DEVELOPMENT OF ENZIMATICALLY RETICULATED EDIBLE FILMS TO BE USED AS ACTIVE PACKAGING

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SUMMARY

The use of plastic packaging is widely diffuse because this material possesses several advantages such as being lightweight, durable, easy to carry and having a low cost production. However, plastic packagings have the problem of not being biodegradable and, therefore, have a global environmental impact. Moreover when in contact with foods, petrol-derived plastics can be harmful for human health especially if the convey plasticizers such as polyvinylclhoride (PVC) that is can cause severe damages to endocrin system. A solution to this problem is to direct attention towards the development of packaging designed primarily for the food industry. (Floros *et al*.,1997). These innovative materials, that could be named edible films, are obtained from natural molecules like proteins, polysaccharides or lipids and are prepared using different techniques such as spray-drying, casting and dip-coating. In some cases, these films can be used as carriers of antimicrobial agents and are defined as active packaging. However, even if edible films are harmless for both human and environment health,, they possess pour mechanical and barriers properties compared to the traditional ones. Such properties can be improved by the inclusion of covalent bonds by transglutaminase (TGase), that is a biotechnological tool that can polymerize proteins through intermolecular cross-links ε-(γ - glutamyl) lysine.

In this work we have obtained edible films made of Citrus pectin and TGase-crosslinked whey proteins, and evaluated their use as active packaging when conveying a peptide with antimicrobial activity. Moreover such films have been used to coat dry biscuits and fryed donuts to establish their effectiveness in extending biscuit shelf-life and reduction of fat up-take in donuts.

The milk whey is the residue of cheese, is a highly polluting waste material despite being a reserve of high biological value food. As source of such proteins we have used a purchased product named Whey Protein Isolate (WPI) contaning: β-lactoglobulin (65%), α-lactoalbumin (25%), and bovine serum albumin (8%). WPI is largely used in the food industry as a milk substitute, for the production of ice cream, for increasing the protein content of foods, and as food for people that practise hard physical activity.

Pectin is a heteropolysaccharide, consists mainly of acid D-galacturonic molecules linked by (-(1-4) and is obtained mainly from the peel of fruits of the Citrus family. In isolated form, pectin is rapidly reassociated to form aggregates or networks may also interact with proteins through hydrogen-type bonds, and ionic bonds.(Liu and Kost, 2009). These interactions can be improved by several treatments to obtain three dimensional complexes with improved mechanical and barrier properties.

At the first beginning, the present research was focused on indentifying the best WPI/Pectin ratio and the pHc in order to obtaining the maximum degree of complexation. These parameters allowed us to obtain a stable colloidal solution that was used as the basis for the production of edible films crosslinked or not by TGase. Results have shown that the films obtained at a 4:1 ratio WPI/Pectin and pH 5.1 (pHc) crosslinked with TGase showed good mechanical properties and barrier water vapor and oxygen. In addition, the crosslinking created by TGase makes them a valid support for the release of molecules with antimicrobial activity.

The application of these films by dip-coating technique has allowed us to create a layer on surface foods such as dry biscuits and donuts. Results obtained have shown that the application of the films on dry biscuits decreases absorption of water prolonging the shell-life, while applying the same coating to fried products like donuts significantly reduces the oil absorption.

In conclusion, the TGase turns out to be a valuable biotechnology tool to reticulate soluble complexes obtained from WPI and Pectin, that allow to obtain edible films with good mechanical and barrier properties useful for valuable applications in the food field.

RIASSUNTO

L'uso della plastica nel packaging è molto diffuso perché tale materiale presenta i vantaggi di essere leggero, resistente, facile da trasportare e di avere un basso costo di produzione. Tuttavia, packaging di tipo plastico hanno il problema di non essere biodegradabilie di rappresentare, quindi un problema per l'ambiente a livello mondiale.. Inoltre quando a contatto con gli alimenti, plastiche di tipo tradizionale possono risultare dannose per la salute dell'uomo specie se contengono elasticizzanti quali il polivinilcloruro (PVC) che è risultato essere dannoso per il sistema endocrino. Per tale ragione un grande interesse è rivolto allo sviluppo di packaging alternativi che possano essere usati principalmente nel settore alimentare. (Floros et al., 1997). Questi materiali innovativi, che possiamo denominare film edibili, sono ottenuti da molecole naturali quali proteine, polisaccaridi o lipidi e vengono preparati utilizzando tecniche diverse come lo spray-drying, il solvent-casting e il dip-coating. In alcuni casi, questi film possono essere utilizzati come carriers di agenti antimicrobici e vengono definiti c"active packaging" Tuttavia, i film edibili, ancorchè innocui per la salute dell'ambiente e dell'uomo, hanno proprietà meccaniche e di barriera limitate rispetto alle pellicole plastiche tradizionali. Tali proprietà possono essere migliorate mediante l'inserimento di legami covalenti e, a tal proposito, l'enzima transglutaminasi (TGasi) risulta essere un ottimo strumento biotecnologico in grado di polimerizzare le proteine attraverso legami crociati intermolecolari ε-(γ-glutamil) lisina.

In questo lavoro di tesi abbiamo ottenuto film edibili utilizzando pectine da *Citrus* e proteine del siero reticolate dall'enzima TGasi e valutato il loro uso come active packaging utilizzandoli come carrier di un peptide ad attività antimicrobica. Inoltre tali films sono stati usati per rivestire prodotti da forno (biscotti secchi) e alimenti fritti (graffe), per stabilire la loro efficacia nel prolungare rispettivamente la *shelf-life* e la capacità di ridurre l'*up-take* dei grassi durante la frittura.

Il siero di latte, é il residuo della produzione del formaggio, rappresenta un materiale di scarto altamente inquinante nonostante sia una riserva di proteine alimentari ad alto valore biologico.Come fonte di tali proteine abbiamo utilizzato un prodotto commerciale denominato Whey Protein Isolate (WPI) contenente: β-lattoglobulina

(65%), α-lactoalbumin (25%), e sieroalbumina bovina (8%). Tale prodotto commerciale è ampiamente utilizzato nell'industria alimentare come sostituto del latte, per la produzione di gelati, per aumentare il contenuto proteico dei prodotti alimentari oltre che come alimento per soggetti impegnati in intensa attività fisica.

La pectina è un eteropolisaccaride costituito principalmente da molecole di acido D-galatturonico unite da legami α-(1-4) che viene ottenuta principalmente dalle bucce di frutti della genere *Citrus*. In forma isolata, le pectine si riassociano rapidamente o aggregano per formare networks. In presenza di proteine le pectine possono associarsi ad esse tramite legami di tipo idrogeno e ionico (Liu e Kost, 2009). Queste interazioni possono essere migliorate mediante vari trattamenti ottenendo reticoli tridimensionali con migliori proprietà meccaniche e di barriera.

Le attività di ricerca della presente tesi si sono concentrate in un primo momento sullo studio volto ad individuare il miglior rapporto WPI:Pectine ed il pHc per ottenere la massima complessazione fra i due componenti. Questi parametri ci hanno permesso di ottenere una soluzione colloidale stabile che è stata utilizzata come base per la produzione di film edibili reticolati e non dalla TGasi. I risultati hanno messo in evidenza che i film ottenuti al rapporto 4:1 WPI:Pectine ed a pH 5.1 (pHc) reticolati con la TGasi mostrano buone proprietà meccaniche e barriera all'ossigeno e al vapore acqueo.

L'applicazione di questi film con la tecnica di dip-coating su prodotti alimentari quali biscotti secchi e graffe diminuisce l'assorbimento d'acqua prolungando la *shelf-life* nei primi, mentre riduce significativamente l'assorbimento dell'olio nei secondi

In conclusione, la TGasi risulta essere un valido strumento biotecnologico in grado di reticolare complessi solubili ottenuti da WPI e Pectine e dar luogo a film edibili con buone proprietà meccaniche e barriera utili per una valida applicazione in campo alimentare.

1. INTRODUCTION

Packaging is one of the most important processes to maintain the quality of food products for storage, transportation and end-use. The main function of food packaging is to achieve preservation and the safe delivery of food products until consumption. Therefore, food packaging contributes to extending the shelf-life and maintaining the quality and the safety of the food products (Han, 2005). The limitation of petroleum resources and the awareness of environmental protection have raised a new prospect on biobased materials. These materials have several advantages because are less dense than metal and some petroleum-derived thermal plastics also are more biodegradable. The latter property enables the end-use products of biobased materials to be disposed of upon completion of their useful life without causing any environmental concerns (Liu and Kost, 2009).

The development of biopolymer films has increased the amount of research on edible packaging. Film-forming biopolymers can be proteins, polysaccharides (carbohydrates and gums) or lipids (Gennadios *et al*., 1997; Han, 2005). Protein and polysaccharide films can enhance food quality by acting as barriers and by providing protection to a food product after the primary package is opened (Wang *et al*., 2009; Cao *et al*., 2007). These films can be also used for individual packaging of small portions of food, particularly products that are currently not individually packaged for practical reasons.

To provide shelf-life extension, and to improve the quality, safety and integrity of the packaged food, innovative active packaging concepts are being developed. An active packaging may be defined as a package that changes the condition of the packaged food to extend shelf-life or improve food safety or sensory properties, while maintaining the quality of the packaged food (de Kruijf, 2004). The use of active packaging has several advantages for the producer, as follows (Scully, 2009):

a) Shelf-life extension.
b) Less expensive packaging materials. Packaging of limited-shelf-life products may require enhancement of only one property for a precise period.

c) Simpler processing. Introduction of additional packaging properties can allow the producer to achieve best quality without use of expensive equipment.
d) Reduction or removal of preservatives from food. This is done to meet consumer demands for ‘‘fresher’’ foods containing fewer additives by transferring preservatives from the food to the packaging.
e) Aspect.

All active packaging technologies involve some physical, chemical, or biological action to alter the interactions between the package, the product, and the package headspace to achieve certain desired outcomes (Rooney, 1995; Brody *et al*., 2001). Protein or polysaccharide-based edible films can be potentially used for active packaging and applied as a coating or thin layer film.

1.1 Edible films & coatings

An edible film or coating may also provide some mechanical protection for a food, reducing breakage and improving food integrity. Edible films and coatings are not necessarily intended to eliminate the need for non-edible protective packaging, but they could be intended to interact with conventional packaging to enhance the shelf-life and product quality. When an edible film or coating prevents exchange of moisture, oxygen, aroma, or oil between the food and the environment, the quality and shelf-life of the food also are increased (Krochta, 2002).

Films can form different shapes like wraps, bags, capsules, or pouches, while coatings are a particular form of films that are applied directly to the surface of materials. This coating or coating layers can be remove; however, coatings are typically not intended for disposal separately from the coated materials. Therefore, coatings are regarded as a part of the final product (Han, 2005). The major benefit of the edible coatings is that they can be consumed along with the food, can provide additional nutrients, may enhance sensory characteristics and may include quality-enhancing antimicrobials (Guilbert *et al*., 1996).The main film-forming materials are biopolymers, such as proteins, polysaccharides and lipids. They can

be used alone or in combinations. The physical and chemical characteristics of the biopolymers greatly influence the properties of resulting films and coatings (Sothornvit and Krochta, 2000). Edible films and coatings can increase the effectiveness of some food processing unit operations. For example, edible coatings on potato slices/strips or fried products can reduce oil absorption during frying (Balasubramaniam *et al*., 1997; Varela and Fiszman, 2011).

1.2 Film & coating formation

Edible films must have specific functional requirements, like mechanical and permeability properties. The properties of these films are dependent on the nature of the components they are made of and the process of formation and application. A plasticizer agent must often be added to the film forming solution to reduce film or coating brittleness.

Edible films and coatings can be formed by the following mechanisms (Gontard, 1992):

a*) Simple coacervation*: where a hydrocolloid dispersed in water is precipitated or undergoes a phase change after solvent evaporation (drying), after the addition of a hydrosoluble non-electrolyte in which the hydrocolloid is insoluble (e.g. ethanol), after pH adjustment of the addition of an electrolyte which induced salting out or cross-linking.

b) *Complex coacervation*: where two hydrocolloid solutions with opposite electron charges are mixed, thus causing interaction and precipitation of the polymer complex.

c) *Gelation or thermal coagulation*: where heating of the macromolecule, which leads to its denaturation, is followed by precipitation, or where cooling of a hydrocolloid dispersion provokes gelation.

1.3 Protein films

The main characteristics of protein-based films derived from the protein-protein interactions. Such are influenced by amino acid composition among those are formed ionic interactions, hydrogen

bonds, together with intramolecular S-S bonds (Gennadios and Weller, 1991).
Thus, protein-based edible films can form bonds at different positions and offer high potential for forming numerous linkages (Ou *et al*., 2005). Such linkages influence mechanical properties that are better than those exhibited by polysaccharide and lipid-based films (Cuq *et al*., 2002). In comparison with synthetic polymers, the protein films limit their application in food packaging due the poor water vapor resistance and lower mechanical strength. Many approaches exist to improve the barrier properties of edible protein films. For example, recurring to chemical, enzymatic, physical methods, or by adding hydrophobic components. Protein films are brittle and susceptible to cracking due to the strong cohesive energy density of the polymer. The addition of compatible plasticizers improves the extensibility and viscoelasticity of the films. According to Ressouany *et al*. (1998), sorbitol is a good plasticizer and significantly increased puncture resistance (Han, 2005).

The principal protein film-forming materials derived from animal sources are whey protein, collagen, gelatin, casein and egg white proteins. Protein film-forming materials derived from plant sources include corn zein, wheat gluten, soy protein, peanut protein, and cottonseed protein (Krochta, 2002). Depending on their origin, the protein fractions used are more or less complex mixtures of different macromolecules and are used directly for film production or as blends, for example, with polysaccharides (Patzsch *et al*., 2010).

1.3.1 Whey protein isolate

Whey protein isolate (WPI) comprises 20% of the milk protein and is the protein that remains soluble after casein has been precipitated at pH 4.6. It is composed by three main proteins: *β*-lactoglobulin (65%), *α*-lactalbumin (25%) and bovine serum albumin (8%). Other minor proteins present are lactoperoxidase, lactoferrin and immunoglobulins (Farrell *et al*., 2004).

β-Lactoglobulin (β-Lg) comprises approximately 57% of the protein in whey (Dybing and Smith, 1991). Its primary structure consists of 162 amino acids with a molecular mass of 18,300 Da. and a pI equal to

5.1. Crystallization analysis has shown that β-Lg exists in a globular form, with stabilizing hydrophobic and SH groups located in the interior. The thiol group is important because it appears to facilitate molecular thiol-disulfide interchange reactions that allow formation of intermolecular disulfide-bonded dimers and polymers upon heating (Kinsella, 1984). The functional properties of these groups can be exploited to form effective edible films. β-Lg undergoes time and temperature-dependent denaturation reactions at temperatures above 65°C, which result in a general molecular expansion, exposure of the internal -SH group, and hydrophobic and $-NH_2$ groups (Brunner, 1977; Kinsella, 1984).

α-Lactalbumin (α-La) is the second most abundant whey protein, accounting for about 19% of the total whey proteins (Dybing and Smith, 1991). It is a globular protein that contains 123 amino acid residues with a molecular mass of 14,000 Da. and pI of 4.1. Conformational changes occur in α-La at pH 4, where the molecule loses the Ca^{+2} that is tightly bound at higher pH. At pH values between 4 and 5, α-La appears to occur as two forms with different thermal stabilities. At pH 6.5, α-La begins unfolding at 62°C; but on cooling, the molecule reverts to its native configuration. This reversibility is lost if the native S-S bonds are broken, for example, by heat-induced thiol-disulfide interchange reactions between α-La and β-Lg (Pérez-Gago and Krochta, 2002).

Because native whey proteins maintain their globular structure with most of the hydrophobic and -SH groups buried in the interior of the molecule, native protein films have a more random structure in which cohesion relies mainly on hydrogen bonding. In contrast, the intermolecular forces that promote cohesion in heat-denatured films also involve intermolecular S-S bonds and hydrophobic interactions among the unfolded protein strands (Pérez-Gago and Krochta, 2002). Formation of whey protein films has mainly involved heat denaturation of whey proteins in aqueous solutions. Heating modifies the three-dimensional structure of the protein, exposing internal -SH and hydrophobic groups (Shimada and Cheftel, 1998), which promote intermolecular S-S bonding and hydrophobic interactions upon drying (McHugh and Krochta, 1994b). The unfolded structure of heat-denatured whey proteins and the covalent S-S bonding during drying lead to film insolubility in water and produce films that are

stronger and that can withstand higher deformations.

WPI is used in the food industry to increase protein content and to enhance flavour or texture. It has also been used as a film and coating former. WPI (containing >90% protein) is characterized by its capacity to create transparent films and coatings that exhibit good oxygen, carbon dioxide, aroma and lipid barrier properties. WPI films may be used as antimicrobial carriers, but they have the drawback of being brittle (Rossi-Márquez *et al*., 2009; Sothornvit and Krochta, 2000).

1.4 Polysaccharides

These natural polymers can prevent the product's deterioration, extending the shelf-life and maintaining the sensory quality and safety of several food products (Robertson, 1993). The main polysaccharide film-forming materials include pectins, chitosan, starch and starch derivatives, cellulose derivatives, alginate, carrageenan, and various gums.
Due to the hydrophilic nature of polysaccharides, polysaccharide-based films exhibit limited water vapor barrier ability (Gennadios *et al*., 1997). However, films based on polysaccharides such as like alginate, cellulose ethers, chitosan, carrageenan or pectins exhibit good gas-barrier properties (Baldwin *et* al., 1997; Ben and Kurth, 1995).

1.4.1 Pectin

Pectin is a cell wall polysaccharide. The majority of the pectin structure consists of homopolymeric partially methylated poly α-(1-4)-D-galacturonic acid residues (“smooth” regions), but there are substantial “hairy” regions of alternating α-(1-2)-L-rhamnosyl-α-(1-4)-D-galacturonosyl sections containing branch-points with mostly neutral side chains (1–20 residues) of mainly L-arabinose and D-galactose (rhamnogalacturonan I). The types and amounts of substructural entities in pectin preparations depend on their source and extraction methodology. Commercial pectin is mainly derived from citrus peels and apple pomace. The pectin molecule does not

adopt a straight conformation in solution, but is extended and curved with a large amount of flexibility.

The carboxylate groups tend to expand the structure of pectin. Methylation of these carboxylic acid groups forms their methyl esters, which are much more hydrophobic and have a different effect on the structure of surrounding water. Thus, the properties of pectin depend on the degree of esterification (D.E.). High D.E. pectin (>50% esterified) tends to gel through the formation of hydrogen-bonding and hydrophobic interactions at low solution pH (pH-3.0) to reduce electrostatic repulsions, or in the presence of sugars (>70% esterified). Low D.E. pectin (<50% esterified) gels by calcium divalent cations that bridge adjacent two-fold helical chains to form the so-called ‘‘egg-box’’ junction zone structures so long as a minimum of 14–20 residues can cooperate. In isolated form, pectin readily reassociates or aggregates to form networks, and it interacts with proteins and other polysaccharides via hydrogen bonding, ionic, or hydrophobic interactions (Liu and Kost, 2009). Although pectinate coatings have poor moisture barriers, they can retard water loss from food by acting as a sacrificial agent. Pectin can also form cross-links with proteins under certain conditions (Thakur *et al*., 1997). Autoclaving enhances pectin-protein interactions, resulting in a three dimensional network with improved mechanical and barrier properties (Lacroix and Le Tien, 2005).

1.5 Protein-Pectin complexes

Mixtures of biopolymers are often unstable. This condition can lead to a separation of the mixture into two phases, as illustrated in Figure 1. On mixing aqueous solutions of proteins and polysaccharides three different results may be reached:

- A liquid two phase system can be obtained in which the two macromolecular components are mainly distributed in different phases. This is due to the limited thermodynamic compatibility (also called “simple coacervation”) of protein and polysaccharides in aqueous media.

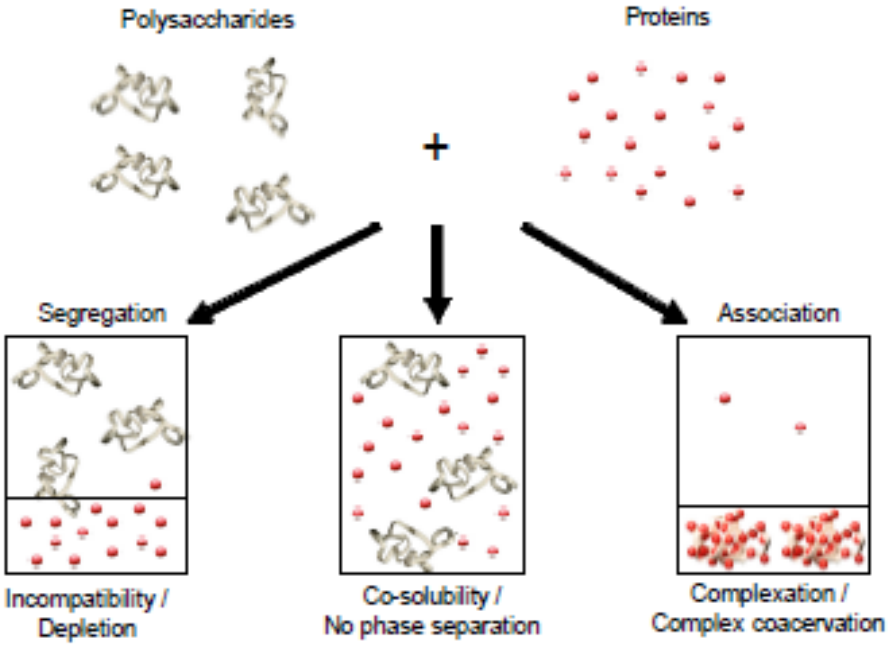


Figure 1. Protein/polysaccharide mixtures.

- A two-phase system may be obtained where both macromolecular components are largely in the same single concentrated phase. This phenomenon (complex coacervation) is attributed to the formation of an insoluble electrostatic protein-anionic polysaccharide complex.

- Homogeneous stable solutions can be obtained in which the two macromolecular components are either co-soluble or exist as soluble complexes (Tolstuguzov, 1991).

Under certain conditions, polysaccharides like pectin may form cross-links with proteins (Thakur *et al*., 1997). Heat treatments may enhance protein-polysaccharide interactions, resulting in a three-dimensional network with improved mechanical properties. The thermodynamic incompatibility of proteins and polysaccharides means that under certain conditions, any protein-polysaccharide-water system is spontaneously separated into two liquid phases. The conditions necessary for phase separation vary according to the biopolymers. Thermodynamic incompatibility appears to be a fundamental property of proteins and polysaccharides. This is comparable to the thermodynamic incompatibility of synthetic

polymers in a common solvent. Unlike synthetic polymers, however, proteins and polysaccharides are only incompatible under certain conditions which inhibit the formation of inter-biopolymer complexes. This mainly occurs at high ionic strengths and/or at pHs higher than the protein pI (Grinberg, & Tolstoguzov, 1997).

A convenient way to prepare covalent conjugates from mixtures of proteins and polysaccharides is through the Maillard reaction (non-enzymic browning) carried out under controlled dry-heating conditions (Dickinson and Galazka, 1991; Nagasawa *et al*., 1996; Akhtar and Dickinson, 2003; Wooster and Augustin, 2006; Akhtar and Dickinson, 2007). During this complex sequence of reactions, the terminal and side-chain amine groups on a protein molecule become linked to the reducing end of a polysaccharide. The conjugate protein–polysaccharide can correspond to one or two polysaccharide molecules (on average) becoming attached to each protein molecule. Because of the large number of potentially reactive groups on each protein molecule, these Maillard-type conjugates are necessarily somewhat polydisperse in size and composition (Dickinson, 2008).

1.6 Complexation and complex coacervation

Depending on the charge density of the biopolymers, complexation may be driven predominantly by the enthalpy change or the entropy change. So, weakly charged biopolymers tend to associate through direct enthalpic electrostatic interactions, whereas the formation of aggregated complexes is entropically driven, as a consequence of the conformational changes of the biopolymers and the release of counterions and water molecules into bulk solution. Coacervation is a peculiar phenomenon, in which a macromolecular aqueous solution separates into two immiscible liquid phases. The denser phase concentrated in colloid components is generally called as the coacervate, which is in equilibrium with the relatively dilute colloid liquid phase. This liquid-liquid phase separation can be divided into either “simple” or “complex” coacervation. The former involves only one macromolecule and may result from the addition of a dehydrating agent that promotes polymer-polymer interactions over polymer-solvent interactions. In the latter case, complex coacervation is usually a physicochemical phenomenon caused by the

electrostatic interaction between two oppositely charged polyions, such as charged polysaccharides and proteins.

General principles of complex coacervation asserted are:

(a) There is mobility of both protein and polysaccharide in the liquid coacervate phase.

(b) If the polysaccharide is a strong polyelectrolyte, precipitation occurs instead of coacervation.

(c) Soluble complexes exist in solution prior to onset of macroscopic phase separation.

(d) At the mixing ratio where coacervation is maximized, the protein–polysaccharide complexes are electrically neutral.

(e) Even when one biopolymer component is in great excess, the complexes are only modestly charged.

(f) Addition of electrolyte has a dissociating effect on coacervate complexes.

(g) Temperature has a minor influence on the phase diagram (de Kruif *et al*., 2002).

Whereas the covalent conjugates are discrete macromolecular entities that maintain their integrity with changes in pH and ionic strength, this is clearly not the case for electrostatic complexes and coacervates. Nevertheless, under certain conditions where the protein polysaccharide interaction is weakly attractive, it can sometimes be difficult to distinguish between the (de)stabilization mechanisms involving complexation and those involving segregative (net repulsive) protein–polysaccharide interactions. This is especially the case of the dispersions or emulsions containing mixtures of milk proteins with weak polyelectrolytes like certain types of pectin around neutral pH conditions, i.e., at pH values similar to the complexation pH (pHc) (for a definition of pHc see below).

One beneficial consequence of complexation is the protection afforded against loss of solubility due to aggregation induced by heating or high-pressure processing (Ledward, 1979; Galazka *et al*., 1997; Galazka *et al*., 1999). This process can be attributed to stabilization of the native protein structure in the complexed state, and also to blocking of potentially hydrophobic binding sites on the partially unfolded globular protein (Dickinson, 2008). Complexing is inhibited at sufficiently high ionic strengths as well as at pHs above

the pI. The latter corresponds to conditions where both biopolymers bear negative net charges.

Soluble complexes between protein and polyelectrolyte are formed at a specific pH, called pHc, which varies with the ionic strength. In a number of cases it was found that at low ionic strength, soluble complexes also form at pH values for which the proteins and polyelectrolytes carry the same net charge. The phenomenon has been ascribed to the attraction between polyelectrolyte charges and oppositely charged “patches” on the protein surface (Dubin *et al.*, 1994; Wen *et al.*, 1997).

1.7 pH complexation

The overall interaction between two biopolymers is made up from an average over the large number of different intermolecular forces (Israelachvili, 1992). Between this intermolecular forces contributing to protein - polysaccharide interactions there are:

- Covalent: very strong linkages formed between specific reactive groups on different macromolecules. (Covalent bonding confers permanence to protein/polysaccharide complexes.).
- Van der Waals: universal weak attractive interactions of electromagnetic origin exhibited by groups with permanent or induced dipoles (acting between all groups to some extent, van der Waals forces rarely have a predominant influence on net protein-polysaccharide interactions).
- Hydrogen bonding: Hydrogen bonding becomes less important with increasing temperature.
- Hydrophobic: moderately strong short-range attractive interaction of the type long-range attractive interaction of entropic origin between non-polar groups separated by water. (Hydrophobic forces increase in strength with increasing temperature up to 60°C.)
- Electrostatic: interaction that may be either repulsive or attractive depending on whether the electrical charges are of the same or opposite sign, and either short-ranged (weak) or long-ranged (strong) depending on the degree of ionization and the background electrolyte concentration. (The overall polymer-polymer electrostatic interaction is very sensitive to variations in pH and ionic strength) (Dickinson, 2008).

pH complexation (pHc) can be defined as the value at which in a protein-polysaccharides system formation of soluble polymers occur (see Montecarlo's diagram Figure 2). The onset of complex formation is controlled by the interaction between a single protein molecule and a single sequence of polymer segments (Weinbreck *et al*., 2004). In a protein–polysaccharide system, generally, the polysaccharide carries negative charge, while the sign of the protein's charge varies with the pH of the solution. Soluble complexes are formed when the protein binds to the polysaccharide, forming a complex of which the charge has the same sign as the polysaccharide. The soluble complexes are stabilized by electrostatic repulsion (Ganzevles *et al*., 2006; Kaibara *et al*., 2000). On the contrary at value below the pHc polymers can be strongly associated causing the formation of aggregates that undergo to precipitation. At value of pH higher that pHc the system contains soluble polymers that are physically distinct from each other (Figure 2).

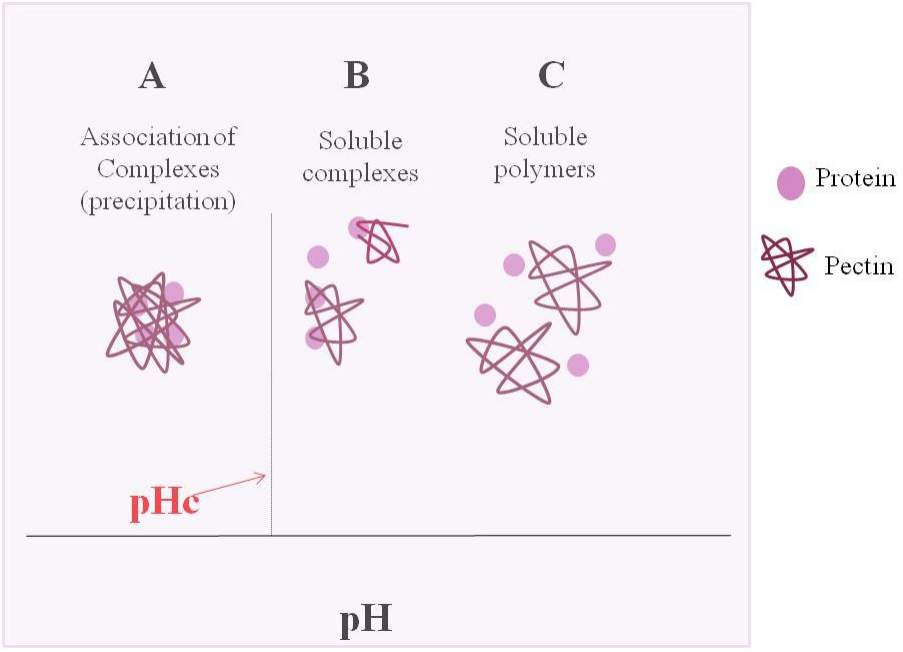


Figure 2.– Montecarlo's diagram (Weinbreck *et al*., 2004).

1.8 Antimicrobial films

Incorporation of antimicrobial agents in food packaging and coatings is one of the most studied methods to control microbial surface growth in food products. An edible film with preservatives can be used as an active packaging on the surface of edible food to improve the microbial stability (Ozdemir and Floros, 2003). This kind of edible films can improve the microbial stability of foods by acting as a barrier against the growth of microorganisms.

Among the molecules with antimicrobial activity, antimicrobial peptides (AMP) and proteins play a very important role. Such are defined peptides and proteins (García-Olmedo *et al*., 2001) involved in the mechanisms of innate immunity, being actively against the pathogen membranes. Antimicrobial peptides and proteins receive raising interest both because of the increase of antibiotic-resistant bacteria and because of the difficulty exhibited by bacteria to overcome AMPs attack. These proteins belong to the antimicrobial peptides class, that, in the last decade, have been isolated from a wide range of organisms (bacteria, plants, insects, amphibians and mammals) where they have an important role in the defense mechanisms (Capparelli *et al*., 2005). They derive from small propeptides, released in a mature form from specific proteases. Two main mechanisms of action have been proposed to explain the effect of the interaction between peptides and membrane: a) "detergent" effect, due to the permeabilization of the double lipid layer, determining the membrane destructuration and the leakage of cytoplasmic components; b) the ion channel or pore formation, due to the peptide monomers aggregation in the double lipid layer (Guerneve, 1998). Properties exhibited by AMPs make their use very promising. The biochemical characteristics of AMPs and their membrane toxic antimicrobial activity could be used either in medicine sector or in food sector.

In particular, AMPs could be sought as a new class of antimicrobial agents in order to obtain new natural antibiotics, thus overcoming the problem of antibiotic resistance. These antimicrobial peptides do not induce resistance and, therefore, represent a potential alternative to conventional antibiotics, in view of their rapid action and highly selective toxicity. The ability of natural AMPs to control bacterial

growth, together with the inability of bacteria to develop resistance against AMPs, make them good candidates for food preservation.

In this thesis a peptide derived from royal jellein protein was used. Such protein occurs in royal jelly produced by the cephalic gland of bees (*Apis mellifera)* Capparelli *et al*. (2009) have demonstrated that this peptide (among others royal jellein-derived), chemically synthesized, is efficient in contrasting Gram negative and Gram positive bacteria both alone and in synergy with temporins , another class of AMPS derived from *Rana temporaria*. Therefore such molecules are promising candidates for designing new antimicrobial agents that can be introduced into active edible films. In fact, in this way, these films could be used as defense tool against different types of microorganisms that negatively affect the food storage and shelf-life.

1.9 Transglutaminase

Cross-linking agents have been used to improve barrier properties and mechanical strength of different materials. Regarding hydrocolloid film containing proteins, these can be cross-linked recurring to chemical methods, i.e. by using glutaraldehyde. Since this agent is not food-grade, the use of enzymatic tool is highly preferred. Among cross-linking enzymes, transglutaminase (TGase) is the most studied.

The complete name of TGase is R-glutaminyl-peptide: amine (γ-glutamyltransferase (EC 2.3.2.13), thus indicating that the catalysis consists of the acyl transfer of γ -glutamyl residues, present in protein or peptide substrates (acyl donor), to an acyl acceptor substrate, resulting in a variety of different products depending on the involved molecules (Sarkar *et al*., 1957; Folk and Finlayson, 1977). The transamidation reaction occurs when the acyl acceptor is either the ε-amino group of an endoprotein lysine residue or a low molecular mass primary amine, thus, generating ε-(γ-glutamyl)lysine crosslinked linear or branched homo- and heteropolymers in the first case (Figure 3, panels A and B), or protein-amino derivatives in the latter (Figure 3, panel C). Since 1957, when Clarke *et al*. (1957) described a transamidating activity in guinea pig liver, research on

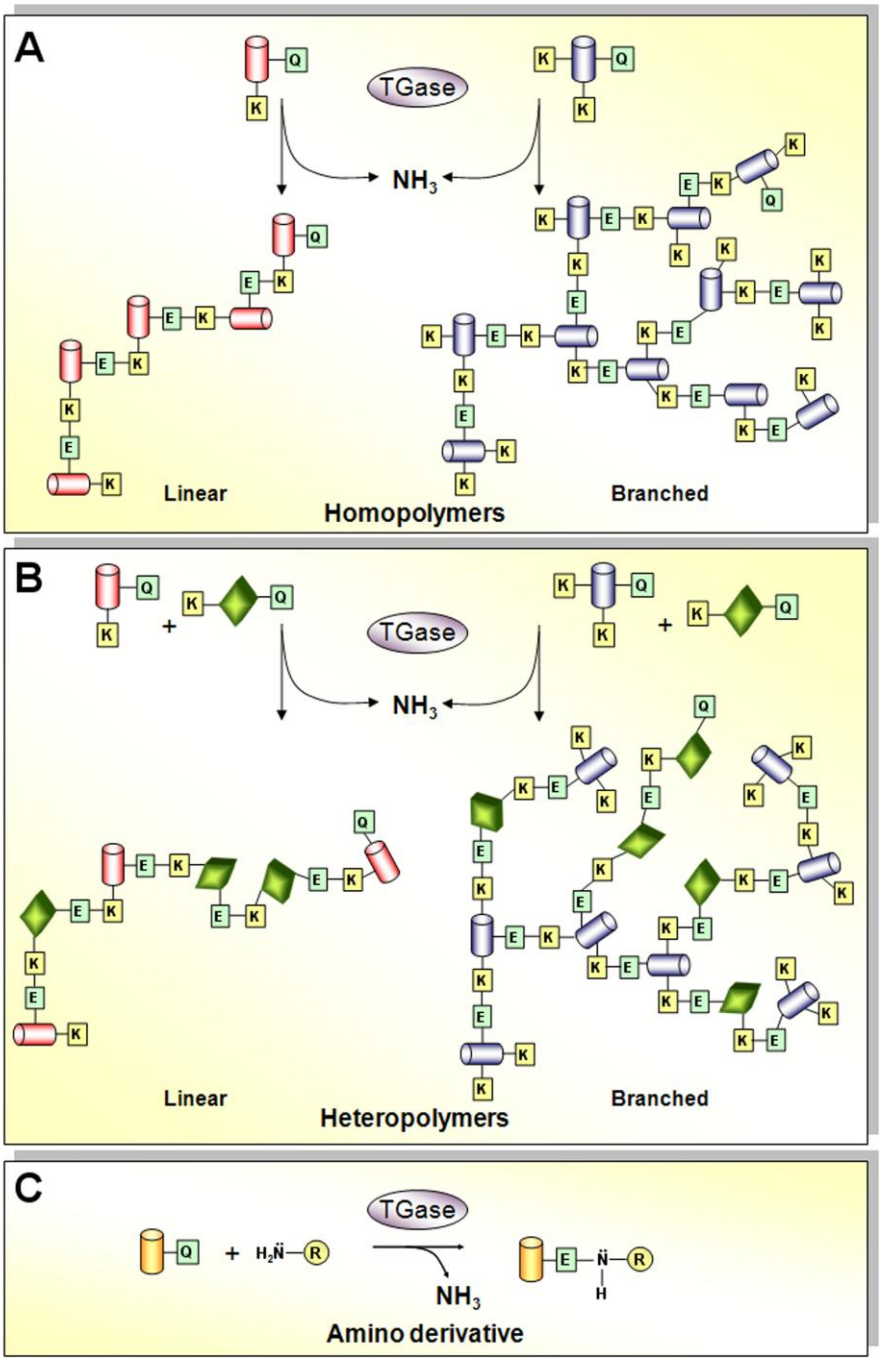


Figure 3. TGase catalyzed reactions.

this class of enzymes has been growing, from human health (biomedicine and cosmetics) to industrial applications in different fields (food, leather,and textile industries). Such a large range of interest is also related to the existence of different isoforms of TGase which are distributed in different organisms, such as bacteria, plants (Villalobos *et al*., 2004), invertebrates (Singh and Mehta, 1994), vertebrates including amphibians (Zhang and Masui, 1997), fish (Yasueda *et al*., 1994), and birds (Puszkin and Raghuraman, 1985). Blood plasma TGase (activated coagulation Factor XIII), nowadays used as a therapeutic agent, was the first isoform used to modify protein and peptide substrates (Nielsen, 1995). Also, the so-called "tissue" TGase (type 2), which has the ability to modify biological properties of some peptides and proteins has been well established (Cordella-Miele *et al*., 1990; Persico *et al*., 1992; Porta *et al*., 1993; Peluso *et al*., 1994; Esposito *et al*., 1995; 1999; Tufano *et al*., 1996; 1998; 2001; Filippelli *et al*., 1997) and which is used as a biotechnological tool in biomedicine, specifically for the diagnosis of an autoimmune pathology like the celiac disease (Dieterich *et al*., 1997).

The preliminary studies on the TGase-catalyzed modifications of proteins of food interest were carried out with the enzyme purified either from guinea pig liver or bovine blood plasma. However, the high costs of their production inhibited the development of technologies involving these TGase iso-forms to enhance texture and emulsion properties of protein based foods. Also, keratinocyte TGase, which needs a complex post-translational modification to be fully active (Chakravarty and Rice, 1989), was never suggested for biotechnological applications. In fact, for its implication in lamellar ichthyosis (Huber *et al*., 1995), this molecular form of the enzyme is only proposed as a possible target for gene therapy. Therefore the large majority of the different TGase isoforms are not attractive for potential biotechnological uses, since their production is expensive and they cannot be easily manipulated outside of their natural environment. In 1989 a microbial TGase (mTGase) was isolated from *Streptoverticillium* sp. and its characterization indicated that this isoform could be extremely useful as a biotechnological tool (Ando *et al*.,1989).

The protein structure of the bacterial enzyme is quite different from that of the mammalian enzymes. Moreover, mTGase has a smaller molecular mass (about 40 kDa despite 80–100 kDa of mammalian TGases) and a very low-sequence identity with other TGases (Micanovic *et al*., 1994). However, the hydrophobic environment of the catalytic site, including a single cysteine residue, was found to be similar to those of other isoforms, while no sequence identity with the calcium-binding domain was detected. In fact, mTGase possesses calcium-independent activity and, furthermore, exhibits a wide substrate specificity being also active over a wide range of temperature and pH values (Yokoyama *et al*., 2004).

Although TGases are known mostly for their role in the post-translational remodelling of proteins in vivo, the catalyzed reaction can be considered an important way to manipulate proteins of different origins in vitro. Therefore, because of the low-cost mass production by traditional fermentation technology and since mTGase has been “Generally Recognized As Safe” and its use is allowed as food additive, mTGase has been largely utilized in the last decade as a biotechnological tool, mostly in the food field (Zhu *et al*., 1995; Kuraishi *et al*., 2001). Thus, Ajinomoto Incorporation actually produces several preparations of mTGase that are commercialized with different names. They differ in stabilizer composition in relation to the type of food for the production of which they have been designed. In agreement with the European legislation (Directive 89/107/EC), Ajinomoto declared that mTGase could be considered as a processing aid and thus its presence does not need to be indicated in the finished products. As a consequence, mTGase is extensively used to improve texture, mechanical properties, and emulsifying characteristics of food proteins for their best utilization as ingredients of complex foods (Kuraishi *et al*., 2001).

Investigations in this field have been carried out by using proteins of different origins, like those extracted from both plants (Mariniello *et al*., 2003; 2007) and animals (Motoki and Seguro, 1998). Moreover, several studies have been also performed by using milk-derived proteins such as both caseins and whey proteins (Mahmoud and Savello, 1992; Yildirim and Hettiarachchy, 1998; Cozzolino *et al*., 2003; Di Pierro *et al*., 2010).

1.10 ζ-potential

It is a physical property which is exhibited by any particle in suspension. It can be used to optimize the formulations of suspensions and emulsions. Knowledge of the ζ- potential can reduce the time needed to produce trial formulations. It is also an aid in predicting long-term stability. Three of the fundamental states of matter are solids, liquids and gases. If one of these states is finely dispersed in another then we have a colloidal system.
The development of a net charge at the particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter ions, ions of opposite charge to that of the particle, close to the surface. Thus an electrical double layer exists round each particle. The liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where they are less firmly associated. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move it. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is the ζ–potential (Figure 4) (Tadros, 2009).

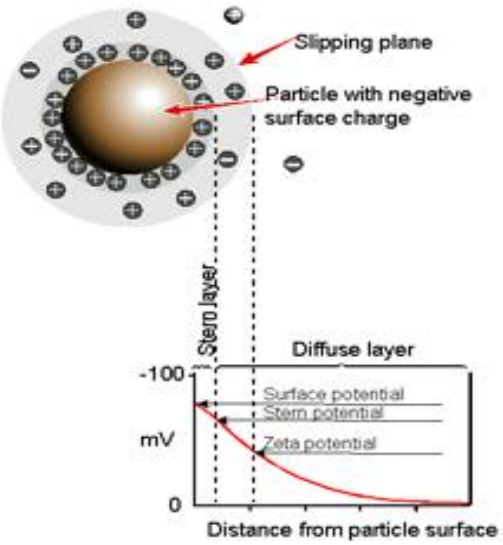


Figure 4. Schematic representation of ζ-potential

2. MATERIALS AND METHODS

2.1 Materials

Commercial Whey protein isolate (90-92% protein on a dry weight basis) was obtained by BioLine (New Zealand). ACTIVE WM (product no.AJ301402, lot no. 00.02.03) containing *Streptoverticillium* CA^{2+} independent TGase was obtained from Ajinomoto Co. (Japan). Sorbitol, pectin from *Citrus* fruits (6.7-9.4% methoxy groups on dry basis) and other reagents were obtained from Sigma (Steinheim, Germany). Royal jelly peptide with antimicrobial activity was kindley provided by Prof. Rosanna Capparelli (Romanelli *et al*., 2011). Products for biscuits and donuts preparation were purchased at the local market.

2.2 Complex formation

WPI/Pectin complexation was followed by potentiometric titration of protein acidic and basic groups (Mattison *et al*., 1998, Wen and Dubin, 1997). WPI and Pectin solutions were separately prepared by dissolving 1.2 g of WPI isolate, plus 0.6 g of sorbitol, and 1.2 g Pectin in 25 mL of distilled water, respectively, and by stirring the solutions for 2 hours at room temperature for a complete hydration of the macromolecules. Then, WPI solution was heated, under continuous stirring, in a bath water at 80°C for 25 min to denature the proteins. Different aliquots of pectin solution were brought to 25 mL with distilled water to obtain variously diluted polysaccharide solutions, heated to 80°C for 3 min and then were added slowly and under stirring to WPI solution at 80°C to obtain WPI/Pectin ratios of 2:1, 4:1, 6:1 and 8:1 (w/w), respectively, with a constant WPI concentration of 20 mg/mL.

Each obtained film forming solution was heated at 80°C for further 2 min and finally cooled under stirring at room temperature (Table 1). The potentiometric titrations were performed at 22°C. The initial pH was adjusted to 8.0 ± 0.05 with 0.01N NaOH and the solutions were titrated with 0.01 N HCl to reach pH 4.0 corresponding to a pH value lower than pectin pKa (4.5).

Table 1. Different WPI/Pectin ratio tested.

Ratio	Proteins (g/25mL)	Pectins (g/25 mL)
8:1	0.075	0.0092
6:1	0.075	0.0125
4:1	0.075	0.0187
2:1	0.075	0.0375

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 pHc was recorded as the pH value corresponding to the inflexion point of the titration curve. To evaluate the influence of both electrostatic interactions and hydrogen bonds on the pHc, we analyzed further film forming solutions containing either 110 mM sodium chloride or 110 mM urea.

2.3 TGase polymerization assay

To evaluate the feasibility of the enzyme to crosslink WPI, 100 μg of WPI were incubated with different amounts of TGase (0.5, 1, 2, 4 and 8U) in 100 μL of 1.5 mM TRIS-HCl pH 7.5 for 1 h at 37°C. At the end of the reaction 25 μL of Sample Buffer 5X were added, then the samples were heated at 100 °C for 5 min to stop the reaction. At the end of reaction 0.8 μg of proteins were analyzed by a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

To evaluate both the effects of heat treatment and reducing agent the assay was performed with or without heat treatment (80°C for 25 min) and with or without 10mM dithiothreitol (DDT) in the absence or presence of the enzyme (8 U/g of WPI).

Pectin effect on TGase crosslinking was evaluated as follows: unheated and heated proteins (80°C for 25 min) were prepared in the presence of pectin. For heated proteins, pectin were pre-heated to 80°C and mixed gently with proteins. The solution was incubated in the absence or presence of TGase (8 U/g of WPI) for 1 h at 37°C. In

both the assay, at the end of incubation time, the samples were analyzed by SDS-PAGE as decribed above.

2.4 Film forming solution preparation

Film forming solutions were prepared as describe in 2.2 and film casting, solubilizing 1.2 g of WPI, 0.6 g of sorbitol and 0.3 g of Pectin in 51 mL of water. The pH of the FFS prepared with a WPI/Pectin ratio of 4:1 was adjusted to the desired value and then TGase (8 U/gr of WPI) was added by stirring overnight a room temperature. Finally, the FFS were cast by pipetting 30 mL of each solution into Petri dishes (150 mm x 15 mm) and the films were obtained by drying at 45°C and 30% HR. Control was made without TGase.

2.5 Charge and size of particles

The electrical potential (ζ-potential) of proteins and complexes was measured using a Zetasizer Nano-ZS (Malvern Instrument Ltd., Worcestershire, UK) using 5 mL of a 4:1 WPI/Pectin solution buffered to the pHc value. Particle size was determinate using a Laser diffraction particle size analyzer LS 13 320 (Beckman Coulter, California, USA).

2.6 Film thickness

Film thickness was measured with a micrometer model HO62 (Metrocontrol Srl, Casoria (Na), Italy) at five random positions over the film area.

2.7 Film mechanical properties

Tensile strength (TS), Young's modulus (YM) and elongation to break (ETB) 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 razor blade, into 10-11 mm wide and 50 mm length strips equilibrated overnight at 50% ±

5% RH and 23 ± 2 °C in an environmental chamber. Ten samples of each film type were tested. Tensile properties were measured according to the ASTM (1991) Standard Method D882 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 a tension mode.

2.8 Water vapour permeability

Was evaluated by a gravimetric test according to ASTM E96 (1993) by means of a Fisher/Payne permeability cup (Carlo Erba, Italy). Three grams of silica gel were introduced in each cup. The film samples having diameter of about 6 cm were put on top of the cup and sealed by means of a top ring kept in place by three tight clamps. The film area exposed to vapor transmission was 10 cm^2.

The cups containing silica gel were weighed and then placed in a desiccator containing a saturated KCl solution which provided a constant water activity at 25 °C equal to 0.8434. The desiccator was stored in a Heareus thermostated incubator at 25.0 ± 0.1 °C. Cups were weighed at scheduled times, and the amount of water vapor 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 g 0.998. The water vapour permeability (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 Δp is the water vapor pressure across the film. By assuming that the vapor pressure inside the cup, due to the presence of silica gel, can be taken equal to zero, Δp becomes equal to the vapor pressure inside the desiccator and was calculated by multiplying water activity and the water tension (P_0) at 25 °C (P_0) 3167 kPa).

2.9 Oxygen permeability

Film permeability to oxygen was determined using a modified ASTM D 3985 (1981) with MultiPerm apparatus (Extrasolution s.r.l., Pisa, Italy). Duplicate samples of each film were conditioned for 2 days at 50% RH before measurement. Aluminum masks were used to reduce film test area to 5 cm^2, whereas the testing was performed at 25 °C under 50% RH.

2.10 Film antimicrobial properties

To evaluate antimicrobial properties of the film prepared in the presence or absence of TGase and to assess the effect of the single compounds (WPI, Pectin and Sorbitol), a statistical custom design 3X3X3 (Table 2) was set up using three different concentrations of the single compounds (0%, 50%, 100%, where 100 correspond to 100% FFS 4:1 ratio) .

Table 2. Statistical design.

Protein	Pectin	Sorbitol
50	0	100
50	50	0
100	0	50
0	50	50
0	100	0
50	100	50
100	50	100

To evaluate this property, a bacterial inhibition growth test was prepared using as a target microorganism *Salmonella enterica* serovar Paratyphi B 0404572. 150 μL of FFS prepared as describe before, at pHc and using 0%, 50% and 100 % of three different compounds in the absence or presence of TGase was casted onto ELISA plate (Microtest™ 96 well plate, Becton Dickinson, USA) and let dry overnight at 45°C and 30% RH.

Then 100 μL of Mueller-Hinton liquid agar was added to each well and let overnight at 25°C and 60%HR. Next, an aliquot of 100 μL of Muller-Hinton was taken from each well and centrifuged for 10 min at 10000 x g and casted onto multi-well plate. Finally a *S. enterica* aliquot (10^5 CFU/mL) was then included into the system and incubated overnight at 37°C. Bacterial growth was calculated reading the O.D. of each well at 655 nm (Microplate reader model 680, Biorad, Hercules, CA). Finally, the inhibition growth % was calculated as describe Capparelli *et al*., (2005). To study the ability of film to act as carrier of antimicrobial molecules, an antimicrobial peptide *Royal Jellein I modified* (0.75 μg) was added to FFS (150 μL) before the casting (MIC= 5μg/mL).

2.11 Dry-biscuits preparation

Ingredients used were wheat flour (1000 g.), olive oil (125 mL), salt (16 gr.), baking powder (5.3 gr.) and water. All ingredients were mixed and the cookie dough was left incubated at room temperature for 1 h. Then the dough was worked with a rolling pin to a final thickness of 1 cm. Cookie dough was cut with a circular cookie cutter (outside diameter 50 mm, inside diameter 15 mm), cooked in a boiling water bath and left cool at room temperature. For the coated biscuits, the coating was applied by dipping biscuits pieces in the FFS or the FFS+TGase. All biscuit pieces, coated and not, were dry at room temperature overnight. Next day, were baked for 19 min at 180°C in an electrical heated oven on a steel baking tray with baking paper. Biscuits were left cooled at room temperature and stored at 25°C and 50% RH for subsequent characterization.

2.11.1 Moisture content

Total moisture contents of the biscuits that were either uncoated or coated with FFS and FFS+TGase were determinate by AOAC standard method 950.46 (AOAC 1990). Biscuits samples were dried for 24 h in a 95°C oven and weight loss is reported as moisture. Moisture content was measured in triplicate.

2.11.2 Mechanical properties

Biscuits hardness was determined using a Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA) equipped with a 2kN load cell in compression mode with a cylindrical probe (55 mm diameter). Pre and post-test speeds were 2.0 mm/s, while test speed was 1.0 mm/s. Biscuits, prepared as above were centered and compressed 20%. Hardness was derived from the positive peak value obtained. Biscuits break strength was measured using the three point bending test. Pre-test, test and post-test were respectively 2.5mm/s, 2.0mm/s and 10mm/s. Test was performed for at least eight biscuits per batch.

2.12 Donuts preparation

Yeast was dissolved in warm milk for 10 min, then commercial wheat flour, sugar, egg and butter (Table 3) were added and mixed until dough was smooth. Dough was cover and let rise for 1 h. The dough was roll around lightly onto floured surface to approx 1 cm and cutted with a donut cutter (outside diameter 50mm, inside diameter 15 mm). Donut pieces were covered and let rise for another 30 min.

Table 3. Donuts preparation.

Ingredient	g
Wheat flour	500
Sugar	80
Egg	63
Butter	40
Yeast	25
Milk	24

2.12.1 Coating application

Donut pieces were submerged for 10 s into FFS solution or FFS enzimatically reticulated (FFS+TGase), then allowed to drip for 5 min before frying. Sunflower seed oil (Carrefour, Italy) was poured into an

electric fryer (GIRMI, Viterbo, Italy) with a capacity of 2.5 L. The oil was heated to the processing temperature (190°C). Donut pieces (with and without coating) were fried for 4 min and let drain on paper towels.

2.12.2 Fat Uptake and Moisture content

After cooling, the fat content and moisture content of samples was measured. While,moisture content was determined as described for Biscuits, fat content were determined using a Soxhlet equipment and hexane as solvent (AOAC 930.39, 1990). Analysis was performed in triplicates using the following calculations:

$$Fat\ reduction\ due\ to\ coating = \frac{(Fat\ non\ coated) - (Fat\ coated)}{Fat\ non\ coated}$$

$$Water\ increase\ due\ to\ coating = \frac{(Water\ coated) - (Water\ non\ coated)}{Water\ non\ coated}$$

$$Water\ increase\ due\ to\ coating = \frac{(Water\ coated) - (Water\ non\ coated)}{Water\ non\ coated}$$

$$Water\ increase\ due\ to\ coating = \frac{(Water\ coated) - (Water\ non\ coated)}{Water\ non\ coated}$$

$$Water\ loss\ during\ frying = \frac{(Initial\ water) - (Water\ after\ frying)}{Initial\ water}$$

$$Decrease\ in\ water\ loss\ due\ to\ coating = \frac{(Water\ loss\ non\ coated) - (Water\ loss\ coated)}{Water\ loss\ non\ coated}$$

$$Fat\ uptake = \frac{(Final\ fat\ content * mass\ after\ frying - initial\ fat\ content * mass\ before\ frying)}{Dry\ mass}$$

$$Reduction\ of\ fat\ uptake\ due\ to\ coating = \frac{(Fat\ uptake\ non\ coated) - (Fat\ uptake\ coated)}{Fat\ uptake\ non\ coated}$$

$$Index = \frac{Reduction\ of\ fat\ uptake}{Decrease\ of\ water\ loss}$$

2.13 Statistical Analysis

JMP software 8.0 (SAS Campus Drive, Building S, Cary, NC) 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

3.1 WPI/Pectin complexation and determination of pHc

To evaluate the pH in which WPI and Pectin are able to form soluble complex (pHc), we have titred the solutions containing only WPI and different ratio of WPI/Pectin (Figure 5). The titration curves obtained from pH 8.0 to pH 5.1 with ratios (w/w) of 8:1, 6:1 and 4:1 respectively are almost superimposed to the titration curve obtained with only WPI. When the pH is below 5.1 no differences were observed in the slope of the curves obtained with ratio of 8:1 and 6:1, while the curve obtained with the ratio 4:1 changes the slope.

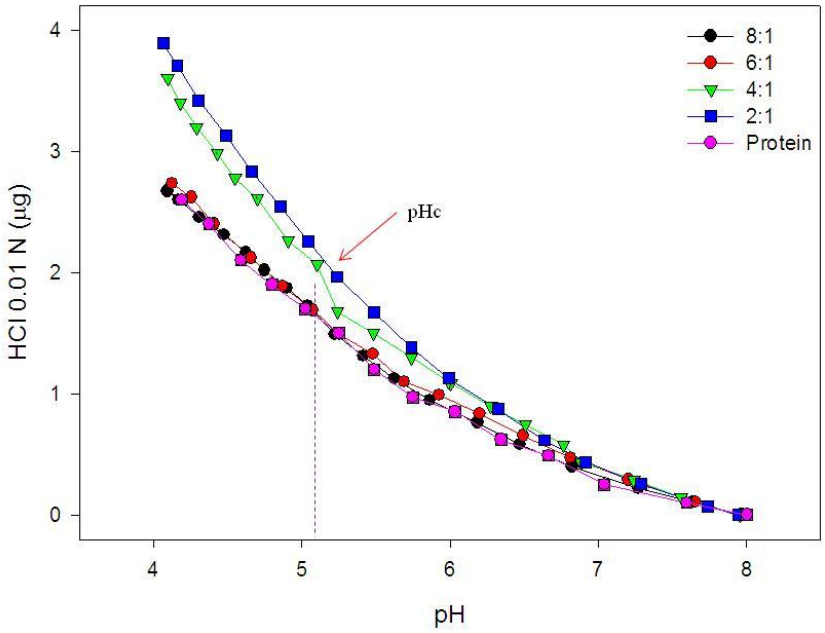


Figure 5. Titration curves of WPI/Pectin solutions at different ratios.

The projection on the abscissa of this inflexion point represents the pH value at which molecules carry equal and opposite charges, leading to optimal complexation and represent the pHc value (Schmitt, 2000). Moreover, because the curve corresponding to 4:1

ratio is the first curve that shifts from the curve of only WPI, it is possible to affirm that this ratio is the best condition in which there is the highest amount of protein molecules linked to polysaccharide molecules (Schmitt *et al.*, 1998, Girard *et al*., 2002).
In order to evaluate whether the nature of protein and pectin interactions involved in the WPI/Pectin complexes were based on electrostatic and/or hydrogen bonds, further titrations were carried out in either 110 mM sodium chloride or 110 mM urea by using 4:1 WPI/Pectin film forming solutions. The results reported in Figure 6 show that, the titration curve obtained with the film forming solution prepared in urea were similar to the control, indicating the absence of hydrophobic interactions.

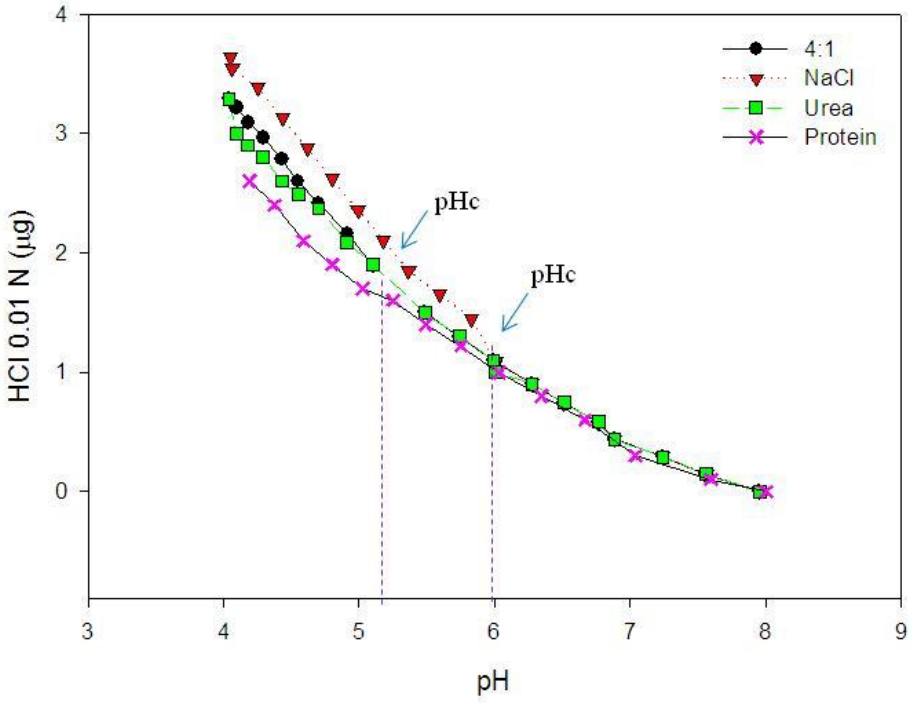


Figure 6. Titration curves obtained with WPI/Pectin.

The titration curve corresponding to the sodium chloride shows a markedly change in the slope of the curve, thus indicating a shift of pHc value that becomes 6.0. This result suggests the occurrence of electrostatic interactions that stabilize WPI/Pectin complexes

(Tolstoguzov, 1991). On the contrary, urea does not affect the curve slope, suggesting that hydrophobic interactions are not important in WPI/Pectin complexes (Girard *et al*., 2002).

3.2 Film casting

Once found the best WPI/Pectin ratio and the pHc, next step was to determine the minimum amounts of solids/cm^2 that allows to peel the films from the plates. Results obtained by casting different amount of film forming solutions and let dry overnight at 45°C and 30% RH give rise to a value of 23.8 mg/cm^2. Films were easy to be removed from the plates but they were brittle, therefore it was necessary to add a plasticizer to the film forming formulation. A plasticizer agent has the characteristic of reduce polymer intermolecular forces, increasing the mobility of the polymeric chains, and improving the mechanical characteristics of the film, such as the film extensibility (Krochta and Sothornvit, 2001). For this purpose, different amounts of sorbitol were tested until the best proportion was found. An amount of sorbitol equal to 50% in respect of protein concentration, allowed to obtain film less brittle, transparent, easy to removed from the plates and very flexible (Figure 7).

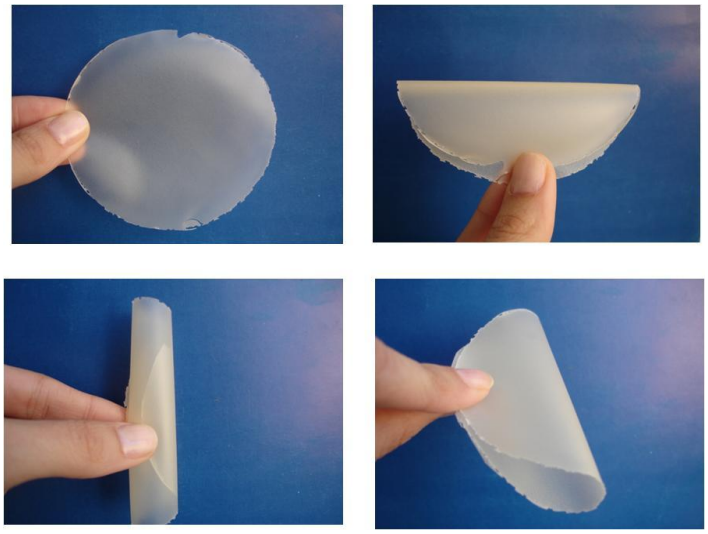


Figure 7. Films prepared at protein/pectin ratio 4:1 at pHc in the presence of 50% of sorbitol.

3.3 Polymerization assay

It is well known that whey proteins are not able to act as substrate of TGase when are in their native state (Oh *et al*., 2004). However, it has been demonstrated that heat treatment and/or the addition of reducing agent, such as dithiothreitol (DTT), leading to protein denaturation make the glutamine and lysine more exposed, increasing the susceptibility of the proteins to the enzyme modification (Hérnandez-Balada *et al*., 2009). Hence, to confirm the ability of WPI to act as a substrate of TGase, WPI preheated at 80°C for 20 min either in the absence or presence of DTT, were incubated with TGase for 1h at 37°C. At the end of incubation, the reaction products were analyzed by SDS-PAGE.

Results shown in Figure 8 confirm that WPI without heat or reducing agent treatment are not able to act as TGase substrate (Figure 8, lanes 1,2), whereas, in the presence of DTT it is possible to observe a reduction of band intensity corresponding to WPI (Figure 8, lanes 3,4), with the concomitant formation of high molecular weight polymers, some of them unable to enter the stacking gel. These results show that the reduction of disulfide bonds improves the reactivity of WPI to enzyme treatment.

The same results were obtained by preheating the proteins (Figure 8, lanes 5,6) while, with both heat treatment and DTT all the proteins present in WPI are completely crosslinked by the enzyme (Figure 8, lanes 7,8). Despite the best results obtained with both heat and DTT (DTT is consider as a non food grade agent), we decided to carry out subsequent experiments by using only the heat treatment.

Other polymerization assays were performed in order to evaluate the effect of FFS components (Sorbitol and Pectin) on the WPI polymerization. The results obtained with SDS-PAGE analysis of heated WPI, incubated in the presence of either pectin or sorbitol, do not show any difference on the ability of TGase to polymerize the WPI (data not shown).

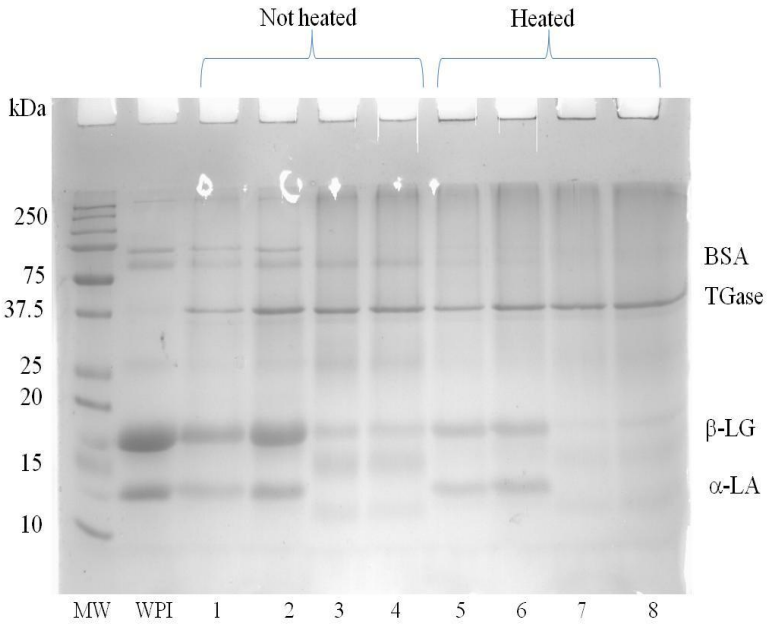


Figure 8. SDS-PAGE of WPI heated or not at 80°C for 20 min and incubated with TGase either in the presence or absence of DTT 10 mM. Samples were run in duplicate. Lanes with not heated WPI: 1-2 WPI+TGase, 3-4= WPI+TGase + DDT; Lanes with heated WPI: 5-6= WPI+TGase, 7-8 = WPI+TGase + DDT; WPI= native WPI; MW= molecular weight standard (Biorad).

3.4 ζ-potential measurements and particle size

The magnitude of ζ-potential gives an indication of the potential stability of the colloidal system. A value of < -30 mV or > 30 mV is the limit for stability of colloidal dispersion. If all particles in suspension have a large negative or positive ζ-potential, then they will tend to repel each other and there is no tendency to flocculate. We have tested the stability of both heat treated-WPI solutions and pectin solutions. WPI solutions (Figure 9, Panel A) show an instability from the beginning (-20mV) and this instability increases until it reaches the complete flocculation (5mV) after 3h, whereas, pectin solutions (Figure 9, Panel B) show a good stability from the beginning to 10 h (- 40 mV). Afterwards pectin solutions reach -30 mV conserving this value of ζ-potential for 20 h (Figure 9, Panel B).

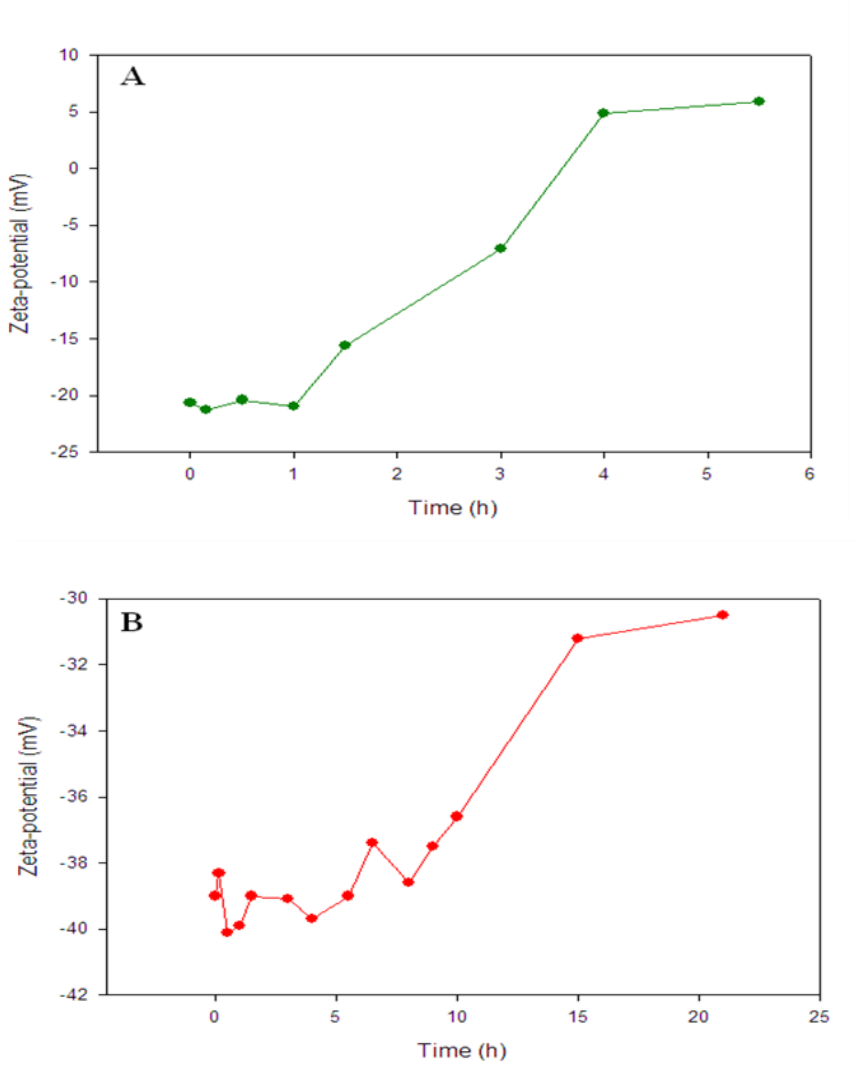


Figure 9. ζ– potential measurements. A) WPI + sorbitol solution. B) Pectin + sorbitol solution.

When WPI and Pectin were mixed to ratio 4:1 and at pHc in the absence or presence of TGase (Figure 10), the obtained complexes have a charge between -30 mV and -40 mV which gives a good stability to colloidal system in the following 8 h.

After this time, the complexes obtained in the absence of TGase reach the value of -30 mV in 2 h, becoming thus instable. It is worth of note that complexes obtained with the aim of TGase became instable in a longer time, reaching the value of -30 mV after 6 h.

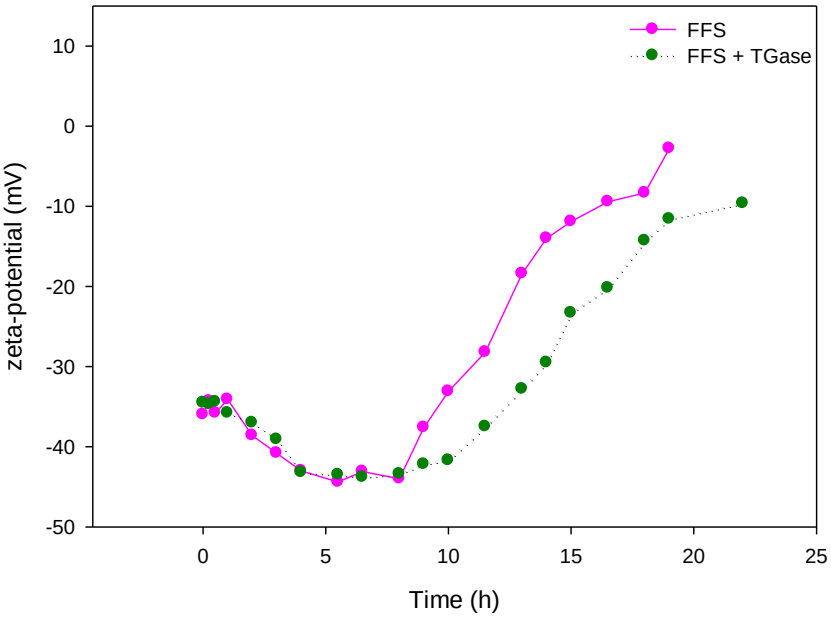


Figure 10. ζ –potential measurements of FFS and TGase-treated FFS.

The light scattering measurements (Table 4) indicated that particle size in the WPI solution was higher than that shown by FFS and FFS + TGase complexes at pHc.

Table 4. Particle size of sample solutions.

Solution	Particle size (μm)
WPI	39.05 ± 1.02
FFS	5.99 ± 0.86
FFS + TGase	7.91 ± 0.93

Values are mean ± standard deviation (SD) of five replicates.

3.5 pH effect on film characteristics

To investigate whether the pH at which FFS are used influence TGase-mediated cross-linking, some experiments were conducted at pH 6.0, thus different from the identified pHc, in the absence and in the presence of the enzyme. Before casting, FFS samples were analyzed by SDS-PAGE in order to evaluate the effect of pH on the enzyme activity. As it is possible to note in Figure 11, no effect was found on WPI crosslinking (Figure 11 lane 3 and 4). This results were not surprising since it is well known that microbial TGase is active in a wide pH range (Ando *et al*. 1989)

To verify how pHc, (the value of pH that allows the formation of soluble WPI/Pectin complexes) really affect mechanical and barrier properties, some films were cast at pH 6.0 , a value at which WPI and Pectin do not have any interactions, but only soluble polymers occur (see Montecarlo's diagram in Figure 2). The film forming solutions prepared at pH 5.1 and pH 6.0 were cast into Petri dishes and let to dry at 45°C and 30% HR. Films prepared at pH 5.1 were transparent and flexible, whereas films at pH 6.0 were not homogeneous and showed an not smooth (rough) surface (Fig. 12).

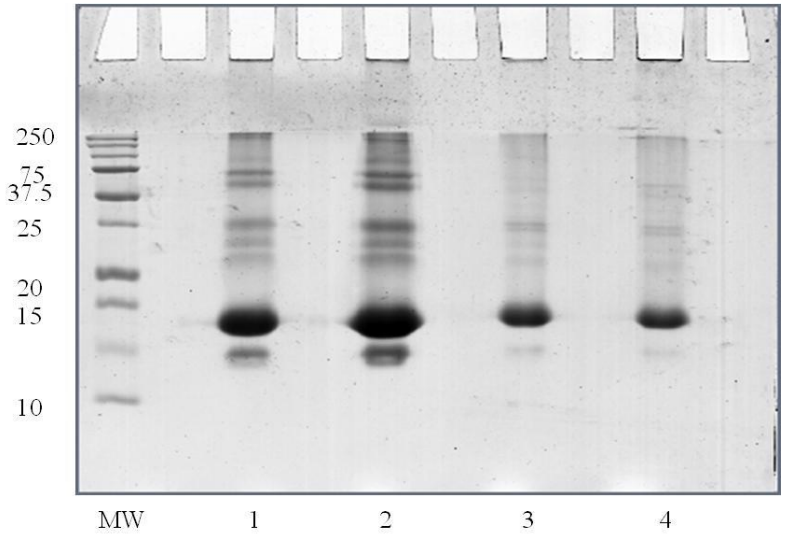
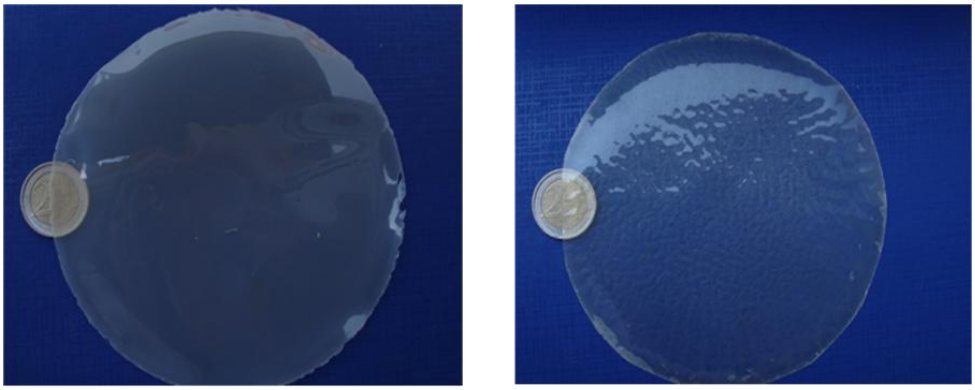


Figure 11. SDS-PAGE patterns of native and TGase treated WPI.Lanes: MW= molecular weight standard (in kDa);1= FFS at pHc; 2= FFS at pH 6.0; 3= FFS at pHc+ TGase; 4= FFS at pH 6.0+TGase



pH 5.1 pH 6.0

Figure 12. Films prepared at different pH.

Regarding film thickness, results shown in Table 5 demonstrate that pH does not affect this film characteristic. On the contrary, films prepared with TGase were thicker, probably due to the presence of covalent bonds that separates the molecules present in the film and, thus, there is an increment on the free volume inside the film matrix.

Table 5. Film Thickness

Film	Thickness (μm)
pHc	152 ± 12
pHc + TGase	216 ± 20
pH 6.0	151 ± 18
pH 6.0 + TGase	214 ± 16

Values are mean ± standard deviation (SD) of five replicates.

The results reported in the panel A of Figure 13 indicate that the films obtained by using WPI/Pectin film forming solution prepared at pHc in the presence of TGase show a tensile strength significantly higher than that of the corresponding films obtained with solutions made at the same pH in the absence of the enzyme, the graph shows that

TGase-made films at pHc are more resistant than films obtained at pH 6.0.

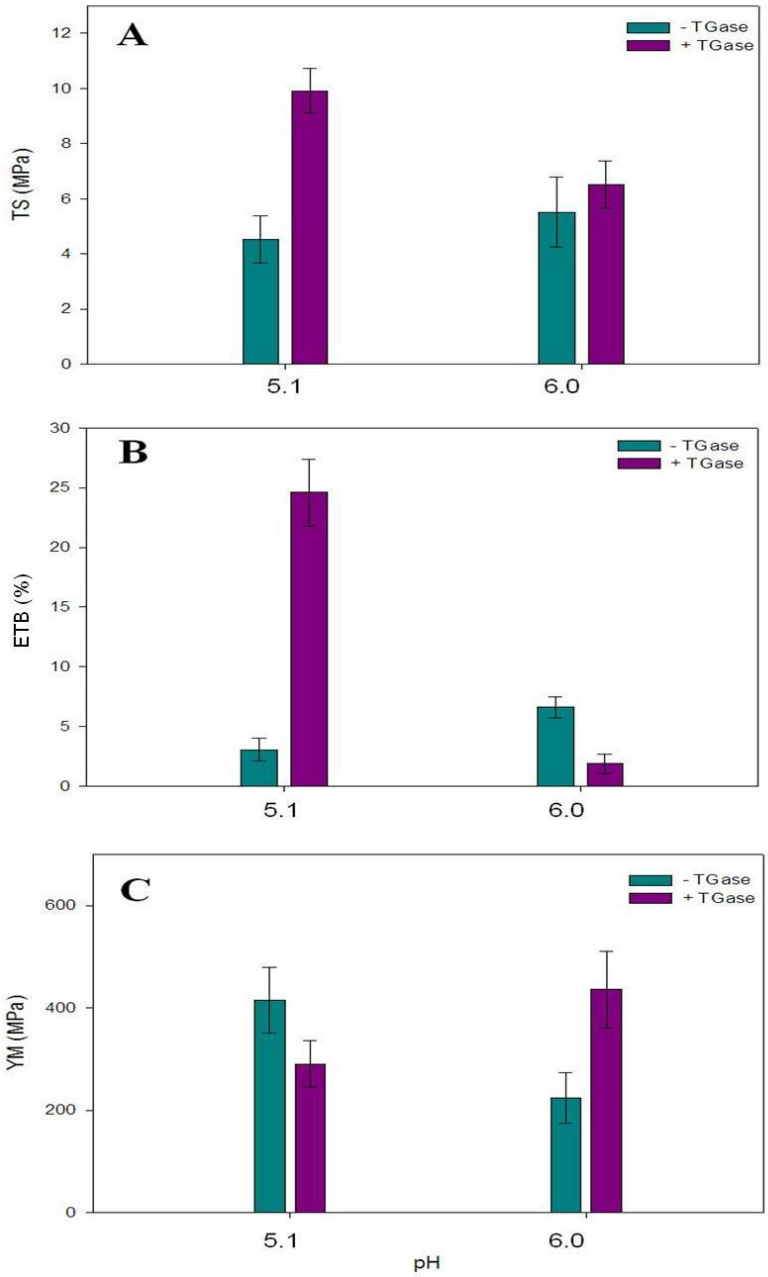


Figure 13. Film mechanical properties. A: Tensile Strength (TS), B: Elongation to break (ETB), C: Young's Modulus (YM).

Panels B and C of Figure 13 show, respectively, the measurements of film Elongation to break, related to the capacity of the different materials to extend, and the Young's modulus that indicates the film stiffness. From the panel B of the Figure 13 it is possible to conclude that, when TGase was absent in the film forming solution, the films prepared at pH 6.0 were two-fold more extensible than those prepared at pH 5.1. Conversely, when the films were prepared in the presence of the enzyme at pH 6.0, a significant reduction of their elasticity, due to the formation of covalent crosslinks between the single soluble WPI molecules, was observed.

Finally, panel C of Fig. 13 shows that the TGase-containing film prepared at pHc exhibited a reduced value of Young's modulus, index of a more flexible material, with respect to the film made in the presence of the enzyme at pH 6.0.

To evaluate the possible influence of TGase on the film barrier properties, we investigated both water vapor and oxygen permeability of the films obtained at pHc in the presence or absence of the enzyme. It is well known that several polymer characteristics concur to characterize the barrier capability of a material to water vapor and different gases (Han, & Scanlon, 2005). Among the various factors, the polymer chemical nature and the specific processing conditions are recognized, however, as the most relevant ones (Jasse *et al*., 1994).

Previous studies, in fact, have shown that an increase in the crystallinity, density, orientation, molecular weight, and also crosslinking, of the different materials tested are generally responsible for a decreased film permeability (Han and Scanlon, 2005; Salame, 2009).

The results of our experiments, reported in Table 6, clearly show that the production of TGase-catalyzed crosslinks among WPI/Pectin supermolecular soluble complexes, obtained at pHc, significantly reduced film permeability to both water vapor and oxygen, this effect being probably due to the marked decrease of interstitial spaces inside the WPI/Pectin soluble complex network (Miller and Krochta, 1997).

Table 6. Film permeability to water vapor (WVP) and Oxygen (P_{OX}).

Films	WVP	P_{Ox}
	(cm^3 mm m^{-2} day^{-1} kPa^{-1})	
pHc	15.38 ± 0.19[a]	0.025 ± 0.006[a]
pHc + TGase	9.90 ± 0.45[b]	0.016 ± 0.002[b]
pH 6.0	15.82 ± 1.53[a]	0.018 ± 0.002[c]
pH 6.0 + TGase	12.95 ± 0.77[b]	----

Values are mean ± standard deviation (SD). Mean followed by the same letters are not significant (Tukey-Kramer test α= 5%).

3.6 FFS antimicrobial properties

Since FFS are made of three different components, a statistical custom design (JMP Version 8.0 software (SAS Institute, Cary, NC, USA)) was planned. In Figure 14 a “dex mean plot” is reported to identify which FFS component has a major effect as antimicrobial agent (NIST/SEMATECH, 2010). Thus O.D. values were plotted versus three different levels of WPI, Pectin and sorbitol preparing FFS at pHc in the absence and in the presence of the enzyme. In particular for each component the following percentages were tested: 0, 50 and 100. In the absence of the enzyme (Figure 14, Panel A) it is possible to observe that sorbitol exhibits the major antibacterial property. In fact when FFS contains 50% sorbitol an O.D. value of around 0.1 is reached, while if sorbitol is absent from the FFS such value arises to 0.3 O.D. In the same manner, when sorbitol is used at 100% in the statistical custom design experiment, the microbial growth is even more inhibited. In the presence of TGase (Panel B of Figure 14) we observed that any sample, despite of each component presence, has a lower effect on growth inhibition since O.D. values are much higher (from 0.5 to 0.7), probably because the presence of TGase-mediated cross-links entrap sorbitol that can not exert its antimicrobial property.

Furthermore, in this case, the more significant component is represented by the proteins (Figure 14). These results suggest also

that the films obtained in the absence of enzyme showed antimicrobial properties and this properties is reduced by the TGase.

To evaluate the ability of films to act as carrier of molecules with antimicrobial properties we added before the casting a peptide named *Royal Jellein I modified* (RJ) (Romanelli *et al.,* 2011) and tested the % of inhibition growth (Capparelli *et al*., 2005; Romanelli *et al*. 2009) (Figure15).The results show that in the absence of TGase the antimicrobial activity of the FFS *per se* is enhanced when RJ peptide is present, confirming a bacteriostatic property of such peptide (Romanelli *et al*., 2009). In the presence of the enzyme the inhibition capacity of the RJ peptide on microbial growth is confirmed, reaching an inhibition percentage of around 80%, much higher than the control which is constituted by the FFS in the presence of TGase and in the absence of the peptide.

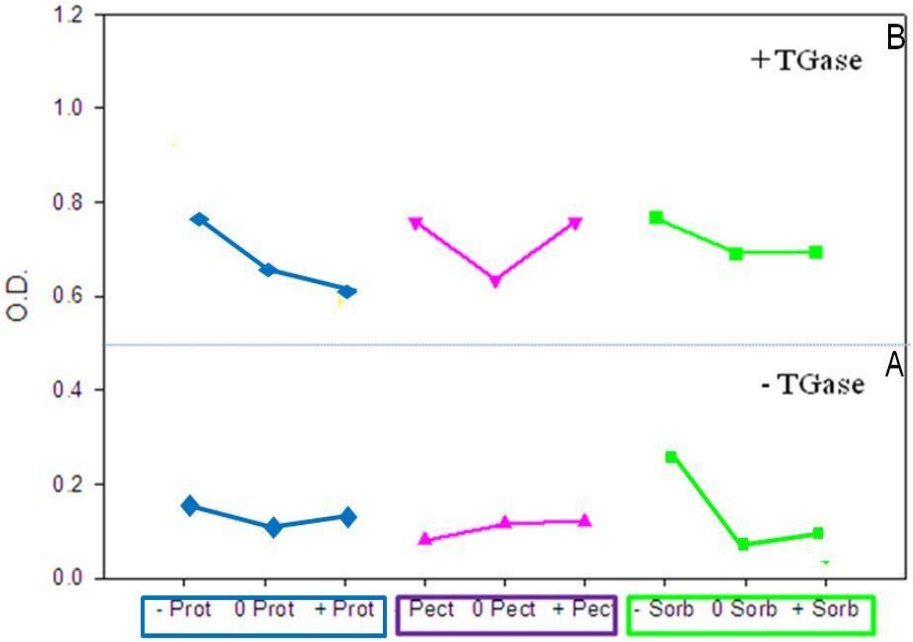


Figure 14. Dex mean plot. -, 0, + indicates 0, 50 and 100% of each component respectively.

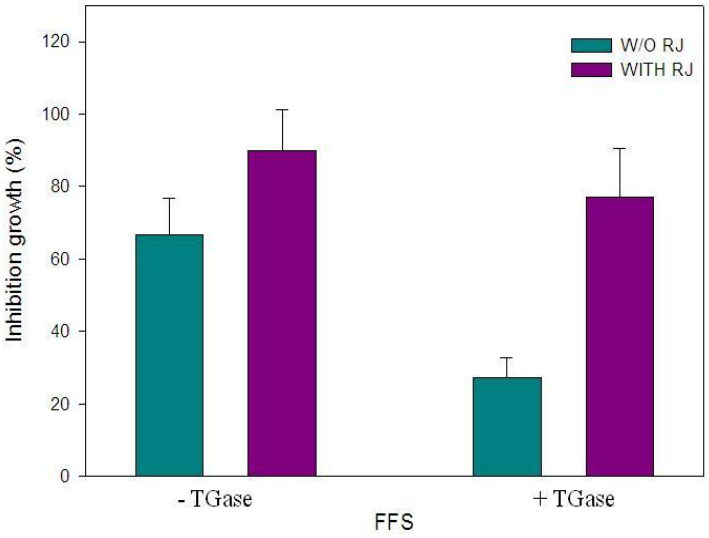


Figure 15. Effect of FFS on microbial growth inhibition when containing or not RJ peptide.

3.7 Food applications

3.7.1 Donuts

Results showed that fried samples coated with FFS improved the donut characteristics respect of fat uptake and water loss (Table 7).

Table 7. Some properties of coated and non-coated donuts.

Sample	Fat reduction due to coating	Water increased due to coating	Water loss during frying	Decrease in water loss due to coating	Reduction of fat uptake
Blank(non coated)	------	------	0.416 ± 0.008^{a}	------	0.269 ± 0.005^{b}
H_2O	0.080 ± 0.009^{b}	0.281 ± 0.005^{a}	0.317 ± 0.017^{b}	0.283 ± 0.007^{a}	0.269 ± 0.005^{b}
FFS	0.422 ± 0.014^{a}	0.218 ± 0.016^{b}	0.4129 ± 0.010^{a}	0.039 ± 0.0009^{b}	0.623 ± 0.005^{a}
FFS + TGase	0.417 ± 0.007^{a}	0.223 ± 0.006^{b}	0.422 ± 0.014^{a}	0.020 ± 0.001^{c}	0.615 ± 0.006^{a}

Different letters mean significant difference at $P < 0.05$ level (Tukey-Kramer test).

Tukey-Kramer test show that there was a significant difference in the reduction of fat uptake and decrease in water loss during frying between the coated and uncoated samples, and, thus, the application of the FFS coating has a significant effect in reducing the food fat uptake (Figure 16). A coating has to be designed to minimized water loss, thus preventing oil from entering.

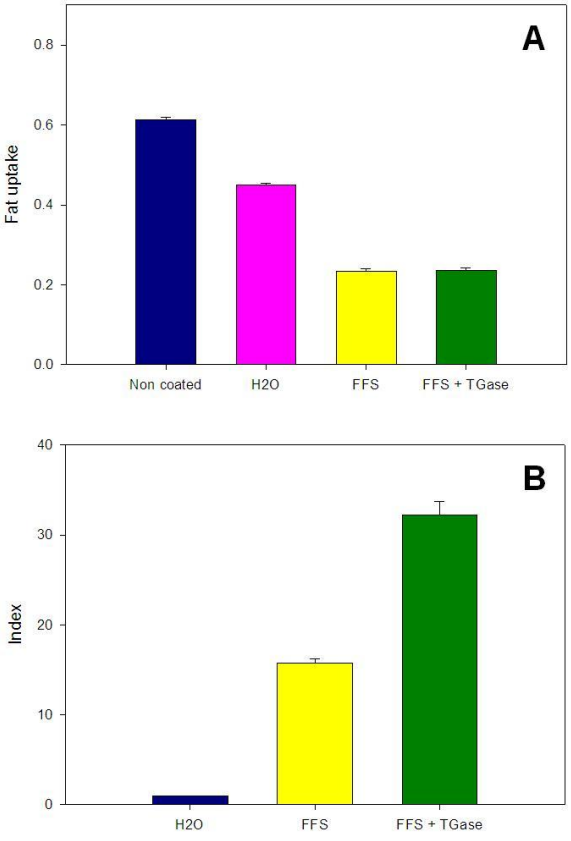


Figure 16. Fat-uptake and Index in fried donuts with and without coating.

The crust derived from the frying may act as a diffusion barrier that limits mass transfer, but inner moisture converted to steam may find selective channels in the structure and escape through open capillaries, pores, and crevasses and oil may enter the voids left by water.

Therefore, hydrocolloid molecules can be added to these coatings as water binders to minimize the water loss and, in certain cases, as physical fat barriers (Varela *et al*., 2011).

3.8 Dry Biscuits

The efficiency of WPI/Pectin based edible coating in controlling moisture transfer in moisture-sensitive products was evaluated by coating dry biscuits. Texture is a primary sensory attribute for these products, and loss of the desired texture resulter in shorter shelf-life. The hardness curve for coated and untreated biscuits is shown in Figure 17A.

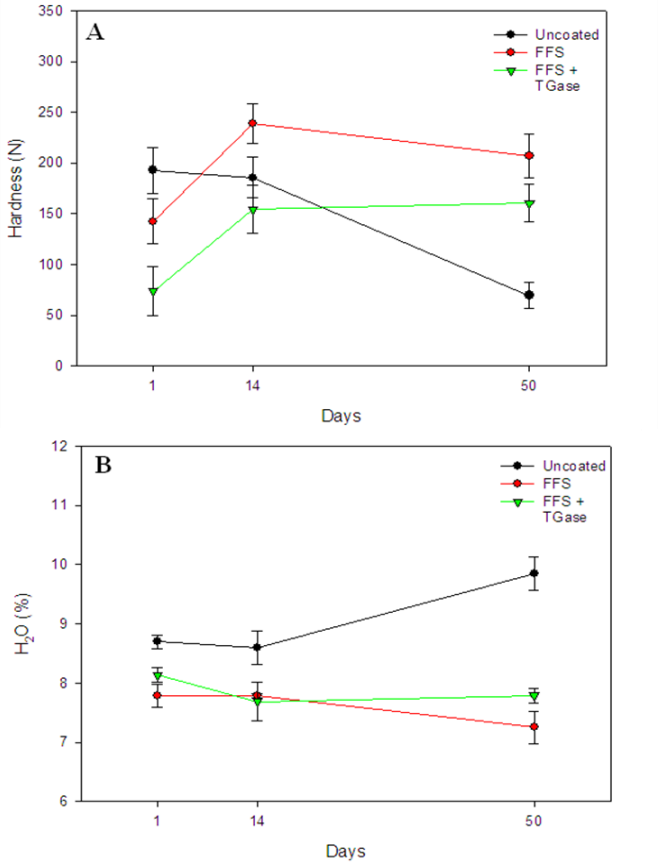


Figure 17. Hardness and water content curves for biscuits over time.

Relative humidity is the main factor that affects edible coating performance and the loss of crispness. The water content curve (Figure 17B) shows a higher uptake for the uncoated biscuits. These results are consistent with Bravin *et al*. (2006), who studied a polysaccharide-lipid edible coating in dry bakery products and concluded that the coating has the capacity to delay water vapor transfer from the environment and the dry bakery product.

In fact, also springiness and chewiness was affected by the coating presence (Table 8). Springiness can be defined as the degree to which the sample returns to its original shape after the first compression. Chewiness is the number of chews necessary to chew a sample to a state ready for swallowing, smaller is the measure crispier are the biscuits. The results shows an increase of springiness in both uncoated and coated with FFS after 50 days while no difference was found in coated with FFS + TGase. Regarding chewiness, the presence of coating both FFS and FFS+TGase decrease this property over time.

Table 8. Some mechanical properties of Biscuits.

Properties	Days	Uncoated	Coated with FFS	Coated with FFS+TGase
Springiness (mm)	1	1.13 ± 0.26	1.23 ± 0.40	1.07 ± 0.26
	14	1.68 ± 0.10	1.10 ± 0.15	1.17 ± 0.31
	50	1.48 ± 0.19	1.58 ± 0.11	1.27 ± 0.30
Chewiness (N*mm)	1	38.07 ± 9.97	68.14 ± 22.86	68.11 ± 28.13
	14	59.19 ± 9.18	53.54 ± 15.89	50.30 ± 11.11
	50	62.88 ± 17.22	45.81 ± 13.38	43.05 ± 17.72

4. DISCUSSION

Hydrocolloid-based films show several advantages compared to petroleum -derived ones in terms of biodegradability and digestibility. However, they have the disadvantage of being less stable. This is true also for composite hydrocolloids films made of different components, i.e. proteins and polysaccharides such are pectin. In this case is useful to find optimal conditions to let components to be associated to each other for giving more stable structures. We have studied WPI and pectin from *Citrus* as hydrocolloid components of our edible films obtained both in the absence and in the presence of the enzyme TGase, that it has been demonstrated an useful tool to reticulate the protein component of edible films (Porta *et al*., 2011). As first approach in this study we have carried out experiments to find the best conditions to promote a complex formation between WPI and pectin.

It is known that the number of protein molecules available per pectin chains is important for the electrostatic complex formation (Girard *et al*., 2002). For this reason, for a given WPI/Pectin ratio there is a specific pH at which electroneutrality of the complexes is reached (Schmitt, 2000). This pH corresponds to the pH at which macromolecules carry equal and opposite charges, leading to optimal complexation (Bédie *et al*., 2008). As shown in Figure 5, the WPI/Pectin ratio had no effect on the initial pH of soluble complexes formation. The titration starts around pH 8 where both the pectin and the WPI are negatively charged and complexation begins with one protein molecule binding to one chain of pectin. For ratios where one of the biopolymers is in excess, soluble complexes are obtained because of the presence of non-neutralized charges. Girard *et al*. (2002) proposed two models to describe the binding between proteins and anionic polysaccharides as a function of pH. The first model arises at pH< pI of the protein. The binding is non-specific and is due to opposite net charges of the two biopolymers. The second occurs at pH>pI of the protein and it is between chain segments of anionic polysaccharides and the positively charged region on the polypeptide chains of protein.

Our experiments devoted to estabilish pHc have demonstrated that this is equal to 5.1, a value that is consistent with pI of proteins occurring in WPI and pectin used that possess in average a pKa near to 4.5. Titration experiments carried out either in the presence of NaCl or urea have demonstrated the nature of the interactions that occur between proteins and pectin.

FFS were prepared at pHc in the presence of TGase to reticulate the proteins that act as substrates when are heated or reduced by DTT. As is it well known, native whey proteins are globular proteins containing most of the hydrophobic and SH groups hidden in the inner part of the molecule. Both heat treatment and DDT modify the three-dimensional structure of the protein, exposing internal glutamine and lysine residues, potential substrate for the enzyme TGase. Incubation of WPI solutions with TGase after heat treatment or under reducing conditions resulted, as expected, in bands at the top of the stacking gel (Figure 8) showing a formation of high molecular weight polymers.

Thus, experiments were conducted preparing FFS using preheated WPI in the ratio 4.1 with pectin at pHc in the presence and in the absence of TGase. To study stability of such complexes ζ-potential measurements were conducted. The results obtained have shown that TGase is able to produce FFS more stable than FFS obtained in the absence of the enzyme (Figure 10). This event is even more significative considering the fact that WPI show a zeta-potential higher that pectin (Figure 9). The importance of TGase-mediated crosslinking is also demonstrated by light scattering experiments that have assessed the particle size in the different systems. WPI particles show a size eight-fold greater than the FFS sample. This result is consistent with the fact that, in the absence of pectin, WPI that have been undergone to heat treatment, form protein-protein aggregates that posses a discrete size. In the presence of pectin, WPI are distributed along the structure of such polysaccharides, thus giving arise to particles with smaller size (Table 4).

The size of particles occurring in TGase-treated FFS increases from 5 to 7 probably because of the presence of isopeptide bonds catalyzed by the enzyme. In fact these bonds can fix a precise distance among the residues involved and thus increasing the

diameter of particles. This result is consistent with the fact that TGase-treatment also affects the thickness of the films prepared in the presence of the enzyme (Table 5). In the same manner, the presence of TGase-mediated crosslinks influences the mechanical and permeability properties of the films.

The mechanical properties of materials are largely associated with distribution and density of intermolecular and intramolecular interactions in the network that produces the protein films. According to Chambi *et al*. (2006) and Cuq (2002), these interactions depend on the arrangement and orientation of the polymer chains. As shown in Figure 13, there was an increased in TS value of films enzimatically reticulated at pHc than those obtained at the same pH without the enzyme, as well as at pH 6.0 both in the absence and presence of TGase.

It is worthy to note that a significant increase of the TS was observed only at pHc, by using films made in the presence of TGase, while no significant difference was observed in the film containing the enzyme but made at pH 6.0. These findings could be explained by considering the possible formation of a supermolecular structural network during the film casting (Dubin *et al*., 1994; Wen and Dubin, 1997). In fact, our data suggest that TGase-catalyzed covalent bonds among soluble ionic complexes of WPI/Pectin, produced at pH 5.1, may be responsible for the observed increase of the film resistance with respect to the one exhibited by films prepared at pH 6.0, in which the enzyme should be able to catalyze the formation of covalent bonds only among not complexed WPI molecules. This observation is in agreement with similar findings reported by Mariniello *et al*. (2003) and Di Pierro *et al*. (2006), who studied pectin/soy flour films and chitosan/WPI films reticulated by TGase respectively. In such studies, TGase-treated films showed an improvement in TS. In particular, Di Pierro *et al*. (2006) attributed this result to that TGase catalyzed formation of intermolecular bonds among glutamine and lysine residues of the different whey proteins.

The introduction of TGase-catalyzed covalent bonds in the WPI/Pectin supermolecular structural network occurring at pH 5.1 surprisingly increased the ETB of the obtained films about six-fold in comparison with films obtained in the absence of the enzyme at the

same pH, and more than ten-fold in comparison with the ones prepared with the enzyme at pH 6.0. These results are in agreement with Oh *et al.* (2004), who studied dairy protein/zein films cross-linked with TGase, and determined that the presence of the enzyme increased the elongation of the film. Finally, the observed high elasticity and low YM lead to define the films deriving from TGase-crosslinked WPI/Pectin complexes as typical elastomers (Ashby and Jones, 2005).

As far as the film barrier properties, the WVP and oxygen permeability value of films decreased as a function of the TGase treatment, when compared with the same films without it. A decrease in free volume within the film matrix induced by the covalent cross-links created by TGase reduces the water diffusion within the film matrix. As a consequence, the network became denser and less permeable. Concerning these barrier properties of the films, it should be considered that this value is supposed to be dependent on the number of available polar groups that the polymer components possess (Di Pierro *et al*., 2006). Chambi and Grosso (2006) reported a contrary tendency for gelatin/casein cross-linked films attributed to the increased in mobility of the chains. However, Carvalho and Grosso (2004) observed similar tendency for gelatin based films cross-linked with TGase. According to the authors, the new conformation acquired due to the action of TGase favored the barrier property.

In order to find a useful application of our hydrocolloid films, we have evaluated the influence of such films on water absorption and fat uptake of some bakery products, such as donuts and biscuits.

Fried foods contained significant amounts of fat, reaching in some cases 1/3 of the total food product (Mellema, 2003). Despite this, fried food remains popular although excess fat consumption is considered as the key dietary contributor to high blood cholesterol, high blood pressure and heart disease. The aim of the frying process is to seal the food surface by immersing it in the hot oil, retaining flavors and juices inside, however, it involves heat and mass transfer, causing oil transfer into the product and water transfer from the product to the oil. In deep-fat-frying, water in the crust evaporates and some water migrates from the core to the crust. Since this water

leaves voids that allow the fat to enter, moisture loss and fat uptake are closely related (Varela and Fiszman, 2011).

Donuts coated with FFS in the presence or absence of TGase showed a decreased in the fat reduction value due to coating compared to the blank and the water coating. This effect is correlated to the water loss during frying. However, there was a significant reduction on the decreased in water loss due to coating on donuts coated with FFS, and this effect was higher on coating where TGase was present.

However, we have found no difference on reduction of fat uptake among FFS coatings. The Index, that represents a relationship between the reduction of fat uptake and the decreased in water loss, was higher for TGase treated coating. Therefore, higher the Index better the coating properties of FFS. Since the coating should reduce the fat uptake but maintain the water loss during frying, this Index is a better measure for the films suitability. Several authors (Albert *et al*. 2002; Freitas *et al*., 2009) studied different edible coatings on fried products, establishing that protein coatings had the best barrier properties reducing the fat-uptake and the water loss in the product.

Several authors demonstrated that sensorial perception of texture attributes can be predicted using an instrumental test, as the dynanometer, featuring a compression cycle (Meullenet, 1998; Meullenet and Gross, 1999; Mochizuki, 2001). The efficiency of WPI/Pectin based coating in controlling moisture transfer was evaluated by coating biscuits. Coated biscuits showed a hardness reduction over time. The drop is associated with a loss of crispness due to hydration, which induces glass transition in polymer amorphous regions, water plasticization transfers the material from the glassy state (crisp texture) to the rubbery state, resulting in sogginess (Roudaut *et al*., 2002). The loss in this texture attribute is the major cause of dry food rejection by consumers. Biscuits usually have below 7-8% of moisture content (Manley, 1998). This may lead to absorption of water from the atmosphere following prolonged exposure to ambient conditions, making the biscuit soft and soggy, thus, coating can be an alternative to proctect food against environmental conditions. Non-coated biscuits absorbed more water, while coating created a film on biscuit surfaces and reduces the

amount of water absorbed. For biscuits covered by TGase-coating, water absorption was reduced but it also remained constant over time. As the water absorption in noncoated biscuits increased as expected, hardeness values dropped down. This is due to the fact that water acts as a plasticizer reducing biscuits hardness. Regarding coated biscuits there was an increased on hardness values and, also, in this case, the presence of TGase-coating keeps the value constant over time. This behavior was also observed by Mandala *et al*. (2006), who studied the textural parameters of commercial biscuits using puncture test. These authors demonstrated that the increment of relative humidity created a decrease on the puncture forces values.

Regarding springiness, there was a decrease for TGase-coated biscuits, while for chewiness test, the presence of coating lead to a reduction of this parameter. Therefore, the presence of such coatings created a barrier against water absorption, the crispness and hardness characteristics being maintaned. Infact, when a compression was applied (to mimic the chewiness), biscuits tended to break in pieces, reducing the amount of chewies necessary to swallow them.

An other feature that was analyzed regards the use of hydrocolloid films as active packaging in order to use them in future to improve the food shel-life. An edible film can be used as a vehicle for the incorporation of food additives such antimicrobial agents, delivering them to the food surfaces where deterioration by microbial growth often begins. Due to the acceptance of “natural foods”, incorporations of biopreservatives, as bacteriocins or antimicrobial peptides, into ebible films is an alternative to preserve food. As shown in Figure 14, sorbitol, used as plasticizer, shows an important effect on the decrease of O.D.. Zactiti and Kieckbusch (2009), mentioned that the film composition, as concentration and presence of components can influence the migrations of antimicrobial agents. In our case, sorbitol exerts this effect since, as a polyalcohol shows a bacteriostatic/bactericidal activity. For films prepared with TGase there was a film components effect, creating an increase on the O.D.. This effect could be attributed to the presence of TGase catalyzed covalent bonds, that in some way trap each single component in the film network.

Regarding the use of film as an antimicrobial agent carrier,we have observed an inhibition growth effect of films without TGase. On the other hand, for TGase-mediated films, there is a discrete inhibition, but the presence of RJ enhance this effect, indicating that altough the presence of covalent bonds could trap film components, the RJ has the capacity to migrate into film network and to be released to food surface.

5. CONCLUSIONS

Our study strongly confirms that TGase is a very useful tool to produce composite bioplastics from renewable biomass sources with improved both mechanical and barrier characteristics. In particular, we demonstrated the crucial importance of the pH value of the film forming solution which is able to dramatically influence the supermolecular structure of soluble P/P complexes able to act as enzyme substrates. The described composite film, constituted by TGase-crosslinked WP/LM-Pec soluble complexes, may represent a new possible candidate to substitute coating non-edible material for both food and pharmaceutical applications.

In fact, our results demonstrated that FFS coating created on the surface of dry biscuits decrease the amount of water absorption, maintaining the characteristic biscuits hardness and crispness over time. Whereas the presence of coating on donuts decrease the water loss during frying process preventing the fat uptake and, thus, create a barrier that reduces oil absorption. These two coating effects where improved by the presence of TGase.

Films components showed an antimicrobial activity against *S. enterica*, although this activity could be reduced by the presence of enzymatic reticulation that block the components within the film matrix. Experiments carried out with antimicrobial peptide *RJ* in films demonstrated that the presence of covalent bonds created by TGase do not influence the peptide ability to migrate within the film and can be release onto food surface protecting against microbial surface.

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ABBREVIATIONS

α-lactoalbumin	α-La
Antimicrobial peptides	AMP
β-lactoglobulin	β-Lg
Degree of esterification	D.E.
Dithiothreitol	DTT
Elongation to break	ETB
Film forming solution	FFS
Isoelectric point	pI
Royal Jellein I mod	RJ
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Tensile strength	TS
Transglutaminase	TGase
Water vapour permeability	WVP
Whey protein isolate	WPI
Young's modulus	YM

APPENDIX

Montana Food Research Award 2010. Special Mention. “L’enzima transglutaminasi come strumento biotecnologico per la preparazione di film edibili da utilizzare nel packaging attivo”. Giosafatto C.V.L., Sorrentino A., **Rossi Marquez G**., Esposito M.

Giornate Scientifiche 2010. Universitá degli Studi di Napoli “Federico II”. Oral presentation. “Proprietà barriera e meccaniche di film edibili a base di Whey protein isolate e pectine preparati in presenza di transglutaminasi”. **Rossi-Márquez G**., Di Pierro P., Fenderico M., Porta R. ISBN 978-88-95028-66-8

Under review. Di Pierro, P., **Rossi Marquez, G**., Mariniello, L., Sorrentino, A., Porta, R. 2011. “Effect of transglutaminase on the mechanical and barrier properties of whey protein/pectin films prepared at complexation pH (pHc)”. Journal of Biotechnology.

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***EFFoST* Annual Meeting 2011. Berlin**

Microfluidic focusing flow technology for micro droplets generation of protein/pectin complexes

Cristian Davalos Saucedo, Prospero Di Pierro, Paolo Masi, Giovanna Rossi Marquez, Fabrizio Sarghini

Highly structured protein/polysaccharide complexes may display better functionality than proteins and polysaccharides alone (Ye, 2008). The manipulation of protein/polysaccharide interactions (Dickinson, 2008) can represent an important tool to modify the microstructure of the composite systems to produce edible films. The use of edible films to produce microcapsules (encapsulation of aroma compounds, vitamins, and additives) confers to them the status of active films. There is a growing demand in the food, personal care, agricultural, and pharmaceutical industries for new encapsulation techniques with defined mechanisms for the release of active ingredients. Microencapsulation defined as “the technology of packaging solid, liquid and gaseous materials in small capsules that release their contents at controlled rates over prolonged periods of time”. We have recently obtained and characterized a whey protein and pectin (P/P) soluble complex with dimensions of 5-7 μm diameter which shown good properties to be used as a material for active films production. The characteristics of adsorbed complexes, the structures of mixed biopolymer interfaces, are still poorly understood. A proper understanding and control of the P/P interactions should help in designing emulsified films by microdroplet. Microfluidic represents a promising tool for generation of micro droplets. Monodispersed microdroplets generation of protein/pectins aggregates introduces additional challenges, due to the molecular structure of the complex subjected to aggregation in presence of an adverse pressure gradient or unbalanced electrical charges. As a consequence, direct extrusion in coaxial borosilicate capillaries resulted to be inappropriate, due to protein structuring. In this work a monodispersed droplets generation of P/P in oil was obtained by using an opposing focusing flows in a coaxial capillaries device (see Fig. 1a) and varying the ratio between P/P inner flow rate and oil and consequently controlling the droplets radius. A typical experimental dripping regime is shown in Fig. 1b. Effect of relative ratio between

P/P-OIL was investigated, and results are presented for different dripping regimes.

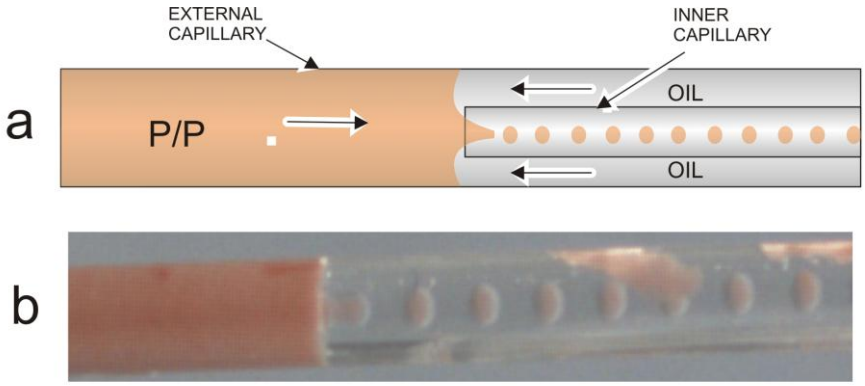

Fig.1. Focusing flow device configuration (a) and experimental dripping regime (b)

Effect of transglutaminase on the mechanical and barrier properties of whey protein/pectin films prepared at complexation pH (pHc)

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Abstract

The behavior of pectin and thermally denatured whey proteins at different both protein/polysaccharide ratios and pH values was preliminarly investigated to determine the best experimental conditions for obtaining composite films. Our findings suggest the formation at pH 5.1 (pHc) of transglutaminase-catalyzed crosslinks among soluble ionic whey protein/pectin complexes, which could be responsible for the observed increase of tensile strength of films obtained in the presence of enzyme, whereas the films prepared at higher pH (6.0) showed a minor resistance probably because the enzyme produced only homo- and/or heteropolymers among free whey protein molecules. Furthermore, the introduction of transglutaminase-catalyzed crosslinks in the whey protein/pectin supermolecular network at pHc also increased the elongation to break of the obtained films in comparison both with films prepared in the absence of the enzyme at the same pH and with the ones prepared with the enzyme at pH 6.0. Conversely, a significant reduction of elasticity, probably due to the formation of covalent bonds among single whey protein molecules, was observed when

the films were prepared in the presence of the enzyme at pH 6.0. Transglutaminase-containing films prepared at pHc also showed an increased stiffness with respect to the films made in the presence of the enzyme at higher pH. Finally, we found that the presence of the enzyme at pHc significantly reduced film permeability to both water vapor and oxygen, this effect being probably due to the marked decrease of the interstitial spaces among the whey protein/pectin soluble complexes following their crosslinking by transglutaminase.

Keywords: *transglutaminase, edible films, bioplastics, pHc, pectin, whey proteins.*

Introduction

Protein/polysaccharide (P/P) complexes widely occur in nature as well as in a large variety of industrial products (Ye, 2008). The study of P/P interactions has, hence, relevance not only for issues concerning biological systems like the living cell organization, but also to improve the drug vehicle or food processing by edible films or coatings (Doublier et al., 2000; De Kruif and Tuinier, 2001) .

Attractive interactions between positively charged proteins and anionic polysaccharides can lead to gelation (MacDougall et al., 2001), coacervation (Turgeon et al., 2003), or multilayer formation (Decher, 1997) and, as a consequence, the overall stability and texture of colloidal systems depend not only on the functional properties of the individual ingredients, but also on the nature and strength of P/P interactions. In fact, highly structured P/P complexes may display better functionality - as hydration, interfacial and adsorption properties - than proteins and polysaccharides alone (Ye, 2008). Therefore, the manipulation of P/P interactions (Dickinson, 2008) can represent an important tool to modify the microstructure and the shelf-life of the composite systems in the edible films, since

the formation of a continuous network strictly depends on the biopolymer behaviour in the film-forming solution. For these reasons the understanding of stability and phase behaviour of the latter solution is crucial for optimizing film performance.

During the titration of a polyanion/protein mixture from high pH, as the charge on the protein is reduced there is a transition - experimentally probed by different techniques (Li et al., 1996; Wen and Dubin, 1997; Hattori et al., 2001) - at a specific pH value, called complexation pH (pHc), where a soluble complex is formed (Weinbreck and de Kruif, 2003). Then, the complex may be further stabilized through other intermolecular forces like hydrophobic ones (Hallberg and Dubin, 1998) and/or hydrogen bonds (Girard et al., 2002). The formation of primary soluble complexes is usually a reversible process, generally occurring at low ionic strength and at a pH value corresponding to the pHc. It is worthy to note that the pHc may shift to lower values with an ionic strength increase that is able to shield the P/P attractive interactions (Weinbreck et al., 2003).

As part of our current research to improve the features of P/P composite edible films (Porta et al., 2011), we examined the interactions between pectin, a strong anionic polysaccharide, and the

globular proteins contained in whey protein (WP) isolate. Pectin is a polyelectrolyte generally associated with the cell wall and the intercellular regions of plants and fruits, and is widely used as a gelling and stabilizing agent in foods (Schols and Voragen, 2002). It is said to be high-methylated when the percentage of the esterified hydroxyl groups is higher than 50%, whereas it is considered low-methylated when the degree of its esterification is less than 50%. Recently, WP based edible films have been extensively investigated for food packaging and coatings (Regalado-González et al., 2006). WP isolate contains about 75% of β-lactoglobulin (β-Lgb) and 15% of α-lactalbumin (α-Lab). The quantitatively major protein occurring in the whey, β-Lgb, is a compact globular protein with molecular mass of 18.3 kDa (Sawyer, 2003) and is extensively used in food industry because of its binding (Kontopidis et al., 2004), emulsifying (Leman et al., 2005), foaming (Bals and Kulozik, 2003), and gelling properties (Kerstens et al., 2005). Although β-Lgb and α-Lab have quite different pI (5.2 and 4.1, respectively), the electrostatic interactions between WP and pectin are dominated by β-Lgb properties since β-Lgb is present in a much larger quantity. As a consequence, P/P complexation must take place at a pH value below 5.2, where pectin

and β-Lgb carry opposite charges. As far as the hydrogen bonds are concerned, it has been reported that they play a significant role, especially in the high-methylated pectin containing complexes, only after the resulting electrostatic interactions (Girard et al., 2002).

Numerous studies have previously characterized different complexes formed by pectin and β-Lgb, demonstrating that it is possible to produce biopolymer systems with different properties by controlling the pH of the mixture solution (Girard et al., 2002; Wang and Qvist, 2000; Girard et al., 2003a; Girard et al., 2004; Ganzevles et al., 2006; Ganzevles et al., 2007; Bedié et al., 2008; Jones et al., 2009; Kováčová et al., 2009; Sperber et al., 2009). When pectin and β-Lgb are mixed at neutral pH and the pH is reduced, the formation of a soluble complex is first observed, then a coacervate is produced and, finally, a precipitate is formed as the strength and number of electrostatic bonds are increased (Jones et al., 2009). In fact, during the formation of a P/P electrostatic complex the overall net charge of the anionic polysaccharide progressively decreases with the gradual attachment of each successive cationic protein. Diminishing of the net opposite charges on the macromolecular reactants reduces both the hydrophilicity and, as a consequence, the solubility of the

resultant complex. Therefore, P/P ratio in the mixture strongly affects both the charge balance of the complex and the behaviour of the latter. Maximum complexation is obtained with a specific P/P ratio at a given condition of pH and ionic strength. Complex formation, thus, is guided not only by the characteristics of the polymers (i.e. chemical nature, charge density, molar mass, concentration and ratio) but also by environmental conditions (Bedié et al., 2008; Ye, 2008). In addition, P/P complex may be influenced by some intrinsic factors such as protein aggregation and crosslinking (Bedié et al., 2008).

In the present study, we prepared hydrocolloid composite films with different ratios of anionic pectin and amphoteric WP, crosslinked or not by the enzyme transglutaminase (TGase) (Sperber et al., 2009; Aeschlimann and Paulsson, 1994; Mariniello and Porta, 2005; Mariniello et al., 2008), by forming electrostatic P/P complexes at the pHc. Our experiments demonstrated that the tested parameters have considerable influence on WP/pectin complex formation and, as a consequence, on the mechanical and barrier properties of the obtained edible films.

Materials and Method

Materials

Commercial WP isolate was obtained by BioLine (New Zealand**).** Low methoxyl pectin (LM-Pec) from *Citrus* fruits, sorbitol and all other reagents were purchased from Sigma (Steinheim, Germany). Microbial TGase (Activa WM; product no. AJ301402, lot no. 00.02.03), derived from the culture of *Streptoverticillium* sp., was supplied by Ajinomoto Co. (Japan).

Methods

Film forming solution

WP and LM-Pec solutions were separately prepared by dissolving 1,2 g of WP isolate, plus 0.6 g of sorbitol, and 1.2 g LM-Pec in 25 mL of distilled water, respectively, and by stirring the solutions for 2 hours at room temperature for a complete hydration of the macromolecules. Then, WP solution was heated, under continuous stirring, in a bath water at 80°C for 25 min to denature the proteins. Different aliquots of pectin solution were brought to 25 mL with distilled water to obtain variously diluted polysaccharide solutions which, after heating at 80°C for 3 min, were then added slowly and

under stirring to various WP solutions. The differently diluted pectin solutions were added to WP solution to obtain WP/LM-Pec ratios of 2:1, 4:1, 6:1 and 8:1 (w/w), respectively, with a constant WP concentration of 20 mg/mL. Each obtained film forming solution was heated at 80°C for further 2 min and finally cooled under stirring at room temperature. The pH of the film forming solutions prepared with a WP/LM-Pec ratio of 4:1 was adjusted to the desired value and then TGase (8 U/gr of WPI) was added by stirring overnight a room temperature. Finally, the film forming solutions were cast by pipetting 30 mL of each solution into Petri dishes (150mm x 15 mm) and the films obtained by drying at 45°C and 30% HR.

Potentiometric titrations

P/P complexation was followed by potentiometric titration of protein acidic and basic groups (Wen and Dubin, 1997; Mattison et al., 1998). The potentiometric titrations were performed at 22°C on all the film forming solutions, obtained as described above, and on a solution containing only WP isolate at a concentration of 20 mg/mL. The initial pH was adjusted to 8.0 ± 0.05 with 0.01N NaOH and the solutions were titrated with 0.01 N HCl to reach pH 4.0 corresponding

to a pH value lower than pectin pKa (4.5). The pH was noted when the value was stable for at last 1 min. The reproducibility of two repeated titrations was ± 0.05 pH units. The pHc was recorded as the pH value corresponding to the inflexion point of the titration curve. To evaluate the influence of both electrostatic interactions and hydrogen bonds on the pHc, we analyzed further film forming solutions containing either 110 mM sodium chloride or 110 mM urea.

Film Characterization

Thickness

Film thickness was measured using an electronic digital micrometer with a sensitivity of 2 μm (Metrocontrol, Srl, model HO62). Film strips were placed between the jaws of the micrometer and the gap was reduced until the minimum friction was measured. Mean thickness (mm) was determined from the average of measurements at five locations.

Mechanical properties

Film tensile strength (TS), elongation to 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 razor blade, into 10-11 mm wide and 50 mm length strips equilibrated overnight at 50% ± 5% RH and 23 ± 2 °C in an environmental chamber. Ten samples of each film type were then tested. Tensile properties were measured according to the ASTM (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 a tension mode.

Water vapor permeability (WVP)

WVP was evaluated by a gravimetric test according to ASTM E96 (1993) by means of a Fisher/Payne permeability cup (Carlo Erba, Italy). Three grams of silica gel were introduced in each cup. The film samples having diameter of about 6 cm were put on top of the cup and sealed by means of a top ring kept in place by three tight clamps. The film area exposed to vapor transmission was 10 cm^2. The cups containing silica gel were weighed and then placed in a desiccator containing a saturated KCl solution which provided a constant water activity at 25 °C equal to 0.8434. The desiccator was

stored in a Heareus thermostated incubator at 25.0 ± 0.1 °C. Cups were weighed at scheduled times, and the amount of water vapor 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:

$$\text{WVP} = X/(A\Delta p)\, \mathrm{d}m/\mathrm{d}t$$

where $\mathrm{d}m/\mathrm{d}t$ 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 Δp is the water vapor pressure across the film. By assuming that the vapor pressure inside the cup, due to the presence of silica gel, can be taken equal to zero, Δp becomes equal to the vapor pressure inside the desiccator and was calculated by multiplying water activity and the water tension ($P0$) at 25 °C ($P0$) 3167 kPa).

Oxygen permeability

Film permeability to oxygen was determined using a modified ASTM D 3985 (1981) with MultiPerm apparatus (Extrasolution s.r.l., Pisa, Italy). Duplicate samples of each film were conditioned for 2 days at 50% RH before measurement. Aluminum masks were used to reduce film test area to 5 cm^2, whereas the testing was performed at 25 °C under 50% RH.

Statistical analysis

JMP software 5.0 (SAS Campus Drive, Building S, Cary, NC) 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 $p<0.05$.

Results and Discussion

To produce edible WP/pectin films with acceptable mechanical and barrier properties, we preliminarly investigated the behavior of LM-Pec in the presence of thermally denatured WP at different both P/P ratios and pH values to determine the best experimental conditions

for obtaining soluble P/P complexes. In fact, it is well known that, according to the model proposed by Weinbreck et al. (2003), proteins and polysaccharides occur in free molecular forms when they are dissolved in water at a pH values higher than their pHc, whereas at the pHc they interact by forming soluble complexes which generally aggregate and precipitate with further pH decreases. Therefore, Fig. 1 shows the titration curves of different denatured WP/LM-Pec film forming solutions prepared at various P/P ratios. The detected pHc value, corresponding to the inflexion point of the different titration curves obtained on the curve observed with WP alone, was about 5.1. Moreover, Fig.1 shows that the titration curves obtained with WP/LM-Pec ratios (w/w) of 8:1, 6:1 and 4:1 were almost superimposable by decreasing the pH from 8.0 to the pHc value, while the titration curve corresponding to WP/LM-Pec ratio of 2:1 shifted at pH 6.0 and the curve corresponding to WP/LM-Pec ratio of 4:1 shifted at pH values lower than pHc. These results indicate, thus, that the optimal ratio to obtain soluble WP/LM-Pec complexes was 4:1 (Girard et al., 2002). To determine the nature of P/P interactions (electrostatic and/or hydrogen bonds) involved in the WP/LM-Pec complexes, further titrations were carried out in either 110 mM

sodium chloride or 110 mM urea by using 4:1 WP/LM-Pec film forming solutions. The results reported in Fig. 2 show that, whereas the titration curve obtained with the film forming solution prepared in urea was superimposable to the control, indicating the absence of hydrophobic interactions, the one corresponding to the sodium chloride containing solution markedly shifted under pH 6.0, clearly indicating the occurrence of electrostatic interactions in WP/LM-Pec complexes (Tolstoguzov, 1997).

To investigate the possible effects of TGase-catalyzed covalent crosslinks on the mechanical and barrier properties of WP/LM-Pec films, we prepared different film forming solutions at pH 5.1 (pHc, when soluble P/P complexes occur) and at pH 6.0 (when the polymers are free in the solution) both in the presence and absence of the enzyme. The results reported in the panel A of Fig. 3 indicate that the films obtained by using WP/LM-Pec film forming solution prepared at pHc in the presence of TGase showed a tensile strength significantly higher than that of the corresponding films obtained with solutions made at the same pH in the absence of the enzyme, as well as at pH 6.0 both in the absence and presence of the enzyme. Moreover, it is worthy to note that a significant increase of the tensile

strength was observed only at pHc, by using films made in the presence of TGase, while no significant difference was observed in the film containing the enzyme but made at pH 6.0. These findings could be explained by considering the possible formation of a supermolecular structural network during the film casting (Dubin et al., 1994; Wen, and Dubin, 1997). In fact, our data suggest that TGase-catalyzed covalent bonds among soluble ionic complexes of WP/LM-Pec, produced at pH 5.1, may be responsible for the observed increase of the film resistance with respect to the one exhibited by films prepared at pH 6.0, in which the enzyme should be able to catalyze the formation of covalent bonds only among not complexed WP molecules.

Panels B and C of Fig. 3 show, respectively, the measurements of film elongation to break, related to the capacity of the different materials to extend, and the Young's modulus that indicates the film stiffness. From the panel B of the Fig. 3 it is possible to conclude that, when TGase was absent in the film forming solution, the films prepared at pH 6.0 were two-fold more extensible than those prepared at pH 5.1. Conversely, when the films were prepared in the presence of the enzyme at pH 6.0, a significant reduction of their

elasticity, due to the formation of covalent crosslinks between the single soluble WP molecules, was observed. Moreover, the introduction of TGase-catalyzed covalent bonds in the WP/LM-Pec supermolecular structural network occurring at pH 5.1 surprisingly increased the elongation to break of the obtained films about six-fold in comparison with films obtained in the absence of the enzyme at the same pH, and more than ten-fold in comparison with the ones prepared with the enzyme at pH 6.0. Finally, panel C of Fig. 3 shows that the TGase-containing film prepared at pHc exhibited a reduced value of Young's modulus, index of a more flexible material, with respect to the film made in the presence of the enzyme at pH 6.0. The observed high elasticity and low Young's modulus lead to define the films deriving from TGase-crosslinked WP/LM-Pec complexes as typical elastomers (Ashby and Jones, 2005).

Finally, to evaluate the possible influence of TGase on the film barrier properties, we investigated both water vapor and oxygen permeability of the films obtained at pHc in the presence or absence of the enzyme. It is well known that several polymer characteristics concur to characterize the barrier capability of a material to water vapor and different gases (Han and Scanlon, 2005). Among the

various factors, the polymer chemical nature and the specific processing conditions are recognized, however, as the most relevant ones (Jasse et al., 1994). Previous studies, in fact, have shown that an increase in the crystallinity, density, orientation, molecular weight, and also crosslinking, of the different materials tested are generally responsible for a decreased film permeability (Han and Scanlon, 2005; Salame, 2009). The results of our experiments, reported in Table 1, clearly show that the production of TGase-catalyzed crosslinks among WP/LM-Pec sovramolecular soluble complexes, obtained at pHc, significantly reduced film permeability to both water vapor and oxygen, this effect being probably due to the marked decrease of interstitial spaces inside the WP/LM-Pec soluble complex network (Miller and Krochta, 1997).

Conclusions

Our study strongly confirms that TGase is a very useful tool to produce composite bioplastics from renewable biomass sources with improved both mechanical and barrier characteristics. In particular, we demonstrated the crucial importance of the pH value of the film

forming solution which is able to dramatically influence the sovramolecular structure of soluble P/P complexes able to act as enzyme substrates. The described composite film, constituted by TGase-crosslinked WP/LM-Pec soluble complexes, may represent a new possible candidate to substitute coating non-edible material for both food and pharmaceutical applications.

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Figure Capture

Figure 1. Titration curves of WP/LM-Pec film forming solutions with different P/P ratios (w/w): 2:1 (♦), 4:1 (■), 6:1 (▼), 8:1 (●), WP only (◊). Experimental details are given in the text.

Figure 2. Titration curves of WP/LM-Pec (4:1, w/w) film forming solutions in the absence (■) and in the presence of either 110 mM urea (●) or 110 mM NaCl (▽). Experimental details are given in the text.

Figure 3. Tensile strength (A), elongation to break (B) and Young's modulus (C) of films obtained by casting WP/LM-Pec (4:1, w/w) mixture at different pH values in the presence (grey bar) or absence (open bar) of TGase. Experimental details are given in the text.

Table 1. Barrier properties of WP/LM-Pec (4:1, w/w) films obtained at pHc value in the absence and presence of TGase.

Films	Thickness (mm)	Water vapor permeability	Oxygen permeability
		(cm^3 mm/m^2 day Kpa)	
WP/LM-Pec	0.152 ± 0.012	15.38 ± 0.19	0,025 ± 0.006
WP/LM-Pec +TGase	0.216 ± 0.021	9.90 ± 0.45	0,016 ± 0.002

Mean ± SD of ten samples. Experimental details are given in the text.

Figure 1

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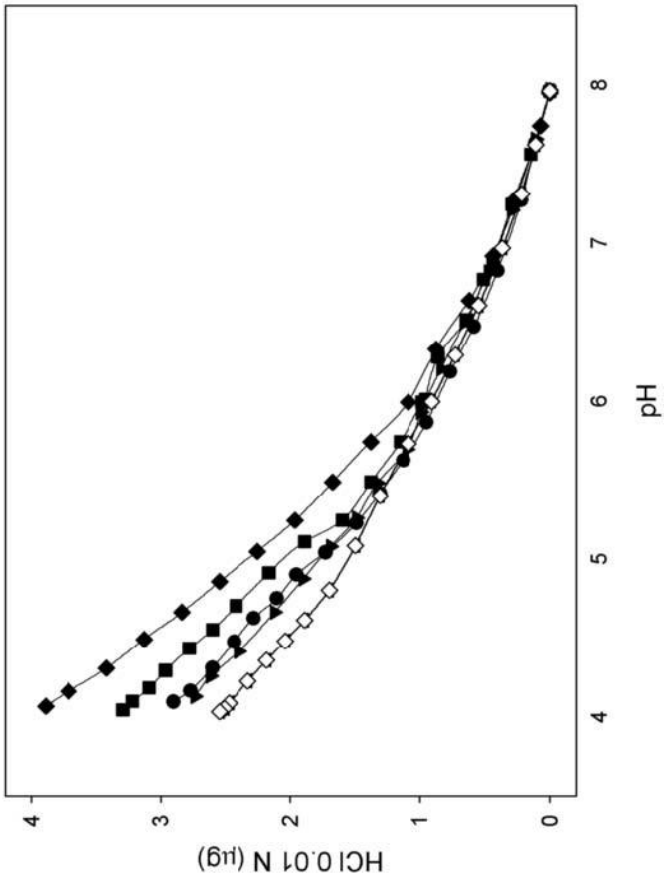


Figure 2
Click here to download high resolution image

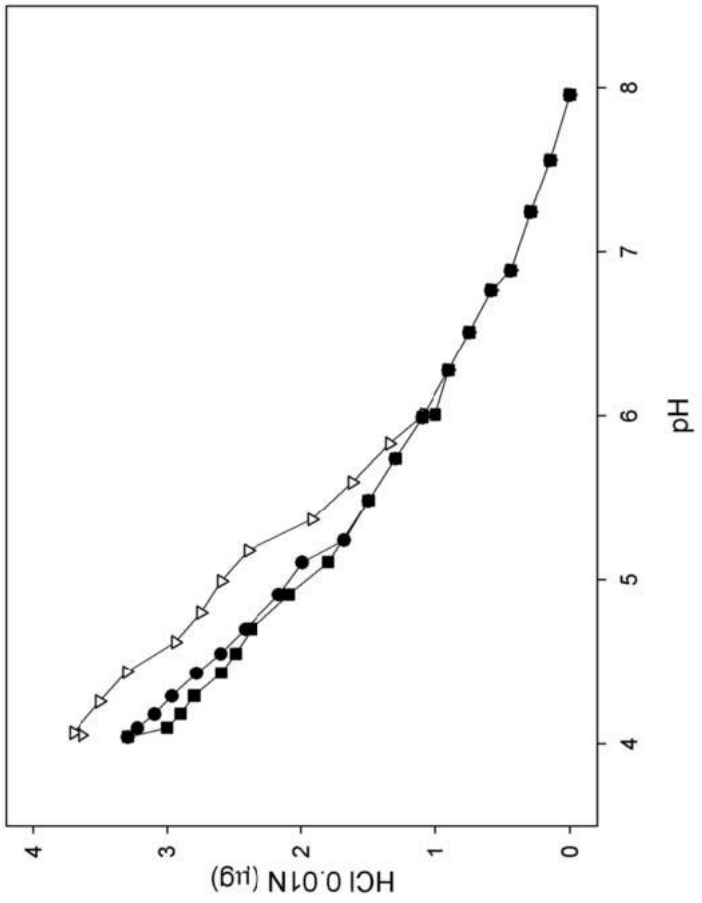
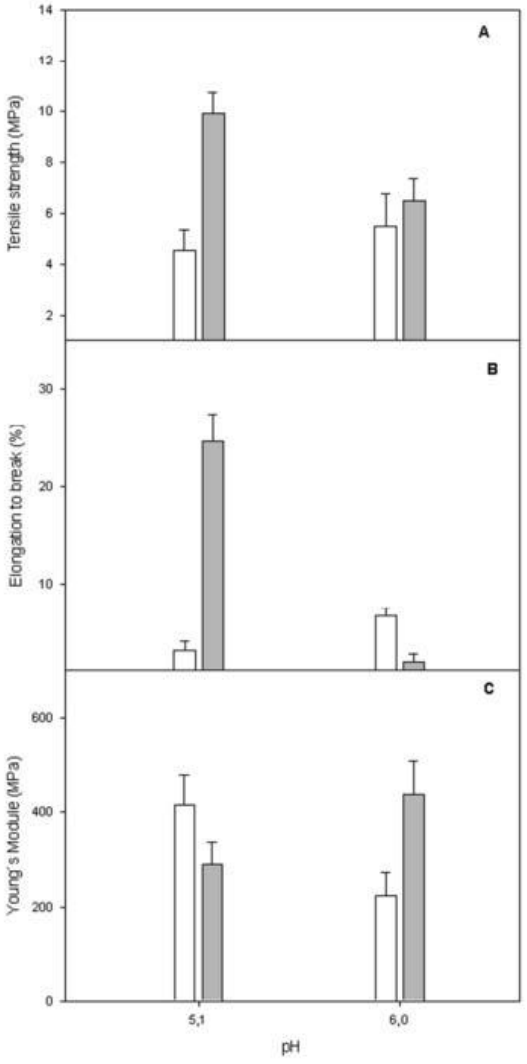


Figure 3
Click here to download high resolution image


A
B
C
Tensile strength (MPa)
Elongation to break (%)
Young's Module (MPa)
14
12
10
8
6
4
2
30
20
10
600
400
200
0
5,1
6,0
pH

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