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The effect of heat and transglutaminase treatment on emulsifying and gelling properties of faba bean protein isolate

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ABSTRACT

The effects of heat treatment (90 °C, 5 or 30 min) and enzymatic crosslinking with transglutaminase (TG, 10, 100 or 1000 nkat/g protein) on emulsifying and gelling properties of faba bean protein isolate (FPI) were studied. Heat treatment of FPI caused a clear shift in far-UV CD spectra towards random coil structure and an increase in surface hydrophobicity from 181 to 504 RFU. TG crosslinked heat-treated FPI more efficiently as compared to native FPI. TG-induced crosslinking caused a reduction of surface hydrophobicity from 504 to 435 RFU. Emulsifying activity and stability indexes of FPI stabilized emulsions ranged between 25 and 30 m²/g and 18–35 min, respectively. Rheological properties of the FPI gels induced by heating, followed by acidification or TG treatment (10 or 100 nkat/g protein) were analyzed. FPI showed gel-like characteristics at 10% concentration ($G' = 180$ Pa). When the heat-treated FPI dispersion was further acidified or TG-treated (100 nkat/g), G' values of >6500 and > 3500 Pa were achieved, respectively. Water holding capacity of the FPI gels were >93% and >98% for acid- and TG-induced gels, respectively. The gel microstructures showed mainly heterogeneously-sized protein aggregates, however, no differences were observed between acid- and TG-induced gels.

1. Introduction

Faba beans (*Vicia faba* L.) are widely consumed worldwide as a rich source of plant protein and energy for humans and animals. The amino acid composition of *Vicia faba* proteins is similar to other grain legumes, i.e. regarded as nutritionally good except for being deficient in sulphur containing amino acids and tryptophane (Vioque, Alaiz, & Girón-Calle, 2012). Faba beans are a versatile crop, which can grow in varied climatic zones, and with its nitrogen fixing ability, like other legumes, it offers excellent agricultural practices for rotation and prevention of soil degradation (Multari, Stewart, & Russell, 2015). For the increasing need by the food industry in seeking alternative protein ingredients to meat, egg and dairy-derived proteins due to the high pressure they exert on the environment and sustainability of food chain, faba beans are potential sustainable protein source with a relatively good nutritional profile.

When compared to animal counterparts such as egg and dairy proteins, the use of plant protein ingredients (isolates and concentrates) is hindered mainly by the characteristic sensory attributes and the poor

techno-functional properties sourced by complex quaternary structure of plant proteins. Last few decades, tremendous amount of studies have focused on improving the nutritional, sensory and techno-functional properties of various plant proteins using approaches such as physico-chemical modifications (Mirmoghtadaie, Kadivar, & Shahedi, 2009; Schwenke, Knopfe, Seifert, Görnitz, & Zirwer, 2000; Yin, Tang, Wen, Yang, & Yuan, 2010), bioprocessing with enzymes (Eckert et al., 2019; Ercili-Cura et al., 2015; Jiang et al., 2015; C.; Liu, Bhattarai, Mikkonen, & Heinonen, 2019; Y.; Liu et al., 2011; Nisov, Ercili-Cura, & Nordlund, 2020; Nivala, Mäkinen, Kruus, Nordlund, & Ercili-Cura, 2017), fermentation (Coda et al., 2015; Rosa-Sibakov, Re, Karsma, Laitila, & Nordlund, 2018; Sözer et al., 2019; Xu et al., 2019), germination (Hsu, Leung, Morad, Finney, & Leung, 1982) or breeding strategies (Le, Chu, & Le, 2016; Wenefrida, Utomo, & Linscombe, 2013). All different approaches deliver valuable insight and lead the way for wider use of plant protein ingredients in the food industry as today technologies allow production of isolates and concentrates for industrial food production, and thus, the number of ingredients on the market with different grades

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; BSA, bovine serum albumin; CD, circular dichroism; CLSM, confocal laser scanning microscopy; EAI, emulsion activity index; ESI, emulsion stability index; FPI, faba bean protein isolate; GDL, D-glucono-δ-lactone; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TG, transglutaminase; UV, ultraviolet; WHC, water holding capacity.

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(from soluble to texturized) is increasing rapidly.

Faba bean seed storage proteins mainly include globulins (7S vicilin-type and 11S legumin-type) and albumins (enzymes, trypsin inhibitors and phytolectins) followed by minor proportions of prolamins and glutelins (Shewry, Napier, & Tatham, 1995; Tandang-Silvas et al., 2010). Generally, globulins comprise about 70–85% of the total seed proteins in *Vicia* species. As in other legumes, *Vicia faba* globulins are oligomeric proteins; hexameric 11S globulins comprise of subunits of 50–60 kDa (each contain acidic and basic polypeptides, ~40 and ~20 kDa, respectively) whereas trimeric 7S globulins comprise of 40–70 kDa subunits. A third type of globulin, convicilin, is also present in *Vicia* species. Convicilin is a 7S globulin which shows different amino acid profile but similar immunological properties to 7S vicilin (reviewed in Multari et al., 2015; Singhal, Can Karaca, Tyler, & Nickerson, 2016). The protein isolates from oil seeds and grain legumes mostly contain 11S (legumin-type) globulins (Schwenke et al., 2000).

Techno-functional properties such as emulsifying, foaming and gelling of faba bean protein isolate (FPI) have been investigated in multiple studies. Can Karaca, Low, and Nickerson (2011) reported that FPI prepared by isoelectric precipitation showed higher amount of soluble protein than that of pea protein isolate at neutral pH, 90% and 61%, relatively. Accordingly, FPI showed better emulsifying properties compared to pea protein isolate (Can Karaca et al., 2011; Tsoukala, Papalamprou, Makri, Doxastakis, & Braudo, 2006). FPI has also shown better water and oil absorption capacity than pea and soy protein isolates however, foaming capacity and stability were found to be poorer than that of pea and soy proteins (Fernández-Quintela, Macarulla, del Barrio, & Martínez, 1997). Nivala et al. (2017) reported inferior foaming properties of FPI compared with oat protein isolate despite higher solubility was shown for FPI at neutral pH. Thus far, there are only few studies reported about the gelation properties of FPI, although gel formation of proteins is a crucial property critical for structuring of many food products including bakery, dairy and meat substitutes. Fernández-Quintela et al. (1997) reported a lower least gelling concentration for FPI (prepared by isoelectric precipitation) compared to that of pea and soy protein isolates. In another study, Cai, Klamczynska, and Baik (2001) reported that faba bean proteins formed bean curds (2.3–3% protein, CaSO₄-coagulated) with comparable textural properties to soy proteins. Recently, Langton et al. (2020) compared two protein extraction methods; water and alkaline extraction on the heat-induced gelation of FPI at acidic and neutral pH. The extraction method did not affect gelation properties while pH of the dispersion during heat treatment led to formation of particulate or fine gel structures at pH 5 and pH 7, respectively.

Enzymatic modification of proteins is effectively used in food industry for improving nutritional and technological properties of protein ingredients. Mild enzymatic hydrolysis for example, has been shown to impact surface active properties of faba bean proteins (Eckert et al., 2019; Liu et al., 2019a; Tsoukala et al., 2006). Enzymatic protein crosslinking has also been elucidated, however in fewer publications. Krause, Dudek, and Schwenke (2000) reported about foaming and emulsifying properties of internally crosslinked faba bean legumin by dimethylsuberimidate and reported that although surface hydrophobicity was increased the foaming and emulsifying properties were impaired likely due to the reduced molecular flexibility. Recently, C. Liu, Damodaran, and Heinonen (2019) reported the emulsifying properties of TG-treated FPI and the oxidative stability of the emulsions. In our previous study, we reported enzymatic crosslinking of faba bean proteins by transglutaminase (EC 2.3.2.13) and tyrosinase (EC 1.14.18.1) with subsequent effects on FPI solubility, dispersion stability and foaming properties (Nivala et al., 2017). When we previously focused on exploring the functionality of native state FPI, in the current study, we aimed to explore the effects of partial protein denaturation on the efficiency of crosslinking catalysed by transglutaminase, and how it effects emulsifying and gelling properties of FPI.

2. Materials and methods

2.1. Materials

FPI was prepared from a protein rich (51.5%) faba bean fraction obtained by dry fractionation of faba bean flour as described in Coda et al. (2015). The FPI was prepared by alkaline extraction (pH 9.0) followed by isoelectric precipitation (pH 4.5), neutralization and freeze drying as described in Nivala et al. (2017). Moisture content of FPI was analyzed by oven drying (105 °C, overnight). Protein content was analyzed based on the total nitrogen content quantified using a Kjeldahl autoanalyser (Foss Tecator Ab, Höganäs, Sweden) according to the AOAC method 2001.11 (N x 6.25) (AOAC, 2000).

A commercial *Streptomyces mobaraensis* transglutaminase (TG) prepartate Activa® WM (Ajinomoto, Tokyo, Japan) was used after removal of maltodextrin (Lantto, Puolanne, Kalkkinen, Buchert, & Autio, 2005). TG activity was determined based on the colorimetric hydroxamate method as described in Nivala et al. (2017). The enzyme activity, assayed on 0.03 mol/L N-carbobenzoxy-L-glutaminyglycine, was 8000 nkat/mL.

2.2. Sample preparation: colloidal protein fraction

FPI was dispersed in water at a concentration of 20 mg/mL by magnetic stirring (2 h at 40 °C). Sodium azide (0.01% w/v) was added to prevent microbial growth. The suspension was then centrifuged (10 000 g, 15 min, 20 °C) to obtain a stable colloidal dispersion. The protein content in the supernatant (colloidal fraction) was analyzed by Bio-Rad DC assay kit as described in (Nivala et al., 2017). The colloidal FPI fraction was heat-treated for 30 min at 90 °C (in sealed tubes placed in water bath) and cooled rapidly to 40 °C. The heat-treated fraction was TG-treated at dosage of 1000 nkat/g protein by incubating (2 h at 40 °C) in a water bath, under magnetic stirring. Native, heat-treated and/or TG-treated colloidal fractions were used in the following analysis (sections 2.3–2.6). The amount of soluble protein was re-analyzed from the colloidal protein fraction after subjecting to heat and/or TG treatments as described in (Nivala et al., 2017).

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

The molecular weight (MW) distribution of the FPI samples with and without TG treatment were analyzed by SDS-PAGE performed under reducing conditions according to (Laemmli, 1970). Precast 4–20% Criterion TGX Stain-Free gels (#567–8093, Bio-Rad, USA) with pre-stained SDS-PAGE standard (#161–0363, Bio-Rad, USA). The bands were visualized using a Criterion Stain Free™ Gel Imaging System (Bio-Rad, USA).

2.4. Circular dichroism (CD) analysis

The protein secondary structural motifs of native and heat-treated FPI samples were analyzed by CD (Aviv 62A DS CD spectrometer, Aviv Biomedical Inc., Lakewood, USA) at 25 °C using a quartz cuvette with a 1 mm path length. The FPI samples were diluted to 0.2 mg/mL in 20 mmol/L sodium phosphate buffer (pH 7.2). The sample solution was clear and no precipitation occurred. The CD spectra were determined at far-ultraviolet (far-UV) regions (190–260 nm). The buffer baseline was subtracted. Three measurements were performed for each sample.

2.5. Surface hydrophobicity

The surface hydrophobicity of FPI samples was determined using 8-anilino-1-naphthalenesulfonic acid (ANS, #10417, Sigma, USA) as a fluorescent probe. The heat- and TG-treated FPI samples were diluted to a range of concentrations from 0.25 to 1 mg/mL in 50 mmol/L sodium

phosphate buffer pH 7.2. Samples were mixed with ANS directly in black polystyrene 96-well microplate wells. The fluorescence intensity was measured with a fluorospectrophotometer (Varioskan, Thermo Fisher Scientific, Germany) with the excitation and emission wavelengths of 390 and 470 nm, respectively. The slope of the curve of the fluorescence intensity vs protein concentration (%) was used as an index of average sample surface hydrophobicity and reported in relative fluorescence units (RFU).

2.6. Emulsion properties

Emulsions were prepared by diluting the FPI samples to a concentration of 10 mg/mL in Milli-Q water. FPI protein dispersions and sunflower oil were mixed at a volume ratio of 4:1 (water:oil) where 4 mL of the protein dispersion was added to 1 mL of oil. The water:oil mixes were emulsified using a laboratory homogenizer (Silent Crusher M, Heidolph Instruments GmbH, Schwabach, Germany) equipped with a 8 mm blade for 1 min at 26 000 rpm. The emulsifying properties were determined using a method based on the turbidity change in the emulsion lower phase (Pearce & Kinsella, 1978). An emulsion aliquot (12.5 μ L) was taken from the bottom of the tube immediately after homogenization and diluted in 0.1% SDS solution (5 mL). The same process was repeated 10 min after homogenization. The optical density of the samples in SDS solution was determined by measuring absorbance at 500 nm using plastic cuvettes with 1 cm path length. The emulsion activity index (EAI) and the emulsion stability index (ESI) were calculated according to equations (1) and (2):

$$\text{EAI (m}^2/\text{g)} = \frac{2 \times 2.303 \times A_0 \times \text{Dilution factor}}{1 \times c \times \Phi \times 10000} \quad (1)$$

$$\text{ESI (min)} = \frac{10 \times A_0}{(A_0 - A_{10})} \quad (2)$$

where dilution factor is 400, l is the path length (cm), c is the weight of protein per unit volume (g/mL) and Φ is the oil volumetric fraction (0.20), A_0 and A_{10} are absorbance values at time 0 and 10 min, respectively (Can Karaca et al., 2011; Pearce & Kinsella, 1978). The authors recognize the fact that the EAI formula holds the error of using Φ instead of $(1 - \Phi)$ in the denominator despite the recognized correction done by Cameron, Weber, Idziak, Neufeld, and Cooper (1991) on the original equation formulated by Pearce and Kinsella (1978). We used the original formula to be able to compare the results with other studies as the original formula is more widely used in the areal literature.

2.7. Gel formation: small deformation oscillatory measurements

2.7.1. Heat-induced gelation

Heat-induced gelation analysis was performed by using the whole FPI protein suspension (i.e. soluble and insoluble proteins, no centrifugation step), and gelation was followed by small deformation oscillatory measurements. The FPI sample was dispersed in water at concentrations of 10 or 15% (w/w) by magnetic stirring (2 h at 40 °C). Sodium azide (0.01%) was added to prevent microbial growth. A heating and cooling cycle was performed in a stress-controlled rheometer (DHR-2, TA-Instruments, Crawley, UK) equipped with a Peltier plate and a plate-plate ($\varnothing$ 40 mm) geometry with 1 mm measurement gap. A solvent trap with vegetable oil was used to prevent drying. An aliquot (1.4 mL) of the FPI suspension was placed within the geometry and subjected to a temperature ramp (25–90 °C, at a constant rate of 2 °C min⁻¹), holding for 5 min at 90 °C and cooling back to 25 °C at a constant rate of 2 °C min⁻¹. The temperature ramp was followed by 15 min conditioning at 25 °C. A constant frequency of 0.1 Hz and a constant oscillatory strain of 0.1% were used, which were determined to be in the linear viscoelastic region (LVR) of the final gels.

2.7.2. Acid and enzyme-induced gel formation

The FPI sample was dispersed in water (10% w/w) by magnetic stirring for 2 h at 40 °C in order to obtain a stable dispersion. Sodium azide (0.01%) was added. A pre-heat treatment was performed at 90 °C for 30 min after which the samples were tempered quickly to 40 °C in an ice bath. The acid-induced protein gels were prepared by addition of 1% (w/w) D-glucono- δ -lactone (GDL, Sigma, St. Louis, USA), stirring for 2 min and incubation (40 °C, 3 h), until final pH of around 5.0. The temperature of 40 °C was selected in order to mimic fermentation using thermophilic starter cultures. The enzyme-induced gelation was achieved by addition of TG to the heat-treated FPI dispersion at the dosages of 10 or 100 nkat/g protein and mixing shortly with magnetic stirrer followed by incubation (40 °C, 3 h). Gel formation and final gel properties were analyzed by dynamic oscillatory measurements using the same rheometer and geometry explained in section 2.7.1. After mixing of GDL or TG, an aliquot of the samples was transferred to the rheometer and a time sweep measurement was performed for 3 h at 40 °C using a constant strain (0.1%) and a constant frequency (0.1 Hz), which were determined to be in LVR. Time sweep was followed by a frequency sweep (0.01–10 Hz, 0.1% strain) and a strain sweep (0.01–100%, 0.1 Hz frequency). pH of the samples during gel formation was followed using a pH meter equipped with a data logger.

2.7.3. Water holding capacity (WHC) and spontaneous syneresis of gels

For the WHC analysis, the GDL or TG-induced gels were formed in the Eppendorf tubes where 1 g of the sample was weighed and incubated (40 °C, 3 h). The samples were incubated overnight at 4 °C before the analysis. The tubes were centrifuged (3000 g, 15 min, 6 °C), the supernatants were removed carefully, and the pellets were weighed. The WHC was determined as a ratio of the remaining pellet weight to the initial weight of the gel and expressed as percentage. The spontaneous syneresis was determined by forming the gels in sealed glass bottles (around 2 g of sample) and weighing the water released on top of the gel after overnight storage at 4 °C. Triplicate analyses were performed.

2.7.4. Confocal laser scanning microscopy (CLSM)

Microstructures of the gels were visualized by CLSM (Bio-Rad, Hemel Hempstead Herts, UK) equipped with argon laser 488 nm/He–Ne laser 543 nm; emission filter E50LP. For preparing the FPI samples for microscopy, GDL (for the acid-induced gels) or TG (for the enzyme-induced gels) was added to FPI suspensions, mixed for 2 min, after which 1 mL of the FPI samples and 10 μ L of the fluorescent dye Rhodamin B aqueous solution (0.1% w/w) were mixed. The stained FPI sample (300 μ L) was pipetted onto microscopy slide with Press-to-Seal silicone isolator with adhesive surface (20 mm diameter and 1 mm deep, Molecular Probes, Eugene, USA), covered with cover glass and sealed with the nail polish to prevent drying. The samples on the slide were incubated at 40 °C, 3 h for gel formation and stored at 4 °C overnight before CLSM. The micrographs were acquired at 512 $\times$ 512 pixel resolution.

3. Results and discussion

3.1. Protein denaturation and solubility

The FPI suspension (2% protein w/w) had a native pH of 7.3. The amount of soluble protein (colloidal protein fraction, derived after dispersing for 2 h at 40 °C with subsequent centrifugation) was analyzed to be 95% (18 mg/mL). Therefore, majority of the proteins of FPI were soluble in the studied conditions. Our previous results had indicated around 80% solubility for FPI (Nivala et al., 2017), however, the samples were dispersed at room temperature, while in the current study 40 °C was used which may have led to increased solubility.

The secondary structural features of the native (non-heated) and heat-treated colloidal faba bean proteins were analyzed by CD. The far-UV CD spectra of the native FPI showed a broad shoulder at 210–220 nm and a positive peak around 195 nm (Fig. 1). The broad negative shoulder

Fig. 1. Far-UV CD spectra of native (line) and heat-treated (dashed line) faba bean proteins. The spectra are the average of triplicate measurements.

at 210–220 nm indicates that the structure consists predominantly of parallel and anti-parallel β -sheet structures however the typical distinct negative peak at 216–218 nm associated with well-defined β -sheets was missing. The dominant β -sheet conformation is generally expected for legumins (Choi & Ma, 2007; Clara Sze, Kshirsagar, Venkatachalam, & Sathe, 2007; Martínez-Velasco et al., 2018; Schwenke et al., 2000; Yin et al., 2010), though there are not many published studies on far-UV spectra of FPI. The protein composition of the FPI used in the current study comprised of legumin and vicilin polypeptides as well as higher MW proteins (Nivala et al., 2017). The multi-protein nature of the plant material may inhibit collection of proper dichroic data. Liu et al. (2019b) recently reported the effect of TG-induced crosslinking on the far-UV spectra of a similar FPI preparation. A wide negative peak at 210–225 nm was evident which showed minor shifts after TG crosslinking at lower dosages than in the current study (Liu et al., 2019b). The far-UV CD spectra of the heat-treated (90 °C, 30 min) FPI sample showed a clear shift towards 200 nm and loss of positive peak around 195 nm (Fig. 1). The shift clearly indicates the loss of ordered structures and the increase of random coil conformation and molecular flexibility. The spectrum for the heat-denatured FPI shows similarity with e.g. molten globule state of bovine β -lactoglobulin (Ercili-Cura et al., 2012). The denaturation temperature of FPI was measured to be 94 °C by differential scanning calorimetry in our previous study (Nivala et al., 2017). Thus, a partially unfolded structure is expected in the current heat treatment conditions.

3.2. Enzymatic crosslinking of the heat-treated proteins

Enzyme treatment with 1000 nkat TG/g protein dosage caused efficient crosslinking of the faba bean proteins to large polymers (>250 kDa) identified by SDS-PAGE (Fig. 2), which was also depicted in (Nivala et al., 2017). FPI was composed mainly of legumin-like 11S proteins (α - and β -polypeptides). Faint bands of vicilin and convicilin were visible. The TG-induced crosslinking of the proteins was intensified after the heat treatment. When the heat-treated (90 °C, 30 min) FPI was incubated with TG, it was observed that both hydrophilic α - and hydrophobic β -polypeptides were crosslinked into large polymers (Fig. 2). Whereas without the heat treatment, β -polypeptides of legumin-like 11S globulins, buried inside the core of the native proteins, stayed mainly intact after TG treatment. The change observed in the secondary structure of faba bean proteins i.e., partial denaturation and molten globule-like conformation formation may have facilitated efficient exposure of β -polypeptides to the enzyme and formation of inter-molecular γ -glutamyl-lysine isopeptide links. Neither heat treatment, nor TG-induced crosslinking caused a dramatic change in the amount of soluble proteins analyzed from the colloidal fraction (data not shown). The results indicate that the colloidal particles resisted extensive particle-particle interactions at the studied concentration of around 18 mg/mL in the colloidal fraction.

Fig. 2. Crosslinked polymers of native and heat-treated FPI visualized by SDS-PAGE under reducing conditions. Soluble fractions of FPI were TG-treated (1000 nkat/g, 2 h at 40 °C) in native state (non-heated) and after heat-treatment at 90 °C, 30 min (heated). The molecular weight (MW) markers (std), crosslinked polymers and protein subunits are marked in the figure.

3.3. Surface hydrophobicity and emulsion properties

The surface hydrophobicity of colloidal FPI sample increased remarkably after the heat treatment (from 181 to 504 RFU) while the TG treatment caused a decrease in surface hydrophobicity of the heat-treated FPI from 504 to 435 RFU (Table 1). Similarly, a decrease in surface hydrophobicity was observed for TG-treated native FPI. Increase in surface hydrophobicity after heat treatment is due to dissociation of subunits and partial unfolding of globulin structures exposing the buried hydrophobic sites. Similar increase in hydrophobicity was reported for e.g. soy protein isolate (Wang, Li, Jiang, Qi, & Zhou, 2014). Decrease of surface hydrophobicity due to enzymatic crosslinking may be explained by decreased structural flexibility of colloidal proteins due to intra-molecular and intra-particle crosslinking (Ercili-Cura et al., 2015; Nivala et al., 2017), meaning that crosslinking reduced availability of hydrophobic sites. ANS is a fluorescent probe, which binds to hydrophobic sites on the protein surface, while a second mechanism of binding involves electrostatic interaction between positively charged amino acid residues (involving lysine) and the negatively charged sulfonate group of ANS (Johnston, Nickerson, & Low, 2015; Lee, 2010). Thus, possible change in spatial organization of the surface hydrophobic sites e.g. observed by loss of β -legumins into larger aggregates (Fig. 2) and even loss of lysine residues into TG-induced formation of γ -glutamyl-lysine bonds may have caused reduction of surface hydrophobicity in crosslinked FPI samples.

The emulsion properties, EAI and ESI, were analyzed following the turbidity change in the bottom of the emulsion tubes (Table 1). EAI values ranging between 25 and 30 m²/g were obtained for all the samples. A similar EAI range was reported for soy protein isolates at pH 6.5 by Pearce and Kinsella (1978). For comparison, EAI values of 149 and 119 were reported for sodium caseinate and whey protein powder, respectively (Pearce & Kinsella, 1978). Heat treatment caused a minor increase in EAI of FPI from 25.1 to 27.4 m²/g. TG treatment also caused increase of EAI in native faba bean proteins (up to 30.5 m²/g). EAI, is an index of the area stabilized per unit weight of protein, and it is a function of the oil volume fraction. Thus, EAI depends on the protein/oil ratio chosen for the study, and is also affected by the methodology and

Table 1

The pH values, surface hydrophobicity and emulsion properties of heat-treated and/or TG-treated faba bean protein isolate dispersions. The results are average of triplicate measurements $\pm$ StdDev.

Treatment	TG dosage (nkat/g protein)	pH	Surface hydrophobicity (RFU)	EAI ^a (m ² g ⁻¹)	ESI ^b (min)
native	0	7.3 $\pm$ 0	181 $\pm$ 8	25.1 $\pm$ 2.5	17.6 $\pm$ 0.8
	1000	7.3 $\pm$ 0	162 $\pm$ 8	30.5 $\pm$ 1.4	34.6 $\pm$ 11.0
heated (90 °C/30 min)	0	7.2 $\pm$ 0	504 $\pm$ 18	27.4 $\pm$ 1.6	26.3 $\pm$ 3.7
	1000	7.3 $\pm$ 0.1	435 $\pm$ 32	28.9 $\pm$ 1.0	22.6 $\pm$ 2.5

^a EAI, emulsifying activity index.

^b ESI, emulsion stability (10 min).

equipment used to prepare the emulsions (Pearce & Kinsella, 1978). Therefore, making comparisons of EAI values between other reported values in literature is challenging. Based on the observed ESI values, no conclusions can be made on stability of the emulsions due to high standard deviations (>10%) observed for each case. ESI is reliable parameter only when 'good enough' emulsion is achieved. The emulsions generated in the current study were not stable in that respect. Similar value range, however, was reported for salt extracted faba bean, lupin, pea, chick pea and soybean protein stabilized emulsions by Can Karaca et al. (2011) and for faba bean proteins for emulsions generated with much lower protein/oil ratio. Johnston et al. (2015) reported improved creaming stability after crosslinking of faba bean proteins with genipin and hypothesized that the interface formed of crosslinked proteins would show higher resistance to destabilization. In addition, optimal (slight) reduction in solubility may provide a thicker interface with improved steric stability. We have previously reported around 70% reduction of protein solubility for native faba bean proteins when crosslinked with TG (1000 nkat/g protein) (Nivala et al., 2017). In the current study, only colloidal protein fraction was used for heat and TG treatments and subsequent emulsification. The differences between EAI values have been minor indicating emulsions were stabilized by similar colloidal particles despite the heat and TG treatments in the studied conditions. No correlation between emulsion properties and surface hydrophobicity was possible either. It would be expected that increased surface hydrophobicity would improve emulsifying activity of proteins as hydrophobicity is one of the driving forces for adsorption of proteins to oil/water interface, however increased protein-protein interactions would be the limiting factor. Molina, Papadopolou, and Ledward (2001) reported similar phenomena for high pressure treated soy protein 11S globulins at pH 6.5. It was indicated that surface hydrophobicity is more indicative for emulsifying properties in the case of highly soluble proteins, whereas for proteins with lower solubility, solubility is more important parameter (Molina et al., 2001). Overall, emulsifying properties of FPI were not affected dramatically by the heat and TG treatments in the current conditions. It should be investigated further using other protein/oil ratios.

3.4. Gel formation

3.4.1. Heat-induced gelation

Rheological changes during heat treatment (90 °C, 5 min) of FPI dispersions at concentrations of 10 and 15% (w/w) were followed by small deformation oscillatory measurements performed during heating and cooling cycles (Fig. 3). The whole FPI suspension without separation of the colloidal fraction was used for the gelation experiments. Despite a heat treatment duration of 30 min was used in the rest of the study, *in-situ* heating experiment was performed for an incubation period of 5 min (at 90 °C) using the plate-plate geometry to minimize the possible evaporation impact. It was observed that at both concentrations, the dispersions started to show gel-like characteristics ($\tan\delta > 1$) at around 83 °C (30 min after the start of heating cycle). The denaturation temperature for FPI was shown to be 94 °C in our previous publication with onset of denaturation temperature being around 83 °C (Nivala et al., 2017). Similar gelation temperature was reported for soy protein isolate

(Renkema & van Vliet, 2002). The modulus continued to increase during the cooling cycle and plateaued at around 25 °C (70 min after the start of heating-cooling cycle). At 10% concentration, FPI dispersion showed gel-like characteristics with a final storage modulus (G') value of 180 Pa. However, the sample was still easily flowing, thus it was possible to use that condition for acidification and TG-induced gelation subsequently. Increasing the protein concentration to 15% resulted in slight increase of final G' to 230 Pa at the studied heating conditions (Fig. 3). Higher G' values were reported for 15% FPI gels at neutral pH when heat-treated at 95 °C, for 30 min and cooled back to room temperature (Langton et al., 2020). It is known that gel stiffness correlates well with the degree of protein denaturation, i.e. the higher the degree of denaturation the higher the G' of heat-induced gel for e.g. soy 11S globulins (Renkema, Gruppen, & van Vliet, 2002; Renkema & van Vliet, 2002). With the heat treatment conditions used in the current study, stiff gels were not achieved at either protein concentration, as the degree of protein denaturation was not extensive. The main target for studying heat-induced changes in rheological properties of the 10 and 15% dispersions in the current study was to show the effects of pre-heat treatment at 90 °C on dispersion state before using such for the subsequent acid- and TG-induced gelation studies.

3.4.2. Acid-induced and TG-induced gelation

Acid-induced and TG-induced gelation of FPI was studied at a concentration of 10% protein. FPI dispersion was heat-treated (90 °C, 30 min) as a pre-treatment before acidification or TG addition. Rheological parameters G' and $\tan\delta$, and pH were recorded for 3 h to follow gelation of acidified or enzyme-treated FPI dispersions (Fig. 4, Table 2). The gelation of the pre-heated and acidified (1% GDL) FPI dispersion resulted in higher G' after 3-h acidification compared to G' of the enzyme-treated (10 or 100 nkat TG/g protein) gels after 3-h cross-linking. The final pH value of 5.1 was reached after 3 h of GDL addition. The final G' of 6853 Pa and the final $\tan\delta$ value of 0.15 was reached after 3 h of GDL addition. The heat-induced gelation of globular proteins

Fig. 3. The rheological parameters obtained from the small deformation oscillatory measurements during temperature ramp, which included heating from 25 to 90 °C (ramp 2 °C/min), soaking at 90 °C for 5 min and cooling back to 25 °C. The storage modulus (circle) and loss modulus (triangle) were depicted for 10% (open symbols) and 15% (filled symbols) faba bean protein dispersions during temperature ramp (solid line).

Fig. 4. The rheological parameters obtained from the small deformation oscillatory measurements during gelation at 40 °C. The FPI dispersion was pre-heated at 90 °C for 30 min and subsequently acidified by GDL addition (circle) or enzyme treatment with 10 nkat TG/g protein (open triangle) or 100 nkat TG/g protein (filled triangle). The storage modulus (G') (A) and $\tan\delta$ (B) were depicted for the acidified and TG-treated protein gels.

Table 2

The storage modulus (G'), $\tan\delta$ and water holding capacity (WHC) values attained at the end of the time sweep (at 180 min) for the pre-heat-treated (90 °C, 30 min) FPI dispersions that were gelled by gradual acidification to pH 5 (by addition of GDL) or TG treatment. Standard error between replicate measurements in case of G' , $\tan\delta$ and pH values are indicated. The WHC results are average of triplicate measurements $\pm$ StdDev.

GDL (%)	TG dosage (nkat/g protein)	G' (Pa)	$\tan\delta$	pH	WHC (%)
1	0	6853 $\pm$ 77	0.15 $\pm$ 0.005	5.1 $\pm$ 0.06	93.7 $\pm$ 0.79
0	10	323 $\pm$ 280	0.08 $\pm$ 0.003	6.9 $\pm$ 0.01	99.9 $\pm$ 0.04
0	100	3534 $\pm$ 294	0.04 $\pm$ 0.001	6.9 $\pm$ 0.05	98.4 $\pm$ 0.40

takes place by denaturation/partial unfolding followed by aggregation due to intermolecular hydrophobic interactions and agglomeration of aggregates into network structure. Physical interaction such as hydrogen bond formation proceeds and further strengthens the gel network (O'Kane, Happe, Vereijken, Gruppen, & van Boekel, 2004). When heat-treated FPI dispersion was acidified, electrostatic interactions strengthened the intermolecular bonds between the protein molecules forming a stronger gel network at pH 5, which is in the range of isoelectric point for faba bean globulins (Nivala et al., 2017). Langton et al. (2020) reported G' values around 10 000 Pa for 15% FPI dispersion heat-treated after pH adjustment to 5.0. Our results indicate similar gel profile can be achieved at a lower protein concentration of 10% after a shorter overall heating and cooling regime and slow acidification to pH 5.

G' values for the TG-induced FPI gels were 323 and 3534 Pa with the enzyme dosages 10 and 100 nkat/g protein, respectively. The $\tan\delta$ values for these samples were 0.08 and 0.04, respectively (Fig. 4). The higher TG dosage of 1000 nkat/g resulted in extremely stiff gels which could not be tested due to the limitations of the rheometer to handle high axial force. There was no change in pH during 3-h TG-induced gelation. The TG addition at a dosage of 100 nkat/g yielded FPI gels with similar G' but dramatically lower $\tan\delta$ values compared to the acid-induced gels. Similar phenomena was observed for heat-treated milk and sodium caseinate gels after TG-induced crosslinking and acidification (Ercili-Cura et al., 2012, 2013). Low $\tan\delta$ was associated with limited potential of the gel network (strands and aggregates) for fast rearrangements (van Vliet & Walstra, 1994) and also associated with a higher gel strength as analyzed by large deformation measurements (Ercili-Cura et al., 2012, 2013) and with a higher WHC (Ercili-Cura et al., 2013). In the current study, none of the gels exhibited spontaneous syneresis. The WHC of the gels were found to be >90% indicating excellent water holding properties of FPI gels (Table 2). The TG-induced gels possessed higher WHC compared to that of acid-induced gels. The gel network induced by lower enzyme dosage (10 nkat/g) could hold nearly all liquid inside the gel structure as shown with a WHC value of 99.92 $\pm$ 0.04%.

The improved viscoelastic nature of TG-induced gel (100 nkat/g dosage) compared with the acid-induced gels was evident in frequency and strain sweeps performed on the final gels (Fig. 5). The dependency of G' to frequency (log-log plot, Fig. 5A) was lower for the gel formed by TG treatment (100 nkat/g dosage) compared to other gels, indicating a more pronounced solid-like character. Strain sweeps were performed after the frequency sweeps (final frequency of 10 Hz was not destructive on the gel structures) (Fig. 5B). The acid-induced gel showed yield strain value at around 27%. The TG-induced FPI gels on the other hand, exhibited increasing modulus values with increasing strain which is referred as strain hardening (Fig. 5B). Strain hardening was earlier observed for the TG-treated dairy milk gels (Ercili-Cura et al., 2013). In fractal colloid gels, strain hardening is associated to intrinsic strength of particles or aggregates forming the strands in a gel network (Gisler, Ball, & Weitz, 1999). The microstructure of both acid- and TG-induced gels, analyzed by CLSM, showed particulate gel structures composed of heterogeneously-sized protein aggregates in a fractal plane (Fig. 6). Aggregates as large as 10–30 μ m were observed within a network of smaller particles. However, no clear differences were evident between acid- and TG-induced gels. It may be concluded that inter-molecular covalent crosslinks formed by TG reinforced the intra-particle and inter-particle structures without causing massive rearrangements in the spatial distribution of the large aggregates. Both strands and aggregates are resistant to yielding upon increasing strain. However, it is not possible to explain the structural similarity between the microstructure of electrostatically-driven acid gels and neutral gels formed by incorporation of covalent crosslinks within and between existing aggregates. Similar particle and fractal dimensions observed between acid- and TG-induced gels may infer that both mechanisms will provide similar mouthfeel properties related to coarseness when used in gelled-type food applications.

4. Conclusions

The emulsification and gelation properties of FPI were studied in connection to heating, acidification and enzyme-aided protein

Fig. 5. The rheological parameter G' of FPI gels depicted during frequency sweep (0.01–10 Hz) (A) and strain sweep (0.01–100%) (B). The FPI dispersions were pre-heated at 90 °C for 30 min and acidified with GDL (circle), or TG-treated at dosages of 10 nkat TG/g protein (open triangle), or 100 nkat TG/g protein (filled triangle). The differences from the mean of replicate measurements are marked in the figure.

Fig. 6. The microstructures of the final FPI gels visualized by CLSM. The micrographs of acid-induced (A) and TG-induced gels with dosages 10 nkat/g protein (B) and 100 nkat/g protein (C) are depicted. The scale bar represents 20 µm.

crosslinking. The changes in the secondary structure of faba bean proteins due to heat treatment was linked with the efficiency of enzymatic crosslinking attained by TG, i.e., the higher the molecular flexibility, the higher the portion of crosslinked proteins was observed. FPI showed similar emulsifying activity to soy protein isolate, and neither the heat treatment (90 °C, 30 min) nor the TG-induced crosslinking (1000 nkat/g) dramatically affected the emulsion formation and stability of FPI emulsions. FPI showed significant gel-forming ability by pre-heating followed by acidification to pH 5 or by TG treatment at pH 7. TG-induced gels were characterized with a high final storage modulus and strain hardening response indicating mechanically strong gels and thus, high potential of TG-treated FPI as a gelling agent at neutral conditions. Significant WHC also indicates a wide range of food applications for cross-linked faba bean proteins.

CRediT authorship contribution statement

Outi Nivala: Conceptualization, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing. **Emilia Nordlund:** Resources, Writing - review & editing. **Kristiina Kruus:** Funding acquisition, Supervision. **Dilek Ercili-Cura:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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