Properties of Transglutaminase-Treated Red Bean Protein Films

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ABSTRACT: The effects of microbial transglutaminase (MTGase) treatment at various enzyme concentrations (4-40 U g^{-1}) on the properties of cast films from red bean protein isolate were investigated. The evaluated properties included mechanical properties (tensile strength, TS and elongation at break, EB), surface hydrophobicity (H_s), moisture content (MC), total soluble matter (TSM), water vapor transmission rate (WVTR) and permeability (WVP), as well as transparency. The results indicated that the TS and EB, and TSM were greatly improved by the MTGase treatment at low enzyme concentrations of 4-10 U g^{-1}, but considerably impaired at higher concentrations 20-40 U g^{-1}. The MTGase treatment also significantly increased the H_s, but decreased MC and WVTR, but the changes were independent of the applied enzyme concentration. The MTGase treatment progressively decreased the moisture loss during the drying of film-forming solutions, upon the increase in enzyme concentration. The protein solubility analyses in various solvents indicated that the contribution of covalent linkages to film network formation progressively increased upon the increase in enzyme concentration. The results suggest that the modifications of the properties of cast films are closely associated with the extent of MTGase-induced covalent polymerization and/or aggregation. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 122: 789–797, 2011

Key words: cast film; red bean protein isolate; transglutaminase; crosslinking

INTRODUCTION

During past decades, there is increasing interest in developing environment-friendly and biodegradable materials from natural biopolymers, including polysaccharides, proteins, lipids, and/or a combination of these materials. To date, films and coatings based on most of food proteins and milk proteins in particular have been widely investigated.[1] These protein-based films generally exhibit good barrier properties against oxygen and organic vapors, but poor barrier properties against water vapor. The mechanical properties of these films are also much weaker than those synthetic biopolymers. These poor properties of protein films greatly limit their applications in food packaging.

A number of physical, chemical and enzymatic treatments have been studied aiming to improve the mechanical and barrier properties of protein films. One extensively applied method to enhance these properties has been modification of film polymer network through crosslinking of the polymer chains. In this aspect, many chemical agents such as glutaraldehyde, glyceraldehyde, formaldehyde, glyoxal, etc. have been confirmed to effectively modify the properties of protein films by means of covalent crosslinking.[2,3] However, these chemical agents are usually toxic and not being edible, which limits the application of chemical crosslinking in the use for edible packaging. Another more potential alternative is enzyme-induced covalent crosslinking, especially using transglutaminase. Transglutaminase (TGase; E.C. 2.3.2.13) can catalyze acyl-transfer reactions between λ-carboxyamide groups of glutamine residues (acyl donor) and ε-amino groups of lysine residues (acyl acceptor) of proteins, thus causing the formation of ε-(λ-glutaminyl)lysine intra and intermolecular crosslinked biopolymers.[4] This covalent crosslinking leads to changes in functionality of food proteins, and thus also causes modification of protein films.

The influence of covalent crosslinking with TGase (especially those from microbial source; MTGase) on

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properties of protein-based films has been investigated for many food proteins, including dairy proteins,[5,6] soya 11S globulin,[6] egg white proteins,[7] deamidated gluten,[8] soy proteins,[5,9,10] gelatin.[2,11] In most of these works, it was generally indicated that tensile strength (TS) or puncture strength of cast films was a variable extent improved by the covalent crosslinking, but elongation at break (flexibility) decreased accordingly (e.g., 9, 10), while water vapor barrier property was also improved (e.g., 11). The extent of modification of properties considerably varied with the type of applied proteins.[5] The differences can be attributed to the differences in nature and extent of crosslinking of proteins.

Recently, the utilization of storage proteins from many *Phaseolus* legumes has attracted much interest from academic research and industrial application, due to their excellent functionality.[12,13]

In our previous work, the film-forming properties of three selected *Phaseolus* legume (kidney bean, red bean, and mung bean) protein isolates have been investigated for the first time and compared with those of soy protein isolate (SPI) films.[14] It was indicated that the mechanical properties of these protein isolate films were much poorer than those of SPI counterparts, while the hydrophobic properties were similar. However, a heating curing treatment after film formation could greatly improve the mechanical properties of cast films from kidney, red, and mung bean protein isolates, with the extent much higher than that of SPI films. The improvement of mechanical properties by the heating was attributed to heat-induced protein aggregation. This suggests that the proteins from those legumes exhibited good potential to be applied in the protein-based film formulations. Except the heat curing, very little information is available addressing the modifications of the films based on this kind of proteins by other treatments, e.g., enzymatic crosslinking techniques. Likewise, the 7S globulins (vicilin) in kidney bean protein isolate were recently confirmed to be good catalytic substrates for the MTGase.[15]

Thus, the objective of the present work was undertaken to investigate the influence of covalent crosslinking using MTGase on the properties of cast films from a specific protein isolate of *Phaseolus* legumes, namely red bean protein isolate (RPI). The dependence of the influence upon the enzyme concentration was specifically characterized. The RPI was chosen for the starting material, due to the two following considerations: (1) the mechanical properties of the corresponding films were poorest among three tested protein isolates from kidney, mung, and red beans[14]; (2) the physicochemical and functional properties of proteins in red bean have been more recognized in literatures relative to other legumes. Furthermore, in a more recent of our work, the influence of chemical succinylation on the properties of red bean protein isolate cast films has been reported.[16] The tested film properties included tensile strength and elongation at break, surface hydrophobicity, moisture content and total soluble matter, as well as water vapor barrier properties. The influence of MTGase treatment on the drying process of film-forming solutions was also evaluated. The protein solubility analysis in various solvents was applied to evaluate the change pattern of interactive force in MTGase-treated films.

EXPERIMENTAL

Materials

Red bean (*Phaseolus angularis*), cultivated in Shandong province of China were purchased in a supermarket in Guangzhou of China. The RPI were prepared from the defatted flour, according to the same process as described by Tang et al.[14] The protein content for the protein isolate was about 92.5% (dry basis), as determined by micro-Kjeldahl method, using a nitrogen conversion factor of 6.25.

L-glutamic acid γ-monohydroxamate and N_α-CBZ-GLN-GLY were purchased from Sigma Chemical Co. (St. Louis, MO). The commercial MTGase was purchased from TAIXIN YIMIN Fine Chemical Industry (Jiangsu province, China). The activity of the enzyme product was 200 units per gram of sample (U g^{-1})as determined by a colorimetric procedure using N_α-CBZ-GLN-GLY as the substrate and L-glutamic acid γ-monohydroxamate as the standard. The stock enzyme solution was prepared based on our previous work.[15] All other chemicals were of analytical or better grade.

Film preparation

The film-forming solutions were prepared by dispersing individual protein products (5%, w/w) and 0.5 g of glycerol per gram of protein in distilled water. The dispersions were magnetically stirred for 30 min at room temperature. The pH of the dispersions was adjusted to 9.0 (±0.1) with 1*M* NaOH. The resultant film-forming solutions were incubated at 90°C for 30 min in a shaking water bath, and then centrifuged at low speed (100 × *g*, 10 min) to remove the bubbles in the solutions (after cooling to room temperature). Various enzyme concentrations of 0 (control), 4, 10, 20, 30, and 40 U g^{-1} of MTGase were added into the film-forming solutions and mixed well, after cooling to room temperature. Last, the film-forming solutions were cast into leveled glass plates coated with polyethylene films (Clorox Cbina, Guangzhou, China). The film thickness was controlled by casting the same volume of the

solutions on each plate (18 × 20 cm^2). The castings were air-dried at room conditions [25°C ± 1°C, 50% ± 5% relative humidity (RH)] for 36 h, and then the films were peeled off the plates and various specimens for physical properties testing cut. Specimens of 2.5 × 10 cm^2 rectangular strips were cut for tensile testing. All the films were stored in a desiccator containing magnesium nitrate saturated solution [$Mg(NO_3)_2 \cdot 6H_2O$] with a RH of 50% for at least two days, before the measurements of individual properties.

Film thickness determination

Film thickness was measured with a hand-held micrometer to the nearest 0.001 mm. Five thickness measurements were taken on each tensile testing specimen along the length of the strip with the mean value used in tensile strength calculations. Similarly, five measurements were taken on each water vapor transmission (WVP) specimen, one at the center and four around the perimeter, and the mean values were used in WVP calculations.

Tensile strength and elongation at break

TS and EB of the films were measured using a TA-XT2i texture analyzer (Stable Micro Systems, London, UK). Initial gap separation and cross-head speed were set at 50 mm and 1 mm s^{-1}, respectively. TS was calculated by dividing the maximum load at break by initial specimen cross-sectional area.[18] EB was calculated by dividing by the extension at break of the specimen by the initial gauge length of the specimen (50 mm) and multiplying by 100.[17] Each data was the mean and standard deviation of at least six determinations.

Surface hydrophobicity

The surface hydrophobicity of the films was evaluated using contact angle (θ) of water upon the film surface by the sessile drop method, based on optical contact angle method.[16] The θ was measured in a static manner at time 0 s when the water drop was just deposited onto the test film surface. The θ measurements were carried out with an OCA 20 AMP (Dataphiscis Instruments GmbH, Germany). A droplet of de-ionized water (4 μL) was deposited on the film surface with a precision syringe. The drop image was recorded by a video camera, and the profile of droplet was numerically solved and fitted to laplace-young equation. Ten parallel measurements were performed for each film. The surface in contact with low-density polyethylene (LDPE) support during drying will be referred as the "bottom side" in this study and the other side in contact with the air during drying will be referred as "top side." At least 10 parallel measurements were performed for each side each film.

Moisture content and total soluble matter

The MC was determined from preweighed film samples (±0.0001 g) dried in an air-circulating oven at 105°C for 24 h, as the percentage of initial film weight lost during drying and reported on wet basis.

The TSM was measured by immersing preweighed film samples (obtained together for MC measurements) in 30 m*M* of distilled water (25°C) for 24 h, with occasional gentle stirring. The insoluble dry matter was measured by removing the film pieces from the beakers, gently rinsing them with distilled water, and then drying them in an air-circulating oven (at 105°C for 24 h). The weight of soluble dry matter was calculated by subtracting the weight of insoluble dry matter from the initial weight of dry matter.

Water vapor transmission rate and permeability

The WVTR and WVP of films were measured using the ASTM method.[17] Circular plastic cups with diameter of 3 cm and depth of 5 cm were used. Three grams of $CaCl_2$ were placed in each cup, and the cups were covered with circular films with diameter of 7 cm. Sealed cups were preweighed with their contents and placed in a desiccator kept at 25°C. One liter of pure water was placed in the bottom for providing 100% RH at 25°C. Then, the cups were weighed every 12 h for a week. The WVTR and WVP of films were measured from the weight gain of the cups. The WVTR (g h^{-1} m^{-2}) and WVP (g mm m^{-2} h^{-1} k Pa^{-1}) was calculated as eqs. (1) and (2):

$$\mathrm{WVTR} = W/t \times S \tag{1}$$

$$\mathrm{WVP} = \frac{\mathrm{WVTR} \cdot L}{\Delta P} \tag{2}$$

where W is the increased water weight (g), t is the time (h), S is the film area covered in the mouth of cups (m^2), L is the mean film thickness (mm); $\triangle p$ was the partial water vapor pressure difference (kPa) across the two sides of the film specimen (the vapor pressure of pure water at 25°C = 3.1671 kPa).

Transparency

Filmstrips cut in 1.2 × 4 cm^2 were attached to one side of a colorimetric cup. The relative transparency of films was measured at 500 nm, while the empty colorimetric cup was used as the control.

TABLE I
Tensile Strength (TS), Elongation at Break (EB), Surface Hydrophobicity (*Hs*), Moisture Content (MC), Total Soluble Matter (TSM), Water Vapor Transmission Rate (WVTR) and Permeability (WVP), as well as Transparency of Untreated and MTGase-Treated RPI Cast Films

Items	Enzyme concentrations (units per gram of protein)					
	0	4	10	20	30	40
Film thickness (mm)	0.084 ± 0.004	0.089 ± 0.004	0.093 ± 0.005	0.103 ± 0.005	0.112 ± 0.004	0.124 ± 0.005
TS (MPa)	1.27 ± 0.10^{c}	2.85 ± 0.30^{b}	3.32 ± 0.23^{a}	1.09 ± 0.18^{c}	0.89 ± 0.18^{d}	0.72 ± 0.20^{d}
EB (%)	84.4 ± 7.5^{c}	97.3 ± 5.3^{b}	108.0 ± 5.5^{a}	80.5 ± 10.5^{c}	61.7 ± 10.9^{d}	58.0 ± 12.7^{d}
H_s (°)						
Top	85.0 ± 2.1^{ce}	89.3 ± 1.9^{be}	93.4 ± 2.0^{ae}	93.0 ± 1.7^{ae}	93.4 ± 1.5^{ae}	92.5 ± 1.7^{ae}
Bottom	79.0 ± 2.2^{cf}	83.8 ± 2.0^{cf}	88.0 ± 1.6^{bf}	91.5 ± 1.5^{ae}	92.7 ± 2.1^{ae}	93.2 ± 1.8^{ae}
MC (%)	24.5 ± 1.2^{a}	21.5 ± 1.0^{b}	21.0 ± 1.0^{b}	21.3 ± 1.2^{b}	18.2 ± 1.1^{c}	18.0 ± 1.1^{c}
TSM (%)	25.9 ± 2.2^{c}	22.2 ± 1.3^{d}	22.0 ± 1.1^{d}	24.8 ± 1.2^{c}	29.5 ± 1.0^{b}	32.2 ± 1.1^{a}
WVTR (g/h.m^2)	23.9 ± 1.8^{a}	19.5 ± 1.4^{b}	17.2 ± 1.1^{b}	17.0 ± 1.8^{b}	17.1 ± 1.5^{b}	17.1 ± 1.2^{b}
WVP (g.mm/kPa.h. m^2)	0.63 ± 0.05^{a}	0.55 ± 0.08^{a}	0.51 ± 0.05^{a}	0.55 ± 0.05^{a}	0.60 ± 0.05^{a}	0.66 ± 0.05^{a}
Transparency (%)	75.2 ± 2.5^{a}	70.3 ± 1.5^{b}	70.1 ± 1.7^{b}	70.3 ± 1.4^{b}	70.2 ± 1.5^{b}	70.0 ± 1.6^{b}

Different superscript letters (a–d) indicate the significant difference at $p < 0.05$ level among various RPI films, in the same row. Different superscript letters (e–f) indicate the significant difference at $P < 0.05$ level between top and bottom sides, at a given enzyme concentration. The data for TS and EB are the means and standard deviations of more than 6 determinations, and that for H_s (contact angle) the means and standard deviations of more than 10 determinations.

Protein solubility of film

The protein solubility of film in various solvents was determined according to our previous work,[14] based on Lupano and colleagues.[18,19] Samples (5 mg mL^{-1}) were dispersed in various solvents as follows: B, Tris-Glycine buffer (0.086*M* Tris, 0.09*M* glycine., and 4 m*M* Na_2EDTA., pH 8.0); BSU, B containing 0.5% sodium dodecyl sulfate (SDS) and 8*M* urea; BSUM, BSU plus 1% (v/v) β-mercaptoethanol (2-ME). The mixtures were incubated for 24 h at 25°C in a shaking water bath. The resultant suspensions were centrifuged at 20,000 × *g* for 20 min at 25°C and the protein concentration in the supernatants were determined by the Lowry method[20] using bovine serum albumin (DingGuo Biochem, Beijing, China) as the standard. The colorimetry was performed at 750 nm in a T6 spectrophotometer (Beijing Purkinje General Instrument, Beijing, China). All determinations were conducted in at least duplicate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The protein samples for Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were prepared by dispersing the films (5 mg) in 1 mL of sample buffer, namely 0.125*M* Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 5% (v/v) 2-ME, 10% (v/v) glycerol, and 0.05% (w/v) bromophenol blue, and then heating at 100°C for 5 min. The SDS-PAGE experiment was performed on a discontinuous buffered system according to the method described by Laemmli[21] using 12% separating gel and 4% stacking gel. Each sample (6 μL) was applied to a single lane. Before the sample entering the separating gel, electrophoresis was performed at 40 mA, and the other was at 80 mA. The gel was stained with 0.25% Coomassie brilliant blue (*R*-250) in 50% trichloroacetic acid, and destained in 7% acetic acid and about 40% (v/v) methanol.

Statistics

Microcal Origin V.8.0 software (Microcal software, Northampton, USA) was used for statistical analysis of means and standard deviations. Duncan's Multiple Range Test ($P < 0.05$) was used to detect significant difference in different mean values.

RESULTS AND DISCUSSION

TS and EB

Table I shows the TS and EB values of untreated (control) and MTGase-treated RPI films. Untreated RPI film had TS and EB of 1.27 MPa and 84.4%, respectively. The data are consistent with our previous work.[14] The MTGase treatment resulted in remarkable changes in TS and EB of the cast films, with the extent of changes depending on the applied enzyme concentration (Table I). With the enzyme concentration increasing from 0 to 10 U g^{-1}, both TS and EB progressively and significantly ($P < 0.05$) increased. Compared with control films, the TS and EB were increased about 1.6 and 0.3 times, respectively, by the MTGase treatment at an enzyme concentration of 10 U g^{-1}. However, the TS and EB values on the contrary considerably decreased upon further increase in enzyme concentration up to 40 U g^{-1}. At

enzyme concentrations of 30–40 U g^{-1}, the TS and EB (especially the EB) were even significantly lower than that of control. These data suggest that there was an optimal concentration range for the enzyme to improve the mechanical properties (TS and EB) of cast RPI films. In the present work, the optimal enzyme concentration was estimated to be around 10 U g^{-1}. A similar enzyme optimal value (about 10 U g^{-1}) has been observed for TS of cast SPI films in our previous work.[10]

Besides the covalent polymerization, the MTGase may also lead to protein aggregation following structural unfolding, due to hydrophobic interactions of exposed hydrophobic groups or clusters initially buried within the protein molecules.[22] Both covalent polymerization and noncovalent aggregation will result in changes in mechanical properties of cast protein films. The improvement of TS of cast films by crosslinking within film structures, or formation of high molecular weight biopolymers using MTGase or guinea pig liver TGase has been widely observed in many previous literatures, e.g., for whey proteins,[23] demidated gluten,[8] soy proteins.[9,10] However, it should be kept in mind that the enzymatically induced protein aggregation accompanying the covalent polymerization would impair the mechanical properties of cast films,[10] which is often neglected in many previous literatures. Hence, the influence of MTGase treatment on the TS of cast protein films can be considered to be the net result from both covalent crosslinking or polymerization (positive) and protein aggregation (negative). In this work, the improvement of TS at low enzyme concentrations (e.g., 4–10 U g^{-1}) reflected that the covalent crosslinking contributed a major influence. In contrast, at high enzyme concentrations (e.g., above 20 U g^{-1}), the negative contribution from MTGase-induced protein aggregation to the TS of cast films seemed to superimpose the positive contribution from covalent polymerization.

The mechanical properties of protein film network are largely associated with distribution and density of intermolecular and intramolecular interactions, which will be dependent on arrangement and orientation of the polymers chains.[24] When more intermolecular interactions between proteins are of low energy, the films are easily stretched.[25] Therefore, the improvement in EB at low enzyme concentrations in this work (Table I) can be attributed to enhanced intermolecular interactions between proteins that are of low energy, especially many weak interactive forces, e.g., van der Waals forces. A similar improvement for elongation by MTGase has been observed for mixed gelatin and casein films.[26] On the other hand, the considerable impairment in EB at high enzyme concentration (Table I) can be attributed to increased intermolecular covalent bonding sites between proteins that are of high energy. In this case, each crosslinkage site can act as a rigid node and tends to restrict the motion of the chain segments adjacent to it.[11]

Concurrent increases in TS and EB values by the MTGase treatment at low enzyme concentrations of 4 and 10 U g^{-1} (Table I) has been similarly observed in deamidated gluten films treated by MTGase,[8] where this phenomenon has been explained as follows: the formation of covalent linkages by MTGase are flexible enough to permit a gain in elongation. However, in our previous works about MTGase-treated SPI films, an increase in TS and a concurrent decrease in EB were observed at similar enzyme concentrations.[9,10] The difference may be associated with the differences in nature of covalently crosslinked polymers between different proteins. In the SPI case, the formed covalent linkages would be much less flexible in nature than those in the current case. In other crosslinking cases, such as formaldehyde-treated peak protein films[27] and glutenin-rich films crosslinked by aldehydes,[28] the increases in TS of films are also accompanied by the decreases in EB.

Surface hydrophobicity (H_s)

Table I displays the initial contact angle (θ) values for air (top) and support (bottom) sides of untreated (control) and MTGase-treated RPI films. The quantitative definition of the relative terms "hydrophobic" and "hydrophilic" surfaces has been done respectively, for surfaces exhibiting a water $\theta > 65^\circ$ and $\theta < 65^\circ$.[14,29] All test films had θ values (on both air and support sides) higher than about 80°, indicating that these films were very hydrophobic in nature. On the whole, the top side θ of any test film was higher than the bottom side θ (Table I). A similar phenomenon has been observed in our previous work,[14] where the difference was attributed to the difference in reorientation of hydrophobic clusters of proteins during film-forming process.

The MTGase treatment at any test enzyme concentration significantly ($P < 0.05$) increased θ values (both sides) of cast films, with an exception for the bottom side at an enzyme concentration of 4 U g^{-1}; however, the differences were not significant among various MTGase-treated films (Table I). The data indicated that the MTGase treatment could improve the surface hydrophobic properties of cast RPI films. Similar improvements have been observed in MTGase-treated SPI films.[9,10] The improvement of film surface hydrophobicity could be attributed to MTGase-induced protein structural unfolding and subsequent exposure of hydrophobic clusters initially buried within the protein molecules. However, a reversal result was observed in MTGase-treated

deamidated gluten films,[8] and in this case, the decrease in θ was attributed to the changes in protein orientation by the covalent linkages. The difference reflects that the modification of surface hydropobicity of protein films by MTGase might be dependent upon the type of applied proteins.

MC and TSM

The MTGase treatment resulted in significant ($P < 0.05$) decrease in MC, and the extent of decrease depending on the applied enzyme concentration (Table I). The extent of decrease (around 14%) in MC was similar at enzyme concentrations in the range 4–20 U g^{-1}, while at concentrations 30–40 U g^{-1}, the extent of decrease (about 26%) was significantly ($P < 0.05$) higher (Table I). The decrease in MC has been observed in MTGase-treated SPI films.[9,10] This phenomenon is consistent with the fact that the covalent crosslinking of the proteins by MTGase usually leads to decreases in charged amino groups (e.g., ε-amino groups of lysine residues) that can bind water by hydrogen bonding.

In contrast, the influence of MTGase treatment on TSM of cast films was strongly dependent upon the applied enzyme concentration (Table I). At low enzyme concentrations (e.g., 4–10 U g^{-1}), the MTGase treatment significantly decreased the TSM of the cast films, while at high concentrations 30–40 U g^{-1}, the treatment on the contrary considerably and significantly increased the TSM. A similar phenomenon about the dependence of the TSM on enzyme concentration has been observed in MTGase-treated SPI films.[10] The TSM data are generally consistent with the TS data, reflecting that the network structure of cast films formed at various enzyme concentrations of MTGase might be different. The network structure of cast films treated at low enzyme concentrations seems to be more compact and homogenous (compared with control), while that at higher enzyme concentrations would be more inhomogeneous and porous. The TSM data at higher enzyme concentrations indirectly confirmed the formation of MTGase-induced aggregates that might impair the mechanical properties of cast films. The MTGase-induced formation of protein aggregates has been confirmed in SPI film-forming solutions treated with MTGase.[10]

WVTR and WVP

The MTGase treatment resulted in significant ($P < 0.05$) decreases in WVTR, but the extent of decrease was similar and insignificant among various MTGase-treated cast films (Table I). The WVTR data are in agreement with the contact angle data of these films, indicating that the MTGase treatment improved the hydrophobic properties (or lessened the hydrophilic properties). The decreases might be associated with the decreases in charged amino groups and the exposure of hydrophobic clusters following the MTGase-induced protein unfolding and subsequent aggregation. In contrast, the WVP of cast RPI films was slightly but insignificantly ($P < 0.05$) decreased by the MTGase treatment, at low enzyme concentrations 4–20 U g^{-1}, while at high enzyme concentrations above 20 U g^{-1}, the WVP was almost the same as that of control (Table I). The result is similar to that observed in MTGase-treated SPI films,[9] confirming that these protein films are poor barriers of water vapor.

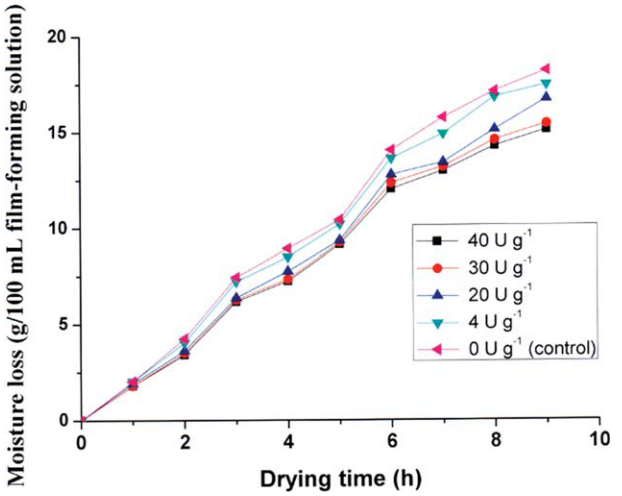

Figure 1 Moisture loss profiles of untreated and MTGase-treated film-forming solutions, during drying process. Each film-forming solution (40 mL) is cast on 21 × 18 cm^2 surface of a glass plate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Transparency

The mean transparency of RPI films was significantly ($P < 0.05$) decreased from 75.2 to about 70.0% by the MTGase treatment, but there were no significant differences among various MTGase-treated films (Table I). The influence of the color of enzyme solution on the film transparency could be considered to be minor, since there were no significant changes between untreated films and the films with the heat-inactivated enzyme solutions (Table I). The decrease in transparency can be attributed to the enzyme-induced formation of protein aggregates.

Moisture loss during drying process of the film-forming solutions

Figure 1 shows moisture loss profiles of untreated and MTGase-treated film-forming solutions during

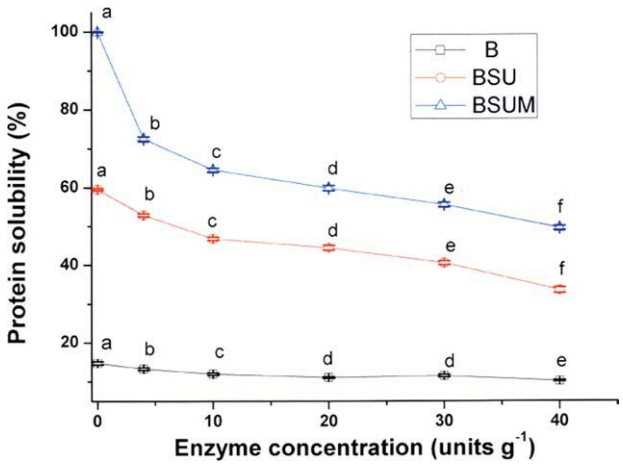

Figure 2 Protein solubility in various solvents for untreated and MTGase-treated RPI films as a function of enzyme concentration (0–40 U g^{-1}). Solvent B: Tris-Glycine buffer (0.086*M* Tris, 0.09*M* glycine, and 4 m*M* Na_2EDTA, pH 8.0); solvent BSU: solvent B with 0.5% SDS and 8*M* urea; solvent BSUM: solvent BSU plus 1% (v/v) 2-ME. Each value represents the mean and standard deviation of three replicates. Different characters (a–f) within the figure represent significant difference at $P < 0.05$ level, in a given solvent. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

drying process, at room temperature. In all cases, the moisture loss progressively increased with the drying time from 0 to 9 h, but the rate of moisture loss was highly dependent upon the applied enzyme concentration. The MTGase treatment led to a gradual decrease in moisture loss rate, in a concentration-dependent manner. A similar influence of MTGase on the moisture loss rate has been observed in MTGase-treated SPI film-forming solutions.[9] In a protein solution, there are basically three forms of moisture, free moisture, "semibound" and "bound" moisture. The moisture loss of film-forming solutions (5%, w/v) during drying of 9 h in the current work can be considered to be prominently from free moisture. Thus, the phenomenon indicated that the MTGase greatly decreased the loss rate of free moisture. Taking the TS and surface hydrophobicity data into account, it can be considered that the decrease in free moisture loss would in some sense be unfavorable for formation of compact and homogenous film network structure, since there would be more time for the formation of insoluble aggregates and/or precipitates induced by the enzyme.

PS in various solvents

To reveal the influence of MTGase treatment on the interactive forces involved in the formation of film network, we analyzed the PS of untreated and MTGase-treated RPI films in three kinds of solvents (B, BSU and BSUM), as shown in Figure 2. Usually, solvent B disrupts the electrostatic interactions, solvent BSU disrupts the hydrogen bonds and hydrophobic interactions, and BSUM can disrupt hydrogen bonds, hydrophobic interactions, as well as disulfide bonds.[18,19]

The PS of untreated RPI film in B was about 14.8%, while that in BSU and BSUM 59.5 and 100%, respectively, (Fig. 2), indicating that the interactive forces maintaining the untreated film network were mainly hydrogen bonds and hydrophobic interactions, and to a similar extent, disulfide bonds. This is consistent with our previous observation of remarkable decrease (about 9.5 μmol per gram of protein) in free sulfydryl (SH) content in film-forming RPI solutions after film formation.[14] The MTGase treatment resulted in progressive decreases of PS in B, BSU, and BSUM, depending on the enzyme concentration, indicating gradual change in interactive force pattern in the films. The PS in B progressively and significantly decreased from 14.8 to 10.2% by the MTGase treatment, when the enzyme concentration increased from 0 to 40 U g^{-1} (Fig. 2), indicating slight decline in electrostatic interactions involved in the film network formation. The slight decrease in electrostatic interactions is in agreement with the fact that the MTGase treatment results in gradual decrease in charged amino groups (e.g., ε-amino groups of Lys resides) of the proteins, due to covalent linkage formation, upon the increase in enzyme concentration. The PS difference between BSU and B also progressively and significantly decreased from 44.7 (for control) to 22.3% (for MTGase-treated film at 40 U g^{-1}) (Fig. 2), suggesting that the contribution of hydrogen bonds and hydrophobic interactions to the film network considerably and progressively decreased upon the increase in enzyme concentration. Furthermore, the PS difference between BSUM and BSM (reflecting the contribution of disulfide bond formation) was also significantly decreased by the MTGase treatment from 40.5 to about 15-19.6%, but there were no significant differences among MTGase-treated films at 20–40 U g^{-1} (Fig. 2). This data suggests that the MTGase treatment considerably lessened the contribution of disulfide bond formation to the film network.

On the other hand, the PS in BSUM progressively decreased from 100 to about 50%, as the enzyme concentration increased from 0 to 40 U g^{-1} (Fig. 2), indicating gradual increase in the contribution of enzymatically induced covalent linkages between the proteins. In the case of 40 U g^{-1}, the PS of the MTGase-treated film in B, BSU, and BSM was 10.2, 33.5, and 49.5%, respectively, suggesting that in this case, the main interactive forces maintaining the film network were covalent linkages (about 50% of total interactive forces), followed by hydrogen bonds and

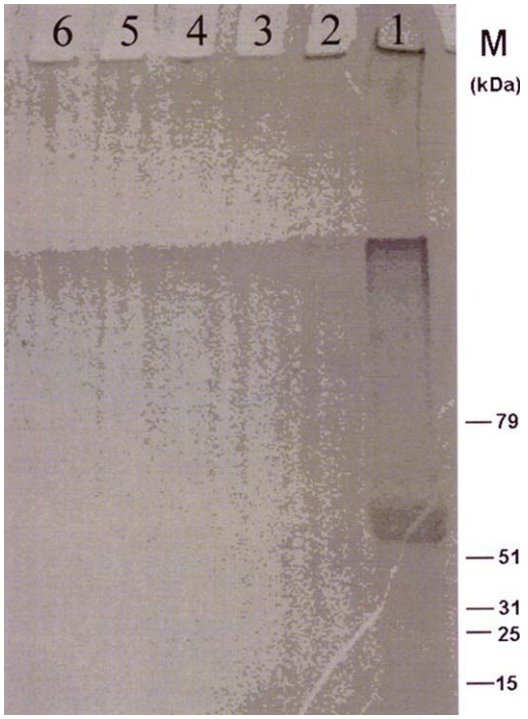

Figure 3 SDS-PAGE profiles of soluble proteins in untreated and MTGase-treated RPI films. Lanes 1–6 represent the RPI films treated by MTGase at enzyme levels of 0, 4, 10, 20, 30, and 40 U g^{-1}, respectively. M, protein markers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

hydrophobic interactions (about 23%), and disulfide bonds (16%), while the electrostatic interactions were still minor (about 10%; similar to the control).

SDS-PAGE analysis

The crosslinkage of the proteins in cast RPI films by MTGase at various enzyme concentrations 0–40 U g^{-1} was evaluated by reducing SDS-PAGE. In the untreated film case, a major band at around 56 kPa corresponding to the polypeptide of vicilin in RPI was observed (Fig. 3, Lane 1). In this case, there were a set of aggregates with molecular weight above 56 kPa observed, and some aggregates even could not enter the stacking gel and the separating gel. In contrast, there were no distinct dyed bands observed in any tested MTGase-treated RPI film (Fig. 3, Lanes 2–6), indicating that the proteins involved in the MTGase-treated film network couldn't be solubilized by 2% (w/v) SDS and 5% (v/v) 2-ME. This phenomenon further confirmed that the film network of MTGase-treated films was mainly maintained by the covalent linkages between the proteins. Similar phenomena have been observed in TGase-treated α_{s1}-casein films,[30] and whey proteins.[23]

CONCLUSIONS

The MTGase addition prior to drying of film-forming solutions resulted in considerable changes in properties of cast RPI films, depending on the applied enzyme concentration. Generally, the mechanical properties (TS and EB) was improved by the MTGase treatment at low enzyme concentrations 4–10 U g^{-1}, but considerably impaired at high enzyme concentrations, e.g., above 20 U g^{-1}. The MTGase treatment also resulted in improvements of surface hydrophobicity, MC, TSM, and WVTR, but most of the improvements were independent of the enzyme concentration, within the test concentration range. The influence of MTGase treatment on the properties seemed to be closely associated with the enzyme-induced covalent polymerization and/or aggregation. The interactive force pattern of proteins involved in the film network formation was remarkably changed by the MTGase treatment. In the MTGase-treated RPI films, the main interactive forces included covalent linkage (major), hydrophobic interactions and hydrogen bonds, as well as disulfide bonds. These results suggest that the covalent crosslinking by MTGase could be applied to improve the properties of legume protein films, but it should be kept in mind that the improvements are closely related to the applied enzyme concentration.

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