic polyamines, in particular the triamine SPD, are able to influence the morphological, mechanical and barrier properties of edible films produced from another polysaccharide, i.e. PEC (Esposito et al., 2016), and that the combination of different concentrations of both SPD and GLY may give rise to biomaterials that possess a wide spectrum of functional characteristics. Therefore, the possible improvement of the physicochemical and biological properties of CH-based films by incorporating various SPD and GLY proportions in the host matrix was investigated. Based on such hypothesis, it is expected that the addition of both plasticizers to polymeric matrix can bring about better features of the CH films so that a new polysaccharide-based biomaterial can represent a valid alternative to the gelatin-based films, such as the well commercialized Viscofan (www.viscofan.com) widely used for food wrapping. In fact, gelatin is one the most controversial ingredients of “kosher and halal” food and it seems advisable to replace it according to the religion-based dietary restrictions of both Muslim and Jewish consumers and the consequent negative impact in their marketplace (Regenstein et al., 2003).

SPD-containing films were found always much more extensible materials exhibiting an EB even higher than that of GLY-plasticized films. In addition they resulted able to be thermo-sealed and to retain the well known antimicrobial CH features. All these results are reported in the following published article.

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# Development and properties of new chitosan-based films plasticized with spermidine and/or glycerol

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Food coating

ABSTRACT

Different chitosan solutions were characterized by evaluating zeta potential and particle size, in the absence or presence of spermidine and/or glycerol, and the physicochemical, morphological and antimicrobial properties of the derived films were determined. An increase of film tensile strength and elongation at break was observed by increasing chitosan amounts, whereas only tensile strength and Young's modulus values were revealed higher at all chitosan concentrations when spermidine was absent. Spermidine-containing films were always more extensible exhibiting an elongation at break even higher than that of glycerol-plasticized films. The concurrent presence of appropriate concentrations of spermidine and glycerol further enhanced the extensibility and plasticity of the biomaterial, conferring to it the ability to be heat-sealed, as well as similar permeability in comparison with Viscofan NDX, widely commercialized as protein-based food casing. Finally, all the prepared films exhibited a clear antimicrobial activity, thus representing credible candidates as food preservative coatings and/or wrappings.

## 1. Introduction

Chitin is the second most abundant biopolymer occurring in nature after cellulose and chitinous waste, mainly produced from sea food processing (crustacean shells), still represents a major environmental issue (Arbia, Arbia, Adour, & Amrane, 2013).

Chitin is not soluble in common solvents, mostly due to its highly cristalline structure, and this property strongly limits the possible re-use of the polysaccharide. Nevertheless, one possible recycling of chitin rich wastes involves the chemical conversion of chitin in chitosan (CH), a random copolymer formed by D-glucosamine and *N*-acetyl-D-glucosamine units, by alkaline deacetylation at high temperatures (Muxika, Etxabide, Uranga, Guerrero, & de la Caba, 2017). Although various factors (e.g. chitin source, alkali concentration, deacetylation temperature and time) may affect its properties, CH (pKa, 6.3) is easily dissolved in acidic solutions, i.e. when its free amino groups are fully protonated (Aljawish, Chevalot, Jasniewski, Scher, & Muniglia, 2015; Babu, O'Connor, & Seeram, 2013; Kaur & Dhillon, 2014; Van den Broek, Knoop, Kappen, & Boeriu, 2015).

The unique physicochemical and biological features of CH make it worthy in regard to various biomedical, pharmaceutical and agricultural applications. Moreover, because of CH broad antibacterial and antifungal properties, CH-based edible films may be promoted as promising "new economy" bio-based plastics (Spierling et al., 2018) also for food coating and protection in addition to the protein-based biomaterials (Han, Yu, & Wang, 2018). In fact, although CH-based films exhibit weak mechanical properties, as well as unsatisfying water vapor (WV) barrier features, they remain the most promising ones among the various hydrocolloid biomaterials so far proposed, because they are biodegradable, biocompatible, non-toxic and obtainable in large quantities from waste products of seafood industries (crustacean shells) (Elsabee & Abdou, 2013; Mayachiew & Devahastin, 2008; Van der Broek et al., 2015). In addition, CH has been considered as a GRAS (Generally Recognized As Safe) food additive for both consumers and the environment (FDA, 2012).

Several advantages have been demonstrated when different food

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products were CH-coated. CH was shown to be able to form a semi-permeable layer on the surface of various fruits and vegetables, and to delay the rate of respiration and their ripening by reducing food moisture and weight loss (Alvarez, Ponce, & Moreira, 2013; Chofer, Sanchez-Gonzalez, Gonzalez-Martinez, & Chiralt, 2012; Gol, Patel, & Rao, 2013; Sun et al., 2014). Moreover, edible CH films have also been used as carriers releasing different bioactive agents like essential oils, as well as antimicrobials and/or antioxidants (Acevedo-Fani, Salvia-Trujillo, Rojas-Graü, & Martín-Belloso, 2015; Avila-Sosa et al., 2012), and to protect fish, red meat, poultry and their processed products, with the aim to decrease color changes, lipid oxidation, growth of pathogenic and spoilage bacteria and to extend product shelf life (Chamanara, Shabanpour, Khomeiri, & Gorgin, 2013; Gómez-Estaca, De Lacey, López-Caballero, Gómez-Guillén, & Montero, 2010; Samelis, 2006).

Many different attempts have been made to improve mechanical, barrier and functionality properties of CH films by blending CH film forming solution (FFS) with other biopolymers like proteins (Baron, Pérez, Salcedo, Córdoba, & Sobral, 2017; Di Pierro et al., 2007, 2006; Escamilla-García et al., 2017). A further way to modify the physicochemical characteristics of the hydrocolloid edible films, and for a subsequent breakthrough in their applications, is the addition of appropriate concentrations of a suitable plasticizer. Generally, plasticizers are added to both synthetic and bio-based polymeric materials to decrease the intermolecular forces along the polymer chains, impart flexibility and lower the glass transition temperature (Mekkonen, Mussone, Khalil, & Bressler, 2013; Vieira, Altenhofen da Silva, Oliveira dos Santos, & Beppu, 2011). Our recent studies have shown that aliphatic polyamines, in particular the triamine spermidine (SPD), are able to influence the morphological, mechanical and barrier properties of pectin- and protein-based films (Esposito et al., 2016; Porta, Di Pierro, Roviello, & Sabbah, 2017). In addition, the combination of different concentrations of both SPD and glycerol (GLY) may give rise to protein-based biomaterials possessing a wide spectrum of functional characteristics (Porta et al., 2017; Sabbah et al., 2017). Since Chanphai and Tajmir-Riahi (2016) recently reported the conjugation of CH nanoparticles with biogenic polyamines SPD and spermine in aqueous solution, we were stimulated to analyze the physicochemical and biological properties of CH-based films by incorporating various SPD and GLY proportions into the host polysaccharide matrix. Based on the present investigation, it is expected that the addition of both plasticizers to polymeric matrix can bring about improved features of the CH films in such a way that a new polysaccharide-based biomaterial can represent a valid alternative to gelatin-based films, such as the well commercialized Viscofan (www.viscofan.com) widely used for food wrapping. In fact, gelatin is one the most controversial of kosher and halal food ingredients and it seems advisable to replace it according to the religion-based dietary restrictions of Muslim and Jewish consumers and the consequent negative impact in their marketplace (Regenstein, Chaudry, & Regenstein, 2003).

## 2. Materials and methods

### 2.1. Materials

CH (mean molar mass of $3.7 \times 10^4$ g/mol) with a degree of 9.0% *N*-acetylation, was a gift from Prof. R.A.A. Muzzarelli (University of Ancona, Italy). The mean molar mass of CH was determined by a viscometric method, as previously described (Costa, Teixeira, Delpech, Sousa & Costa, 2015), by dissolving 0.2 g of CH in 10 mL of 0.1 M acetic acid, containing 0.2 M sodium chloride, and obtaining five different dilutions of the original solution. The degree of *N*-acetylation was determined by the first derivative ultraviolet spectrophotometric method, as described by Muzzarelli and Rocchetti (1985), based on recording of the first derivative of the CH UV spectra at 202 nm by using a standard curve obtained by varying *N*-acetylglucosamine concentrations. Citrus peel low-methylated (7.0%) pectin (Aglupectin USP) was purchased from Silvateam srl (San Michele Mondovi, CN, Italy). Viscofan NDX edible casings were from Naturin Viscofan GmbH (Tajonar-Navarra, Spain). GLY (about 87%) was supplied from the Merck Chemical Company (Darmstadt, Germany), whereas SPD was from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were analytical grade.

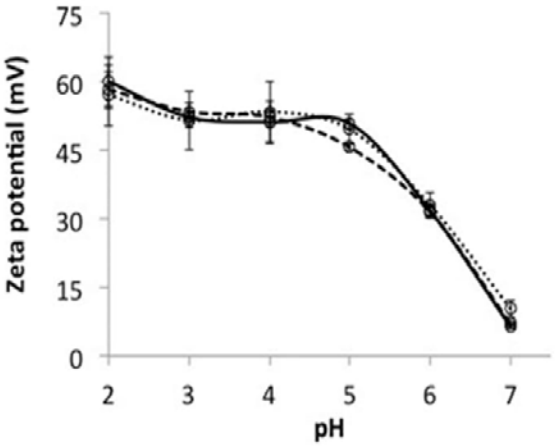


**Fig. 1.** Effect of either 5 mM SPD and/or 25 mM GLY on zeta potential of 0.2% CH FFSs measured at different pH values. The different FFSs contained only CH (solid line), CH + SPD (point line), CH + GLY (dashed line). The results are expressed as mean ± standard deviation. Further experimental details are given in sections 2.2 and 2.5.

### 2.2. Titration of CH FFSs

Zeta potential values of different CH solutions (0.2% CH containing or not 5 mM SPD and/or 25 mM GLY) were determined by a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) equipped with an automatic titrator unit (MPT-2). The device was equipped with a helium-neon laser of 4 mW output power operating at the fixed wavelength of 633 nm (wavelength of laser red emission). The instrument software programmer calculated the zeta potential through the electrophoretic mobility by applying a voltage of 200 mV using the Henry equation. CH FFSs were prepared at pH 2.0 by using 1.0 N HCl and then the titration was carried out from pH 2.0 to pH 7.0 by adding 1.0, 0.5, and 0.1 N NaOH as titrant solutions under constant stirring at 25 °C. Zeta potential values were measured at each pH in triplicate.

### 2.3. CH FFS and film preparation

CH stock solution (2%) was prepared by dissolving the polysaccharide in 0.1 N HCl at room temperature under overnight constant stirring at 700 rpm (Di Pierro et al., 2006). FFSs and films were obtained at pH 4.5 by using CH (0.1–0.6%) mixed or not with different concentrations of SPD (2–10 mM; 5–24%, w/w with respect to maximal CH concentration used) and/or GLY (2–40 mM; 3–60%, w/w with respect to maximal CH concentration used). All FFSs were characterized for their zeta potential, Z-average and conductivity by a Zetasizer Nano-ZSP as described above. FFSs were then poured onto polystyrene plates (1 mL x $cm^2$), most experiments being performed by using 8 cm diameter polystyrene Petri dishes. FFSs were allowed to dry in an adjusted environmental chamber at 25 °C and 45% RH for 48 h and, finally, the dried films were peeled from the casting surface and stored at 25 °C and 50% RH.

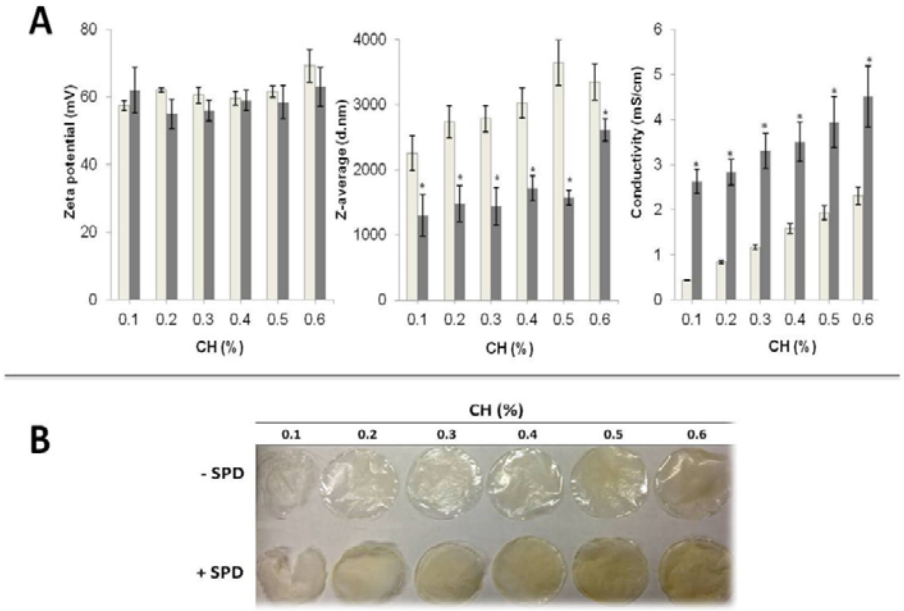


**Fig. 2.** Zeta potential, Z-average and conductivity values of FFSs prepared at pH 4.5 with different CH concentrations in the absence (light bars) or presence (grey bars) of 5 mM SPD (panel A), and the images of the derived films (panel B). The results are expressed as mean ± standard deviation. *Values significantly different compared to the ones obtained at the same CH concentration in the absence of SPD ($p < 0.05$). Further experimental details are given in sections 2.2–2.5.

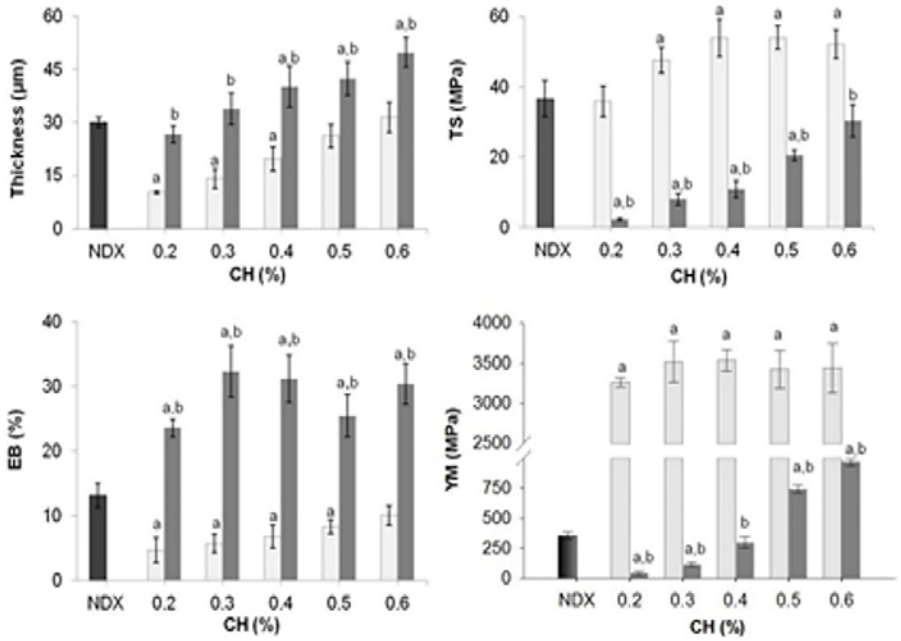


**Fig. 3.** Effect of different CH concentrations on thickness and mechanical properties of films prepared in the absence (light bars) or presence (grey bars) of 5 mM SPD. The results are expressed as mean ± standard deviation. The values significantly different from those obtained by analyzing Viscofan NDX (black bars) are indicated by "a", whereas the values indicated by "b" were significantly different from those obtained at the same CH concentration but in the absence of SPD. Further experimental details are given in sections 2.3–2.5.

### 2.4. Film characterization

All films, cut into 1 cm × 8 cm strips by using a sharp scissor, were conditioned at 25 °C and 50% RH for 2 h by placing them into a desiccator over a saturated solution of $Mg(NO_3)_2.6\ H_2O$ before being tested. Film thickness was measured in six different points with a micrometer (Electronic digital micrometer, DC-516, sensitivity 0.001 mm) and their tensile strength (TS), elongation at break (EB) and Young's modulus (YM) were determined on five specimens of each sample (5 cm gage length, 1 kN load and 1 mm/5 min speed) by using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). One specimen of each sample (1 × 30 cm strip) was always prepared and analyzed with a specimen gage length of 25 cm (ASTM D882-97, 1997) to confirm the data obtained with

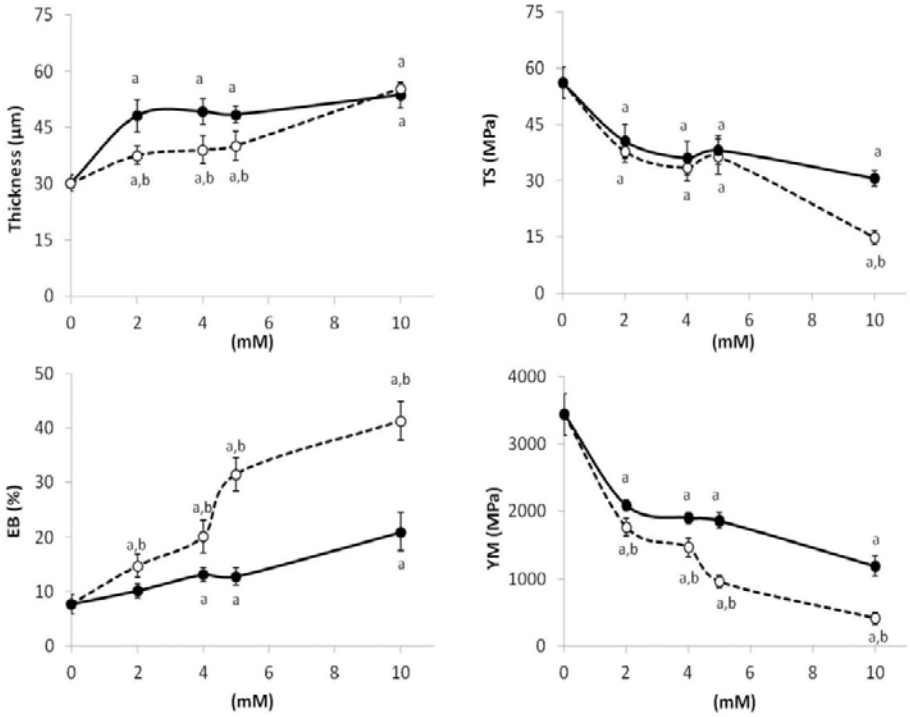


**Fig. 4.** Effect of different concentrations of SPD (dashed line) and GLY (solid line) on the mechanical properties of 0.6% CH films. The results are expressed as mean ± standard deviation. The values significantly different from those obtained with films made by only CH were indicated by "a", whereas the values of the SPD-containing films indicated by "b" were significantly different from those obtained with films prepared in the presence of GLY. Further experimental details are given in sections 2.3–2.5.

**Table 1**
Effect of different concentrations of GLY on the mechanical properties of 0.6% CH films containing different amounts of SPD.

| Addition | | Thickness (µm) | TS (MPa) | EB (%) | YM (MPa) |
|---|---|---|---|---|---|
| SPD | GLY | | | | |
| mM | | | | | |
| - | - | 31.50 ± 4.23 | 52.19 ± 4.15 | 10.07 ± 2.53 | 3438.10 ± 506.30 |
| - | 10 | 53.71 ± 3.46 | 30.57 ± 2.04 | 20.95 ± 3.43 | 1194.38 ± 150.87 |
| - | 20 | 76.00 ± 5.30 | 15.60 ± 2.21 | 73.10 ± 3.48 | 146.30 ± 10.00 |
| - | 30 | 84.67 ± 4.04 | 10.20 ± 0.38 | 109.97 ± 5.51 | 27.70 ± 2.94 |
| - | 40 | 91.00 ± 3.60 | 10.12 ± 2.24 | 136.04 ± 8.15 | 19.24 ± 2.67 |
| 1 | - | 36.70 ± 3.25 | 46.06 ± 5.23 | 12.19 ± 2.07 | 2114.01 ± 206.86 |
| 1 | 10 | 39.33 ± 3.79 | 25.78 ± 3.26 | 25.38 ± 0.24 | 1191.72 ± 46.35 |
| 1 | 20 | 48.00 ± 1.00 | 20.08 ± 3.18 | 73.10 ± 3.40 | 143.11 ± 22.04 |
| 1 | 30 | 74.67 ± 2.52 | 18.12 ± 2.40 | 139.33 ± 8.40 | 42.46 ± 4.33 |
| 1 | 40 | 92.30 ± 3.60 | 11.41 ± 2.42 | 134.60 ± 8.07 | 19.06 ± 1.14 |
| 2 | - | 37.60 ± 4.51 | 37.80 ± 2.90 | 14.73 ± 3.13 | 1874.03 ± 186.30 |
| 2 | 10 | 34.67 ± 3.51 | 30.63 ± 0.38 | 42.33 ± 2.72 | 1613.10 ± 19.03 |
| 2 | 20 | 57.34 ± 2.52 | 31.09 ± 1.69 | 116.78 ± 7.91 | 195.97 ± 62.86 |
| 2 | 30 | 74.34 ± 2.08 | 16.74 ± 1.46 | 113.46 ± 2.44 | 69.56 ± 1.65 |
| 2 | 40 | 95.30 ± 3.50 | 7.80 ± 1.52 | 113.02 ± 5.30 | 29.52 ± 3.72 |
| 5 | - | 49.83 ± 4.21 | 30.33 ± 4.50 | 30.35 ± 3.53 | 965.36 ± 36.56 |
| 5 | 10 | 52.00 ± 2.65 | 28.92 ± 1.99 | 101.32 ± 6.04 | 221.73 ± 2.57 |
| 5 | 20 | 76.33 ± 3.51 | 8.97 ± 2.65 | 118.34 ± 17.22 | 36.90 ± 5.00 |
| 5 | 30 | 86.00 ± 3.61 | 7.86 ± 2.50 | 109.70 ± 10.21 | 26.44 ± 3.25 |
| 5 | 40 | 97.70 ± 2.50 | 7.80 ± 1.52 | 117.70 ± 16.14 | 20.76 ± 2.16 |
| 10 | - | 55.33 ± 1.53 | 14.68 ± 0.92 | 41.36 ± 3.57 | 412.00 ± 90.00 |
| 10 | 10 | 71.00 ± 3.61 | 13.53 ± 1.32 | 111.12 ± 4.88 | 48.28 ± 2.42 |
| 10 | 20 | 82.33 ± 3.79 | 5.95 ± 0.41 | 109.80 ± 4.61 | 31.61 ± 3.54 |
| 10 | 30 | 90.30 ± 2.50 | 5.76 ± 0.57 | 113.57 ± 10.42 | 27.78 ± 3.94 |
| 10 | 40 | 102.00 ± 4.40 | 5.03 ± 0.75 | 109.09 ± 9.75 | 20.84 ± 1.92 |

The results are expressed as mean ± standard deviation. Further experimental details are given in sections 2.3–2.5.

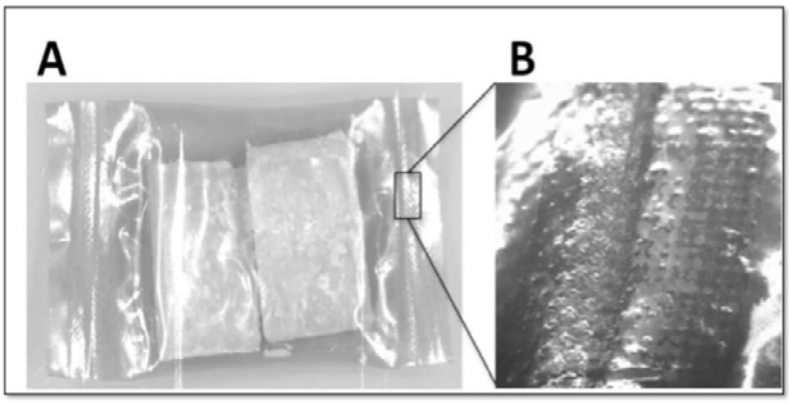


**Fig. 5.** Food wrapping by a heat-sealed CH film containing 5 mM SPD and 25 mM GLY (panel A), and the magnification (50 ×) under optical microscope of the film heat-welding (panel B).

**Table 2**
Gas and WV permeability of 0.6% CH films prepared at pH 4.5 in the presence of 5 mM SPD and/or 25 mM GLY.

| Film | Permeability ($cm^3$ mm $m^{-2}$ $day^{-1}$ $kPa^{-1}$) | | |
|---|---|---|---|
| | $CO_2$ | $O_2$ | WV |
| Viscofan NDX | 3.71 ± 0.16[b] | 0.03 ± 0.01[b] | 0.08 ± 0.01 |
| CH film | 15.81 ± 2.06[a] | 14.33 ± 0.29[a] | 0.05 ± 0.02 |
| CH film + SPD | 2.84 ± 0.01[ab] | 3.67 ± 0.01[ab] | 0.17 ± 0.01[ab] |
| CH film + GLY | 0.71 ± 0.07[ab] | 0.11 ± 0.02[ab] | 0.28 ± 0.02[ab] |
| CH film + SPD + GLY | 2.40 ± 0.13[ab] | 0.05 ± 0.01[ab] | 0.37 ± 0.01[ab] |

Significantly different values compared to those observed analyzing Viscofan NDX ([a]) and to those observed analyzing CH film obtained in the absence of plasticizers ([b]). Further experimental details are given in sections 2.3–2.5.

1 × 8 cm film strips.

To determine film permeabilities to $O_2$ (ASTM D3985-05, 2010), $CO_2$ (ASTM F2476-13, 2013) and WV (ASTM F1249-13, 2013) aluminum masks were used with the aim to reduce the film test area to 5 $cm^2$. The measurements were carried out in triplicate at 25 °C and 50% RH by using a TotalPerm apparatus (Extra Solution s.r.l., Pisa, Italy).

CH films surface and cross section ultrastructure was analyzed by using a Scanning Electron Microscope (SEM). Films were cutted using scissors, mounted on stub and sputter-coated with platinum-palladium (Denton Vacuum Desk V), before observation with Supra 40 ZEISS (EHT = 5.00 kV, detector inlens). To test CH film ability to be heat-sealed, a routine device to thermo-weld polyethylene film strips was purchased from Citynet Medical (Milano, Italy) (C.A. 50 Hz. 200–250 V) and utilized by subjecting double layers of CH films containing different GLY concentrations to a pressure at high temperatures for 10 s. The thermo-sealed film strips were analyzed by an optical microscope at 50× magnification.

To evaluate the antimicrobial activity of CH films, *Salmonella typhimurium* ATCC 14028 was grown in Tryptic Soy Broth (TSB, Becton Dickinson Difco, Franklin Lakes, NJ) and on Tryptic Soy Agar (TSA; Oxoid Ltd., Hampshire, UK). In all the experiments, bacteria were inoculated and grown overnight in TSB at 37 °C and, the day after, were transferred into a fresh TSB tube and grown to mid-logarithmic phase. Bacterial cells were then diluted in TSB to approximately $2 \times 10^7$ CFU/mL and inoculated by surface streaking into TSA plates using a swab. A 1 $cm^2$ strip of CH film, containing or not 5 mM SPD and/or 25 mM GLY, was finally placed onto the center of the inoculated plate and pressed to ensure full contact with the agar surface. Plates were incubated at 37 °C for 24 h and the presence or absence of bacterial growth under the film was evaluated. Parallel experiments by testing the antimicrobial activities of pectin-based films, prepared as previously described (Esposito et al., 2016) in the absence or presence of 5 mM SPD and/or 25 mM GLY, were carried out. Negative controls were carried out by placing pectin and CH films in contact with the agar surface in the absence of bacterial cells.

### 2.5. Statistical analysis

JMP software 10.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. One-way ANOVA and the least significant difference (LSD) test for mean comparisons were used. Differences at $p < 0.05$ were considered significant and are indicated with different letters.

## 3. Results and discussion

Since the ability of the aliphatic triamine SPD to act as plasticizer of pectin-based films (Esposito et al., 2016), as well as of protein-based films (Porta et al., 2017; Sabbah et al., 2017), has been recently demonstrated, we were stimulated to investigate the effects of SPD on the properties of CH-based films. Therefore, preliminary experiments aimed to find adequate conditions for the development of stable FFSs were carried out by determining the zeta potential of CH aqueous solutions, containing or not either SPD or GLY, as a function of pH using an apparatus capable of separating by microelectrophoresis and measuring dynamic light scattering. The zeta potential of 0.2% CH solutions, prepared both in the absence and presence of 5 mM SPD and/or 25 mM GLY, showed a value ranging from +60 mV to +50 mV between pH 2.0 and 5.0 (Fig. 1). The value was found to progressively decrease with further increases of pH and approach to CH pKa, up to +10 mV at pH 7.0. These findings clearly indicated that neither the polyamine, even though positively charged, nor GLY influenced the zeta potential and the stability of CH solution.

Moreover, FFSs containing lower or higher CH concentrations (0.1–0.6%), prepared at pH 4.5 both in the absence and presence of 5 mM SPD, were found to have significantly different zeta potential values (all higher than +50 mV) (Fig. 2, panel A). Conversely, the same panel shows that the Z-average values increased in parallel with the enhancement of CH concentration and FFS conductivity. However, SPD containing CH FFS samples always showed markedly lower Z-average values (< 3000 d nm), probably due to their higher conductivity (Sabbah et al., 2016). Finally, handleable films were formed starting from 0.2% CH and their yellowish color was more intense by both increasing CH concentration and, mostly, in the presence of SPD (Fig. 2, panel B).

Fig. 3 shows the effects of CH concentration on the thickness and

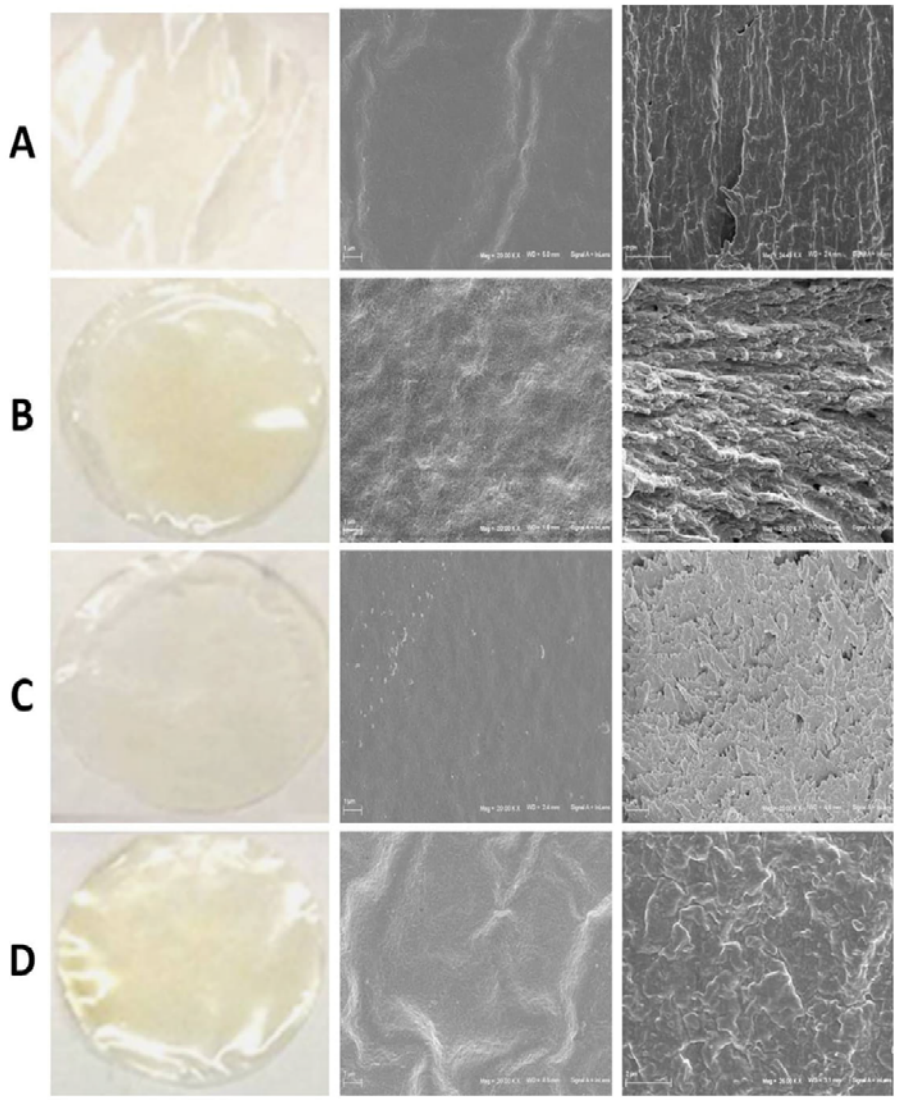


**Fig. 6.** Film images (left) and SEM micrographs of the film surfaces (20,000 × magnification, center) and cross sections (25,000 × magnification, right) obtained with 0.6% CH films prepared in the absence of plasticizers (A) and in the presence of either 25.0 mM GLY (B), 5.0 mM SPD (C) or both (D). The images shown were chosen as the most representative of each sample. Further experimental details are given in sections 2.3–2.5.

mechanical properties of the films prepared in the absence and presence of 5 mM SPD, in comparison with those detected by using a commercial biomaterial widely used for meat casing (Viscofan NDX). Increasing CH amounts were found to determine a parallel increase in film thickness, TS and EB both in the absence and presence of SPD, whereas the EB values were found much higher, and YM much lower, at all CH concentrations when films were prepared in the presence of SPD.

Therefore, the polyamine was confirmed to act as plasticizer not only in anionic polysaccharide (pectin) based films, as previously reported by Esposito et al. (2016), but also in cationic polysaccharide (CH) based ones, being able to increase their EB and reducing both TS and YM. Since also SPD is positively charged under the experimental conditions used to prepare CH FFSs, the plasticizing effect of the polyamine may be explained only by assuming the formation of non-ionic interactions between CH and SPD during FFS drying. This conclusion is supported by spectroscopic, thermodynamics and docking experiments recently reported by Chanphai and Tajmir-Riahi (2016), indicating the occurrence of hydrophobic and H-bonding interactions between the polyamine and CH nanoparticles. However, regardless of any other consideration, it is worthy to note that by adding the polyamine to the FFS, and by varying CH concentration, it is possible to prepare a polysaccharide-based material possessing mechanical properties very similar to those exhibited by Viscofan NDX, a widely commercialized protein-based casing.

To compare SPD effects on the mechanical properties of the CH-based films with respect to those determined by another well known plasticizer, i.e. GLY, we measured the TS, EB and YM values, as well as the thickness, of the CH films containing different concentrations of either SPD or GLY (Fig. 4).

Our findings indicate that SPD-containing CH films were always more extensible, exhibiting a higher EB than that of CH films plasticized by GLY. Conversely, they showed a similar TS, but lower thickness and YM, exhibiting thus also a major elasticity with respect to the GLY-containing films.

Finally, we investigated the mechanical properties of CH films containing different concentrations of both SPD and GLY. Table 1 shows

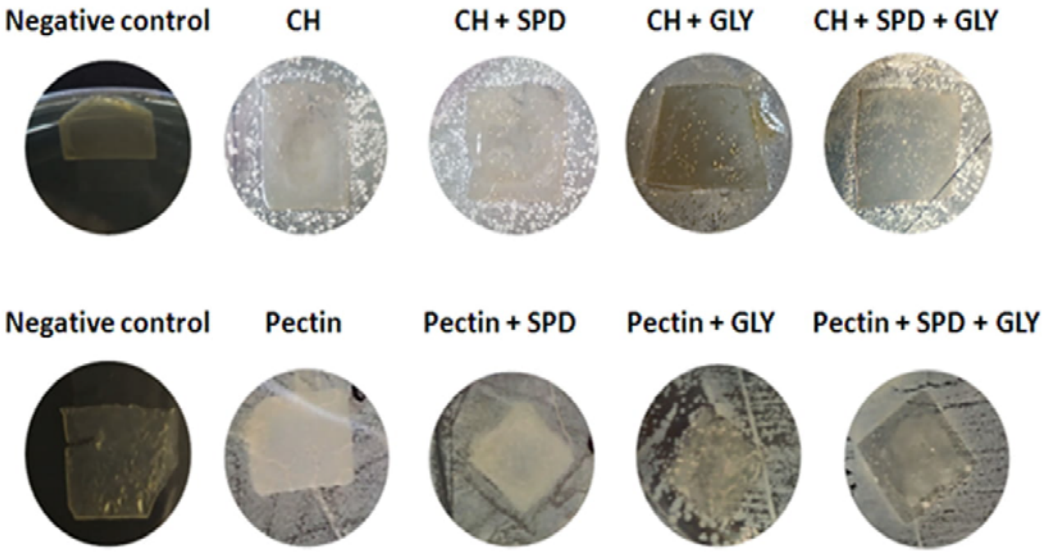


**Fig. 7.** Antimicrobial activity of CH and pectin films prepared in the absence or presence of 5 mM SPD and/or 25 mM GLY. The biological effect was evaluated on *Salmonella typhimurium* ATCC 14028 foodborne pathogenic bacterium by analyzing the growth of bacterial cells in direct contact with the film. CH and pectin negative controls were obtained in the absence of bacterial cells. Further experimental details are given in sections 2.3–2.5.

that the concomitant presence of increasing concentrations of both SPD and GLY significantly enhanced film EB as well as their thickness. More in particular, it seemed sufficient to blend at least 5.0 mM SPD with 10.0 mM GLY to reach a film EB three times higher than that observed with films containing only 5.0 mM SPD. Moreover, to obtain a similar so high EB value with lower SPD concentrations, higher amounts of GLY were needed (20 mM GLY with 2 mM SPD or 30 mM GLY with 1.0 mM SPD).

Conversely, very marked reductions of TS and YM values of the CH films were detected only when GLY was both present in the FFS. In particular, very low YM values began to be observed by mixing 5.0 mM SPD with only 10 mM GLY. Therefore, the obtained CH films blended with both SPD and GLY resulted to possess a capacity to withstand loads tending to elongate not much lower than that of Viscofan NDX but with an ultimate elongation and elasticity markedly improved with respect to those of the widely commercialized biomaterial (see Fig. 3).

Furthermore, we investigated the ability of the CH films, prepared in the absence or presence of different SPD and/or GLY concentrations, to be heat-sealed. Our results indicate that only the films containing at least 25 mM GLY, prepared both in the absence and presence of SPD, were able to be heat-sealed (Fig. 5). Therefore, we decided to continue our studies by analyzing the morphological features, as well as the barrier and antimicrobial properties, of 0.6% CH films obtained in the presence of SPD and/or GLY at concentrations of 5 and 25 mM, respectively.

Also the permeability tests of the CH films blended with SPD and GLY indicated interesting features of the new biomaterials. In fact, Table 2 reports that whereas the CH films obtained in the absence of plasticizers showed, with respect to Viscofan NDX, barrier properties toward $O_2$ and $CO_2$ markedly lower but similar WV permeability, CH films blended with both SPD and GLY exhibited similar gas barrier effect but increased WV permeability. Therefore, once again, SPD and/or GLY addition may represent a useful strategy to give rise to different edible films specifically tailored for desired applications.

Surface and cross-sectional morphology of the selected CH films, prepared in the absence or presence of SPD and/or GLY are illustrated in Fig. 6. According to the SEM images it seems that SPD contributes to maintain the film surface structure of the original CH matrix (Fig. 6 C, left), whereas GLY increases film surface roughness (Fig. 6 B, left). Furthermore, film cross section analyses indicate that, even though all matrices have ordered structures, GLY and SPD alone give rise to fibrous-like and exfoliated layers (Fig. 6 B and C, right), respectively, whereas the concomitant presence of both SPD and GLY generates amorphous structures (Fig. 6 D, right).

Finally, the antimicrobial activity of the selected CH films was assayed on *Salmonella typhimurium* strain by placing small pieces of each film into the center of the inoculated plate in full contact with the agar surface (Sagoo, Board, & Roller, 2002). By analyzing the agar surface in contact with the film after 24 h at 37 °C we observed that the CH film itself exhibited an evident antimicrobial activity that was not influenced by the presence of SPD and/or GLY. Neither the controls carried out without microorganism inoculation nor the films prepared with a different polysaccharide, such as pectin, showed inhibitory effects on the bacterial cell growth similar to those exhibited by all the CH-based films (Fig. 7).

## 4. Conclusions

The results reported in the present study open new possibilities in the applications of bio-based materials derived from CH and, as a consequence, in chitin waste recycling. CH-based films prepared in the presence of SPD and GLY were shown to possess newsworthy mechanical and permeability properties compared to the films obtained with CH alone. The plasticized films are able to be thermo-sealed and retain the well known antimicrobial CH features. The use of these new biomaterials as alternative to Viscofan, a well known and commercialized edible casing, as well as in coatings and wrappings of selected food products, is suggested.

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### 3.3.1 FT-IR analysis of CH films

The FT-IR of pure CH powder, as well as of CH films prepared in the presence or absence of 5 mM SPD and/or 25 mM GLY, were shown in Fig. 13. After film formation, some differences were observed in the CH spectrum, probably due to the effect of film matrix, in comparison with that of pure CH. The O-H bonds band, recorded at approximate wavenumber region 3100-3500 $cm^{-1}$ due hydrogen bonds, was found more intense in films containing 25 mM GLY with respect to the films prepared without plasticizer (Cerqueira et al., 2012). As a consequence, the resulting area of the band grew in the presence of GLY, indicating a higher intensity of hydrogen bonds related with both more OH groups provided by GLY and moisture content. Moreover, the C-H stretching peaks of CH films plasticized with GLY or SPD were detected at 2879 and 2877 $cm^{-1}$, whereas the bands at 1649 $cm^{-1}$, 1568 $cm^{-1}$, and 1372 $cm^{-1}$ can be assigned to the C=O stretching modes amide-I, N-H deformation modes amide-II, and C-N stretching modes amide-III, respectively (Homez-Jara et al., 2018; de Morais Lima et al., 2017; Liu et al., 2013 and Hu et al., 2016). Furthermore, the bands range detected between 1400 to 1600 $cm^{-1}$ correspond to the superposition of vibrations of amide, protonated amino groups ($–NH_3^+$), carboxylate groups ($–COO^-$), and free amino groups ($–NH_2$) (Mauricio-Sánchez et al., 2018). Hu et al., (2016) reported that when different compounds are mixed together, changes in the infrared spectrum reflect the chemical interactions between them. The main differences among the CH films prepared in the presence or absence of SPD and/or GLY were observed mostly in the amide region (Branca et al., 2016).

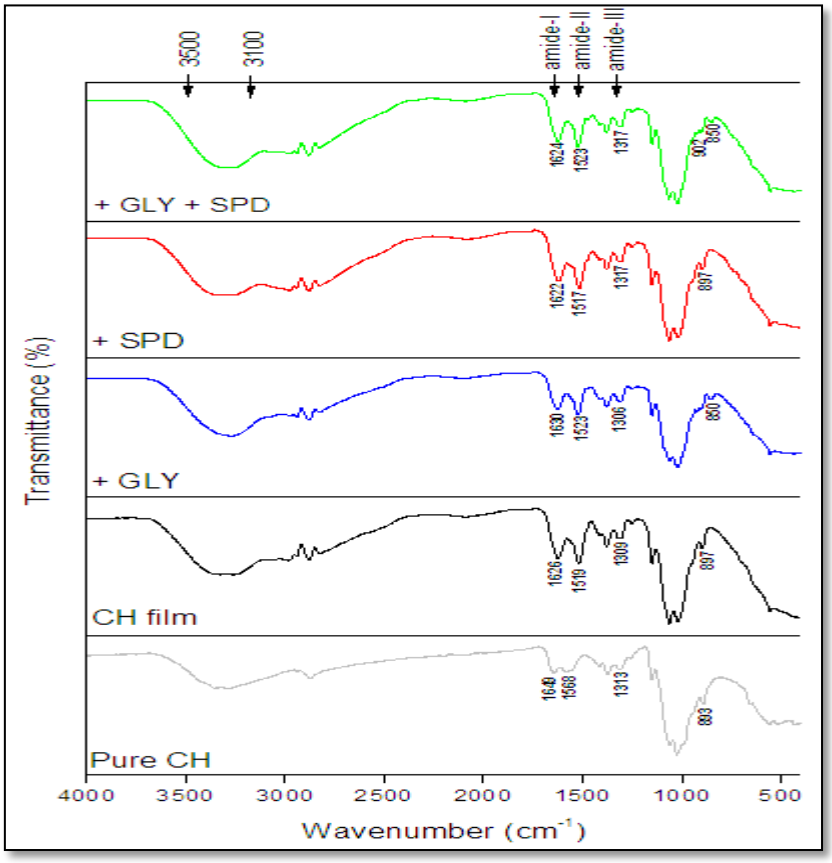


***Fig. 13. FT-IR spectra of pure CH and of 0.6% CH films prepared at pH 4.5 in the presence or absence of 25 mM GLY and/or 5 mM SPD.***

The interaction effects due to the presence of SPD and/or GLY in CH films are inferred from the characteristic bands reported in Table 1. By GLY addition we observed the shifting in the peak corresponding to the amide-I from 1626 $cm^{-1}$ (CH film prepared without plasticizer) to 1630 $cm^{-1}$. However, by adding SPD to CH films prepared both in the absence or presence of GLY, only an increase in the amide-I band intensity was observed. Moreover, a similar shifting from 1519 $cm^{-1}$ to 1523 $cm^{-1}$ was observed in the amide-II band by adding GLY alone or in the presence of SPD. In contrast, a shifting of the amide-III from 1309 $cm^{-1}$ to 1317 $cm^{-1}$ was observed by analyzing CH films prepared in the presence of SPD alone or by adding SPD to the GLY containing films. These changes indicate more hydrogen interactions between CH and both plasticizers due to the availability of a higher number of hydroxyl groups (Liu et al., 2013). Moreover, the shift of the peak detected at 898 $cm^{-1}$ to 919 $cm^{-1}$ after the GLY addition, as well as to 909 $cm^{-1}$ after GLY addition to the SPD containing films, should be related to the symmetric stretching vibrations of the alcoxyl groups (Jamróz et al., 2007), while the interaction between CH and SPD by H-bonds should be responsible for the observed shifting occurring in the presence of polyamine. These conclusions are in agreement with Chanpai and Tajmir-Riahi (2016) results indicating the occurrence of hydrophobic and H-bonding interactions between polyamines and CH chains.

***Table 1. The characteristic bands of pure CH and of 0.6% CH films plasticized by 25 mM GLY and/or 5 mM SPD.***

| **Chemical groups influenced** | **Powder** | **Film** | | | |
|---|---|---|---|---|---|
| | **Pure CH** | **CH** | **+25mM GLY** | **+5mM SPD** | **+25mM GLY + 5 mM SPD** |
| C=O stretching modes (amide-I) | 1649 | 1626 | 1630 | 1622 | 1624 |
| N-H deformation modes (amide-II) | 1568 | 1519 | 1523 | 1517 | 1523 |
| $–NH_3^+$, $–COO^-$ and free $–NH_2$ | 1490 | 1414 | 1465 | 1473 | 1436 |
| $CH_3$ symmetrical deformation mode | 1372 | 1379 | 1379 | 1379 | 1381 |
| amide-III and $CH_2$ wagging | 1313 | 1309 | 1306 | 1317 | 1317 |
| C–O group. | 1251 | 1251 | 1247 | 1249 | 1234 |
| C–O–C– in glycosidic linkage | 1061 | 1061 | 1063 | 1063 | 1065 |
| C–O–C– in glycosidic linkage | 1024 | 1016 | 1020 | 1016 | 1016 |
| comprise vibration | 894 | 898 | 919 | 898 | 902 |
| modes of C–C and C–O stretching and the bending mode of C–H bonds | - | - | 851 | - | 841 |

### 3.4 Food wrapping by different edible films

In order to study the effects of BVPC- and CH-based film wrappings on food shelf-life, heat sealability of different films was investigated by determining the seal strength according to ASTM (2007). Table 2 shows that both BVPC- and CH-based films, prepared under different experimental conditions, may be effectively heat sealed, even though the seal strength never reached the values observed with LDPE or HDPE materials, used as controls, with the exception of CH films prepared in the presence of 25 mM GLY. Fig. 14, shows different food products (sausage, Za'atar and peanuts) wrapped under vacuum with BVPC, CH or LDPE.

***Table 2. Effect of GLY and/or SPD plasticizers the BVPC and CH films on the seal strength.***

| Film | Seal strength (N/m) |
|---|---|
| BVPC + 42 mM, GLY | 33.35 ± 4.61 |
| BVPC + 5 mM, SPD | 57.44 ± 3.54 |
| BVPC + 42 mM, GLY + 5 mM, SPD | 43.61 ± 8.20 |
| CH | 110.75 ± 7.23 |
| CH + 25 mM,GLY | 196.42 ± 8.46 |
| CH + 5 mM, SPD | 65.06 ± 1.84 |
| CH + 25 mM, GLY + 5 mM, SPD | 82.80 ± 6.14 |
| LDPE* | 216.80 ± 4.52 |
| HDPE* | 205.52 ± 7.12 |
| Mater-Bi* | 201.46 ± 6.61 |

*****Control materials, LDPE= low density polyethylene, HDPE= high density polyethylene.

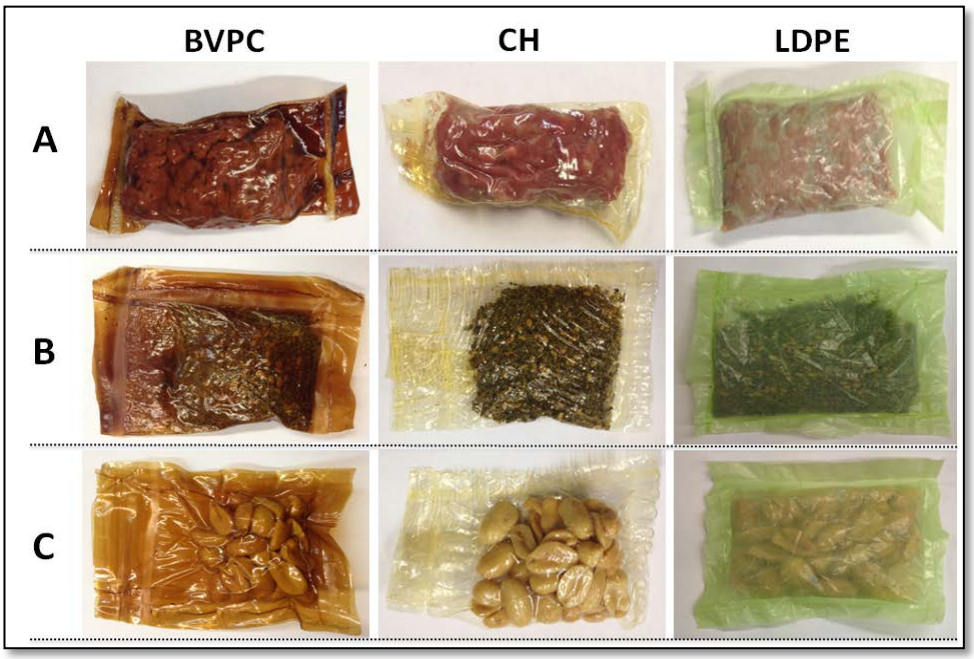


***Fig. 14. Different food products (A) sausage, (B) Za'atar and (C) salted peanuts wrapped under vacuum by BVPC, CH or LDPE films.***

### 3.5 Effects of Nabulsi cheese wrapping with different edible films

Nabulsi cheese is a typical soft and fresh Palestinian cheese generally consumed as such or used as an ingredient in sweet or savory foods. Many undesirable changes such as discoloration, off-flavor production, slime and gas formation, bitterness and textural problems may occur to the Nabulsi cheese produced by traditional methods during its storage in large cans, also in spite of high brine concentration (Mazaherh et al., 2009; Yamani, 1997). The chemical composition of the Nabulsi cheeses varies considerably depending on the source of milk, conditions of cheese production, seasonal variations, feed, degree of curd pressing and whey drainage (Al-Dabbas et al., 2014).

Since most packaging films are heat sealed as final step to create a finished food package, we wrapped (W) under vacuum conditions UNC and SNC samples, prepared in our labs from cow milk, with heat–sealed either LDPE films or hydrocolloid edible films (CH films containing 25 mM GLY or BVPC films containing 42 mM GLY) (Fig.15). These hydrocolloid edible films were selected because of their ability to be sealed under vacuum with high seal strength (Table 2). Unwrapped (UW) cheese samples were used as controls. All W and UW cheeses were then stored in a refrigerator at 4°C and finally analyzed after 3, 6 and 9 days for both pH and TA changes.

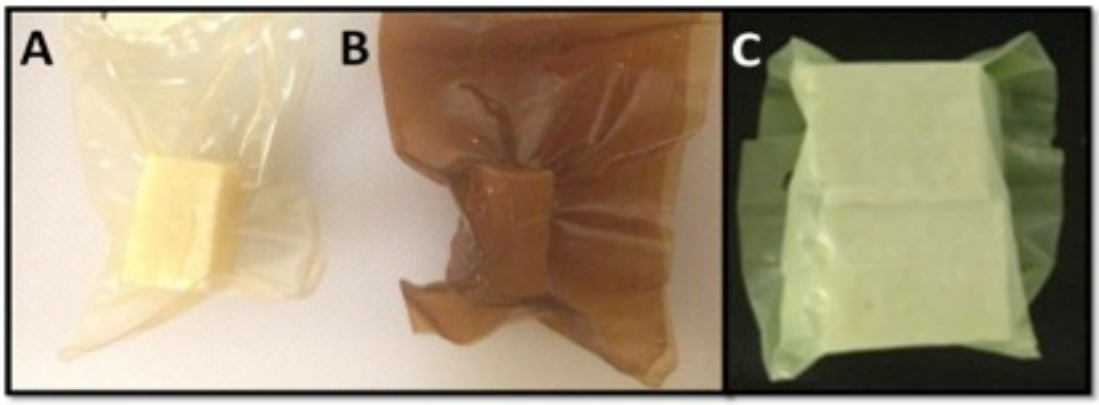


***Fig. 15. Nabulsi cheese samples wrapped under vacuum with CH (A), BVPC (B) or LDPE (C) films before their storage at 4 °C for different times.***

Fig. 16 A shows that the pH value of both W and UW UNC was around 7.1 and that both LDPE- and BVPC-wrappings of UNC samples were effective to maintain such pH value until day 9$^{th}$, whereas the pH of UW cheese progressively decreased to 6.9, 6.7 and 6.4, respectively, after 3, 6 and 9 days of storage. Conversely, a significant effect of the CH-wrapping able to counteract the pH decrease was observed only after 9 days of UNC storage. Furthermore, Fig 16 B shows that the pH values of all W and UW SNC samples did not significantly vary during storage since the salt addition alone was effective to hinder the lowering of cheese pH. CH, BVPC and LDPE wrappings of UNC markedly reduced the TA of the products. The phenomenon was extraordinarily evident after 9 days of storage, when the TA was observed to be lower than 7-fold in W cheeses with respect to the UW counterparts (Fig. 17 A). By contrast, Fig 17 B shows that no significant differences were observed between the TAs of W and UW SNC samples at the different storage times, thus demonstrating that the salt addition alone was sufficient to preserve quite effectively the products until the 9$^{th}$ day.

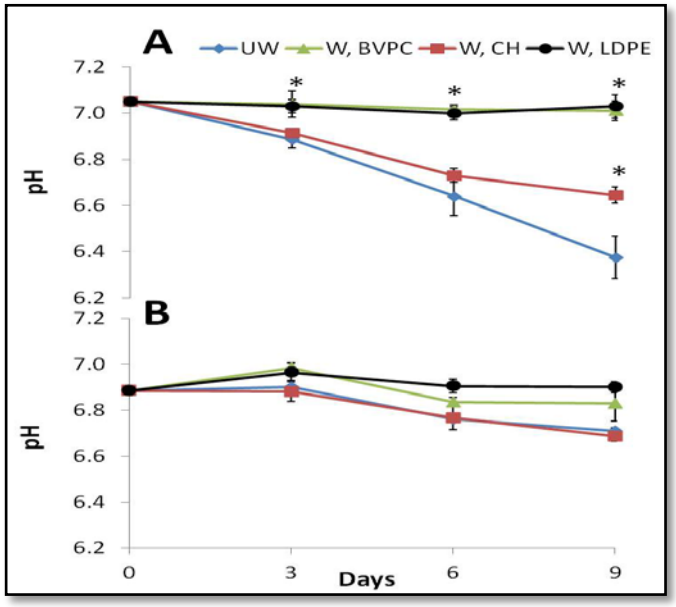


***Fig. 16. Effect of wrapping with BVPC, CH or LDPE films on the pH of "unsalted" (A) and "salted" (B) Nabulsi cheese samples at different storage times. *Significantly different values compared to the unwrapped cheese samples (p<0.05).***

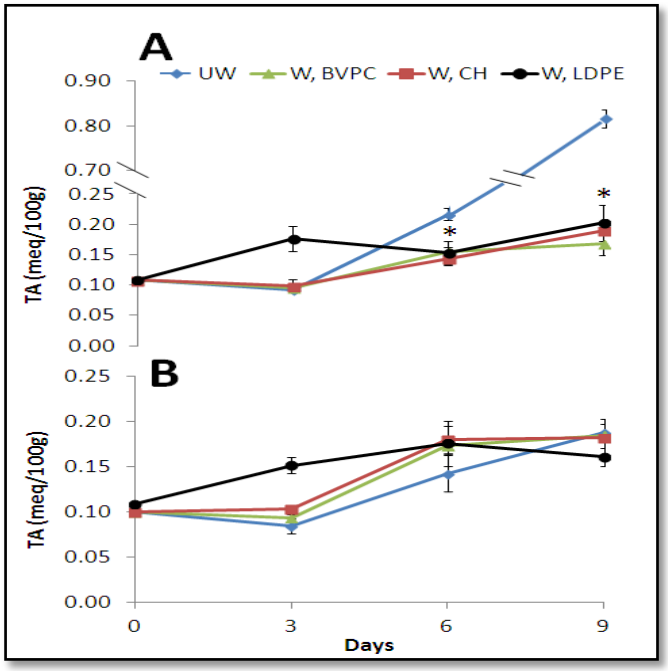


***Fig. 17. Effect of wrapping with BVPC, CH or LDPE films on TA of "unsalted" (A) and "salted" (B) Nabulsi cheese samples at different storage times. *Significantly different values compared to the unwrapped cheese samples (p<0.05).***

Finally, the percentage of W and UW Nabulsi cheese weight loss was calculated at different storage times. The results reported in Fig. 18 showed that both UNC and SNC lost weight in a similar way (about 40% at 9$^{th}$ day) during their storage, both when they were W with BVPC and CH films and when they were UW. Conversely, LDPE wrapping of both UNC and SNC hindered almost completely the weight loss of the product during its storage.

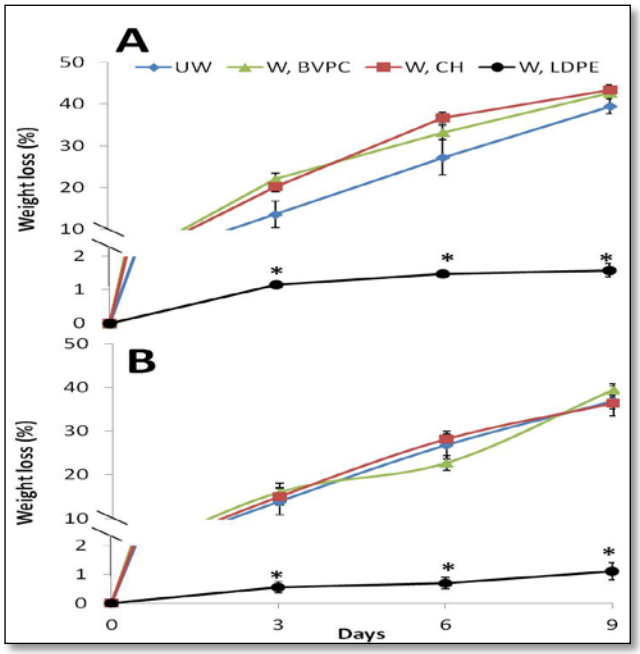


***Fig. 18. Effect of wrapping with BVPC, CH or LDPE films on the weight loss of "unsalted" (A) and "salted" (B) Nabulsi cheese samples at different storage times. *Significantly different values compared to the unwrapped cheese samples (p<0.05).***

Therefore, our findings demonstrate that the Nabulsi cheese wrapping by hydrocolloid films (mostly BVPC-based ones) has the same effects of LDPE wrapping, as well as of the salting treatment, in preventing the lowering of pH and the increase of TA observed during the storage of the dairy product. In addition, since such wrapping was shown to have no effect on the normal weight loss of the cheese occurring during storage, it should be useful to prevent cheese spoilage without influencing water loss and the consequent regular cheese ripening. This result cannot be reached by cheese LDPE wrappings because of the well known barrier action toward WV exerted by the plastic film. Therefore, due to the well known short shelf-life of UNC, also under refrigeration, the observed hydrocolloid film wrapping would allows to preserve the quality of the dairy product during storage without any prior addition of salts.

UNC with a prolonged shelf-life might be particularly required for several arabian confectioneries, such kunafeh and other sweet cakes. Moreover, people at high risk of developing health problems related to salt consumption, such as patients with elevated blood pressure and/or diabetes, might consume unsalted cheese appropriately stored in a W form. In fact, the only way used still today by the industry to market Nabulsi cheese is by immersing it in plastic/glass jars or cans containing high concentrations of brine solution. Consequently, Nabulsi cheese is usually consumed as it is or after partial or complete removal of salts by soaking it in water at least for 12 hours.

In conclusion, a possible future industrial production of UNC W with a hydrocolloid edible film, such as a BVPC- or CH-based one, would present the advantages to increase the shelf-life of a fresh dairy product, avoid any postprocess contamination, and enhance the following possible demand for a cheese healthy and ready-to-eat.

## 4. CONCLUSIONS

The development of biopolymer-based films, mechanically resistant and exhibiting tailored barrier properties, are receiving an increasing attention for their possible industrial application as alternatives to oil-derived polymers. Due to their low cost and high yield, bitter vetch proteins present undoubted advantages for developing new edible eco-friendly biomaterials. On the other hand CH, synthetically derived following the deacetylation of chitin, the second most abundant polysaccharide occurring in nature, may also represent an interesting biopolymer source deriving from the polluting waste of crustacean shells. Our findings suggest the use of SPD, alone or in combination with a primary plasticizer such as glycerol, as additives of either BVPC- or CH-based edible hydrocolloids endowed with specific mechanical and/or barrier properties suitable for coating or wrapping different food products. In fact, one of the main technical challenges in food processing and storage, today, is the development of tailor-made packaging materials with appropriate characteristics, according to the selective requirements of the various fresh or processed foodstuffs: meat, fish, dairy products, fruit, vegetables, as well as ready-to-eat meals. Desirable mechanical and permeability properties remain, thus, strategical for any edible coating in the processing and end use of food products, representing the main parameters to ensure food integrity against mechanical damage, microbial spoilage, and duration of the guarantee term. Furthermore, the blended edible film obtained by adding PEC and crosslinking BVPC by mTGase is also a promising biomaterial for food coatings as well as effective drug delivery systems. The improved mechanical features exhibited by BVPC/PEC films prepared in the presence of the crosslinking enzyme, together with their high barrier activity to both $CO_2$ and $O_2$, confer to these innovative materials potential interesting applications in both food and pharmaceutical industries.

The ability of the obtained films to give rise to heat-sealed under vacuum wrappings opens to possible new types of future industrial production of unsalted Nabulsi cheese, offering the advantages to increase the shelf-life also of other fresh dairy products, avoiding any post process contamination, and enhancing the following demand for a healthy and ready-to-eat cheese. Finally, the obtained edible materials are suggested as alternative to gelatin-based edible casings, widely used thus far for sausage production.

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## 6. APPENDIX

### Appendix 1

**Experience in foreign laboratories**

1. Stage in Department of Analytical Chemistry, Complutense University of Madrid (Spain)
Supervisor: Prof. Reynaldo Villalonga Santana
Topic: Physicochemical characterization of bitter vetch protein- and chitosan-based films.
Date: May/2018- July/2018.

**REYNALDO VILLALONGA SANTANA, DNI 54523793Q**
**PROFESOR TITULAR DE UNIVERSIDAD**
**DEPARTAMENTO DE QUIMICA ANALITICA**
**FACULDAD DE CIENCIAS QUIMICAS**
**UNIVERSIDAD COMPLUTENSE DE MADRID**

**July 29th, 2018**

**TO WHOM IT MAY CONCERM**

This is to certify that **Dr. Mohammed T. M Sabah** from Università degli Studi di Napoli "Federico II", has been working in my research group at Complutense University of Madrid, Department of Analytical Chemistry from 28/4/2018 to 29/7/2018.

Please do not hesitate to contact me. I wish him all the best in his future endeavor.

Yours Sincerely
Prof. Reynaldo Villalonga
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Complutense University of Madrid, 28040 – Madrid, Spain
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## Appendix 2

**Awards**

1- Best Poster Award at the "*Second Palestinian International Conference on Material Science and Nanotechnology (PICNM2016),"* An-Najah National University, Nablus, Palestine, 2016.

Second Palestinian International Conference on
Material Science and Nanotechnology
(PICNM2016)

**Best Poster Award**

This is to certify that the poster presentation of

*Dr. Mohammed Sabah*

Has been selected as one of the best posters presented in the Second Palestinian International Conference on Material Science and Nanotechnology (PICNM2016) held at An-Najah National University, Nablus, Palestine

*March 23-24, 2016*

Prof. Dr. Ghassan Saffarini
Conference Chairman
Dean, Faculty of Science

2. Best Poster at the *"Advanced Training Course on Emerging Biotechnologies for Sustainable Waste Management and Biorefinery Development",* University of Naples "Federico II", Napoli, Italy, 2016.

BIOASSORT

*Winner Certificate*

*This is to certify that*

MOHAMMED SABBAH

*has been awarded at the*

ADVANCED TRAINING COURSE ON EMERGING BIOTECHNOLOGIES FOR SUSTAINABLE WASTE MANAGEMENT AND BIOREFINERY DEVELOPMENT
4 - 5 April at University of Naples Federico II
Sala Azzurra-Complesso Universitario Monte S. Angelo

*with a free registration to the 6th International IUPAC Conference on Green Chemistry in Venice*

IN CONNECTION WITH XXI IUPAC CHEMRAWN CONFERENCE
SOLID URBAN WASTE MANGEMENT
April 5|7|8,2016

*Bioassort Coordinator*
*Prof. Vincenza Faraco*

## Appendix 3

### Contributions to scientific meetings

1. Contribution: Oral Presentation
   XV FISV Congress ("La Sapienza" University of Rome), 18-21 September. 2018.

### O17.2 - Innovative chitosan-based biodegradable plastics as food coating and wrapping

Mohammed Sabbah[1,2], P. Di Pierro[1], M. Cammarota[3], A. Arciello[1], E. Dell'Olmo[1], Carlos Regalado Gonzales[4], R. Porta[1]

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Chitin is the second most abundant polysaccharide occurring in nature and its wastes represent a major environmental issue. One possible chitin recycling involves its conversion in chitosan (CH) by alkaline deacetylation. Because of its biodegradability, low toxicity, antibacterial and antifungal properties, as well as possible production in large quantities from seafood industry wastes, CH has been recently promoted as matrix of promising "new economy" bio-based plastics for food coating and protection. The results reported in the present study open new possibilities in the applications of CH-based biomaterials and, as a consequence, in chitin waste recycling. In fact, CH edible films prepared in the presence of spermidine and glycerol were shown to possess newsworthy mechanical and permeability properties compared to the films obtained with CH alone. In addition, these films are also able to be thermo-sealed and retain the antimicrobial and antifungal CH features. Therefore, their use as alternative to the traditional plastics in coatings and wrappings of selected food products is suggested. (*Supp. by Ital. Min. For. Aff. & Inter. Coop.; IV Progr. Quadro Coop. Italia/Messico*).

2. Contribution: Poster Presentation
43rd FEBS Congress, Prague, 7-12 July, 2018

# Preparation and characterization of bitter vetch (Vicia ervilia) seed protein films reinforced by nanoparticles and transglutaminase-catalyzed crosslinks

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Since the environmental impact of plastic wastes is escalating rising widespread global concern, it is crucial to find enduring plastic alternatives. Among the biodegradable polymers useful to substitute the oil-based polymers, some plant proteins may represent a possible renewable raw source. Although the limited mechanical and barrier properties of hydrocolloid films so far produced, their use might be extended by adding reinforcement agents as nanoparticles (NPs) as well as chemical or enzymatic matrix cross-linkers. We suggest here a new strategy to produce nano-reinforced biomaterials by using as polymer matrix a protein mixture extracted from bitter vetch (BV) seeds and, as filler, mesoporous silica NPs functionalized or not with (3-aminopropyl)-triethoxysilane. Hence, nanostructured edible films were prepared from BV seed proteins, before and after their crosslinking by microbial transglutaminase (mTG), and characterized for their physicochemical, morphological and bioactive properties. Film tensile strength and elongation at break significantly increased in the presence of both kinds of NPs, even though the amino-functionalized ones resulted more effective, determining a two-fold increase of the mechanical properties. mTG-catalyzed protein crosslinking counteracted these NP-induced effects while, conversely, it further increased film barrier properties to gases and water vapour observed with NP alone. AFM and SEM analyses indicated a more compact structure of the nanocomposite film matrix with more evident continuous zones compared to control films, as well as an effect of mTG in including more homogenously both NPs into the crosslinked protein network. Finally, the antimicrobial and antifungal activities of the obtained biomaterials, possibly increased by nisin addition, suggest their potential application as a bio-preservative active packaging able to improve the shelf life of a variety of different food products.

3. Contribution: Invited Speaker
59th Congress of the Italian Society of Biochemistry and Molecular Biology (Caserta), 20-22. September, 2017.

Abstract

SIB 2017

Invited Speaker

# Hydrocolloid materials: reinforcement and new plasticizers

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Hydrocolloid materials derived from proteins and/or polysaccharides are increasingly used to produce edible films and coatings for food and pharmaceutical applications. Biomaterials need to have good mechanical resistance, elasticity and flexibility, low brittleness, and sufficient toughness to prevent its cracking during handling and storage. Although the limited mechanical and barrier properties of hydrocolloid films, their use may be extended by adding some reinforcement agents to the film forming solutions (FFSs), such as nanoparticles, chemical or enzymatic cross-linkers, and plasticizers. The advancement of nanotechnology has boosted interest to new types of composite materials in which the filler has at least one dimension smaller than 100 nm. Furthermore, compared to chemical cross-linking agents, enzymes offer undoubted advantages, first of all the absence of toxicity. Among the biocatalysts, *Streptoverticillium mobaerense* transglutaminase is a powerful tool, being able to produce protein inter- ε-(γ-glutamyl)lysine isopeptide bonds, to modify soluble substrate proteins into high molecular weight polymers inside the FFSs, and to improve film water vapor barrier features to allow effective food coatings [1,2]. Finally, plasticizing compounds are generally added to hydrocolloid FFSs to obtain the desired deformability of biomaterials. Our recent studies demonstrated the effectiveness of polyamines as a valid alternative to the conventional plasticizers (glycerol, sorbitol, propylene glycol, polyethylene glycol, oligosaccharides), being able in preventing both polysaccharide [3] and protein [4] chains to be closer and allowing them to slip one over the other and, consequently, in increasing film elongation and flexibility.

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4. Contribution: Poster Presentation
59th Congress of the Italian Society of Biochemistry and Molecular Biology (Caserta), 20-22. September, 2017.

Abstract
Poster



## Spermidine, a New Cationic Plasticizer for Polysaccharide- and Protein-Based Films

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The use of bio-based polymers for manufacturing biodegradable/edible materials, both to protect pharmaceuticals and to improve the shelf life of food products, is greatly increasing. Among them, several plant polysaccharides and proteins, representan abundant, inexpensive and renewable raw source. In addition, a major component of such hydrocolloid films is the plasticizer. The presence of a plasticizer generally reduces the intermolecular forces and increases the mobility of the polymeric chains, thereby improving the flexibility and the extensibility of the derived biomaterial. The most commonly studied plasticizers are polyols, like glycerol (GLY) and some mono or oligosaccharides. In particular, GLY not only increases film extensibility, but also migrates inside the film network causing often the loss of desirable mechanical properties of the material. Therefore, replacing GLY with a different plasticizer might help to improve film characteristics allowing potential industrial applications. To improve film properties it seemed of interest to test as plasticizers hydrophilic small molecules, like spermidine (SPD), containing amino instead of hydroxyl functional groups, thereby able to trigger ionic interactions with either polysaccharides or proteins. Pectin and bitter vetch (*Vicia ervilia*) seed proteins (BVP) were used to prepare hydrocolloid films, whereas GLY and SPD were added as film plasticizers, either singly or in combination, at various concentrations. Our results indicate that SPD increased the tensile strength and reduced the elongation at break of both pectin and BVP films, whereas blending of different amounts of both plasticizers were able to give rise to hydrocolloid films with mechanical properties tailored for specific applications.

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5. Contribution: Poster Presentation
   2nd Innovations in Food Packaging Shelf-°Life and Food Safety Conference (Munich-Germany), 3-6. October, 2017.

Abstract
Poster

**[P016]**
**Innovative Hydrocolloid Bioplastics Triggered By Polyamines**

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The interest in hydrocolloid bioplastics increased during the last twenty years due to their noticeable advantages with respect to the conventional petroleum-derived materials. However, even though protein-based films generally exhibit better gas barrier and mechanical properties than the ones of polysaccharide origin, both generally possess poor water vapor barrier characteristics as a consequence of their hydrophilic nature. Polyamines (PAs) putrescine (PT), spermidine (SPD) and spermine (SPM) are small mol.wt. aliphatic polycations able to ionically interact with negative charges under pH 8.0, as well as to link proton acceptors by intermolecular hydrogen bonds at pH values where their amino groups are undissociated. With the aim to generate innovative bioplastics with improved mechanical and barrier features, we investigated the possible influence of PAs on film generating solutions made from either citrus peel low-methylated pectins (PEC) or proteins extracted from bitter vetch (BV) seeds. Our results showed that a) PAs decreased the negative zeta potential of all samples under pH 8.0 as a consequence of their ionic interaction with the negativelly charged biopolymers; b) PA addition led to obtain handleable films by using either PEC or BV proteins also in the absence of plasticizers; c) PT and SPD gave rise to a structural organization of PEC different from that determined by calcium ions, previously described as *"egg box"* model; d) increasing PT and SPD concentrations progressively reduced PEC film tensile strength and markedly enhanced film thickness, elongation at break and permeability to water vapor and $CO_2$, both in the presence and absence of plasticizer; e) high concentrations of both SPD and SPM are able to act as *"glycerol-like"* plasticizers in the protein-based films when their amino groups are undissociated. Our findings suggest the possible application of PAs as plasticizers and open new perspectives in obtaining hydrocolloid films and coatings with different features.

Keywords: Bioplastics, Bitter vetch, Pectin, Polyamines

6. Contribution: Poster Presentation
   Workshop at An-Najah National University (Nablus, Palestine), 23- 24 March, 2016.

## Poster Presentations

### Effect of polyamines on low methoxyl pectin-based films

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**Abstract**

Low methoxyl pectin (LMPEC) contains a high amount of free carboxylic acid groups able to interact with $Ca^{2+}$ originating a gel network. LMPEC gelation primarily involves electrostatic forces between the cation and the negative charged cavity formed by the polymer chains where $Ca^{2+}$ are inserted. These structures, called *egg-boxes*, are stabilized by both Van der Waals interactions and hydrogen bonds. They are of great importance both in the area of fruit and vegetable processing as well as for the use of LMPEC in various food products. Polyamines (PAs) are low mol.wt organic cations known to mimic the action of divalent cations both *in vitro* and *in vivo*. The different length of PA aliphatic chains, thus, stimulated us to investigate their effect on the mechanical and barrier properties of LMPEC-based edible films. In fact, LMPEC represents also a suitable polymeric matrix for the preparation of coating films potentially useful for food active packaging for its biodegradability and biocompatibility. One of the main additives of the bio-based edible films is the plasticizer, generally a small molecule of low volatility, like glycerol or sorbitol, able toimprove film extensibility and flexibilityby increasing both free volume and polymer chain mobility. Therefore, our research focused on the specific comparison among calcium, and the two PAs putrescine (PT) and spermidine (SPD) as possible agents influencing the functionality of LMPEC-based films prepared in the presence or absence of glycerol. Zeta potential and particle size were determined on LMPEC aqueous solutions as a function of pH and the effect of calcium ions, PT and SPD on LMPEC-based films were studied. $Ca^{2+}$ and PAs were found to differently influence thickness, as well as mechanical and barrier properties, of films prepared at pH 7.5 either in the presence or absence of the plasticizer glycerol. In particular, $Ca^{2+}$ was found to increase film tensile strength and elongation to break only in the presence of glycerol and did not affect film thickness and permeability to both water vapor and $CO_2$. Conversely, increasing PA concentrations progressively reduced film tensile strength and markedly enhanced film thickness, elongation to break and permeability to water vapor and $CO_2$, both in the presence and absence of glycerol. Our findings suggest that PAs give rise to a LMPEC structural organization different from that determined by calcium ions, previously described as "*egg box*" model, and that PAs can be used as effective plasticizers to obtain more flexible and less brittle hydrocolloidal films.

7. Contribution: Poster Presentation

Advanced Training Course on Emerging Biotechnologies for Sustainable Waste Management and Biorefinery Development (University of Naples Federico II, Monte S. Angelo Via Cintia, Napoli), 4- 5. April, 2016.

## PREPARATION AND CHARACTERIZATION OF BIOPLASTICS FROM GRASS PEA PROTEINS CAST IN THE PRESENCE OF TRANSGLUTAMINASE

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The aim of this work was to prepare bioplastics from renewable and biodegradable polymers. Bioplastics are alternative to petroleum-based plastics, the latter extremely pollutant since their combustion contributes to the $CO_2$ enrichment in the atmosphere. In particular, we used proteins from grass pea (*Lathyrus sativus*), a low production cost legume cultivated specially in the past for seeds for animal and human consumption. It adapts to harsh environments, being resistant to drought and low quality of soil. It is characterized by advantageous biological and agronomic characters such as resistance to insects and pests and high protein content of its seeds. We demonstrated that proteins from grass pea seeds act as effective substrate for microbial transglutaminase (TG), an enzyme able to catalyze isopeptide bonds between glutamines and lysines. This enzyme has been widely proposed for improving technological features of several protein-based edible films (1,2). After assessing the film forming properties of grass pea proteins, the bioplastics were produced in absence and presence of TG. The bioplastics were characterized according to their mechanical and optical properties. Gas permeability studies will be also performed in order to generate a set of crucial information needed for industrial applications.

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8. Contribution: Poster Presentation
XXI IUPAC Chemrawn Conference on Solid Urban Waste Management Conference (CNR, Piazzale Aldo Moro 7 - 00185 Rome, Italy), 6- 8. April, 2016.



# PROTEIN EDIBLE FILMS REINFORCED WITH NANOPARTICLES ACTING AS TRANSGLUTAMINASE SUBSTRATES

## Transglutaminase and aminated nanoparticles for improving properties of edible films

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### INTRODUCTION
The development of nanocomposites is increasingly employed to improve physical properties of polymer films, (1). We suggest a new strategy to reinforce edible films containing proteins able to act as acyl donor substrates of transglutaminase (TGase) by using nanoparticles functionalized with amino moieties as acyl acceptors for the enzyme.

### METHODS
To improve the structural network of bitter vetch protein (BVP) films reinforced by TGase (2), different nanoparticles were functionalized with aliphatic chains endowed with primary amino groups. This aim was obtained by treating either mesoporous silica nanoparticles (MSN) with 3(2-aminoethylamino) propyltrimetoxysilane (AEAPTMS) and (3-aminopropyl) triethoxysilane (APTES) or gold nanoparticles (AuNP) with aminated polyethylene glycol (PEG). Finally, BVP films were prepared in the presence of both TGase and functionalized nanoparticles.

### RESULTS & DISCUSSION
BVP edible films containing aminated nanoparticles and TGase were analyzed for their mechanical as well as barrier properties, and their morphological features were observed by electron microscopy. All the BVP films reinforced with TGase and functionalized nanoparticles were compared to the BVP edible ones as coatings effective to increase food shelf-life.

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9. Contribution: Poster Presentation
XIV° FISV Congress ("La Sapienza" University of Rome, Rome), 20-23 September, 2016.



their nutritional nitrogen requirements, constitutes a tool that could help the researchers and professionals of alimentary industries to produce foods with optimal qualities. Phenotype Microarray (PM) analysis, which is a high throughput method for microbial characterization, has been applied to the chemical sensitivity and carbon metabolism analysis of LAB. Nevertheless, such approach failed when nitrogen metabolism was investigated. Consequently, the aim of the present research is to define an efficient protocol for analysing LAB nitrogen metabolism using PM, evaluating appropriate tetrazolium dye, used as a reporter of metabolic activity, carbon sources, and buffer conditions. The results obtained will be showed and discussed.

### P1.15
### Anti-virulence activity of niclosamide in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients

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The antibiotic-resistant chronic lung infection caused by *Pseudomonas aeruginosa* is the major cause of death in cystic fibrosis (CF) patients. The anti-helmintic drug niclosamide (NCL) inhibits the *P. aeruginosa* quorum sensing (QS) pathways based on *N*-3-oxododecanoyl-homoserine lactone (3OC12-HSL) and *N*-butanoyl-homoserine lactone (C4-HSL) as signals, hence reducing virulence *in vitro* and in an insect model of infection. However, since *P. aeruginosa* CF isolates show high genetic variability, the effect of NCL must be assessed in clinical isolates in order to support repurposing of this drug for CF therapy. Here 100 *P. aeruginosa* isolates from intermittent and chronic CF infections were collected and analyzed. Results showed that 63 strains produced both QS signals, while 22 and 6 strains produced only C4-HSL or 3OC12-HSL, respectively. QS proficient strains were found to be overall sensitive to NCL, with no effect on growth. However, great variability in the extent of NCL-mediated QS inhibition was observed among strains. Our results highlight that anti-QS molecules such as NCL have a good potential for CF therapy, though upon strain-specific susceptibility analysis.

### P1.16
### Immobilization of *Aspergillus niger* Cellulase on epoxy beads

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Cellulase (E.C. 3.2.1.4) is an important enzyme useful to obtain glucose from biomass and, thus, it is important for the circular economy, also known as bioeconomy. In fact, biomass can be used as substrate for the enzyme with the aim to obtain biofuel. Microorganisms provide powerful enzymes for different application. In this work *Aspergillus niger* cellulase has been chosen to be immobilized on epoxy beads. Enzyme immobilization is highly desired for industrial application to obtain high efficiency of the process at lower costs. Of course studies are need to find an efficient immobilization method that provides an enzymes exhibiting high performances. In this study we have used beads from ChiralVision (Immobeads-COV-2), made of a support that immobilizes covalently the enzyme. To quantify enzymatic activity we have used the 3,5-dinitrosalicylic acid based method (Ghose, 1987), while to determine the protein concentration, BioRad method was used (Bradford, 1976). Immobilization yield was equal to 42% while protein loading was in average 1 mg of enzyme per gr of beads. Experiments are in progress to establish the stability of the immobilized enzyme over the time.

### P1.17
### Environmental spread of antibiotic resistance genes (ARGs) in aquatic systems with different level of microbial contamination

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Aquatic ecosystem is considered as a significant reservoir of antibiotic resistance genes (ARGs), which could potentially be transferred from environmental microorganisms to human pathogens. In order to mitigate possible health problems, the environmental spread of the ARGs should be characterized and monitored. The purpose of the study was the assessment of ARGs occurrence in various water environments in Italy with different levels of microbial contamination (i.e. raw and treated wastewaters, surface waters and groundwaters). By using PCR and qPCR, the presence and/or abundance of 13 ARGs, selected on the basis of their environmental spread and clinical relevance, were investigated in comparison to total bacteria (16S rDNA gene) and a fecal contamination indicator (*E.coli* uidA gene). ARGs, comprising clinically relevant extended spectrum beta lactamases, were frequently detected in water environment. Overall, higher ARGs levels were associated to more contaminated waters (e.g. wastewater or contaminated surface water). Instead, limited or no correlation was found among the ARGs levels and *E.coli* levels, indicating different contamination sources and fate in the environments.

### P1.18
### Biodegradable films and containers from bitter vetch (*Vicia ervilia*) seed proteins

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Bitter vetch (*Vicia ervilia*; BV) seeds were analyzed as source to produce protein-based edible films and properly shaped biodegradable containers. Seed protein concentrates were prepared and analyzed for proteins, carbohydrates, phenols and other organic compounds, and protein film forming solutions were cast in the presence of different glycerol concentrations. Both lower plasticizer concentration and lamination by additional corn zein layer were found to reduce film moisture content and elongation at break, while both film tensile strength and water vapor barrier properties resulted enhanced. The obtained bioplastics were finally processed by a new laboratory plastic moulding equipment specifically designed and fabricated to convert films to shaped containers. The use of either lower glycerol concentration or zein lamination gave rise to satisfactory vacuum thermoformed containers with acceptable resistance and stability. These findings open new perspectives in using BV proteins as a sustainable alternative to fossil fuel based plastics to produce a variety of properly shaped biodegradable articles.
*Supported by "Ministero degli Affari Esteri e della Cooperazione Internazionale"*

### P1.19
### Profiling microbial communities iIn hyperalkaline waters of the Kizildag ophiolite complex (Turkey)

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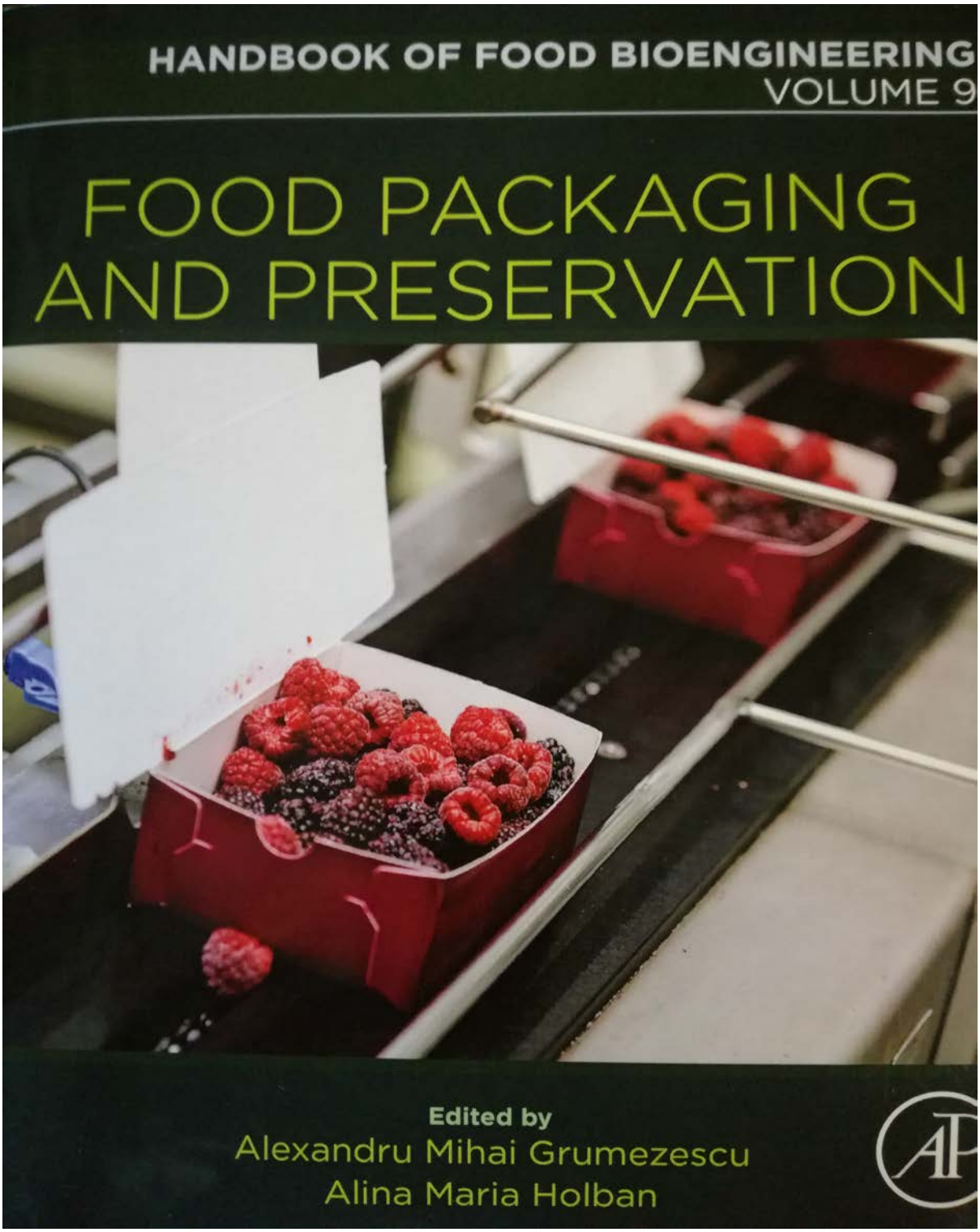
It has recently been hypothesized that life on Earth could have been originated in hyperalkaline waters related to serpentinization of ophiolitic rocks, despite their extreme conditions (high pH and very low levels of nutrients). Five hyperalkaline springs of the Kizildag

## Appendix 4

### Chapters in Books

1. **Dairy Whey Protein-Based Films and Coatings for Food Preservation,** P. Di Pierro, L. Mariniello, V.L. Giosafatto, M. Esposito, M. Sabbah, R. Porta. In: *Handbook of Food Bioengineering*, *vol 9, Food Packaging and Preservation*, 1st Edition. ISBN: 9780128115169 (A. Grumezescu & A.M. Holban eds.), Elsevier-Academic Press, pp.439-456.



CHAPTER 13

# Dairy Whey Protein-Based Edible Films and Coatings for Food Preservation

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## 1 Introduction

It is well known that the dairy industries generate large volumes of a liquid waste as a by-product during coagulation of the casein process. This liquid, known as milk or dairy whey (DW), represents the residue that remains after recovery of the curd obtained from the acid or proteolytic milk clotting. DW derives from all types (cow, goat, sheep, and camel) of milk, even though bovine DW is the most common whey produced in the western countries, shares about 85%–95% of the originating milk volume, and contains about 55% of the whole milk nutrients (Yadav et al., 2015). An approximate comparative analysis of the different components of whole bovine milk and DW, respectively, is reported in Table 13.1, since the precise composition depends mainly on the specific processing techniques used in casein removal, as well as on the milk animal source.

DW protein fraction is a heterogeneous mixture of different proteins—β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobulins (Igs), lactoferrin (LF), lactoperoxidase (LP), proteose-peptone (PP), and glycomacropeptide (GMP)—endowed with specific functional and neutraceutical properties. All these components occur naturally in the milk, with the only exception of GMP, which derives from casein proteolysis following the first step of the enzymatic cheese processing. β-LG (18.3 kDa) is the major component of DW proteins, accounting for more than half of the total DW proteins. It shows an isoelectric point of 5.4 and exists in a dimeric form made by two identical subunits (162 amino acids each), which forms aggregates at around 80°C. After thermal denaturation, the Cys-121 thiol group is exposed and available for intermolecular disulfide bond formation. Conversely, α-LA (14 kDa) is a globular protein of a single polypeptide chain (123 amino acids and isoelectric point of 4.4), represents about 10% of the total DW proteins, and is more resistant to heat than β-LG, its denaturation being also reversible (Hernández-Ledesma et al., 2011). Calcium strongly binds to α-LA providing, thus, stabilization of its molecular conformation. DW contains also

Food Packaging and Preservation
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**Table 13.1: Comparative analysis of whole bovine milk and its whey components (% w/v).**

| Components | Milk | Milk Whey |
|---|---|---|
| Total solids | 13 | 6.5 |
| Caseins | 3.0 | 0.2 |
| Other proteins | 0.8 | 0.8 |
| Lactose | 5.0 | 5.0 |
| Fat | 4.0 | 0.2 |
| Salts | 0.8 | 0.8 |
| Ash | 0.8 | 0.7 |

*Source: From Yadav, J.S.S., Yan, S., Pilli, S., Kumar, L., Tyagi, R.D., Surampalli, R.Y., 2015. Cheese whey: A potential resource to transform into bioprotein, functional/nutritional proteins and bioactive peptides. Biotechnol. Adv. 33, 756–774.*

5% of BSA (66.3 kDa and isoelectric point 5.1), a protein of 582 amino acids endowed with a high capacity to reversibly bind several ligands (Hernández-Ledesma et al., 2011; Mollea et al., 2013; Walstra et al., 2006). The total content of Ig (150–1000 kDa and isoelectric points between 5.5 and 8.3) in DW is around 0.7 g/L and IgG, present in a monomeric form, shares up to 80% (w/w) of the total Igs. Conversely, IgA and IgM occur in polymeric forms (Mollea et al., 2013). The main role of Ig is to neutralize toxins, inactivate viruses, and agglutinate bacteria. In addition, IgA also protects from proteolysis. Further reported roles played by Ig are blood pressure lowering and cholesterol level reduction (Mollea et al., 2013). The iron-binding glycoprotein LF (76.5 kDa) consists of a single polypeptide chain of 700 amino acids, with a very high isoelectric point (9.5–10), containing one or two carbohydrate chains. LF, occurring in amounts lower than 2% in DW at low concentrations, is heat-, acid-, trypsin-, and chymotrypsin-resistant, while it is easily hydrolyzed by pepsin (Modler, 2000), and is known for its antimicrobial and antifungal activity against a wide range of bacteria and yeasts (Hernández-Ledesma et al., 2011), as well as for its immunomodulatory activity. In fact, it acts on cell growth and differentiation, embryonic development, myelopoiesis, endothelial cell adhesion, and cytokine generation (Cornish et al., 2004; Ulber et al., 2001). LP (78 kDa and isoelectric point 9.5), a single polypeptide chain of 612 amino acids present in very low quantities in DW (0.5%), is a natural enzyme of the mammal host defense system (Hernández-Ledesma et al., 2011). This enzyme, containing one heme group and about 10% (w/w) of carbohydrate moieties, completely loses its catalytic activity after treatment at 70°C for 15 min or at 85°C for a few seconds (Modler, 2009; Mollea et al., 2013). PP is the protein fraction (4–22 kDa) remaining soluble when milk is heated at 95°C for 20 min at pH 4.7. PP has 4 components and the component-3, deriving from the fat globule membrane, is the most abundant (25%). The other three PP components originate from β-casein following plasmin action (Krissansen, 2007). Finally, the thermostable polypeptide called GMP (6.8 kDa), originating from κ-casein during cheese processing by the action of the milk-clotting chymosin, may represent 10%–20% of the total DW protein fraction. It contains 64

amino acid residues, many of them rich in branched chains, and does not show absorption at 280 nm because of the absence of aromatic amino acids. GMP isoelectric point varies from 4.3 to 4.6 depending on the different glycosylation degree (from 0 to 5 trisaccharide units of *N*-acetyl-neuraminic acid) although, however, up to 50% of GMP can be found unglycosylated (Krissansen, 2007; Rajput et al., 2013).

The total worldwide DW production is estimated to be more than 180 million tons/year, the major amount (approximately 70%) coming from EU and USA (Mollea et al., 2013; Yadav et al., 2015). DW is responsible for relevant environmental problems due to its both large volume and high organic content and, thus, its disposal into municipal sewers is almost everywhere forbidden. On the other hand, land dumping creates severe pollution concerns for the environment by negatively influencing soil physicochemical characteristics. Therefore, since the DW components are difficult to degrade, an ecofriendly treatment of DW, when it is not recycled, is required before its disposal, even because the occurrence of numerous nutrients in DW is considered as a potential resource for the production of different value-added products. Various advanced technologies are currently in use and a large fraction of DW is utilized and transformed into valuable products.

The current DW processing involves two main procedures to transform such liquid waste into value-added product(s). The first one is its physical or thermal treatment to obtain mainly DW powder (DWPD, the simplest dried whey product obtained by spray-drying water removal), DW with reduced lactose (DWRL, product containing less than 60% lactose), DW protein concentrate (DWPC, resulting from the removal of nonprotein components and defined with different numbers indicating the percentage of protein content, that is, DWPC34 = 34% protein, DWPC80 = 80% protein), DW protein isolate (DWPI, containing protein concentrations higher than 90% and lactose and fat less than 1%) (USDEC, 2006). The most preferred method to concentrate whey proteins in their native state is membrane ultrafiltration or nanofiltration, energy saving technologies producing high quality products in terms of protein functional properties (Limsawat and Pruksasri, 2010). DW proteins may have, in fact, several applications in the pharmaceutical industry, being therapeutically used to control blood pressure, as well as to induce sleep (Korhonen, 2009; Modler, 2009). Moreover, DW proteins have also the potential to be transformed into bioactive peptides via protease or microrganism treatments. The second procedure involves biotechnological treatments, where DW components are used as substrates for various enzymatic processes to obtain animal feed, probiotics, biopreservatives, and bioplastics (Mollea et al., 2013; Panesar et al., 2013; Tarhan et al., 2016).

However, large amounts of DW remain generally unutilized and, thus, DW still deserves attention by researchers to develop further innovative processes able to provide maximal benefits from this by-product and to limit its environmental pollution impact (Carvalho et al., 2013; Prazeres et al., 2012).

One possible recycle of DW is the use of its protein content as biopolymer source to prepare food edible coatings. In fact, the packaging and food industries are joining increasing efforts to

use biodegradable materials as an environmental friendly alternative to nonrecyclable traditional plastic materials. One of these is represented by biopolymer films, which are made out of renewable resources containing natural raw materials, such as polysaccharides and proteins. Biodegradable polymers are well known for their applications as edible films either for individual coating of small food products or placed within a dual texture food to prevent moisture migration and maintain the texture of each of the layers. On the other hand, edible films can also be used as carrier agents for several types of additives. Moreover, the incorporation of antimicrobial agents into biopolymer films has been extensively studied with the aim to create inside the package an environment that will delay or prevent the growth of microorganisms on the product surface, extending food shelf life, and improving their safety. Other additives, such as antioxidants, antibrowning agents, nutraceuticals, texture enhancers, flavor, and color ingredients can also be added to enhance the functional and organoleptic properties of the packaged foods.

Among hydrocolloids protein-based edible films have interesting properties and various biomaterials have been tested so far as film protein source, including soya (Mariniello et al., 2003), beans (Giosafatto et al., 2014a,b), corn (Cho et al., 2010), bitter vetch (Arabestani et al., 2016a; Porta et al., 2015), egg (Giménez et al., 2012), and quinoa (Valenzuela et al., 2013). However, in spite of their excellent gas barrier properties, protein-based films, as well as the polysaccharide-based ones, are mechanically weaker and poor moisture barriers because of their hydrophilic nature (Wihodo and Moraru, 2013). An improvement of the functional properties of protein-based films, blended or not with polysaccharides, was obtained by cross-linking either with physical, chemical, or enzymatic methods and the resulting film properties strictly depend on the specific protein structure, as well as on treatment conditions and methodologies (Benbettaieb et al., 2016). In particular, cross-linking yield is related to the amount of reactive amino acid residues (mostly cysteine, lysine and arginine) and whether it occurs in a film forming solution (FFS) or in a dry film. Physical treatments, such as heating, are generally the most common methods used. The mechanism of cross-linking by UV irradiation involves double bonds and aromatic rings via free radical formation in amino acids and these unstable products, reacting together, induce the intermolecular covalent bond formation. This treatment is generally performed in both FFS and dry film, and the tuning of the irradiation dose allows tailoring film characteristics. Finally, the combination of γ-irradiation and thermal treatment was shown to significantly improve protein-based film functional properties due to disulfide and bityrosine cross-link formation. Furthermore, also chemical cross-linkers were reported effective in promoting improvements of both mechanical and barrier properties of protein films. However, the results were greatly dependent on the operating conditions during film formation, and the main limitations are related to the toxicity of these agents. As a consequence, due to toxicity of aldehyde residues remaining inside the protein network, chemical cross-linkers are generally not welcome for film food applications. Conversely, enzymatic methods have attracted more attention, particularly following the use of peroxidase and, mostly transglutaminase (TGase), to catalyze the formation of covalent cross-links into protein-based films (Di Pierro et al., 2010; Porta et al., 2011a).

## 2 Properties of DW Protein Edible Films

DW is a suitable starting source for obtaining edible films (Khanzadi et al., 2015). Its excellent nutritional value and the reported properties of DW proteins originating from the waste stream from the cheese industry, such as solubility in water and ability to act as emulsifiers, enable the production of transparent, flexible, colorless, and odorless edible films with a poor moisture barrier, good aroma barrier, and low $O_2$ permeability ($O_2P$). Such films are usually obtained by casting and drying of aqueous DWPI (Galietta et al., 1998), even though fluidized-bed coating, a method used commonly by the pharmaceutical industry to coat tablets, has been successfully applied to coat nuts and peanuts (Lin and Krochta, 2006). Also extrusion and compression-molding, common industrial methodologies used to form films and containers, could be adapted to produce DW protein-based films, avoiding the need to add and then removing the solvent. However, despite some promising preliminary results, the properties of extruded and compression-molded DW protein films need to be better investigated (Henriques et al., 2016).

Plasticizers are generally added to hydrocolloidal films to improve their characteristics. DW protein films produced without addition of any plasticizers are very brittle, whereas the addition of plasticizers provides flexibility to the films but also increases their water vapor permeability (WVP). Moreover, increased concentration of plasticizers decreased the tensile strength and Young's modulus of DW protein films increasing their elongation (Shaw et al., 2002). Among the various plasticizers, increased glycerol content was demonstrated to enhance DW film WVP and solubility in water, decreasing its mechanical resistance, apparent Young modulus, and glass transition temperature (Galietta et al., 1998; Sothornvit and Krochta, 2000a). Similar effects were observed by adding sorbitol to DWPI FFS. In fact, increasing sorbitol content led to increases in moisture content, WVP, and % elongation, while it decreased film tensile strength, elastic modulus, and glass transition temperature. In contrast, increasing levels of xylitol had no effect on WVP, moisture content and glass transition temperature of the films, but decreased % elongation, tensile strength, and elastic modulus. These differences in the film physical properties measured in the presence of the different plasticizers at various concentrations have been attributed to differences in the hygroscopic and crystalline properties of the plasticizers (Shaw et al., 2002). The effect of different plasticizers on β-LG film $O_2P$ and mechanical properties was also studied. Sucrose-plasticized films showed the best $O_2$ barrier, whereas glycerol- and propylene glycol-plasticized films had similar $O_2P$ values, and both had higher $O_2P$ than sorbitol-plasticized films. Polyethylene glycol-plasticized films were found to be the poorest $O_2$ barriers (Sothornvit and Krochta, 2000b). Propylene glycol-plasticized β-LG films resulted the most brittle, exhibiting mechanical properties independent of plasticizer content, whereas films containing other plasticizers (glycerol, sorbitol, polyethylene glycol, and sucrose) exhibited negative exponential dependence on plasticizer concentration for both tensile strength and elongation at break (Sothornvit and Krochta, 2001).

A significant reduction in WVP of DW protein films was achieved by adding different lipids to FFSs (Shellhammer and Krochta, 1997), as well as by emulsifying DW FFS with beeswax and casting it at pH 7.0 (Pérez-Gago and Krochta, 1999). However, the incorporation of lipid materials into edible film formulations could adversely affect the sensory characteristics of films. More recently DWPI and mesquite gum were shown totally compatible to form FFSs and films. In fact, the grafting of different amounts of mesquite gum into DW proteins allowed to obtain composite films with improved flexibility without increasing plasticizer content and, consequently, without affecting negatively other film features, such as WVP (Osés et al., 2009).

To try to improve film properties, several DW protein cross-linking methods were employed. First, it was demonstrated that, while native DWPI produces totally water-soluble films, DW heat-denatured solutions give rise to films made with insoluble proteins endowed with higher tensile properties than native DW protein films but with similar WVP (Pérez-Gago et al., 1999). DW protein film solubility was shown to decrease as FFS heating time and temperature increased, while films became at the same time stiffer, stronger, and more stretchable. During thermal denaturation, the Cys-121 thiol group of β-LG becomes exposed and available for intermolecular disulfide bond formation. Consequently, edible films made from denatured DW protein are stronger and more cohesive than those made from native protein (Pérez-Gago et al., 1999). In addition, $O_2P$ was always observed to be lower for films made from heat-denatured whey protein with respect to controls. These results indicate that covalent cross-linking due to heat denaturation of DW proteins is accountable for film water insolubility, higher both mechanical resistance, and $O_2$ barrier properties, without affecting WVP of the films (Pérez-Gago and Krochta, 2001; Sharma et al., 2016). When cross-linked by heating or by γ-irradiation and entrapped in cellulose, DW proteins were shown to generate insoluble films with good mechanical properties, decreased WVP, and high resistance to attack by proteolytic enzymes. X-ray diffraction analysis suggested that cross-linking by γ-irradiation modified conformation of DW proteins, which became more ordered and more stable (Le Tien et al., 2000).

In addition, the incorporation of formaldehyde as a cross-linking agent was shown to enhance film insolubility behavior, mechanical properties, and glass transition temperature (Galietta et al., 1998). Solubility of DWPI films significantly decreased upon treatment of FFS, and also with other chemical cross-linkers (glutaraldehyde, dialdehyde starch, and carbonyldiimidazole), whereas film tensile strength increased and the percentage of elongation was not affected. Conversely, chemical cross-linking increased film WVP and decreased its $O_2P$. No effect was observed by UV treatment of the films on both their solubility and permeabilities (Ustunol and Mert, 2004).

Also TGase was used to produce films from DW proteins. Solubility of TGase cross-linked films was lower than that of control films, whereas their tensile strength values were two times

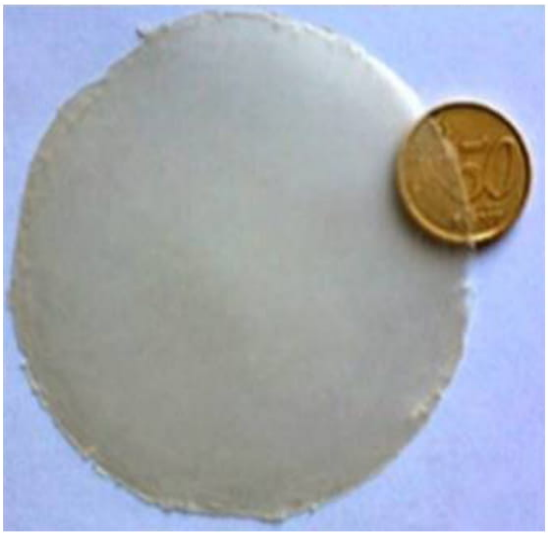

**Figure 13.1: Typical Film Obtained by Grafting Chitosan With Spray-Dried Whey From Bovine Milk Both in the Absence and Presence of TGase.**

greater. Conversely, the thickness of cross-linked films, as well their ability to be hydrolyzed within 24 h by trypsin and α-chimotripsin, were similar to those of control films (Yildrim and Hettiarachchy, 1998). Apparently very similar flexible, transparent, smooth in their surface, and slightly yellowish films were obtained by grafting spray-dried whey from bovine milk with chitosan both in the absence and presence of TGase (Fig. 13.1). The films prepared in the presence of the enzyme showed low solubility at a wide range of pH, a lower degree of swelling, and good biodegradability following protease treatments (Di Pierro et al., 2006). The addition of TGase to FFS also induced an enhancement in film mechanical resistance and a reduction in its deformability, and even the barrier efficiency toward $O_2$ was found to be markedly improved in the cross-linked films, which also showed a lower WVP (Table 13.2).

**Table 13.2: Differences in mechanical and barrier properties between blended polysaccharide/DW films prepared in the absence or presence of TGase.**

| Properties | Chitosan/DW Film[a] | | Pectin/DW Films[b] | |
|---|---|---|---|---|
| | Without TGase | With TGase | Without TGase | With TGase |
| Tensile strength (MPa) | 9.5 ± 0.6 | 26.2 ± 0.9 | 4.5 ± 1.1 | 9.7 ± 1.3 |
| Elongation at break (%) | 14.1 ± 0.6 | 3.1 ± 0.3 | 2.1 ± 0.8 | 25.2 ± 2.3 |
| $O_2P$ ($cm^3$ µm/$m^2$ d kPa) | 20.6 ± 0.7 | 7.8 ± 0.8 | 0.025 ± 0.006 | 0.016 ± 0.002 |
| WVP ($cm^3$ µm/$m^2$ d kPa) | 3.2 ± 0.1 | 0.9 ± 0.1 | 15.4 ± 0.2 | 9.9 ± 0.5 |

DW, Dairy whey; $O_2P$, $O_2$ permeability; WVP, water vapor permeability.

[a]From Di Pierro, P., Chico, B., Villalonga, R., Mariniello, L., Damiao, A.E., Masi, P., Porta, R., 2006. Chitosan-whey protein edible films produced in the absence or presence of transglutaminase: analysis of their mechanical and barrier properties. Biomacromolecules 7, 744–749.

[b]From Di Pierro, P., Rossi Marquez, G., Mariniello, L., Sorrentino, A., Villalonga, R., Porta, R., 2013. Effect of transglutaminase on the mechanical and barrier properties of whey protein/pectin films prepared at complexation pH. J. Agric. Food Chem. 61, 4593–4598.

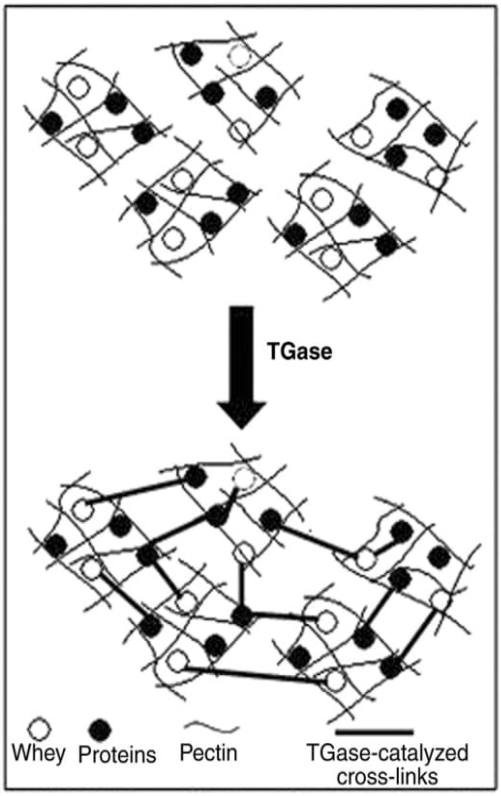


**Figure 13.2: TGase-Catalyzed Cross-Links Among Soluble Ionic DW Protein/Pectin Complexes Occurring at pH 5.1 (Complexation pH).**

Since several studies have characterized different complexes formed by pectin and β-LG (Sperber et al., 2009), demonstrating that it is possible to produce biopolymer systems with different properties, the behavior of blend films derived from thermally denatured DW proteins and pectin was also investigated at both different ratios and pH values (Di Pierro et al., 2013). The data obtained suggested the formation at pH 5.1 (complexation pH) of TGase-catalyzed cross-links among soluble ionic DW protein/pectin complexes (Fig. 13.2), which could be responsible for the recorded increase of both tensile strength (2-fold) and elongation to break (10-fold) of films obtained in the presence of enzyme, as well as of the reduced film permeability (Table 13.2). Conversely, a significant decrease in film elasticity, probably due to the formation of covalent bonds among single DW protein molecules, was observed when the blended films were prepared in the presence of TGase at pH 6.0 (Fig. 13.3). Moreover, although no macroscopic differences were observed between pectin/DW protein films prepared in the absence or presence of TGase (Fig. 13.4), scanning electron and atomic force microscopies revealed significant morphological changes, their images showing an increased homogeneity and compactness of the microstructure of the enzyme-containing film and a much rougher film surface (Di Pierro et al., 2013).

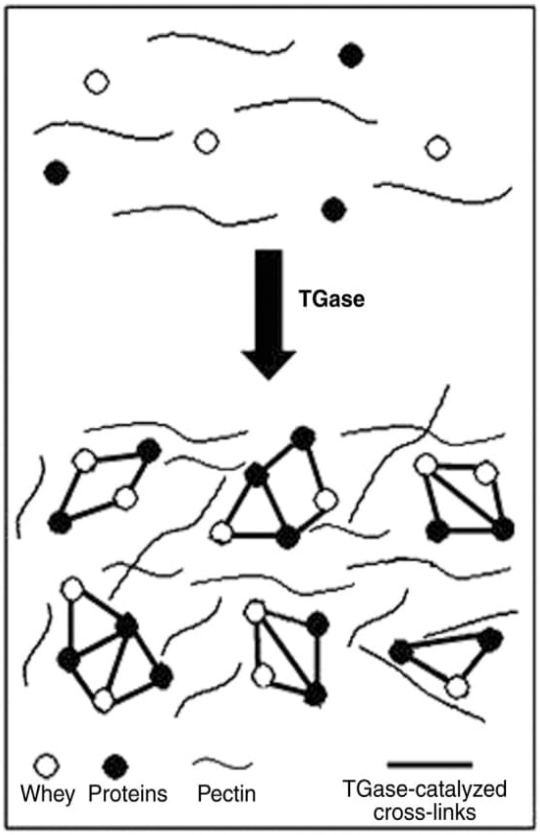


**Figure 13.3: TGase-Catalyzed Cross-Links Among Single DW Protein Molecules Occurring at pH 6.0.**

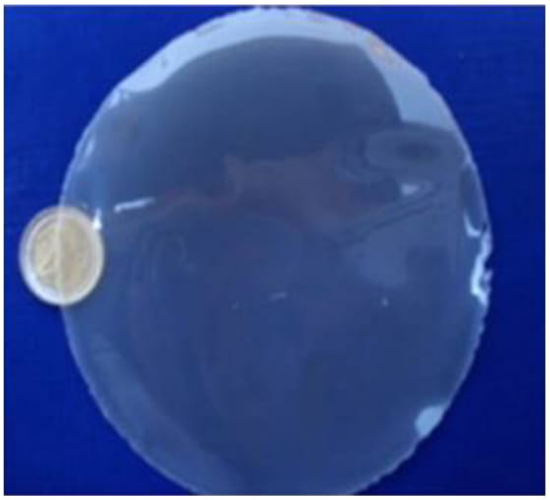

**Figure 13.4: Typical Pectin/DW Protein Film Obtained Both in the Absence and Presence of TGase.**

## 3 DW Protein Film Applications

Since almost 20 years it is known that edible coatings made of DW proteins confer good aroma, humidity, and $O_2$ barrier on various foods. These edible coatings were first shown to be advantageous when used on meat products with the benefit of moisture loss reduction during storage of fresh or frozen meats, retention of juices from packed fresh meat and poultry, reduction of surface spoilage and pathogen microorganisms, and restriction of volatile flavor loss and foreign odor pick up (Gennadios et al., 1997). DW protein coatings also helped to avoid peanut rancidity by lipid oxidation delay (Khwaldia et al., 2004). In fact, the shelf life of peanuts could be extended to 273 days by coating them with DW protein compared to only 136 days for uncoated samples. Moreover, a study by Caner (2005) showed that the shelf life of grade-A eggs coated by DW was extended one week longer than uncoated eggs. It is worthy to note that DW protein films mostly do not modify the aspect and the sensory attributes of the coated goods, as reported by Wagh et al. (2014) that prepared DW protein-based films to protect Cheddar cheese.

However, it is well known that the main cause of spoilage for many food products is surface microbial growth. Growth of yeasts, molds, and bacteria during food storage and distribution can drastically reduce their quality and safety. Thus, the protection of food products with moisture-proof packaging may be an effective methodology to prevent this phenomenon, and edible coatings have been studied as antimicrobial carriers because of their effectiveness in retaining additives on food surfaces. Of course, the choice of an active additive depends on the desired objective, the nature of the food product, and the specific application. Antimicrobial films based on DWPI containing sorbic acid or *p*-aminobenzoic acid were developed by Cagri et al. (2004), who observed that both films inhibited *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella typhimurium* DT104 on a nonselective plating medium and decreased their populations on bologna and sausage slices after 21 days of aerobic storage at refrigerated temperature. Moreover, the growth of mesophilic aerobic and lactic acid bacteria, mold, and yeast on meat slices was inhibited by the same films. Oregano or garlic essential oil added to DWPI edible films exhibited larger inhibitory zones on *Staphylococcus aureus*, *S. enteritidis*, *L. monocytogenes*, *E. coli* O157:H7, *and Lactobacillus plantarum* as compared to rosemary essential oil containing films (Seydim and Sarikus, 2006). Furthermore, DWPI films prepared by incorporating oregano oil into sorbitol-plasticized films were effective in increasing the shelf life of beef meat with minimal changes in the color of the latter (Zinoviadou et al., 2009), whereas the use of lysozyme and lactoperoxidase in DWPI films was shown to extend the shelf life of smoked salmon (Min et al., 2008). More recently, Henriques et al. (2013) report the effectiveness of edible DWPC coatings with antimicrobial properties produced by heat denaturation and UV irradiation applied to ripened cheese as alternatives to commercial cheese coatings.

Chitosan, a hydrophilic biopolymer obtained by alkaline *N*-deacetylation of the polysaccharide chitin, is known to possess broad-spectrum antimicrobial properties against fungi, bacteria, and viruses (Elsabee and Abdou, 2013). We prepared and characterized DW protein films grafted with chitosan (Di Pierro et al., 2006) and coated fresh Ricotta cheese with these blended films to reduce microbial growth during cheese storage under 40% $CO_2$/60% $N_2$ modified atmosphere packaging (MAP) conditions (Di Pierro et al., 2011). Cheese microbiological and physicochemical changes throughout a 30-day storage period were monitored to determine whether these films, compared to the films made from chitosan alone, could lead to an extension of shelf life. Thus, the chitosan/DW protein film was shown to exhibit lower gas permeability and about three times higher WVP than films prepared only with chitosan. In addition, over a 30-day storage period, no differences in the pH of control and coated Ricotta cheeses were observed. While the titratable acidity of the control increased linearly during the first two weeks and remained constant for the rest of the storage period, the corresponding values for coated Ricotta cheese did not change significantly during the first 21 days and reached the control acidity level only on day 30. Moreover, the viable numbers of lactic acid bacteria and mesophilic and psychrotrophic microorganisms were observed to be significantly lower in the chitosan/DW protein coated cheese at each storage time. Finally, no differences in visual appearance, texture, flavor, and odor between uncoated and coated Ricotta cheese samples were detected during sensory evaluation. Therefore, since the prepared chitosan/DW protein film delayed the development of indesirable acidity, reduced the growth of microbial contaminants and did not modify sensory characteristics of the coated cheese, its potential application for extending the shelf life not only of Ricotta cheese but even of other fresh dairy products was suggested.

In the case of fresh-cut fruit and vegetable products, since browning and ripening times are major factors determining their shelf life, edible films have great advantages over MAP or treating the cut surfaces with ascorbic acid, a well-known and frequently utilized browning inhibitor agent. In fact, MAP is effective to slow oxidation, but if $O_2$ levels are reduced too much, the risk of anaerobic bacterial growth becomes severe. Moreover, the amount of added ascorbic acid is generally so high that the product flavor is often negatively affected (Pérez-Gago et al., 2003). Protein-based edible coatings exhibiting moderate WVP, as well as $O_2$ and $CO_2$ permeabilities can be, thus, effectively applied to the surfaces of many fresh-cut fruits and vegetables with the aim to inhibit enzymatic browning, reduce water loss, minimize aroma loss, and delay ripening (Olivas and Barbosa-Canovas, 2005). In a study by Le Tien et al. (2001), DW protein coatings were shown to significantly delay browning of fresh-cut apples and potatoes. In addition, DW protein coatings containing different antibrowning agents were shown to effectively preserve apple slices color during storage and to extend the shelf life of the latter for two weeks when stored in packed trays (Lee et al., 2003). Further studies indicate that addition of antibrowning agents to DW protein coatings, combined with proper storage conditions, significantly extend shelf life of fresh-cut apples (Pérez-Gago et al., 2005).

Similar results were recently obtained in our laboratories (Porta et al., 2013; Rossi Marquez et al., 2017) by coating fresh-cut apples, carrots, and potatoes with a DW protein film grafted with pectin and containing TGase. The ability of such hydrocolloid film to act as an effective barrier to water vapor allowed coated fruit and vegetables to markedly decrease their weight loss and to counteract the microbial growth during their storage at 4°C for 10 days. In addition, food texture was assessed both at the harvest of the products and after storage by recording their mechanical profiles. An almost stable physiological situation was depicted between the two stages (at harvest and after storage), with a practically unmodified pattern of both product hardness and chewiness, when apples, potatoes, and carrots were coated with the blended edible films. The obtained results clearly showed the different browning and softening degrees between uncoated and coated apple and potato samples after 10 days of storage at 4°C, thus confirming that DW could effectively be used as a natural component of antioxidative and moisture barrier coatings to enhance the shelf life of fresh-cut fruits and vegetables.

Pectin/DW protein edible films prepared in the presence of TGase were also recently tested as water barrier coatings of both fried and baked foods (Rossi Marquez et al., 2014). The results of these studies demonstrated an undoubted effect of the produced hydrocolloidal films, due to their capability to reduce WVP, in decreasing moisture loss in both doughnuts and french fries when applied before deep-fat frying. At the same time, a significant decrease in oil content was observed in the coated fried foods (about 50% in doughnuts and 25% in french fries) with respect to both uncoated controls and DW/soy protein coated samples. Conversely, no difference was observed between uncoated and coated both doughnuts and french fries with regard to their texture and sensory properties. The proposed methodology resulted to be effective to hinder moisture absorption also by taralli biscuits during 50 days of storage, thus preventing moisture absorption and the conversion of food matrix from a glassy state to a rubbery state, which is the major cause of the rejection of baked foods by consumers.

More recently, DW proteins were suggested even as a possible source for the production of alternative edible casings, the soft cylindrical containers used to contain sausage mixtures. In fact, the supply of natural casings, traditionally obtained from animal intestines derived from slaughtering, is becoming no longer sufficient for continuously growing consumer demand. Synthetic casings have been found to contain phthalates that are harmful for the endocrine system (Sheikh et al., 2016), whereas the biobased casings would offer fewer risks. In particular, DW proteins are of high quality, having a biological value higher than egg or casein proteins, and possess also some useful functional properties, such as solubility, as well as emulsifying and foaming activities. When compared to hot dogs prepared with collagen or natural casings, sensory panelists gave hot dogs prepared with DWPI–*p*-aminobenzoic acid casings superior scores for texture, juiciness, flavor, and overall desirability (Cagri et al., 2003). However, it was established that even though DW is suitable for preparing effective casings, the production is still not as economical as that of the nonbiodegradable synthetic casings (Mubururu et al., 2014).

## 4 Concluding Remarks and Future Trends

For further and continued improvements of waste management practices, many agencies in the world, such as the US Environmental Protection Agency (EPA), advise reducing the initial amount of packaging and promote composting. For reduction, EPA suggests designing packaging systems with decreased amounts of environmentally toxic materials to make them easier to reuse or generate compost. In particular, EPA stresses packaging with lower danger of damage or spoilage to food products. Protein-based edible films and coatings fit both criteria. In fact, they can reduce the complexity of packaging systems, making them either easier to recycle or compostable, and can also protect food from chemical or biological damage, thereby lengthening product shelf life. In a global food industry, DW components compete with many other molecules for market share. The trading of these ingredients is subject to the cyclical variation in world prices of commodities and, to meet consumer demands for healthy and tasty foods, has led to new and receptive markets (e.g., functional foods and nutraceuticals). Therefore, new opportunities for innovative applications of high-value DW-derived components might open up. Although the DW surplus amount has been so far utilized mainly to obtain value-added products, such as different nutritional proteins and bioactive peptides, the development of edible films and coatings to be applied in both food and nonfood sectors remains a very attractive perspective to tackle DW environmental problems. However, new advances are needed in DW-processing technologies, including efficient and cost-effective unit processes for DW concentration, transformation and fractionation together with the introduction of modern biochemical techniques, some from outside the dairy and food industries, for the manufacture of new DW-based products for increasingly receptive markets. In this context it should be noted that no DW utilization strategy would succeed without a suitable attention being paid to lactose, since this component represents more than 75% of DW solids. The best potential for a possible commercial success of DW derived proteins is, in our opinion, for food coatings able to protect against chemical and biological damage, thus lengthening the shelf life of products, such as cheese, fruit, and vegetables. As multinational companies, such as Wal-Mart and McDonald's began to make use of biobased materials to fulfill their packaging needs, more coatings derived from natural biopolymers with the functionality of petroleum-based packaging have been produced. In this respect, DW protein-based coatings have proved effective gas barriers being able also to act as vehicles for a variety of compounds, such as antioxidants, antimicrobials, or various nutrients. Moreover, the coating methodology may be combined with additional thermal or nonthermal food processing, as well as with further food storage systems, such as MAP. However, even though all the protein-based films can be by now considered acceptable replacements for synthetic gas barriers, their mechanical properties still need improvement. Thus, chemical, physical, or enzymatic protein cross-linking (Arabestani et al., 2016b; Benbettaieb et al., 2016; Porta et al., 2011b), as well as blending options and nanotechnology, seem possible viable routes to improve both tensile

strength and elongation at break characteristics of DW protein-based films. Nanoparticles, in particular, are known to affect electronic and atomic interactions without changing the chemistry (Lagaron et al., 2005), as shown by tensile strength and elastic modulus increase of wheat gluten films following montmorillonite clay nanoparticle grafting (Olabarrieta et al., 2006). More recently, sodium laurate-modified $TiO_2$ nanoparticles were successfully incorporated into DWPI films (Zhang et al., 2016), as well as nanocellulose (Qazanfarzadeh and Kadivar, 2016) or zein nanoparticles (Oymaci and Altinkaya, 2016), and the prepared nanocomposite materials exhibited improved mechanical features. Application of a complex science approach will lead to a greater understanding of how DW components may interact with other polymer particles and how they can be manipulated and exploited in supramolecular assemblies, leading to new microstructures and architectures and, consequently, to new biomaterials. In this context, edible nanostructures constitute a major challenge in food industries since the application of nanocoatings can be of great usefulness for food packaging. In fact, the reduction of film thickness may improve its barrier properties while exchanging its resistance, flexibility and tension, as reported by several studies describing DW nanocoatings with different interesting functionalities (Matalanis et al., 2011; Zuniga et al., 2010). The potential applications to different foods have been already suggested, even including that of delivery vectors of functional and bioactive compounds able to enhance safety, as well as nutritional and sensory attributes of food. However, for this and other possible industrial relapses, the cost effectiveness will always remain a driver in present and future DW processing developments.

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CHAPTER

# 21

# Transglutaminase Cross-Linked Edible Films and Coatings for Food Applications

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## 21.1 INTRODUCTION

Transglutaminases (EC 2.3.2.13, TGase) are a family of intra- and extracellular enzymes that are able to catalyze the posttranslational modification of proteins by introducing isopeptide bonds, which are highly resistant toward enzymatic proteolysis and mechanical stress, between *endo*-protein Lys ε-amino (acyl acceptor substrate) and Gln γ-carboxamide (acyl donor substrate) groups. The resulting reaction products range from peptides and polypeptides containing either intramolecular cross-links (Fig. 21.1A) or intermolecular cross-links, the latter classified as linear (Fig. 21.1B) or branched homo- (Fig. 21.1C) and heteropolymers (Fig. 21.1D), respectively. Free ammonia derived from γ-carboxamide group is released during the reaction. Although protein cross-linking is the reaction that has always drawn the greatest attention, the significance of TGase-mediated structural modifications by primary amine or polyamine covalent incorporation into polypeptides is also well documented. In addition, TGase is able to catalyze Gln deamidation in the absence of free amines, with water acting as an acyl acceptor (Eckert et al., 2014; Kuraishi et al., 2001; Motoki and Seguro, 1998). Therefore, all the reactions catalyzed by this enzyme result in significant changes, not only of the structure but also of the physical and chemical characteristics of the proteins acting as substrates, such as viscosity, thermal stability, and elasticity (Kieliszek and Misiewicz, 2014).

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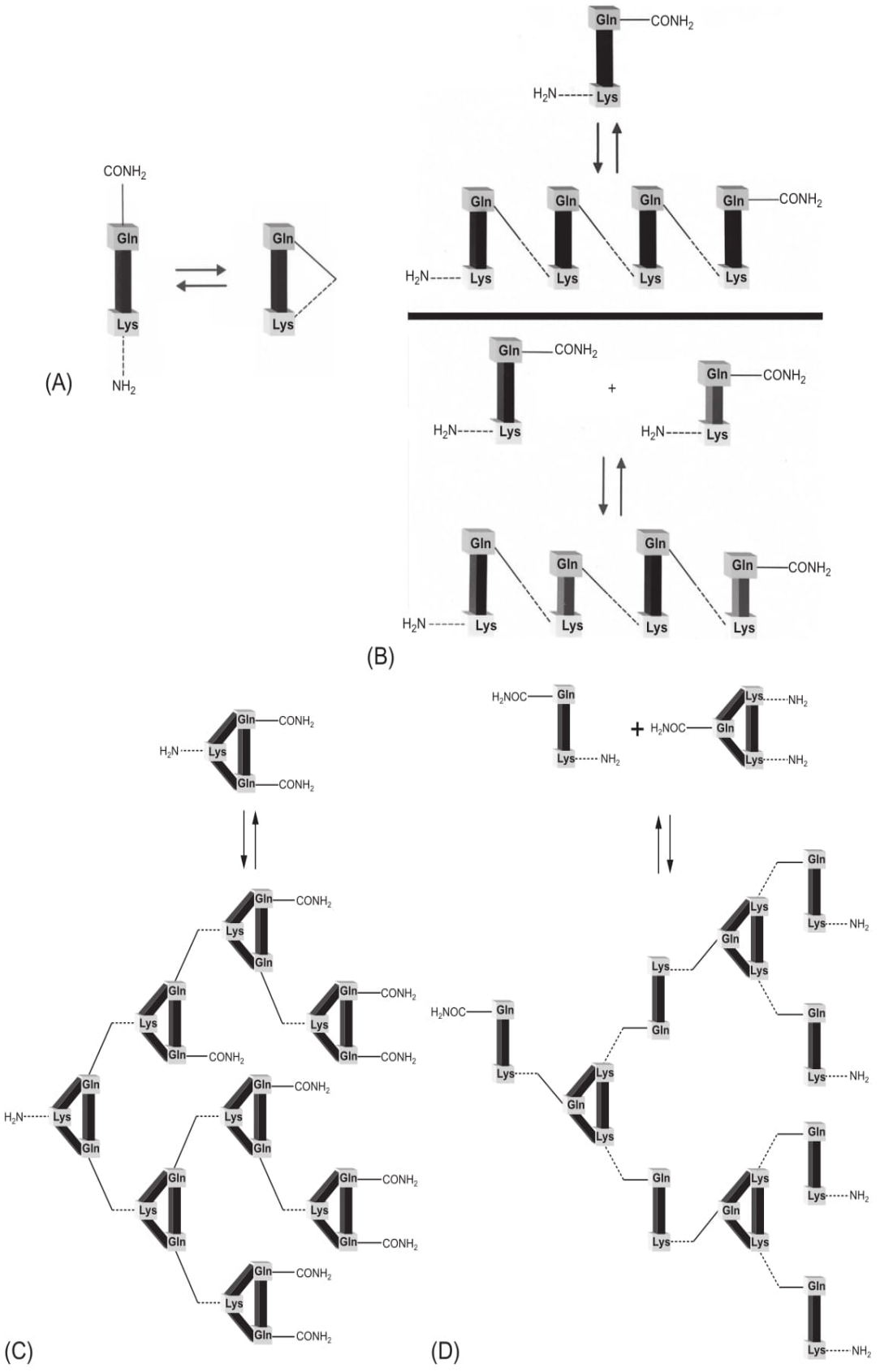


FIG. 21.1 (A) TGase-catalyzed intramolecular cross-link; (B) TGase-catalyzed formation of linear homo- (upper) and hetero- (under) polymers by intermolecular cross-links; TGase-catalyzed formation of branched homo- (C) and hetero- (D) polymers by intermolecular cross-links.

TGases are widespread in nature (Kashiwagi et al., 2002). Since 1959 discovery of a transamidating activity in guinea pig liver extracts due to the most widely distributed and extensively studied molecular form of the enzyme, afterwards designated as TGase2, several additional proteins exhibiting the same catalytic activity were identified in unicellular, invertebrate, and vertebrate organisms as well as in plants (Griffin et al., 2002; Yasueda et al., 1994; Yu et al., 2008). Nine TGase genes have been demonstrated in humans, eight of them able to give rise to catalytically active proteins (the best known is the blood coagulation Factor XIII responsible for fibrin clot stabilization), whereas that relative to the erythrocyte membrane protein band 4.2 leads to an enzymatically inactive product. The first TGase primary structure established was that of Factor XIII (Ichinose et al., 1990) and only a faint sequence homology was then observed among the different enzyme molecular forms. However, all the multiple forms of TGase share an identical sequence at the level of the active site, that is, a catalytic triad of cysteine, histidine, and aspartate (Cys-314/His-373/Asp-396 and Cys-276/His-334/Asp-358 in Factor XIII and TGase2, respectively), with a proceeding of the reaction through an intermediate product linked to the Cys–SH group and where hydrogen bonds between Asp and His residues contribute to maintain a catalytic orientation of the protein (Strop, 2014). Six mammalian TGases with a total mass ranging from 77 to 90 kDa have been well characterized. All of them show structures consisting of a β-sandwich core domain, which contains both active and regulatory sites, and two C- terminal β-barrels (Strop, 2014).

In higher organisms, TGases have been suggested or demonstrated to play different roles by modifying different proteins in different organs (Eckert et al., 2014; Lorand and Graham, 2003). Other than Factor XIII, involved in blood coagulation, TGase2 has been proposed to have a function in cellular differentiation, tissue stabilization, and apoptosis as well as to promote cell adhesion, whereas keratinocyte and epidermal enzymes cross-link proteins on the outer surface of the squamous epithelium. In addition, some molecular forms of the enzyme have also been suggested to be involved in additional specific human physiological as well as pathological conditions, including immunosuppression, neurodegeneration, and coeliac and neoplastic diseases, so as to be considered as potential therapeutic targets (Eckert et al., 2014; Paonessa et al., 1984; Peluso et al., 1994). Finally, the eukaryotic enzyme is regulated by guanosine-5′-triphosphate and requires calcium concentrations above physiological conditions to be catalytically active. Therefore, its reaction products are formed only under conditions disrupting cellular homeostasis, such as those occurring during blood coagulation and wound healing (Eckert et al., 2014). In contrast to the extensively studied mammalian TGases, there have been so far limited studies on the presence and role played by such enzyme in plants, where it was first detected in pea seedlings (Icekson and Apelbaum, 1987). It then was showed in further tissues, from soy to topinambour, from fodder beet to apple, and was immunodetected in the protein extracts of maize meristematic calli and their isolated chloroplasts (Campos et al., 2010; Falcone et al., 1993; Luciano and Arntfield, 2012; Sobieszczuk-Nowicka et al., 2008). As far as the occurrence of TGase in unicellular organisms, it was investigated by screening different microorganisms with the aim mainly to have available an inexpensive and stable source of the enzyme for possible biotechnological applications (Kieliszek and Misiewicz, 2014; Rachel and Pelletier, 2013; Strop, 2014). The enzyme, isolated from *Streptoverticillium* sp. and *Physarum polycephalum*, has also been detected in *Bacillus subtilis* spores and, as an extracellular molecular form, it was shown to be produced by *Streptoverticillium cinnamoneum* subsp., *Streptoverticillium griseocarneum*, *Streptoverticillium*

*ladakanum*, *Streptomyces netropsis*, and *Streptomyces lydicus* (Aidaroos et al., 2011; Duran et al., 1998; Færgemand and Qvist, 1997; Gerber et al., 1994; Ho et al., 2000; Yu et al., 2008). Although microbial TGase (mTGase) catalyzes the same reaction catalyzed by the other molecular forms of the enzyme, it does not share with them a sequence or structural homology, is not regulated by calcium or guanosine-5′-triphosphate, and has a broader substrate specificity and pH dependence (Kieliszek and Misiewicz, 2014). In fact, mTGase, the isoelectric point of which is 8.9, is active over a wide range of pH with an optimum value between 5 and 8. Moreover, the enzyme is active at high temperatures up to 70°C with an optimum at 55°C, although at this temperature it loses catalytic activity over time (Kieliszek and Misiewicz, 2014; Jaros et al., 2006; Yokoyama et al., 2004). mTGase (38 kDa, a single polypeptide chain of 331 amino acids) has a globular structure determined by 11 α-helices and eight β-strands, with the active site located in a central pocket composed of five acidic residues (Asp-3, -4, -255 and Glu-249, -300) and numerous aromatic residues (Tyr-62, -75, -278, -291, -302 and Trp-59, -69) (Kashiwagi et al., 2002; Yokoyama et al., 2004). A Cys protease-like mechanism of reaction, in which Asp-255 plays the role of His residue in Factor XIII and TGase2, has been hypothesized for mTGase, with the formation of a tetrahedral enzyme/substrate complex following the thioester binding between Cys-64 and the Gln γ-carboxamide carbonyl group of the protein acyl donor substrate. Then, a proton is donated by Asp-255 to the oxyanion hole and an enzyme-bound thioamide is formed with the release of ammonia. Finally, an acyl acceptor substrate (either an endoprotein Lys or a free primary amine) attacks the thioamide carbonyl group, facilitated by Asp-255 deprotonation, and the cross-linked or aminated protein is released while the enzyme is regenerated in its native form (Kashiwagi et al., 2002; Yokoyama et al., 2004). mTGase specificity toward the acyl donor substrates is determined by a combination of primary and secondary structure of the protein domain containing the reactive Gln residue, as well as by protein substrate flexibility around its reactive γ-carboxamide group (Strop, 2014). Conversely, and similarly to the other TGases, minor structural requirements have been demonstrated for the reactive sites of acyl acceptor substrates. However, mTGase molecular mass, which is about half with respect to the one of its counterparts, the single domain and the absence of sequence homology with the other enzyme molecular forms suggest that mTGase is probably a product of convergent evolution (Ando et al., 1989; Kanaji et al., 1993; Jaros et al., 2006). The only feature similar to that of all TGases is its obvious posttranslational activation because undesired protein cross-linking might be harmful to both unicellular and multicellular organisms. Finally, the biological function of mTGase still remains largely unknown, even though it has been hypothesized that in *Streptomyces mobaraensis* the enzyme might play the role to cross-link inhibitory proteins participating in the formation of an antibiotic shield against host proteases (Sarafeddinov et al., 2011).

Applied research carried out up to now with TGase involved almost exclusively two molecular forms of the enzyme, i.e., TGase2 and mTGase. These two forms have been extensively studied both in academia and industry but, while the interest in TGase2 started from a medical perspective to better understand the role of the enzyme in various diseases, the microbial molecular form of the enzyme encountered great attention in many different food applications (Góes-Favoni and Bueno, 2014; Kieliszek and Misiewicz, 2014; Kuraishi et al., 2001; Mariniello and Porta, 2005; Mariniello et al., 2008; Motoki and Seguro, 1998; Motoki and Kumazawa, 2000; Rachel and Pelletier, 2013). In fact, mTGase, considered to be "generally recognized as safe" (GRAS) for human intake, has been widely proposed as a processing aid

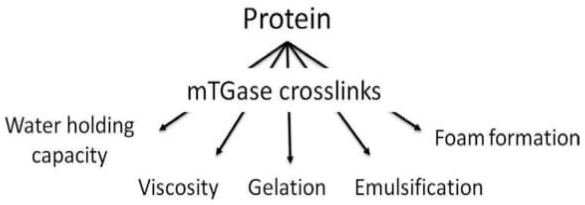


FIG. 21.2 Main functional properties of proteins of food interest influenced by mTGase.

for modifying the functional properties of many different food products. With proteins being one of the main classes of food components, their structural modification via chemical, physical, or enzymatic methods is an alternative available for the improvement and/or development of new functional properties of specific protein-based foods. In view of the considerable interest of the food industry in using enzymes due to their specificity, mild reaction conditions, and lower risk of formation of toxic products (Özrenk, 2006), gaining an understanding of their effects and mechanism of action in the modification of the functional properties of proteins is the first step in promoting their industrial use. On the other hand, according to the requirements of Article 6.4 (c) of Directive 2000/13/EC and Article 20 (b) of Regulation (EU) N. 1169/2011, food enzymes used as processing aids are not required to be declared in the list of ingredients on prepacked food. Therefore, many attempts were performed with the aim to improve the physical and biological properties of food proteins by mTGase (Fig. 21.2) because a variety of them have been shown to act as effective acyl donor and/or acceptor substrates for the enzyme (Bönisch et al., 2006; Giosafatto et al., 2012; Hong and Xiong, 2012; Mariniello and Porta, 2005, Mariniello et al., 2007, 1993, 2014; Porta et al., 2011a).

## 21.2 TRANSGLUTAMINASE AND FOOD EDIBLE COATINGS

The current scenario of plastic pollution shows that >700 thousand plastic shopping bags and 400 thousand plastic bottles are consumed worldwide every minute. Consequently, about 35 million tons of plastic waste are annually produced in the entire world while only 7% of that is recycled (Sabbah and Porta, 2017). One possible solution to reduce the pollution due to plastics of petrochemical origin is their increasing replacement with biodegradable materials derived from aliphatic polyesters (e.g., polylactates and polyhydroxyalkanoates) and/or natural biopolymers such as polysaccharides and proteins (Gómez-Estaca et al., 2016; Pathak et al., 2014; Song and Zheng, 2014). A great number of agroresources rich in proteins, such as soybeans (38%–44%), sunflower seeds (28%–42%), peas (22%–28%), cereal grains (8%–15%), and milk whey (1%) may be available and possibly recycled to produce biomaterials useful for food biodegradable and/or edible coatings and packaging (Rhim, 2007). The application of such "bioplastic" films in the food industry was proposed in the late 1960s with the aim to prolong the shelf life of different fresh and manufactured foods and to improve their quality (Krochta and De Mulder-Johnston, 1997). Then, the use of edible coatings increased with time, showing to be a simple and effective technology in preventing the textural deterioration of several food products (Porta et al., 2013; Zink et al., 2016). Coatings can be obtained in several ways, generally by dipping or spraying foods with specifically tailored film-forming solutions (FFSs) (Coltelli et al., 2016; Song and Zheng, 2014). However, films made of polysaccharides and/or proteins, while possessing good gas barrier features toward oxygen and carbon

dioxide, usually exhibit poor mechanical properties and, due to their hydrophilic nature, are too water sensitive to be applied to the majority of foods having high or intermediate moisture (Krochta and DeMulder-Johnston, 1997; Nisperos-Carriedo, 1994). These disadvantages can be resolved by the preparation of blended films, as protein-polysaccharide films and/or by adding lipids or other components such as nanoparticles able to reinforce the film network (Porta et al., 2011b; Rhim, 2007; Rostamzad et al., 2016). An additional strategy to improve the characteristics of protein-based films is to create a cross-linked structure of its network either chemically or enzymatically. Among the enzymes able to create protein cross-linkings, TGase is certainly the most efficient, being able to catalyze isopeptide bonds between reactive Gln and Lys residues existing in the polypeptide sequence. Mahmoud and Savello (1992, 1993) were the first to utilize TGase as a cross-linker to produce whey protein (WP) homo- and heteropolymers containing films. Afterwards, different authors proposed further proteins and methodologies to produce TGase cross-linked films with specific properties suitable for coating specific food products (Cui et al., 2017; Elango et al., 2017; Mariniello and Porta, 2003; Porta et al., 2011b, c; Rossi Marquez et al., 2014, 2017). This kind of food coating needs, of course, to be produced by using proteins able to act as acyl donors and/or acceptor substrates of the enzyme. Therefore, most of the studies were mainly focused on a few specific proteins able to easily form cross-linked polymers in the presence of mTGase. An overview of the main effects of mTGase-catalyzed reactions on the properties of different protein-based films and food coatings is given hereafter and summarized in Table 21.1.

**TABLE 21.1** Effects of mTGase-Catalyzed Reactions on the Properties of Different Edible Films and Food Coatings

| Protein Film[a] | Effect | References |
|---|---|---|
| *White (milk) proteins* | | |
| Casein | Mechanical strength and flexibility increases, solubility decrease | Oh et al. (2004) and Al-Saadi et al. (2014) |
| $\alpha_{s1}$-Casein | Tensile strength increase and water solubility decrease | Motoki et al. (1987) |
| Sodium caseinate/hydroxy-propylmethylcellulose | Solubility decrease and stiffness increase | Perone et al. (2014) |
| Casein/type I collagen | Improvement of thermal stability | Wu et al. (2017) |
| Succinylated casein/egg-white protein | Improvement of mechanical properties, water resistance, and thermal stability | Peng et al. (2017) |
| Sodium caseinate/WP | Water solubility decrease and mechanical/water barrier properties increase | Qiao et al. (2014) |
| WP | Tensile strength and elongation at break increase. Delayed lipid oxidation | Rodriguez-Turienzo et al. (2013) |
| WP/chitosan | Lower permeability to water vapor | Di Pierro et al. (2006) |
| WP/carboxymethylated chitosan | Improvement of water vapor barrier and mechanical properties | Jiang et al. (2016) |

**TABLE 21.1** Effects of mTGase-Catalyzed Reactions on the Properties of Different Edible Films and Food Coatings—cont'd

| Protein Film[a] | Effect | References |
|---|---|---|
| WP/pectin | Tensile strength, elongation at break, and gas barrier increase. Oil content decrease in coated fried foods. Prolonged shelf life of coated fruits and vegetables | Di Pierro et al. (2013) and Rossi Marquez et al. (2014, 2017) |
| *Green (plant) proteins* | | |
| WG | Increased solubility and integrity. Improved mechanical properties and water vapor permeability | Larré et al. (2000) and Jinshui et al. (2005) |
| WG/α-polylysine | Improved mechanical properties and water stability, more compact network structures and higher hydrophobicity | Cui et al. (2017) |
| Zein hydrolysate/WPs | Increased integrity and flexibility | Oh et al. (2004) |
| Zein/oleic acid | Improved mechanical and water barrier properties | Masamba et al. (2016) |
| SPI | Increased tensile strength and surface hydrophobicity thermostability | Tang et al. (2005) |
| SPI/pectin soy flour proteins/pectin | Higher homogeneity, increased tensile strength, reduced flexibility, and lower gas exchange | Mariniello et al. (2003) and Di Pierro et al. (2005) |
| Soy flour proteins/ putrescine-pectin | Improved mechanical properties and decreased water vapor permeability | Di Pierro et al. (2010) |
| Skimmed soy proteins/WPI | High water-keeping capacity and strong elasticity | Su et al. (2007) |
| SPI/gelatin films | Improved thermal stability, water resistance, and tensile strength properties | Weng and Zheng (2015) |
| SPI/type I collagen | Improved thermal stability and mechanical properties | Wu et al. (2017) |
| Phaseolin/grapefruit albedo | Improved mechanical characteristics and barrier properties to carbon dioxide and water vapor | Mariniello et al. (2010) |
| Phaseolin/citrus pectin | Easy digestion by the human gut, lower $CO_2$ and $O_2$ permeability than LDPE and MATER-BI | Giosafatto et al. (2014a) |
| Phaseolin/pectin/trehalose | Smoother surface and antioxidant properties | Giosafatto et al. (2014b) |
| BVP | Decreased $CO_2$ and $O_2$ permeability and increased resistance and stiffness | Porta et al. (2015) |
| BVP/pectin | Decreased gas permeability and improved mechanical properties | Porta et al. (2016) |
| *Red (animal) proteins* | | |
| Type I collagen/casein, keratin or SPI | Improved thermal stability and mechanical characteristics | Wu et al. (2017) |

*Continued*

**TABLE 21.1** Effects of mTGase-Catalyzed Reactions on the Properties of Different Edible Films and Food Coatings—cont'd

| Protein Film[a] | Effect | References |
|---|---|---|
| Shark catfish skin collagen | Higher tensile strength, stiffness, water solubility, and stability against proteolysis | Elango et al. (2017) |
| Gelatin | Improved water vapor barrier, mechanical properties, water resistance, and thermal stability | Babin and Dickinson (2001), Carvalho and Grosso (2004), and Liu et al. (2016, 2017) |
| Fish-skin gelatin | Decreased tensile strength, elongation at break, and solubility | Piotrowska et al. (2008) |
| Catfish gelatin | Increased elongation at break and decreased tensile strength | Oh (2012) |
| Gelatin/calcium carbonate | Reduced water vapor permeability, increased mechanical properties, and facilitated film formation | Wang et al. (2015) |
| Gelatin/casein | Increased elongation at break and decreased water vapor permeability | Chambi and Grosso (2006) |
| Fish gelatin/nanoclay | Decreased tensile strength and elongation at break | Bae et al. (2009) |
| Fish gelatin/chitosan | Increased deformation and water vapor permeability, decreased tensile strength and solubility | Alvarado et al. (2015) |
| Fish gelatin/starch | Increased tensile strength and Young's modulus, decreased solubility and elongation at break | Al-Hassan and Norziah (2017) |
| Pork gelatin-coated paper | Reduced water vapor permeability | Battisti et al. (2017) |
| Fish myofibrillar protein | Increased tensile strength, barrier properties, and thermal stability. Decreased elongation at break and solubility | Kaewprachua et al. (2017) |
| Fish myofibrillar protein/nanoclay | Improved water gain, water vapor permeability, solubility, tensile strength, and elongation at break | Rostamzad et al. (2016) |
| Ovalbumin/chitosan | Improved mechanical properties | Di Pierro et al. (2007) |

[a] *WPI = Whey protein isolate, WG = Wheat gluten, SPI = Soy protein isolate, BVP = Bitter vetch proteins.*

## 21.2.1 White (Milk) Proteins

### *21.2.1.1 Caseins*

Caseins are the most abundant proteins occurring in milk, accounting for about 80% of the total milk protein content. Native caseins exist as large highly hydrated colloidal micelles that coagulate under the effect of specific proteolytic enzymes to give rise to many different dairy products. However, since caseins present in their structures many acyl acceptor (Lys) and donor (Gln) reactive sites, they could be easily polymerized by TGase (Schorsch et al., 2000).

The obtained casein-based films have a hydrophilic character and, consequently, they exhibit high water vapor permeability (Chick and Hernandez, 2002). Oh et al. (2004) tried to improve their properties by adding TGase to FFSs containing casein alone or in combination with other proteins, such as zein hydrolysates, to obtain films with higher mechanical strength and flexibility, whereas Al-Saadi et al. (2014) observed a lower solubility of the casein films prepared in the presence of the enzyme. Among the different composite films prepared with other proteins or polysaccharide derivatives (Perone et al., 2014; Wu et al., 2017), Qiao et al. (2014) observed that water solubility decreased while both mechanical and water barrier properties increased when sodium caseinate film was blended with WPs and cross-linked by TGase. These results are in agreement with the previous ones showing that the presence of the enzyme increased the mechanical properties and decreased the film water solubility of $\alpha_{s1}$-casein-based films (Motoki et al., 1987). Furthermore, Peng et al. (2017) recently investigated the effect of TGase on succinylated casein films blended with egg white protein. They observed that the TGase-containing films were more homogeneous and smoother and possessed better water resistance and thermal stability compared to the ones prepared in the absence of the enzyme. In addition, the spatial conformation and degree of crystallinity of such composite films were also found to be positively affected by TGase.

#### *21.2.1.2 Whey Proteins*

Nevertheless, the main interest in milk-derived protein films and coatings has been focused on the protein soluble fraction occurring in the milk whey, that is, the liquid by-product of cheese manufacturing that is available in large amounts and whose annual production increases year over year (Khwaldia et al., 2004). There is great concern in the food industry to find out multiple and suitable methodologies for recovering this valuable by-product that, otherwise, may represent a dangerous polluting waste for its lactose and protein content (Di Pierro et al., 2006; Siso, 1996; Smithers, 2008). The milk WP fraction has been widely tested for the preparation of biodegradable films, due to its unique nutritional and functional properties (Ferreira et al., 2009; Schmid, 2013; Schmid et al., 2014). Depending on the WP manufacturing process, various proteins such as immunoglobulins, bovin serum albumin, and several low mol. wt. polypeptides may occur in different proportions in the WP preparations, either as isolates or concentrates, in addition to the two main components α-lactoalbumin and β-lactoglobulin (Andersson, 2008; Herrmann et al., 2004; Lent et al., 1998). Although WP-derived bioplastics, as the other protein-based films, have excellent gas barrier properties, they show low tensile strength and high water vapor permeability due to the high proportion of hydrophilic amino acids occurring in the polypeptide sequences of both α-lactoalbumin and β-lactoglobulin (Ghanbarzadeha and Oromiehi, 2008). WP films are generally formed in aqueous solutions in the presence of different plasticizers. Their properties have been shown to be significantly influenced by the presence of lipids, salts and TGase (Hamman and Schmid, 2014). In particular, the addition of mTGase to WP FFSs was demonstrated to induce an enhancement of film mechanical resistance and a reduction in its deformability. Even the barrier efficiency toward $O_2$ was found to be markedly improved in the cross-linked films, which also showed a lower permeability to water vapor (Di Pierro et al., 2006, 2013; Rossi Marquez et al., 2014). Studies were also carried out by blending WP with different polysaccharides, such as free (Di Pierro et al., 2006) or carboxymethylated (Jiang et al., 2016) chitosan and pectin (Di Pierro et al., 2013; Rossi Marquez et al., 2014,

2017), with the aim to improve film coating properties. In particular, Rossi Marquez et al. (2014) demonstrated a clear effect of the TGase-cross-linked WP/pectin films in decreasing moisture loss in both doughnuts and French fries when applied as coating before food frying. A concomitant decrease in oil content was also observed in the coated fried foods with no difference in their texture noted during the sensory evaluation tests recorded between uncoated and coated foods. The same coating methodology was effective at hindering moisture absorption by biscuits during their storage, being able to prevent the conversion of the food matrix from a glassy state to a rubbery state. Furthermore, Rodriguez-Turienzo et al. (2013) evaluated the effect of the enzyme on either heated or ultrasound-treated WP films. In particular, they investigated mTGase ability to improve the quality parameters of coated frozen Atlantic salmon (*Salmo salar*). The authors concluded that the enzyme addition to the heated WP coatings delayed lipid oxidation, even though it did not significantly modify the yields, drip losses, color, or chemical composition of the fish fillets. Finally, Jiang et al. (2016) reported that mTGase was effective at improving the water vapor barrier and mechanical properties of WP/carboxymethylated chitosan films, whereas Rossi Marquez et al. (2017) showed that WP/pectin films obtained in the presence of mTGase significantly increased the shelf life of fresh cut apples, potatoes, and carrots without affecting their sensory properties.

## 21.2.2 Green (Plant) Proteins

### *21.2.2.1 Wheat Gluten*

Wheat gluten (WG) is a term generally used to define >50 different salt water-insoluble wheat flour proteins of different classes. It includes the glutelin and prolamine fractions of wheat flour proteins, typically referred to as glutenin and gliadin, respectively (Coltelli et al., 2016). WG is well known to form a unique viscoelastic network that gives integrity to wheat dough, which has been used worldwide for centuries for a myriad of bakery and other food products. WG films are typically prepared from aqueous/alcoholic FFSs. Because casting is the method widely used for gluten film formation, homogeneous FFSs are required, aided by mechanical mixing, heating, and adjusting to alkaline or acidic pH values (Zhang and Mittal, 2010). Larré et al. (2000) reported that TGase is effective in introducing isopeptide covalent bonds into films obtained from slightly deamidated gluten. Protein cross-linkings induce the formation of polymers of high molecular weight that are responsible not only for the greater insolubility of the treated films, but also for the reduced film surface hydrophobicity. The determination of film mechanical properties showed that the addition of the enzyme increased film integrity and resistance as well as its capacity to stretch. In addition, Jinshui et al. (2005) showed that the films prepared from TGase-modified gluten exhibited higher tensile strength but lower elongation at break and water vapor permeability, compared to the native gluten films. They also showed that, when the modified gluten films were immersed in water at 25°C, their water resistance result increased. Although gluten proteins can be easily manufactured into various types of biological materials, the lack of primary amino groups in their sequence limits the possibility of modification. Recently, Cui et al. (2017) investigated the effect of α-polylysine in gluten TGase-mediated cross-linking and the consequences on the properties of the derived films. The mechanical properties as well as the water stability of the obtained films were found to

be significantly improved in the presence of the enzyme. The films also exhibited rougher surfaces, more compact network structures, and higher hydrophobicity.

#### *21.2.2.2 Zein*

Zein, an alcohol-soluble protein enriched in the endosperm of corn, belongs to the class of prolamine proteins and is one of the best studied plant biomacromolecules (Lawton, 2002; Momany et al., 2005). In the absence of plasticizers, zein is known to form brittle films with poor flexibility (Lawton, 2002). Interestingly, Gln is an abundant amino acid at the zein surface, forming polyGln strands. Due to the existence of these polyGln turns and to the fact that some Gln are particularly exposed, it was first hypothesized and then demonstrated that zein is an effective acyl donor substrate of TGase (Cui et al., 2016). The addition of TGase and WPs to zein hydrolysate decreased the solubility of the derived films, which exhibited lower tensile strength and higher elongation at break values. Therefore, enzyme-catalyzed cross-linkage between zein hydrolysate and WPs markedly increased the integrity and flexibility of the protein films (Oh et al., 2004). Furthermore, the effect of different drying temperatures and pH values on the properties of TGase-cross-linked zeine/oleic acid composite films has been recently reported (Masamba et al., 2016). The results of these investigations showed that low pHs and high drying temperatures are ideal processing conditions in improving the mechanical and water barrier properties of TGase cross-linked films. Conversely, high pHs and low drying temperatures did not improve tensile strength, solubility, or water vapor permeability, instead only improving elongation at the break. The effects observed at low pHs were explained with protein aggregation as evidenced by the enzyme-catalyzed increase in zein molecular weight.

#### *21.2.2.3 Soy Proteins*

Soy protein isolate is a soybean oil by-product containing a high amount of proteins (≥90%). It is abundantly available, cheap, and easily modified by TGase (Guoa et al., 2015). Moreover, having a good film-forming ability, soy proteins have been extensively studied for food coating applications, leading to the formation of films with high thermostability as packaging material, owing to both hydrogen and disulfide bonds as well as to hydrophobic interactions (Li et al., 2008, 2016). Tang et al. (2005) demonstrated that mTGase was able to increase soy protein film tensile strength and surface hydrophobicity while decreasing film elongation at break, moisture content, and transparency. Microstructural analyses indicated that the cross-linked films had a rougher surface and a more homogeneous and compact cross-section compared to the controls. The mTGase-catalyzed cross-linking of soy protein films was also investigated in the presence of pectins, which were shown to facilitate the formation of high molecular weight protein polymers due to polysaccharide/protein electrostatic interactions (Di Pierro et al., 2005; Mariniello et al., 2003). Film homogeneity was improved and its tensile strength was increased whereas gas permeability was reduced. Furthermore, a putrescine/pectin conjugate was tested to prepare blended soy flour protein films in the presence of mTGase. The obtained cross-linked films were found to possess a decreased water vapor permeability as well as improved mechanical properties, with respect to the ones obtained with not aminated pectin (Di Pierro et al., 2010). Soy protein blended films were also prepared by adding to FFSs other proteins of different origin. Su et al. (2007) used skimmed soybean protein powder to investigate the formation of soy protein/WP blended films by a purified mTGase

produced from a new strain of *Streptomyces* sp. They found that the tensile strength values of cross-linked films were much greater than those of the control while the films had high elasticity and the prevention rates against the permeability of water vapor and oxygen were also upgraded by >70%. TGase-cross-linked soy protein films were also obtained in the presence of gelatin, and these blended films showed better thermal stability as well as improved water resistance properties with respect to the ones prepared in the presence of only mTGase. In addition, SEM morphological characterization revealed that the enzyme also produced a more compact network structure of the blended films (Weng and Zheng, 2015). Recently, also Wu et al. (2017) observed that the cross-linking not only improved the thermal stability of soy protein/collagen complex but enhanced the mechanical properties of the combined films in terms of both tensile strength and elongation at break.

#### 21.2.2.4 Phaseolin

Phaseolin, a bean (*Phaseolus vulgaris* L.) protein having structural properties very similar to those of the globulins occurring in soybean and other legumes, has been shown to act as both an acyl donor and acceptor substrate of mTGase (Mariniello et al., 2007). Accordingly, albedo-phaseolin films prepared in the presence of the enzyme have been suggested as promising candidates to be used to obtain edible food wraps because of their suitable mechanical characteristics and barrier properties to carbon dioxide and water vapor (Mariniello et al., 2010). Afterwards, Giosafatto et al. (2014a, b) characterized the hydrocolloid films made by using *Citrus* pectin and TGase-cross-linked phaseolin. For the first time, trehalose, a nonreducing homodisaccharide into which two glucose units are linked together by a α-1,1-glycosidic linkage, was used as a component of hydrocolloid films constituted of both proteins and carbohydrates. These investigations demonstrated that the obtained films acted as very effective barriers to gases and exhibited a high antioxidant capability.

#### 21.2.2.5 Bitter Vetch Proteins

Bitter vetch (BV, *Vicia ervilia*) is an annual *Vicia* genus cultivated for forage and seed yield. In particular, BV seeds, with up to 25% protein, are an abundant, inexpensive, and renewable source of both protein and energy (Sadeghi et al., 2009). Thus, BV proteins might represent an affordable alternative protein source to produce edible films for both pharmaceutical and food applications. In particular, Arabestani et al. (2013, 2016), Porta et al. (2017), Sabbah et al. (2017), and Fernandez-Bats et al. (2018) recently described edible films obtained from BV protein concentrate (BVPC), showing promising barrier and mechanical properties that are useful to give rise to nanocomposite or bilayered films, as well as biodegradable containers. Porta et al. (2015) determined some properties of BVPC films reinforced by TGase cross-linking. The surface of films prepared in the presence of the enzyme appeared more compact and smoother and the film cross-sections showed the disappearance of the discontinuous zones observed in the control films and, on the contrary, a very homogeneous structure. TGase cross-linked films also exhibited a markedly decreased oxygen (700-fold) and carbon dioxide (50-fold) permeability compared to the controls, as well as significantly different mechanical properties being increased their resistance and stiffness. Moreover, BVPC/pectin blended films have been shown to exhibit a tensile strength double the one observed by using films containing only BV proteins, with an increase of about 3-fold observed in the presence of TGase (Porta et al., 2016). Also, the elongation at break resulted higher in the films containing

the enzyme, leading to the conclusion that films were more extensible mostly when both pectins and TGase occurred in the FFSs. A direct correlation between the improved film mechanical properties and the negative increase of the zeta-potential of the originating FFSs was recorded. Conversely, gas permeability of BVPC/pectin films markedly decreased and TGase addition determined a further enhancement of their barrier properties. Finally, cross-sectional SEM analysis of the same samples showed that BVPC/pectin films cross-linked by TGase possessed a more compact and homogeneous microstructure in comparison with the reticular structure observed in the control samples.

## 21.2.3 Red (Animal) Proteins

### *21.2.3.1 Collagen*

Collagen is the main protein component in animal connective tissues such as skin, cartilage, and bone, appearing in a fibrous triple helical structure consisting of three protein molecules (Coltelli et al., 2016; Sahithi et al., 2013). Type I collagen is the most common among 28 different types of collagen and it has been widely used in biomedical materials, pharmaceuticals, and foods (Shoulders and Raines, 2009; Sinthusamran et al., 2013). Few studies have been made on the formation of collagen films cross-linked by TGase. Only recently, Wu et al. (2017) investigated the thermal stability and mechanical properties of type I collagen enzymatically cross-linked with casein, keratin, and soy proteins. The obtained results indicated that the high molecular weight particles produced could be effective for collagen film application as food packaging material. On the other hand, Elango et al. (2017), by studying the effects of TGase on mechanical and functional properties of shark catfish skin collagen films, concluded that the enzyme containing films showed improved tensile strength and stiffness. In addition, the cross-linked collagen films exhibited a high stability against in vitro biodegradation by proteolytic enzymes. Gelatin is a water-soluble protein obtained from collagen by acid or alkaline hydrolysis. Babin and Dickinson (2001) showed that the gelatin treatment with TGase determined either negative or positive effects on the protein film stiffness, depending on the order in which the enzyme-catalyzed cross-linkages were formed, and also if they appeared before or after the formation of junction zones induced by the cooling of the solution. Afterwards, Carvalho and Grosso (2004) reported an improvement of the gelatin film water vapor barrier and mechanical properties by TGase treatment, whereas Piotrowska et al. (2008) showed that enzymatic cross-linking of fish skin gelatin significantly decreased film tensile strength and elongation at break as well as film solubility in an aqueous medium without improving water vapor permeability. Conversely, Oh (2012) observed that the mechanical properties of catfish (*Ictalurus punctatus*) gelatin film were significantly affected by TGase cross-linking, with the tensile strength significantly decreasing and elongation at break increasing while no significant difference in film water vapor permeability was detected. More recently, Wang et al. (2015) successfully utilized the enzyme in the preparation of gelatin-calcium carbonate composite films, showing that TGase improved both mechanical and barrier properties of gelatin films as well as the protein thermal stability. Finally, Liu et al. (2016, 2017) determined the effect of drying temperature and glycerol on the properties of gelatin films modified with TGase. They demonstrated that drying temperature might be used to tailor the physical properties of enzyme-modified gelatin films for specific applications and that an improvement of the mechanical properties was associated with film glycerol content. As far as the gelatin composite films are concerned, Chambi

and Grosso (2006) reported that enzymatic cross-linking induced a substantial increase in the high molecular weight protein components of gelatin/casein FFSs. The derived films (casein/gelatin, 3:1) showed a significant increase in the elongation at break values and a significant decrease in water vapor permeability as compared to films made from gelatin or casein alone and from gelatin/casein blended films untreated with TGase, while no difference was detected in the tensile strength values. A gelatin/nanoclay (unmodified Na-montmorillonite) composite film was also produced, and its mechanical and barrier properties were investigated after treatment with mTGase (Bae et al., 2009). The viscosity of the originating FFS was shown to increase in the presence of enzyme because of protein cross-linking, whereas both tensile strength and elongation at break of the derived films decreased; no significant effect on either oxygen or water vapor permeability was observed. Furthermore, Alvarado et al. (2015) reported that films made from fish gelatin and chitosan, in the presence of glycerol and mTGase, showed adequate properties as packaging material. In fact, by using a 3:1 (chitosan:gelatin) ratio, a decrease in tensile strength and an increase in deformation and water vapor permeability were observed. Conversely, the addition of TGase to fish gelatin/starch blended films was recently shown to significantly increase both tensile strength and Young's modulus and to reduce elongation at break as a result of gelatin enzymatic polymerization (Al-Hassan and Norziah, 2017). Finally, one recent application of gelatin film prepared in the presence of mTGase was developed by Battisti et al. (2017). These authors produced a paper sheet coated with a polymeric film based on enzyme cross-linked gelatin containing citric acid. The coated papers, endowed with antimicrobial and antioxidant properties, might be used as active packaging of fresh beef because the obtained material was shown to be able to reduce water vapor permeability.

#### *21.2.3.2 Myofibrillar Proteins*

Myofibrillar proteins of fish muscle are generally used as a film-forming material. In fact, although these proteins are insoluble in water, they become soluble by adjusting the pH of the solution. The obtained films exhibit a homogeneous and smooth surface, similar to the commercial wrap films of polyvinyl chloride. However, as compared to the latter, myofibrillar protein films have relatively poor mechanical and barrier properties (Kaewprachua et al., 2016). Therefore, different attempts have been performed to improve these features to render such films comparable to the commercial ones through optimization of various parameters such as the addition of different amounts and types of plasticizer and/or nanoparticle as well as of cross-linking agent. To this aim, Kaewprachua et al. (2017) investigated mTGase effects on the properties of fish myofibrillar protein films. They observed an increased thickness, thermal stability, tensile strength, and gas barrier properties whereas film lightness, transparency, elongation at break, water vapor permeability, moisture content, solubility, and degree of swelling all decreased. Furthermore, investigations on the combination of montmorillonite nanoclay addition to myofibrillar protein FFS and TGase treatment showed a significant improvement of water gain and water vapor permeability of the obtained films, as well as of their solubility. Moreover, these nanocomposite films also exhibited an improved tensile strength and elongation at break (Rostamzad et al., 2016).

#### *21.2.3.3 Ovalbumin*

Egg white contains many globular functionally important proteins (main solutes present in egg white representing 10% of its weight) with high potential for industrial applications.

Ovalbumin, the most abundant of these (54% of the total egg white proteins), is widely used as a nutrient supplement (Abeyrathne et al., 2013). Flexible, smooth, transparent, and slightly yellowish films have been obtained by mixing ovalbumin with chitosan, but the addition of mTGase to the chitosan/ovalbumin blended FFS gave rise to biomaterials characterized by a lower solubility at a wide range of pH and better enzymatic hydrolysis by trypsin (Di Pierro et al., 2007). The degree of swelling was also reduced and the mechanical resistance of the cross-linked films was shown to be significantly improved, whereas the barrier efficiency toward water vapor was only slightly affected. The protein enzymatic cross-linking of such composite material confirms once again that the approach in modifying the protein film network by TGase described throughout the present chapter represents a very useful strategy in preparing edible films with tailored characteristics for specific food coatings.

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## Appendix 5

- Courses, training courses and seminars followed during PhD course.

***A. Courses***

1. Piero Pucci “Advanced mass spectrometry”, 11-15/7/2016.
2. De Rosa “Nanostructures and nanotechnology”, 21-23/9/2016.
3. Eugenio Notomista “Protein structure visualization, analysis and modeling”, 5,9,16/5/2017.
4. Valeria Giosafatto “Enzymes in food biotechnology”, 25-26/5/2017.
5. Moh Mousori “Communicating and disseminating your research work”, 5-7/6/2017.
6. Valeria Giosafatto “Bioplastics”, 21,27/6/2017.
7. Cinzia Pezzella “Microbial cell factories in industrial biotechnology”, 22-23/6/2017.
8. Daniele Naviglio “The techniques of solid-liquid extraction used in the preparation of the sample for chemical analysis and production of extracts for industrial uses”, 10-13/7/2017.
9. Raffaele Velotta “Introduction to data analysis”, 25-28/9/2017.
10. Bedini Emilianno “Glycochemistry”, 4-7/12/2017.
11. Valeria Giosafatto “Bioplastics”, 5-6/10/2018.
12. Valeria Giosafatto “Enzymes as additives or processing aids”, 8-9/10/2018.
13. Rachelle Isticato “Enzymatic and microbial applications in biotransformations and nanotechnology”, 10-11/10/2018.

***B. Training Courses***

1. Guido Pintacuda “Training in NMR solid state”, 22-24/2/2016.
2. Vinceza Faraco “Advanced training course on emerging biotechnologies for sustainable waste management and biorefinery development”, 4-5/4/2016.
3. Francesce “Training in NMR solid state”, 25-27/6/2016.
4. Maarten Wirix “TEM training course”, 25-28/9/2017.

***C. Seminars***

1. Giovanni Sannia “Bioeconomy in the circular economy”, 29/06/2016.
2. Enrico Cappellini “Paleoproteomics: the state of the art of ancient proteins in art and archaeology”, 29/06/2016.
3. Carlos Regalado “Effect of antimicrobial edible coating based on starch and beewax nanoemulsion on the shelf life of tomato (*Solanum Lycopersicum*)”, 14/07/2016.
4. Tomaso Zambelli “Development of the Fluid FM and its applications for 2D patterning as well as 3D microprinting” 17/10/2016.
5. Tomaso Zambelli “Fluid FM for single cell manipulation” 18/10/2016.
6. Francesca Viganò “Biomolecule analytics using microscale thermophoresis (MST)”, 26/10/2016.
7. E. Filippone “From plants to microalgae, the biotec world is tinged with green (and not only!)” 26/10/2016.
8. Aurelio Teleman “Regulation of mitochondrial function by a lipid metabolite”, 22/11/2016.

9. Raffaele Scoccianti “Effective communication in industry and tips for building a strong CV”, 23/11/2016.
10. Caserta S. ““How “smart" a cell can be? Do transport phenomena play a role in dynamic evolution of cell systems?”, 8/2/2017.
11. Sacha Lucchini “From the farm to the gut: mechanisms of bacterial adaptation”, 22/2/2017.
12. Andrea Strazzulli “Discovery novel hyperthermophilic carbohydrate active enzymes for plantbiomass degradation: a metagenomic approach”, 8/3/2017.
13. Giovanni Libralato “Hygiene research and implications of emerging compounds: personal care products, pharmaceuticals and engineered nanomaterials”, 14/3/2017.
14. Giuseppe Nicotra “Challenges beyond the nanoscale, and the new Sub –A S/TEM microscope at CNR”, 28/4/2017.
15. Angharad M. R. Gatehouse “The Role of RNA interference in crop protection”, 11/5/2017.
16. Thierry Tron “On the use of surface functionalized enzymes.”, 11/5/2017.
17. Aurel Radulescu “Elucidation of the morphology of hydrocarbon polymer electrolyte membranes by small-angle neutron scattering technique”, 15/6/2017.
18. Gabriel Luna-Barcenas “Nanocomposites for biomedical & environmental applications”, 5/7/2017.
19. Mohammad Altamini “Anti adhesive effect of oligosaccharide micro flora using cell culture model”, 7/7/2017.
20. Henk Haasgmann “Immunomosulatory antimicrobial peptides: biology and applications”, 15/02/2018.
21. Charlotte Blom “Enzyme discovery at novozymes”, 13/3/2018.
22. Sabine Flitch “The golden age of glycoscience-new tools in synthesis and analysis”, 24/9/2018.
23. Carmen Galan “Novel synthetic glycol-tools for biology research”, 26/9/2018.

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