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Characterization of oil-in-water emulsions stabilized by tyrosinase-crosslinked soy glycinin

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ABSTRACT

The effect of crosslinked soy glycinin with tyrosinase from *Bacillus megaterium* (TyrBm) on o/w emulsion properties was studied. The ability of TyrBm to crosslink soy glycinin was evaluated in the presence or absence of three phenolic mediators. It was observed that crosslinking of glycinin is facilitated by a phenolic mediator and is negligible in its absence. Subsequently, the glycinin-stabilized emulsions were evaluated in two systems: (i) homogenization after crosslinking in the presence of a mediator, caffeic acid, and (ii) homogenization prior to crosslinking in the absence of caffeic acid. Emulsions were prepared using a high-pressure homogenizer and their particle size, creaming resistance, viscosity and microstructure were measured. Results indicate that the method of emulsion preparation affected the emulsion physical stability, thus, the first system led to a decrease in emulsion stability against creaming while the second system resulted with improved properties after the enzymatic treatment; Crosslinking after homogenization eliminated the need for a phenolic mediator and led to a lower creaming velocity and higher viscosity. In addition, fluorescence microscopy observations demonstrated that the crosslinking reaction of TyrBm after homogenization led to the formation of cold-set gel-like structures of small droplets linked by covalent bonds.

1. Introduction

Soy proteins are widely used in various food products, due to their high nutritional value and their ability to improve texture as emulsifying and gelation agents (Friedman & Brandon, 2001; Hughes, Ryan, Mukherjea, & Schasteen, 2011; Liu, 1997; Mujoo, Trinh, & Ng, 2003; Renkema, Lakemond, de Jongh, Gruppen, & van Vliet, 2000; Zhang, Wu, Lan, & Yang, 2013). They are correlated with many health benefits, such as reduced LDL cholesterol uptake and prevention of diabetes (Friedman & Brandon, 2001). They were also approved by the US Food and Drug Administration (FDA) as a health claim in foods stating that soy proteins reduce the risk of coronary heart disease (Zhang et al., 2003). Glycinin and β-conglycinin, which account for 65–85% of the total seed protein, are found to be the most important soy proteins and have been utilized and modified for various purposes, such as for food functionalities (Liu, 1997; Mujoo et al., 2003). Glycinin is an oligomeric protein with a molecular mass of ~350 kDa. The individual 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). When compared to other soy fractions, glycinin showed a lower emulsifying ability than soy protein isolate or than the other major protein, β-conglycinin. This is probably due to its high molecular mass and globular structure that limits its ability to adsorb at the oil droplet surface (Keerati-u-rai & Corredig, 2010; Kinsella, 1979; Wagner & Guéguen, 1999). Better control of the final soy product can be obtained by understanding the influence of each protein alone on the emulsion properties. Glycinin has been investigated for its emulsifying properties and different methods have been used to improve its emulsification ability and emulsion stability, such as: heat and pH treatment, high pressure and chemical crosslinking with formaldehyde (Keerati-u-rai & Corredig, 2009; Tang, Chen, & Foegeding, 2011; Yasir, Sutton, Newberry, Andrews, & Gerrard, 2007). A different approach for altering protein structure and improving its properties is enzymatic crosslinking. The great advantage of enzymatic modifications over chemical

Abbreviations: TyrBm, tyrosinase from *Bacillus megaterium*; SPB, sodium phosphate buffer; AP%, percentage of adsorbed proteins.

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modifications is the mild conditions of enzymatic reactions, the ability to control the reaction through slight changes of temperature and pH, and finally, the higher specificity of enzymatic reactions, which allow the avoidance of possible side products. To date, enzymatic crosslinking has been investigated in some detail using the enzyme transglutaminase that catalyzes acyl transfer reactions, resulting in formation of ε-(γ-glutaminyl) lysine interactions (Bao et al., 2011; Castro-Briones et al., 2009; Hu, Zhao, Sun, Zhao, & Ren, 2011; Min & Green, 2008; Muguruma et al., 2003; Siu, Ma, Mock, & Mine, 2002). Transglutaminase treatment of soy proteins-stabilized emulsions led to an increase in droplet size and improved emulsion stability against creaming (Hu, Xu, Fan, Cheng, & Li, 2011; Yang, Liu, & Tang, 2013).

Tyrosinase from the soil bacteria *Bacillus megaterium* (TyrBm) was isolated and characterized in our lab and was investigated for its monophenolase and diphenolase activities (Goldfeder, Egozy, Shuster Ben-Yosef, Adir, & Fishman, 2012; Sendovski, Kanteev, Shuster Ben-Yosef, Adir, & Fishman, 2010; Shuster & Fishman, 2009). TyrBm is a "type-3-copper" enzyme, which contains two copper ions in its active site that are necessary for its activity. It is the enzyme responsible for the production of melanin in organisms and its native substrate is L-tyrosine. Tyrosinases from different origins have been investigated for their protein crosslinking ability on milk and wheat proteins, but not on soy glycinin (Heijnis, Wierenga, van Berkel, & Gruppen, 2010; Selinheimo, Autio, Kruus, & Buchert, 2007; Thalmann & Lötzbeyer, 2002). Studies demonstrated that the crosslinking ability of tyrosinase is limited by the structure of the substrate protein and the exposure of its tyrosine residues and in most cases, required a low molecular weight phenolic agent as a mediator (Fig. 1) (Heck, Faccio, Richter, & Thöny-Meyer, 2013; Thalmann & Lötzbeyer, 2002).

In this study, we used TyrBm for crosslinking soy glycinin in order to improve its emulsion properties. The crosslinking reaction was tested both in the presence and in the absence of a phenolic agent. In order to overcome the need for a phenolic reagent, the crosslinking reaction in the absence of a reagent was carried out after the homogenization. We assumed that during homogenization, some of the hidden tyrosine residues become more exposed to the tyrosinase active site. By introducing covalent bonds within the soy protein network, we have improved emulsion stability against creaming and delayed phase separation.

2. Experimental

2.1. Materials

The commercial soy glycinin standard (98% purity), sodium bisulfite, caffeic acid, chlorogenic acid, coumaric acid and Nile red were obtained from Sigma Chemical Co. (Rehovot, Israel). Refined corn oil was purchased from the local supermarket (Haifa, Israel).

2.2. Methods

2.2.1. Purification of TyrBm

TyrBm was isolated in our laboratory from soil samples, and the gene encoding for the tyrosinase was cloned into *Escherichia coli* BL21, expressed and purified as previously described, and the enzyme activity was determined on L-tyrosine, and L-dopa (Shuster & Fishman, 2009). In this work, the enzyme activity on caffeic acid was determined as well.

2.2.2. Isolation of soy protein fractions

Glycinin-rich fraction was isolated from defatted soybean flakes, kindly provided by Shemen Industries Ltd. (Haifa, Israel). The isolation was done according to the method of Nagano et al. (Nagano, Hirotsuka, Mori, Kohyama, & Nishinari, 1992), with slight

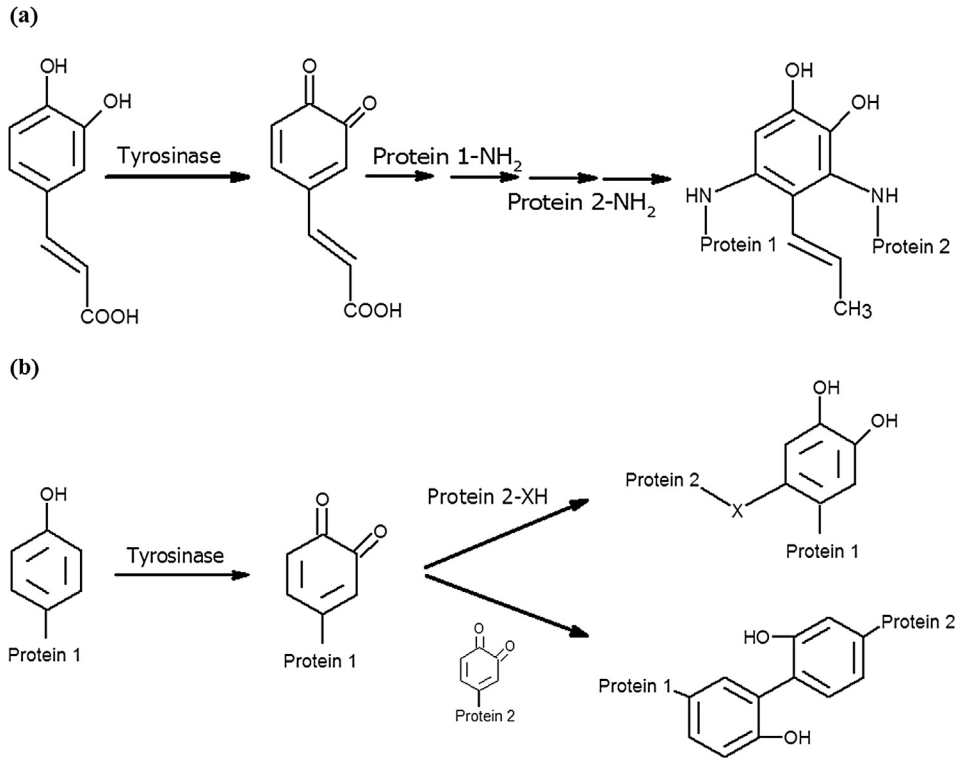

Fig. 1. Reaction scheme of a tyrosinase generated crosslinking of proteins (adapted from (Heck et al., 2013)); (a) in the presence of caffeic acid as a low molecular weight phenolic mediator; (b) in the absence of a low molecular weight phenolic reagent.

changes. The defatted soybean flakes were dispersed in distilled water (1:15, w/w), adjusted to pH 8 and stirred at room temperature for 1 h. Solids were removed by a stainless steel screen (120 mesh) and then centrifuged at 13,000 *g* for 30 min at 15 °C. Dry sodium bisulfite (NaHSO3) was added to the supernatant (0.98 g L^{-1}), pH was adjusted to 6.4 and the mixture was kept overnight at 4 °C, followed by centrifugation at 6800 *g* for 20 min at 4 °C. The precipitate (glycinin) was suspended in distilled water, adjusted to pH 7.8, dialyzed against distilled water (24 h, 4 °C) and lyophilized. All pH adjustments were done using 2 M HCl or NaOH. The purity of the glycinin was evaluated on SDS-PAGE gel in comparison to a commercial standard (Fig. s1). 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).

2.2.3. Preparation of oil-in-water emulsions

Glycinin dispersion was formed in 100 mM sodium phosphate buffer (SPB) pH = 7.4 (1%, w/v), was stirred at ambient temperature for 30 min to ensure full hydration, and 10% (w/v) corn oil was added. The mixture was then pre-homogenized using a shear dispersing unit (Pro 200, Biogen series pro scientific, Oxford, CT, USA) for 1 min at 35,000 rpm following by a high pressure homogenization (EmulsiFlex-C3, Avestin Inc., Ottawa, ON, Canada) for 3-5 passes at 20 kPsi. The crosslinked and control emulsions prepared in later experiments (Section 3.2) were homogenized as above for 3 passes at 20 kPsi.

2.2.4. Enzymatic crosslinking of soy glycinin

0.5% (w/v) glycinin was suspended in 100 mM SPB pH = 7.4 and stirred for 30 min at ambient temperature. 0.005% (w/v) TyrBm was added. Distilled water or low molecular weight phenolic agent (caffeic acid, *p*-coumaric acid or chlorogenic acid) were added to 1 mM concentration. The reaction mixture was incubated at 37 °C with shaking at 200 rpm in an incubator shaker (TU-400 Orbital Shaker Incubator, MRC, Holon, Israel) for various times (0, 30, 60, 120, 180 and 240 min, respectively), then stopped by directly mixing reaction mixture with electrophoresis sample buffer (*x*4) at 1:1 ratio (v/v). The samples were analyzed by SDS-PAGE.

2.2.5. Preparation of crosslinked protein solutions and emulsions

1% (w/v) glycinin was suspended in 100 mM SPB pH = 7.4 and stirred for 30 min at ambient temperature. For emulsions made with crosslinked protein, the suspended protein solutions were incubated with 0.01% TyrBm and 2 mM caffeic acid at 37 °C with shaking at 200 rpm in an incubator shaker (TU-400 Orbital Shaker Incubator, MRC, Holon, Israel) for 120 min. For control emulsions made with non-crosslinked glycinin, protein solutions were incubated for 120 min under the same conditions, with distilled water instead of enzyme. Reaction was stopped by adding EDTA to a final concentration of 1.5 mM, which was previously shown to inhibit TyrBm (unpublished results). Emulsions with 10% corn oil were then prepared as described in Section 2.2.3. For crosslinking after homogenization, emulsions were prepared using 1% (w/v) native glycinin and corn oil (10% w/v). Immediately after homogenization, 0.01% TyrBm (or distilled water, for the control) was added and the emulsions were incubated for 120 min at 37 °C. The reaction was stopped by EDTA addition.

2.2.6. 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 10 min at 95 °C, after addition of sample buffer (4*x*), 1:1 (v/v). Samples were mixed with reducing (5% β-mercaptoethanol) or non-reducing (0.5% β-mercaptoethanol) sample buffer, as specified. 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)].

2.2.7. Determination of volume-average droplet size ($d_{4,3}$)

Particle size was determined using static laser light scattering (SLS) (Malvern Mastersizer 2000, Malvern Instruments, Worcestershire, U.K.). Refractive indices used for the phosphate buffer and corn oil phases were 1.33 and 1.47 respectively (Maneephan, Zebin, & Milena, 2011; Sandra, Decker, & McClements, 2008), and size information is given by the mean volume weighed droplet diameter (1):

$$d_{4,3} = \sum_i n_i d_i^4 \Big/ \sum_i n_i d_i^3 \quad (1)$$

where n_i is the number of droplets of diameter d_i. A few drops of sample were dispersed in approximately 150 ml phosphate buffer in the sample chamber with mild stirring until approximately (15 ± 1)% obscuration was obtained.

2.2.8. Determination of creaming stability

Physical stability of the emulsions against creaming was monitored using an analytical centrifugal analyzer (LUMisizer, L.U.M. GmbH, Berlin, Germany). For this purpose, rectangular cells were loaded with emulsion samples whose time and space-resolved transmission extinction profiles were monitored under analytical centrifugation. This has been applied to evaluate emulsion creaming velocities, as previously described (Detloff, Sobisch, & Lerche, 2007; Shimoni, Shani Levi, Levi Tal, & Lesmes, 2013). Basically, 400 μL of non-diluted emulsions were loaded into rectangular cells whose transmission extinction profiles were recorded at 500 g, 23 °C for 5.5 h.

2.2.9. Measurements of percentage of adsorbed proteins (AP%)

The AP% of freshly prepared emulsions was determined using the method described previously (Shao & Tang, 2014), with a few modifications. Briefly, 1 ml of fresh emulsion was centrifuged at 10,000 g for 30 min at 20 °C. Two phases were observed after centrifugation: the creamed oil droplets at the top of the tube and the aqueous phase of the emulsion at the bottom. The subnatant was extracted using a syringe and then filtered through a 0.22 mm filter (Millipore Corp.). The protein concentration of the filtrate (C_f) was determined with the Bradford method using BSA as the standard (Bradford, 1976). The starting protein solution was also centrifuged at the same conditions to determine the protein concentration (C_s) in the supernatant. The AP% was calculated as follows (2):

$$\mathrm{AP\%} = \left(C_s - C_f\right) \times 100/C_0 \quad (2)$$

where C_0 (mg/ml) is the initial protein concentration applied for the emulsion preparation (10 $mgml^{-1}$ in the present work). For the evaluation of the amount of crosslinked protein in the bulk and the interface during the reaction, 1 ml of the emulsion was evaluated at 1, 2, 3 and 4 h into the enzymatic reaction as follows: 1 ml emulsion was centrifuged, the subnatant was extracted and the supernatant was dissolved in 1 ml buffer. The two phases were analyzed by SDS-PAGE and the Bradford method to evaluate the crosslinking extent, and the amount of protein, respectively.

2.2.10. Measurements of shear viscosity

Shear viscosity measurements of the emulsions using parallel plates (d = 60 mm) were carried out in a Discovery Hybrid Rheometer (DHR-2, TA Instruments, DE, USA). The crosslinked or control emulsion was placed between parallel plates at 25 °C and the gap between the two plates was set to 1.0 mm. The sample temperature was monitored through the lower plate. Excess sample was trimmed off the plate. The equipment was driven using the Trios program (TA Instruments, DE, USA). Shear rate was increased from 0 to 300 s^{-1} and viscosity was recorded.

2.2.11. Direct observation of emulsions

Emulsions were visually inspected using a Cell Observer inverted microscope (Zeiss Axiovert 200, Jena, Germany). The lipid phase of the emulsions was labeled using Nile red (0.1 mg ml^{-1} Nile red in ethanol which is diluted 1:50 before use with the tested emulsion) and incubated in ice for 10 min before being placed on a glass microscope slide and covered. Deflocculation was performed by adding 1% SDS. Images were processed by the AxioVision (Zeiss) image analysis software for acquisition and image processing.

2.2.12. Experimental design and analysis

All the results presented in this paper are the average of at least three repetitions. Statistical analyses were performed using Microsoft Excel 2010 data analysis tool pack and mainly relied on paired sample *t*-tests assuming equal variances. The level of significance used was 99.5%.

3. Results & discussion

3.1. Glycinin-stabilized o/w emulsion fabrication

The purified glycinin fraction was used to fabricate o/w emulsions with 1% (w/v) protein and 10% corn oil. A 10% oil emulsion was chosen as it is considered a good model for foods (Keerati-u-rai & Corredig, 2009, 2010). Particle size measurements indicated the formation of particles with $d_{4,3}$ of 8.0–8.5 μm at all homogenization conditions (Fig. 2a). Creaming velocity was also similar for emulsions prepared using 3–5 passes of homogenization, and determined as 3.4 μm/s (Fig. 2b). Emulsion physical stability was confirmed by visible evaluations (results not shown) that indicated no phase separation in the emulsions over a period of 48 h. There was no significant difference in droplet size or creaming resistance between the emulsions as a result of increasing passes in the homogenizer.

3.2. TyrBm-catalyzed crosslinking of soy glycinin

Before using tyrosinase with the emulsion system, its effect on native soy glycinin was evaluated. TyrBm was incubated with glycinin solutions for different periods of time. Preliminary trials were carried out in order to establish standard conditions; these confirmed that soy glycinin is not a good substrate for TyrBm when it maintains its quaternary structure in an aqueous dispersion (Fig. 3a). This is probably due to the position of the tyrosine residues in the core of the glycinin globular structure which makes them unexposed and unavailable to the tyrosinase active site (Jee, Park, & Kim, 2000). In order to obtain a higher crosslinking rate, three low molecular weight phenolic reagents were tested: caffeic acid, chlorogenic acid and *p*-coumaric acid. Crosslinking of glycinin was significantly higher in the presence of 1 mM phenolic agents than in its absence as observed by the formation of high molecular weight bands in the SDS-PAGE gel (Fig. 3b–d, Fig. s2). Caffeic acid led to the highest crosslinking rate, presumably because of the high diphenolase activity of tyrosinase on caffeic acid compared to its monophenolase activity on *p*-coumaric acid. Chlorogenic acid has a very large side chain which might limit the substrate availability to the active site thus leading to a smaller activity rate. The reaction mixture in the presence of a phenolic reagent contained a strong pigment attributed to self-polymerization of the oxidized phenolic reagent and the formation of melanin (Goldfeder et al., 2012; Shuster & Fishman, 2009). In the control tubes, which were incubated under the same conditions with distilled water rather than enzyme, a low amount of pigment was observed, indicating that the reactive diphenols (caffeic acid and chlorogenic acid) spontaneously oxidized to quinones and further polymerized. However, the pigmentation in the absence of enzyme was minor compared to the pigmentation in the presence of TyrBm. Different caffeic acid concentrations were tested and it was found that optimal crosslinking is achieved with 2 mM caffeic acid, and 1% enzyme to glycinin ratio (results not shown).

3.3. Properties of crosslinked-glycinin stabilized emulsions

3.3.1. Crosslinking prior to homogenization, in the presence of caffeic acid

The emulsion prepared with an initially-crosslinked glycinin and caffeic acid had a grainy texture and contained a dark pigment as a result of self-polymerization of oxidized caffeic acid. Phase separation was visible after a few hours for the crosslinked

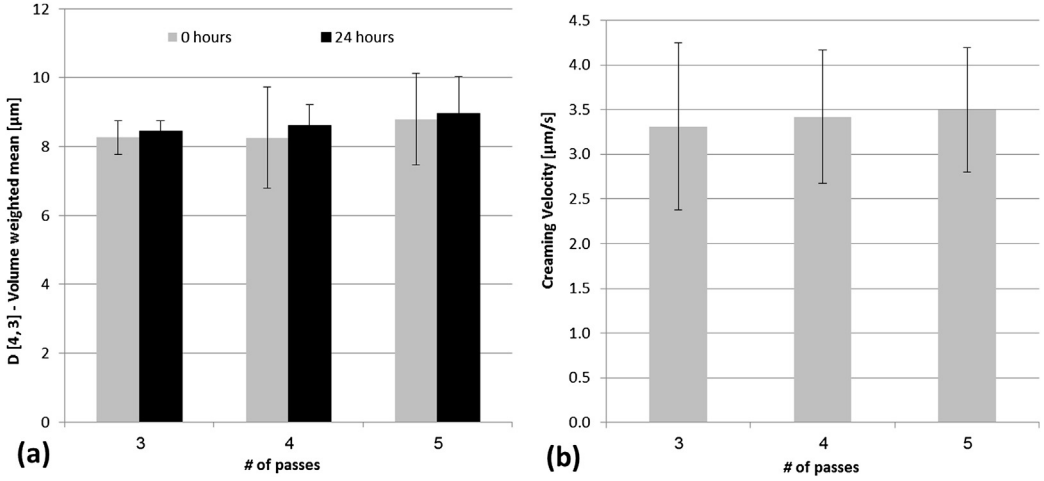

Fig. 2. Characterization of o/w glycinin emulsions after homogenization for 3–5 passes at 20 kPsi. (a) Volume weighted mean diameter measured using SLS and (b) Creaming velocity measured using LUMisizer. Emulsions contained 1% glycinin and 10% corn oil (w/v).

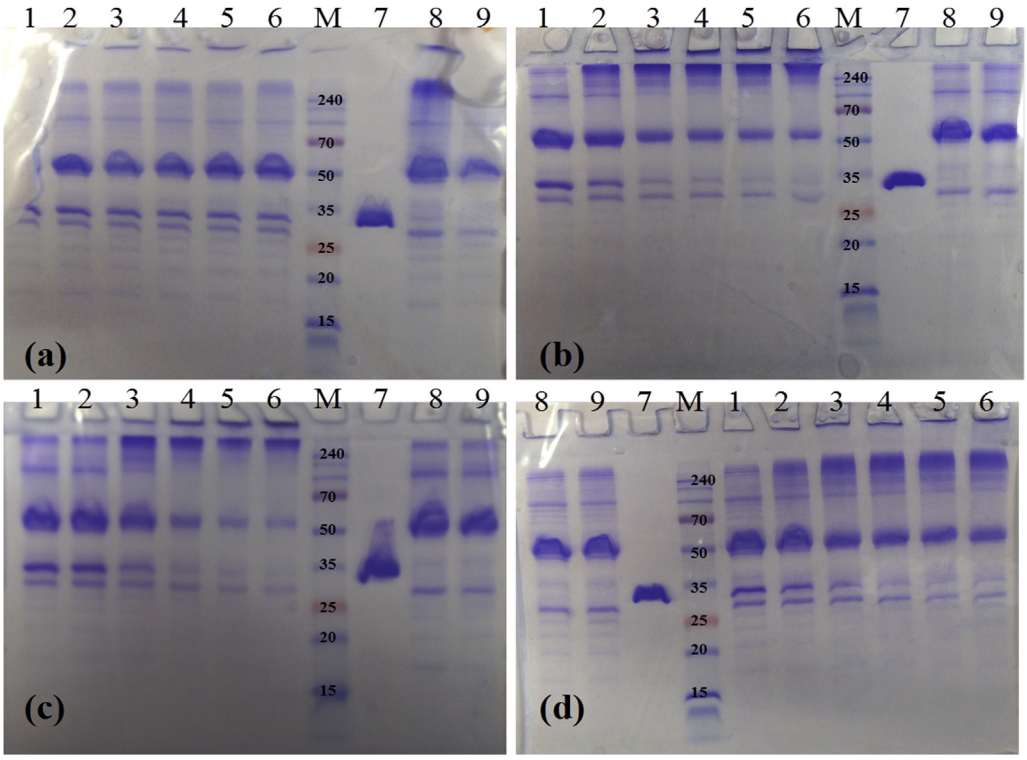

Fig. 3. SDS-PAGE gels describing crosslinking of TyrBm (a) in the absence of a phenolic agent; in the presence of (b) caffeic acid, (c) *p*-coumaric acid and (d) chlorogenic acid. M – Molecular size marker. Lanes 1–6: TyrBm polymerized glycinin after incubation at 37 °C for 0–240 min. Lane 7: TyrBm. Lanes 8–9: Glycinin control without TyrBm, at 0 and 240 min incubation, respectively.

emulsions, while the emulsions prepared with native glycinin remained stable (Fig. s3). Particle size measurements indicated a 4-fold increase in the droplet size (Fig. 4a), and the creaming velocity also increased by 3.5-fold (Fig. 4b) as a result of 120 min crosslinking with TyrBm. All in all, the crosslinking reaction before homogenization in the presence of caffeic acid led to a less stable emulsion. The deterioration in emulsion stability may be attributed to the large protein network that was formed during the crosslinking reaction. As observed, crosslinking in the presence of caffeic acid is extensive. We assume that the oxidized phenol and the protein form a large and branched network that cannot adsorb to the oil droplet surface properly and sufficiently.

3.3.2. *Crosslinking after homogenization, in the absence of caffeic acid*

Since color formation is not necessarily desirable in food products, crosslinking in the absence of a phenolic reagent is preferable. Improved crosslinking without a mediator may be more likely obtained after homogenization of the protein. During homogenization, the protein adsorbs to the oil droplet surface and tyrosine

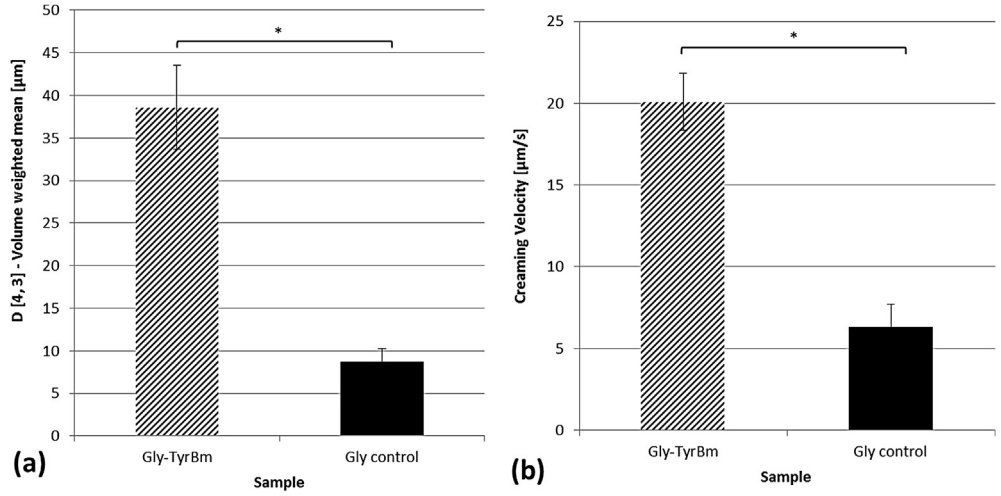

Fig. 4. (a) Volume weighted mean diameter and (b) creaming velocity of o/w emulsions that were crosslinked for 2 h at 37 °C with TyrBm before homogenization. Stripped bars: Crosslinked protein, Black bars: native protein. *p-value<0.005.

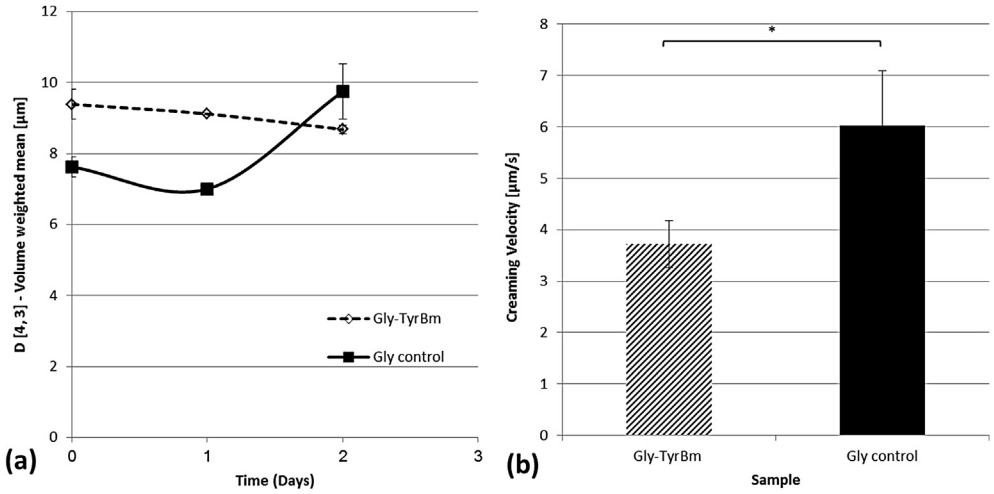

Fig. 5. (a) Volume weighted mean diameter as function of storage time and (b) creaming velocity of glycinin-stabilized o/w emulsions that were crosslinked for 2 h at 37 °C with TyrBm after homogenization. Stripped bar or line: Crosslinked protein, black bar or line: native protein. *p-value<0.005.

residues can become more exposed and available to the enzyme's active site, leading to a higher crosslinking rate (Færgemand, Otte, & Qvist, 1998). Therefore, homogenization was performed prior to crosslinking in the absence of caffeic acid. The o/w emulsion stabilized with native glycinin and followed by crosslinking with TyrBm after homogenization, showed a slightly larger particle size compared to the control emulsion (Fig. 5a). This may be due to a few inter-droplet crosslinks which led to larger droplet aggregates. However, the control particle size increased over time while the crosslinked emulsion particles remained nearly constant over 48 h. Space and time profiles measured using the analytical centrifuge demonstrated that performing the crosslinking reaction after homogenization led to a lower creaming velocity in comparison to control emulsions (Fig. 5b). The decrease in creaming velocity is in contrast to the larger particle size existing in the enzymatically-treated emulsion. According to Stokes law, which is given as (3):

$$\upsilon_s = 2d^2(\rho_0 - \rho)g/9\eta_0 \quad (3)$$

where υ_s is the droplet velocity and d is the radius, ρ_0 and ρ are the density of the continuous and the dispersed phase, respectively, and η_0 is the dynamic viscosity of the continuous phase, the droplet velocity in laminar flow is dependent on the square of the droplet radius (Dickinson, 1992; Lamb, 1994). Therefore, the improved creaming resistance can be attributed to increased viscosity of the continuous phase by the formation of covalent bonds between peptide chains in the bulk. Indeed, the dynamic viscosity of the emulsion stabilized by crosslinked glycinin, measured using a rheometer, was 1.9-fold higher than the native glycinin-stabilized emulsion at a shear rate up to 140 s^{-1} (Fig. s4). Visible stability tests (Fig. 6) confirmed that the crosslinked-glycinin stabilized-emulsion (TyrBm-Gly) remained stable over a 48-h period at ambient temperature, while the control emulsion showed phase separation during the first 24 h. The increase in stability might be partially due to a steric interference as shown by a higher percentage of adsorbed proteins (AP%) to the oil droplets in the enzymatically treated emulsions, in comparison to the control emulsions (AP% increased by 12 ± 2%, p-value < 0.005). Moreover, in diluted emulsions containing 10% oil, the amount of non-adsorbed protein in the aqueous phase is relatively large. This was proven by measuring the amount of protein in the bulk and in the interface at different time points during the crosslinking reaction. The crosslinking reaction takes place not only in the interface but also in the non-adsorbed protein in the bulk, contributing to an increased viscosity of the continuous phase and a lower creaming rate (results not shown). The results correlated with other studies on the crosslinking of soy protein-stabilized emulsions by transglutaminase (Yang et al., 2013).

Microscopy images were used to further study the microstructure of the emulsions. Analyses revealed that flocculation occurs in both the crosslinked and the control emulsions (Fig. 7). After a short treatment with 1% SDS to deflocculate the emulsions, the control

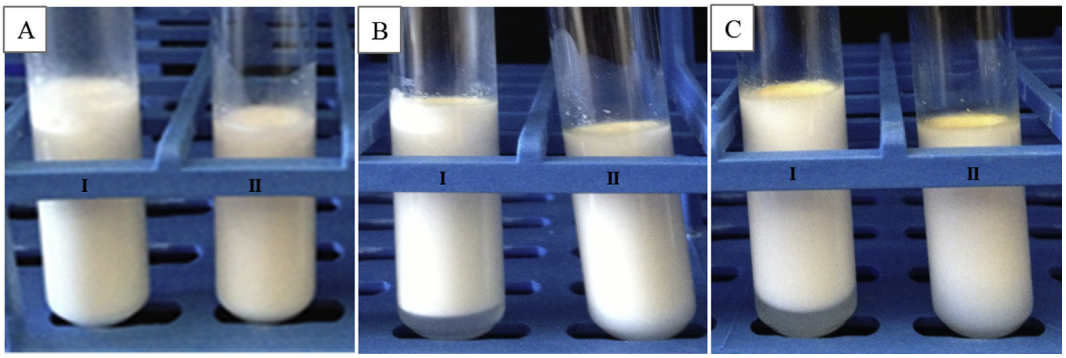

Fig. 6. Typical visual images of glycinin-stabilized o/w emulsions that were crosslinked for 2 h at 37 °C with TyrBm after homogenization (II) and control emulsions with native glycinin (I) at different times: (A) immediately after crosslinking, (B) 24 h and (C) 48 h after crosslinking.

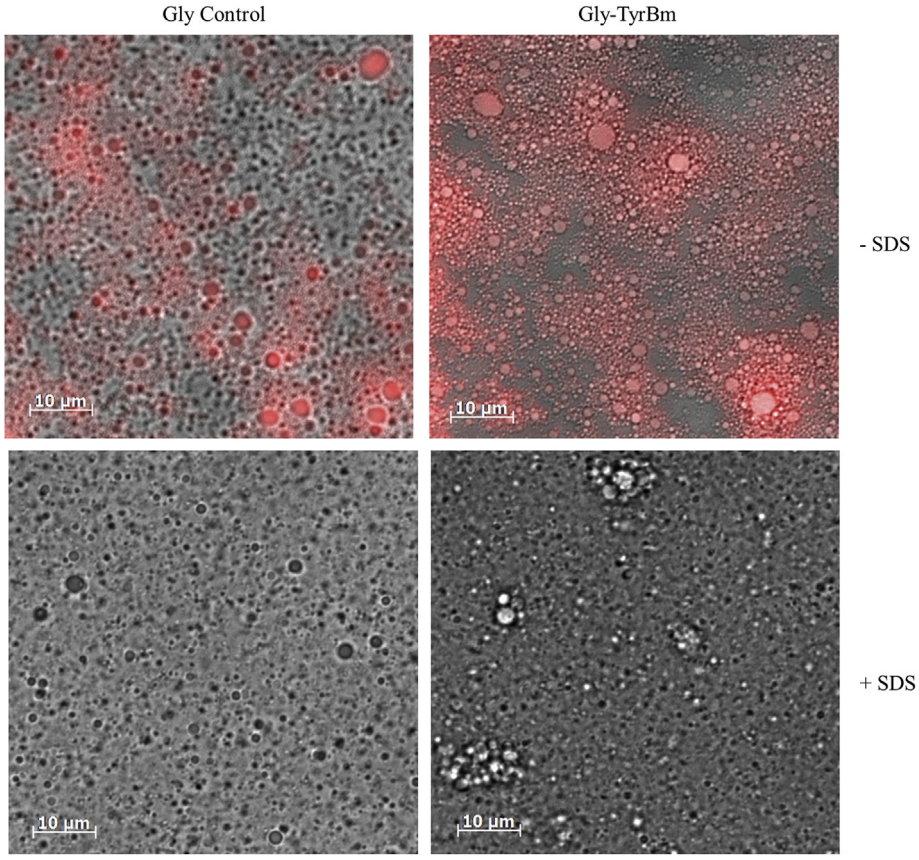

Fig. 7. Inverted micrographs of 24 h o/w emulsions that were crosslinked for 2 h at 37 °C with TyrBm after homogenization and control emulsions with native glycinin before and after deflocculating with 1% SDS. Scale-bar: 10 µm.

emulsion contained mostly single droplets of 1.0–1.5 micron. The crosslinked-glycinin based-emulsion however, still contained aggregates. The remaining aggregates were smaller and more spherical compared to the flocculation observed prior to the SDS treatment. It is assumed that the remaining aggregates are the result of covalent bonds between the protein chains on the droplet surface, induced by tyrosinase crosslinking activity. The crosslinking reaction promoted the formation of cold-set gel-like structures in the emulsion. These structures have increased the viscosity and decreased the mobilization of the droplets, thus, contributing to emulsion stability. Similar structures were reported by Tang et al. for soy protein isolate stabilized emulsion gels formed by transglutaminase crosslinking. In their work, they reported the formation of gel like emulsions as a result of the long (6 h) enzymatic activity which led to increase in the emulsion viscosity (Tang & Liu, 2013; Yang et al., 2013).

4. Conclusions

The aim of the present study was to improve the stability of glycinin-based o/w emulsions using crosslinking with tyrosinase. It was proved that crosslinking of soy glycinin by TyrBm altered the properties of o/w emulsions compared to native glycinin-stabilized emulsions. Crosslinking in the presence of caffeic acid prior to homogenization was substantial and rapid, but led to a less stable emulsion with undesired properties such as dark pigmentation. Crosslinking after homogenization diminished the need in caffeic acid, decreased creaming velocity in the emulsion and increased the viscosity. Microscopy imaging in the presence of SDS suggested that covalent bonds within the protein network resulting from tyrosinase crosslinking activity, assisted in the formation of cold-set gel-like structures in the emulsion, thus leading to the improved physical stability. This work contributes to our understanding of the emulsion properties of glycinin and suggests that crosslinking with tyrosinase is a feasible approach to improving glycinin-stabilized o/w emulsions without the need for a phenolic mediator.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2014.07.007.

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