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Structuring colloidal oat and faba bean protein particles via enzymatic modification

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ABSTRACT

Oat and faba bean protein isolates were treated with transglutaminase from *Streptomyces mobaraensis* and tyrosinase from *Trichoderma reesei* to modify the colloidal properties of protein particles in order to improve their colloidal stability and foaming properties. Transglutaminase crosslinked faba bean protein extensively already with 10 nkat/g enzyme dosage. Oat protein was crosslinked to some extent with transglutaminase with higher dosages (100 and 1000 nkat/g). Transglutaminase increased the absolute zeta-potential values and reduced the particle size of oat protein particles. As a result, the colloidal stability and foaming properties were improved. Tyrosinase had limited crosslinking ability on both plant protein materials. Tyrosinase greatly reduced the solubility of oat protein despite limited crosslinking. Tyrosinase did not have effect on zeta-potential or colloidal stability of either protein, but it impaired foaming properties of both. Thus, the crosslinking enzymes studied caused significantly different end product functionality, presumably due to the different mechanism of action.

1. Introduction

Plant proteins possess high potential as sustainable protein source in the diet of next generations, but their poor solubility and technological functionality restrict their applicability in food products. In-solution behaviour of proteins is critical for their use in food applications, as homogenous dispersability is prerequisite for the stabilisation of interfaces in foams and emulsions, and formation of structures. Solubility and functionality usually goes hand in hand, but the assumption that molecular solubilisation is needed often leads in the necessity of hydrolysis treatment, where formation of peptides is associated with off-flavour formation considered as a serious drawback in exploitation of these proteins. Instead, structuring plant proteins into stable and functional colloidal protein particles may be pursued. As an example, water-insoluble colloidal particles from a corn zein protein have been shown to function as stabilisers in oil-in-water Pickering emulsions (de Folter, van Ruijven, & Velikov, 2012). Structuring the plant proteins by enzymatic crosslinking is an attractive option due to mild reaction conditions needed and the specificity of the enzymes. Enzymatic crosslinking has been studied in modifying textural and structural properties in protein-based food matrices such as gels (Ercili-Cura et al., 2010), emulsions (Mohamed, Biresaw, Xu, Hojilla-Evangelista, & Rayas-Duarte, 2009) and foams (Ma & Harwalkar, 1984; Mohamed et al., 2009; Partanen et al., 2009). Most widely studied and commercially available crosslinking enzyme today is transglutaminase (EC 2.3.2.13) that catalyzes acyl transfer reaction between a protein-bound glutamine residue (acyl donor) and an ε-amino group of a protein-bound lysine residue (acyl acceptor), leading to (γ-glutamyl)-lysine isopeptide linkages. In the absence of amines, water serves as acyl acceptor, leading to deamidation of glutamines into glutamic acid. The schemes of reactions catalyzed by transglutaminase can be followed for example from (Griffin, Casadio, & Bergamini, 2002; Kuraishi, Yamazaki, & Susa, 2001). Introduction of covalent crosslinks between/within protein molecules with transglutaminase, has been shown to alter the structure and stability of colloidal casein micelles in milk (Huppertz & de Kruif, 2008) and oat proteins (Ercili-Cura et al., 2015). Several oxidoreductases (oxidases, oxygenases, peroxidases) such as tyrosinases have also been studied in food matrices (Ercili-Cura et al., 2010; Selinheimo et al., 2006). Tyrosinase (EC 1.14.18.1) is an oxidoreductase catalysing

Abbreviations: BSA, bovine serum albumin; DSC, differential scanning calorimetry; FPI, faba bean protein isolate; L-DOPA, L-3,4-dihydroxyphenylalanine; OPI, oat protein isolate; SC-CO_2, supercritical carbon dioxide; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TG, transglutaminase; TrT, Trichoderma reesei tyrosinase; Z-GLN-GLY-OH, N-carbobenzoxy-L-glutaminylglycine.

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ortho-hydroxylation of monophenols to o-diphenols, which are further oxidized to o-quinones In proteins, phenolic ring of tyrosine residues serve as substrate to tyrosinase. The quinones formed in the oxidation reaction can further react non-enzymatically with other tyrosinyl, cysteinyl, lysyl or histidinyl moieties leading to formation of inter- and intra-molecular covalent crosslinks. Tyrosinase catalysed reactions on proteinaceous substrates can be followed from e.g. Kim and Uyama (2005), Monogioudi et al. (2009).

Oat (*Avena sativa* L.) and faba bean (*Vicia faba*) are potential sustainable protein sources with relatively good nutritional profile. Oat is a unique cereal due to its relatively high protein content and its distinct protein composition compared to other cereals. The salt-soluble globulins are the major protein fraction in oat grain, comprising about 70–80% of the total proteins (Robert, Nozzolillo, Cudjoe, & Altosaar, 1983). Oat globulins (12S) are similar to legume-like 11S globulin proteins in terms of the quaternary structure, molecular weight and amino acid sequence (Klose & Arendt, 2012; Shotwell, Afonso, Davies, Chesnut, & Larkins, 1988; Tandang-Silvas et al., 2010). The quaternary structure of a mature oat 12S protein is a hexamer with a molecular weight of 322 kDa consisting of six subunits (each 54 kDa) containing α and β-chains (reviewed by Klose & Arendt, 2012). The globulins are the major storage proteins also in faba bean (*Vicia Faba* L.) seeds, and they consist about 85% of total seed proteins (Müntz, Horstmann, & Schlesier, 1999). The globulins are synthetized in the endoplasmic reticulum (ER) and the signal peptide is cleavaged when proteins are translocated in the lumen of ER (Shewry, Napier, & Tatham, 1995; Tandang-Silvas et al., 2010). Vicilin (7S) and legumin (11S) are the major globulin proteins found in all *Vicia* species (Müntz et al., 1999). Vicilin and legumins do not have obvious sequence similarities but they form a quaternary structure and mature proteins have a trimeric or hexameric structure, respectively (reviewed by Peter R Shewry et al., 1995). The molecular mass of vicilins is between 150 and 200 kDa and their three subunits have masses each 40–70 kDa. Legumins have molecular mass 300–400 kDa and each subunit have mass 50–60 kDa and contains α and β-chains (Tandang-Silvas et al., 2010).

The main goal of this work was to study enzyme-aided formation of protein particles with good solution stability and superior functionality in terms of stabilising food colloids. TG-catalysed modification of the colloidal oat protein particles was recently shown to affect dispersion characteristics and the surface activity of the proteins (Ercili-Cura et al., 2015). It was shown that TG treatment resulted in formation of intermolecular covalent bonds but also affected the surface charge properties (zeta-potential) of oat protein particles indicating possible deamidation. The aim of the present study was to investigate the effects of transglutaminase (TG) from *Streptomyces mobaraensis* and tyrosinase from *Trichoderma reesei* (TrT) induced modifications on the physicochemical properties and foaming properties of colloidal oat and faba bean protein particles.

2. Materials and methods

2.1. Preparation of protein isolates

High protein fractions obtained by dry fractionation of faba bean (cv. Kontu; Profood Ltd, Vihanti, Finland) and supercritical carbon dioxide (SC-CO_2) extracted oat flour (Sibakov et al., 2011) were used for the production of faba bean protein isolate (FPI) and oat protein isolate (OPI). Proteins were isolated by alkaline extraction (OPI pH 10 and FPI pH 9), followed by an isoelectric precipitation (OPI pH 5.5 and FPI 4.5) (Fernández-Quintela, Macarulla, del Barrio, & Martinez, 1997; Ma & Harwalkar, 1984). The isolates were lyophilised (Christ Alpha 2-4, B. Braun Biotech International GmbH, Melsungen, Germany) and stored in desiccators at 4 °C.

2.2. Chemical analysis

Analyses of the protein isolates were done as follows: nitrogen content was determined using Kjeldahl autoanalyser (Foss Tecator Ab, Höganäs, Sweden), and total protein concentration was calculated using N × 6,25 as a conversion factor according to 90/496/EEC, total fibre was quantified using the enzymatic-gravimetric AOAC method 991.43 (AOAC, 1995), total lipid content was determined with gravimetric method after defatting the protein isolate with heptane extraction for five hour in a Soxhlet extractor (Büchi B-811, Labortechnik AG, Flawil, Switzerland), ash content was quantified gravimetrically after combustion at 550 °C for 23 h in a muffle furnace (model N11, Nabertherm GmbH, Lilienthal/Bremen, Germany), and moisture was determined by drying at 105 °C for 24 h. Duplicate measurements were performed for each analysis. The qualitative analysis of starch were done by dying the samples with Lugol solution and visualising with microscopy according to Andersson et al. (2011).

2.3. Enzymes and activity measurements

TG was obtained as a commercial preparate from Ajinomoto (Tokyo, Japan), and it was used after removal of maltodextrin (Lantto, Puolanne, Kalkkinen, Buchert, & Autio, 2005). TG activity was determined by the colorimetric hydroxymate method (Folk, 1970) using 0.03 M N-carbobenzoxy-L-glutaminylglycine (Z-GLN-GLY-OH, Sigma-Aldrich) as substrate at pH 6.0. TG activity on Z-GLN-GLY-OH substrate was 7000 nkat/ml.

TrT was earlier produced and purified at VTT as described by (Selinheimo et al., 2006). TrT activity was measured using 15 mM L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate at pH 7.0 (Gasparetti, Nordlund, Jänis, Buchert, & Kruus, 2012). The measured enzyme activity on L-DOPA was 600 nkat/ml.

2.4. Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed according to (Ercili-Cura et al., 2015). The peak temperatures and enthalpies of OPI and FPI were analysed by DSC equipment (Mettler Toledo DSC820, Dietikon, Switwerland) with liquid nitrogen cooling system. Samples (10 mg) were weighed in 100 µl stainless steel pans with excess water (75%). Samples were equilibrated for two hours and heated (0–150 °C) at a rate of 10 °C/min. Two measurements were performed for each sample.

2.5. Soluble protein content

Protein isolates were dispersed in water at a concentration of 10 mg/mL and stirred for 2 h at room temperature. For determination of soluble protein content as a function of pH, prepared protein suspension was divided to aliquots and the pHs were adjusted in a pH range 3–10 by using HCl or NaOH. After 30 min of magnetic stirring the suspensions were centrifuged at 10 000g × 15 min at 20 °C. The protein contents of the supernatants (dispersion of colloidal proteins, defined as the soluble fraction in this study) were analysed by Bio-Rad Lowry Assay kit after trichloroacetic acid (TCA) precipitation and quantified using Bovine serum albumin (BSA, #A8022, Sigma) as the protein standard. Soluble protein content in the enzyme-treated samples was analysed using the same centrifugation procedure at the end of the reactions.

2.6. Enzyme treatment

Protein isolates were dispersed in water at a concentration of 10 mg/mL and stirred for 2 h at room temperature. The enzymes TG or TrT were added to the protein suspensions at the dosages of 10, 100 and 1000 nkat/g protein. The protein suspensions were incubated for 20 h at 40 °C at 400 rpm in a water bath.

2.7. Zeta-potential and particle size

Protein suspensions were centrifuged (10 000$g \times$ 15 min at 20 °C) and the supernatant (dispersion of colloidal proteins) was used for the zeta-potential and the particle size analysis. Zeta-potential and the particle size distribution of the colloidal protein particles were measured using a Zetasizer nano ZS (Malvern Instruments, Malvern, UK). Disposable folded capillary cells and disposable cuvettes were used to measure the zeta-potential and the particle size, respectively.

2.8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Enzyme-treated protein suspensions were centrifuged (10 000$g \times$ 15 min at 20 °C), and the supernatants (stock solutions) were analysed by SDS-PAGE performed under reducing conditions. Precast 12 % Tris-glycine polyacrylamide gels (#567-1043, Bio-Rad, USA) with prestained SDS-PAGE standard (#161-0318, Bio-Rad, USA) were used. The gel was Coomassie stained to visualise the protein bands.

2.9. Dynamic surface tension at air-water interface

The surface tension at the air-water interface was measured using a force tensiometer (Sigma 701, Attension, Espoo, Finland) as described in (Ercili-Cura et al., 2015). The protein isolates (stock solutions, i.e. initial samples after centrifugation) were diluted to 0.1 mg/ml in 10 mM sodium phosphate buffer pH 7.2. The plate was wetted up to 6 mm, and the immersed height of the plate was 2 mm. The dynamic surface tension was measured at 10 s intervals for 2 h.

2.10. Foaming properties

Protein suspensions (10 mg/ml in water) were used to analyse foaming activity and foam stability. The samples were aerated with a laboratory homogenizer (Silent Crusher M, Heidolph Instruments GmbH, Schwabach, Germany) equipped with a 8 mm blade (type 8F) at 25 000 rpm for 5 min. Foam heights were measured immediately after aeration. The foaming activity was expressed as height of the fresh foams. The foam stability was followed 1 h, 2 h and 1 day after aeration. The protein suspensions were used as such for foaming, centrifugation to remove insoluble proteins was not performed as it was assumed that only the colloidal proteins will contribute to foam formation. The insoluble large protein particles were sedimented in time. The sample was withdrawn from the fresh foam with a disposable pipet and the pictures from fresh intact foams were taken by light microscopy (Olympus BX40F, Olympus optical Co, Ltd., Japan) to analyse foam structure.

2.11. Viscosity

Viscosity of the non-enzyme-treated and enzyme-treated (1000 nkat/g) OPI samples were determined with a stress controlled rheometer (AR-G2, TA instruments Ltd., Crawley, UK) equipped with a 40 mm parallel plate. Samples were taken from the OPI suspensions after sedimentation of the insoluble proteins. The liquid portion containing the colloidal proteins was placed on steel plate. Viscosity was measured at a shear rate from 0.1 to 100 s^{-1}.

3. Results

3.1. Characterization of protein isolates

OPI contained 92.1% protein, 2.3% lipids, 5.3% total dietary fibre and 2.4% ash (on dry weight basis). FPI showed a similar composition with 92.2% protein, 2.7% lipids, 5.8% total dietary fibre and 6.4% ash. The isolates did not contain starch as verified with the iodine staining followed by imaging with the light microscopy. The preservation of native state of the protein isolates were characterised by DSC analyses. The OPI and FPI showed single endothermic peaks at 111 °C and 94 °C, respectively (Supplementary data 1). The thermograms (associated with the denaturation of storage proteins) of OPI and FPI indicated that the proteins were in their native state after the alkaline extraction (Ercili-Cura et al., 2015; van Vliet, Martin, & Bos, 2002).

Protein solubility and zeta-potential were measured as a function of pH for aqueous dispersions of OPI and FPI. OPI showed bell shaped curve with solubility-minimum at around pH 5.5 typical for plant globulins (Fig. 1a). At this pH, the zeta-potential was zero. FPI showed minimum solubility at around pH 4.5. Oat proteins showed rather low solubility at neutral pH. Only 10–20% of oat proteins were soluble at around pH 7, whereas FPI had more than 65% of proteins soluble at pH 7 (Fig. 1b). Between pH 7 to 10, zeta-potential values ranged from −30 mV to −35 mV for both isolates. Also the solubility of proteins was markedly higher within this pH range (Fig. 1b).

3.2. Enzymatic crosslinking of protein isolates

The effect of TG and TrT on protein crosslinking was visualised by SDS-PAGE. Crosslinking, visible as high molecular weight aggregates, was intensified as TG dosage increased. Both acidic and basic polypeptides were prone to crosslinking (Fig. 2a). In TrT-catalysed reactions, crosslinking was most evident at dosages of 100 and 1000 nkat/g, and large polymers (>200 kDa) were observed at the highest enzyme dosage (Fig. 2a). In faba bean protein, α-legumin and vicilin were highly prone to crosslinking by TG, and even at the lowest dosage of 10 nkat/g, a clear decrease in intensities of both bands were observed. On the other hand, β-legumin was not prone to crosslinking (Fig. 2b). TrT did not efficiently crosslink faba proteins. Only the vicilin band at 70 kDa disappeared and a smear in the higher molecular weight area was observed at 1000 nkat/g (Fig. 2b).

3.3. Colloidal properties of protein particles

Size and zeta-potential of the protein particles after enzyme treatments were analysed. These measurements were performed on the soluble portion (remains in suspension at 10 000 g) of the initial suspensions, which was defined as the supernatant after centrifugation. The dispersions were stable and showed no sedimentation during the course of the measurements. The zeta-potential of protein dispersion controls incubated overnight at 40 °C without enzyme were −24 mV and −30 mV for OPI and FPI, respectively. TG treatment caused gradual increase in the absolute value of zeta-potential with increasing enzyme dosage in both protein dispersions (Table 1). The zeta-potentials for TG-treated (1000 nkat TG/g of protein) OPI and FPI samples were highly negative, −32 mV and −38 mV. TrT treatment did not have any effect on zeta-potential in the OPI or FPI dispersions (Table 1).

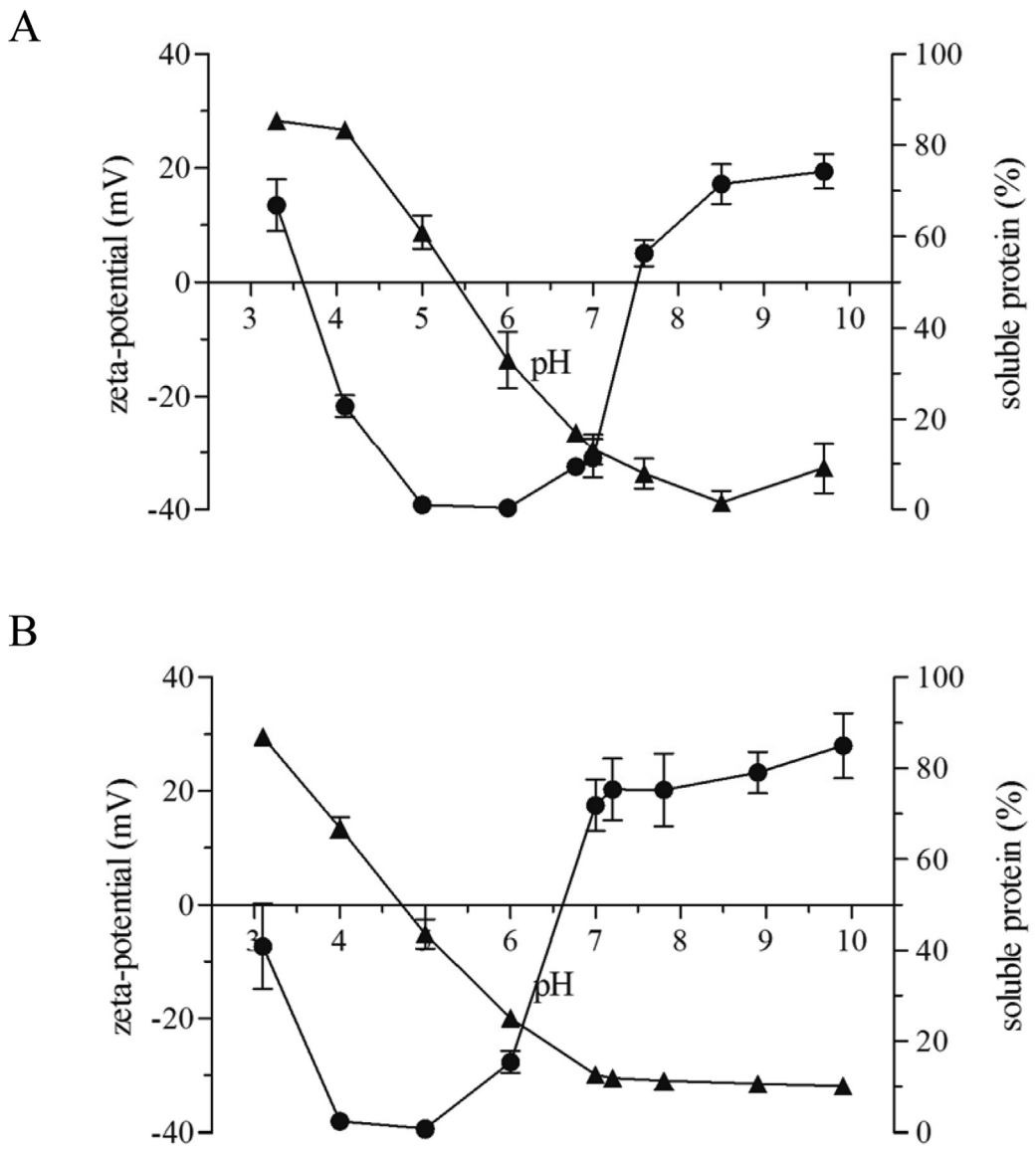

Fig. 1. Zeta-potential and protein-solubility of OPI and FPI at pH 3–10. Zeta-potential values (filled triangle) and the protein solubility (filled circle) for OPI (A) and FPI (B) dispersions.

In TrT treatment, pH decreased in both non-enzyme-treated and TrT-treated OPI samples during incubation from 7.2 to about 6.8. With the action of TG, the decrease in pH of OPI suspension was prevented. The pH of high dosage-TG treated OPI suspensions remained circa 7.2. The OPI solubility was increased from 16% to 19% after TG treatment (Table 1), however this subtle change was due to the pH difference between two solutions. TG-treatment decreased the solubility of FPI from 83 to 60%. TrT treatment decreased the solubility of OPI and FPI, from 16 to 6% and from 83 to 75%, respectively.

The particle size of oat and faba bean protein dispersions decreased as a result of both enzyme treatments (Table 1). The average of particle size of OPI decreased from 255 to 103 nm in TG-catalysed reaction (1000 nkat/g) and to 141 nm in TrT-catalysed reaction (1000 nkat/g). The particles size of FPI also decreased with enzyme treatment, from 145 to 52 nm and to 90 nm in TG- and TrT-catalysed reactions, respectively. FPI solutions were more polydispersed than OPI with polydispersity indices of 0.6 compared to 0.2 of OPI.

Despite a profound increase in absolute zeta-potential values upon TG-treatment of both OPI and FPI dispersions, no increase in soluble protein content was observed. However, TG treatments influenced the colloidal stability of OPI, and during one month storage at 4 °C TG-treated OPI dispersions were found to be stable against sedimentation, while the control sample without enzyme treatment did sediment heavily (Fig. 3). In the control sample, protein content of the supernatant decreased from 1.8 to 0.1 mg/ml during storage, while all protein remained in suspension in the sample treated with 1000 nkat TG/g. Faba bean proteins retained their solubility during storage even without enzyme, and no effect of enzyme treatment was observed.

3.4. Adsorption to air-water interface

Dynamic surface tensions at air-water interface were determined for OPI and FPI solutions before and after enzyme treatments. Untreated OPI dispersions had lower surface tension than FPI dispersions, 41 and 53 mN/m in equilibrium, respectively. Both

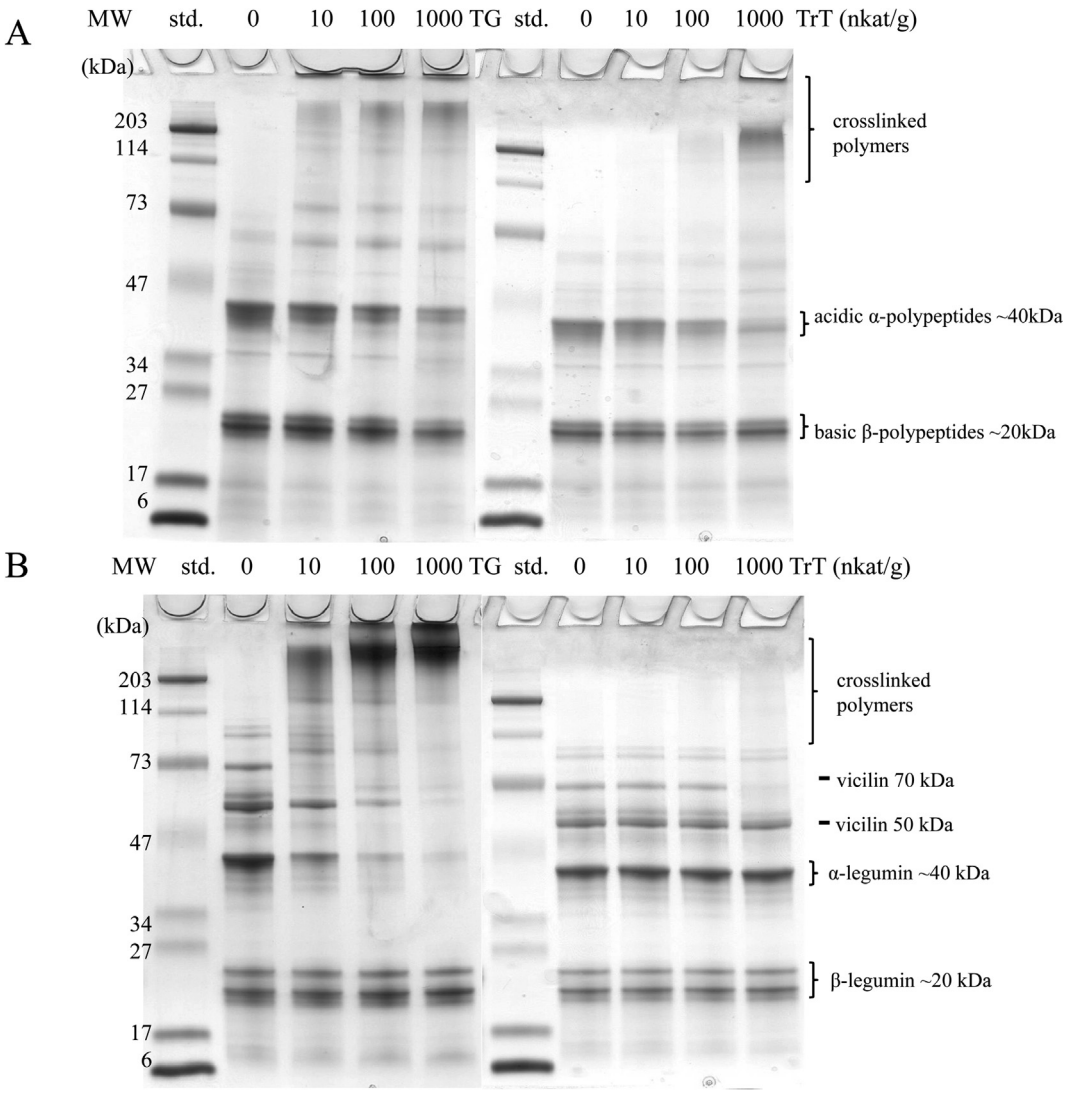

Fig. 2. Crosslinked polymers of OPI and FPI visualised by SDS-PAGE. SDS-PAGE under reducing conditions of OPI (A) and FPI (B) dispersions in water (pH 7.2) enzyme-treated with TG or TrT with dosages of 10–1000 nkat/g for 20 h at 40 °C. Soluble proteins after centrifugation at 10 000 g were enzyme-treated. The molecular weight markers, crosslinked polymers and protein subunits are marked to the figure.

Table 1
The zeta-potential, particle size distribution and protein solubility of TG- and TrT-treated oat and faba bean protein isolate solutions. Triplicate measurements were done, and the results are average of three consecutive readings.

Protein isolate	Enzyme	Enzyme dosage (nkat/g)	Zeta-potential (mV)	Soluble protein (%)	Particle size (nm)[a]	PdI[b]	pH
OPI		0	−24 ± 0.9	16 ± 3.6	255 ± 18.7	0.26 ± 0.03	6.8 ± 0
	TG	10	−27 ± 0.8	21 ± 3.7	135 ± 19.4	0.21 ± 0.05	6.9 ± 0.07
		100	−29 ± 1.5	19 ± 2.8	101 ± 4.7	0.21 ± 0.01	7.1 ± 0.07
		1000	−32 ± 0.7	19 ± 1.7	103 ± 4.6	0.26 ± 0.01	7.2 ± 0
	TrT	10	−23 ± 1.7	14 ± 3.9	242 ± 16.2	0.25 ± 0.01	6.8 ± 0
		100	−24 ± 0.6	9 ± 1.3	197 ± 10.5	0.23 ± 0.01	6.8 ± 0
		1000	−24 ± 0.3	6 ± 1.9	141 ± 11.1	0.22 ± 0.01	6.7 ± 0
FPI		0	−30 ± 0.3	83 ± 4.5	145 ± 7.7	0.56 ± 0.03	7.2 ± 0.07
	TG	10	−31 ± 1.2	77 ± 1.3	100 ± 10.7	0.61 ± 0.03	7.2 ± 0
		100	−36 ± 0.6	64 ± 7.8	64 ± 5.6	0.61 ± 0,02	7.3 ± 0.07
		1000	−38 ± 0.3	60 ± 2.3	52 ± 3.2	0.57 ± 0.02	7.3 ± 0
	TrT	10	−30 ± 0.3	83 ± 3.8	133 ± 9.8	0.58 ± 0.02	7.1 ± 0
		100	−29 ± 1.3	78 ± 4.3	106 ± 6.5	0.63 ± 0.04	7.1 ± 0
		1000	−29 ± 0.8	75 ± 0.2	90 ± 7.7	0.63 ± 0.02	7.0 ± 0

[a] Particle size distribution was determined by the Z-average size (the intensity weighted harmonic mean).
[b] PdI, polydispersity index

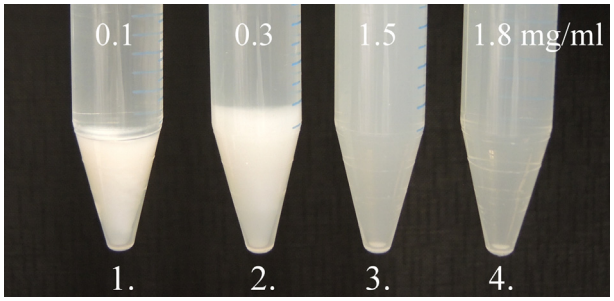

Fig. 3. The colloidal stability of TG-treated OPI. Photos of non-enzyme-treated (1.) and TG-treated OPI with 10 (2.), 100 (3.) and 1000 nkat/g (3.) enzyme dosages. The colloidal stability of TG-treated oat protein particles with 100 and 1000 nkat/g dosages was preserved after one month cold storage at +4 °C.

enzyme treatments (1000 nkat/g) slightly increased the surface tension values: TG or TrT-treated OPI had equilibrium value of 44 mN/m and FPI a value of 56 mN/m.

3.5. Foaming

Foaming properties of enzyme-treated samples were evaluated in terms of foaming activity, expressed as a height of fresh foam, and foam stability. Low protein concentration (1 %) was used in foaming experiments to eliminate viscosity effect on the colloidal stability, and viscosity was determined to be same in both, non-enzyme-treated and TG-treated, OPI dispersions. The viscosity of OPI control and TG-treated OPI (1000nkat/g) was 0.001 Pa s with 25 s^{-1} shear rate. TG treatment increased foam height of oat protein solutions, while TrT treatment reduced it (Fig. 4). TG treatment (1000 nkat/g) of OPI increased the foam height by 29 %, whereas TrT treatment (1000 nkat/g) decreased the foam height by 40 %. Untreated faba bean protein dispersion foamed poorly as the solution height of the FPI solution increased 18 % compared to initial height with aeration, while in the case of OPI it increased by 31%. The FPI foam was unstable and no foam remained after overnight storage. Enzyme treatments did not improve the foaming properties of FPI (Fig. 4). TG treatment (1000 nkat/g) of FPI decreased foam height by 14%, and TrT treatment (1000 nkat/g) decreased foam height by 5 % compared to control sample. The stability of OPI foams improved upon TG treatment (100 and 1000 nkat/g). TG–treated OPI sample (1000 nkat/g) had some foam left even after one day (stored at 23 °C). Light microscopic evaluation of fresh foams revealed a reduction in the bubble size between OPI and TG-treated OPI samples but no clear trend was seen according to the enzyme dosage, and the results were not conclusive (Fig. 5).

4. Discussion

Enzymatic modification and its effects on the colloidal properties and foaming properties of oat and faba bean protein particles were studied to create enzyme-treated protein particles with good solution stability and improved functionality in terms of stabilising food colloids.

4.1. Crosslinking of faba bean and oat proteins

Oat globulins were readily crosslinked by TG. Oat globulin polypeptides, acidic α-polypeptides and basic β-polypeptides, were both crosslinked to polymers as visualised by SDS-PAGE in reducing conditions. This is in line with the results from (Ercili-Cura et al., 2015). Faba bean proteins, vicilin and legumin, were also found to be highly prone to crosslinking by TG; with the highest studied TG dosage (1000 nkat/g), α-legumin and vicilins were almost completely crosslinked to higher oligomers while β-legumins stayed intact. Similar crosslinking behaviour in the case of legumins has been shown on soy proteins (Tang, Wu, Chen, & Yang, 2006), and it can be explained by the folding of the legumin proteins: hydrophilic α-legumins are exposed at the surface and more hydrophobic β-legumins are buried inside in the core (Plietz, Drescher, & Damaschun, 1987). Vicilins (7S) are more soluble than legumins (11S), which could explain their availability for crosslinking (Suchkov, Popello, Grinberg, & Tolstogusov, 1990).

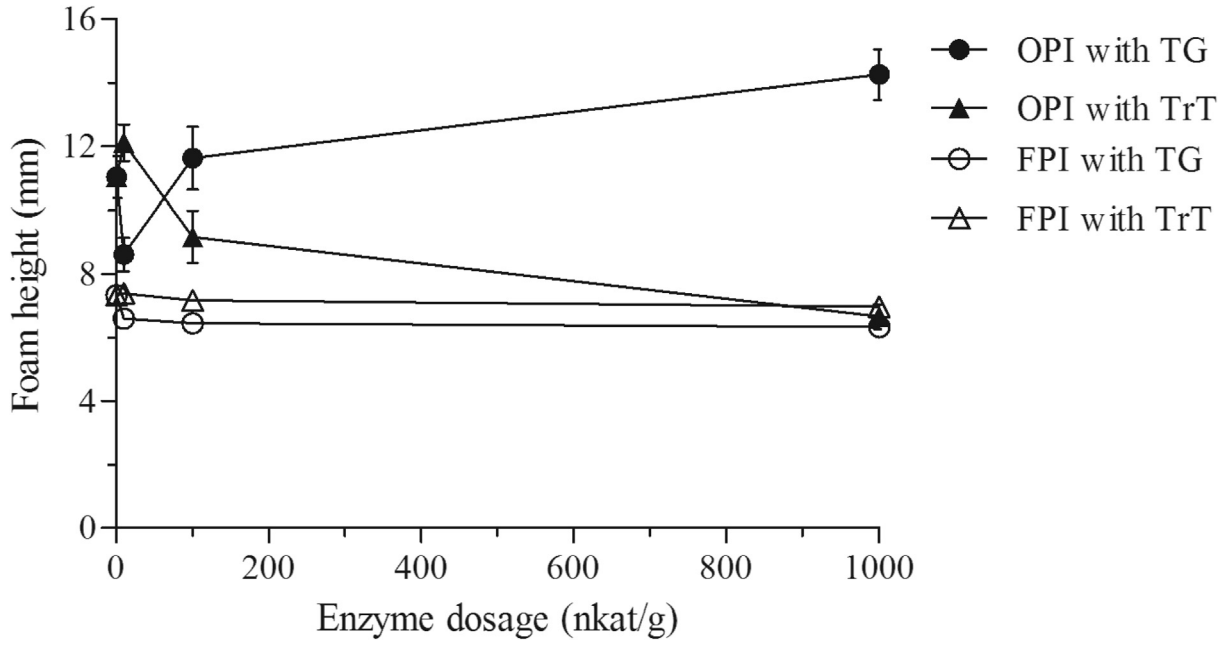

Fig. 4. Foaming activity of protein isolates. Foaming activity of OPI and FPI expressed as foam height versus enzyme (TG or TrT) dosage. The standard deviation was smaller than 10%.

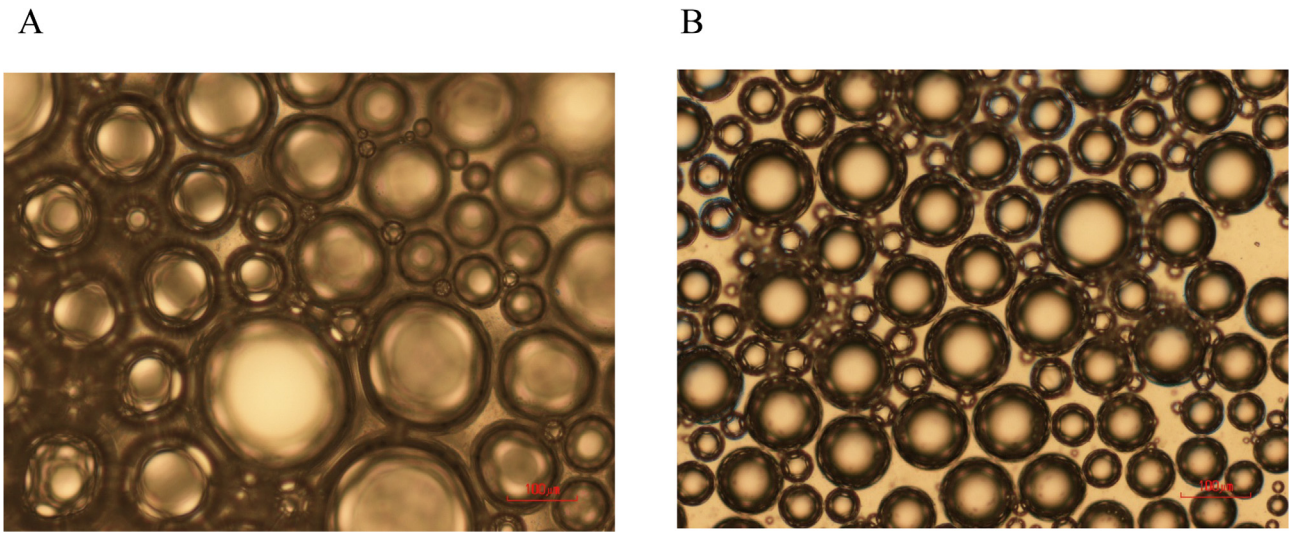

Fig. 5. Light microscope pictures of TG-treated OPI. OPI (A) and TG-treated (1000 nkat/g) OPI (B) in light microscope pictures. Ten time magnification was used when taking pictures, and the red scale bar (100 μm) is shown in pictures.

In contrast to TG, TrT had limited crosslinking ability, especially on faba bean proteins. The target amino acid residues of these two enzymes are different. TG forms enzyme/substrate complexation with protein-bound glutamine residues (acyl donor) to initiate acyl transfer and crosslink formation between glutamine and lysine residues (Griffin et al., 2002; Kuraishi et al., 2001), whereas TrT can oxidase protein-bound tyrosine residues which further reacts non-enzymatically with other tyrosines, lysines or cysteins (Buchert et al., 2010). Glutamines are more abundant than tyrosines in oat and faba bean storage proteins (oat globulin and faba bean legumin protein sequences in Swiss-Prot: P12615.1 and P05190.1, respectively). Thus, it could be expected that those proteins are better substrates for TG than for tyrosinase. However, not only the abundancy but also availability/accessibility of the target residues to the solvent/enzyme which is related to their position in the globular structure is crucial for enzymatic catalysis. Only limited tyrosyl succinylation has been observed in the tyrosine residues of faba bean legumin which implies that tyrosine residues were presumably structurally not available for TrT reaction (Schwenke, Knopfe, Mikheeva, & Grinberg, 1998). The steric hindrance in the folded globular protein structure affected also the TG reactions. FPI was extensively crosslinked by TG, but there were differences between the α- and β-chain crosslinking. In legumin polypeptide chains most of the lysine residues are located in exposed α-chain, although different legumin types A and B have alteration in the distribution of lysine residues. In α-A-chains 29% of lysines and in α-B-chains 23% are located in the flexible, unstructured C-terminus of α-chain (Schwenke et al., 1998). The β-chains contain also lysine residues but they are likely available only after unfolding of the globular structure (Schwenke et al., 1998).

The low activity of TrT on faba bean can partly be explained by contamination in the substrate co-passengers. The reaction of oxidative TrT enzyme can be inhibited by antioxidants like ascorbic acid (reviewed by Faccio, Kruus, Saloheimo, and Thöny-Meyer (2012)), and faba bean is especially rich in polyphenolic tannins that is reported to be responsible for the antioxidative properties of faba bean seeds (Sinha, Kumar, Kumar, Bharti, & Shahi, 2013). These compounds are however largely removed during the protein isolation process (Fernández-Quintela et al., 1997), but some residual antioxidants, such as tannins, might have been interfering TrT catalysed crosslinking in FPI samples.

Tyrosinase is involved in the melanogenesis and in the formation of mixed-melanins (Kobayashi et al., 1995). The colour of melanins varies from black to yellowish-brown eg. in case of eumelanins (Sánchez-Ferrer, Neptuno Rodríguez-López, García-Cánovas, & García-Carmona, 1995). The formation of pink colour was observed with the high tyrosinase dosages (100 and 1000 nkat/g protein) for both oat and faba bean dispersions. The colour formation due to TrT-induced protein crosslinking was previously attributed to the formation of localized conjugated double bonds between the aromatic rings of tyrosine-residues (Monogioudi et al., 2009).

4.2. Functional characteristics of the enzymatically treated proteins

TG-catalysed reactions increased absolute zeta-potential values of OPI which is in line with the previous report of Dilek Ercili-Cura et al., 2015. The effect was proposed to be caused by deamidation that occurred as a side reaction during TG treatment which was highly intensified at high enzyme dosage. Glutamine residues are more abundant compared to lysines in oat globulin and faba bean legumin protein structures. Additionally, oat has glutamine-rich repeats near the C-terminus of the acidic α-polypeptide (Shotwell et al., 1988) which may serve as a special domain for deamidation of glutamines to glutamic acid by TG catalysis. Zeta-potential gradually increased from −24 to −32 mV and from −30 to −38 mV for OPI and FPI, respectively, as a function of TG dosage. Despite observed increase in net negative charge, the amount of soluble protein in FPI suspension decreased from 83% to 60%, increasing with enzyme dosage. TG treatment did not largely change the protein solubility in OPI suspension. In non-enzyme-treated OPI solution pH decreased from 7.2 to 6.8 during overnight incubation at 40 °C, but in TG-treated OPI solution pH was preserved during incubation. Ammonia is generated during the TG-catalysed reactions, which may be the reason for pH stability in 1000 nkat TG/g OPI samples (Kashiwagi et al., 2002). TrT did not have any effect on zeta-potential of OPI or FPI solutions.

The particle sizes of OPI and FPI dispersions decreased as a function of TG and TrT dosage. TG treatment reducing particle size more than TrT treatment. OPI samples were not very polydisperse and the soluble protein content was similar between the untreated and TG-treated OPI samples. Intra-particle crosslinks were probably formed in TG-catalysed reactions, which prevented aggregation

and increased colloidal stability. In the case of FPI, decrease in particle size was observed but also the solubility decreased. Larger particles may have been formed due to inter-particle crosslinking, that were removed during centrifugation at 10 000 g. Only smaller, less crosslinked particles would have remained in the sample for particle size analysis. In a similar way particle size was reduced in TrT-treated samples but protein solubility was also decreased.

Despite no effect on protein solubility, colloidal stability of TG-treated OPI dispersion was greatly improved. The colloidal stability of TG-treated crosslinked oat protein dispersions was likely improved due to the stability against dissociation/re-association, which is in line with the previous results by (Ercili-Cura et al., 2015), where TG-treated OPI was stable against dissociation/re-association upon dilution. Dissociation/re-association leading to sedimentation was prevented both due to improved intra-particle integrity due to crosslinking and also due to altered surface charge properties. At the highest enzyme dosage (1000 nkat/g), zeta-potential was higher than −30 mV in absolute value, which may contribute to improved electrostatic stability of the colloidal protein dispersion leading to no sedimentation.

Foamability of OPI and FPI was found to be limited in those native conditions. Foaming of oat proteins was clearly superior to foaming of faba bean proteins as was observed by foam height and stability observed over time. The composition of impurities in the isolates were rather similar. Both isolates contained 5–6 % dietary fibre, most of which was insoluble fibre and around 2.5% fat which comprised of non-polar lipids (extracted with hexane). Despite the low concentration, both insoluble fibre and non-polar lipids may have had minor detrimental effects on the foaming properties of the protein isolates. In fact, Kaukonen et al. (2011) have previously shown impaired foam properties after addition of minor amounts of non-polar lipids to water extracts of SC-CO_2 extracted oat powder. Despite limited foamability, the effect of enzyme treatments was followed thoroughly. TG treatment increased foam height of OPI samples but TrT treatment decreased foam height greatly. This was possibly caused by a decrease in soluble protein that was greatly decreased by TrT treatment. The protein concentration should have been adjusted same prior foaming to deduce whether lowered foam height (decreasing with enzyme dosage) was due to crosslinked proteins with altered colloidal properties or merely due to reduced protein solubility caused by crosslinking. Faba bean proteins showed highly limited foamability, and enzyme treatment with TG or TrT even reduced foaming ability. Enzyme treatments with TG or TrT increased the dynamic surface tensions of OPI and FPI and impaired adsorption at the air-water interface. A similar effect of TG treatment on dynamic surface tension of oat proteins has been reported earlier (Ercili-Cura et al., 2015). The reason for decreased foaming ability in enzyme-treated FPI samples could be explained by lowered adsorption at air-water interface due to the reduced structural flexibility. For soluble proteins, like FPI at neutral pH, increased molecular weight hinders efficient adsorption to air-water interfaces (Partanen et al., 2009). The surface activity of proteins is highly related to structural flexibility. Crosslinking hinders that even further for soluble proteins (Krause, Dudek, & Schwenke, 2000; van Vliet et al., 2002).

5. Conclusions

This study aimed to improve the colloidal stability and foaming properties of oat and faba bean protein enzymatically. The colloidal stability and foaming properties of oat protein suspension were improved by TG treatment. The increased colloidal stability was likely due to improved electrostatic stability and intra-particle crosslinking causing stability against dissociation/re-association in oat protein particles. Faba bean proteins were intensively crosslinked by TG as visualized by SDS-PAGE and electrostatic stability was improved, but soluble protein concentration decreased, which implied to inter-particle crosslinking. The foaming properties of FPI decreased with increasing enzyme dosage. TrT crosslinked OPI and FPI to some extent, but did not improve foaming properties or have effect on colloidal stability. Proneness to crosslinking by TrT could be improved by partial denaturation or limited hydrolysis, that could loosen the structure of globular proteins. This has been shown for β-lactoglobulin (Partanen et al., 2011). FPI did not have the same tendency to sediment under quiescent conditions as OPI, and FPI had good colloidal stability even without an enzyme treatment. The colloidal stability of OPI was drastically improved with TG treatment. This could be exploited when developing protein-based foams that need long shelf-life at cold storage before use.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.03.114.

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