

# Putrescine–polysaccharide conjugates as transglutaminase substrates and their possible use in producing crosslinked films

Prospero Di Pierro · Loredana Mariniello ·  
Angela Sorrentino · Reynaldo Villalonga ·  
Belkis Chico · Raffaele Porta

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**Abstract** Putrescine (1,4-diaminobutane) was covalently linked to alginate and low-methoxyl pectin to synthesize new aminated polysaccharides. Both putrescine–pectin and –alginate conjugates, although the latter at higher concentrations, were found to be able to act as effective acyl acceptor transglutaminase substrates in vitro using both dimethylated casein and soy flour proteins as acyl donors. Monodansylcadaverine, a well known acyl acceptor transglutaminase substrate, dose-dependently counteracted the covalent binding of the aminated polysaccharides to the proteins. Putrescine–pectin conjugate was also tested to prepare, in combination with soy flour proteins, edible films in the presence of purified microbial transglutaminase. Characterization of the enzymatically crosslinked films showed a significant decreased water vapor permeability, with respect to the ones obtained with non-aminated pectin in the presence of transglutaminase, as well as improved mechanical properties, such as high extensibility. Possible biotechnological applications of hydrocolloid films containing putrescine–polysaccharide derivatives enzymatically crosslinked to proteins were suggested.

**Keywords** Putrescine · Transglutaminase · Edible films · Pectin · Alginate · Crosslink

## Abbreviations

ALG	Alginate
ALGPt	Alginate–putrescine conjugate
DMC	Dimethylated casein
MDC	Monodansylcadaverine
PEC	Pectin
PECPt	Pectin–putrescine conjugate
Pt	Putrescine
SF	Soy flour proteins
mTGase	Microbial transglutaminase from <i>Streptovercillium mobaraense</i>
WVP	Water vapor permeability

## Introduction

Putrescine (1,4-diaminobutane, Pt) is a diamine widely occurring in nature deriving from the enzymatic decarboxylation of the amino acid ornithine and the precursor of the polyamines spermidine and spermine (Cohen 1971). It was demonstrated that in mammals Pt is also able to act as an effective acyl acceptor substrate for the enzyme transglutaminase (protein-glutamine  $\gamma$ -glutamyl transferase, E.C. 2.3.2.13; TGase) being incorporated into proteins producing mono- and bis( $\gamma$ -glutaminy)Pt derivatives of the latter (Folk and Chung 1985). In the absence of exogenous amines, this enzyme catalyzes the formation of intra- or inter-molecular  $\varepsilon$ -( $\gamma$ -glutamyl)lysine crosslinks into proteins via an acyl-transfer reaction, the  $\gamma$ -carboxamide group of endoprotein glutamine serving as acyl donor and the  $\varepsilon$ -amino group of endoprotein lysine serving as acyl acceptor (Folk 1980; Aeschlimann and Paulsson 1994; Aeschlimann and Thomazy 2000). In this sense, TGase was

P. Di Pierro · L. Mariniello · A. Sorrentino · R. Porta (✉)  
Department of Food Science, University of Naples  
“Federico II”, Portici, Naples, Italy  
e-mail: raffaele.porta@unina.it

R. Villalonga · B. Chico  
Center for Enzyme Technology,  
University of Matanzas, Matanzas, Cuba

successfully used to modify the biological activities of peptides and proteins by covalently linking polyamines to their reactive endo-glutamines residues (Persico et al. 1992; Esposito et al. 1995, 1999; Tufano et al. 1996; Josten et al. 1998; Mancuso et al. 1998; Bechtold et al. 2000; Mancuso et al. 2001). In addition, TGase was employed for immobilizing enzymes (Villalonga et al. 2003a, b), as well as for improving the properties of several protein (such as casein, egg white, and whey proteins)-based edible films (Motoki et al. 1987; Mahmoud and Savello 1993; Lim et al. 1998). In the last few years, the microbial isoform of the enzyme (mTGase) isolated from *Streptovorticillium mobaraense* possessing wide substrate specificity, calcium independence and high thermostability, has been largely utilized as a biotechnological tool, mostly in the food field (Mariniello and Porta 2005; Mariniello et al. 2008).

In recent papers (Mariniello et al. 2003; Di Pierro et al. 2005), we examined the potential use of mTGase as a biotechnological tool for preparing films containing pectin (PEC) and soy flour proteins (SF). The obtained hydrocolloid films were characterized for their mechanical and barrier properties to oxygen, carbon dioxide and water vapor. In the present study, we describe the procedure to prepare Pt derivatives of both PEC (PECPt) and alginate (ALGPt), their ability to act as acyl acceptor substrates of mTGase, and the preparation of hydrocolloid films containing PECPt crosslinked to soy flour proteins by the enzyme. Finally, some functional characteristics of the obtained films (mechanical properties and water vapor permeability) were also investigated.

## Materials and methods

### Materials

PEC from citrus fruits [mol. wt.  $1.0 \times 10^5$  (Anger and Berth 1986); degree of de-esterification, 79% (Grasdalen et al. 1988)] was purchased from Fluka. Sodium alginate (ALG) from *Laminaria hyperborean* [mol. wt.  $1.9 \times 10^5$  (Smidsrød 1970), 37% mannuronate and 63% guluronate (Grasdalen et al. 1979)] was from BDH (Poole, UK). Defatted soy flour (type 1) and dimethylated casein (DMC) was purchased from Sigma Chem. Co., (St. Louis, MO). mTGase (Activa WM), derived from the culture of *Streptovorticillium* sp., was supplied by Ajinomoto Co Inc., Japan. The enzyme was prepared dissolving the commercial preparation (containing 1% TGase and 99% maltodextrins) in distilled water. The specific activity of the enzyme was 92 U/g (Mariniello et al. 2007a, b). All other chemicals were analytical grade.

### PECPt and ALGPt synthesis

Polysaccharides were oxidized by dissolving 100 mg of both PEC and ALG in 5 mL of H<sub>2</sub>O and adding, at 4°C in the dark, 400 mg of sodium metaperiodate to each solution under continuous stirring. After 2 h, the reactions were stopped by addition of 100 µL of ethylene glycol. The activated polymer solutions, after stirring for 1 h and dialysis against distilled H<sub>2</sub>O, were treated with Pt (10 mmol) for 1 h at 25°C and then NaBH<sub>4</sub> (20 mg) was added during 4 h of continuous stirring at 25°C. At the end, the obtained Pt-conjugates of PEC and ALG were dialyzed against distilled H<sub>2</sub>O and freeze-dried.

The molecular weights of the native polysaccharides were determined by analytical gel permeation chromatography on TESEK Hema-bio columns 40, 100, 300 and 1,000 (4 × 30 cm) calibrated with pullulan standards. The degree of modification of the polymers was determined by measuring the amount of free amino groups with *o*-phthalaldehyde using glycine as standard (Bruneel and Schacht 1993).

### mTGase acyl acceptor substrate activity of PECPt and ALGPt

The occurrence of mTGase-reactive amino donor residues in both PECPt and ALGPt was assayed by testing the ability of the polysaccharide derivatives to be incorporated into acyl donor substrate proteins in the presence of the enzyme. Therefore, 200 µg of DMC were incubated with different amounts (10–500 µg) of PECPt or ALGPt for 1 h at 37°C in 100 µL of 125 mM Tris-HCl, pH 7.5, in the presence of 20, 50, 100 and 200 mU of mTGase. When SF (280 µg) were tested as substrate, the maximal mTGase amount (200 mU) was present in the assay. Controls were simultaneously run by incubating either the acyl donor protein substrates, both alone and in the presence of 200 mU of mTGase but in the absence of polysaccharide Pt-conjugates, or the polysaccharide Pt-conjugates with 200 mU of enzyme in the absence of acyl donors. At the end of incubation, 3 µL of each sample were dissolved in the 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) blue of bromophenol] and analyzed by SDS-PAGE (5% stacking and 12% separating gels) as described by Laemmli (1970). Electrophoresis was performed at constant current (40 mA for 1 h and 70 mA for another 2 h) and the proteins stained with Coomassie Brilliant Blue R250, whereas polysaccharides were revealed with Schiff's reagent (0.05 M sodium meta-periodate) (Thornton et al. 1994). Bio-Rad Precision Protein Standards were used as molecular weight markers.

### Counteracting of mTGase-catalyzed PECPt incorporation into DMC by monodansylcadaverine (MDC)

200 µg of DMC and 65 µg PECPt were incubated for 1 h at 37°C with 200 mU of mTGase in 100 µL of 125 mM Tris–HCl, pH 7.5, in the absence or presence of 0.2, 0.4, and 1.6 mM of MDC. Control was performed by incubating DMC and PECPt with mTGase in the presence of the maximal MDC concentration (1.6 mM) after heating of the reaction mixture at 100°C for 5 min as soon as the enzyme was added. At the end of incubation, the assays were stopped by addition of 0.9 mL of a solution of 9 M urea, 2% (w/v) SDS, and 40 mM dithiothreitol in 50 mM Tris–HCl buffer, pH 7.1, and aliquots (20 µL) of each sample analyzed by 12% SDS–PAGE. Proteins were visualized by Coomassie staining while ultraviolet illumination revealed protein bound MDC. Bio-Rad Precision Protein Standards were used as molecular weight markers.

### Determination of $K_m$ values of mTGase toward PECPt and ALGPt

The affinity of mTGase toward the two polysaccharide Pt-conjugates was evaluated by varying their concentrations in an assay carried out to determine the enzyme activity by a continuous spectrophotometric method (Day and Keillor 1999). mTGase (2 U) was incubated in 1 mL of 200 mM MOPS buffer, pH 7.0, containing 0.5 µM NADH, 10 mM  $\alpha$ -ketoglutarate, 2.4 U of glutamate dehydrogenase, 10 mM Z-Gln-Gly, acting as acyl donor substrate, and increasing concentrations (between 10 and 100 µM) of either PECPt or ALGPt as acyl acceptors. The assays were performed at 37°C using 1 mL quartz cuvettes with a 1 cm light path, and the decrease in absorbance due to the oxidation of NADH was followed against a blank at 340 nm in a Perkin Elmer spectrophotometer.  $K_m$  was calculated with the Lineweaver–Burk method by reporting in the plot the reciprocal of the obtained absorbance values ( $\Delta OD/\text{min}$ ) versus the ones of acyl acceptor substrate concentration occurring in the reaction mixtures.

### Film-making procedure

Defatted soy flour, containing all proteins occurring in soy seeds, was dissolved in distilled water at a concentration of 13 mg/mL and the resulting solution was centrifuged at 20,000×g for 20 min. PEC and PECPt were dissolved in distilled water at a concentration of 16 mg/mL. To obtain films, 4.3 mL of SF solution and 7.0 mL of either PEC or PECPt solutions were gently mixed. mTGase-polymerized films were obtained by adding 5 mU of the enzyme to the mixtures of SF and either PEC or PECPt, ensuring that the

enzyme was evenly dispersed throughout the aqueous phase within 1 min. All the film-making mixtures were de-aerated under vacuum, in order to prevent small bubbles which could form pinholes in the finished films, and then transferred into polystyrene Petri dishes (60 × 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). Film thickness was measured using a micrometer model HO62 with sensitivity of  $\pm 2$  µm (Metrocontrol Srl, Casoria (Na), Italy).

### Film mechanical properties

Film tensile strength and elongation to break were measured with an Instron universal testing instrument (Instron Engineering Corp., Canton, MA; model 4301) according to 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. 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  $50 \pm 5\%$  RH and  $23 \pm 2^\circ\text{C}$  in an environmental chamber. Ten samples of each film type were tested.

### Water vapor permeability

Film water vapor permeability (WVP) was evaluated by a gravimetric test according with ASTM (1993) by means of a Fisher/Payne permeability Cup (Carlo Erba, Italy) as previously described by Di Pierro et al. (2005). Three grams of silica gel were introduced into each cup and a film sample disk, 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 vapor transmission was 10 cm<sup>2</sup>. The cups containing silica gel were weighed 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  $25.0 \pm 0.1^\circ\text{C}$ . 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 the last four data points resulted in  $r^2 \geq 0.998$ .

The WVP was calculated from the equation:

$$\text{WVP} = X/(A\Delta P)dm/dt$$

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  $\Delta P$  is the water vapor pressure across the film. Assuming that the vapor pressure inside the cup, due to the presence of silica gel, as equal to zero,  $\Delta P$  becomes equal to the vapor pressure inside the desiccator given by the product of the water activity and water pressure ( $P_0$ ) at 25°C ( $P_0 = 3.167$  kPa).

#### Protein determination

Protein determination was carried out by the Kjeldahl method using a DK6 Heating Digester equipped with a UDK 126A distillation unit (Velp Scientifica, Milan, Italy). The titration was performed by using 0.2 N HCl acid and Tashiro's indicator solution.

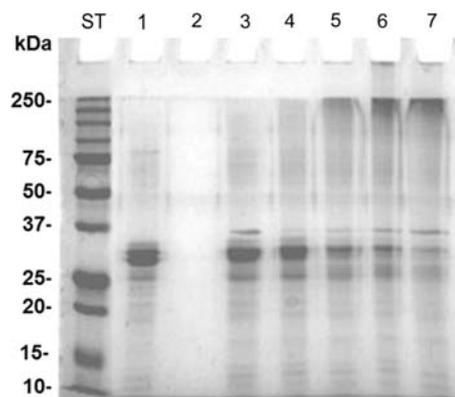
#### Statistical analysis

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

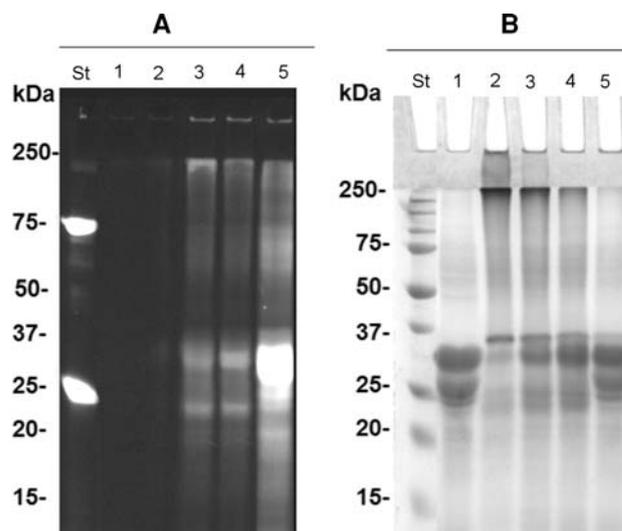
## Results

Pt-derivatives of two different polysaccharides, PEC and ALG, were chemically synthesized in the attempt of obtaining sugar polymers able to act as mTGase acyl acceptor substrates. Such synthesis was possible by a preliminary oxidation with metaperiodate of the C2–C3 alcoholic groups of uronic acid units occurring in PEC and ALG polymers, respectively, followed by reaction with Pt and the final  $\text{NaBH}_4$  reduction of the diamine–Schiff's base in C2 obtained from each polysaccharide. In this way, we obtained PECPt and ALGpt conjugates of 1.11 and  $1.94 \times 10^5$  Da containing 130 and 46 mol of Pt/mol of PEC and ALG, respectively.

In order to analyze the ability of the two polysaccharide derivatives to act as mTGase acyl acceptor substrates, we investigated their possible covalent binding to an effective acyl donor protein substrate of the enzyme, namely DMC, that is completely devoid of acyl acceptor activity due to the complete methylation of all the  $\epsilon$ -amino groups of its endoprotein lysyl residues. After the incubation of the polysaccharide Pt-derivatives with both mTGase and DMC, the formation of crosslinked polymers and the disappearance of DMC bands were evaluated by SDS-PAGE. The electrophoretic pattern reported in Fig. 1 clearly shows that, when incubated with mTGase, DMC gave rise to crosslinked polymer(s) only when PECPt was present in the reaction mixture and that the intensity of the high mol. wt. bands was dependent on the amount of the enzyme

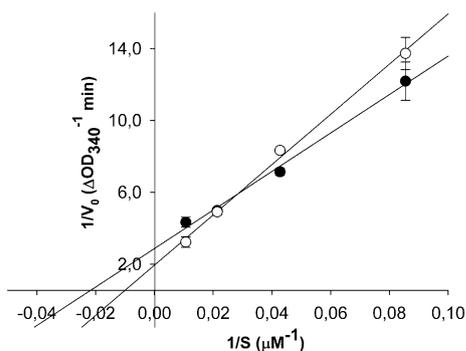


**Fig. 1** PECPt incorporation into DMC catalyzed by mTGase. SDS-PAGE (12%) analysis of the samples previously incubated with mTGase at different concentrations. DMC (lane 1) and PECPt (65 µg) (lane 2) incubated alone; DMC incubated with the maximal mTGase amount in the absence of PECPt (lane 3) or in the presence of PECPt and increasing mTGase amounts (lanes 4–7). Further experimental details are given in the text

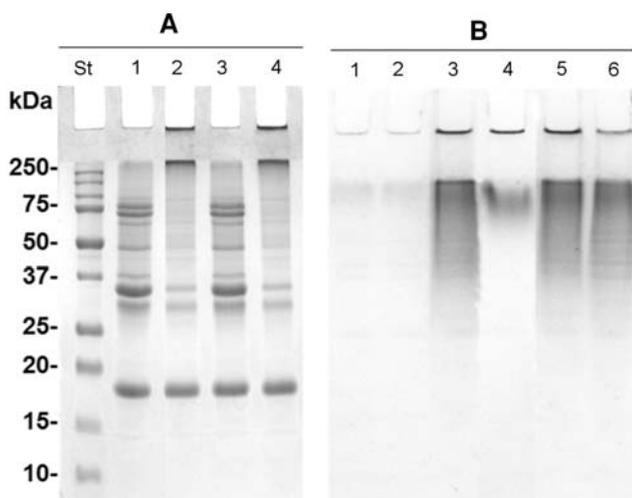


**Fig. 2** Counteracting of mTGase-catalyzed PECPt incorporation into DMC by MDC. SDS-PAGE (12%) analysis of the samples previously incubated with different MDC concentrations. DMC was incubated alone (lane 1) or with PECPt (65 µg) and mTGase either in the absence (lane 2) or presence (lanes 3–5) of increasing MDC concentrations. Ultraviolet visualization (a) and Coomassie staining (b) of the gel were performed at the end of the electrophoretic run. Further experimental details are given in the text

added (lanes 4–7) to the mixture. These data were confirmed by the results obtained from the SDS-PAGE experiments shown in Fig. 2 indicating that the addition to the reaction mixture of increasing concentrations of MDC, a well-known acyl acceptor substrate of the enzyme, effectively counteracted the formation of crosslinked polymer(s) between DMC and PECPt (lanes 3–5, Fig. 2b). In this case, conversely, the formation of a dose-dependent MDC–DMC derivative, with a mol.wt. very similar to that



**Fig. 3** Determination of mTGase  $K_m$  values for both PECPt (open circle) and ALGPt (filled circle) by double reciprocal plot. Experimental details are described in the text



**Fig. 4** PECPt incorporation into SF catalyzed by mTGase. SDS-PAGE (12%) analysis of the samples previously incubated under different experimental conditions. SF incubated alone (lane 1), with only mTGase (lane 2) or only PECPt (65 μg) (lane 3), or with both mTGase and PECPt (lane 4). PECPt was also incubated alone (lane 5) or in the presence of mTGase (lane 6). Coomassie (a) and Schiff's reagent (b) staining of the gels were performed at the end of the electrophoretic run. Further experimental details are given in the text

of DMC (lanes 3–5, Fig. 2a), was revealed by ultraviolet illumination of the gel. Experiments performed using ALGPt as mTGase acyl acceptor substrate indicated that higher amounts (fivefold) of ALGPt gave results similar to the ones reported for PECPt (data not shown), although the detected  $K_m$  values of the enzyme toward ALGPt ( $0.37 \times 10^{-4}$  M) and PECPt ( $0.71 \times 10^{-4}$  M) were of the same order of magnitude (Fig. 3).

Moreover, in order to investigate the possibility to use PECPt, instead of PEC, to prepare hydrocolloid films containing SF, we tested the ability of the Pt-conjugate of PEC to crosslink with SF in the presence of mTGase. In fact, the hypothesized capacity of PECPt to covalently bind to SF via mTGase might open new strategies in the production of edible films in which polysaccharides and

proteins could constitute a tight and resistant network with predictable and desired features. Therefore, PECPt was also tested as mTGase acyl acceptor using SF as acyl donors (Fig. 4). Although no difference was found in the Coomassie-stained SDS-PAGE pattern when SF were incubated with mTGase both in the absence (Fig. 4a, lane 2) and presence (Fig. 4a, lane 4) of PECPt, the lane 4 of Fig. 4b, showing the gel stained with Schiff's reagent to reveal the presence of carbohydrates, clearly indicated that PECPt crosslinked with SF producing high mol.wt. mixed PECPt-SF polymer(s) in the presence of the enzyme. The similar SDS-PAGE profiles visualized by Coomassie in lanes 2 and 4 of Fig. 4a can be easily explained with the well-known SF capacity to produce, in the presence of mTGase, high mol. wt. polymer(s) also in the absence of exogenous acyl acceptors, since SF contains in addition to endoprotein reactive glutamyl residues (acyl donors) also endoprotein reactive lysyl residues (acyl acceptors) (Mariniello et al. 2003). Similar results (data not shown) were obtained using ALGPt instead of PECPt even though at higher amounts.

The ability of PECPt to covalently bind to SF and to produce carbohydrate–protein heteropolymers stimulate us to prepare SF-based films containing PECPt crosslinked to protein(s) in substitution of PEC reticulated (Mariniello et al. 2003; Di Pierro et al. 2005). To establish whether mTGase treatment modified mechanical properties of SF/PECPt-based films in a different way from that detected when unmodified PEC was used (Mariniello et al. 2003), the tensile strength and the elongation to break of both types of film were investigated and compared. As shown in Table 1, whereas the tensile strength values resulted not to be significantly different, the average elongation to break of PECPt containing films obtained in the presence of mTGase was almost five times higher than that previously detected with SF/PEC-based films. Conversely, both tensile strength and elongation to break of the films did not change when PEC was substituted with PECPt and the films were prepared in the absence of the enzyme. These findings clearly indicate that the films obtained following mTGase-catalyzed PECPt crosslinking to SF were more extensible than the corresponding controls prepared with PEC and SF both in the presence and absence of enzyme. Furthermore, to investigate whether PECPt-SF crosslinks produced by mTGase also influence the barrier properties of the films, WVP of the latter was examined and compared to that exhibited by films prepared with PEC (Di Pierro et al. 2005). The results reported in Table 1 demonstrate that WVP was also clearly influenced by the PECPt-SF crosslinks produced by mTGase, since the films prepared with PECPt in the presence of the enzyme showed a significant decrease in WVP compared to the controls obtained either with PECPt in the absence of the enzyme or with PEC both

**Table 1** Comparison of the functional properties of PEC and PECPt containing films obtained in the absence or presence of mTGase

Film components	WVP (cm <sup>3</sup> mm m <sup>-2</sup> day <sup>-1</sup> kPa <sup>-1</sup> )	Tensile strength (MPa)	Elongation to break (%)
SF + PEC	3.5 ± 0.1	6.8 ± 0.9	11.6 ± 1.1
SF + PEC + mTGase	3.0 ± 0.1	12.4 ± 1.1	7.2 ± 1.0
SF + PECPt	3.4 ± 0.2	4.9 ± 0.9	10.8 ± 1.3
SF + PECPt + mTGase	2.1 ± 0.2 <sup>a</sup>	14.3 ± 2.3	32.6 ± 1.9 <sup>a</sup>

The results are expressed as means of ten samples ± SD. Experimental details are given in the text

<sup>a</sup> Significantly different for  $P < 0.05$

in the presence and absence of enzyme. Therefore, it is reasonable to hypothesize that the PECPt-SF crosslinks significantly reduces the free volume inside the film, thus influencing its barrier properties to water vapor.

## Discussion

Several attempts to produce films containing both polysaccharides and proteins crosslinked by TGase were recently carried out in our laboratories, and all of them were based on the principle of the ability of the polypeptide chain to act as both acyl donor and acceptor substrate for the enzyme (Di Pierro et al. 2005, 2006, 2007; Mariniello et al. 2003, 2007a, b, 2008). Therefore, all the films prepared so far were constituted by a polysaccharide network in which TGase-crosslinked proteins were entrapped and non-covalently bound to the sugar polymers. Conversely, the data reported in the present paper represent the results of the first study devoted to create a network of a polysaccharide covalently linked to proteins by TGase. To reach this aim we chemically synthesized Pt-derivatives of two different polysaccharides, namely PEC and ALG, and demonstrated that their aminated conjugates are able to effectively act as acyl acceptor TGase substrates using both DMC and SF as acyl donors. Both PEC, a linear galacturonic acid homopolymer, and ALG, a binary linear heteropolymer containing 1,4-linked  $\alpha$ -L-guluronic and  $\beta$ -D-mannuronic acids, easily reacted with the primary amino group of Pt following metaperiodate oxidation of -uronic acid C2-C3 alcoholic groups. The final NaBH<sub>4</sub> reduction of the diamine-Schiff's bases, obtained in C2, stabilized PECPt and ALGPt conjugates containing, thus, a covalently bound aminobutane-moiety potentially capable to act as acyl acceptor site in TGase-catalyzed reactions. Our experiments showed that mTGase effectively crosslinks PECPt and ALGPt with well recognized acyl donor substrates of the enzyme (DMC and SF) and that a well-known alternative acyl acceptor substrate (MDC) is able to counteract with the binding of both sugar polymers to proteins. Furthermore, we confirmed (data not shown)

previous results of Chen et al. (2003) indicating that chitosan, a C2-aminated polysaccharide derived from chitin N-deacetylation, is not able to function as amino donor for TGase. Therefore, we assume that the polysaccharide-bound primary amino group must be localized at a relative distance from the sugar cyclic structure to be recognized by the enzyme active site.

Since, earlier we obtained films made of TGase crosslinked SF proteins and unmodified PEC (Mariniello et al. 2003; Di Pierro et al. 2005), we decided to prepare hydrocolloid films made by PECPt and SF in the presence of TGase, and to analyze their mechanical and moisture barrier properties compared to the ones detected with either SF/PEC films obtained in the presence of TGase or SF/PECPt films prepared in the absence of the enzyme. The obtained results clearly indicate that the films prepared with PECPt and SF in the presence of the enzyme are more extensible than the controls since the observed elongation to break values was found about fivefold higher, whereas their mechanical resistance, measured by tensile strength, was shown not to be significantly changed. Moreover, also the moisture barrier capacity of SF/PECPt films crosslinked by TGase resulted significantly higher, WVP decreasing of about 1/3 with respect to the corresponding values measured by analyzing the control films.

In conclusion, our findings demonstrate that the chemically synthesized Pt-derivatives of PEC and ALG are effective TGase acyl acceptor substrates in vitro, and that these sugar amino-conjugates can be successfully used to create a variety of polysaccharide-protein heteropolymers by means of the enzyme. Films produced by mixed crosslinked networks between proteins (SF) and a polysaccharide Pt-conjugate (PECPt) showed mechanical and WVP properties significantly different from those exhibited by similar films in which sugar and amino acid polymers were non-covalently bound to each other. These results open, in our opinion, a new chapter in the biodegradable/edible polymer field finalized to obtain hydrocolloid films with marked extensibility and increased moisture barrier capacity.

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## References

- Aeschlimann D, Paulsson M (1994) Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thromb Haemost* 71:402–415
- Aeschlimann D, Thomazy V (2000) Protein cross-linking in assembly and remodelling of extracellular matrices: the role of transglutaminases. *Connective Tissue Res* 41:1–27
- Anger H, Berth G (1986) Gel permeation chromatography and the Mark-Houwink relation for pectins with different degrees of esterification. *Carbohydr Polym* 6:193–202
- ASTM (1991) Standard test method for tensile properties of thin plastic sheeting D882. In: Annual book of American standard testing methods. American Society for Testing and Materials, Philadelphia, PA, pp 313–321
- ASTM (1993) Standard test method for water vapour transmission of materials E96-93. In: Annual book of American standard testing methods. American Society for Testing and Materials, Philadelphia, PA, pp 701–708
- Bechtold U, Otterbach JT, Pasternack R, Fuchsbauer HL (2000) Enzymic preparation of protein G-peroxidase conjugates catalysed by transglutaminase. *J Biochem* 127:239–245
- Bruneel D, Schacht E (1993) Chemical modification of pullulan: 2. Chloroformate activation. *Polymer* 34:2633–2637
- Chen T, Embree HD, Brownc EM, Taylorc MM, Paynea GF (2003) Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. *Biomaterials* 24:2831–2841
- Cohen SS (1971) Introduction to the polyamines. Prentice-Hall, Englewood Cliffs, NJ
- Day N, Keillor JW (1999) A Continuous spectrophotometric linked enzyme assay for transglutaminase activity. *Anal Biochem* 274:141–144
- Di Pierro P, Mariniello L, Giosafatto CVL, Masi P, Porta R (2005) Solubility and permeability properties of edible pectin-soy flour films obtained in the absence or presence of transglutaminase. *Food Biotechnol* 19:37–49
- Di Pierro P, Chico B, Villalonga R, Mariniello L, Damiao AE, Masi P, Porta R (2006) Chitosan-whey protein edible films produced in the absence or presence of transglutaminase: analysis of their mechanical and barrier properties. *Biomacromolecules* 7:744–749
- Di Pierro P, Chico B, Villalonga R, Mariniello L, Masi P, Porta R (2007) Transglutaminase-catalyzed preparation of chitosan-ovalbumin films. *Enzyme Microb Technol* 40:437–441
- Esposito C, Mancuso F, Calignano A, Di Pierro P, Pucci P, Porta R (1995) Neurokinin receptors could be differentiated by their capacity to respond to the transglutaminase-synthesized  $\gamma$ -(glutamyl<sup>15</sup>) spermidine derivative of substance P. *J Neurochem* 65:420–426
- Esposito C, Costa C, Amoresano A, Mariniello L, Sommella MG, Caputo I, Porta R (1999) Transglutaminase-mediated amine incorporation into substance P protects the peptide against proteolysis in vitro. *Regul Pept* 84:75–80
- Folk JE (1980) Transglutaminases. *Annu Rev Biochem* 49:517–531
- Folk JE, Chung SI (1985) Transglutaminases. *Methods Enzymol* 113:358–364
- Grasdalen H, Larsen B, Smidsrød O (1979) A p.m.r. study of the composition and sequence of uronate residues in alginates. *Carbohydr Res* 68:23–31
- Grasdalen H, Bakøy OE, Larsen B (1988) Determination of the degree of esterification and the distribution of methylated and free carboxyl groups in pectins by <sup>1</sup>H-NMR spectroscopy. *Carbohydr Res* 184:183–191
- Josten A, Meusel M, Spener F (1998) Microbial transglutaminase-mediated synthesis of hapten–protein conjugates for immunoassays. *Anal Biochem* 258:202–208
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Lim LT, Mine Y, Tung MA (1998) Transglutaminase cross-linked egg white protein films: tensile properties and oxygen permeability. *J Agric Food Chem* 46:4022–4029
- Mahmoud R, Savello PA (1993) Solubility and hydrolyzability of films produced by transglutaminase catalytic cross-linking of whey protein. *J Dairy Sci* 76:29–35
- Mancuso F, Costa C, Calignano A, Mariniello L, Rossi F, Porta R, Esposito C (1998) Transglutaminase-synthesized  $\gamma$ -(glutamyl<sup>15</sup>) spermidine derivative of substance P is a selective tool for neurokinin-2 receptors characterization. *Peptides* 19:683–690
- Mancuso F, Porta R, Calignano A, Di Pierro P, Sommella MG, Esposito C (2001) Substance P and its transglutaminase-synthesized derivatives elicit yawning behaviour via nitric oxide in rats. *Peptides* 22:1453–1457
- Mariniello L, Porta R (2005) Transglutaminase as biotechnological tools. In: Metha K, Eckert R (eds) Transglutaminases: family of enzymes with diverse functions. Karger Press, Basel CH, pp 174–191
- 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. *J Biotechnol* 102:191–198
- Mariniello L, Giosafatto CVL, Moschetti G, Aponte M, Masi P, Sorrentino A, Porta R (2007a) Fennel waste-based films suitable for protecting cultivations. *Biomacromolecules* 8:3008–3014
- Mariniello L, Giosafatto CVL, Di Pierro P, Sorrentino A, Porta R (2007b) Synthesis and resistance to in vitro proteolysis of transglutaminase cross-linked phaseolin, the major storage protein from *Phaseolus vulgaris*. *J Agric Food Chem* 55:4717–4721
- Mariniello L, Di Pierro P, Giosafatto CVL, Sorrentino A, Porta R (2008) Transglutaminase in food biotechnology. In: Porta R, Di Pierro P, Mariniello L (eds) Recent research developments in food biotechnology. Enzymes as additives or processing aids. Research Signpost, Kerala, India
- Motoki M, Aso H, Seguro K, Nio N (1987) Casein film prepared using transglutaminase. *Agric Biol Chem* 51:993–996
- Persico P, Calignano A, Mancuso F, Marino G, Pucci P, Esposito C, Mariniello L, Porta R (1992) Substance P inactivation by transglutaminase in vitro. *Peptides* 13:151–154
- Smidsrød O (1970) Solution properties of alginate. *Carbohydr Res* 13:359–372
- Thornton DJ, Carlstedt I, Sheehan JK (1994) Identification of glycoproteins on nitrocellulose membranes and gels. In: Walker JM (ed) Methods in molecular biology, vol 32. Humana Press Inc, Totowa, NJ, pp 119–128
- Tufano MA, Porta R, Farzati B, Di Pierro P, Rossano F, Catalanotti P, Baroni A, Metafora S (1996) 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* 168:148–157
- Villalonga R, Fernández M, Fragoso A, Cao R, Di Pierro P, Mariniello L, Porta R (2003a) Transglutaminase-catalyzed synthesis of trypsin–cyclodextrin conjugates. Kinetics and stability properties. *Biotechnol Bioeng* 81:732–737
- Villalonga R, Fernández M, Fragoso A, Cao R, Mariniello L, Porta R (2003b) Thermal stabilization of trypsin by enzymatic modification with  $\beta$ -cyclodextrin derivatives. *Biotechnol Appl Biochem* 38:53–59