Chitosan−Whey Protein Edible Films Produced in the Absence or Presence of Transglutaminase: Analysis of Their Mechanical and Barrier Properties

Prospero Di Pierro,[†] Belkis Chico,[‡] Reynaldo Villalonga,[‡] Loredana Mariniello,[†] Angelo E. Damiao,[‡] Paolo Masi,[†] and Raffaele Porta*[,†]

Dipartimento di Scienza degli Alimenti, Universita' di Napoli "Federico II", Parco Gussone 80055, Portici, Naples, Italy, and Enzyme Technology Group, Center for Biotechnological Studies, University of Matanzas, Autopista a Varadero Km 3 1/2, Matanzas, C.P. 44740, Cuba

Received September 9, 2005; Revised Manuscript Received January 10, 2006

Chitosan−whey protein edible films with different protein concentrations were prepared in the absence or presence of microbial transglutaminase as cross-linking agent. The films prepared in the presence of the enzyme showed low solubility at a wide range of pH, a lower degree of swelling, and good biodegradability following protease treatments. The presence of transglutaminase induced also an enhancement in film mechanical resistance and a reduction in their deformability. Finally, the barrier efficiency toward oxygen and carbon dioxide was found to be markedly improved in the cross-linked films which showed also a lower permeability to water vapor. Some potential practical applications of transglutaminase-treated chitosan−whey protein films are suggested.

Introduction

The large majority of the polymers presently utilized are of synthetic origin, their biocompatibility and biodegradability being extremely limited with respect to the biopolymers such as cellulose, chitin, chitosan, and their derivatives. Chitosan [poly-β-(1-4)-2-amino-2-deoxy-D-glucose], a hydrophilic polyelectrolyte prepared by N-deacetylation of chitin,[1] has attracted great attention since chitin is the second most abundant polysaccharide existing in nature. Typically, chitosan is produced from wastes generated from crustacean processing (shrimp and crabs), even though it is also possible to obtain it from the chitin component of fungal cell walls.[2] Chitosan shows physical, chemical, and biological properties which have suggested its use for several industrial, both biomedical and biotechnological, applications.[1−7] In fact, the high content of primary amino groups, the high biocompatibility,[8] and other peculiar biological properties, such as wound healing and antibacterial activity, confer to this biopolymer different potential utilizations.[4,9−13]

Chitosan films are usually prepared by solvent evaporation, chemical cross-linking, or physical interactions with other compounds such as proteins.[9,11−15] However, polymeric films produced by physical methods often result in poor mechanical and permeability properties when compared with those obtained through chemical reactions. On the other hand, chemical cross-linking agents frequently induce toxicity or confer other undesirable effects to these materials. Therefore, the possibility to use enzymatic methods both to prepare polymeric films and to improve their features has been the object of extensive studies.[16]

In a recent paper we examined the potential use of transglutaminase as a biotechnological tool for preparing pectin−soy protein films through an enzymatic cross-linking reaction.[17] Transglutaminase (TGase, protein−glutamine γ-glutamyl transferase, E.C. 2.3.2.13) catalyzes the introduction of ϵ-(γ-glutamyl)-lysine cross-links into proteins via an acyl transfer reaction. The γ-carboxamide group of glutamine serves as the acyl donor, whereas the ϵ-amino group of lysine acts as the acyl acceptor.[18] In addition, endoprotein reactive lysine may be substituted by several compounds containing primary amino groups, giving rise to a variety of protein−(γ-glutamyl) derivatives.[18,19] In this sense, TGase was previously and successfully used to modify the biological activities of peptides and proteins by covalently linking polyamines to their reactive *endo*-glutamine residues.[20−28] Moreover, TGase was employed for immobilizing enzymes,[29,30] as well as for improving the properties of several protein (such as casein, egg white, and whey proteins) based films.[31−33]

Liquid whey, a byproduct of cheese manufacturing containing proteins (α-lactoalbumin (α-LA), β-lactoglobulin (β-LG), whey albumin (BSA), immunoglobulin (Ig), and macropeptide), lactose, fat, and inorganic minerals, is produced in large amounts, and its annual production is continuously rising. Even though recycled in several ways, too much liquid whey is still wasted in the environment, and consequently, there is a significant interest in finding new applications to avoid the pollution mostly due to the whey proteins.[34] In the present study we report the preparation, in the absence or presence of TGase, of edible films constituted by chitosan and whey proteins (CWP). Several properties of both types of film were also analyzed and compared to those of the main films presently utilized as packing materials and bioplastics.

Materials and Methods

Materials. Chitosan from lobster shells (degree of N-acetylation = 9.0%) was obtained by Professor R. A. A. Muzzarelli (Ancona University, Italy).[35] The enzymatic preparation, named ACTIVA WM (product no. AJ301402, lot no. 00.02.03) and containing *Streptoverticillium* Ca^{2+}-independent TGase, was obtained from Ajinomoto Co.

* Corresponding author. Phone: 39 081 2539470. Fax: 39 081 2539473. E-mail: portaraf@unina.it.

[†] Università di Napoli "Federico II".

[‡] University of Matanzas.

10.1021/bm050661u CCC: $33.50
Published on Web 02/25/2006

(Japan). As indicated by the purchaser, WM product contains 1% enzyme and 99% maltodextrins, the latter used as stabilizer. Spray-dried whey from bovine milk (product no.W1500, lot no. 81K0279) containing 19% w/w proteins (biuret) and 65% w/w lactose was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were analytical grade.

Film Making. A concentrated chitosan solution (2.5% w/v) was prepared by adding chitosan (hydrochloride salt) to 0.1 M phosphate buffer, pH 6.0, and stirring overnight. The whey solution (5% w/v) was prepared by adding lyophilized milk whey to distilled water and stirring up to complete solubilization. The protein concentration (2.7 mg/mL) was determined by Bradford's method.[36] TGase solution (enzyme specific activity = 5.8 units/mL, as determined according to Mariniello et al.[17] where one unit of TGase was defined as the amount of enzyme able to incorporate 0.8 pmol of [^{3}H] spermidine into N,N-dimethylated casein in 1 h at pH 8.0 and 37 °C) was prepared by dissolving 180 mg of ACTIVA WM in 1.0 mL of distilled water.

Film-forming solutions with different amounts of whey proteins (0.125, 0.25, 0.37, 0.5, and 1.0 mg of protein/cm^2) were prepared in polystyrene Petri dishes (60 mm × 15 mm) by mixing 110 mg of glycerol (δ = 1.26 g mL^{-1}), chitosan (9.2 mg/cm^2 final concentration), different amounts of whey proteins, and when present, TGase (22.5 units) ensuring that the enzyme was evenly dispersed throughout the aqueous phase by gently mixing for 1 min. The solutions were deaerated under vacuum, prior to casting films, to prevent pinholes formation, then transferred into dishes, and finally left at 50 °C overnight under air circulation. The obtained films were peeled from the Petri dishes and stored at 20 °C in a desiccator (50% RH). The thickness of each film was measured using a micrometer model HO62 with a sensitivity of ±2 μm (Metrocontrol Srl, Casoria (Na), Italy). Film strips were placed between the jaws of the micrometer, and the gap was reduced until the first indication of contact. Mean thickness (μm) of the films were determined from the average of measurements at 10 locations.

Film Solubility. Film solubility was tested in buffered water solutions. The procedure was similar to that described by Stuchell and Krochta.[37] Small pieces of films (20−25 mg) were dried at 70 °C and 50 torr in a vacuum oven for 24 h and then weighed to the nearest 0.0001 g to determine the initial dry weight of the film. Each film piece was incubated at 25 °C for 24 h in a screw-top tube (150 mm × 15 mm) with 10 mL of 0.1 M acetate (pH 4.0), phosphate (pH 6.0), or Tris-HCl (pH 8.0) buffer solutions. At the end of the incubation the samples were poured onto Whatman no. 1 qualitative filter papers. The nondissolved materials, taken off by the filters with 10 mL of distilled water, were dried at 70 °C and 50 torr in a vacuum oven for 24 h and then weighed. The percentage of soluble matter was calculated as follows:

$$\text{soluble matter (\%)} = (\text{initial dry weight} - \text{final dry weight}) \times 100/\text{initial dry weight}$$

Twenty microliters of each supernatant solution was also analyzed by SDS−PAGE according to Laemmli.[38]

Film Swelling and Degradability Properties. The swelling studies of CWP films were carried out by incubating the samples at 25 °C in solutions of 20 mM sodium phosphate buffer, pH 7.0, for a period of 6 h under continuous shaking. The films were removed at scheduled times, gently dried on filter papers, and weighed by an analytical balance. The degrees of swelling were calculated by using the following equation

$$\text{degree of swelling} = (W_s - W)/W$$

where W_s and W are the weight (g) of the swollen and dry films, respectively.

Degradability studies were performed by first swelling the CWP films at 25 °C in solutions of 20 mM sodium phosphate buffer, pH 7.0, for a period of 2 h under continuous shaking, and further treatment with trypsin and α-chymotrypsin (1.0 μg of protease/g film) during 20 h under the same conditions. Aliquots (500 μL) were removed from the solutions at scheduled times, mixed with 500 μL of 1.0 M NaOH, and quantified for L-tyrosine with the Folin−Ciocalteau reagent.

Film Mechanical Properties. Two film mechanical properties, its tensile strength and elongation to break, were measured by using an Instron universal testing instrument model no. 4301 (Instron Engineering Corp., Canton, MA). Film samples were cut, using a sharp razor blade, into 10−11 mm wide and 100 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 (1991) Standard Method D882 using Test Method A, the static weighing, constant rate-of-grip separation test. The initial grip separation was 90 mm, and the crosshead speed was 30 mm/min in a tension mode.

Film Permeability Properties. Water vapor permeability (WVP) of films was evaluated by a gravimetric test according to ASTM E96 (1993) by means of a Fisher/Payne permeability cup (Carlo Erba, Italy) as previously described.[39] 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 (P_0) at 25 °C (P_0 = 3167 kPa).

Permeability of films to oxygen (P_{O_2}) and carbon dioxide (P_{CO_2}) were examined at 30 °C by using a modified manometric standard method using the equipment described by Di Pierro et al.[39] The tests were performed at 0% RH and a ΔP of 100 kPa for every gas. Ten independent tests for each film were performed.

Statistical Analysis. Microsoft Excel-2002 (Microsoft Co., Redmond, WA) was used for all statistical analyses. The data were subjected to the analysis of variance, and the means were compared using Student's t-test. Differences were considered to be significant at $P < 0.05$.

Results

Flexible, transparent, smooth in their surface, and slightly yellowish films were obtained after drying the film-forming solutions containing chitosan and whey proteins, both in the presence and absence of TGase. The concentration of chitosan in the films was kept constant at 9.2 mg/cm^2, while protein concentration varied from 0 to 1.0 mg/cm^2. Moreover, the cross-linking reaction catalyzed by TGase was performed at pH 6.0, to facilitate the solubility of chitosan.

As reported in Figure 1, the thickness of the films increased with the increase of the amount of whey proteins added, but the presence of TGase in the film-forming solution significantly reduced the entity of the thickness enhancement. CWP films prepared in the presence of TGase were totally insoluble after

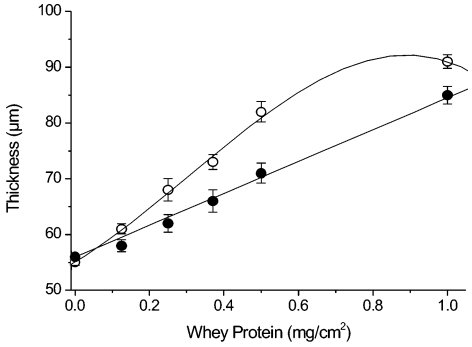

Figure 1. Influence of protein concentration on the thickness of CWP films prepared in the absence (○) and presence (●) of TGase.

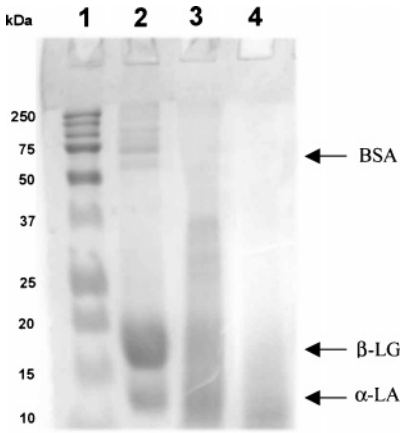

Figure 2. SDS−PAGE analysis (12%) of the protein solubilized from CWP films prepared in the absence (lane 3) and presence (lane 4) of TGase after their incubation for 24 h at 25 °C and pH 4.0. Molecular weight (Precision Plus protein standards dual color, Bio-Rad Laboratories, Inc.) and whey protein standards are reported in lane 1 and lane 2, respectively.

their incubation at 25 °C for 24 h in water solutions buffered at different values of pH. Conversely, about 88%, 55%, and 36% of the initial components were released from the films prepared in the absence of enzyme during their incubation at pH 4.0, 6.0, and 8.0, respectively, under the same experimental conditions (data not shown). The higher resistance of CWP films prepared with TGase was also confirmed by SDS−PAGE analysis illustrated in Figure 2. In fact, a high amount of whey proteins was released from the polymeric matrix after acidic treatment of the films prepared in the absence of TGase (lane 3), whereas the enzyme-catalyzed cross-linking of whey proteins significantly reduced the disruption of the film structure (lane 4). However, the remarkable resistance of CWP films prepared in the presence of TGase to their disruption in water at different pH values was unrelated to their resistance to the action of proteolytic enzymes. In fact, when CWP films prepared with TGase were incubated for 20 h at 25 °C in the presence of two different serine proteases, trypsin and α-chymotrypsin, a high amount of soluble peptides was released from the film materials, as revealed by the quantification of L-tyrosine in the incubation solution (Table 1). Statistically not significant differences were detected when CWP films prepared in the absence of TGase were subjected to proteolysis (data not shown).

Swelling kinetics of CWP films, evaluated in solutions of 20 mM sodium phosphate buffer, pH 7.0, at 25 °C, indicated a maximum degree of swelling for all types of films after 2 h incubation (data not shown). Figure 3 shows the effect of protein concentration on the maximum degree of swelling. In comparison with films made with only chitosan, those prepared by

Table 1. Amount of L-Tyrosine Released from CWP Films Prepared in the Presence of TGase after Treatment with Serine Proteases at pH 7.0 and 25 °C

protein content in the films	L-tyrosine released (μmol/g of film)	
(mg/cm²)	trypsin	α-chymotrypsin
0		
0.125	0.96 ± 0.06	1.01 ± 0.05
0.25	1.02 ± 0.03	1.07 ± 0.07
0.37	1.29 ± 0.04	1.13 ± 0.05
0.5	1.45 ± 0.07	1.14 ± 0.06
1.0	1.60 ± 0.04	1.29 ± 0.05

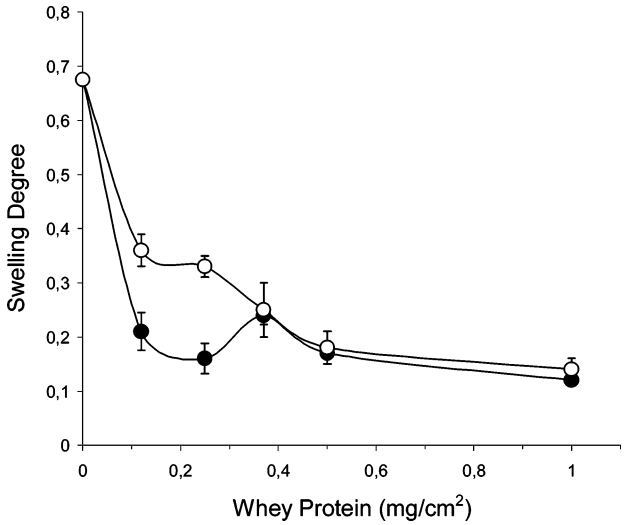

Figure 3. Effect of protein concentration on the maximum degree of swelling of CWP films, prepared in the absence (○) and presence (●) of TGase, determined at 25 °C in 20 mM sodium phosphate buffer, pH 7.0.

mixing chitosan and whey proteins showed, at the equilibrium, a degree of swelling decreasing with the increase of the protein concentration. In addition, CWP films prepared in the presence of TGase showed a significantly lower degree of swelling in comparison with those prepared at low protein concentrations in the absence of the enzyme.

Panel A of Figure 4 shows the effects of protein concentration on the tensile strength of CWP films prepared both in the presence and absence of TGase. In the absence of the enzyme the addition of whey proteins significantly reduced the mechanical resistance of the films obtained with chitosan alone. On the contrary, a marked improvement in the mechanical resistance of CWP films was observed when they were prepared in the presence of TGase. In fact, a progressive marked increase of the tensile strength up to 0.25 mg of protein per cm² of film was observed. A further increase of the proteins provoked a reduction of the tensile strength that, however, remained to values significantly higher than those measured with pure chitosan film.

The influence of the addition of different amounts of whey proteins on the maximum elongation at break of the chitosan films is shown in panel B of Figure 4. A marked decrease of flexibility dependent on the increase in protein amount was determined by analyzing the films prepared both in the presence and absence of TGase, even though the effect resulted significantly higher in the films obtained in the presence of the enzyme.

Finally, to investigate whether the cross-linking action of TGase influenced also the barrier properties of CWP films, their permeability to oxygen, carbon dioxide, and water vapor was determined and compared to that exhibited by un-cross-linked

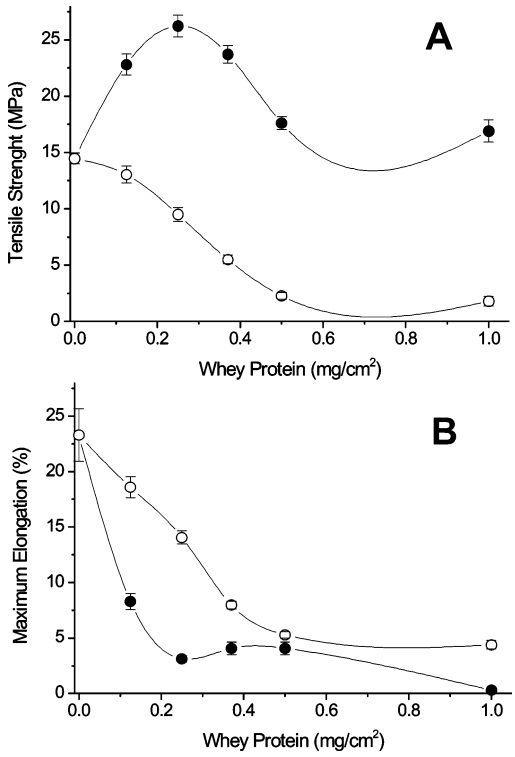

Figure 4. Influence of protein concentration on the tensile strength (A) and maximum elongation at break (B) of CWP films prepared in the absence (○) and presence (●) of TGase.

materials. The addition of protein to the chitosan matrix slightly decreased film permeability to oxygen, whereas the barrier properties were greatly improved by the concurrent presence of TGase (Figure 5, panel A). The low permeability exhibited by CWP films containing 0.25−0.37 mg of whey protein per cm^2 is noteworthy. Similar film permeability behavior was observed toward carbon dioxide for the materials prepared in the presence of the enzyme (Figure 5, panel B). Conversely, the CWP films prepared in the absence of TGase showed a higher permeability toward CO_2 in comparison with that of the pure chitosan-based films. Finally, the addition of whey proteins into the polysaccharide matrix increased its WVP, and this effect was found slightly but significantly lower in materials prepared in the presence of TGase (Figure 5, panel C). However, it should be noted that films containing 0.125−0.37 mg of whey proteins per cm^2 prepared in the presence of the enzyme showed a barrier activity against water vapor similar to that of films constituted by chitosan only.

Discussion

In the present work we described the preparation of several CWP films with different protein concentrations by casting the polymeric solutions both in the presence and absence of the enzyme TGase. Chitosan was selected as the macromolecular matrix for its well-known film-forming and biodegradability properties.[8,9,11−15] The enzymatic formation of intermolecular protein cross-linking in the films was catalyzed since whey proteins are able to act as both acyl donor and acceptor substrates of TGase.[33] The production of these covalent cross-links was supported by several experimental evidences, such as the absence of whey protein native bands in the SDS−PAGE profiles after incubation at pH 4.0 for 24 h of the film prepared in the presence of enzyme, as well as by the higher degree of

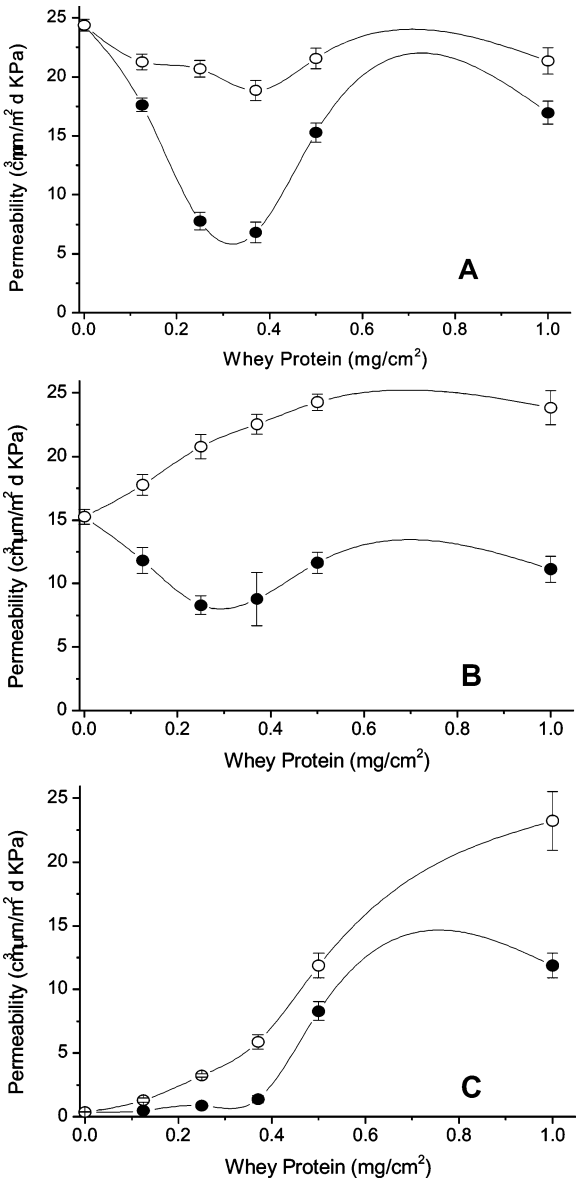

Figure 5. Influence of protein concentration on the permeability to oxygen (A), carbon dioxide (B), and water vapor (C) of CWP films prepared in the absence (○) and presence (●) of TGase.

packing of the latter as revealed by its lower thickness compared to that of the film prepared in the absence of TGase.

All the CWP films showed good optical characteristics, and the edible properties of the ones prepared in the presence of TGase were demonstrated by their easy degradation with serine proteases.

The suitable use of edible packaging strongly depends on their favorable mechanical and barrier properties. Therefore, two different mechanical properties, i.e., tensile strength and elongation to break, were investigated for CWP films cross-linked or not by TGase. The tensile strength provides a measure of film strength, whereas the elongation to break is an indicator of the flexibility of the materials. The results of our experiments indicated that the mechanical resistance of the chitosan films was affected by adding whey proteins. This phenomenon should not be associated with the plasticization of the materials with the polypeptide structures, in agreement with the lower elastic properties exhibited by these films compared to those constituted by pure chitosan (Figure 4, panel B). Conversely, it might be due to the disruption of the three-dimensional structure of the chitosan film following the introduction of globular protein

Table 2. Mechanical Properties of CWP (0.25 mg/cm^2 of Proteins) Films Compared to Those of Other Edible Protein Films

film	tensile strength (MPa)	elongation (%)
chitosan (9.2 mg/cm^2)	14.4 ± 0.58	23.3 ± 2.9
CWP	9.5 ± 0.6	14.1 ± 0.59
CWP + TGase	26.2 ± 0.9	3.1 ± 0.3
pectin−soy flour protein[a]	6.89 ± 0.92	11.619 ± 1.09
pectin−soy flour protein + TGase[a]	12.49 ± 1.05	7.29 ± 1.03
soy[b]	8.5	31.9
whey protein[c]	6.9	41
pea protein[d]	7.3	46.8
formaldehyde cross-linked soy protein[e]	12.6	25
zein[f]	8.7	11.9

[a] Ref 17. [b] Ref 44. [c] Ref 45. [d] Ref 46. [e] Ref 47. [f] Ref 48.

structures into the polysaccharide matrix. But, the surprising result was that the protein covalent cross-linking catalyzed by TGase strongly improved CWP films' mechanical resistance and that this effect was markedly higher for the films containing a low amount of whey protein. These data could be explained with the possible TGase-catalyzed formation of intermolecular linkages among active glutamine and lysine residues of the different whey proteins. Such kind of covalent cross-linking might reduce the intermolecular chain mobility of the chitosan matrix, thus increasing the tensile strength and reducing the extensibility of the films with lower protein concentration.

In this context, it should be noted that chitosan was not found to be able to act as a TGase amino donor substrate during an enzymatic assay of incorporation of the peptide *N*-CBZ-Glu-Gly into the polymeric chain and visualization of the product by ^{1}H NMR analysis (data not shown). Therefore, our experiment confirmed previous data of Chen et al. showing that TGase was not able to cross-link acyl donor protein directly to chitosan amino groups.[40]

As reported in Table 2, CWP film prepared in the presence of the enzyme at 0.25 mg/cm^2 possesses higher mechanical resistance and lower flexibility with respect to several different edible protein films previously studied as potential food packing materials. On the other hand, when higher amounts of whey protein were used, the TGase-mediated formation of high molecular weight protein adducts into the film network could disrupt the three-dimensional structure of the film, thus affecting its mechanical characteristics. This explanation is supported by the results obtained in the experiments of film swelling. It is well-known that the maximum degree of swelling of a polymeric material strongly depends on the amount and nature of the intermolecular chain interactions.[41] Our studies showed that the maximum degree of intermolecular chain interactions was obtained by using CWP films prepared in the presence of TGase and containing 0.25 mg of protein per cm^2. In fact, increasing whey protein concentrations swelling properties resulted identical to those exhibited by the films prepared in the absence of the enzyme.

Finally, we analyzed the barrier properties to the main environmental gases of CWP films since they are important characteristics to be considered for a packing material finalized to increase the shelf life of foods.[42] Our findings showed that the permeability to oxygen and carbon dioxide of CWP films containing 0.25−0.37 mg of whey protein per cm^2 and prepared in the presence of TGase was markedly low, this feature being probably associated with the more compact structure of these materials determined by the enzymatic cross-linking. It should be noted, however, that a reverted permeability behavior, similar to that observed in the mechanical and swelling experiments, was detected by analyzing films containing highest amount of proteins.

Scheme 1. Reduction of Polar Groups in the Films Prepared via TGase-Catalyzed Reaction

Table 3. Barrier Properties of CWP (0.25 mg/cm^2 of Proteins) Films Prepared in the Presence or Absence of TGase Compared to Those of Other Tested Films[a]

film	thickness (μm)	permeability (cm^3 μm/m^2 d kPa)		
		O_2	CO_2	H_2O vapor
CWP	68 ± 0.9	20.6 ± 0.7	20.7 ± 0.95	3.24 ± 0.15
CWP + TGase	62 ± 2	7.8 ± 0.8	8.3 ± 0.7	0.88 ± 0.06
HDPE[b]	11.2 ± 0.3	60 ± 12.4	117 ± 11.5	14 ± 1.5
Mater-BI[b]	12.8 ± 0.6	722 ± 24.6	1355 ± 8.3	1961 ± 12.7
PSF[b]	79 ± 2.2	450 ± 24.2	854 ± 14.2	550 ± 14.6
PSF + TGase[b]	95 ± 3.3	39 ± 4.3	75 ± 6.1	316 ± 22.5

[a] The results are expressed as means of 10 replicates ± standard deviation. Experimental details are given in the text. HDPE, high-density polyethylene; MATER-BI, film made of starch and biodegradable polyesters as poly-ϵ-caprolactone, commercially available from Novamont Spa. (Novara, Italy); PSF, Pectin−soy flour film. [b] Ref 39.

Concerning the WVP characteristics of the films prepared in the presence or absence of TGase, it should be considered that this property is supposed to be dependent on the number of ''available" polar (−OH, −COOH, −NH_2) groups that the polymeric components possess.[42] Thus, the increased WVP showed by the chitosan-based materials after addition of increasing amounts of whey proteins could be directly related to the availability of new polar groups due to the higher content of polypeptide structures. Consequently, the changes in the hydrophilic properties of CWP films associated with the disappearance of primary amino and amide groups and the formation of less hydrophilic secondary amide linkages through the TGase-catalyzed reaction (Scheme 1) should be the main factor contributing to the lower WVP exhibited by these films, in comparison with those prepared in the absence of enzyme.

Finally, the barrier properties of some synthetic (high-density polyethylene), biodegradable (Mater-Bi), and edible (pectin−soy flour protein) films were determined and compared to the ones detected in the present study with the CWP films prepared at 0.25 mg/cm^2 (Table 3). The concurrent markedly low permeability to oxygen, carbon dioxide, and water vapor exhibited by the CWP films prepared in the presence of TGase confer to these new bioplastics interesting potential practical applications, taking into account that poor barrier abilities toward the main environmental gases were considered so far to be the major limitations to a wider use of both biodegradable and edible films.[43]

Acknowledgment. This research was supported by the Ministero dell' Istruzione dell' Università e della Ricerca (former MURST) through a Grant to R. Porta for "Piani di Potenziamento della Ricerca Scientifica e Tecnologica".

References and Notes

(1) Majeti, N. V.; Ravi, K. A review of chitin and chitosan application. *React. Funct. Polym.* **2000**, *46*, 1−27.
(2) Knorr, D. Recovery and utilization of chitin and chitosan in food processing waste management. *Food Technol.* **1991**, *45*, 114−122.
(3) Muzzarelli, R. A. A. Chitosan-based dietary foods. *Carbohydr. Polym.* **1996**, *29*, 309−316.
(4) Prudden, J. F.; Migel, P.; Hanson, P.; Friedrich, L.; Balassa, L. The discovery of a potent pure chemical wound-healing accelerator. *Am. J. Surg.* **1970**, *119*, 560−564.

(5) Minami, S.; Okamoto, Y.; Matsuhashi, A. Application of chitin and chitosan in large animal practice. In *Advances in Chitin and Chitosan*; Brine, C. J., Sanford, P. A., Zikakis, J. P., Eds.; Elsevier: New York, 1992; pp 61−69.
(6) Okamoto, Y.; Minami, S.; Matsuhashi, A. Application of chitin and chitosan in small animals. In *Advances in Chitin and Chitosan*; Brine, C. J., Sanford, P. A., Zikakis, J. P., Eds.; Elsevier: New York, 1992; pp 70−78.
(7) Hirano, S. Some ecologically friendly applications of chitin and chitosan in biotechnology. *Ind. Biotechnol. Polym.* **1999**, *12*, 189−203.
(8) Muzzarelli, R. A. A. Biochemical significance of exogenous chitins and chitosans in animals and patients. *Carbohydr. Polym.* **1993**, *28*, 7−16.
(9) Khan, T. A.; Peh, K. K.; Chang, H. S. Mechanical, bioadhesive strength and biological evaluations of chitosan films for wound dressing. *J. Pharm. Pharm. Sci.* **2000**, *3*, 303−311.
(10) Tsai, G. J.; Su, W. H. Antibacterial activity of shrimp chitosan against *Escherichia coli*. *J. Food Prot.* **1999**, *62*, 239−243.
(11) Ouattara, B.; Simard, R. E.; Piette, G.; Bégin, A.; Holley, R. A. Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *Int. J. Food Microbiol.* **2000**, *62*, 139−148.
(12) Coma, V.; Martial-Gros, A.; Garreau, S.; Copinet, A.; Salin, F.; Deschamps, A. Edible antimicrobial films based on chitosan matrix. *J. Food Sci.* **2002**, *67*, 1162−1169.
(13) Begin, A.; Van Calsteren, M. R. Antimicrobial films produced from chitosan. *Int. J. Biol. Macromol.* **1999**, *26*, 63−67.
(14) Nakatsuka, S.; Andrady, A. L. Permeability of vitamin B-12 in chitosan membranes. Effect of cross-linking and blending with poly(vinyl alcohol) on permeability. *J. Appl. Polym. Sci.* **1992**, *44*, 17−28.
(15) Thacharodi, D.; Rao, K. P. Propanolol hydrochloride release behaviour of cross-linked chitosan membranes. *J. Chem. Technol. Biotechnol.* **1993**, *58*, 177−181.
(16) Kumar, G.; Bristow, J. F.; Smith, P. J.; Payne G. F. Enzymatic gelation of the natural polymer chitosan. *Polymer* **2000**, *41*, 2157−2168.
(17) Mariniello, L.; Di Pierro, P.; Esposito, C.; Sorrentino, A.; Masi, P.; Porta, R. Preparation and mechanical properties of edible pectin−soy flour films obtained in the absence or presence of transglutaminase. *J. Biotechnol.* **2003**, *102*, 191−198.
(18) Folk, J. E. Transglutaminases. *Annu. Rev. Biochem.* **1980**, *49*, 517−531.
(19) Aeschlimann, D.; Paulsson, M. Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thromb. Haemostasis* **1994**, *71*, 402−415.
(20) Esposito, C.; Mancuso, F.; Calignano, A.; Di Pierro, P.; Pucci, P.; Porta, R. Neurokinin receptors could be differentiated by their capacity to respond to the transglutaminase-synthesized γ-(glutamyl^5) spermidine derivative of substance P. *J. Neurochem.* **1995**, *65*, 420−426.
(21) Esposito, C.; Costa, C.; Amoresano, A.; Mariniello, L.; Sommella, M. G.; Caputo, I.; Porta, R. Transglutaminase-mediated amine incorporation into substance P protects the peptide against proteolysis in vitro. *Regul. Pept.* **1999**, *84*, 75−80.
(22) Mancuso, F.; Costa, C.; Calignano, A.; Mariniello, L.; Rossi, F.; Porta, R.; Esposito, C. Transglutaminase-synthesized γ-(glutamyl^5) spermidine derivative of substance P is a selective tool for neurokinin-2 receptors characterization. *Peptides* **1998**, *19*, 683−690.
(23) Mancuso, F.; Porta, R.; Calignano, A.; Di Pierro, P.; Sommella, M. C.; Esposito, C. Substance P and its transglutaminase-synthesized derivatives elicit yawning behavior via nitric oxide in rats. *Peptides* **2001**, *22*, 1453−1457.
(24) Persico, P.; Calignano, A.; Mancuso, F.; Marino, G.; Pucci, P.; Esposito, C.; Mariniello, L.; Porta, R. Substance P inactivation by transglutaminase in vitro. *Peptides* **1992**, *13*, 151−154.
(25) Bechtold, U.; Otterbach, J. T.; Pasternack, R.; Fuchsbauer, H. L. Enzymic preparation of protein G-peroxidase conjugates catalysed by transglutaminase. *J. Biochem.* **2000**, *127*, 239−245.
(26) Josten, A.; Meusel, M.; Spener, F. Microbial transglutaminase-mediated synthesis of hapten−protein conjugates for immunoassays. *Anal. Biochem.* **1998**, *258*, 202−208.
(27) Mancuso, F.; Costa, C.; Calignano, A.; Mariniello, L.; Rossi, F.; Porta, R.; Esposito, C. Transglutaminase-synthesized γ-(glutamyl^5) spermidine derivative of substance P is a selective tool for neurokinin-2 receptors characterization. *Peptides* **1998**, *19*, 683−690.
(28) Tufano, M. A.; Porta, R.; Farzati, B.; Di Pierro, P.; Rossano, F.; Catalanotti, P.; Baroni, A.; Metafora, S. Rat seminal vesicle protein SV-IV and its transglutaminase-synthesized polyaminated derivative SPD2-SV-IV induce cytokine release from human resting lymphocytes and monocytes in vitro. *Cell. Immunol.* **1996**, *168*, 148−157.
(29) Villalonga, R.; Fernández, M.; Fragoso, A.; Cao, R.; Di Pierro, P.; Mariniello, L.; Porta, R. Transglutaminase-catalyzed synthesis of trypsin−cyclodextrin conjugates. Kinetics and stability properties. *Biotechnol. Bioeng.* **2003**, *81*, 732−737.
(30) Villalonga, R.; Fernández, M.; Fragoso, A.; Cao, R.; Mariniello, L.; Porta, R. Thermal stabilization of trypsin by enzymatic modification with β-cyclodextrin derivatives. *Biotechnol. Appl. Biochem.* **2003**, *38*, 53−59.
(31) Motoki, M.; Aso, H.; Seguro, K.; Nio, N. Casein film prepared using transglutaminase. *Agric. Biol. Chem.* **1987**, *51*, 993−996.
(32) Lim, L. T.; Mine, Y.; Tung, M. A. Transglutaminase cross-linked egg white protein films: tensile properties and mechanical permeability. *J. Agric. Food Chem.* **1998**, *46*, 4022−4029.
(33) Mahmoud, R.; Savello, P. A. Solubility and hydrolyzability of films produced by transglutaminase catalytic cross-linking of whey protein. *J. Dairy Sci.* **1993**, *76*, 29−35.
(34) Kinsella, J. E. Milk proteins: physiochemical and functional properties. *CRC Crit. Rev. Food Sci.* **1984**, *21*, 197−262.
(35) Baxter, A.; Dillon, M.; Taylor, K. D. A.; Roberts, G. A. F. Improved method for IR determination of the degree of N-acetylation of chitosan. *Int. J. Biol. Macromol.* **1992**, *14*, 166−169.
(36) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **1976**, *76*, 248−250.
(37) Stuchell, Y. M.; Krochta, J. M. Enzymatic treatment and thermal effects on edible soy protein films. *J. Food. Sci.* **1994**, *59*, 1332−1337.
(38) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(39) Di Pierro, P.; Mariniello, L.; Giosafatto, C. V. L.; Masi, P.; Porta, R. Solubility and permeability properties of edible pectin−soy flour films obtained in the absence or presence of transglutaminase. *Food Biotechnol.* **2005**, *19*, 37−49.
(40) Chen, T.; Embree, H. D.; Brown, E. M.; Taylor, M. M.; Payne, G. F. Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. *Biomaterials* **2003**, *24*, 2831−2841.
(41) Flory, P. J. *Principles of Polymer Chemistry*; Cornell University Press: Ithaca, NY, 1953.
(42) Miller, K. S.; Krochta, J. M. Oxygen and aroma barrier properties of edible films: A review. *Trends Food Sci. Technol.* **1997**, *8*, 228−237.
(43) Krochta, J. M.; de Mulder-Johnston, C. Edible and biodegradable polymer films: Challenges and opportunities. *Food Technol.* **1997**, *51*, 61−74.
(44) Kunte, L. A.; Gennadios, A.; Cuppett, S. L.; Hanna, M. A.; Weller, C. L. Cast films from soy protein isolates and fractions. *Cereal Chem.* **1997**, *74*, 115−118.
(45) Pérez-Gago, M. B.; Nadaud, P.; Krochta, J. M. Water vapour permeability, solubility and tensil properties of heat-denatured versus native whey protein films. *J. Food Sci.* **1999**, *64*, 1034−1037.
(46) Choi, W. S.; Han, J. H. Physical and mechanical properties of pea-protein-based edible films. *J. Food Sci.* **2001**, *66*, 319−322.
(47) Rhim, J. W.; Weller, C. L. Properties of formaldehyde adsorbed soy protein isolate films. *Food Sci. Biotechnol.* **2000**, *9*, 228−233.
(48) Lai, H. M.; Padua, G. M. Properties and microstructure of plasticized zein films. *Cereal Chem.* **1997**, *74*, 771−775.

BM050661U