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Gelling of microbial transglutaminase cross-linked soy protein in the presence of ribose and sucrose

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ABSTRACT

Soy protein isolate (SPI) was incubated with microbial transglutaminase (MTGase) enzyme for 5 (SPI/MTG(5)) or 24 (SPI/MTG(24)) h at 40 °C and the cross-linked SPI obtained was freeze-dried, and heated with 2% (w/v) ribose (R) for 2 h at 95 °C to produce combined-treated gels. Longer incubation period resulted in more compact and less swollen SPI particle shape when reconstituted with sugar solution. Thus, this MTGase treatment affected samples in terms of flow behaviour and gelling capacity. Rheological study showed different gelling profiles with the cross-linking treatments and combined cross-linked SPI gave a higher G' value compared to single treated samples. These are due to the formation of additional ε-(γ-glutamyl)lysine bonds and "Maillard cross-links" within the SPI protein network during the MTGase incubation and heating in the presence of ribose (i.e. reducing sugar). Network/non-network protein analysis found that network protein increased with cross-linking treatment, which also resulted in different SDS–PAGE profiles. As in non-network protein fraction, A_4 subunit was suggested to become part of the network protein as a result of combined cross-linking.

1. Introduction

Protein modification involving the application of transglutaminase enzyme has attracted attention in the food industries (MdYasir, Sutton, Newberry, Andrews, & Gerrard, 2007a; Tang, 2007). Microbial transglutaminase (MTGase; protein–glutamine: amine γ-glutamyltransferase, E.C. 2.3.2.13) works by catalyzing an acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutamine residues (acyl donors) and variety of primary amines (acyl acceptors), including the ε-amino group of lysine residues to form an ε-(γ-glutamyl)lysine isopeptide bond (Motoki & Seguro, 1998). This treatment has been used in meat products (Trespalacios & Pla, 2007), fish products (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2006), dairy products (Jaros, Partschefeld, Henle, & Rohm, 2006; Lorenzen, 2007), legume products (Tang, Li, Wang, & Yang, 2007) and wheat products (Caballero, Gómez, & Rosell, 2007) in enhancing the texture and functional properties.

Another potential modification method, which is the "Maillard cross-linking" or Maillard reaction comprises the reaction between reducing sugars and amino groups of amino acids and proteins, has been shown to produce cross-linked protein and improved protein gel strength and texture of protein-based products (Gerrard, Brown, & Fayle, 2002; Hill & Easa, 1998; Md Yasir, Sutton, Newberry, Andrews, & Gerrard, 2007b).

MTGase-cross-linked high protein products (e.g. surimi, yogurt and sausage) are available in the market. To further enhance the functionalities of cross-linked protein such as gelling capacity and water holding capacity, it is possible for technologists to use a combination of two cross-linking treatments. On the other hand, there could be situations when the cross-linked products are subjected to heating treatments (cooking, retort cooking, blanching or drying) in the presence of reducing sugars or other ingredients capable of undergoing the Maillard reaction. A previous paper (Gan, Cheng, & Easa, 2008) has shown the effect of this on physicochemical properties and microstructure of the soy protein isolate (SPI) gel formed. Other studies also showed that this combined treatment not just improved the mechanical properties (i.e. viscoelasticity and gel strength) (Gan et al., 2008), also lowered the undesirable browning effect (Gan, Cheng, & Easa, 2009). Surprisingly, the resulting SPI gel was also proved to be more easily digested by digestive enzymes than the control Maillard gel of SPI with ribose alone (Gan, Cheng, Azahari, & Easa, 2009). Therefore, the effects of MTGase pre-incubation in the behaviours of SPI particles prior to ribose-induced "Maillard cross-linking" were investigated.

The objective of this paper was to further investigate the gelling of SPI in terms of gelling capacity, rheological properties and the network/non-network protein formed, as a result of the combined cross-linking treatments using microbial transglutaminase, followed by a heating treatment with ribose that induces "Maillard cross-linkings". Ribose, an emerging nutraceutical ingredient

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(Hellsten, Skadhauge, & Bangsbo, 2004), is also known for its high reactivity in terms of reacting with protein via the Maillard reaction (Ashoor & Zent, 1984) and capability to cross-link proteins (Graham, 1996).

2. Materials and methods

2.1. Materials

Commercial grade soy protein isolate (SPI) with 90% protein content and nitrogen solubility index of 88% was purchased from Sim Company Sdn. Bhd., Penang, Malaysia. Other compositions are: moisture (<8.0%); ash (<6.0%) and fat (<0.7%). A Ca^{2+} independent microbial transglutaminase (ACTIVA TG-K) was a gift from Ajinomoto Co., Inc. (Tokyo, Japan). D-(-)-ribose was purchased from Sigma–Aldrich company. Other chemicals (analytical grade) used in this study were obtained from Sigma–Aldrich company and Fluka company.

2.2. Sample treatments

2.2.1. MTGase cross-linking

SPI dispersions were prepared by adding 10 g of SPI into 100 mL of distilled water and mixed using magnetic stirring (100 rpm, 10 min). MTGase (4.0 U) was subsequently added to the SPI dispersions. Sodium azide (0.01%) was also added to prevent microbial growth. The mixtures were then incubated at 40 °C with constant shaking (90 rpm) in LM-570R Orbital Shaker (Yih Der, Taiwan) for 5 and 24 h. The non-MTGase incubated samples (0 h) considered as a control sample (SPI/C). The enzyme was subsequently deactivated by heating at 95 °C for 30 min in a water bath. The mixtures were then cooled to room temperature in running water followed by freezing at −20 °C prior to lyophilization (Labconco, Kansas City, US). The lyophilized samples were ground and sieved (width = 0.5 mm, diameter = 0.32 mm). All samples were stored in glass sample bottles in a desiccator prior to further treatment.

2.2.2. Maillard cross-linking

MTGase-incubated (SPI/MTG(5) or SPI/MTG(24)) and non-incubated SPI (SPI/C) dispersions (0.1 g/mL) with 2% (w/v) ribose or 2% (w/v) sucrose (as control) were prepared and subjected to a heating treatment at 95 °C for 2 h in WB22–16X2501 water bath (Memmert, Germany). The mixtures were then cooled to room temperature in running water. The gels formed were stored in SJ-D21T refrigerator (SHARP, Osaka, Japan) at ~2 °C overnight prior to analysis.

The sample treatments/preparations and their designations are summarized in Fig. 1. Sample definitions and descriptions are: SPI/C/S(2): non-MTGase incubated sample heated with 2% (w/v) sucrose solution for 2 h; SPI/MTG(5)/S(2): MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) sucrose solution for 2 h; SPI/MTG(24)/S(2): MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) sucrose solution for 2 h; SPI/C/R(2): non-MTGase incubated sample heated with 2% (w/v) ribose solution for 2 h; SPI/MTG(5)/R(2): MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) ribose solution for 2 h; SPI/MTG(24)/R(2): MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) ribose solution for 2 h.

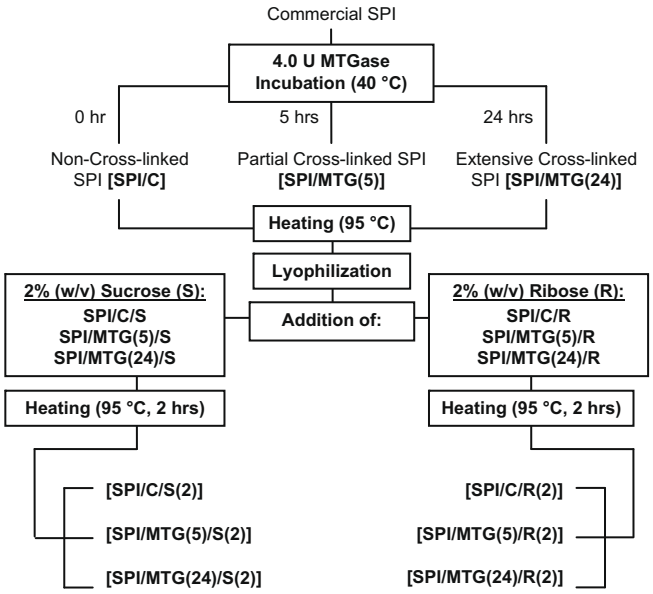

Fig. 1. Summary of sample preparations/treatments and their designations.

2.3. Analysis

2.3.1. Enzyme activity determination

Enzyme activity determination was performed according to the method of Folk (1970). A reaction mixture (2 mL) containing 200 μL MTGase solution, 1400 μL of 0.1 M tris–acetate buffer (pH 6.0), 100 μL of 2.0 M hydroxylamine and 300 μL of 0.1 M CBZ-L-glutaminyl-glycine was prepared. After incubation for 10 min at 37 °C, the reaction was stopped by the addition of 2 mL of TCA solution prepared from equal portions of 15% TCA, 5% $FeCl_3$ in 0.1 N HCl and 2.5 N HCl. The precipitate was removed by centrifugation (Hettich, Germany) at 4000 *g* for 15 min. The absorbance of the supernatant at 525 nm was then obtained using UV-1601PC UV–vis recording spectrophotometer (Shimadzu, Japan).

Standard curve was obtained using L-glutamic acid-γ-monohydroxamic acid as standard with a concentration range from 0 to 0.25 mM.

One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of peptide-bound γ-glutamyl hydroxamate per min. Specific activity is the number of units of activity per milligram of protein.

2.3.2. Flow analysis of SPI suspensions

The flow behaviours of MTGase-incubated and non-incubated SPI suspensions were examined using AR1000-N rheometer (TA Instrument, UK). A parallel plate measuring geometry was used (20 mm diameter) with a gap width of 1 mm. Samples were loaded onto the rheometer and allowed to equilibrate to measuring temperature (25 °C) for 5 min. Shear stress and viscosity of the suspensions were obtained over shear rate ranging from 0.01 to 1000 s^{-1}.

2.3.3. Microscopic observation of SPI suspensions

Microscopic appearance of the MTGase-incubated suspensions were recorded with a light microscope (Olympus, Japan). One drop of the diluted sample was spread to a very thin layer on microscope slide. A cover glass was placed on the smears. Samples were observed under magnification of 100×. Micrographs were obtained using DIMAS (Digital Image Measurement and Analysis System version 4.0, USA).

2.3.4. Gelling capacity

Aqueous suspensions (8–14% w/v) of SPI/C/S(2), SPI/C/R(2), SPI/MTG(5)/S(2), SPI/MTG5/R(2), SPI/MTG(24)/S(2) and SPI/MTG(24)/R(2) samples were heated at 95 °C for 2 h and then cooled overnight at 4 °C. The formation of self-supporting gel was examined by visual observation (Sorgentini, Wagner, & Añón, 1995).

2.3.5. Dynamic rheological measurement

The gelation profiles of 15% (w/v) suspensions of SPI/C/S, SPI/C/R, SPI/MTG(5)/S, SPI/MTG(5)/R, SPI/MTG(24)/S and SPI/MTG(24)/R during heating were investigated by dynamic oscillatory rheometry using a AR1000-N rheometer (TA Instrument, UK). Linear viscoelastic ranges of the samples were determined at two different temperatures (i.e. 25 °C and 95 °C) prior to the analysis. The rheometer was set up with 2 cm parallel plate geometry (1 mm gap). Each sample was loaded into the rheometer and the perimeter was covered with a thin layer of paraffin oil to prevent dehydration and the plate was insulated to reduce heat dissipation. Subsequently, the sample was allowed to relax and equilibrate at initial temperature (25 °C) for 5 min prior to assessment of its rheological properties in different analysis as below.

2.3.5.1. Temperature ramp analysis. The samples were heated at a scan rate of 5 °C/min by circulating water system. After reaching the final temperature (95 °C), the samples were held at the temperature for 1 h and subsequently cooled down at a rate of 5 °C/min from 95 to 25 °C. The measurement was performed at fixed frequency of 1 Hz with maximum strain of 0.05% (within the linear viscoelastic region). Note that the holding period was reduced to 1 h instead of 2 h is due to the equipment limitation (i.e. to prevent the "overheat circumstances" of the temperature control system).

2.3.5.2. Frequency sweep analysis. Frequency sweep experiment was carried out on the same sample from temperature ramp test at frequencies range from 0.01 to 10 Hz with 0.05% strain at 25 °C.

2.3.6. Network and non-network protein content of the gels

Isolation of network and non-network protein within the gel was conducted according to a modified method of Lakemond et al. (2003). SPI/C/S(2), SPI/C/R(2), SPI/MTG(5)/S(2), SPI/MTG(5)/R(2), SPI/MTG(24)/S(2) and SPI/MTG(24)/R(2) gels were centrifuged at 15,800*g* for 30 min at 20 °C. As estimated from Stokes equation protein aggregates in the supernatant is smaller than ~0.2 μm. The protein in the supernatant obtained was defined as non-network protein. The precipitates obtained from centrifugation were transferred to acetate phosphate buffer (250 times excess w/v) according to the final pH of the gel. Sodium azide (0.01%) was added to prevent microbial growth. The precipitate was mixed and soluble protein in the samples was allowed to diffuse out for 24 h. The precipitates were subsequently centrifuged (870 *g* for 15 min) and washed twice with the same buffer. The supernatant was accumulated as non-network protein whereas the final residue obtained was defined as network protein. The protein contents of network/non-network protein were then determined using macro-Kjeldahl method (AOAC., 1990).

2.3.6.1. Network and non-network protein fractions of the gels. The fraction of the protein which contributed to network and non-network protein was determined using SDS–PAGE according to the method of Laemmli (1970) using 4% stacking gel and 12% separating gel. Gel samples were mixed with sample buffer containing 2% SDS and 5% β-ME (ratio 1:4). The mixtures were then heated at 90 °C for 5 min before loading. The samples were run at 150 V and 50 mA in the Mini-PROTEAN II Electrophoresis Cell for 1.5 h. Subsequently, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid mixed solution, and destained in solution containing 40% methanol and 10% acetic acid. The gel was then photographed with a CANON IXUS 430 digital camera. The protein fractions were identified using Sigma's recombinant molecular weight standard mixture with molecular masses of 15, 25, 35, 50, 75, 100 and 150 kDa.

2.4. Statistical analysis

An analysis of variance (ANOVA) of the data was performed and Duncan's Multiple-Range Test with a confidence interval of 95% was used to compare the means.

3. Results and discussion

Previous study (Gan et al., 2008) showed that combined treatments enhanced the gelling properties of SPI gels by increasing the compressive gel strength, viscoelasticity properties (K_1 and K_2), solidity (E_a), and by lowering the% deformation as compared to single-treated or control gels (gels produced without cross-linking treatment). However, different gel morphologies were found as MTGase incubation time prolonged. Therefore, the effects of MTGase pre-incubation in the behaviours of SPI particles prior to ribose-induced "Maillard cross-linking" were investigated.

Flow analysis was first conducted using MTGase pre-cross-linked and non-pre-crosslinked samples (i.e. SPI/MTG(5), SPI/MTG(24) and SPI/C with degree of cross-linking of 78%, 93% and 0%, respectively, as reported previously (Gan et al., 2009)). Fig. 2a and b show that these samples exhibited thixotropic behaviour (shear thinning) and SPI/MTG(5) gave the highest viscosity followed by SPI/C and SPI/MTG(24). Variables affecting the rheological behaviour of a protein suspension include molecular size, shape, conformation, protein–solvent interactions, hydrodynamic volume, and molecular flexibility in the hydrated state (Damodaran, 1996; Rha & Pradipasena, 1986). In concentrated protein suspension, the "interactive volume" of the hydrated protein plays an important role in the viscosity, which includes the effect of hydrodynamic and/or potential interactions. As the concentration of protein increases at a characteristic concentration the interactive volume comes into contact and further crowding compacts the "interactive volume" (Rha & Pradipasena, 1986).

As observed under microscope (Fig. 3), proteins in SPI/C exhibited irregular shape of protein particles. This change of protein particles resulted from the heating and the grinding process which leads to swelling and breakdown down of protein particles, respectively, during the preparation of the powdered samples. The partial cross-linked samples (SPI/MTG(5)) seems to retain a swollen globular shape. This occurrence might be due to the formation of MTGase cross-linking in SPI that resisted from chain opening as reported by Tang, Chen, Li, and Yang (2006). These swollen particles of SPI/MTG(5) gave higher interactive volume and thus increased the viscosity of the suspension. A more prominent effect was observed in SPI/MTG(24) that undergone prolonged incubation (i.e. extensive cross-linked) which retained the compact globular shape without or with slight swelling. The viscosity of the suspension containing larger aggregates is higher than that of suspension containing small particles and that the hysteresis effect is more pronounced (Rha & Pradipasena, 1986). However, particle size is not the only factor responsible for the high viscosity and hysteresis effect. It also depends on particle shape and the interactions between the particles. A breakdown in particle size and shape was observed in SPI/C. This would explain the differences between SPI/MTG(5) and SPI/C, which caused the latter obtained lower viscosity.

On the other hand, prolonged MTGase incubation significantly lowered the viscosity of the sample (SPI/MTG(24)). Tanimoto and Kinsella (1988) has shown that β-lactoglobulin cross-linked by MTGase contains intra-molecular bonds that impedes unfolding of the molecules upon heating, hence suggesting a compact nature of the polymerized SPI molecules leading to a smaller hydrodynamic volume. In addition, their study revealed that the formation of MTGase catalyzed ε-(γ-glutamyl)lysine bonds attenuates

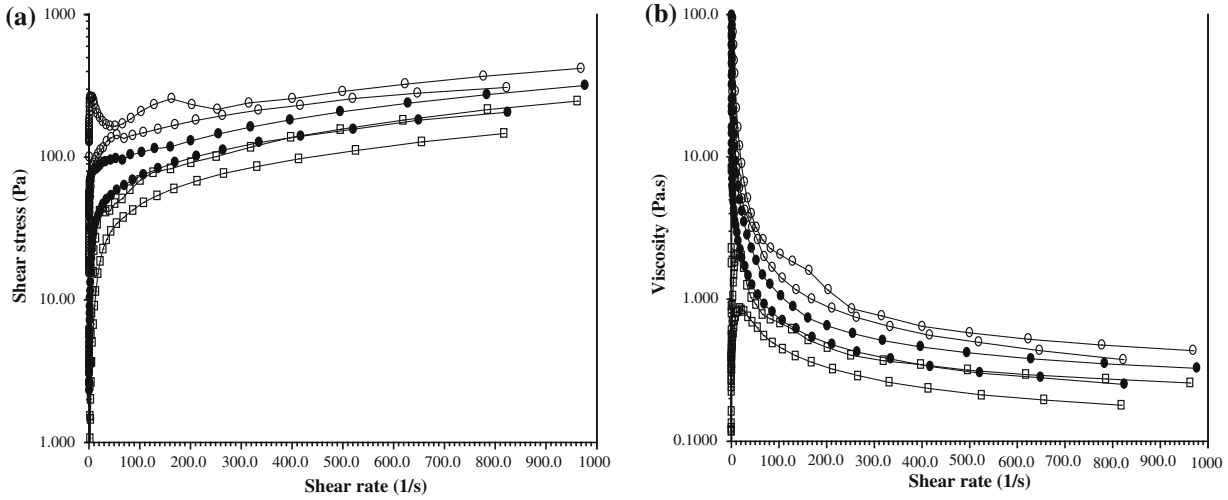

Fig. 2. Flow behaviours [(a) shear stress vs. shear rate and (b) viscosity vs. shear rate] of SPI/C (●), SPI/MTG(5) (○) and SPI/MTG(24) (□) suspension at a concentration of 15% (w/v) after ageing for 24 h. **Sample definitions and descriptions:** SPI/C: non-MTGase incubated sample. SPI/MTG(5): MTGase incubated sample (incubated for 5 h). SPI/MTG(24): MTGase incubated sample (incubated for 24 h).

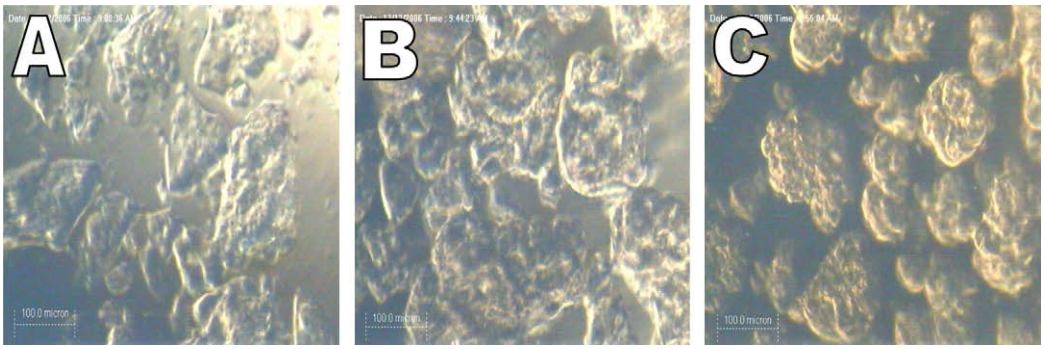

Fig. 3. Microstructure of (A) SPI/C, (B) SPI/MTG(5) and (C) SPI/MTG(24) suspensions at a concentration of 15% (w/v) observed at magnification 100×. Sample definitions and descriptions: SPI/C: non-MTGase incubated sample. SPI/MTG(5): MTGase incubated sample (incubated for 5 h). SPI/MTG(24): MTGase incubated sample (incubated for 24 h).

hydrophobic interaction through steric hindrance and formation of compact molecules that limits exposure of the hydrophobic moieties. Thus, the compact protein structures confined the hydrophobic residues in the interior part of the aggregates and prevent hydrophobic association.

Heating a protein solution causes molecular unfolding which leads first to aggregation and then to gelation, when the amount of aggregated protein exceeds a critical concentration (Clark, 1992; Clark & Lee-Tuffnell, 1986). Therefore, inclusion of additional non-disulphide covalent bonds catalyzed by MTGase incubation and/or by heating in the presence of ribose (i.e. "Maillard cross-linking" occurred) would decrease the protein requirement. Note that self-standing gel was studied instead of gelation alone. Thereby, the threshold concentration of SPI in producing self-standing gel in protein concentration at a range of 8–14% (w/v) was investigated.

Table 1 shows that SPI/C/S(2) did not produce self-standing gel even at a concentration of 14% (w/v), as for SPI/C/R(2) self-standing gel was obtained at a concentration of 12% (w/v). This is due to ribose-induced "Maillard cross-linking" enhanced the interactions between protein chains thus increased the gelling capacity. On the other hand, the protein concentration requirement has been reduced to 10% (w/v) after the MTGase incubation for 5 (SPI/MTG(5)/S(2)) or 24 h (SPI/MTG(24)/S(2)) which showed that MTGase-catalyzed ε-(γ-glutamyl)lysine bonds gave a more pronounced effect in enhancing gelling capacity. However, incorporation of ribose in MTGase pre-crosslinked samples (SPI/MTG(5)/R(2) and SPI/MTG(24)/R(2)) gave insignificant effect and there were two significant different gel structures formed, namely a true three-dimensional filamentous network and a particulate non-homogeneous random aggregated gel with different gel properties were observed in SPI/MTG(5)/S(2) or SPI/MTG(5)/R(2) and SPI/MTG(24)/S(2) or SPI/MTG(24)/R(2), respectively, as reported in previous paper (Gan et al., 2008).

Therefore following the result obtained from the gelling capacity analysis, gelling behaviour of SPI/C, SPI/MTG(5) and SPI/MTG(24) in the presence of sucrose (S) or ribose (R) during

Table 1
Estimation of minimum protein concentration required in producing self-standing gel.

Samples	Protein concentration (%, w/v)
SPI/C/S(2)	$\geqslant 15^{a}$
SPI/C/R(2)	$\geqslant 12^{b}$
SPI/MTG(5)/S(2)	$\geqslant 10^{c}$
SPI/MTG(5)/R(2)	$\geqslant 10^{c}$
SPI/MTG(24)/S(2)	$\geqslant 10^{c}$
SPI/MTG(24)/R(2)	$\geqslant 10^{c}$

Note: Comparison within the column in the table with the data written as mean ($n = 3$). Means within the same column not followed by the same letter are significantly different at $p < 0.05$ level of significance, according to Duncan's Multiple-Range Test.

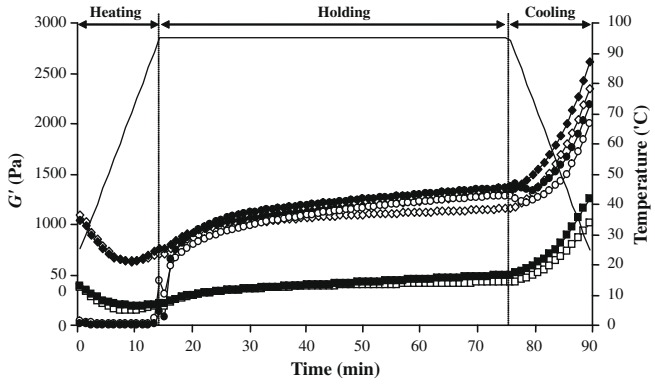

Fig. 4. Changes of the G' of (□) SPI/C/S, (■) SPI/C/R, (◇) SPI/MTG(5)/S, (♦) SPI/MTG(5)/R, (○) SPI/MTG(24)/S and (●) SPI/MTG(24)/R at a concentration of 15% (w/v) in the heating–holding–cooling cycle (heating from 25 °C to 95 °C at 5 °C/min; holding at 95 °C for 1 h; cooling from 95 °C to 25 °C at 5 °C/min). All samples were tested at a frequency of 1 Hz and strain of 0.05%. The line shows the temperature against time. Sample definitions and descriptions: SPI/C/S: non-MTGase incubated sample heated with 2% (w/v) sucrose solution. SPI/MTG(5)/S: MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) sucrose solution. SPI/MTG(24)/S: MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) sucrose solution. SPI/C/R: non-MTGase incubated sample heated with 2% (w/v) ribose solution. SPI/MTG(5)/R: MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) ribose solution. SPI/MTG(24)/R: MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) ribose solution.

heating–holding–cooling cycle using a small deformation oscillation test (i.e. temperature ramp) was investigated. Fig. 4 shows the time–temperature profiles of gelation for different SPI samples. Protein suspensions were heated from 25 °C to 95 °C, then held at 95 °C for 1 h and subsequently cooled down to 25 °C, at which storage modulus (G') was recorded as a function of heating time.

During the ramping up (heating) period, different G' change patterns were evident. A significant drop in G' with heating time and heating temperature was observed in SPI/C/S, SPI/C/R, SPI/MTG(5)/S and SPI/MTG(5)/R. This could be attributed to the gain in entropy during protein denaturation that resulted from a higher opening and diminishing rate of hydrogen bonds and electrostatic cross-links. As a result, an apparent increase in mobility was followed (Chronakis, 1996; Tang, Wu, Chen, & Yang, 2006). Conversely, the extensively MTGase cross-linked samples (i.e. SPI/MTG(24)/S and SPI/MTG(24)/R) did not respond ($G' \approx 0$) to the oscillation test within a threshold of 13 min heating, after which G' of the samples increased drastically. This again indicates that the sample has been highly cross-linked till it resists to hydration upon heat treatment. However, once this cross-linked region was "invaded", a co-operative dissociation of the protein molecules followed and subsequently re-associated. This was supported by a drastic increase in G' shown in Fig. 4.

Growth region followed by a plateau region could be observed after the 13 min where the subsequent heating to 95 °C and holding at the temperature for 1 h involved. In these regions, formation of disulphide cross-linking and/or ongoing incorporation of protein and rearrangement in network structure took place in samples without addition of ribose (i.e. SPI/C/S, SPI/MTG(5)/S and SPI/MTG(24)/S). However, SPI/C/R, SPI/MTG(5)/R and SPI/MTG(24)/R obtained a slight higher value in G' profile than that of heated in sucrose suggesting the occurrence of additional "Maillard cross-linking" throughout the heating/holding processes (Cabodevila, Hill, Armstrong, De Sousa, & Mitchell, 1994; Easa, 1996). At any particular heating time and heating temperature, the partially MTGase cross-linked samples (i.e. SPI/MTG(5)/S and SPI/MTG(5)/R) show relatively higher G' as compared to the SPI/C/S or SPI/C/R. This again indicates that the MTGase cross-linked samples are highly associated via inter- and/or intra-molecular interactions that subsequently account for higher G' observed.

Much apparent increase in G' was observed on cooling. This increase in G' could completely be reversed on heating in which no formation of covalent bonds took place at this stage (Renkema, Knabben, & van Vliet, 2001). Those exposed hydrophobic groups would associate together again to form new aggregates with larger size, subsequently reconstructing the gel network in this stage (Tang et al., 2006). Note that the gelation of SPIs that treated with MTGase and/or ribose-induced Maillard reaction was not mainly due to association and dissociation of protein chains alone. The cross-linked protein chains via MTGase and/or "Maillard cross-linking" would gave a larger and more compact protein aggregates compared to non-cross-linked gel samples (Gan et al., 2008). Thus, gave higher G' values to gels produced.

The mechanical spectra of the gels obtained from the heating–holding–cooling cycle were subsequently characterized via frequency sweep at a constant strain (0.05%) and temperature (25 °C). The mechanical spectras of the gels formed as a function of oscillation frequency (0.01–10 Hz) are shown in Fig. 5. G' values of all samples were slightly frequency dependent and thus the loss tangent are low (result not shown), illustrating predominantly solid-like response with more elastic network. Combination of both MTGase and Maillard cross-linking had markedly increased the G' values. The highest G' value was shown in SPI/MTG(5)/R, followed by SPI/MTG(5)/S, SPI/MTG(24)/R, SPI/MTG(24)/S, SPI/C/R and SPI/C/S. Interestingly, the G' of extensively cross-linked SPIs (i.e. SPI/MTG(24)/S and SPI/MTG(24)/R) seemed to be lower than partially cross-linked SPIs (i.e. SPI/MTG(5)/S and SPI/MTG(5)/R). Damodaran (1988) stated that heating the protein solution above the denaturation temperature is not the only critical step in thermal gelation process. The tendency of the protein to refold during the cooling regime of the gelation process seems to play a critical role in the formation of the gel network. Therefore, it is suggested that the partial cross-linked SPIs (SPI/MTG(5)/S or SPI/MTG(5)/R) were able to unfold upon denaturation that optimized the protein–protein and protein–solvent interactions. Thus, an ordered-strand gel network could be formed which resists to deformation. As for the extensively cross-linked systems (SPI/MTG(24)/S or SPI/

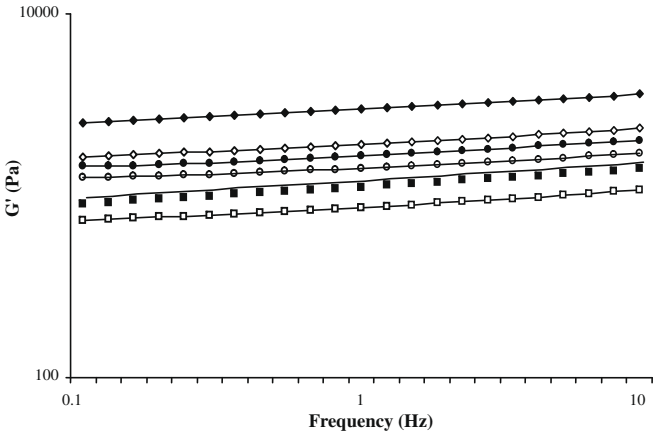

Fig. 5. Mechanical spectra of (□) SPI/C/S, (■) SPI/C/R, (◇) SPI/MTG(5)/S, (♦) SPI/MTG(5)/R, (○) SPI/MTG(24)/S and (●) SPI/MTG(24)/R at a concentration of 15% (w/v) as a function of oscillation frequency after the heating–holding–cooling cycle (heating from 25 °C to 95 °C at 5 °C/min; holding at 95 °C for 1 h; cooling from 95 °C to 25 °C at 5 °C/min). All samples were tested at a strain of 0.05%. Sample definitions and descriptions: SPI/C/S: non-MTGase incubated sample heated with 2% (w/v) sucrose solution. SPI/MTG(5)/S: MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) sucrose solution. SPI/MTG(24)/S: MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) sucrose solution. SPI/C/R: non-MTGase incubated sample heated with 2% (w/v) ribose solution. SPI/MTG(5)/R: MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) ribose solution. SPI/MTG(24)/R: MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) ribose solution.

Table 2
Distribution of % network protein and % non-network protein over (A) SPI/C/S(2), (B) SPI/C/R(2), (C) SPI/MTG(5)/S(2), (D) SPI/MTG(5)/R(2), (E) SPI/MTG(24)/S(2) and (F) SPI/MTG(24)/R(2) gels at a concentration of 15% (w/v).

Samples	% Network protein	% Non-network protein
SPI/C/S(2)	75.4 ± 0.6^{a}	$24.6 \pm 0.6^{e*}$
SPI/C/R(2)	77.2 ± 1.2^{b}	$21.8 \pm 0.6^{d*}$
SPI/MTG(5)/S(2)	82.7 ± 0.4^{c}	$17.3 \pm 0.4^{c*}$
SPI/MTG(5)/R(2)	83.8 ± 0.4^{cd}	$16.2 \pm 0.4^{a*b*}$
SPI/MTG(24)/S(2)	83.1 ± 0.1^{cd}	$16.9 \pm 0.1^{b*c*}$
SPI/MTG(24)/R(2)	84.4 ± 0.8^{d}	$15.5 \pm 0.8^{a*}$

Note: Comparison within the column in the table with the data written as mean ± standard deviation ($n = 3$). Means within the same column not followed by the same letter are significantly different at $p < 0.05$ level of significance, according to Duncan's Multiple-Range Test.

MTG(24)/R), it is difficult to be hydrated as mentioned previously, hence resist to denaturation. As a result, the protein molecules did not unfold completely and this would deter protein–protein and protein–solvent interactions. Thus, lower G' value was obtained. Further support is shown in the morphology of the gels formed in a previous paper (Gan et al., 2008). Accordingly, the result indicates that combination of both MTGase incubation and ribose-induced "Maillard cross-linking" cause rheological modification more than single treatment. Thus, these treatments gave rise to a more solid-like behaviour due to formation of cross-links, i.e. ε-(γ-glutamyl)lysine and/or Maillard cross-links.

The isolation of the network and non-network protein of the gels was carried out in order to study the gel protein constituents and network structure. Network protein was defined as protein aggregates that obtained molecular size larger than 0.2 μm via centrifugal method according to Lakemond et al. (2003). The proportion of the network and non-network protein in the SPI gels is presented in Table 2. Result shows that SPI/C/S(2) had the lowest percentage of network protein as compared to those of other gels and yet the value is considered relatively high. This is due to the high concentration (15%, w/v) of SPI used in forming self-standing gel. The structures within these aggregates were mainly made up of disulphide bonds, hydrophobic interactions and electrostatic bonds (Mori, Nakamura, & Utsumi, 1986; Utsumi & Kinsella, 1985). The order of the% network protein in the samples were as follows: SPI/C/S(2) < SPI/C/R(2) < SPI/MTG(5)/S(2) < SPI/MTG(5)/R(2), SPI/MTG(24)/S(2) < SPI/MTG(24)/R(2). It became evident that% network protein increased with the extent of cross-linking treatments. A longer incubation in MTGase resulted in higher% network protein within the gel, and a higher% network protein was obtained with the incorporation of ribose that caused additional cross-linking. These results further support the discussion in the rheological studies.

Two major fractions of SPI, namely 11S globulin (glycinin) and 7S globulin (β-conglycinin) that are known to have different compositions and structures (Kilara & Sharkasi, 1985) play important roles in network formation and gelation. The profiles of the network and non-network protein constituents obtained from the analysis above are shown in Figs. 6 and 7, respectively. The nature of the primary network particles differed between the gels. Generally, incubation treatment with MTGase and/or heating treatment with ribose changed the protein constituents of the gels. In SPI/C/S(2) gels (Fig. 6, lane 2), the network was mainly formed from the subunits of β-conglycinin and glycinin, which were almost the same as that of native SPI (Tang et al., 2006). The network of SPI/MTG(5)/S(2) gels (Fig. 6, lane 4) showed the absence of β-conglycinin whereas SPI/MTG(24)/S(2) gels (Fig. 6, lane 6) composed predominantly of basic subunits. The gels produced using ribose, were predominantly formed by basic subunits and A_4 subunit. The profiles of SPI/C/R(2) and SPI/MTG(5)/R(2) are depicted in Fig. 6, lane 3 and lane 5, respectively, which consisted 77.2% and 83.8% of the total protein incorporated in the gel network. Disappearance of all bands were observed in the profiles of SPI/MTG(24)/R(2). The SDS–PAGE profiles obtained in this study were similar to the cross-linking profiles reported previously (Gan et al., 2009). However, the basic subunits of glycinin were more dominant in contributing to the network protein of these gels. The fact that protein fractions were virtually absent in cross-linked gels, may be due mainly to the formation of additional covalent cross-links as a result of the single or combined cross-linking treatment, in which large complexes were formed (Easa, 1996; Tang et al., 2006; Utsumi & Kinsella, 1985). The combined cross-linking treatment was able to cross-link the acidic subunits of glycinin and the α-, α′- and β-conglycinin to form high molecular weight biopolymer complexes as part of the network protein. It is thought that these complexes were too large to enter the stacking and separating gels.

In the non-network protein (Fig. 7), all protein fractions were also indicated in the profile of SPI/C/S(2) gels (Fig. 7, lane 2), which subunit of A_4 is more dominant. Protein subunit A_3 has been reported to contribute to the formation of the network structure of 11S globulin gels, whereas A_4 subunit contributes only marginally (Mori, Nakamura, & Utsumi, 1981; Nakamura, Utsumi, & Mori, 1984). It has also been reported that A_3 played an important role in increasing the hardness of the 11S globulin gels and coincide with the observation where A_4 is liberated during the formation of soluble aggregate (transient intermediate) during heating (Mori

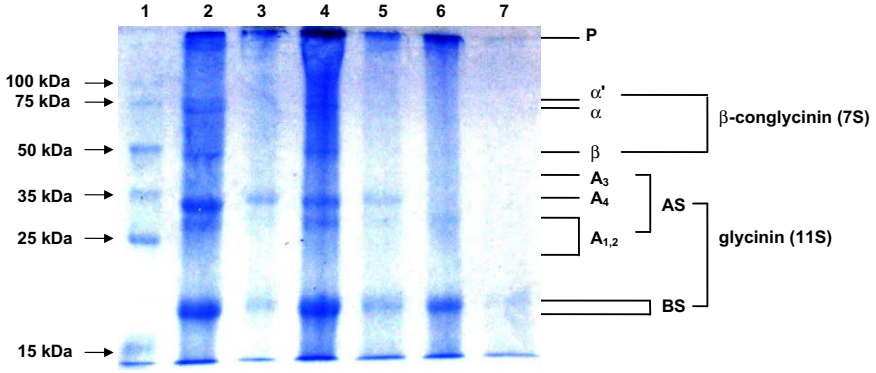

Fig. 6. SDS–PAGE profile of network protein composition of various SPI gels. Lane 1: standard protein marker; lane 2: SPI/C/S(2); lane 3: SPI/C/R(2), lane 4: SPI/MTG(5)/S(2); lane 5: SPI/MTG(5)/R(2); lane 6: SPI/MTG(24)/S(2); lane 7: SPI/MTG(24)/R(2). P: polymer; AS: acidic subunit; BS: basic subunits. Sample definitions and descriptions: SPI/C/S(2): non-MTGase incubated sample heated with 2% (w/v) sucrose solution for 2 h. SPI/MTG(5)/S(2): MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) sucrose solution for 2 h. PI/MTG(24)/S(2): MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) sucrose solution for 2 h. SPI/C/R(2): non-MTGase incubated sample heated with 2% (w/v) ribose solution for 2 h. SPI/MTG(5)/R(2): MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) ribose solution for 2 h. SPI/MTG(24)/R(2): MTGase incubated sample (incubated for 24 h) heated with 2% (w/v) ribose solution for 2 h.

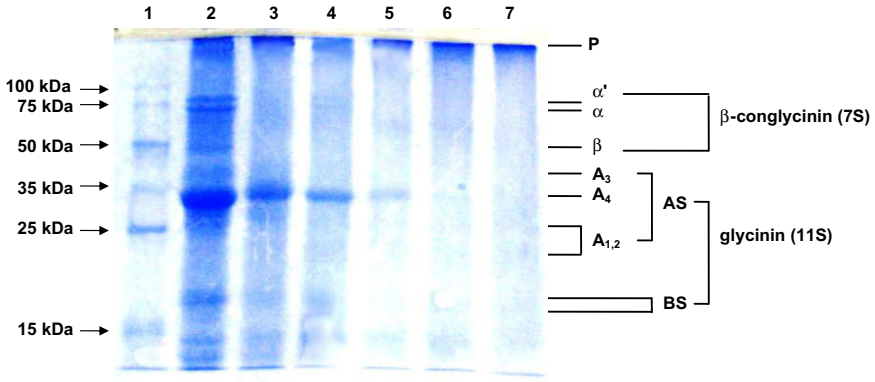

Fig. 7. SDS–PAGE profile of non-network protein composition of various SPI gels. Lane 1: standard protein marker; lane 2: SPI/C/S(2); lane 3: SPI/C/R(2), lane 4: SPI/MTG(5)/S(2); lane 5: SPI/MTG(5)/R(2); lane 6: SPI/MTG(24)/S(2); lane 7: SPI/MTG(24)/R(2). P: polymer; AS: acidic subunit; BS: basic subunits. Sample definitions and descriptions: SPI/C/S(2): non-MTGase incubated sample heated with 2% (w/v) sucrose solution for 2 h. SPI/MTG(5)/S(2): MTGase incubated sample (incubated for 5 hrs) heated with 2% (w/v) sucrose solution for 2 h. SPI/MTG(24)/S(2): MTGase incubated sample (incubated for 24 hrs) heated with 2% (w/v) sucrose solution for 2 h. SPI/C/R(2): non-MTGase incubated sample heated with 2% (w/v) ribose solution for 2 h. SPI/MTG(5)/R(2): MTGase incubated sample (incubated for 5 h) heated with 2% (w/v) ribose solution for 2 h. SPI/MTG(24)/R(2): MTGase incubated sample (incubated for 24 hrs) heated with 2% (w/v) ribose solution for 2 h.

et al., 1981; Nakamura et al., 1984). Therefore, subunit of A_4 was found predominantly in SPI/C/R(2) (Fig. 7, lane 3), SPI/MTG(5)/S(2) (Fig. 7, lane 4) and SPI/MTG(5)/R(2) (Fig. 7, lane 5) in non-network protein of the gels where the band intensity decreased with cross-link density. The fact that the protein bands were virtually absent in SPI/MTG(24)/S(2) (Fig. 7, lane 6) and SPI/MTG(24)/R(2) gels (Fig. 7, lane 7) may indicate that the covalent cross-links formed from the MTGase and/or the Maillard reaction treatments were not restricted in the non-network protein which might resulted as part of the network protein.

Despite the occurrence of ε-(γ-glutamyl)lysine cross-link within the gels produced by the MTGase incubation, a quantity of lysine was still available for the Maillard reaction to occur during subsequent treatment. The Maillard cross-links formed as a result of the Maillard reaction were able to cross-link the network and non-network proteins that were already stabilized via ε-(γ-glutamyl)lysine bonds. Since the% network protein increased and% non-network protein decreased upon heating of SPI/C, SPI/MTG(5) and SPI/MTG(24) with ribose, it would be possible to suggest that the network and non-network proteins of the gels produced using ribose or the combined cross-linking treatment were linked via the Maillard cross-links.

4. Conclusions

This paper evaluates the gelling of combined-treated SPI gels produced by pre-cross-linking treatments of SPI via MTGase mediated ε-(γ-glutamyl)lysine bonds followed by ribose-induced "Maillard cross-linking". The rheological study showed different gelling behaviours of MTGase pre-cross-linked SPI during the heating in the presence of sucrose or ribose, and gave higher G' values in combined cross-linked samples compared to non-cross-linked or single-treated samples. Also, profiles of network and non-network protein fractions of the gel formed were different. Non-network protein in native SPI was suggested to become part of the network protein after the cross-linking treatments were introduced as formation of additional cross-links within the different protein fractions of SPI occurred thus increased the network protein content.

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