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Modulating the gel properties of soy glycinin by crosslinking with tyrosinase

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ABSTRACT

The gelation progression and gel properties of enzymatically crosslinked soy glycinin were evaluated in comparison to non-crosslinked glycinin. Glycinin was initially crosslinked using tyrosinase from *Bacillus megaterium* (TyrBm) and was later used to form gel upon heating. Gelation was evaluated by small deformation rheological measurements and revealed a significant increase in storage modulus (G′) obtained in the crosslinked gel. This was confirmed by temperature sweep and frequency sweep measurements that supported the results and proved that the difference in modulus was not frequency dependent. Texture profile analysis showed an increase in hardness and decrease in elasticity of the crosslinked gels. Scanning electron microscopy (SEM) images displayed a more structural network with larger pore size in the crosslinked gel. The less dense structure of the crosslinked glycinin gel network led to a slight decrease in the water holding capacity. Finally, thermal analysis using differential scanning calorimetry (DSC) confirmed no change in the gelation point induced by denaturation, however thermal gravimetric analysis (TGA) did show a difference in the decomposition profile of the crosslinked protein compared with non-crosslinked glycinin. The results suggest that by applying TyrBm mediated crosslinking we may modulate the protein gel properties for tailoring the texture of food products.

1. Introduction

With increasing awareness to the environment and the search for non-animal protein sources, soy proteins have become more popular and are being used in various food products as emulsifiers, gelation agents and protein additives (Friedman & Brandon, 2001; Hughes, Ryan, Mukherjea, & Schasteen, 2011; Liu, 1997; Mujoo, Trinh, & Ng, 2003; Renkema, Lakemond, de Jongh, Gruppen, & van Vliet, 2000; Thomson, Brinkworth, Noakes, & Buckley, 2016; Zhang, Wu, Lan, & Yang, 2013). They have a high nutritional value and are correlated with reduced LDL cholesterol uptake, prevention of diabetes and other health benefits (Friedman & Brandon, 2001; Zhang, Shu, Gao, et al., 2003). Glycinin is one of the storage soy proteins, which accounts for 30–35% of the total seed proteins. It is responsible for the gel properties of soy protein systems, and has been utilized and modified for various purposes such as Tofu production and milk substitutes (Liu, 1997; Mujoo et al., 2003; Utsumi, Matasumura, & Mori, 1997; Yasir, Sutton, Newberry, et al., 2007a, 2007b). Glycinin is a hexamer with a total molecular mass of ~350 kDa which comprises several sub-units. The glycinin monomer consists of an acidic polypeptide subunit (AS) with a size of ~38 kDa and a basic polypeptide subunit (BS) with a size of ~20 kDa, linked by a single disulfide bridge. At least six acidic polypeptides (A1a, A1b, A2–A4, and A5) and five basic polypeptides (B1a, B1b and B2–B4) have been isolated (Mujoo et al., 2003).

The properties and characteristics of glycinin gels obtained by heating have been widely investigated (Renkema, Knabben, & van Vliet, 2001; Renkema, Lakemond, de Jongh, et al., 2000). Other treatments have also been applied to induce gel formation of soy proteins, including acidification, conjugation with a carbohydrate and crosslinking by transglutaminase (Bhattacharya & Jena, 2007; Jian, Xiong, Guo, et al., 2014; Kang, Matsumura, Ikura, et al., 1994; Tang, Wu, Chen, & Yang, 2006; Zhang et al., 2013). Transglutaminase treatment on glycinin led to the formation of a strong, turbid gel at 40 °C. It was suggested however, that heat treatment might strengthen the gel due to formation of hydrophobic and hydrogen bonds as well as disulfide bonds that are involved in the gelation process of glycinin during heating (Tang et al., 2006). As the biological approach offers a higher specificity of the reaction preventing formation of side products, there is constant need for new and effective biological crosslinkers. This research offers a new enzymatic crosslinking mechanism by tyrosinase.

Bacterial tyrosinase from *Bacillus megaterium* (TyrBm) was previously characterized in our lab (Sendovski, Kanteev, Ben-Yosef, et al., 2011; Shuster & Fishman, 2009). TyrBm is a type-3-copper enzyme, which contains two copper ions in its active site that are necessary for its oxidation activity. It performs two successive reactions:

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hydroxylation of monophenols and subsequent oxidation of the diphenols into quinones which polymerize spontaneously to form insoluble polymers such as melanin. Tyrosinases from bacterial or fungal origins have been investigated for their protein crosslinking ability on milk and wheat proteins (Heijnis, Wierenga, van Berkel, & Gruppen, 2010; Selinheimo, Autio, Kruus, & Buchert, 2007; Thalmann & Lötzbeyer, 2002). In previous work we have demonstrated the use of TyrBm crosslinking on glycinin to improve the properties of glycinin-stabilized oil-in-water emulsions (Isaschar-Ovdat, Rosenberg, Lesmes, & Fishman, 2015).

In the present study soy glycinin gels obtained after crosslinking with TyrBm were evaluated for their rheological behavior and texture properties. By introducing covalent bonds within the protein network we have modulated the gel properties and structure, allowing the potential use of this system in food applications.

2. Experimental

2.1. Materials

Soy glycinin standard (98% purity), sodium bisulfite and all other chemicals were obtained from Sigma Chemical Co. (Rehovot, Israel). Tissue freezing medium for the SEM samples was purchased from TED PELLA Inc. (Redding, CA, USA). Defatted soybean flakes were kindly provided by Shemen Industries Ltd. (Haifa, Israel). Protein molecular weight marker was purchased from m.biotech (Hanam, Korea).

2.2. Methods

2.2.1. Purification of TyrBm and activity determination

TyrBm was isolated as previously described (Isaschar-Ovdat et al., 2015).

2.2.2. Isolation of soy protein fractions

Glycinin-rich fraction was isolated from defatted soybean flakes as previously described (Isaschar-Ovdat et al., 2015).

2.2.3. Enzymatic crosslinking of soy glycinin

Glycinin at 2% (w/v) was suspended in 50 mM Tris-HCl buffer pH 7.5 (reaction buffer) for 30 min at ambient temperature and TyrBm was added at 1:25 ratio (0.08% w/v) (Isaschar-Ovdat et al., 2015). The non-crosslinked glycinin was treated similarly but without the enzyme. The reaction mixtures were incubated at 37 °C with shaking at 250 rpm in an incubator shaker (TU-400 Orbital Shaker Incubator, MRC, Holon) for 4 h, then stopped by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 1.5 mM, which was previously shown to inhibit TyrBm by chelation of copper (unpublished results). The reaction mixtures were lyophilized and kept at −20 °C until further use.

2.2.4. SDS-PAGE analysis

SDS-PAGE was performed on a discontinuous buffered system (Laemmli, 1970) using 12% separating gel and 4% stacking gel. The samples were heated for 5 min at 95 °C, after adding reducing sample buffer (4×), 1:1 (v/v). The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% ethanol and 10% acetic acid, and destained in 10% acetic acid [methanol:acetic acid:water, 20:10:70 (v/v/v)].

2.2.5. Preparation of heat-induced gels

Gel samples (5 mL) were prepared by mixing 10% (w/v) non-crosslinked or crosslinked glycinin dispersion with distilled H_2O in sealed glass cylinders (15 mm diameter, 50 mm height). The mixture was incubated for 30 min at 95 °C followed by cooling under running tap water and overnight at 4 °C. Before measurements the gels were equilibrated to room temperature for 30 min.

2.2.6. Thermal gravimetric analysis of dried non-crosslinked and crosslinked glycinin

Thermal gravimetric analysis (TGA) was performed using TGA-Q5000 system (TA Instruments, USA). Analysis was done on the lyophilized non-crosslinked or crosslinked protein powder. Samples were heated under nitrogen atmosphere from room temperature to 600 °C at a heating rate of 10 °C·min^{-1}. The results were analyzed using Universal Analysis 200 version 4.5A build 4.5.0.5 software.

2.2.7. Differential scanning calorimetry

The protein denaturation was analyzed by differential scanning calorimetry (DSC) equipped with a HSS7 high-sensitivity sensor (DSC1 system, Mettler-Toledo, USA). Measurements were carried out under a nitrogen atmosphere. 45 μL of 10% (w/v) non-crosslinked or crosslinked glycinin dispersion in medium buffer were loaded into a sealed stainless steel pan to prevent mass loss during the experiment. An empty, hermetically sealed stainless steel pan was used as the reference. The samples were scanned from 25 to 150 °C at 5 °C·min^{-1}. The temperature at which denaturation starts, the onset temperature (T_o), was calculated by taking the intercept of the baseline and the extrapolated slope of the peak. The peak denaturation temperature (T_p) was taken as the temperature of maximum heat flow. The enthalpy of denaturation (ΔH) was integrated by the peak area between the starting point of transition to the end point of transition. Values presented are the mean ± SD (standard deviation) of 7 repetitions.

2.2.8. Rheological measurements at small deformation

Rheological measurements using parallel plates ($d = 40$ mm) were carried out in a Discovery Hybrid Rheometer (DHR-2, TA Instruments, DE, USA). 10% (w/v) non-crosslinked or crosslinked glycinin were dispersed in reaction buffer and stirred at 4 °C overnight. The mixture was equilibrated to room temperature for 30 min before analysis. The sample was placed between parallel plates at 25 °C and the gap between the two plates was set to 1.0 mm. The temperature was monitored through the lower plate. Excess sample was trimmed and exposed edges were covered with a thin layer of silicone oil to prevent solvent evaporation. The equipment was controlled using the Trios program (TA Instruments, DE, USA).

2.2.8.1. Temperature sweep measurements of non-crosslinked and crosslinked glycinin gels. Temperature sweep curves were recorded, after reaching equilibrium, by heating from 25 °C to 95 °C at 5 °C·min^{-1}, holding for 30 min at 95 °C and then cooling back to 25 °C at the same rate and holding for 15 min at 25 °C. The storage modulus (G′) and loss modulus (G″) were recorded as a function of time. Experiments were conducted using a fixed frequency of 0.1 Hz and 4 Pa stress within the linear viscoelastic region (LVR) (determined prior at the relevant temperature range).

2.2.8.2. Frequency sweep measurements of non-crosslinked and crosslinked glycinin. The storage modulus (G′) and loss modulus (G″) were recorded as a function of frequency. Frequency sweep measurements were performed using a frequency range of 0.01–10.0 Hz with a constant strain of 2% within the LVR region (determined prior at the relevant temperature range). Frequency sweep experiments were conducted at different temperatures using 5 °C intervals during heating and cooling between 25 and 95 °C. The samples were manually heated or cooled to the desired temperature and a frequency sweep experiment was conducted.

2.2.9. Textural profile analysis (TPA) of non-crosslinked and crosslinked glycinin gels

The textural characteristic of the prepared gels was analyzed according to a texture profile analysis (TPA) using a Texture Analyzer (Model LRX-5 K, Lloyd Instruments, UK). The gel samples were prepared in a glass cylinder with dimensions of 15 mm diameter × 50 mm height and remained in the glass cylinder during the measurement. The

samples were penetrated twice by a cylinder probe (5 mm diameter) to 50% of the initial height at constant speed of 1 mm/s with a 10 N load transducer. From the TPA curves (Fig. 1), the following texture parameters were obtained: hardness at 50% of deformation, cohesiveness, gumminess and resilience. Hardness was defined by maximum peak force (g) during the first compression cycle. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve (area 2/area 1). Gumminess was obtained by multiplying hardness and cohesiveness. Resilience was obtained by dividing the area during the first withdrawal by the area of the first penetration (area 5/area 4). All parameters were calculated from the raw data obtained from the texture analyzer, using "Wolfram Mathematica" program (Wolfram, Champaign, IL, USA). Results shown in this study are the mean value of at least four repetitions.

2.2.10. Scanning electron microscopy (SEM) and pore size analysis

Microstructural features of the formed glycinin gels were analyzed by scanning electron microscopy (Phenom ProX desktop SEM). A small slice (2 × 5 mm) of the gel was placed on a drop of tissue freezing medium on the temperature controlled sample holder and the temperature was decrease to −25 °C. When the sample was frozen, it was placed inside the SEM device and images were taken. Three gel preparations of each type were evaluated. At least five images were captured per gel. The SEM images were further analyzed for pore size using PHENOM PoroMetric software. Results are the mean of >9 images of the non-crosslinked and crosslinked gels.

2.2.11. Water holding capacity

Water holding measurements were performed as described by Urbonaite et al. (Urbonaite, de Jongh, van der Linden, & Pouvreau, 2014). In short, gels were cut in 15 mm height and 4.8 mm diameter cylinders and carefully placed on the bottom of the spin tube. An ultrafiltration unit (Merck Millipore, Ultrafree-CL Filters, Darmstadt, Germany) consisted of an Eppendorf tube (5 mL) and an inner tube (2 mL) with an ultrafiltration membrane of 3000 NMWL. Centrifugation was performed at different g-force values ranging from 100 *g* to 10,000 *g* for 10 min at 20 °C (Sarvall LYNX 4000 centrifuge, rotor A27-8 × 50, Rochester, NY). Excluded water from the gel was collected at the bottom of the Eppendorf tube and the removed weight was determined. The water holding capacity (WHC) was calculated as the percentage remaining water in the gel after centrifugation, as followed (1):

$$WHC = \left(\frac{W_T - W_R}{W_T}\right) \times 100\% \quad (1)$$

where W_T is the total quantity (g) of water in the sample and W_R is quantity (g) of water released.

2.2.12. Statistical analysis

The significance of differences between means was determined by paired sample Student's *t*-test. The level of significance used was 95% or higher.

3. Results & discussion

3.1. Composition of glycinin-rich fractions

The purity of the glycinin-rich fractions was evaluated by SDS-PAGE gel in comparison to a commercial standard. The total protein content was 97.8% as determined by the Bradford method (Bradford, 1976). The relative content of glycinin in the glycinin-rich fraction was 94.5% (vs. commercial standard, 98%) as determined by band intensity using ImageJ software (http://rsb.info.nih.gov/ij/index.html). This is comparable to previous results obtained in our lab (Isaschar-Ovdat et al., 2015).

3.2. TyrBm-catalyzed crosslinking of soy glycinin

In a previous study, we showed that TyrBm-crosslinking of soy glycinin is poor when it maintains its quaternary structure in an aqueous dispersion and may need an addition of a low molecular weight phenolic reagent as a mediator (Isaschar-Ovdat et al., 2015). In order to obtain a crosslinked-glycinin powder without an added phenolic mediator, the reaction conditions (medium buffer and enzyme to protein ratio) were adjusted. The higher enzyme to protein ratio (changed from 1:100 to 1:25) led to the formation of crosslinked protein aggregates observed in the SDS-PAGE without the inclusion of a phenolic mediator (Fig. 2). The crosslinked system contains a heterogeneous mixture of high molecular weight glycinin fractions as well as a non-

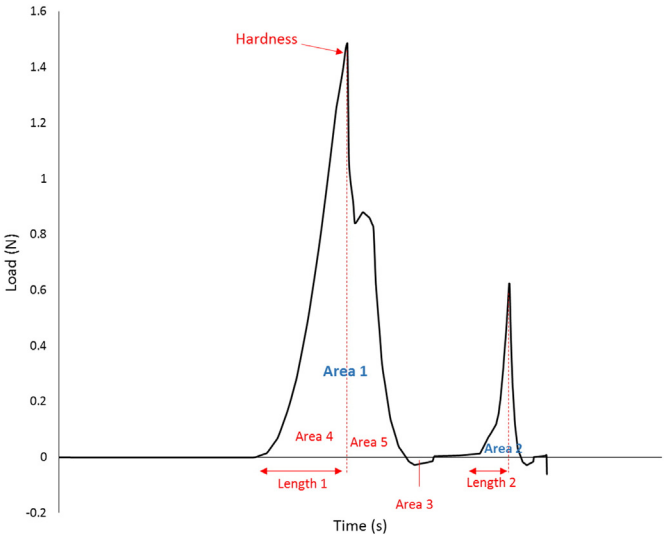

Fig. 1. Typical texture profile analysis curve of glycinin gel. The different designated "areas" in the graph were used to calculate the texture parameters as described in the experimental section.

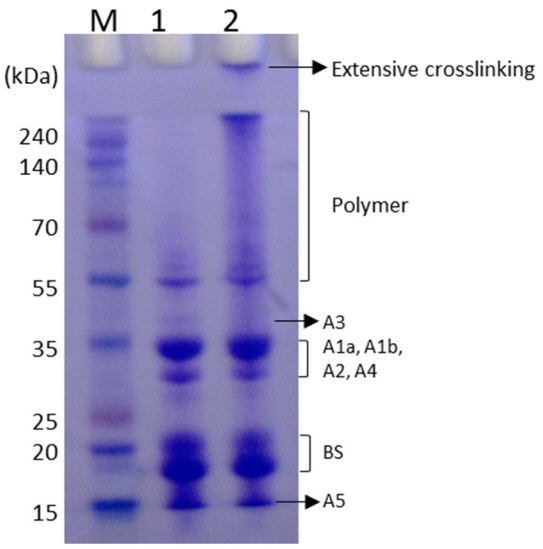

Fig. 2. SDS-PAGE pattern of non-crosslinked glycinin (lane 1) and TyrBm-crosslinked glycinin (lane 2) after 240 min incubation at 37 °C. M indicates the molecular weight marker. A small reduction in the intensity of the bands representing the acidic subunit (AS) and basic subunit (BS), concomitant with the formation of high molecular weight bands in the resolving and the stacking gel (polymer), indicate on crosslinking.

crosslinked glycinin. Similar results were described by Lantto et al. that studied the effect of tyrosinase crosslinking on chicken breast myofibrils (Lantto, Puolanne, Kruus, et al., 2007).

3.3. Thermal gravimetric analysis of dried non-crosslinked and crosslinked glycinin

To further characterize the influence of enzymatic crosslinking on glycinin, thermal gravimetric analysis was utilized. Dry powder of TyrBm-crosslinked glycinin was compared to dry powder of non-crosslinked glycinin. Fig. 3a presents a characteristic TGA curve of non-crosslinked and TyrBm-crosslinked glycinin and Fig. 3b shows the corresponding derivative-TG (DTG) curves. Non-crosslinked glycinin presents two clear weight loss stages. The first one is around 50 °C and is attributed to water loss. The second major weight loss starts at approximately 220 °C and peaks at 280 °C, resulting from thermal dissociation of the quaternary structure of the protein and the breakage of the peptide backbone (Guerrero, Retegi, Gabilondo, & de la Caba, 2010; Swain, Rao, & Nayak, 2005). The crosslinked glycinin presents similar two weight loss stages with an additional major weight loss stage that starts around 150 °C and peaks at 210 °C. This weight loss can be attributed to the dissociation of the quaternary structure and covalent bonds induced by TyrBm activity. Another major effect of crosslinking is associated with the width of the dissociation peak, which is larger for the crosslinked glycinin. As the peak is wider, higher energy is required for breakage of the amino acid polymer chain. Studied regarding chemical crosslinking of SPI showed similar influence of crosslinking on the thermal decomposition. The crosslinked SPI showed higher weight loss at a temperature range of 150–210 °C correlated with the covalent bonds, whereas the native non-crosslinked SPI did not presented these weight loss values (Swain et al., 2005).

3.4. Differential scanning calorimetry

Denaturation is essential for protein gel formation, since the gel network of glycinin is formed by hydrophobic interactions, hydrogen bonding and disulfide bridges during heating (Catsimpoolas & Meyer, 1970; Kinsella, 1979). Differential scanning calorimetry (DSC) was used to examine the thermal stability of non-crosslinked and crosslinked glycinin. Fig. 4 shows typical DSC thermograms of 10% (w/v) non-crosslinked and TyrBm-crosslinked glycinin suspensions while the onset (T_o) and peak (T_p) denaturation temperatures obtained from those thermograms are presented in Table 1. Similar onset denaturation values were reported in the literature for the glycinin-rich fraction at pH 7.6 (Kim, Kim, Yang, & Kwon, 2004; Renkema et al., 2000). No significant difference was found in the thermal transition temperatures

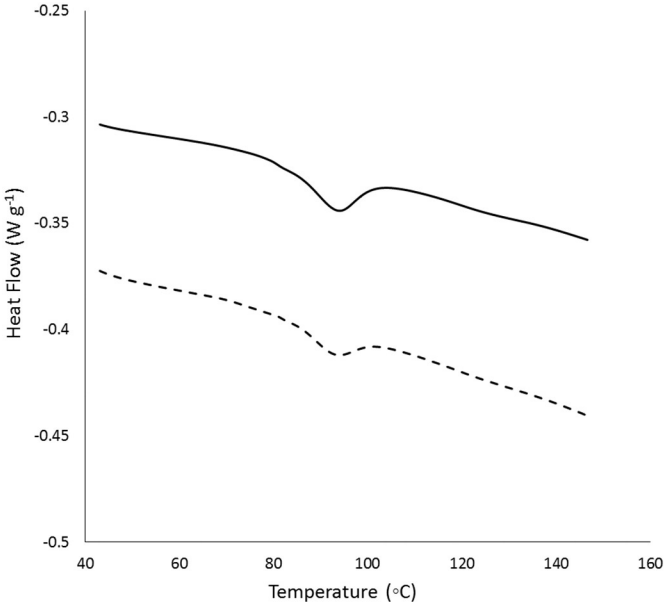

Fig. 4. DSC-thermograms of 10% (w/v) non-crosslinked (solid line) and crosslinked (dashed line) glycinin gels. TyrBm-crosslinked glycinin thermogram was shifted by 1.2-fold.

(T_o and T_p) in comparison between non-crosslinked and crosslinked glycinin. The enthalpy values of non-crosslinked glycinin were 10-fold lower than reported in the literature due to prior subjection of the soy flakes to extreme heating during the oil extraction process indicating significant protein denaturation (Kim et al., 2004; Sessa, 1993). This earlier subjection to heat and denaturation of a large percentage of the glycinin led to lower detection values and therefore made it more difficult to visualize significant changes in the thermal transition temperatures and enthalpy values. The TyrBm-crosslinked gel thermal transition behavior is characterized by a lower enthalpy in comparison with non-crosslinked glycinin gel (Table 1, $p > 0.05$). This may be the result of TyrBm-catalyzed polymerization of glycinin to larger but less dense and therefore less energy consuming structures (Lantto et al., 2007). The crosslinked suspension contains a heterogeneous mixture of modified glycinin as well as a non-crosslinked glycinin, as presented in Fig. 2. Thus, differences in denaturation may be more significant in a homogenous mixture of crosslinked product by the same level of crosslinking. In conclusion, the thermal analysis shows that crosslinking did not affect the unfolding state of the overall protein tertiary structure.

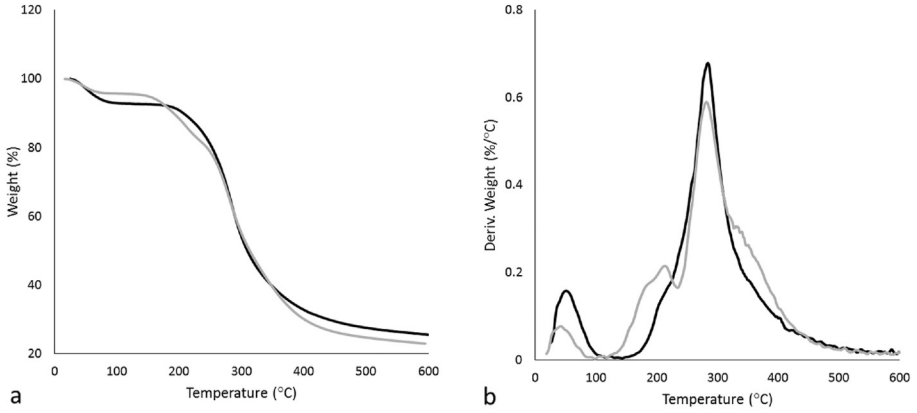

Fig. 3. TGA (a) and derivative-TG curves (b) of non-crosslinked (black line) and TyrBm-crosslinked (grey line) glycinin.

Table 1
Onset (T_o) and peak (T_p) denaturation temperature and enthalpy (ΔH) of non-crosslinked and TyrBm-crosslinked glycinin gels (10% w/v).

	Non-crosslinked glycinin	TyrBm-crosslinked glycinin
T_o (°C)	86 ± 2	86 ± 2
T_p (°C)	92 ± 3	94 ± 2
ΔH (J/g)	1.6 ± 0.8	1.1 ± 0.2

3.5. Small deformation rheological measurements

3.5.1. Temperature sweep measurements

The gelation process of crosslinked and non-crosslinked glycinin dispersion (10% w/v) was monitored by rheology measurements during a heating/cooling cycle using a temperature sweep. Fig. 5 shows the behavior of the storage modulus, G′, and loss modulus, G″, as a function of time (Fig. 5a) and temperature (Fig. 5b and c) during the gelation process. According to Fig. 5a, as the temperature increases, a small decrease in the modulus values is observed. This behavior is characteristic of a shear thinning behavior and is typical of many concentrated protein solutions (Jian et al., 2014; Keerati-u-rai & Corredig, 2010). As the heating proceeds and reaches 60 °C, a crossover point between G″ and G′ is observed whereby G′ exceeded G″. The crossover point at 60 °C is observed more clearly in Fig. 5b and c. Further heating and incubation at 95 °C, which is considered to be the gelation temperature or gel point (Renkema et al., 2000), led to sharp increase in G′ that exceeded G″ by 5-fold in both the non-crosslinked glycinin gel and the crosslinked glycinin gel. At this temperature (95 °C), the storage modulus of the TyrBm-crosslinked gel increased more rapidly than the non-crosslinked gel modulus and reached a 100-fold higher value. The increase in G′ values upon heating was attributed to the unfolding of the protein quaternary structure followed by the formation of new inter and intra-molecular interactions in the sequential cooling. These interactions lead to the sol-gel transition and are attributed to hydrogen bonding, hydrophobic and electrostatic interactions as well as sulfhydryl-disulfide interchange (Catsimpoolas & Meyer, 1970; Renkema et al., 2000). These mechanisms are similar for the non-crosslinked and crosslinked glycinin gel, therefore, no difference in the gelation point was observed. Fig. 5b and c present the crossover point of G′ over G″ of both the non-crosslinked and crosslinked glycinin gels around 60 °C at approximately 0.02 Pa. The values increase and at 95 °C, G′ of both the non-crosslinked and crosslinked glycinin gel reaches approximately 0.5 Pa. The significant difference mentioned is obtained only during incubation at 95 °C. The crosslinked protein contains covalent bonds, as seen by SDS-PAGE analysis (Fig. 2), that strengthen the network and lead to a higher G′ value at the end of the gelation process. While the enzymatic crosslinking prior to gelation did not change the gelation mechanism, the viscoelastic properties of the formed gel were modulated and led to a firmer product.

3.5.2. Frequency sweep measurements

The behavior of storage modulus (G′) and loss modulus (G″) of crosslinked and non-crosslinked glycinin dispersion (10% w/v) was monitored during a frequency sweep experiment at different temperatures. Frequency sweep experiments were conducted at different thermal conditions by changing the temperature of the suspension manually during heating/cooling at 5 °C ramps. The suspension was allowed to equilibrate at each temperature for precisely 2 min before the experiment was initiated. Fig. 6 demonstrates the effect of frequency on the modulus values obtained at different temperatures. The data is presented as a function of temperature where each frequency sweep run was converted to one curve. According to our results, the modulus values (G′, G″) increase as frequency increases. G′ increased by 100-fold during the heating cycle. The rapid increase in modulus during heating is induced by the gel network formation and transition of sol to sol-gel. Cooling strengthens the network even further as it allows the formation of new physical bonds (Catsimpoolas & Meyer, 1970). G′, which represents the "solid" properties of the matrix is less affected by the frequency during cooling, while G″ is affected more significantly as a result of the change in frequency. In both cases, G′ is higher than G″ at the end of the gelation step at all frequencies. The gelation point (defined as an increase in >0.5 Pa K^{-1} in modulus) is not dependent on frequency and can be seen at approximately 90 °C for the non-crosslinked glycinin gel (Fig. 6a,b), while the sudden increase in slope for the crosslinked gel is observed at 80 °C (Fig. 6c,d) (Renkema et al., 2000). This is coherent in all frequency range. The crosslinked glycinin gel

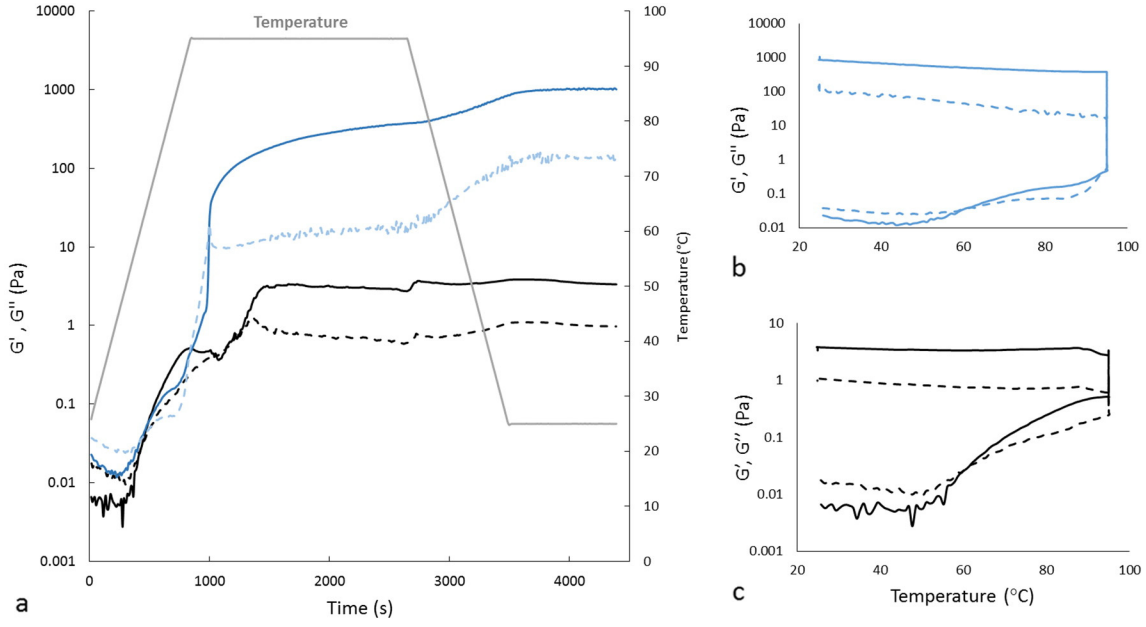

Fig. 5. Changes in storage modulus (G′, solid line) and loss modulus (G″, dashed line) as a function of time (a) and temperature (b, c) of non-crosslinked (black) and crosslinked (blue) glycinin. Temperature sweep rate was 5 °C·min^{-1} while stress and frequency remained constant (4 Pa, 0.1 Hz). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

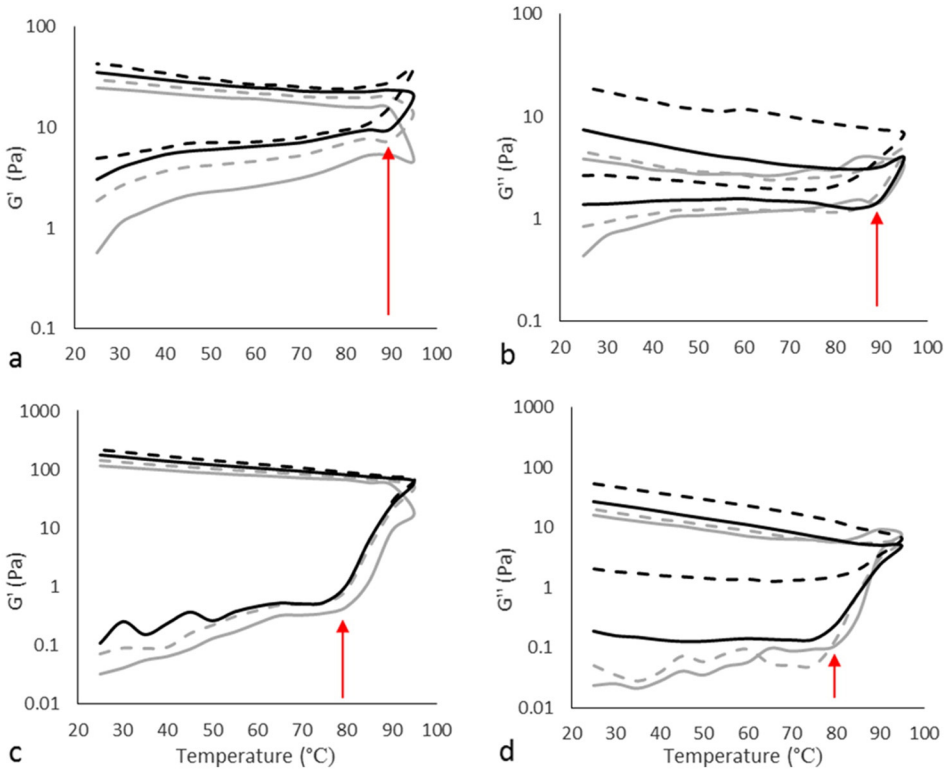

Fig. 6. Changes in storage modulus (G′; a, c) and loss modulus (G″; b, d) as a function of temperature of non-crosslinked (a, b) and crosslinked (c, d) glycinin at different frequency values. Solid grey - 0.01 Hz, dashed grey - 0.1 Hz, solid black - 1.0 Hz and dashed black - 10.0 Hz. The increase in slope is marked by a red arrow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

presents a more rapid increase in both G′ and G″ values and higher final values, as seen in the temperature sweep experiments.

3.6. Textural profile analysis of non-crosslinked and crosslinked glycinin gels

Texture profile analysis mimics the natural conditions during food consumption in the mouth, i.e. chewing, by compressing or penetrating a product twice (Guerrero et al., 2010; Urbonaite et al., 2014). Four textural parameters were evaluated by the TPA curves: hardness, cohesiveness, gumminess and resilience. The TPA results shown in Table 2 demonstrate that TyrBm-crosslinking led to 2-fold increase in the gel hardness and gumminess (which is calculated from the hardness). Hardness is used to estimate the maximum force of the first compression and is related to the strength of the gel (Fig. 1) (Swain et al., 2005). The hardness is affected by the formation of the gel network. As the enzymatic activity preceded the gel formation, the covalent bonds changed the protein network elasticity, thus, influenced the rearrangement of the network. The covalent bonds form a more rigid network. The rigidity limits the protein unfolding upon heating and therefore influences the physical interactions that are formed (such as hydrophobic interactions and H-bonding). In contrast, covalent bonds induced by the enzyme are present in the network and may serve as a replacement for the hydrophobic and hydrogen interactions. The gelation process is induced by physical bonds formed upon heating and is expressed in the similar cohesiveness values of the non-crosslinked and the crosslinked gels. The rheological measurements in small deformation strengthen these results with the similar gelation point obtained, and higher G′ value for the crosslinked glycinin gel. The non-crosslinked glycinin gel hardness, cohesiveness and gumminess values obtained were similar to values reported by Tang et al. (Tang et al., 2006) that investigated the formation of 7% glycinin-rich SPI gels by transglutaminase crosslinking. The non-crosslinked glycinin gel's higher elasticity is also characterized by a 4-fold increase in resilience compared with the crosslinked gel. The non-crosslinked gel's dense and clustered structure enabled it to maintain its form much better in comparison with the crosslinked gel that collapsed under high force, due to the covalent bonds induced by TyrBm.

Table 2
TPA parameters of formed gels of 10% (w/v) non-crosslinked or TyrBm-crosslinked glycinin.

Parameter	Non-crosslinked glycinin[a]	TyrBm-crosslinked glycinin[a]
Hardness (N)	0.76 ± 0.08[c]	1.40 ± 0.14[c]
Cohesiveness (−)	0.36 ± 0.05	0.35 ± 0.02
Gumminess (N)	0.28 ± 0.06[b]	0.49 ± 0.03[b]
Resilience (−)	4.13 ± 0.29[c]	0.98 ± 0.11[c]

[a] All data were the mean values ± SD ($n = 3$).
[b] Indicate significant ($P < 0.05$) difference between the non-crosslinked and crosslinked gel.
[c] Indicate significant ($P < 0.005$) difference between the non-crosslinked and crosslinked gel.

3.7. Scanning electron microscopy (SEM) and pore size analysis

There is a strong correlation between the physical and textural properties of a gel and its network structure. If a gel has a well-organized three-dimensional network structure, the gel is harder. If a gel has a poorly developed structure, specifically an aggregation-type network, it is softer (Kang et al., 1994). The SEM micrographs showed a more structured and organized protein network in the crosslinked glycinin gel than in the non-crosslinked glycinin gel (Fig. 7). Glycinin gelation mechanism is based on the aggregation of the denaturated protein upon heating (Renkema et al., 2000). The native glycinin gel was characterized as a dense clustered network with repeated long, rod shape junctions. Hermansson reported a similar structure when evaluating a heat induced gel of glycinin using transmission electron microscopy. Furthermore, visualization of protein strands built up as hollow

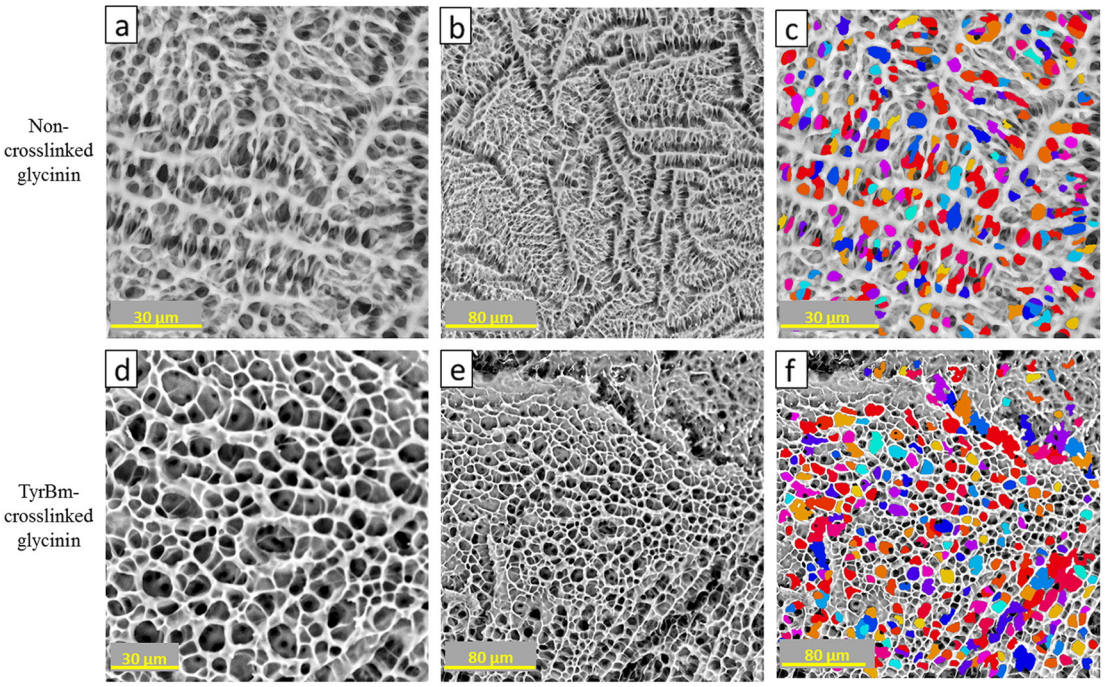

Fig. 7. SEM micrographs of non-crosslinked glycinin (a, b, c) and TyrBm-crosslinked glycinin (d, e, f) formed gels. Gels were formed during 30 min incubation at 95 °C from previously crosslinked glycinin (4 h at 37 °C) or native glycinin. Images a, d and b, e are with different scale and images c and F were used for pore size analysis. Images were captured using SEM PHENOM and pore analysis was done by the PHENOM PoroMetric program.

cylinders were obtained using a higher magnification (Hermansson, 1985). The crosslinked glycinin gel lacks this structure. It is built as a honeycomb without visible junctions. The protein network itself is thinner and more rigid and the pore diameter is 2.7-fold larger than the non-crosslinked gel pores (Table 3). Subsequently, pore volume is increased by 20.4-fold in the crosslinked gel compared with the non-crosslinked gel and therefore, makes it more suitable as a matrix for encapsulation.

3.8. Water holding capacity (WHC)

The water holding capacity (WHC) of non-crosslinked and crosslinked glycinin gels is presented in Fig. 8. With increasing g-force, the WHC of both systems decreased as expected, whereas the decrease was more pronounced for the TyrBm-crosslinked glycinin gel. Lower WHC was related to the coarser microstructure and lower elasticity (Urbonaite et al., 2014) and was therefore indirectly related to aggregate size and density. Moreover, the larger pore size in the crosslinked gel as evidenced by SEM images, and the presence of a less dense network have led to the lower WHC values of this gel. The non-crosslinked gel presents a fine-stranded network, built up by small pores and dense aggregates. This more fine-stranded gel structure was related to a higher WHC which indicates the ability of the matrix to maintain its properties.

Table 3
Pore size analysis of formed gels of 10% (w/v) non-crosslinked or TyrBm-crosslinked glycinin.

	Non-crosslinked glycinin[a]	TyrBm-crosslinked glycinin[a]
Circle equivalent diameter (μm)	3.77 ± 0.04[b]	10.8 ± 0.1[b]
Circumference (μm)	14.3 ± 0.2[b]	41.8 ± 0.7[b]
Area (μm^2)	13.2 ± 0.3[b]	117 ± 4[b]
Volume by area (μm^3)	46 ± 2[b]	1390 ± 80[b]

[a] All data were the mean values ± SD (n = 1855 (non-crosslinked); 3738 (crosslinked)).
[b] Indicate significant ($P < 0.0001$) difference between the non-crosslinked and crosslinked gel.

4. Conclusions

This work studied the physical properties of glycinin gels modulated by crosslinking with tyrosinase. The denaturation transition point, observed by DSC and rheology measurements, was not significantly modified by the enzymatic crosslinking, while a change in the protein decomposition pattern was observed, as determined by TGA. TyrBm-crosslinking of glycinin led to firmer and less elastic gels as evidenced by small deformation measurements. SEM pictures enabled the

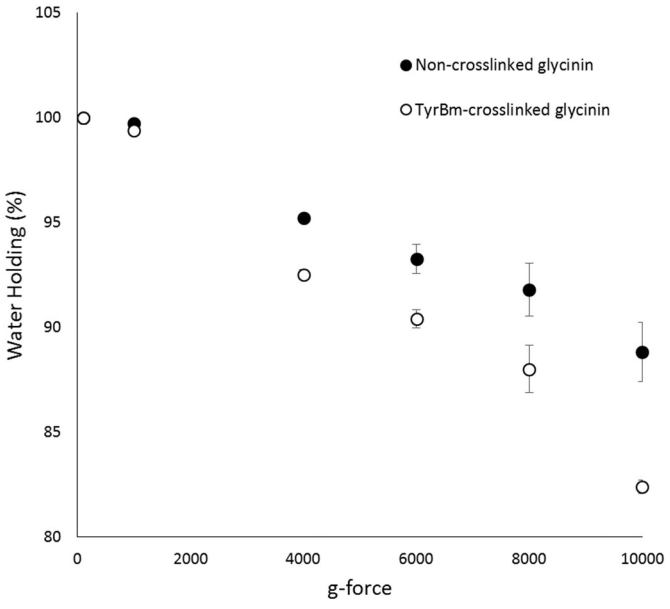

Fig. 8. Water holding capacity of 10% (w/v) non-crosslinked and crosslinked glycinin gels.

visualization of the structural changes in the gel network density and strands diameter. These directly affected the gel pore size and were correlated to both gel hardness and elasticity. Furthermore, gel network rigidity characterized by larger, less dense aggregates, led to coarser gels and decrease in water holding capacity. As gel texture and microstructure together determine the sensory perception and shelf life of foods, understanding of how gel structure can be modulated by enzymatic crosslinking offers the opportunity for texture design of food products with tailored sensory perception.

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