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Transglutaminase-catalyzed preparation of chitosan–ovalbumin films

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Abstract

Microbial transglutaminase was employed as catalyst for preparing chitosan–ovalbumin films. The films showed low solubility at a wide range of pH and underwent to a good enzymatic hydrolysis with trypsin. The degree of swelling was reduced and the mechanical resistance of the chitosan–ovalbumin films increased from 24 to 35 MPa after enzymatic treatment with transglutaminase. The barrier efficiency toward water vapour was slightly improved for the films prepared by transglutaminase-mediated cross-linking.

Keywords: Edible films; Chitosan; Egg white proteins; Transglutaminase; Mechanical strength; Permeability

1. Introduction

Synthetic polymeric films are widely employed as food packing materials and protective coating for pharmaceutical products. However, their industrial production and use are currently associated with a negative environmental impact. For this reason, the demand for biodegradable polymeric films produced from renewable natural resources has increased during last decades [1–3].

Biopolymers including proteins, polysaccharides, lipids or their combinations have been used to prepare edible films [3–6]. Nevertheless, these biopolymer-based materials often lack mechanical strength and cost advantage. That is why the development of new methods for preparing resistant and cost effective edible films receives considerable attention.

Recently we employed transglutaminase as biocatalyst for preparing polysaccharide-protein films through enzymatic cross-linking reactions [4,7]. Transglutaminase (TGase, protein-glutamine:amine 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-carboxyamide group of glutamine serves as the acyl donor and the ε-amino group of lysine serves as the acyl acceptor [8]. Moreover, reactive lysine may be substituted by several compounds containing primary amino groups, giving rise to a variety of protein-(γ-glutamyl) derivatives [8,9]. In this sense, TGase was previously and successfully used to modify biological activities of peptides [10,11] and proteins [12–14] by covalently linking amines to their reactive endo-glutamine residues. Moreover, TGase was employed for improving the properties for many protein-based films [15–17].

In the present study we report the use of TGase for producing films of chitosan and egg white proteins. These materials were characterized according to their mechanical, digestibility and permeability properties. Chitosan [poly-β-(1-4)-2-amino-2-deoxy-D-glucose], a hydrophilic polyelectrolyte prepared by *N*-deacetylation of chitin [18], is produced from wastes generated from the crustacean processing (shrimp and crabs). This natural polymer has been referred as un unique materials for preparing films.

2. Materials and methods

2.1. Materials

Chitosan from lobster shells (degree of *N*-acetylation = 9.0%) [19] was obtained by Prof. R.A.A. Muzzarelli (Ancona University, Italy). *Streptoverticillium* Ca^{2+}-independent TGase was obtained from Ajinomoto Co. (Japan). Albumin from chicken egg (ovalbumin) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were analytical grade.

2.2. Film making

A concentrated chitosan solution (2.5%, w/v) was prepared by adding 2.5 g chitosan (hydrochloride salt) to 90 ml phosphate buffer 0.1 M pH 6.0 and stirring

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overnight. The solution was then adjusted to 100 ml with phosphate buffer 0.1 M pH 6.0. Egg white proteins containing solution was prepared by adding 5 g of protein to 90 ml distilled water and stirring up to complete solubilization. The solution was then adjusted to 100 ml with distilled water and the amount of proteins was determined by Bradford's method [20]. TGase was prepared by dissolving 180 mg of the commercial preparation in 1 ml of distilled water. The specific activity of the enzyme preparation, determined according to Mariniello et al. [4], was 5.8 U/ml. 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.

Film-forming solutions were obtained on polystyrene Petri dishes (60 mm × 15 mm) by mixing 176 μl of 50% glycerol (v/v) with chitosan (9.2 mg/cm^2 final concentration) and egg white proteins (1.0 mg/cm^2), respectively. The solutions were de-aerated under vacuum prior to casting films, to prevent pinhole formation in the film and then transferred into dishes. The solutions were allowed to dry at 50 °C overnight under dry air circulation. For films obtained in the presence of TGase 22.5 U of the enzyme were added, ensuring that the catalyst was evenly dispersed throughout the aqueous phase by gently mixing for 1 min. The obtained film was peeled from the Petri dish and stored at 20 °C in a desiccator (50% RH). 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 was determined from the average of measurements at 10 locations.

2.3. Film solubility

Film solubility was tested in buffered water solutions. The procedure was similar to that described by Stuchell and Krochta [21]. 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 in the nearest 0.1 mg to determine the initial dry weight of the film. Each film piece was incubated at 25 °C for 24 h into 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 #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:

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

2.4. Swelling and digestibility properties

The swelling studies of the 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 5 h under continuous shaking. The films were removed at scheduled times, gently dried on filter paper, and weighed in analytical balance. The degrees of swelling were calculated by using the following equation:

$$\text{degree of swelling} = \frac{W_s - W}{W}$$

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

Degradability studies were performed by first swelling the 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 (1.0 μg protease/g film) for 20 h under the same conditions. Aliquots (500 μl) were then removed, mixed with 500 μl 1 M NaOH and quantified for L-tyrosine with the Folin–Ciocalteau reagent for phenols.

2.5. Film mechanical properties

The tensile strength of the films was measured by using an Instron Universal Testing Instrument Model No 4301 (Instron Engineering Corp., Canton, MA). Film samples were cut into 10–11 mm wide and 100 mm length strips using a sharp razor blade. The strips were equilibrated overnight at 50 ± 5% RH and 23 ± 2 °C in an environmental chamber. Ten samples of each film type were tested. Tensile properties of the films 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 crosshead speed was 30 mm/min in a Tension Mode.

2.6. Water vapour permeability properties

Water vapour permeability of films was evaluated by gravimetric test according to ASTM E96 (1993) by means of a Fisher/Payne permeability Cup (Carlo Erba, Italy) as previously described [4]. Three grams of silica gel were introduced in each cup. Film sample having diameter of about 6 cm was placed 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 vapour transmission was 10 cm^2. The cups containing silica gel were weighed and then placed in desiccators containing a saturated KCl solution which provided a constant water activity at 25 °C equal to 0.8434. The desiccators were stored in a Heareus thermostated incubator at 25.0 ± 0.1 °C. At time intervals of 24 h the cups were weighed until a constant increment in weight was achieved. The amount of water vapour transmission rate through the film was estimated by the linear portion of the diagram obtained by plotting the weight increment of the cup as a function of time. It was assumed that steady-state was reached once the regression analysis made by using the last four data points resulted in $r^2 \geq 0.998$.

The water vapour permeability was calculated from the equation:

$$\text{water vapour permeability} = \frac{X}{(A\text{Dp})} \frac{\mathrm{d}m}{\mathrm{d}t}$$

where dm/dt is the slope of the cup weight *versus* time curve once steady-state was reached, X is the film thickness, A the film exposed area and Dp 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, Dp becomes equal to vapour pressure inside the dessicator and was calculated by multiplying water activity and water tension (P_0) at 25 °C (P_0 = 3.167 kPa). Ten independent tests for each film were performed.

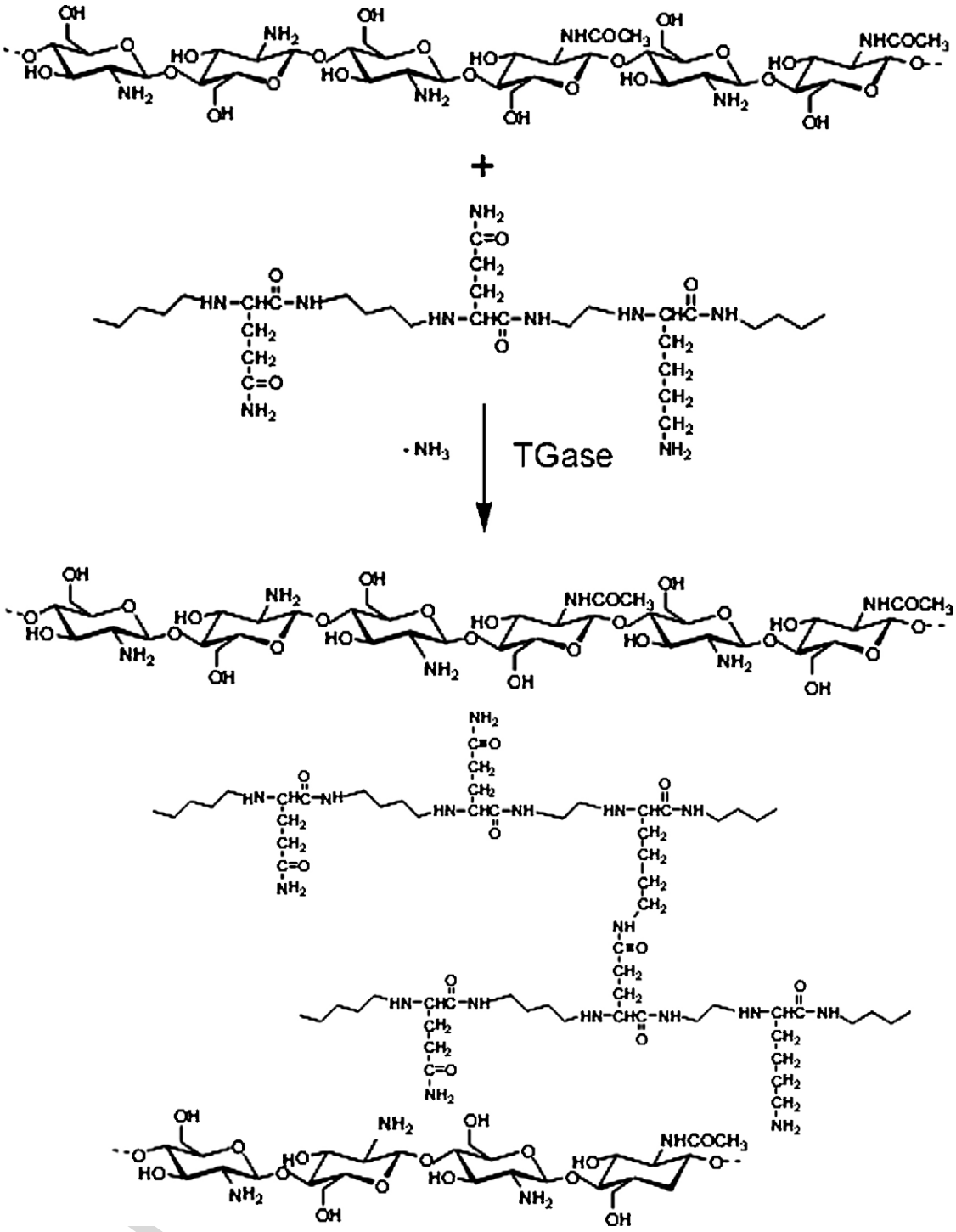
2.7. Statistical analysis

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

3. Results and discussion

In the present work chitosan–ovalbumin films were prepared by casting the polymeric solutions in the presence or absence of the enzyme TGase. Cross-linking of the polysaccharide and protein solutions was performed at pH 4.6 in order to facilitate the solubility of chitosan. Under these conditions, *Streptoverticillium* sp. TGase showed high catalytic activity. Through this process, flexible, smooth, transparent and slightly yellowish films were obtained. Scheme 1 represents the enzymatic approach employed for preparing the polysaccharide-protein films. As controls, films of both chitosan and chitosan–ovalbumin without addition of TGase were also prepared.

The thickness of the chitosan films was increased from 76.3 ± 5.7 to 92 ± 8.6 μm after addition of the protein component. However, the treatment with TGase yielded films with average thickness of 76.38 ± 9 μm. This reduction could be explained by the contraction of the three dimensional film matrixes after the cross-linking reaction. The films prepared with



Scheme 1. Enzymatic cross-linking of egg white proteins in the presence of chitosan.

TGase were practically insoluble after 24 h incubation at 25 °C in solutions at different values of pH.

The chitosan–ovalbumin films, prepared via TGase-catalyzed reaction, were treated with bovine pancreatic trypsin in order to evaluate their digestibility. High amounts of soluble peptides were released from the film materials after 20 h of trypsin treatment at 25 °C, according to the amount of soluble L-tyrosine quantified in the incubation media. In this sense, 1.8 μmol L-tyrosine was released per cm^2 film after 20 h incubation with the serine protease. This result shows the film protein component capability to act as protease substrate even in the presence of chitosan.

The swelling kinetics of the films was studied in solutions at pH 7.0 and 25 °C (Fig. 1). Under these conditions, maximum degree of swelling was achieved for all films after 2 h incubation. The addition of ovalbumin to the chitosan matrix yielded films with lower degree of swelling at equilibrium, and this degree of swelling was significantly reduced by treatment with TGase.

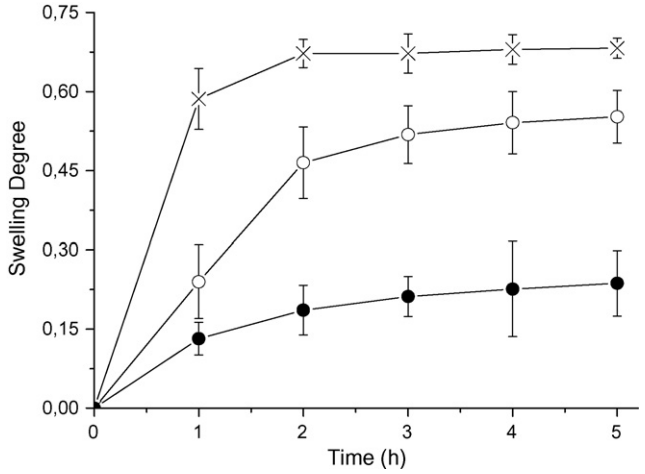


Fig. 1. Degree of swelling at 25 °C and pH 7.0 of the chitosan films (×) and chitosan–ovalbumin films prepared in the presence (●) and absence (◯) of TGase.

It is well-known that the maximum degree of swelling of a polymeric material strongly depends on the amount and nature of intermolecular chain interactions [22]. In this context, our results could be justified by the formation of physical linkages between the chitosan matrix and the protein molecules TGase-cross-linked or not, respectively.

Fig. 2 shows the tensile strength and the elastic modulus of the prepared films. The materials made by chitosan and ovalbumin showed higher tensile strength and lower elastic modulus when compared with those prepared with the polysaccharide alone. This effect could be associated with the formation of non-covalent intermolecular interactions between the protein molecules and the polysaccharide chains. On the other hand, a noticeably improvement in the mechanical resistance and elasticity of the films was found in those prepared by cross-linking with TGase. This result could be related to the formation of intermolecular covalent linkages between the active glutamine and lysine residues occurring in the protein chains. Such kind of TGase-mediated covalent protein-protein cross-linking could reduce the intermolecular chain mobility of the chitosan matrix, thus increasing the tensile strength.

Fig. 3 shows the water vapour permeability of the films. The addition of protein to the chitosan matrix noticeably increased the water vapour permeability of these materials, but this effect was significantly lower in the materials prepared with TGase. The increase in the water vapour permeability properties of the chitosan–ovalbumin films could be directly associated with the introduction into the polymeric matrix of new polar groups belonging to the polypeptide structure [23]. Consequently, the changes in the hydrophilic properties of the films, associated with the disappearance of primary amino and amide groups and the formation of less hydrophilic secondary amide linkages (Scheme 1), should be the main factor contributing to the lower water vapour permeability showed by the films made in the presence of TGase. Similar behaviour was previously reported for chitosan–whey protein films prepared via a TGase-catalyzed process [7].

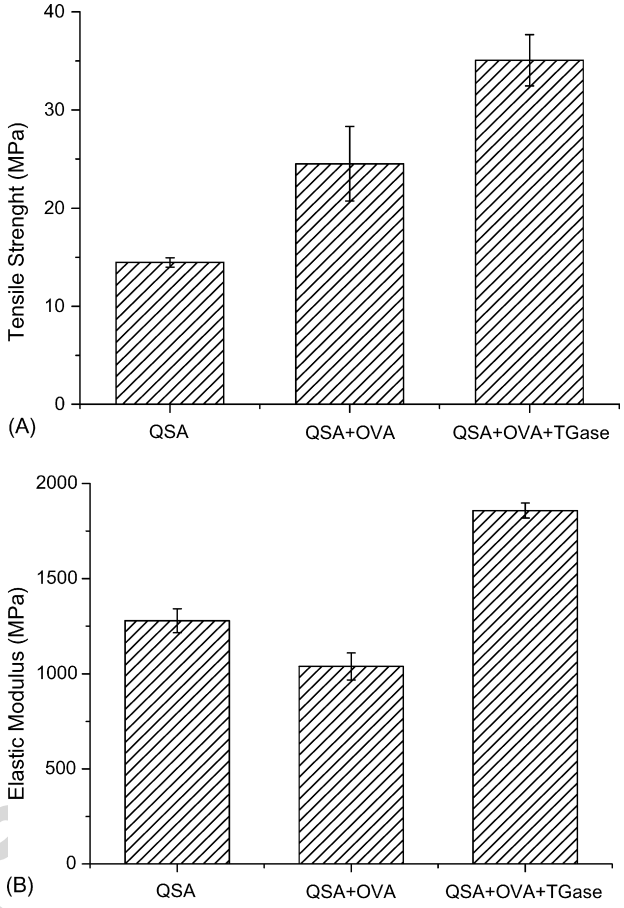


Fig. 2. Tensile strength (A) and elastic modulus (B) of the chitosan and chitosan–ovalbumin films prepared in the presence and absence of TGase (QSA, chitosan; OVA, ovalbumin).

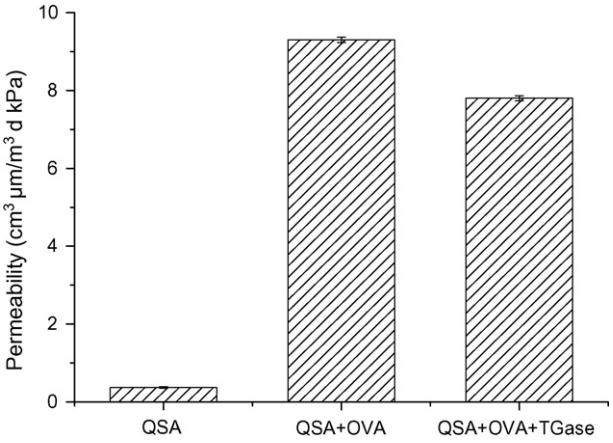


Fig. 3. Water vapour permeability of the chitosan and chitosan–ovalbumin films prepared in the presence and absence of TGase (QSA, chitosan; OVA, ovalbumin).

4. Conclusions

In the present paper we describe the preparation of chitosan–ovalbumin films through a TGase-catalyzed reaction. The improved mechanical and solubility properties of these new materials confirm that this enzymatic approach could be a useful

tool for preparing edible films for food coating and pharmaceutical applications.

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References

[1] Kester JJ, Fennema OR. Edible films and coating: a review. Food Technol 1986;40, 47-59.3.
[2] Krochta JM, De Mulder-Johnston C. Edible and biodegradable polymer films: challenges and opportunities. Food Technol 1997;51:61–74.
[3] Ryu SY, Rhim JW, Roh HJ, Kim SS. Preparation and physical properties of zein-coated high-amylose corn starch film. Lebensm Wiss Technol 2002;35:680–6.
[4] 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;102:191–8.
[5] Coma V, Martial-Gros A, Garreau S, Copinet A, Salin F, Deschamps A. Edible antimicrobial films based on chitosan matrix. J Food Sci 2002;67:1162–9.
[6] Mauer LJ, Smith DE, Labuza TP. Water vapour permeability, mechanical, and structural properties of edible β-casein films. Int Dairy J 2000;10:353–8.
[7] Di Pierro P, Chico B, Villalonga R, Mariniello L, Damiao AE, Masi P, et al. Chitosan-whey protein edible films produced in the absence or presence of transglutaminase: analysis of their mechanical and barrier properties. Biomacromolecules 2006;7:744–9.
[8] Folk JE. Transglutaminases. Ann Rev Biochem 1980;49:517–31.
[9] Aeschlimann D, Paulsson M. Transglutaminases: protein cross-linking enzymes in tissues and body fluids. Thromb Haemost 1994;71:402–15.
[10] Mancuso F, Porta R, Calignano A, Di Pierro P, Sommella MC, Esposito C. Substance P and its transglutaminase-synthesized derivatives elicit yawning behaviour via nitric oxide in rats. Peptides 2001;22:1453–7.
[11] Esposito C, Costa C, Amoresano A, Mariniello L, Sommella MG, Caputo I, et al. Transglutaminase-mediated amine incorporation into substance P protects the peptide against proteolysis in vitro. Reg Pept 1999;84:75–80.
[12] Valdivia A, Villalonga R, Sommella MG, Pérez Y, Mariniello L, Porta R. Transglutaminase-catalyzed site-specific glycosidation of catalase with aminated dextran. J Biotechnol 2006;122:326–33.
[13] Villalonga R, Fernández M, Fragoso A, Cao R, Di Pierro P, Mariniello L, et al. Transglutaminase-catalyzed synthesis of trypsin–cyclodextrin conjugates. Kinetics and stability properties. Biotechnol Bioeng 1996;81:732–7.
[14] 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;38:53–9.
[15] Motoki M, Aso H, Seguro K, Nio N. Casein film prepared using transglutaminase. Agric Biol Chem 1987;51:993–6.
[16] Lim LT, Mine Y, Tung MA. Transglutaminase cross-linked egg white protein films: tensile properties and mechanical permeability. J Agric Food Chem 1998;46:4022–9.
[17] Mahmoud R, Savello PA. Solubility and hydrolyzability of films produced by transglutaminase catalytic cross-linking of whey protein. J Dairy Sci 1993;76:29–35.
[18] Majeti NV, Ravi K. A review of chitin and chitosan application. React Funct Polym 2000;46:1–27.
[19] Baxter A, Dillon M, Taylor KDA, Roberts GAF. Improved method for i.r. determination of the degree of *N*-acetylation of chitosan. Int J Biol Macromol 1992;14:166–9.
[20] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;76:248–50.
[21] Stuchell YM, Krochta JM. Enzymatic treatment and thermal effects on edible soy protein films. J Food Sci 1994;59:1332–7.
[22] Flory PJ. Principles of polymer chemistry. Ithaca, New York: Cornell University Press; 1953.
[23] Miller KS, Krochta JM. Oxygen and aroma barrier properties of edible films: a review. Trends Food Sci Technol 1997;8:228–37.