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Solubility and Permeability Properties of Edible Pectin-Soy Flour Films Obtained in the Absence or Presence of Transglutaminase

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The permeability characteristics and water-solubility of edible pectin–soy flour films obtained in the absence or presence of the enzyme transglutaminase were investigated and compared with those possessed by both the commonly used high density polyethylene film and the biodegradable Mater-Bi® film. The soy protein transglutaminase-catalyzed crosslinking was found to determine a marked decrease in the solubility of the pectin–soy flour films both at different pH and in different denaturing conditions with respect to the films obtained in the absence of the enzyme, even though their solubility remains higher than that of the commercially available polyethylene and Mater-Bi® films. Transglutaminase treatment was also shown to significantly increase pectin–soy flour film barrier properties to oxygen, carbon dioxide and water vapour. In particular, the films obtained in the presence of the enzyme exhibited a permeability to oxygen and carbon dioxide even lower than that possessed by polyethylene films. Our results suggest a possible use of the transglutaminase-polymerized pectin–soy flour films as wrapping of food products requiring a packaging allowing low gas exchange with the environment. Furthermore, the application of these films as coatings to conventional oral dosage forms could provide a viable means of delivering drugs to the colon.

Key Words: edible films; pectin; permeability; soy flour; transglutaminase

INTRODUCTION

The increasing consumer demand for ready-to-eat foods having high quality and long shelf-life characteristics requires the availability of preserved products

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that keep their natural and fresh appearance as long as possible and, at the same time, are safe to eat. Packaging is important for both food physical protection and the right physico-chemical conditions that are essential to obtain a satisfactory shelf-life. Thus, a proper choice of a packaging material endowed with appropriate gas- and water-vapour-barrier properties is crucial to prevent product deterioration due to both chemical and microbiological factors, and to maintain the hygiene status despite frequent handling. The production of films with selective gas permeability is potentially of great interest for controlling gas exchanges during food storage. As is well known, gas permeability of films depends on several factors. Besides the chemical–physical characteristics of the gas and the environmental conditions such as temperature and relative humidity (Gontard et al., 1996), the composition of the material of which the film is made is extremely important. Recently, the use of edible films as packaging material has been proposed as an alternative to chemically synthesized biodegradable or non-degradable polymeric films and, among these, several films constituted by polysaccharides and/or proteins have been extensively studied (Guilbert, 1986; Aydt et al., 1991; Cuq et al., 1995). Some of these investigations have demonstrated that the mechanical and the barrier properties in relation to carbon dioxide of protein-based films are better than those possessed by polysaccharide-based films (Aydt et al., 1991; Cuq et al., 1995), whereas the latter show a more effective barrier to oxygen (Nisperos-Carriedo, 1994). It is well known that in both kinds of films electrostatic, dipole–dipole and hydrophobic interactions, as well as covalent and hydrogen bonds, are responsible for the structure of the film and, consequently for its properties. In particular, it has been established that a high number of crosslinks decreases film permeability and solubility (Rogers, 1985). In the case of protein films, crosslinking can be accomplished either chemically or enzymatically (Fenney and Whitaker 1988). For example, proteins can be crosslinked via glutaraldehyde, but, due to its high toxicity typical of many chemical agents, its use is not recommended for preparing films for biomedical and food applications. For this reasons, edible film protein crosslinking by enzymatic tools is receiving more considerable attention lately. Many reports refer to the use of peroxidase, which has been reported to reduce both tensile strength and extensibility of films derived from thermally denatured soy or gluten (Michon et al., 1999). Another enzyme, named transglutaminase (TGase, E.C. 2.3.2.13), has also been studied to obtain films derived from different proteins of food origins, such as α_{s1}-casein (Motoki et al., 1987), whey proteins (Mahmoud and Savello, 1993; Yildirim and Hettiarachchy, 1997), 11S globulin (Yildirim and Hettiarachchy, 1997) and egg-white protein (Lim et al., 1998). TGase catalyzes the introduction of ε–(γ–glutamyl)-lysine crosslinks into proteins. In the reaction, which causes release of ammonia, the enzyme promotes the acyl transfer of the γ-carboxyamide group of glutamine into the ε-amino group of lysine (Folk, 1980).

Recently we reported (Mariniello et al., 2003) that the treatment with TGase improved tensile strength and extensibility of a film constituted by pectin and soy flour proteins. In this paper, we describe the experiments carried out to determine the solubility in water under different experimental conditions, as well as the barrier properties to oxygen, carbon dioxide and water vapour, of pectin–soy flour (PSF)-based films obtained in the absence or presence of the enzyme.

MATERIALS AND METHODS

Materials

Streptoverticillium Ca^{2+}-independent TGase was obtained from Ajinomoto Co. (Japan). The enzyme was prepared by dissolving the commercial preparation in distilled water. The specific activity of the enzyme preparation was 45 U mL^{-1} as determined using the following procedure: 10, 20, 40, 80 μg of enzyme were incubated in 100 μL of 125 mM Tris-HCl, pH 8.0, in the presence of 200 μg of N, N-dimethylated casein (Sigma) and 6.5 pmol [^{3}H] spermidine (specific activity 15.3 nCi $pmol^{-1}$) (Amersham) for 1 h a 37°C. One unit of TGase was defined as μg of enzyme able to incorporate 0.8 pmol of [^{3}H] spermidine into N,N-dimethylated casein under the described conditions. Defatted soy flour (type I), pectin USP from apple and all other reagents were purchased from Sigma Chemical Co. (St Louis, MO). Mater-Bi® film, made of starch and biodegradable polyesters as poly-ε-caprolactone, was purchased by Novamont Spa (Novara, Italy), whereas high density polyethylene (HDPE) films were derived from common plastic bags.

Film-Making Procedure

Defatted soy flour, in which all soy seed proteins occur, was dissolved (13 mg mL^{-1}) into distilled water brought to pH 9.0 by NaOH addition. Pectin was dissolved in distilled water at a concentration of 16 mg mL^{-1}. To obtain films with the desired ratio of each component, 4.3 mL of soy flour solution and 7 mL of pectin solution were gently mixed and HCl was added to the solution until a pH value of 4.0 was obtained. The final solution was de-aerated under vacuum before film casting to prevent pinhole formation and then transferred into polystyrene Petri dishes (60 mm × 15 mm) to be dried 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). TGase polymerized PSF films were obtained by adding 1.5 U of the enzyme to the final solution of pectin and soy flour, ensuring that the enzyme was evenly dispersed throughout the aqueous phase within 1 min.

Film thickness was measured using a micrometer model HO62 with sensitivity of ±2 μm (Metrocontrol Srl, Casoria (Na), Italy). Film strips were placed between the jaws of the micrometer and the gap reduced until the first indication of contact. Mean thickness (μm) of films were determined from the average of measurements at 10 locations.

Film Solubility

Film solubility was tested both in water solutions that were buffered and contained denaturing agents. The procedure was similar to that described by Stuchell et al. (1994). Small pieces of film (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 × 15 mm) with 10 mL of 0.1 M citrate (pH 3.0), acetate (pH 4.0), phosphate (pH 6.0) or Tris-HCl (pH 8.0) buffer solution. In different experiments, the film pieces were incubated under the same conditions in 10 mL of 10% SDS, 10% β-mercaptoethanol or 6.6 M urea. At the end of the incubation the samples were poured onto Whatman No.1 qualitative filter paper. The non-dissolved material, taken off by the filter with 10 mL of distilled water, was 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:

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

Film Water Vapour Permeability

Film water vapour permeability (WVP) was evaluated by a gravimetric test according to ASTM E96 (1993) by means of a Fisher/Payne permeability cup (Carlo Erba, Italy). Briefly, 3 g of silica gel were introduced in each cup and a disk of film sample with a diameter of about 6 cm was placed on the top of the cup and sealed by means of a ring kept in place by three tight clamps. The film area exposed to vapour 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 of 0.8434. The desiccator was stored in a Heareus thermostated incubator at 25.0 ± 0.1°C. Each 24 h the cups were weighed until a constant increase in weight was achieved. Water vapour transmission rate through the film was estimated by the linear portion of the diagram obtained by plotting weight increment of the cup as a function of time. It was assumed that steady-state was reached once the regression analysis made by using the last four data points resulted in $r^2 \geq 0.998$.

WVP was calculated from the equation:

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

where dm/dt is the slope of the cup weight vs time once steady-state was reached, X is the film thickness, A the film exposed area and Δp the water vapour pressure across the film. By assuming that the vapour pressure inside the cup, due to the presence of silica gel, can be taken equal to zero, Δp becomes equal to vapour pressure inside the desiccator given by the product of the water activity and the vapour pressure (P_0) at 25°C (P_0 =3.167 kPa).

Film Gas Permeability

Film permeability to oxygen and carbon dioxide was examined at 30°C and 0% relative humidity by using a modified manometric method by means of the equipment schematized in Fig. 1. The film sample was set in the gas transmission cell across which a differential pressure was established. Chamber A from the upper part of the gas transmission cell to valve H was a high pressure zone, whereas chamber B from the lower part of the gas transmission cell to valve C was a low pressure zone.

Before each test, the equipment was equilibrated in the following way. All the valves were opened except valves H, B, and A. Valve A was opened to reduce the internal pressure from 100 kPa to 4 kPa in both high and low pressure zones. Then valve A was closed and valve H was opened until the pressure of gas (CO_2 or O_2) reached 100 kPa. This procedure was repeated four times every 20 min. Both CO_2 and O_2 gases were dried by passing through a gas-drying column containing anhydrous calcium chloride before entering the system. Then the pressure was brought back to 4 kPa, valves C and F were closed and the gas was introduced into chamber A by opening valve H until the pressure reached 100 kPa. Due to the high volume of the gas reservoir occurring in chamber A the upstream pressure during the test was assumed constant. The gas transmission rate through the film was determined by measuring the variation of the pressure in chamber B by means of a low-pressure transducer whose sensitivity was quite high because of the low volume of the downstream chamber. It was assumed that the steady-state condition was reached once the regression analysis, made by using the last five data points, resulted in $r^2 \geq 0.998$.

The gas transmission rate was determined according to the equation

$$J = (slope / A)(V_{bp} / RT)$$

where J was the gas transmission rate through the film (mol day^{-1} $m^{-2)}$, A the area of gas transmission (m^2), R the gas universal constant (kPa $cm^3 mol^{-1}$ K^{-1}), T the

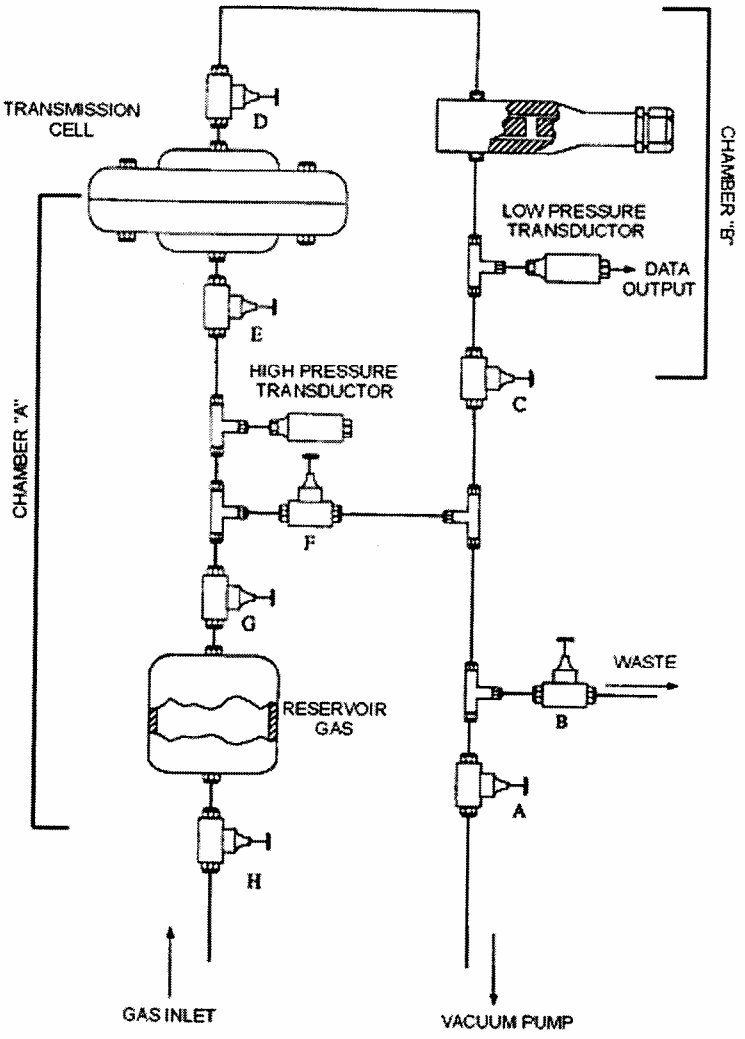

Figure 1: Diagram of the manometric equipment used to determine the permeability of the films to oxygen and carbon dioxide. Further details are reported in the text.

absolute temperature (K), V_{bp} the volume of the low pressure zone (volume between the film and valve C) (cm^3), *slope* the slope of the linear portion of the curve representative of the pressure variation in the low pressure zone (kPa day^{-1}).

Gas permeability through the film samples P was calculated as follows:

$$P = V_m \cdot JX/(\Delta p)$$

where P has a dimension of cm^3 μm m^{-2} day^{-1} kPa^{-1}, Δp (kPa) is the gas differential pressure across the film which coincides with the gas pressure in the

high pressure zone , X is the thickness of the film (μm) and V_m is the molar volume of carbon dioxide and oxygen at STD conditions (22263 and 22393 cm^3 mol^{-1} respectively).

Statistical Analysis

Pharmacologic Calculation System 4.2 (PCS) was used for all the statistical evaluations. The data were analyzed with one-way analysis of variance (ANOVA) and means were compared using the Newman–Keuls Test to determine the significant effect of the TGase-mediated crosslinking on the permeability and solubility properties. Differences between means were considered to be significant at $p < 0.05$.

RESULTS

The water solubility of edible PSF films, prepared in the presence or absence of TGase, was investigated after 24 h incubation both at different values of pH (Fig. 2) and in the presence of different denaturing agents (Fig. 3). Solubility studies were conducted also with HDPE and Mater-Bi® films which resulted in complete insolubility in water following the two treatments tested (data not shown). A significantly lower solubility of PSF films obtained in the absence of TGase was observed at pH 4 (Fig. 2). This result is in agreement with the model of pectin–protein interaction proposed by Takada and Nelson

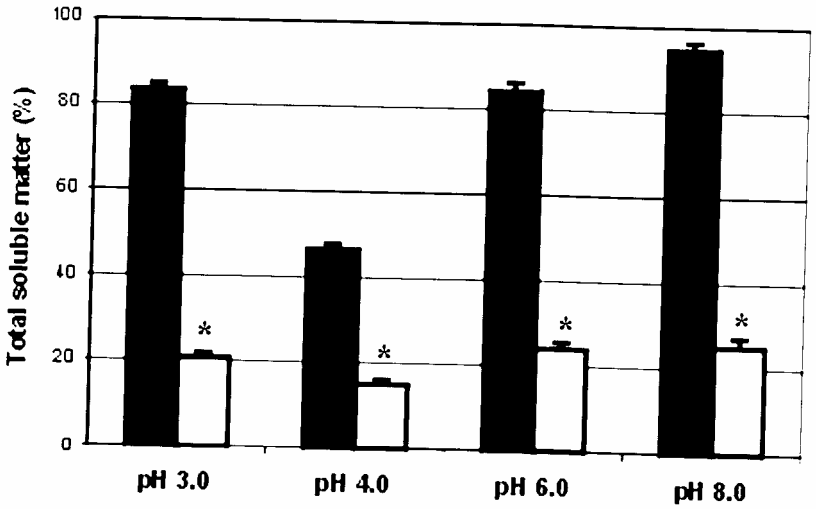

Figure 2: Total soluble matter (% dry weight) recovered after 24 h incubation at different pH of PSF films obtained in the absence (■)or presence (□) of TGase. The results are expressed as means of ten replicates ± standard error. *PSF with TGase film vs PSF without TGase film significant at $p < 0.01$. Further experimental details are given in the text.

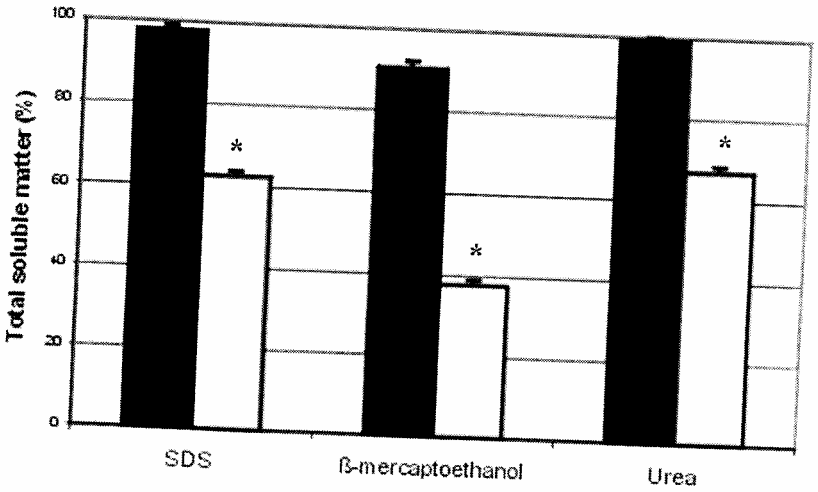

Figure 3: Total soluble matter (% dry weight) recovered after 24 h incubation in different denaturing agents of PSF films obtained in the absence (■) or presence (□) of TGase. The results are expressed as means of ten replicates ± standard error. *PSF with TGase film vs PSF without TGase film significant at $p < 0.01$. Further experimental details are given in the text.

(1983) which refers to the importance of pectin pKa and protein pI to establish the formation of electrostatic complex. Since soy protein pI is 4.5 and pKa of pectin is 3.55–4.10, electrostatic interactions occur at pH 4.0 between the carboxyl group of pectin and ammonia groups of the polypeptide chain, thus stabilizing the network of the film and reducing its solubility.

Conversely, PSF films prepared in the presence of TGase showed a significant decrease in solubility ($p < 0.01$) compared to uncrosslinked controls following 24 h incubation at the different pH (Fig. 2), certainly because of the presence of intermolecular soy protein isopeptide bonds produced by the enzyme (Mariniello et al., 2003). The solubility of the PSF films obtained in the presence of TGase resulted lower than that of the controls also following treatment with SDS and urea, and much lower after incubation in the presence of β-mercaptoethanol (Fig. 3). Similar findings were obtained by Mahmoud and Savello (1993) with TGase-polymerized whey protein films, and by Yildirim and Hettiarachchy (1997) with films containing whey proteins and soybean 11S globulin crosslinked by the enzyme. The lower solubility in β-mercaptoethanol suggests that, in agreement with Yildirim and Hettiarachchy (1997), the contribution of disulphide bonds to the film integrity is less important than both hydrophobic interactions and hydrogen bonds.

To investigate whether soy protein crosslinks produced by TGase also influence the barrier properties of PSF films, permeability to oxygen, carbon dioxide, and water vapour was examined and compared to that exhibited not only by uncrosslinked PSF, but also by HDPE and Mater-Bi® films. It is well known that many factors affect the film barrier properties besides the

Table 1: Barrier properties of the tested films.[a]

Film	Tthickness (μm)	Permeability (cm^3 μm m^{-2} day^{-1} kPa^{-1}) Oxygen	Carbon dioxide	Water vapour
HDPE	11.2 ± 0.3	60.1 ± 12.4	117.4 ± 11.3	13.5 ± 1.0
Mater-Bi®	12.8 ± 0.6	721.5 ± 24.0	1354.6 ± 8.4	1961.4 ± 12.3
PSF	79.3 ± 2.3	449.8 ± 23.6	854.3 ± 14.4	549.5 ± 13.8.
PSF + TGase	94.8 ± 3.3	38.5 ± 4.2[b]	74.6 ± 6.1[b]	316.3 ± 22.46[b]

[a]The results are expressed as means of ten replicates ± standard deviation. Experimental details are given in the text.
[b]PSF + TGase vs HDPE significant at ($p < 0.01$); PSF + TGase vs Mater-Bi® significant at ($p < 0.01$); PSF + TGase vs PSF significant at ($p < 0.01$).

intermolecular crosslinking. Among these the polarity and the density of the molecules constituting the film, as well as the high level of chain-to chain packing, are the most important. In fact, these factors determine the film free volume that is a measure of the interstitial space among the different molecules (Miller and Krochta 1997). The results reported in Table 1 demonstrate that the film permeability to oxygen and carbon dioxide were strongly influenced by the TGase-produced soy protein crosslinks, since the PSF films obtained in the presence of the enzyme showed a significant decrease in permeability to both gases compared to control PSF films ($p < 0.01$). In particular, we found extremely interesting that the edible PSF films obtained in the presence of TGase showed a reduced permeability to both oxygen and carbon dioxide when compared to HDPE films ($p < 0.01$). In the same manner, permeability to water vapour was founded to be influenced by TGase-mediated crosslinks, even though a modest decrease was observed. Therefore, it is reasonable to hypothesize that TGase-mediated soy protein crosslinking markedly reduces the free volume inside the PSF film thus influencing its barrier properties.

DISCUSSION

Petrochemical based plastics (polyesters, polyolefines, polyamides, etc.) have been so far extensively used as packaging materials mostly because of their availability in large amounts at low cost. Although they possess favourable mechanical and permeability properties they are totally non-biodegradable, thus leading to dramatic environmental pollution. As a consequence, the use of synthetic plastics in any form or shape should be more and more restricted and, therefore, their gradual replacement with biodegradable and edible materials, especially those derived from natural resources, appears not only desirable but also predictable.

There has been considerable research on development of biological films for various packaging or coating applications (Tharanathan, 2003), but few have

been applied commercially due to the non-competitive costs and limitations on their performance and physicochemical properties. However, among bioplastics originating from naturally occurring biopolymers polysaccharide/protein/lipid mixtures are very promising (Tharanathan and Saroja, 2001) as they can give rise to different kinds of biodegradable and edible wraps with different characteristics, so that their formulations can be tailor-made to suit to the needs of a specific commodity.

The results reported in the present paper describe the solubility and the permeability properties for water vapour, oxygen, and carbon dioxide of an edible film constituted by pectin and soy flour proteins enzymatically crosslinked by TGase. Pectin, a complex anionic polysaccharide composed by α-1,4-linked D-galacturonic acid residues wherein the uronic acid carboxyls are either fully or partially methyl esterified, is known to produce excellent films with potential commercial uses as water soluble pouches for detergents and different medical delivery systems and devices. On the other hand, proteinaceous hydrocolloids of plant (Meixueir et al., 2000) or animal (Kaya and Kaya, 2000) origin are sometimes utilized in coating formulations to supplement the nutritional value of wrapped food. Since the degree of cohesion depends on polymer structure, which determines the mechanical and barrier properties of the resulting coating, film formation normally involves inter- and intramolecular associations of the polymer chains which form a semi-rigid three-dimensional network that entraps and immobilizes the solvent. For these reasons, we thought to produce a hydrocolloid PSF film in which the occurring proteins were crosslinked by TGase, an enzyme able to produce isopeptide bonds among the soy polypeptide chains. In this way TGase appears a very promising film-forming agent which could be potentially a useful tool in biodegradable/edible packaging of certain foods, to extend their shelf-life, and in providing drug coatings to produce conventional oral dosage forms.

In a recent investigation (Mariniello et al., 2003) we demonstrated by microstructural analyses that the PSF film obtained in the presence of TGase has a smoother surface and higher homogeneity in comparison with the PSF film obtained in the absence of enzyme, whereas studies on its mechanical properties indicated that the enzyme was able to increase film strength and to reduce its flexibility. In the present work we completed the general characterization of such a film by demonstrating that TGase-produced soy protein crosslinks decreased the water solubility and the permeability to water vapour, oxygen, and carbon dioxide of PSF film. Hydrocolloids, being hydrophilic, are known to be poor moisture barriers, a property often partially compensated by lipid addition. Therefore, the major limitation of hydrocolloids in packaging, compared to synthetic polymers, remains their relatively lower water resistance. Data reported in this study indicate that TGase treatment of our hydrocolloid films improves water resistance, which is comparable to the that of synthetic films. In fact, both the reduced water vapour transmission and the decreased total solu-

ble matter recovered after film incubation at different pH values, or in the presence of various denaturing agents, make the TGase-crosslinked PSF film quite different from the biodegradable ones so far proposed. This difference was still more evident after analysis of the barrier properties toward oxygen and carbon dioxide which were also stronger than of widely used commercial polyethylene film. Hence, to our knowledge, this is the first time that an hydrocolloid film is reported to be endowed with permeability features on the same order of magnitude of synthetic ones. Although our data are in line with previously reported results demonstrating that a high level of crosslinks provokes a decrease in polymer permeability (Guilbert, 1986), conversely, more recently Yildirim and Hettiarachchy (1997) demonstrated that permeability to water vapour increases in biopolymers obtained with proteins modified by TGase as a consequence of the larger size of the pores generated in the film by enzyme-produced crosslinks. This discrepancy with our findings could be explained by the concurrent presence in the film of pectin molecules which, entrapped in the protein network, might consistently reduce the size of the pores generated by TGase.

In conclusion, the results reported in the present paper suggest a possible use of PSF films obtained in the presence of TGase as edible wraps for food packaging. In particular, because of their barrier properties, they would find application in preventing quality changes in products, like meat pieces or high-moisture low-sugar cakes, that require envelopment by films with low permeability to oxygen and carbon dioxide and moderate permeability to water vapour. Furthermore, since the use of natural hydrophilic polymers as drug carriers is receiving growing attention, especially from the viewpoint of environmental pollution and safety, TGase-crosslinked PSF films might be also considered as potential pharmaceutical coatings for a controlled release of drugs and/or macromolecules such vaccines and peptides (Bodmeier, 1989). In certain circumstances, different rates of delivery as the dosage form traverses the gastrointestinal tract may be advantageous (Lemmer, 1991). In fact, colonic drug delivery has important implications in a number of key therapeutic areas, including the topical treatment of colonic disorders such as irritable bowel syndrome, Crohn's disease, ulcerative colitis, and carcinomas (Siew et al., 2000). The low solubility at different pH values, together with their predictable degradation when exposed to bacterial pectinolytic enzymes at the colon level (Ashford et al., 1993; Ofori-Kwakye and Fell, 2001), could provide the TGase-crosslinked PSF film even as a potential coating for colonic drug delivery.

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