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Characterization of *Citrus* pectin edible films containing transglutaminase-modified phaseolin

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ABSTRACT

The growing social and economic consequences of pollution derived from plastics are focusing attention on the need to produce novel bioprocesses for enhancing food shelf-life. As a consequence, in recent years the use of edible films for food packaging is generating a huge scientific interest. In this work we report the production of an edible hydrocolloid film made by using *Citrus* pectin and the protein phaseolin crosslinked by microbial transglutaminase, an enzyme able to covalently modify proteins by formation of isopeptide bonds between glutamine and lysine residues. The films were characterized and their morphology was evaluated by both atomic force microscopy and scanning electron microscopy. Mechanical properties and barrier properties to CO_2, O_2 and water vapor have demonstrated that these films possess technological features comparable to those possessed by commercial plastics. It is worth noting that these characteristics are maintained even following storage of the films at 4 °C or −20 °C, suggesting that our bioplastics can be tailored to protect food at low temperature. Moreover, gastric and duodenal digestion studies conducted under the same conditions found in the human digestion system have demonstrated that transglutaminase-containing films are regularly digested encouraging an application of the proposed materials as food coatings.

1. Introduction

As the pollution levels derived from commercial plastics rise rapidly in the EU, the food industry is heavily pushing the development of new innovative products which allow consumers to better sustain their health and well-being. Consumers are becoming increasingly aware of the relationship between pollution and health, emphasizing a need for the production of novel biodegradable plastics with the double aim of enhancing the shelf-life of many food-based products and of reducing the amount of plastic packaging that causes environmental concerns. The challenge is to target nanosciences toward the development of natural structures for food protection.

For this reason edible films based on biopolymers are gaining a great interest in the scientific community because of their biodegradable nature and for their potential use in food industry (Cagri, Ustunol, & Ryser, 2004). In recent years, in fact, several research projects have been carried out to develop edible films based on natural products coming from both agriculture and food industry wastes (Giosafatto, Mariniello, & Ring, 2007; Mariniello, Giosafatto, Di Pierro, Sorrentino, & Porta, 2007; Mariniello, Giosafatto, Moschetti, et al., 2007).

Packaging systems exert the function of food protection by influencing the transport with which low molecular weight substances, responsible for the deterioration of the product, permeate through the packaging. These systems promote an extension of the shelf-life of the product, improving its quality and organoleptic characteristics. Edible films have long been known to exert a protective effect for fruit and vegetables delaying dehydration, reducing respiration, improving the structural quality, helping to preserve the volatile compounds and reduce microbial spoilage (Campos, Gerschenson, & Flores, 2011). Another interesting feature of these materials is their ability to reduce bacterial and fungal infections of semi-processed meat products as well as of ready-to-eat fish (Campos et al., 2011). From a structure point of view, the edible films can be classified as (1) hydrocolloid, (2) lipid and (3) composite (Donhowe & Fennema, 1994). Hydrocolloid films are composed of proteins, cellulose derivatives, pectin (Pec) and other polysaccharides; lipid films consist of waxes, acylglycerols and fatty acids, while the composite films generally contain both lipid and

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hydrocolloid components. Among the polysaccharides, cellulose, starch, alginate, chitosan, Pec and their derivatives are commonly used for the synthesis of these types of films (Donhowe & Fennema, 1994).

In this work we have developed hydrocolloid edible films made of *Citrus* Pec and the protein phaseolin (Ph) modified or not by the enzyme microbial transglutaminase (TG). The use of transglutaminases (EC 2.3.2.13) has been proposed for their ability to catalyze intra and/or intermolecular isopeptide bonds between the γ-carboxamide group of glutamine (acyl donor) and ε-amino group of lysine residues (acyl acceptor) (Cozzolino et al., 2003; Di Pierro, Sorrentino, Mariniello, Giosafatto, & Porta, 2011; Porta, Mariniello, Di Pierro, Sorrentino, & Giosafatto, 2011; Valdivia et al., 2006). In fact, in our previous investigations we have demonstrated that Ph is able to act as an effective substrate for TG (Mariniello, Giosafatto, Di Pierro, et al., 2007; Mariniello, Giosafatto, Di Pierro, Sorrentino, & Porta, 2010; Mariniello, Giosafatto, Moschetti, et al., 2007). The Pec/Ph films were characterized according to their mechanical and barrier properties to water vapor, CO_2 and O_2. The films were analyzed also by Atomic Force Microscopy (AFM), a powerful tool used to evaluate film surface topography (a qualitative parameter) and roughness (a quantitative parameter) (Ghanbarzadeha, Oromiehib, & Razmi-Radb, 2008). Nanoscale measurements by AFM allow to study the influence of different factors on film hardness, elasticity and permeability, extremely useful for designing high-performance edible food packaging. Film morphology characterization was also performed by Scanning Electron Microscopy (SEM). In addition, digestibility studies, carried out under physiological conditions were performed in order to propose these innovative edible films as new candidates for protecting different kinds of food addressed to the human consumption.

2. Experimental procedures

2.1. Materials

Phaseolus vulgaris L. beans were purchased from a local supermarket. Chemicals for electrophoresis were from Bio-Rad (Segrate, Milano, Italy). Microbial TG (Activa WM), derived from the culture of *Streptoverticillium* sp., was supplied by Ajinomoto Co. (Japan). *Citrus* Pec, trypsin from porcine pancreas (product T0303, activity 17,000 U/mg protein), chymotrypsin from bovine pancreas (product C7762, activity 58 U/mg protein), pepsin from porcine gastric mucosa (product P6887, activity 4220 U/mg protein), soybean Bowmann-Birk trypsin-chymotrypsin inhibitor, bile salts, and all other reagents were purchased from Sigma Chemical Company (Pool, Dorset, UK). Chemicals were of analytical grade, unless specified.

2.2. Methods

2.2.1. Ph purification

Ph was isolated from *P. vulgaris* beans by using the ascorbate-NaCl procedure described by Sun and Hall (1975) and modified by Mariniello, Giosafatto, Di Pierro, et al. (2007). To achieve maximum extraction of phaseolin, the extraction steps were repeated three times, and to maximize precipitation of phaseolin, the samples were kept in the dark at 4 °C for 30 min and then centrifuged for 20 min. The purified protein was dissolved into distilled water at a concentration of 7 mg mL^{-1}.

2.2.2. TG preparation

The enzyme solution was prepared by dissolving the commercial preparation (containing 1% of TG and 99% of maltodextrins) in distilled water at a concentration of 180 mg mL^{-1} and the mixture was centrifuged at 10,000 × *g* for 2 min to remove precipitates. The specific activity of the enzyme was 92 U/g. Estimation of enzymatic activity was carried out by a colorimetric hydroxamate assay according to Pasternack et al. (1998).

2.2.3. Citrus Pec preparation

3.2 g of *Citrus* Pec were dissolved in 200 mL of distilled water. The solution was stirred until the Pec was completely solubilized. Then the pH of the solution was adjusted to 5 by using HCl 3 N.

2.2.4. Film forming procedure

Two different kinds of films were prepared: Pectin and Phaseolin based-films (Pec/Ph films) and Pectin and Phaseolin-based films made in the presence of TG (Pec/Fas/TG films). Films were cast by pouring the solution into 5 cm diameter polystyrene Petri dishes. For Pec/Ph films, 12.5 mL of Pec solution (2%, w/w) were mixed with 2.6 mL of Ph solution and spread into the plates. Pec/Ph/TG films were obtained by adding 0.35 U of the enzyme to the final solution of Pec and Ph. All the samples were prepared in the presence of 6% glycerol (with respect to protein content). In fact, preliminary experiments, aimed to study the effect of different amounts (6%, 12% and 24%) of plasticizer, demonstrated no differences on film features at higher glycerol concentrations. The solutions were allowed to dry at 37 °C for 18 h under air circulation. Dried films were peeled intact from the casting surface and conditioned at 50% RH and at 25 °C for 48 h before being tested.

2.2.5. Protein determination

Protein determination was carried out by the Bio-Rad Protein Assay (Bio-Rad), using bovine serum albumin as standard (Bradford, 1976).

2.2.6. 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.

Film water vapor permeability: Film water vapor permeability (WVP) was evaluated by a gravimetric test according to ASTM E96 (1993) by means of a Fisher/Payne permeability cup (Carlo Erba, Italy) as described by Di Pierro, Mariniello, Giosafatto, Masi, & Porta (2005). Silica gel (3 g) was introduced into each cup, and a film sample disk with a diameter of about 6 cm was placed on top of the cup and sealed by means of a ring kept in place by three tight clamps. The film area exposed to vapor transmission was 10 cm^2. The assembled cups were weighted and then placed in a desiccator containing a saturated KCl solution that provided a constant water activity of 0.8434 at 25 °C. The desiccator was stored in a Heareus thermostated incubator at 25.0 ± 0.1 °C. Cups were weighed at scheduled times and the 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 steady-state was reached once the regression analyses made by using the last four data points resulted in $r^2 \geq 0.998$. The WVP was calculated from the equation

$$\mathrm{WVP} = \frac{X}{A\Delta p}\frac{dm}{dt}$$

where dm/dt is the slope of the cup weight *versus* time 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 as equal to zero, Δp becomes equal to the vapor pressure inside the desiccator given by the product of the water activity and water saturation pressure (P0) at 25 °C (P0) 3.167 kPa).

CO_2 and O_2 permeability: CO_2 and O_2 permeabilities were determined using a modification of ASTM Standard Method D 3985-8129 (1981) with MultiPerm apparatus (ExtraSolution s.r.l., Pisa, Italy). The samples, duplicates 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. The testing was performed at 25 °C under 50% RH.

Film mechanical properties: Tensile strength and elongation to break were measured by using an Instron Universal Testing Instrument (Instron Engineering Corp., Canton, MA, model 5543A) according to ASTM (1997) and following the procedure described by Mariniello, Giosafatto, Moschetti, et al. (2007). Film samples were cut into 10 mm wide and 100 mm length strips using a sharp razor blade. The strips were equilibrated overnight at 50% RH and 23 °C in an environmental chamber. Five samples of each film type were tested. Some films before being tested were kept for 8 days at 25 °C, 4 °C or −20 °C in order to study the effect of the storage temperature on film mechanical performances. Each film strip was placed between the pneumatic jaws of the Instron that were previously preset to give an original gauge of 90 mm and the strips were then stretched at a rate of 30 mm min^{-1} until sample failure. Measurements of the load (N) and deformation (mm) were used to calculate tensile strength (maximum load placed on the sample divided by the cross-sectional area) and the elongation to break (deformation of the sample at maximum load divided by original gauge length).

Simulated gastric and duodenal proteolysis: 5 mg of each type of films were incubated in 600 μL of simulated salivary fluid (SSF, 150 mM NaCl, 3 mM urea, pH 6.9) for 5 min at 170 rpm. Afterwards the samples were subjected to gastric and duodenal digestion as described by Giosafatto et al. (2012) with some modifications. Briefly, aliquots (100 μL) of simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5) were placed in 1.5 mL microcentrifuge tubes and incubated at 37 °C. 75 μL of films previously incubated with SSF, the pH of which was adjusted to 2.5 with 6 MHCl, were added together pepsin (1:20 Ph, w/w) to each of the SGF vials to start the digestion reaction. The ratio of pepsin to test protein was 20:1 (w/w). At intervals of 1, 2, 5, 10, 20, 40, 60 min 40 μL of 0.5 M ammonium bicarbonate (NH_4HCO_3) were added to each vial to stop the pepsin reaction. The control was set up by incubating the sample for 60 min without the protease.

Duodenal digestions were performed using, as the starting material, the gastric digests after the 60 min, adjusted to pH 6.5 with 0.5 M bis-Tris HCl, pH 6.5. Bile salts (sodium taurocholate and sodium glycodeoxycholate) dissolved in simulated duodenal fluid (SDF, 0.15 M NaCl pH 6.5) were added to a final concentration of 4 mM. After preheating at 37 °C for 10 min, trypsin, chymotrypsin (the ratio of trypsin and chymotrypsin to test protein was 1:400: (w/w) and 1:100: (w/w), respectively) were added to the duodenal mix. Aliquots were removed over the 60 min digestion time course and proteolysis stopped by addition of a two-fold excess of soybean Bowmann-Birk trypsin-chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsin in the digestion mix. The control was carried out by incubating the sample without the proteases for 60 min. The samples were then analyzed using the SDS-PAGE procedure described below.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): 5 μL of sample buffer (15 mM Tris–HCl, pH 6.8, containing 0.5% (w/v) SDS, 2.5% (v/v) glycerol, 200 mM β mercaptoethanol, and 0.003% (w/v) bromophenol blue) were added to aliquots of 20 μL of each protolysed film sample and analyzed by 12% SDS-PAGE, as described by Laemmli (1970). Electrophoresis was performed at constant voltage (80 V for 2–3 h), and the proteins were stained with Coomassie Brilliant Blue R250. Bio-Rad Precision Protein Standards were used as molecular weight markers.

2.2.6.1. Atomic force microscopy (AFM). Sections of the films were stuck to glass slides using double-sided adhesive tape and the top surface imaged by AFM. The AFM used in this study was an MFP-3D-BIO (Asylum Research, Goleta, CA, USA). Imaging was performed in tapping mode in air in the repulsive regime using Olympus AC160TS cantilevers (Olympus, Japan). The cantilevers were driven into oscillation at 10% below their fundamental resonant frequency (typically around 320 kHz) and the feedback loop operated using amplitude control. The set-point was kept at the minimal value (~90% of the free amplitude) that enabled stable tracking of the sample surface in order to eliminate any sample damage. Each scan was carried out at a relatively slow rate of 0.5 Hz to allow proper imaging of the corrugated surfaces of the films.

2.2.6.2. Scanning electron microscopy (SEM). Film samples were cut into of 9 mm^2 pieces using a sharp razor blade. Then, the film pieces mounted on aluminum stubs, were covered with gold layer and observed with a FEI Quanta 200 ESEM environmental scanning electron microscope working in High Vacuum mode, with 30 KV accelerating voltage, 60 Pa pressure and 10 mm working distance. Three different samples of each type of film were subjected to SEM and five different micrographs of each sample were taken. Micrographs were obtained for sample surface at 1313× magnifications and for sample cross-sections at 5250× magnifications.

2.2.7. Statistical analysis

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

3. Results

3.1. Film mechanical properties as function of glycerol concentration

In this work we have investigated the capability of *Citrus* Pec and TG-modified Ph to form edible films prepared by casting. Previous investigations have demonstrated that Ph is an effective substrate of TG (Mariniello et al., 2010; Mariniello, Giosafatto, Di Pierro, et al., 2007; Mariniello, Giosafatto, Moschetti, et al., 2007). Moreover Ph is a globular protein able to form complexes with different carbohydrate matrices, *e.g.* Pec either purified or not from fennel cell wall and grapefruit albedo (Mariniello et al., 2010; Mariniello, Giosafatto, Di Pierro, et al., 2007; Mariniello, Giosafatto, Moschetti, et al., 2007). In order to make the film structure more flexible and handled, glycerol has been used as plasticizer. Therefore, the film solutions were stratified into Petri dishes and allowed to dry for 48 h in a climate chamber at a temperature of 37 °C and 50% RH. Prior of any characterization, the films were equilibrated for 4 h at 50 ± 5% RH and 23 ± 2 °C in an environmental chamber. In the first instance the experiments were carried out in order to choose the best glycerol concentration. Films were prepared also in the absence of glycerol but the samples were too brittle and, therefore, very difficult to be analyzed. Glycerol was, thus, added to the film solution at a concentration of 6%, 12% or 24% with respect to Ph content. From the macroscopic point of view all films were very handled, flexible and easily removed from the plates and their performance were very similar regardless of the glycerol content. In fact, in all cases the tensile strength and elongation to break values were in average equal to 20 MPa and 1.2%, respectively (data not shown). Thus, the lowest glycerol concentration (6%) was chosen for further experiments.

Table 1
Effect of storage temperature on the mechanical properties of Pec/Ph films prepared in the absence or presence of TG.

	Pec/Ph (25 °C)	Pec/Ph (4 °C)	Pec/Ph (−20 °C)	Pec/Ph/TG (25 °C)	Pec/Ph/TG (4 °C)	Pec/Ph/TG (−20 °C)
Tensile strength (MPa)	21.91 ± 3.1^{c}	23.64 ± 3.4^{bc}	24.6 ± 5.5^{bc}	21.3 ± 3.1^{c}	36.71 ± 3.4^{a}	32.7 ± 9.3^{ab}
Elongation to break (%)	1.17 ± 0.38^{c}	1.84 ± 0.3^{abc}	1.24 ± 0.4^{bc}	1.2 ± 0.36^{bc}	2.4 ± 0.3^{a}	2.00 ± 0.56^{ab}

The films have been kept for 8 days at 25 °C, 4 °C or −20 °C and then analyzed. Values are expressed as means ± SD and those followed by same letters are considered not significantly different (Tukey–Kramer test $\alpha = 5\%$). Further experimental details are given in the text.

3.2. Film mechanical properties as function of temperature

To investigate the ability of films to protect food products that require the cold chain or storage at temperatures close to 0 °C, the mechanical properties were investigated not only after keeping the film at 25 °C, but also at 4 °C and at −20 °C. More in detail, films were maintained to the above temperatures for 8 days. As it is possible to note from Table 1, the mechanical properties of films prepared in the presence of TG seem to improve at low storage temperatures (mostly at 4 °C), suggesting that these films could be particularly suitable for the protection of refrigerated foods.

3.3. Film barrier properties

The wide majority of packaged foods require a barrier to gases, flavors or odors to maintain product quality and provide an acceptable shelf-life. There are some limitations to the application of polysaccharides and proteins from vegetal origin for food packaging, because of their high sensitivity to moisture. Therefore water vapor, CO_2 and O_2 permeability studies of the prepared films were carried out. As it is possible to note from Table 2 Pec/Ph films exhibit quite low permeability values to water vapor, CO_2 and O_2. These data are potentially important for their application in the food packaging. In fact, barrier properties influence food product shelf-life and consequently contribute to preserve the qualitative features of food during the storage. It is worth of noting that, while both type of investigated films are 5-fold less permeable to CO_2 compared to the biodegradable and commercially available Mater-Bi (http://www.novamont.com.ing/hatml/prodotto/tecnologie/film.html), the film reticulated by TG shows a permeability to O_2 that is 240-fold and 620-fold lower than Mater-Bi and the synthetic LDPE, respectively (Salame, 1986).

3.4. Film digestion

Gastric and duodenal proteolytic experiments were performed under physiological conditions in order to study the possible digestion of the films by the human gut. As it is possible to note from Fig. 1 (upper panel A) unmodified Ph is not digested in the gastric environment even after 60 min of incubation with pepsin. These findings are in agreement with what has already been reported by several authors (Mariniello, Giosafatto, Di Pierro, et al., 2007; Montoya et al., 2008) and it is interesting to note that this also occurs when the protein is part of a complex matrix represented

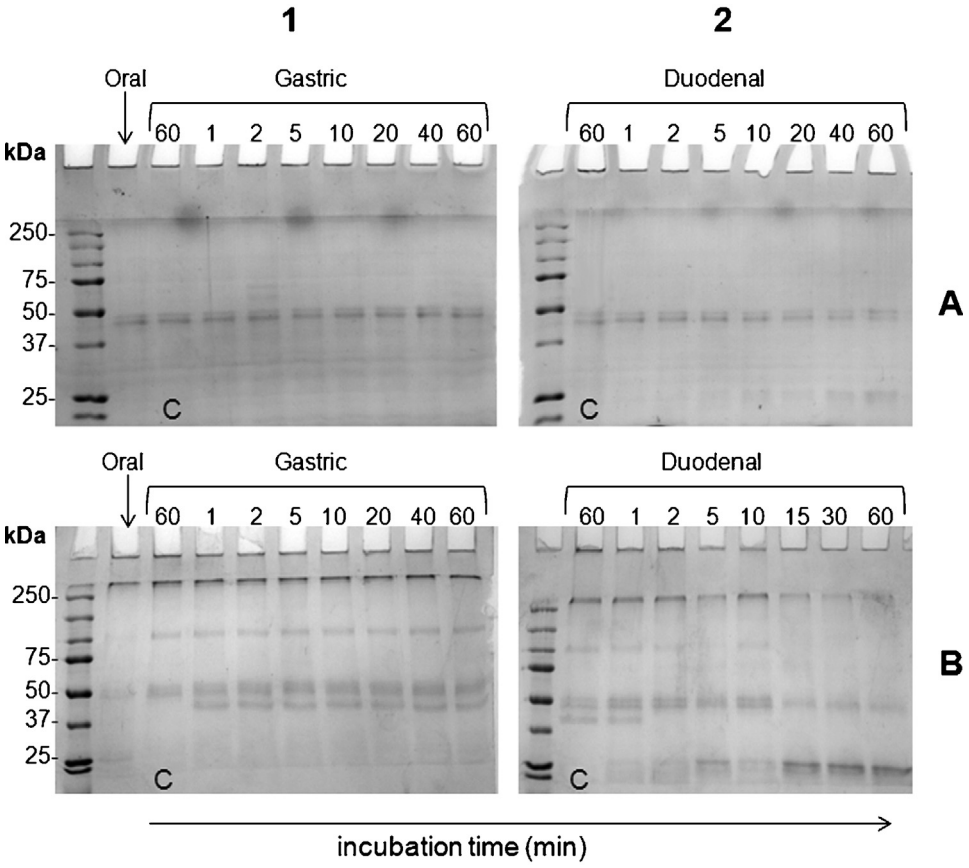

Fig. 1. Time course (min) of gastric and duodenal digestion of Pec/Ph films (upper panels) and of Pec/Ph/TG films (lower panels) carried out under physiological conditions. C, sample incubated for 60 min without proteases. Further experimental details are given in the text.

Table 2
Permeability properties of Pec/Ph films prepared in the absence or presence of TG.

Film	WVP (cm^3 mm m^{-2} day^{-1} kPa)	CO_2 (cm^3 mm m^{-2} day^{-1} kPa)	O_2 (cm^3 mm m^{-2} day^{-1} kPa)	Thickness (μm)
Pec/Ph	6.83 ± 2.42^b	0.29 ± 0.03^c	1.23 ± 0.05^a	57 ± 3
Pec/Ph/TG	6.79 ± 2.76^b	0.24 ± 0.02^c	0.003 ± 0.0002^b	53 ± 07

Values are expressed as means ± SD and those followed by same letters are considered not significantly different (Tukey–Kramer test $\alpha = 5\%$). Further experimental details are given in the text.

by the edible films. The samples obtained after 60 min of pepsin digestion were further processed by recurring to trypsin and α-chymotrypsin with the aim of mimicking duodenal digestion (Fig. 1, upper panel B). We found that unmodified Ph is quite resistant to the duodenal enzymes, being only partially degraded after 20 min of treatment, as indicated by the concomitant presence of a 20 kDa band and of the intact monomeric Ph form still detectable after 60 min of incubation (Fig. 1, upper panel B). On the other hand, the susceptibility to proteolysis of Ph modified in the presence of TG seems to be enhanced in both gastric and duodenal environment (lower panels A and B). In particular, although the produced TG-mediated polymer(s) show to be quite resistant to pepsin, the TG intra-crosslinked monomeric form (Mariniello, Giosafatto, Di Pierro, et al., 2007) is hydrolyzed after 1 min of incubation, as shown by the presence of a faster migrating band at ≈40 kDa. However the pepsin-dependent proteolysis does not proceed even if the incubation time is extended to 60 min (Fig. 1, lower panel A). Conversely, as far as the duodenal digestion, TG-derived polymer(s) are broken down within 1 min as well as the pepsin proteolyzed product of TG intra-crosslinked Ph and both were further gradually digested by the duodenal enzymes to release low molecular weight fragments (Fig. 1, lower panel B). The increased susceptibility of TG-modified macromolecules, both inter- and intra-crosslinked is likely due to the fact that the enzymatic modification causes a change in the three-dimensional structure of the protein by exposing some amino acid residues that are sensitive to either gastric or duodenal enzymatic hydrolysis.

3.5. AFM and SEM analyses

The films were characterized also by AFM (Figs. 2 and 3) and SEM (Fig. 4). Fig. 2 shows two sets of three complimentary AFM images alongside one another. The top set was obtained on films containing TG and the bottom set on films prepared in the absence of the enzyme. The complimentary images show different aspects of the film ultrastructure. In the left hand images surface topography is depicted by brightness. Low lying regions are dark, and tall regions are bright. In terms of visualization by the human eye the principal origin of the contrast seen in these images is simply due to the overall corrugation of the film so it is difficult to discern fine details. However, this information is present within the topography data and can be analyzed mathematically, as discussed later. The middle set of images presents the fine structural detail more clearly due to the mechanism of data capture: they are 'error signal images' (esi) which eliminate the low frequency sample undulations from the image contrast meaning that the tonal range is fully utilized for just the fine structure. The right hand set of images shows another aspect of contrast generation unique to tapping mode imaging; so-called 'phase images'. The AFM cantilever (upon which the AFM tip is mounted) is driven into oscillation very close to its fundamental

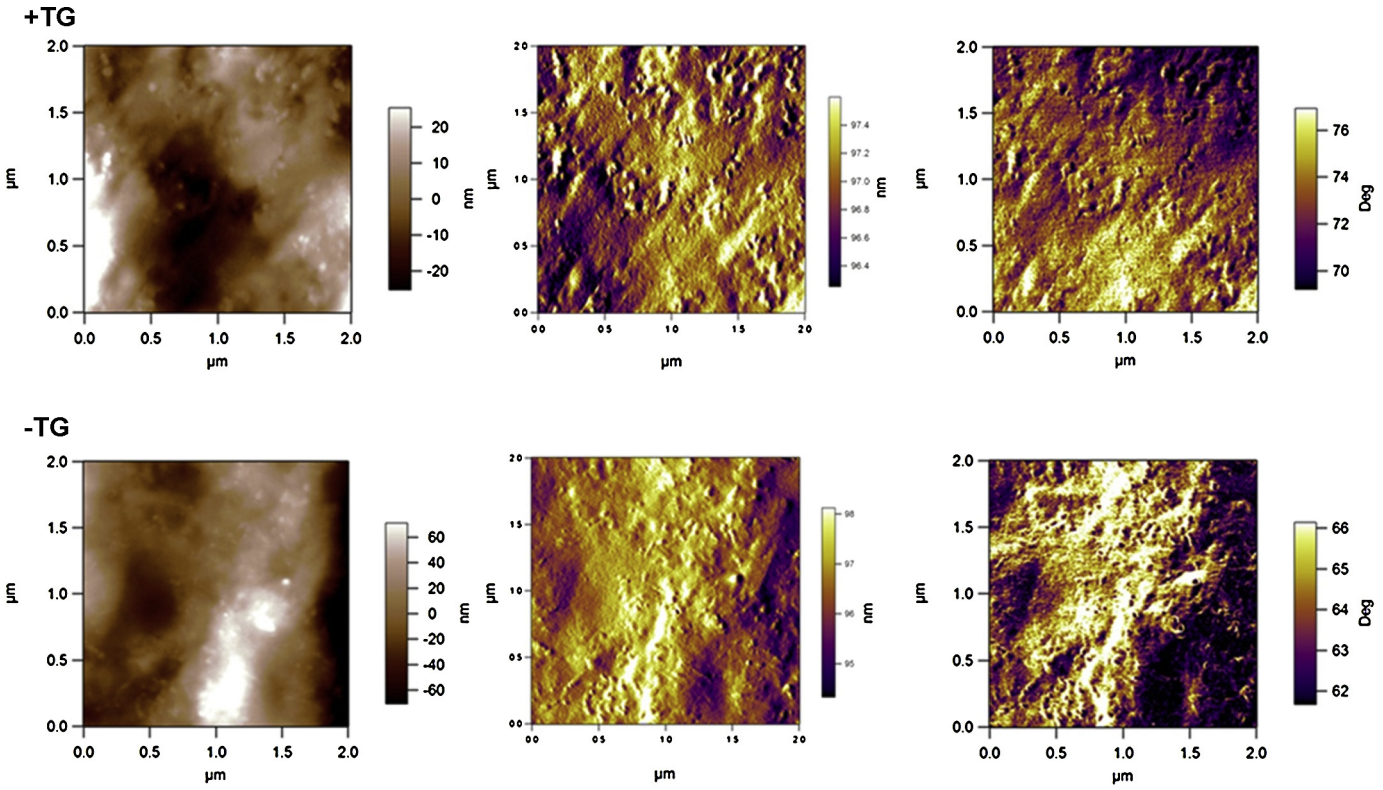

Fig. 2. AFM images of Pec/Ph films prepared in the presence or absence of TG. The images shown were chosen as the most representative from each sample. Experimental details are given in the text.

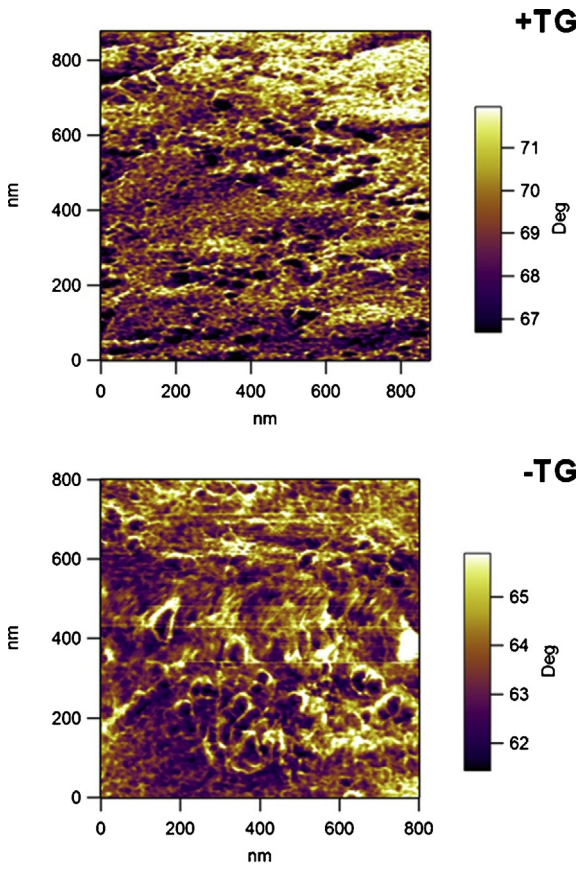

Fig. 3. Higher magnification AFM images of Pec/Ph films prepared in the presence or absence of TG. Experimental details are given in the text.

resonant frequency, in this case 10% below the first Eigen mode. This means that any energy loss as the AFM tip strikes the sample surface will cause a phase change in the cantilever's oscillation (Garcia & Perez, 2002). The amount of energy loss is affected by several factors, with many being due to the nature of the sample itself. These include variations in mechanical modulus, hydrophobicity and the presence of differing adhesive interactions across the sample surface (Garcia & Perez, 2002). Thus, the fact that the ultrastructural contrast is the most pronounced in the phase images of the films suggests that there is a segregation of the material properties of the films on a nanometer length scale and hence microscopic examination should provide important insights. The most striking observable difference in the AFM images obtained on the two different film preparations appears to be a consistent difference in the grain size of the globular features. This can be seen more clearly in Fig. 3 which shows representative areas from each scanned at higher magnification. Mathematical analysis of the surface roughness confirms this observation, with the values derived being significantly higher in the films obtained in the absence of TG (33.44 ± 1.48 nm) compared to those prepared in the presence of the enzyme (15.46 ± 2.01).

To further characterize the films for their morphological changes in relation to the action of TG, SEM analyses of both film surfaces and cross-sections were also performed (Fig. 4). The micrographs show significant differences in the film structure. On the surfaces of both types of films it is possible to observe the presence of phaseolin crystals of quasi-cubic symmetry as already reported by Mariniello, Giosafatto, Moschetti, et al. (2007). It is worth of note that the surface of films prepared in the presence of TG appears more compact and smoother than the ones prepared in the absence of the enzyme. Moreover, the film cross-sections show the presence of several cracks in the Pec/Ph films, totally absent in the Pec/Ph/TG films that, on the contrary, show a very homogeneous structure.

4. Discussion

Edible films are an opportunity for the food industry being an economic and biodegradable tool for coating or packaging a wide range of products. In this paper we have reported the properties of edible films prepared from *Citrus* Pec and the protein Ph modified or not TG. The enzyme has been extensively used by our group in order to change the biological properties of different proteins *in vitro* (Porta, Giosafatto, Di Pierro, Sorrentino, & Mariniello, 2013; Sorrentino et al., 2012) and also to improve several features of hydrocolloid edible films (Giancone et al., 2011; Mariniello, Di Pierro, Giosafatto, Sorrentino, & Porta, 2008; Porta et al., 2012). The protein Ph is an effective substrate of TG (Mariniello, Giosafatto, Di Pierro, et al., 2007), and it is interesting to note that the modification of the protein occurs even in the presence of Pec, as it was previously demonstrated by solubilizing the films and the following analysis by SDS-PAGE (Mariniello et al., 2010; Mariniello, Giosafatto, Di Pierro, et al., 2007; Mariniello, Giosafatto, Moschetti, et al., 2007). In fact, polymers with a molecular mass higher than 250 kDa and the concomitant disappearance of Ph monomer band were found in the TG-containing films. From the macroscopically point of view crosslinking treatment did not lead to any change in the transparency of the films in comparison with those prepared without the enzyme. The films were also obtained in the presence of glycerol, used as plasticizer, that was able to confer to the film a good flexibility and manageability. In fact, the addition of glycerol prevents cracking of the film during handling and storage (Barreto, Pires, & Soldi, 2003; Farahnaky, Saberi, & Majzoobi, 2013). Moreover, to produce a biomaterial able to preserve the shelf-life of coated food, it is important that the film maintains certain performances even when subjected to the temperatures used for food storage. Therefore, the effect of storage temperature on the mechanical properties of films, mainly tensile strength and elongation to break, was investigated. In particular, the materials were kept at room temperature 25 °C, 4 °C and −20 °C for 8 days and both tensile strength and elongation to break of Pec/Ph films were found to remain fairly constant as the temperature changed. Conversely, it is worthy to note that films prepared in the presence of TG seem to become more resistant at low temperatures (mostly at 4 °C) suggesting that these bioplastics can also be used for the protection of food products stored at low temperatures. This is likely due to the fact that the crystallization allows increased molecular interactions in the film protein, and, thus, changes in the mechanical properties of the films (Osés, Fernández-Pan, Mendoza, & Mate, 2009). This phenomenon is more intense in films prepared with TG, where the protein network is already strengthened by the enzyme.

For potential application of the edible films in food packaging sector it is necessary that the films have, in addition to good mechanical properties, barrier features comparable to the commercial products of chemical synthesis. For example, baked foods often require moisture protection, while fresh meats and vegetables require low or controlled oxygen exposure to maximize shelf-life and consumer appeal. Therefore we have characterized these films according to their barrier properties toward water vapor, CO_2 and O_2. Commercially available products have been proposed by BASF with Ecoflex and by Novamont with starch-based Mater-Bi and both are bioplastics containing different additives to improve mechanical and permeability performances (Mariniello, Giosafatto, Moschetti, et al., 2007). In fact, Mater-Bi is made of corn extracted starch to which different synthetic polymers (*i.e.*, poly-(ε-caprolactone) and/or polyvinyl alcohol) are added in order to increase the flexibility and resistance to moisture whereas Ecoflex

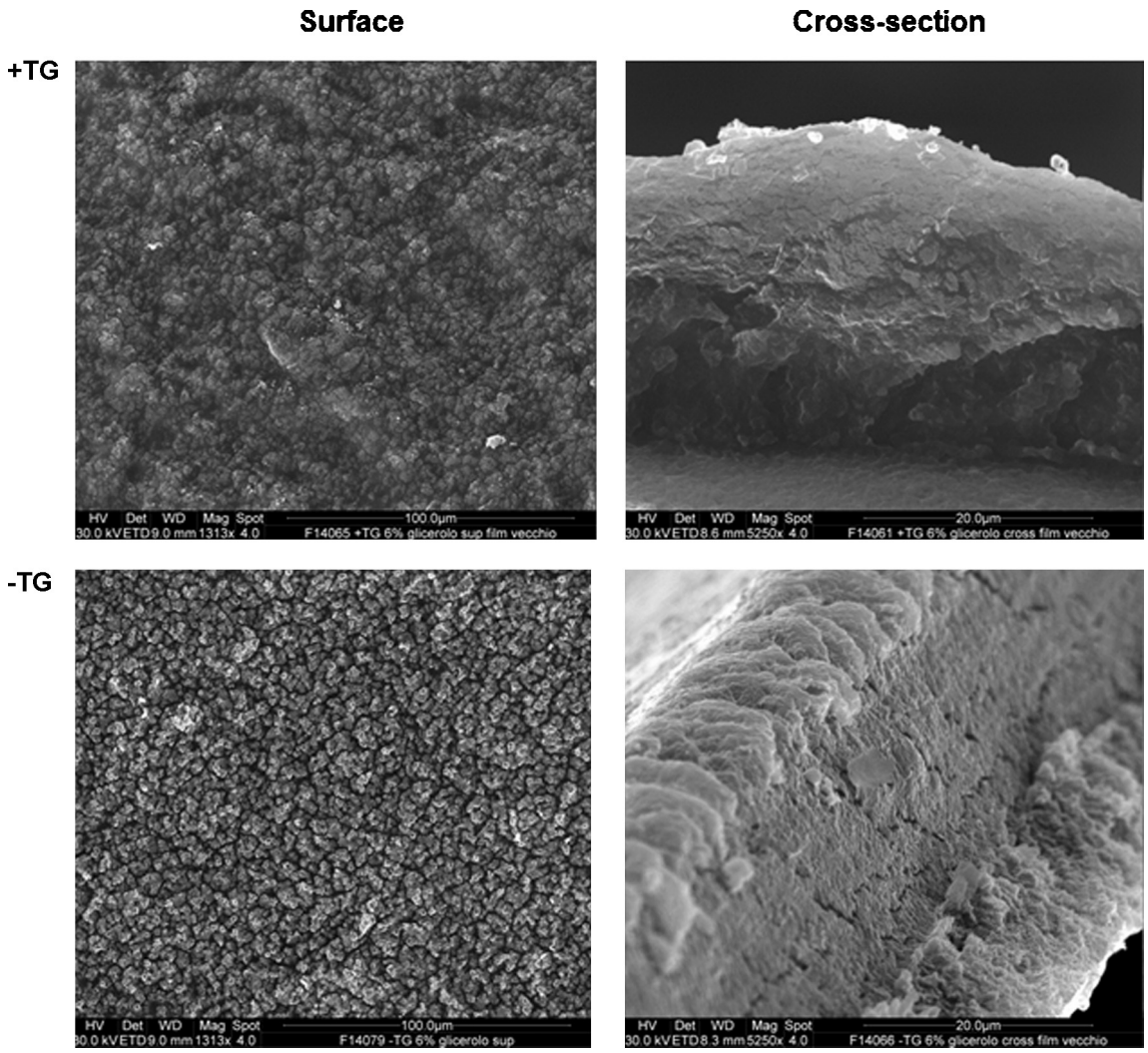

Fig. 4. SEM micrographs of surfaces (at 1313× magnification) and cross-sections (at 5250× magnification) of Pec/Ph films prepared in the presence or absence of TG. The images shown were chosen as the most representative of each sample. Experimental details are given in the text.

is an aliphatic–aromatic copolyester made of modular units including 1,4-butanediol, adipic acid, and terephthalic acid. Our results indicate that the Pec/Ph based-materials are permeable to water vapor, showing characteristics very dissimilar to the cited commercial materials (basf, 2013; Yamamoto, Witt, Skupin, Beimborn, & Müller, 2003; http://www.bioplastics.basf.com/ecoflex.html). As far as the O_2 permeability our results show that the films prepared in the presence of TG offer a very high barrier to this gas. In respiring-foods the O_2 permeability of the edible-film is extremely relevant since foods consume O_2 to produce an equilibrium condition. O_2 uptake by food often results in deleterious reactions which affect its flavor, nutritional quality and acceptability. Furthermore, both films prepared with and without TG exhibit a good CO_2 barrier property being almost 5 times lower than that exhibited by Mater-Bi. This result is of especial importance for fruit and vegetable storage because both kind of foods increase their respiration rate and CO_2 production with temperature and, inside the package, high CO_2 concentrations can be harmful for their shelf-life (Mujca-Paz & Gontard, 1997).

The films, containing or not TG, have been also subjected to experiments of sequential *in vitro* pepsin and trypsin digestion carried out under physiological conditions. In the literature there are only few studies on the simulated digestibility of edible films (López de Lacey et al., 2012). Before the treatment with these enzymes, the films were incubated in a solution mimicking the saliva (SSF). As it is reported by several authors, Ph is very resistant to the enzymatic proteolysis (Mariniello, Giosafatto, Di Pierro, et al., 2007; Montoya et al., 2008), although Ph digestion is markedly improved by thermal treatment (Deshpande & Damodaran, 1991). In fact, heat treatment influences structural changes and favors enzymatic hydrolysis by decreasing the percentage of α-helices and increasing random structures in the protein (Deshpande & Damodaran, 1991). We have found that unmodified Ph entrapped into film matrix is quite resistant to digestion by the gastric enzyme, as it is evident by the presence of intact Ph monomer still detectable after 60 min incubation with pepsin. The observed digestion kinetic is even slower than that observed by previous investigations proteolysis (Mariniello, Giosafatto, Di Pierro, et al., 2007; Montoya et al., 2008). This is probably due to the fact that food matrix, represented in this case by the *Citrus* Pec may affect Ph digestion in the upper gastrointestinal tract (Mandalari, Bisignano, & Whicham, 2011). After the following treatment with the duodenal enzyme trypsin, protein digestion seems to start only after 20 min incubation as shown by the formation of Ph polypeptides with a molecular mass of ≈20 kDa, the latter forms appearing resistant to further proteolysis. Similar undigested Ph polypeptides were reported in previous *in vitro* and *in vivo* studies (Mariniello, Giosafatto, Di Pierro, et al., 2007). The scenario completely changed in the sample prepared in the

presence of TG where the digestion pattern was greatly enhanced. In fact, the Ph monomeric forms, which contain both unmodified and intra-crosslinked protein form, as we demonstrated in previous investigations (Mariniello, Giosafatto, Di Pierro, et al., 2007) were promptly digested by the gastric enzyme upon only 1 min, as shown by the presence of a SDS-PAGE migrating polypeptide at ≈40 kDa. When the duodenal digestion started also the TG-modified polymers (with molecular mass > 250 kDa) became susceptible to the hydrolysis. In fact new fragments, having a molecular mass of ~20 kDa, were released quite rapidly after 1 min incubation. These fragments, further digested during trypsin incubation, were accompanied by the reduction of both high molecular weight polymers and of 50 kDa monomer. Thus, it is possible to suggest that the changes in structural conformation of the protein caused by TG treatment significantly improved the Ph susceptibility to digestive proteases.

Finally, the morphology of the edible films was evaluated by AFM and SEM. The analyses of topography through roughness demonstrated how AFM can be useful to identify structural changes of the film matrix. Additionally, the knowledge of the morphology could be an important parameter to identify structural changes in the films and to predict their porosity, permeability, flexibility and resistance (de Paula Herrmann, Cristiana, Pedroso Yoshida, Antunes, & Marcondes, 2004). Although both kinds of films seem to be composed of nanoparticles, the studied materials exhibit quite different particle aggregation structures. In fact, the films prepared without TG were characterized by a heterogeneous and non-compact network that was also evident by an increase in the roughness values (R_α), calculated using the data from the images with an appropriate software. On the other hand, reorganization of the aggregation structure occurred in TG-containing samples, indicate that the crosslinking agent makes smoother the film surface with evenly distributed and well-shaped particles, demonstrated also by the roughness factor of the surface (15.46 nm). This result is different from those found in our previous experiments carried out with Pec/whey protein films containing TG (Di Pierro et al., 2013) in which supramolecular soluble complexes suggested to determine an increase in the film surface roughness (Di Pierro et al., 2013). On the contrary in this work the results indicate that the enzyme is responsible for a well shaped particle aggregation structure determining a reduction of the roughness values. The different result could be likely due to the fact that whey proteins was a heterogeneous protein source, whereas Ph, was purified to homogeneity from *P. vulgaris* beans before being used for film preparation. The effect of TG on the structure of films was observed also by SEM analyses that show that the enzyme is responsible of a smoother surface and more compact cross-section. These results are in agreement with results already obtained by our group (Mariniello et al., 2003; Mariniello, Giosafatto, Moschetti, et al., 2007), suggesting the importance of TG-catalyzed isopeptide bonds in influencing the film morphology detectable by SEM.

Therefore, our findings are of potential applicative interest, since the structure of edible films and derived food coatings might appear uniform and attractive for the consumers. Moreover the Pec/Ph films prepared in the presence of TG should be also more easily digested by the human gut with respect to the ones prepared in the absence of the enzyme.

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References

ASTM. (1993). *Annual Book of ASTM Standards*. Philadelphia, PA: American Society for Testing and Materials. E96-93

ASTM. (1981). *Annual Book of ASTM Standards*. Philadelphia, PA: American Society for Testing and Materials. D3985-81.

ASTM. (1997). *Annual Book of ASTM Standards*. Philadelphia, PA: American Society for Testing and Materials. D882-97.

Barreto, P. L. M., Pires, A. T. N., & Soldi, V. (2003). Thermal degradation of edible films based on milk proteins and gelatin in inert atmosphere. *Polymer Degradation and Stability*, *79*, 147–152.

Bradford, M. A. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Cagri, A., Ustunol, Z., & Ryser, E. T. (2004). Antimicrobial edible films and coatings. *Journal of Food Protection*, *67*, 833–848.

Campos, C. A., Gerschenson, L. N., & Flores, S. K. (2011). Development of edible films and coatings with antimicrobial activity. *Food and Bioprocess Technology*, *4*, 849–875.

Cozzolino, A., Di Pierro, P., Mariniello, L., Sorrentino, A., Masi, P., & Porta, R. (2003). Incorporation of whey proteins into cheese curd by using transglutaminase from *Streptoverticillium mobaraense*. *Biotechnology and Applied Biochemistry*, *38*, 289–295.

de Paula Herrmann, P. S., Cristiana, M., Pedroso Yoshida, C. M., Antunes, A. J., & Marcondes, J. A. (2004). Surface evaluation of whey protein films by atomic force microscopy and water vapour permeability analysis. *Packaging Technology and Science*, *17*, 267–273.

Deshpande, S. S., & Damodaran, S. (1991). Denaturation behavior of phaseolin in urea, guanidine hydrochloride, and sodium dodecyl sulfate solutions. *Journal of Protein Chemistry*, *10*, 103–115.

Di Pierro, P., Mariniello, L., Giosafatto, C. V. L., Masi, P., & Porta, R. (2005). Solubility and permeability of edible pectin-soy flour films obtained in the absence or presence of transglutaminase. *Food Biotechnology*, *19*, 37–49.

Di Pierro, P., Rossi-Marquez, G., Mariniello, L., Sorrentino, A., Villalonga, R., & Porta, R. (2013). Effect of transglutaminase on the mechanical and barrier properties of whey protein/pectin films prepared at complexation pH. *Journal of Agricultural and Food Chemistry*, *61*, 4593–4598.

Di Pierro, P., Sorrentino, A., Mariniello, L., Giosafatto, C. V. L., & Porta, R. (2011). Chitosan/whey protein film as active coating to extend Ricotta cheese shelf-life. *LWT – Food Science and Technology*, *44*, 2324–2327.

Donhowe, G., & Fennema, O. (1994). Edible films and coatings: Characteristics, formation, definitions and testing methods. In J. M. Krochta, E. A. Baldwin, & M. O. Nisperos-Carriedo (Eds.), *Edible coatings and films to improve food quality* (pp. 1–24). Lancaster: Technomic Publishing.

Farahnaky, A., Saberi, B., & Majzoobi, M. J. (2013). Effect of glycerol on physical and mechanical properties of wheat starch edible films. *Journal of Texture Studies*, *44*, 176–186.

Garcia, R., & Perez, R. (2002). Dynamic atomic force microscopy methods. *Surface Science Reports*, *47*, 197–301.

Ghanbarzadeha, B., Oromiehib, A., & Razmi-Radb, E. (2008). Surface evaluation of resin zein films containing sugar plasticizers by permeability and atomic force microscopy analysis. *Iranian Journal of Pharmaceutical Science*, *4*, 23–30.

Giancone, T., Torrieri, E., Di Pierro, P., Cavella, S., Giosafatto, C. V. L., & Masi, P. (2011). Effect of surface density on the engineering properties of high methoxyl pectin-based films. *Food and Bioprocess Technology*, *4*, 1228–1236.

Giosafatto, C. V. L., Rigby, N. M., Wellner, N., Ridout, M., Husband, F., & Mackie, A. R. (2012). Microbial transglutaminase-mediated modification of ovalbumin. *Food Hydrocolloids*, *26*, 261–267.

Giosafatto, C. V. L., Mariniello, L., & Ring, S. (2007). Extraction and characterization of Foeniculum vulgare pectins and their use to prepare biopolymer films in the presence of phaseolin protein. *Journal of Agricultural and Food Chemistry*, *55*, 1237–1240.

http://www.bioplastics.basf.com/ecoflex.html (accessed 10.10.13).

http://www.novamont.com.ing/hatml/prodotto/tecnologie/film.html (accessed 10.10.13).

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of Bacteriophage T_4. *Nature*, *227*, 680–985.

López de Lacey, A. M., Giménez, B., Pérez-Santín, E., Faulks, R., Mandalari, G., Lopez-Caballero, M. E., et al. (2012). Bioaccessibility of green tea polyphenols incorporated into an edible agar film during simulated human digestion. *Food Research International*, *48*, 462–469.

Mandalari, G., Bisignano, G., & Whicham, M. (2011). Food matrix and processing affect almond protein release during simulated digestion. *Clinical and Translational Allergy*, (Suppl. 1), P20.

Mariniello, L., Di Pierro, P., Giosafatto, C. V. L., Sorrentino, A., & Porta, R. (2008). Enzymes as additives or processing aids. In R. Porta, P. Di Pierro, & L. Mariniello (Eds.), *Recent research developments in food biotechnology* (pp. 185–212). Trivandrum, Kerala, India: Research Signpost.

Mariniello, L., Giosafatto, C. V. L., Moschetti, G., Aponte, M., Masi, P., Sorrentino, A., et al. (2007). Fennel waste-based films suitable for protecting cultivations. *Biomacromoleules*, *8*, 3008–3014.

Mariniello, L., Giosafatto, C. V. L., Di Pierro, P., Sorrentino, A., & Porta, R. (2007). Synthesis and resistance to in vitro proteolysis of transglutaminase-crosslinked phaseolin, the major storage protein from *Phaseolus vulgaris*. *Journal of Agricultural and Food Chemistry*, *55*, 4717–4721.

Mariniello, L., Giosafatto, C. V. L., Di Pierro, P., Sorrentino, A., & Porta, R. (2010). Swelling, mechanical and barrier properties of albedo-based films prepared in the presence of phaseolin crosslinked or not by transglutaminase. *Biomacromolecules*, *11*, 2394–2398.

Mariniello, L., Di Pierro, P., Esposito, C., Sorrentino, A., Masi, P., & Porta, R. (2003). Preparation and mechanical properties of edible pectin-soy flour films obtained in the absence or presence of transglutaminase. *Journal of Biotechnology*, *102*, 191–198.

Montoya, C. A., Leterme, P., Beebe, S., Souffrant, W. B., Molle, D., & Lallès, J.-P. (2008). Phaseolin type and heat treatment influence the biochemistry of protein digestion in the rat intestine. *British Journal of Nutrition*, *99*, 531–539.

Mujca-Paz, H., & Gontard, N. (1997). Oxygen and carbon dioxide permeability of wheat gluten film: Effect of relative humidity and temperature. *Journal of Agricultural and Food Chemistry*, *45*, 4101–4105.

Osés, J., Fernández-Pan, I., Mendoza, M., & Mate, J. I. (2009). Stability of the mechanical properties of edible films based on whey protein isolate during storage at different relative humidity. *Food Hydrocolloids*, *23*, 125–131.

Pasternack, R., Dorsch, S., Otterbach, J. T., Robenek, I. R., Wolf, S., & Fuchsbauer, H. L. (1998). Bacterial pro-transglutaminase from *Streptoverticillium mobaraense*: Purification, characterisation and sequence of the zymogen. *European Journal of Biochemistry*, *257*, 570–576.

Porta, R., Giosafatto, C. V. L., Di Pierro, P., Sorrentino, A., & Mariniello, L. (2013). Transglutaminase-mediated modification of ovomucoid. Effects on its trypsin inhibitory activity and antigenic properties. *Amino Acids*, *44*, 285–292.

Porta, R., Mariniello, L., Di Pierro, P., Sorrentino, A., & Giosafatto, C. V. L. (2011). Transglutaminase crosslinked pectin- and chitosan-based edible films: A review. *Critical Reviews in Food Science and Nutrition*, *51*, 223–238.

Porta, R., Mariniello, L., Di Pierro, P., Sorrentino, A., Giosafatto, C. V. L., Rossi-Marquez, G., et al. (2012). Water barrier edible coatings of fried foods. *Journal of Biotechnology & Biomaterials*, *2*, e116. http://dx.doi.org/10.4172/2155-952X.1000e116

Salame, M. (1986). Barrier polymers. In M. Bakker (Ed.), *The Wiley encyclopaedia of packaging technology* (pp. 48–54). New York: John Wiley and Sons.

Sorrentino, A., Giosafatto, C. V. L., Sirangelo, I., De Simone, C., Di Pierro, P., Porta, R., et al. (2012). Higher susceptibility to amyloid fibril formation of the recombinant ovine prion protein modified by transglutaminase. *BBA Molecular Basis of Disease*, *1822*, 1509–1515.

Sun, S. M., & Hall, T. C. (1975). Solubility characteristics of globulins from Phaseolus seeds in regard to their isolation and characterization. *Journal of Agricultural and Food Chemistry*, *23*, 184–189.

Valdivia, A., Villalonga, R., Di Pierro, P., Perez, Y., Mariniello, L., Gomez, L., et al. (2006). Transglutaminase-catalyzed site-specific glycosylation of catalase with aminated dextran. *Journal of Biotechnology*, *122*, 326–333.

Yamamoto, M., Witt, U., Skupin, G., Beimborn, D., & Müller, R.-J. (2003). Biodegradable aliphatic-aromatic polyesters: "Ecoflex®". In A. Steinbüchel (Ed.), *Biopolymers* (Vol. 4) (pp. 521–553). Weinheim: Wiley-VCH.