



Oil-in-water emulsions stabilized by tyrosinase-crosslinked potato protein



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ABSTRACT

Potato protein (PP) holds great promise as a non-allergenic food ingredient with high nutritional value. Attempts to modulate its functional properties by crosslinking have not been reported to date. The effect of tyrosinase-mediated crosslinking of PP on the properties of o/w emulsions was studied in the present work. Among the various PPs, protease inhibitors were efficiently crosslinked by the enzyme as determined by SDS-PAGE analysis. Concentrated emulsions comprising 40% olive oil and 6.1% PP (w/w) were fabricated by a shear stress homogenizer. The PP-stabilized emulsions were evaluated after one and 4 h of incubation with tyrosinase. Emulsions were characterized by their droplet size distribution, rheological behavior, creaming resistance and microstructure. The crosslinked emulsion had a self-standing elastic gel-like structure after 1 h of incubation. Unlike the Newtonian non-crosslinked emulsion, the crosslinked emulsion exhibited a shear-thinning behavior with a 20-fold increase in viscosity. The longer incubation time coupled with shaking at 250 rpm for up to 4 h resulted in the disruption of the droplets structure and led to a 2-fold decrease in viscosity, compared to the 1-hour crosslinked emulsion. Droplet size distribution showed formation of large particles in the crosslinked emulsion. Microscopy imaging demonstrated formation of aggregated and dense emulsion droplets network, which also contributed to the emulsion gel-like behavior. In the case of the non-crosslinked emulsion, severe flocculation and coalescence was observed, regardless of incubation time. The results suggest that tyrosinase crosslinking is a useful method to modulate the properties of PP-based food formulations.

1. Introduction

Among the various non-animal protein sources, plant proteins have been widely applied as food ingredients due to their functional properties (Vaz Patto et al., 2015) and abundant supply. Potato proteins (PP) are becoming more prevalent in food products due to their high nutritional quality comparable to the protein of whole egg (Friedman, 1996; Liedl, Kosier, & Desborough, 1987) and high essential amino acid index (Alting, Pouvreau, Giuseppin, & van Nieuwenhuijzen, 2011; Pouvreau et al., 2001). They are considered GRAS and non-allergenic and can be incorporated in vegetarian, vegan and Kosher Parve products (David & Livney, 2016). Potato proteins have been found as a valuable component in fining of red wine and white musts (Gambuti, Rinaldi, & Moio, 2012; Gambuti, Rinaldi, Romano, Manzo, & Moio, 2016), cheese making (Spelbrink, Lensing, Egmond, & Giuseppin, 2015), or gluten-free bread making (Witczak, Juszczak, Ziobro, & Korus, 2017). Additionally, their emulsifying (Ralet & Guéguen, 2000; Romero et al., 2011; van Koningsveld et al., 2006), foaming (Baier & Knorr, 2015; van Koningsveld et al., 2002) and

gelling (Creusot, Wierenga, Laus, Giuseppin, & Gruppen, 2011) properties have been previously reported. In terms of health benefits, potato patatin has been shown to possess the ability to regulate serum cholesterol levels (Liyanaage et al., 2008), and to act as a potential cancer chemo-preventive agent (Sun, Jiang, & Wei, 2013). Hydrolyzed potato proteins have shown remarkable antioxidative properties (Cheng, Xiong, & Chen, 2010; Nieto et al., 2009; Udenigwe, Udechukwu, Yiridoe, Gibson, & Gong, 2016).

Potato proteins include three major classes: patatin (up to 40%), protease inhibitors (up to 50%) and others (mostly high molecular weight proteins) (Løkra, Helland, Claussen, Strætkvern, & Egeland, 2008; Pouvreau et al., 2001). Patatin, a globular glycoprotein with an isoelectric point between 4.5 and 5.2, is a dimer that appears as a monomer (40–45 kDa) in the presence of the sodium dodecyl sulfate (Racusen & Weller, 1984). Protease inhibitors are the most abundant and heterogeneous group of proteins with molecular sizes varying between 5 and 25 kDa (Pots et al., 1999). The most common members are serine protease inhibitors (PI-1 and PI-2 from the Kunitz family), potato cysteine protease inhibitors (PCPI), potato aspartate protease inhibitors

Abbreviations: TyrBm, tyrosinase from *Bacillus megaterium*; PP, potato protein; SPB, sodium phosphate buffer; PFJ, potato fruit juice

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(PAPI), other Kunitz type protease inhibitors (PKPI) and potato carboxypeptidase inhibitors (Pouvreau et al., 2001). PI-2 is a dimeric protein with one disulfide bridge and molecular mass of 22 kDa, while PI-1 is a pentameric protein composed of five 7–8 kDa isoforms (Pouvreau et al., 2001). PCPI is considered to be a beta-II protein based on structural properties which are similar to those of potato serine protease inhibitors and Kunitz type soybean trypsin inhibitors (Pouvreau, Gruppen, van Koningsveld, van den Broek, & Voragen, 2005). Potato protease inhibitors vary in their inhibiting effects among the groups, but generally inhibit trypsin, chymotrypsin and human leukocyte elastase (Pouvreau et al., 2001).

It has been shown that potato proteins as a crude extract exhibited poor emulsification properties (Ralet & Guéguen, 2000), thus different methods have been used to improve its emulsification ability and emulsion stability such as: hydrolyzation (Nieto et al., 2009), pH treatment or ionic strength modulation (Delahaije, Gruppen, van Nieuwenhuijzen, Giuseppin, & Wierenga, 2013), succinylation (Delahaije, Wierenga, Giuseppin, & Gruppen, 2014), phosphorylation (Miedzianka & Peksa, 2013), addition of chitosan (Calero, Muñoz, Cox, Heuer, & Guerrero, 2013), addition of κ -carrageenan (O'Sullivan, Kurukji, Norton, & Spyropoulos, 2017), addition of guar gum (Santos, Calero, Guerrero, & Muñoz, 2015), and conjugation with galactose, galactooligosaccharides, or galactan (Seo, Karboune, & Archelas, 2014). Furthermore, it has been reported that fractionated PP isolate possess better emulsifying properties than the raw PP mixture (Ralet & Guéguen, 2000).

Modifying the properties of proteins by enzymatic crosslinking, by introducing new covalent bonds, can expand their applications. The enzymatic approach provides a safe biological technique to improve food properties (Zorn & Li, 2017). Protein crosslinking by tyrosinases from different origins have been demonstrated to increase gel firmness in meat products (Lantto, Puolanne, Kruus, Buchert, & Autio, 2007) and improve the physical stability of milk protein emulsions (Selinheimo et al., 2007).

Bacterial tyrosinase from *Bacillus megaterium* (TyrBm) was isolated, characterized and crystallized in our lab (Goldfeder, Kanteev, Isaschar-Ovdat, Adir, & Fishman, 2014; Sendovski, Kanteev, Ben-Yosef, Adir, & Fishman, 2011). Tyrosinases are dicopper enzymes, responsible for generation of melanin by converting the native substrate L-tyrosine to dopaquinone which spontaneously undergoes polymerization to melanin. Recently, it was found that TyrBm-mediated crosslinking improved soy glycinin emulsion physical stability (Isaschar-Ovdat, Rosenberg, Lesmes, & Fishman, 2015). There are no previous reports describing the modulation of PP properties by chemical or enzymatic crosslinking. The goal of this study was to evaluate the ability of tyrosinase to crosslink PP and investigate the effect of the enzymatic treatment on emulsion stability.

2. Experimental

2.1. Materials

Ammonium sulfate, sodium metabisulfite, and Nile red were obtained from Sigma Chemical Co. (Rehovot, Israel). Refined olive oil and raw potatoes were purchased from the local supermarket (Haifa, Israel), while the commercial Solanin® potato proteins 206P (patatin; 80 kDa) and 306P (protease inhibitors; 5–25 kDa) were obtained from Avebe (Avebe A.U., Foxhol, Netherlands).

2.2. Methods

2.2.1. Purification of TyrBm

TyrBm was isolated and characterized in our laboratory as previously described (Shuster & Fishman, 2009).

2.2.2. Isolation of potato protein fractions

Potato proteins were isolated from fresh potatoes (*Solanum tuberosum* L.) purchased from the local supermarket (Haifa, Israel). The isolation was done according to the method of van Koningsveld et al. (2001) with slight changes. Briefly, potato tubers were peeled and cut into small pieces and frozen at -80°C for 30 min. The frozen potato samples were suspended in buffer (25 mM SPB, pH 7.0, 4 mM sodium metabisulfite) (1:1 ratio) and homogenized using a commercial blender on low speed for 5 min. The potato slurry was subjected to cheese cloth filtration and the turbid filtrate was centrifuged at 14,000g for 30 min at 4°C . The supernatant was then filtered using 1.2 μm GF/C Whatman filters to obtain a clear yellowish filtrate which is known to be similar to industrial potato fruit juice (PFJ).

Protein precipitation was carried out at 60% ammonium sulfate saturation. The mixtures were incubated for 90 min at 100 rpm and 4°C . The recovered precipitates upon centrifugation (14,000g/30 min/ 4°C) were suspended in 25 mM sodium phosphate buffer (SPB, pH 7.0) and dialyzed against the same buffer for 24 h at 4°C (100 rpm) to remove the ammonium sulfate, followed by lyophilization. The purity of the potato protein was evaluated on SDS-PAGE gel in comparison to commercial Solanin® Avebe PP isolates 206P and 306P. The total protein content was determined by the Bradford method (Bradford, 1976) and elemental analysis (FlashSmart CHNS, Thermo Scientific Inc. Massachusetts, US).

2.2.3. Water content

Water content was determined by drying lyophilized potato protein concentrates in triplicates in an oven at 150°C for 16 h, and determining the weight difference before and after drying (Løkra, Schüller, Egeland, Engelsen, & Strætkvern, 2009).

2.2.4. Total phenolic content

The total phenolic content of the extract was determined by the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). 20 μL of different concentrations - depending on solubility - of potato protein extracts or fractions in water was mixed with 100 μL of Folin–Ciocalteu reagent (1:10). After 7 min, 100 μL of Na_2CO_3 solution (200 g/L) was added, followed by a 60 min incubation in the dark (30°C), and the optical density was measured at 765 nm. The total phenolic content was calculated on the basis of a calibration curve of gallic acid and expressed as gallic acid equivalents (GAE), in milligrams per liter of the sample.

2.2.5. Enzymatic crosslinking of potato proteins

1% (w/v) PP was suspended in 25 mM SPB pH = 7 and stirred for 30 min at ambient temperature. The crosslinking reaction was carried out as previously described (Isaschar-Ovdat et al., 2015) with slight changes. TyrBm was added in a protein ratio of 1:30 and the reaction mixture was incubated at 37°C with shaking at 250 rpm in an incubator shaker (TU-400 Orbital Shaker Incubator, MRC, Holon, Israel). Samples were taken at various times (0, 30, 60, 120, 180 and 240 min, respectively) and the reaction was stopped by directly mixing the reaction mixture with electrophoresis sample buffer ($\times 4$) at 1:1 ratio (v/v). The samples were analyzed by SDS-PAGE.

2.2.6. Preparation of crosslinked protein solutions and emulsions

6.1% (w/v) PP was suspended in 25 mM SPB pH = 7 and stirred overnight at 4°C to ensure full hydration. The reaction conditions were set as described in Section 2.2.5 and immediately 40% (w/v) olive oil was added and homogenized using a shear dispersing unit (Pro200, Pro-Scientific, Oxford CT, USA) for 5 min at 35,000 rpm. The emulsions were then incubated for 60 or 240 min at 37°C with shaking at 250 rpm in an incubator shaker (TU-400 Orbital Shaker Incubator, MRC, Holon, Israel). For the non-crosslinked emulsions all details remain identical while SPB was added instead of enzyme.

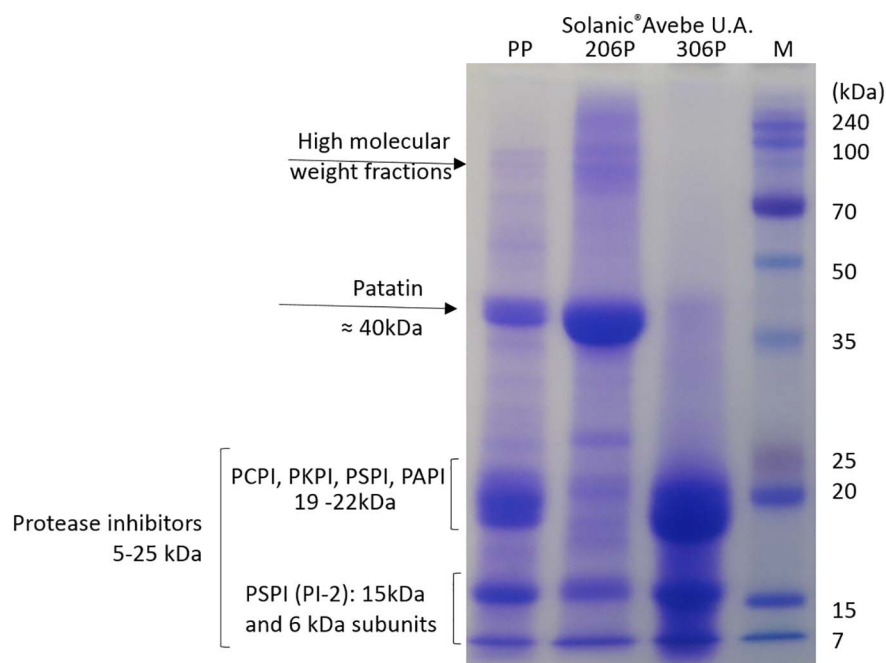


Fig. 1. SDS-PAGE visualization of purified potato protein rich fraction (PP), Solanic®Avebe high molecular weight fraction (206P) and low molecular weight fraction (306P). M indicates the molecular weight marker.

2.2.7. SDS-PAGE analysis

SDS-PAGE was performed on a discontinuous buffered system (Laemmli, 1970) using 15% separating gel and 4% stacking gel. The samples were heated for 10 min at 95 °C, after addition of sample buffer (4 ×), 1:1 (v/v). Samples were mixed with reducing (5% β-mercaptoethanol) sample buffer. The gels were 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)].

2.2.8. Determination of particle size distribution (PSD)

Particle size was determined using laser diffraction particle size analyzer MasterSizer 3000 (Malvern Instruments Ltd., Malvern, Worcestershire, UK) equipped with a wet sample dispersion unit (Malvern Hydro MV, UK). The background and sample integration times were 20 and 10 s, respectively. The optical properties were defined as refractive index 1.549 (olive oil) and 1.330 (dispersant water) and absorption index 0.001, with the normal instrument. In the sample port, the samples were dispersed in distilled water at 1500 rpm until an obscuration of 6–8% was obtained. All measurements were done in triplicate.

2.2.9. Determination of accelerated physical stability

The accelerated physical stability of the emulsions was evaluated by an analytical centrifugal analyzer (LUMisizer, L.U.M. GmbH, Berlin, Germany). In this study, 400 μL of non-diluted emulsions were loaded into rectangular cells whose transmission extinction profiles were recorded at 2000g, 23 °C for 20 h. Integral transmission of each profile was calculated by software SepView 4.1 (LUMisizer, L.U.M. GmbH, Berlin, Germany) and is shown as a function of time.

2.2.10. Rheological measurements at small deformation

Shear viscosity measurements of the emulsions using parallel plates ($d = 40$ mm) were carried out in a Discovery Hybrid Rheometer (DHR-2, TA Instruments, DE, USA). The crosslinked or non-crosslinked 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). For dynamic viscosity measurements, shear rate

was increased from 0 to 300 s^{-1} as a function of shear stress and viscosity was recorded.

Additionally, 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 0.01–100.0 Hz with a constant strain of 1% within the LVR region determined in advance.

2.2.11. Direct observation of emulsions

Cell Observer inverted microscope (Zeiss Axiovert 200, Jena, Germany) was utilized to study the microstructure of the emulsions. 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. Images were processed by the AxioVision (Zeiss) image analysis software for acquisition and image processing.

2.2.12. Experimental design and analysis

Assays were conducted in triplicate, and results were expressed at mean $\pm$ standard deviation. Statistical analyses were performed using Microsoft Excel 2013 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. Protein purification and characterization

Three main groups of potato proteins were clearly distinguished in the PP rich fraction: patatin, protease inhibitors and high molecular weight proteins (Fig. 1). The identity of the proteins was confirmed by comparison with two commercial standards and by proteomic analysis. Protease inhibitors are a wide, heterogenic group of seven different classes of proteins (Pouvreau et al., 2001). Serine protease inhibitor (PI-2) from the Kunitz family is the most abundant group in PFJ prepared from cultivar Elkana (Pouvreau, Gruppen, van Koningsveld, van den Broek, & Voragen, 2003). It is a dimeric protein, with two subunits (16.5 kDa and 4.5 kDa) (Valueva, Revina, Kladnitskaya, & Mosolov, 1998). It was assumed that the band observed on the SDS-PAGE gel (Fig. 1) with molecular mass of ~ 15 kDa is the larger subunit of PI-2.

Differences in molecular weight could be due to different cultivar, storage condition and developmental stage, as previously reported (Løkra et al., 2008; Løkra et al., 2009). Other abundant groups are potato cysteine protease inhibitors (PCPI), potato aspartate protease inhibitors (PAPI) and other Kunitz type protease inhibitors (PKPI), all of which are monomeric proteins (20–21 kDa) (Fig. 1) (Pouvreau et al., 2001).

According to elemental analysis, the PP rich fraction obtained by ammonium sulfate precipitation contained 62.6% protein. Similar results were obtained using Bradford protein analysis. The moisture content of the isolate was determined as $11.35\% \pm 1.31$ and polyphenolic content was determined as 1055 ± 78 mg GAE/L (2.11%). According to previous studies the expected lipid, saccharide and ash contents are in the range 3–13.6%, 0.6–6% and 1.2–6%, respectively (Løkra et al., 2008; Vikelouda & Kiosseoglou, 2004).

Various precipitation methods such as heat, acid, ethanol, ferric chloride and ammonium sulfate were previously studied to isolate potato proteins (Bartova & Barta, 2009; Knorr, Kohler, & Betschart, 1977; van Koningsveld et al., 2001; Waglay, Karboune, & Alli, 2014). The researchers reported a high protein yield and good solubility, with low purification factor when ammonium sulfate precipitation was used as a precipitating agent. PP is known to possess low solubility which may be a major limitation in their purification and application (Cheng et al., 2010; Santos et al., 2015; van Koningsveld et al., 2001; van Koningsveld et al., 2006). When simple tap water extraction was used, purification factor was low as 46% protein and solubility was poor (Ralet & Guéguen, 2000), while by ultrafiltration, 61% was obtained (Baier & Knorr, 2015). In comparison, the protein concentration of commercial samples 206P and 306P, purified by protein expanded bed chromatography method was 83.8% and 88.1%, respectively. Protein expanded bed chromatography is a more efficient process of PP extraction with expected purities ranging between 72 and 96% (Løkra et al., 2008; Løkra et al., 2009; Strætkvern & Schwarz, 2012). However, this method is used for an industrial scale and it is very costly.

3.2. TyrBm-catalyzed crosslinking of potato protein

Before applying tyrosinase on the complex emulsion system, its effect on native potato proteins was evaluated. TyrBm was incubated with PP solutions for different time periods. Preliminary trials were carried out in order to establish standard conditions. SDS-PAGE analysis demonstrated the formation of high-molecular weight fragments after 30 min from the enzyme addition, followed with the concomitant decline of the protein monomer bands (Fig. 2). Patatin is a poor substrate for TyrBm, as its band intensity remained constant with time, while protease inhibitors with molecular weight of ~20 kDa seems to be rapidly crosslinked by TyrBm, as shown by the decline in their band intensity as the reaction proceeded (Fig. 2). The crystal structure of patatin of *Solanum cardiophyllum* (PDB code 4PKA; 88% sequence identity with *S. tuberosum* patatin) revealed that most of the tyrosine residues are located in the core of the globular structure which can explain the low availability of those residues to the tyrosinase active site (Fig. S1A). In contrast, structures of *S. tuberosum* Kunitz-type cysteine protease inhibitor PCPI (PDB code 5DVH) and cathepsin D inhibitor (PDB code 5DZU) disclosed many tyrosine residues on the surface of the protein, positioning outward, suggesting they have higher probability to be crosslinked by TyrBm (Fig. S1B, C). Proteomics analysis of the protease inhibitors with molecular weight of ~20 kDa confirmed that indeed the most abundant fractions which were crosslinked by TyrBm belong to PCPI, cathepsin D inhibitor and other Kunitz-type protease inhibitors. Protease inhibitors crosslinking may influence PP digestibility and energy uptake (Liener, 1994; Vaz Patto et al., 2015). With the formation of protein aggregates by TyrBm-crosslinking, the inhibitors activity may be reduced, since the active site and binding domains could potentially be distorted or unavailable. Their gastric digestibility may also be decreased as seen in digestibility models of milk and peanut proteins after

tyrosinase-crosslinking (Radosavljevic et al., 2014; Stojadinovic, Pieters, Smit, & Velickovic, 2014).

3.3. Characterization of PP stabilized emulsions

PP-stabilized emulsions were incubated for 1 or 4 h with TyrBm (or buffer, for the non-crosslinked system) to evaluate the influence of the polymerization degree on emulsion properties. The crosslinked PP emulsions, incubated for 1 or 4 h, were stable for > 24 h, while the non-crosslinked emulsions showed phase separation shortly after incubation. TyrBm crosslinking of PP emulsions induced the formation of a highly dense and solid-like structure after 1 h of incubation (Fig. 3). It is presumed that TyrBm-crosslinked potato protease inhibitors contributed to the formation of a self-standing elastic gel-like structure, while the non-crosslinked emulsion remained in liquid state, regardless of incubation time (Fig. 3A). A similar effect of emulsion gel formation by enzymatic crosslinking was previously reported when applying transglutaminase to soy-stabilized emulsions (Dickinson & Yamamoto, 1996; Kang, Kim, Shin, Woo, & Moon, 2003; Lee, Choi, & Moon, 2006; Tang, Sun, & Foegeding, 2011).

3.3.1. Emulsion particle size distribution

The droplets diameter distribution of crosslinked and non-crosslinked emulsions incubated for 1 or 4 h are shown in Fig. 4. The non-crosslinked PP-stabilized emulsion showed a bimodal particle size distribution with a minor population at mean diameter of 2.13 μ m, and a major population with diameter of 12.7 μ m, after 1 h of incubation (Fig. 4). When incubation time was prolonged to 4 h the presence of the major population with the mean diameter of 11.2 μ m, was more evident, while the minor population did not change, indicating coalescence and flocculation in the emulsion, which are known as instability factors. The bimodal particle size distribution became trimodal as a result of TyrBm crosslinking of the PP emulsions (Fig. 4). After 1 h of TyrBm crosslinking, a new population with large particle size and mean diameter around 111 μ m was formed. As the crosslinking time was prolonged, this large population shifted towards a smaller particle size and its volume decreased. It is assumed that the large particles visible after 1 h incubation are the result of covalent bonds between droplets induced by TyrBm activity. The longer incubation of 4 h may have caused a structural disruption that led to decreased particle size.

3.3.2. Accelerated physical stability of crosslinked PP emulsions

Investigation of the creaming/sedimentation velocity of the emulsions using analytical centrifugation was carried out. Due to changes in the matrix structure, it was impossible to calculate time and space resolved transmission extinction profiles, since they are no longer practical for the new TyrBm crosslinked PP gel-like emulsion. Thus, integration transmission analysis was applied, clarification rates were calculated (Table S1) and photocentrifuge transmission profiles are presented in Fig. 5. The initial transmission (red colored lines) showed particles movement while the final transmission profile (green colored lines at the top of the graph) represents the cream layer of oil droplets. The colloid instability phenomena appearing as creaming or sedimentation (Lerche & Sobisch, 2011), was visible from the tubes after centrifugal analysis, as well as from transmission profiles (Fig. 5). The high density of red colored lines in the profiles indicate slower separation and allegedly higher stability, which can be seen in the non-crosslinked emulsions (Fig. 5A, C), while for crosslinked samples, profiles revealed progressive movement and fast particle separation and creaming (Fig. 5B, D). However, according to transmission profiles, thickness of the creaming layer was reduced in TyrBm-crosslinked PP stabilized emulsion compared to non-crosslinked emulsions (Fig. 5). Thus, the bigger gaps between red colored lines cannot represent extensive and progressive creaming, but could indicate on a new phenomenon in this specific emulsion system. The clarification rate was higher for crosslinked emulsions compared to the non-crosslinked

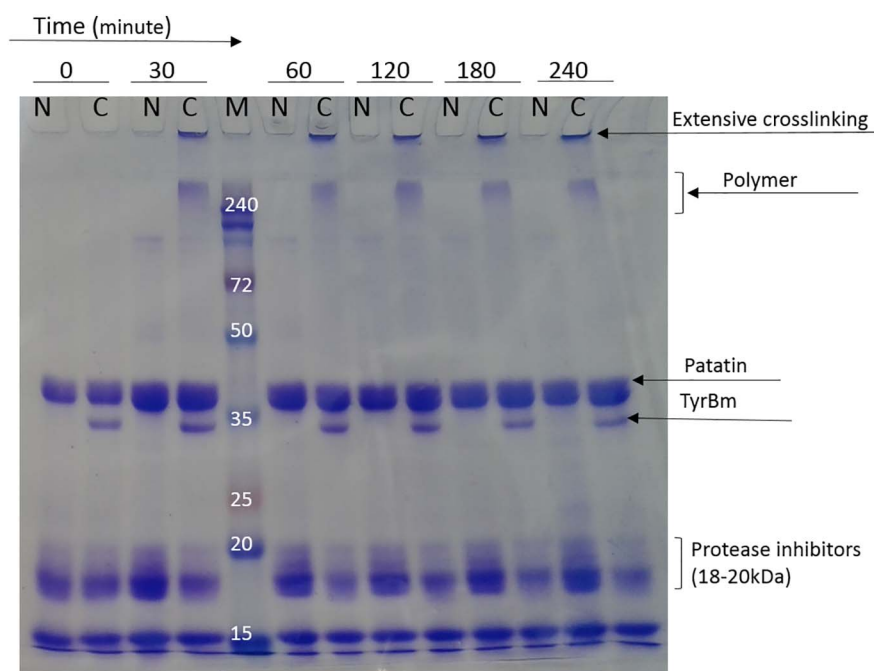


Fig. 2. SDS-PAGE visualization of non-crosslinked (N) and TyrBm-crosslinked potato protein rich fraction (C) during 240 min incubation at 37 °C. M indicates the molecular weight marker.

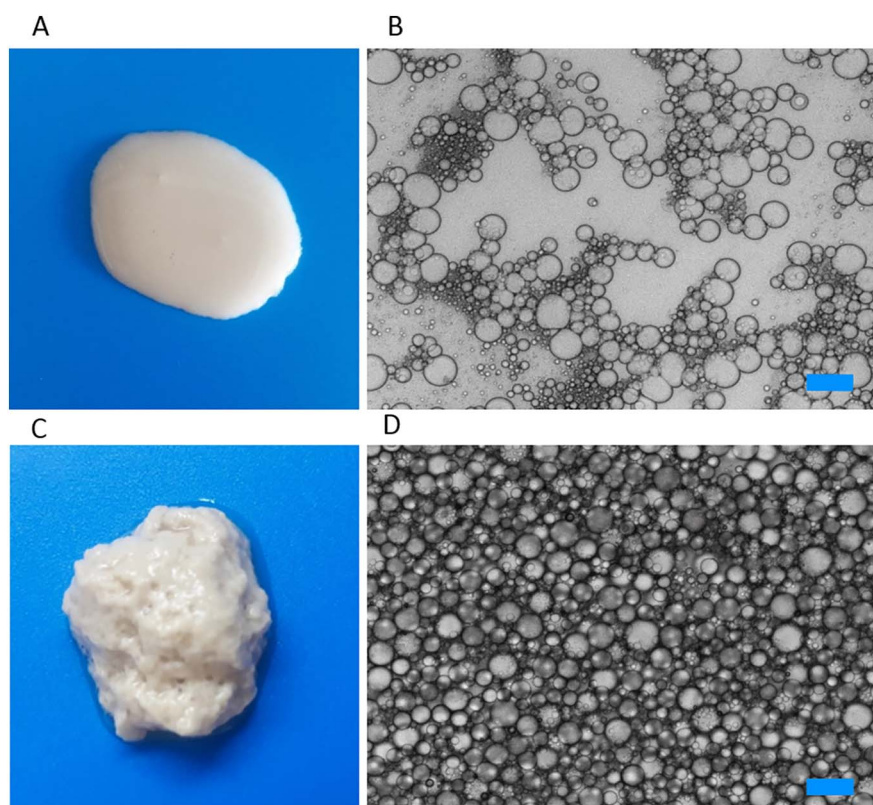


Fig. 3. Photographs and micrographs (20 × magnification) of non-crosslinked (A, B) and TyrBm-crosslinked PP stabilized o/w emulsion (C, D) for 1 h. Bars are 40 μm.

emulsion (Table S1). As the particle sizes of crosslinked emulsions are large ($r > 100$ nm), gravitational forces tend to dominate droplet movement (Piorkowski & McClements, 2014) resulting in sedimentation and creaming, followed by higher clarification rate (Table S1). It is believed that during the centrifugation, disruption of the large particles leads to the breakdown of the structure and not to phase separation. The vast phase separation, can be clearly observed from transmission profiles of the non-crosslinked emulsion after 1 h of incubation (Fig. 5A), while crosslinking with TyrBm led to decreased phase separation (Fig. 5B). As creaming or sedimentation were not completely

eliminated by tyrosinase crosslinking, more work is necessary for in-depth understanding and reducing of these instabilities in PP-stabilized emulsions.

3.3.3. Rheological properties of emulsions

The rheological behavior of crosslinked and non-crosslinked emulsions was evaluated by two small deformation tests. The effect of shear stress over shear rate imparted the viscosity values, while frequency sweep provided an indication to changes in storage modulus (G') and loss modulus (G'') which are indicative of gelation. The crosslinked and

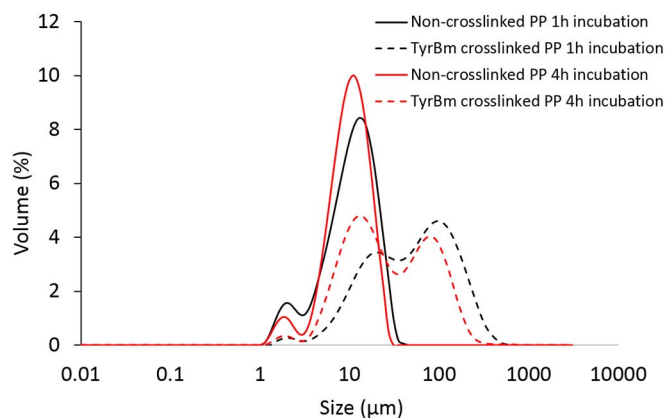


Fig. 4. Particle size distribution of oil droplets in the fresh non-crosslinked (solid line) and TyrBm-crosslinked PP stabilized o/w emulsion (dashed line) after 1 h (black) or 4 h (red) of crosslinking. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

non-crosslinked emulsions showed distinctly different rheological behavior over the shear-rate range tested. The flow curve for TyrBm-crosslinked PP emulsions, regardless of incubation time, exhibited pronounced shear-thinning behavior whereas that of non-crosslinked emulsions showed an approximate Newtonian behavior (Fig. 6). The shear thinning characteristic is most likely related to the formation of aggregates due to crosslinking of PP by TyrBm. Flow curves obtained for crosslinked emulsion fitted to the power law model ($R^2 > 0.998$), while for non-crosslinked emulsion the power law relationship was not distinct ($R^2 < 0.88$). Previously, shear thinning behavior has been observed for PP emulsions in combination with guar gum or chitosan (Calero et al., 2013; Santos et al., 2015) which are known as powerful thickening agents. The high initial viscosity of the crosslinked emulsion progressively decreased upon the increase in the shear rate (Fig. 6), probably due to de-flocculation of the droplets under shearing conditions (Liu & Tang, 2011), but remained significantly greater even at higher shear rate (200 s^{-1}) compared to the non-crosslinked emulsion (Table S2). A comparable phenomena was observed for soy protein gel-like emulsions induced by heat treatment or salt addition (Luo, Liu, & Tang, 2013; Tang & Liu, 2013). At shear rate of 300 s^{-1} , viscosity

of the crosslinked emulsion was 20-fold higher compared to the non-crosslinked emulsion, after 1 h of incubation (Fig. S2, Table S2). With the longer incubation time, the viscosity of the crosslinked emulsion, at the highest shear rate, was 10-fold higher in comparison with the non-crosslinked emulsion (Table S2). The crosslinked emulsion showed shear thinning behavior, thus a decrease in viscosity was expected due to longer incubation coupled with shaking and water loss, which cause disruption of crosslinked PP aggregates and aggregated oil droplet network. Contrary to the crosslinked emulsion, the non-crosslinked emulsion showed low viscosity, despite the fact that it is a concentrated emulsion. The flow curve of the non-crosslinked emulsion was characterized by a decrease in viscosity at higher shear rate (up to 50 s^{-1}) and subsequent stabilization in viscosity, which is common for Newtonian behavior (Fig. S2). In concentrated emulsions, the oil droplets are close enough to interact with each other and as a result, a network of aggregated oil droplets is formed (McClements, 2007). The difference in the interaction mechanism of the oil droplets between the cross-linked and non-crosslinked emulsions may be associated with different rheological behavior and morphology. Tyrosine crosslinking of PP mainly contributed to formation of protein-stabilized oil droplets and flocculated emulsion network followed with the characteristics of the gel like emulsion.

The frequency sweep test supported the result and demonstrated a clear change in the matrix rheological behavior due to the enzymatic reaction. As evident in Fig. 7, the crosslinked emulsions' storage modulus values (G') are initially higher than loss modulus (G'') (one order), and both moduli are frequency independent for a higher range of frequencies, presenting a formation of a crosslinked elastic gel network (Clark & Ross-Murphy, 1987). Crosslinked gels are strong and have permanent covalent networks and little frequency dependence, which was previously observed for glycinin gel crosslinked by TyrBm (Isaschar-Ovdat, Davidovich-Pinhas, & Fishman, 2016) or soy emulsions crosslinked by transglutaminase (Lee et al., 2006; Tang, Chen, & Foegeding, 2011). Storage modulus (G') was 1.5 order of magnitude higher compared to the non-crosslinked emulsion after 1 h of incubation, whereas after longer incubation time it was even more pronounced (two orders) (Fig. 7, Table S2). Thus, TyrBm-crosslinking of PP-stabilized emulsions seems to be a prerequisite to form an elastic gel-like structure. However, the emulsion gel rigidity was not found to depend very strongly on the time of crosslinking. In fact, prolonged

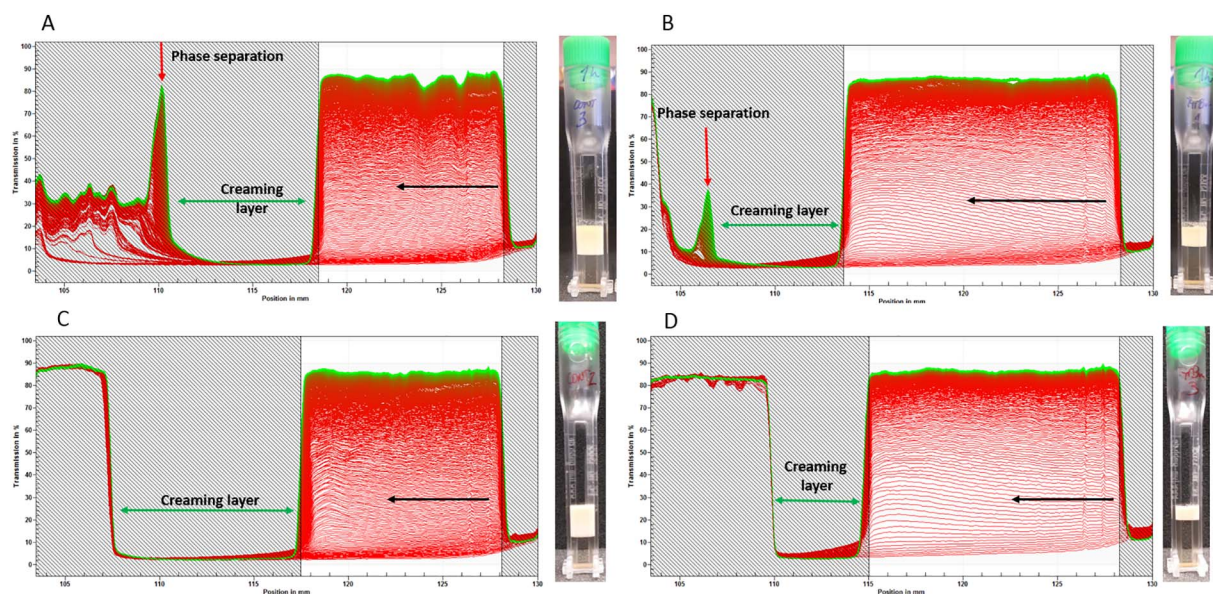


Fig. 5. Photocentrifuge transmission profiles as a function of length of sample cuvettes of the non-crosslinked (A, C) and TyrBm-crosslinked (B, D) PP-stabilized o/w emulsion that were crosslinked for 1 h (A, B) or 4 h (C, D). The color of the transmission profiles changed from red to green as the time of centrifugation progressed from 0 to 20 h, respectively. Black arrows indicate movement of the transmission profiles as a function of time. Green arrows indicate the creaming layer, while the red arrows indicate a peak that represents phase separation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

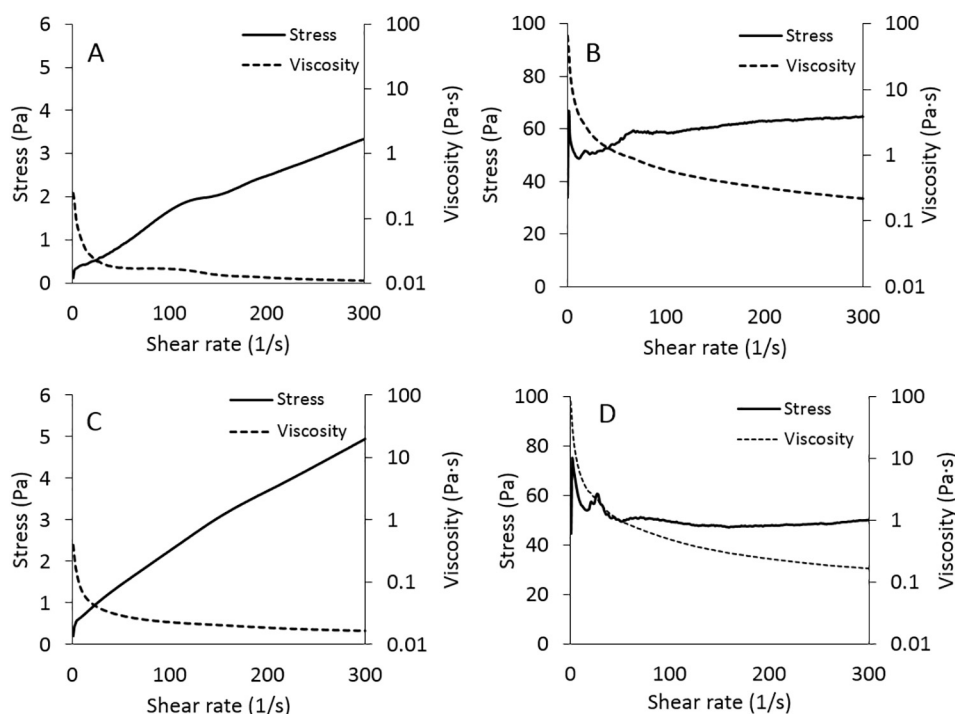


Fig. 6. Shear viscosity (dashed line) and stress (solid line) of non-crosslinked (A, C) and TyrBm-crosslinked PP stabilized o/w emulsion (B, D) that were cross-linked for 1 (A, B) or for 4 h (C, D).

crosslinking revealed comparable emulsion stability and characteristics. Similar values for G' were observed for PP (2 wt%) stabilized 40% o/w emulsion with the addition of 1 wt% of chitosan (Calero et al., 2013) or 0.5 wt% guar gum (Santos et al., 2015).

The non-crosslinked PP emulsions moduli (G' , G'') both presented a marked dependency on frequency, which contribute to the weaker gel strength and indicate typical behavior of weakly flocculated emulsions (Manoi & Rizvi, 2009). This corresponded to the early breakage of G' in the non-crosslinked emulsion (Fig. 7). Furthermore, from Table S2 it can be seen that the moduli of the non-crosslinked emulsion were significantly lower compared to TyrBm-crosslinked emulsions.

3.3.4. Microstructure of emulsions

The microstructure of the TyrBm-crosslinked or non-crosslinked PP-stabilized emulsions was characterized by a Cell Observer inverted microscope, as shown in Fig. 8. The micrographs clearly indicated that the emulsions exhibited remarkable differences as a result of the enzymatic activity. The micrographs of a non-crosslinked emulsion present severe flocculation and coalescence, regardless of incubation time (Fig. 8). As the micrographs were taken 3 h after the formation of the emulsions, these larger particles (flocs) were not observed by particle size measurements that were taken immediately after emulsion

formation. The micrograph of TyrBm-crosslinked PP-stabilized emulsions showed that all oil droplets were present in a closely associated form with a maximum droplet size of 40 μm (Fig. 8). This is characteristic of a particle-gel structure with protein-coated emulsion droplets comprising the primary structural units (Dickinson, 2012). However, the presence of large particles in the crosslinked emulsion (111 or 86.4 μm) previously detected by static light scattering (Fig. 4) can be explained by the formation of a network of aggregated emulsion droplets, which indicate an emulsion gel-like structure (Dickinson, 2012). The formation of a gelled emulsion can be largely attributed to enhanced inter-droplet interactions, particularly between tyrosinase crosslinked PP at the interface. Furthermore, introducing new covalent bonds within the PP fraction increased the connectivity or cohesiveness of aggregated oil droplets. These observations are consistent with the rheological data (Fig. 7), suggesting that increased viscosity and G' values might be due to aggregated oil droplets and crosslinked PP at the oil surface.

4. Conclusions

Gel-like emulsions can be formed by tyrosinase crosslinking of potato proteins. The formation of the gel-like network was largely

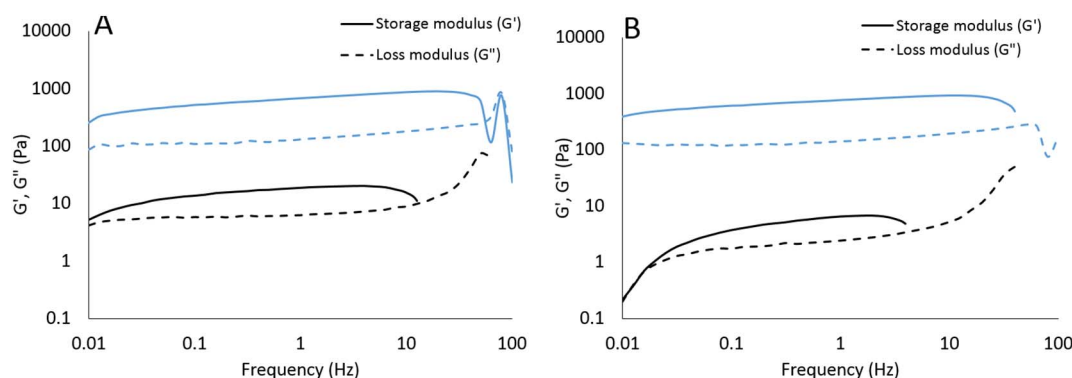


Fig. 7. Frequency sweep of non-crosslinked (black) and TyrBm-crosslinked PP stabilized o/w emulsion (blue) after 1 (A) or 4 h (B) of crosslinking. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

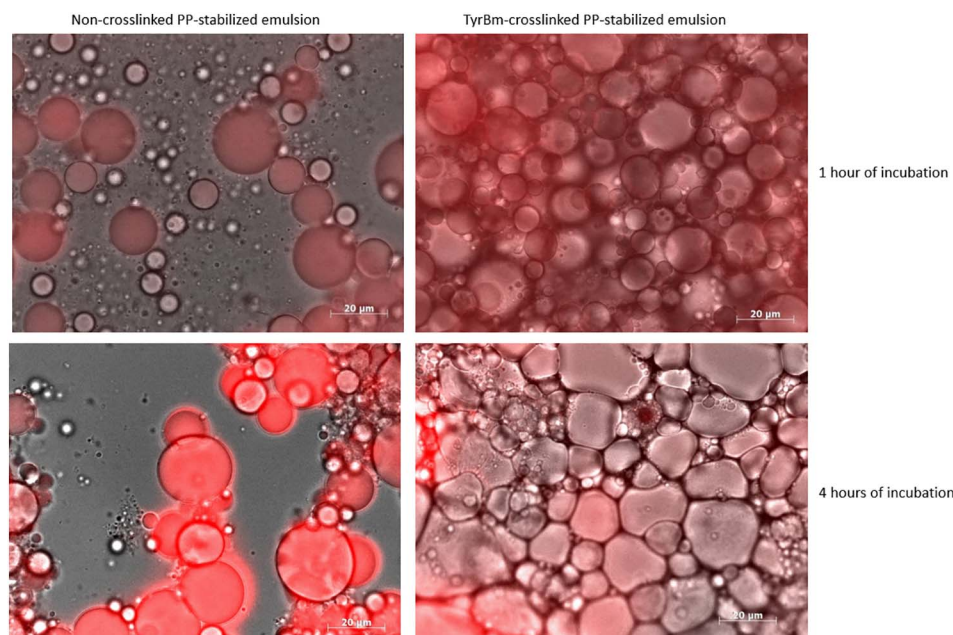


Fig. 8. Inverted micrographs of 3-h o/w emulsions stabilized with PP that were crosslinked for 1 or 4 h with TyrBm or not-crosslinked. Bars are 20 µm.

attributed to the introduction of new covalent bonds among PP polypeptide chains. The present data demonstrated that protease inhibitors, particularly Kunitz type protease inhibitors PCPI and cathepsin D inhibitor, crosslinked by TyrBm play a crucial role in the gel-like network formation of PP-stabilized emulsions. The crosslinked emulsion had a self-standing elastic gel-like structure and was characterized by large particles, increased viscosity and G' value, in comparison with the non-crosslinked PP emulsion. Micrographs of crosslinked PP-stabilized emulsions showed formation of an aggregated emulsion droplets network. While the phase separation mechanism remained similar, its rate decreased, indicating higher physical stability of the crosslinked emulsion. These findings expand our understanding of PP-stabilized emulsions and provide means for modifying them. The increase in matrix viscosity and gel-like behavior can potentially serve as the base for vegan and vegetarian PP based food products as spreads, creams and meat or cheese replacements.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.foodres.2017.07.034>.

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Supplementary file

Oil-in-Water Emulsions Stabilized by Tyrosinase-Crosslinked Potato Protein

Jovana Glusac, Sivan Isaschar-Ovdat, Biljana Kukavica and Ayelet Fishman

Table S1. Clarification rate (%/s) of non-crosslinked and TyrBm-crosslinked PP stabilized o/w emulsion after 1 or 4 hours of incubation. Values are the average of three experiments, and error bars represent the standard deviation of three separate runs.

	1 hour of incubation		4 hours of incubation	
	Non-crosslinked	Crosslinked	Non-crosslinked	Crosslinked
Rate in %/s	0.0152 ± 0.0017^a	0.0254 ± 0.0023^b	0.0140 ± 0.0003^a	0.0405 ± 0.0026^b

a, b: Different letters indicate significant differences at $p < 0.05$ according to t- test. Comparison was made between non-crosslinked and crosslinked sample for the same incubation hour.

Table S2. Shear viscosity at 200 s^{-1} (η_{200}) and at 300 s^{-1} (η_{300}) shear rate, storage modulus (G') and loss modulus (G'') (at 1 Hz) of non-crosslinked and TyrBm-crosslinked PP stabilized o/w emulsion after 1 or 4 hours of incubation. Values are the average of three experiments, and error bars represent the standard deviation of three separate runs.

	1 hour of incubation		4 hours of incubation	
	Non-crosslinked	Crosslinked	Non-crosslinked	Crosslinked
$\eta_{200} \text{ (Pa}\cdot\text{s)}$	0.011 ± 0.002^a	0.340 ± 0.039^b	0.019 ± 0.001^a	0.229 ± 0.019^b
$\eta_{300} \text{ (Pa}\cdot\text{s)}$	0.009 ± 0.002^a	0.231 ± 0.023^b	0.017 ± 0.001^a	0.162 ± 0.013^b
Storage modulus (Pa)	18.99 ± 4.59^a	677.94 ± 48.78^b	8.83 ± 3.22^a	830.60 ± 79.40^b
Loss modulus (Pa)	6.26 ± 2.01^a	131.25 ± 30.63^b	4.63 ± 3.11^a	164.50 ± 32.34^b

a, b: Different letters indicate significant differences at $p < 0.05$ according to t- test. Comparison was made between non-crosslinked and crosslinked sample for the same incubation hour.

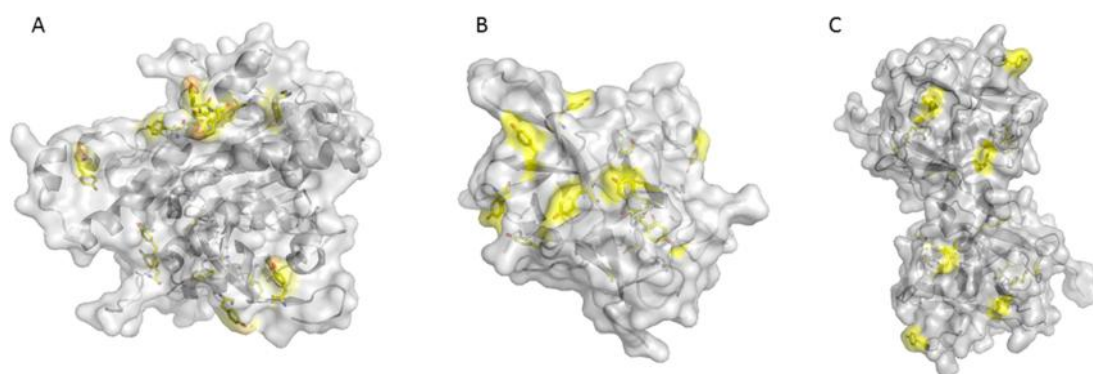


Fig. S1: Structures of (A) *Solanum cardiophyllum* patatin (PDB code 4PKA; 88% sequence identity with *S. tuberosum* patatin), (B) potato (*Solanum tuberosum*) Kunitz-type cysteine protease inhibitor PCPI-3 (PDB code 5DVH), (C) potato (*Solanum tuberosum*) cathepsin D inhibitor structure (PDB code 5DZU). The protein surface is in grey. Tyrosine residues are in yellow and the hydroxyl group is colored in red, while the amino group is colored in blue. All the structures were generated using PyMOL

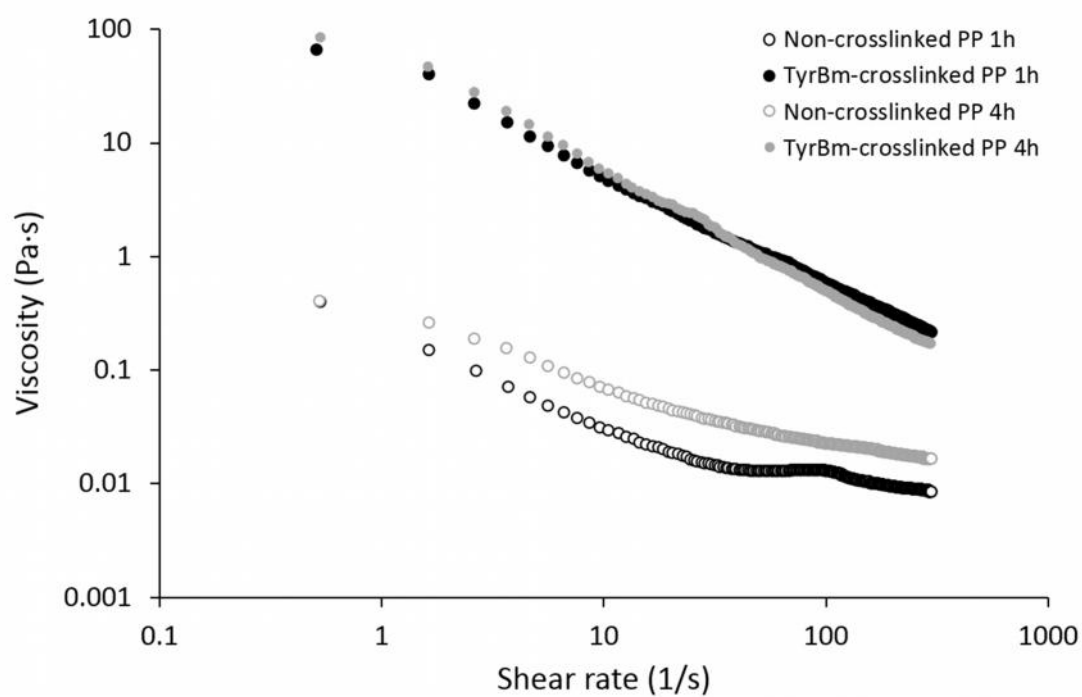


Figure S2. Changes in shear viscosity as a function of shear rate for the non-crosslinked and TyrBm-crosslinked PP stabilized o/w emulsion that were crosslinked for 1 or for 4 hours.