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Heat-induced interactions of hemp protein particles formed by microfluidisation with β-lactoglobulin

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ABSTRACT

This study explored the effect of microfluidization on the dispersibility of hempseed protein (HP) and the interactions of microfluidised HP particles with β-lactoglobulin (β-lg) after heat treatment. Microfluidization increased the dispersible protein fraction from 10% (non-microfluidised) to a maximum of 58% (200 MPa, 6 passes) in HP dispersions. Dispersible HP particles were within the micro-sized range ($d_{4,3} \leq 2$ μm) after microfluidization. Heat treatment (95 °C, 10–60 min) of HP particles with β-lactoglobulin (β-lg) induced protein association by sulphydryl-disulphide exchange reactions; β-lg association with HP particles initiated within the first 20 min. Additionally, the particle size ($d_{4,3}$) values of co-heated HP particles with β-lg were significantly smaller than those found in HP particle dispersions heated alone, results that were in line with microscopy analysis. This suggests that β-lg could have restricted HP particle aggregation. In conclusion, combining microfluidization and heat treatment could offer a venue to modify the physical properties of plant/milk protein mixtures.

1. Introduction

Consumers' demand for dairy alternatives is increasing rapidly and is largely driven by the ongoing changes in dietary patterns towards more sustainable diets. Although plant proteins have been proposed as new ingredients for dairy alternatives, it is very challenging to match the functionality and nutritional benefits of milk proteins. One of the approaches to balancing the nutritional composition and improving the techno-functionality of plant proteins is to combine plant proteins with milk proteins. In fact, recent studies suggest that in some instances plant/milk protein interactions during processing may lead to functional synergies (Martin, de los Reyes Jiménez, & Pouvreau, 2016; Roesch, Juneja, Monagle, & Corredig, 2004). A previous study from our group indicated that the addition of sodium caseinate to hempseed protein (HP) globulins improved the thermal stability of HP globulins at a molecular level (Chuang, Wegrzyn, Anema, & Loveday, 2019).

HP is attracting attention as novel protein ingredient because of its high nutritional value and credible sustainability credentials (Wang, Jiang, & Xiong, 2018). Hempseed proteins have excellent digestibility (about 90%) (Callaway, 2004) and are considered of high quality due to their amino acid profiles (including high levels of arginine) being comparable with those of soybean and egg white, which are recognised as good protein sources (Tang, Ten, Wang, & Yang, 2006). The hempseed proteins are comprised of 60–80% globulin (edestin) and 20–30% albumin (Kim & Lee, 2011). Edestin is a homohexamer (~300 kDa) that is composed of six subunits (~52 kDa) linked by non-covalent interactions. Each subunit is composed of a basic subunit (~18 or 20 kDa) and an acid subunit (~34 kDa). Two cysteine residues form a disulphide bond linking the basic subunit and the acid subunit. Another two cysteine residues form the intrachain disulphide bond in the acid subunit while one cysteine residue remains as a free thiol group (Potin & Saurel, 2020; Wang & Xiong, 2019).

The emulsification and gelation properties of HP are considered to be relatively poor (Shen, Gao, Fang, Rao, & Chen, 2021) and the use of HP powder as a food ingredient has been limited due to its poor solubility in water. This could be due to the inherent insolubility of HP globulin at neutral pH, and the adverse effects of extraction methods on protein structure. Heat treatment during oil extraction and following drying processing leads to complete or partial protein denaturation (Shi, Li, Stone, Guldiken, & Nickerson, 2021). Hempseed proteins undergo extensive aggregation upon thermal treatments above their denaturation temperature. The free thiol groups in HP are involved in protein thermal aggregation through thiol-disulphide exchange reactions and cause large aggregates (Aluko, 2017; Chuang et al., 2019;

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Dapčević-Hadnađev, Hadnađev, Dizdar, & Lješković, 2020; Patel, Cudney, & McPherson, 1994; Wang, Tang, Yang, & Gao, 2008).

In general, several approaches have been applied to improve the solubility of HP. These include enzymatic hydrolysis to remove non-protein components and extract proteins (Malomo & Aluko, 2015), pH cycling to change the tertiary structure of proteins (Jiang, Chen, & Xiong, 2009) and high-pressure processing to disrupt the quaternary and tertiary structure of proteins (Galazka, Dickinson, & Ledward, 2000). However, the functionality improvements using different pre-treatments depend on the intrinsic properties of individual proteins within each plant source. It has been previously reported that hempseed globulin (the major HP) becomes completely insoluble after pH cycling (Chuang, Ye, Anema, & Loveday, 2021).

Microfluidization is a non-thermal process that can modify protein structure or aggregation state by applying ultra-high pressure, hydrodynamic cavitation and intense shearing at the same time. In the microfluidization process, the fluid is divided into two or more microstreams and pumped into the interaction chamber where the collision of microstreams occurs. Before the collision, the dimension of micro-tubes decreases to provide a high shear rate and intensive disruption effect. The collision creates high energy to disrupt the protein aggregates and creates smaller particles (Chen et al., 2012; Mert, 2020). Upon microfluidization of pea protein solution, for example, the solubility of pea protein was increased by 3.78 fold, along with a significant decrease in particle size (He et al., 2021).

β-Lactoglobulin (β-lg) was chosen in this study because it is the major protein in commercial whey protein products. It has a globular structure that is stabilised by hydrogen bonding, van der Waal forces, electrostatic interactions and hydrophobic interactions (Singh & Havea, 2003). Unfolding of β-lg occurs above 70 °C and aggregation begins at 78 °C mainly through disulphide bonding and hydrophobic interactions (Sava, Van der Plancken, Claeys, & Hendrickx, 2005). The unfolding of β-lg exposes the buried hydrophobic side chains and free sulphydryl groups that tend to interact with other protein molecules (Singh & Havea, 2003).

Heat-induced aggregation is one of the most important properties of food proteins which will give food products with different structural and textural features (Chihi, Mession, Sok, & Saurel, 2016). The development of plant-whey protein aggregates may produce novel protein ingredients. Previous studies have shown soy proteins could interact with whey proteins through disulfide bonds (Anuradha & Prakash, 2009; Roesch & Corredig, 2005). It has also been reported that β-lg can interact with pea globulin during heat treatment, leading to smaller aggregate sizes compared with single pea globulin aggregates (Chihi et al., 2016). However, there are no systematic studies on the heat-induced interactions between HP and β-lg, especially interactions at a molecule/-particle level.

According to previous studies on plant-whey aggregates, we hypothesise that the heat treatment can induce interactions between HP and β-lg, which could lead to the formation of composite protein particulated materials. This study explored the effect of microfluidization on the dispersibility of hempseed protein (HP) and the interactions of microfluidised HP particles with β-lactoglobulin (β-lg) after heat treatment.

2. Materials and methods

2.1. Materials

HP concentrate powder was purchased from a local supermarket (Davis Trading Company Ltd., Palmerston North, New Zealand). It contained 59.8% protein, 2.4% fat, 10.7% ash, 6.8% moisture and 20.2% carbohydrate. The protein content was determined using the Kjeldahl method (AOAC 991.20, Nitrogen conversion factor 5.21; AOAC, 2006). The fat, ash and moisture contents were analysed according to AOAC 922.06, AOAC 942.05 and AOAC 925.10, respectively (AOAC, 2006). The carbohydrate content was calculated by subtracting the sum of the protein, ash and fat from the total solid. β-Lactoglobulin was purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA). Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Ltd., and the reagents were made up in Milli-Q water (Milli-Q apparatus; Millipore Corp., Bedford, MA, USA).

2.2. Preparation of HP microparticles

HP concentrate powder was dissolved at room temperature by stirring in Milli-Q water at 0.5, 1, 2 and 3 g/100g protein concentrations. The HP dispersions (pH 7) were passed through a microfluidiser (M-110P, Microfluidics, Newton, MA, USA) at 200 MPa with 2, 4 and 6 passes and then centrifuged at 500×*g* for 15 min at 20 °C to remove the large particles (such as insoluble fibre). According to preliminary experiments, the microfluidization pressure (200 MPa) chosen in this study aimed to get minial particle size. The resulting microfluidised HP supernatant was used for further work. Sodium azide (0.02 g/100g) was added to HP dispersions to inhibit microbial growth.

The protein content in the supernatant was determined by measuring total nitrogen content using the Kjeldahl method (AOAC 991.20, Nitrogen conversion factor 5.21; AOAC, 2006) to calculate the percentage of dispersible protein by the following equation (Eq. (1)):

$$\textit{Dispersible protein}\ (\%) = \frac{\textit{PC microfluidised HP sup}}{\textit{PC original HP}} \times 100 \quad (1)$$

Where *PC Microfluifised HP* sup is the protein content (g/100g) of the supernantant fraction obtained after centrifugation of the microfluised HP dispersion, whereas *PC original HP* is the protein content (g/100g) of the HP dispersion prior microfluidisation.

2.3. Heat treatment of protein solution

β-Lactoglobulin was dissolved in Milli-Q water with magnetic stirring for at least 2 h to prepare the β-lg (0.5 g/100g) stock solutions. The microfluidised HP supernatant and β-lg solution were mixed to achieve the different final protein concentrations. The pH of the protein mixture was adjusted to pH 7. The protein samples were heated at 95 °C for different holding times (10–60 min) in the water bath and were then cooled in ice to 20 °C immediately. 95 °C was chosen as heating temperature is because it was above the denature temperatures of both β-lg (74–76 °C) (Kim, Wang, & Selomulya, 2020) and HP (92 °C) (Wang et al., 2008) To simplify sample nomenclature, the g/100g sign was omitted; as examples, 0.25 g/100g HP is referred to as 0.25HP, 0.25 g/100g β-lg is referred to as 0.25β-lg and the mixture of 0.25 g/100g HP and 0.25 g/100g β-lg is referred to as 0.25HP/0.25β-lg.

2.4. Particle size analysis

The particle size of HP dispersions and their supernatant was measured using static light scattering on a Mastersizer 2000 (Hydro MU, Malvern, Worcestershire, UK). The refractive indices of hempseed protein and water were 1.45 and 1.33, respectively. The data were reported in volume-mean diameter ($d_{4,3}$) and Sauter-average diameter ($d_{3,2}$) and were calculated as the average of triplicate measurements.

2.5. Transmission electron microscopy

Sample preparation and negative staining for transmission electron microscopy (TEM) were performed as described by Vincekovic, Curlin, and Jurasin (2014). 80 μL of protein solution were placed on a formvar/carbon-coated 200 mesh copper grid for 4 min. The excess sample was removed by filter paper. About 80 μL of uranyl acetate (2 g/100g) were placed on the grid for another 4 min and the excess staining solution was removed with filter paper. The stained sample was

imaged by a Philips CM10 electron microscope at 100 kV (Eindhoven, the Netherlands).

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protein composition was studied by Tris-HCl sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as per the protocol described by Dave, Ye, and Singh (2019) and Manderson, Hardman, and Creamer (1998). The protein sample was mixed with non-reducing or reducing sample buffer to a final protein concentration of 1 mg/mL. Dithiothreitol was used as a reducing agent in the reducing sample buffer (200 mM) and the reducing samples were heated at 56 °C for 15 min. Samples (10 μL) were loaded onto Mini-Protean gels (Bio-Rad Laboratories, Richmond, CA, USA) and run at 150 V followed by Coomassie brilliant blue staining and destaining (10% isopropanol and 10% glacial acetic acid in water, v/v). The destained gel was scanned using a Gel Doc XR (Bio-Rad Laboratories) molecular imager and the densitometric analysis of protein composition was carried out with ImageLab software.

2.7. Determination of the proportion of β-lg associated with HP particles

The heat-treated samples were centrifuged at 20,000×*g* for 15 min to separate the supernatant (unassociated β-lg) and sediment (associated β-lg). The original protein mixture (untreated) and supernatants (heat treated) were analysed using SDS-PAGE under reducing conditions. The resulting gels were scanned using a Gel Doc XR (Bio-Rad Laboratories) molecular imager and integrated using ImageLab software for densitometric analysis. The band intensity (%) of β-lg in the centrifugal supernatant was calculated as a percentage of that in the original protein mixture samples.

2.8. Statistical analysis

Experiments were carried out in triplicate, and the results are reported as mean ± standard deviation. Statistical analysis was performed using SPSS software for Windows (version 29.0, SPSS Inc., Chicago, IL, USA). The data were analysed by independent t-tests for between two groups,and one-way analysis of variance (ANOVA) for multiple comparisons, using Duncan's test with the level of significance set at $P < 0.05$.

3. Results and discussion

3.1. Effect of microfluidization on HP dispersibility

HP powder was dispersed in water at different protein concentrations (0.5, 1, 2 and 3 g/100g) and then subjected to microfluidization at 200 MPa with a number of passes. The original and the microfluidised HP dispersion were centrifuged at 500×*g*. The supernatant represents the "dispersible" protein that does not sediment at room temperature (Fig. 1).

In the original HP sample, approximately 10% of total protein was found in the supernatant, at all concentrations tested (0.5–3 g/100g), indicating very low protein solubility/dispersibility of the HP powder. The low water solubility of hempseed proteins may arise from the extraction process and drying conditions used in the manufacture of commercial HP concentrate powder (e.g., alkaline-isoelectric precipitation, salt-dialysis extraction and spray drying), which may cause protein denaturation and aggregation (Cui et al., 2020). Moreover, HP globulin proteins in their native state have low water solubility at neutral pH, whereas HP albumins tend to be soluble in water at neutral pH (Aluko, 2017).

The protein content of the supernatant obtained from microfluidised HP increased after 2 passes (especially 0.5 and 1 g/100g) indicating that the breakdown of the large HP aggregates into smaller aggregates/particles improved the protein dispersibility. Therefore, the smaller HP particles tended to remain in the supernatant. This is consistent with other studies, in which microfluidization was shown to reduce particle size from 180 μm to 20 μm ($d_{4,3}$) and enhance protein solubility in pea protein from 22.6% to 85.3% (Moll, Salminen, Schmitt, & Weiss, 2021) by dissociating large protein aggregates into small particles.

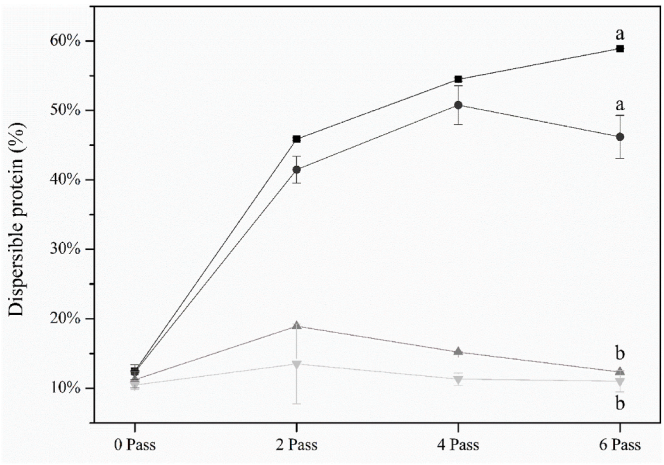

Fig. 1. Dispersible protein (%) of the supernatant of 0.5 (■), 1 (●), 2 (▲) and 3 g/100g (▼) M-HP, with up to 6 microfluidization passes at 200 MPa; bars show standard deviation (n = 3). Different lowercase letters indicate significant differences between dispersions with various protein concentrations ($p < 0.05$).

Interestingly, the protein content of supernatants from HP microfluidised at high concentrations (i.e., 2 and 3 g/100g) decreased from 4 passes onwards, indicating possible reaggregation of proteins. It has been suggested that intense shear and turbulence during microfluidization could expose the buried hydrophobic groups, free thiol groups and disulphide bonds, leading to large aggregates and low solubility (Gong et al., 2019; Moll et al., 2021; Shen & Tang, 2012). Structural changes have been reported in peanut protein dispersion; microfluidization induced an increase in β-sheet and random coil at the expense of α-helices and β-turns to create a loose and unfolded structure (Hu, Zhao, Sun, Zhao, & Ren, 2011). Microfluidization of soya protein was shown to increase surface hydrophobicity and disulphide bonds, enhancing protein-protein interactions (Shen & Tang, 2012).

Thus, it appears that during the microfluidization process, large protein aggregates were broken down into small particles, but simultaneously soluble HP molecules may unfold, exposing hydrophobic residues or thiol groups. At some critical points in the microfluidization process (i.e., the number of passes and pressures), protein molecules may interact with each other or with the protein particles generated during microfluidization (Gong et al., 2019; Hu et al., 2011; Moll et al., 2021; Shen & Tang, 2012). This effect would be expected to be more pronounced at high protein concentrations, i.e., 2 and 3 g/100g HP, as higher protein would provide more active groups and probability of interactions and protein reaggregation. The increase in the number of passes also aggravated the reaggregation by providing more energy input and offering more chances for protein interactions.

Interestingly, in 1 g/100g HP with four microfluidization passes, almost half of the HP was transferred into the supernatant and the dispersion was relatively stable under mild centrifugation conditions; hence the HP particles obtained by this processing condition were chosen as the material for further characterisation.

3.2. Particle size, microstructure and protein composition of HP particles

The 1 g/100g HP dispersion before microfluidization had a bimodal size distribution characterised by a small peak at around 0.2–1 μm and a large peak from 3 to 100 μm (Fig. 2A). After microfluidisation, the

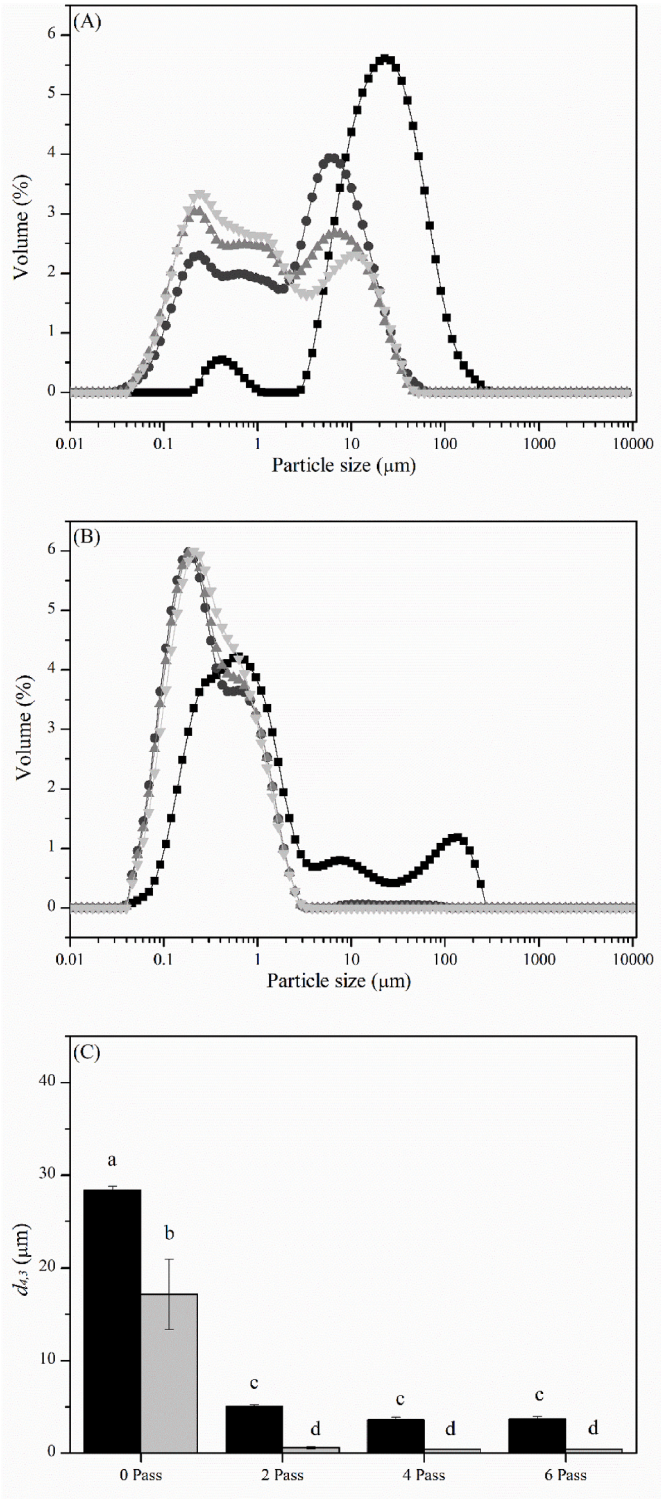

Fig. 2. Particle size distributions of (A) 1 g/100g HP dispersions and (B) corresponding supernatants after up to 6 microfluidization passes (200 MPa): ■, no passes; ●, 2 passes; ▲, 4 passes; ▼, 6 passes; and (C) their corresponding volume-weighted mean diameter ($d_{4,3}$, μm): ■, dispersion and ■, supernatant. Different lowercase letters indicate significant differences ($p < 0.05$).

disintegration of large HP aggregates was evidenced by a shift of the large-sized peak to the lower particle size range, ranging from <0.1 μm to 30 μm. The proportion of two peaks with small particles increased with microfluidization passes, while the proportion of the other peak decreased gradually.

All supernatant fractions (Fig. 2B) had narrower size distributions compared with the original microfluidised dispersions (Fig. 2A). The size distributions of the supernatant obtained from different passes were similar to each other, with the main peak at about 0.2 μm and a shoulder at about 0.6 μm. The narrower size distributions and smaller particle size (0.4 μm, $d_{4,3}$) (Fig. 2C) indicated that the HP particles that remained in the supernatant were relatively mono-disperse and uniform.

TEM examination showed the morphology of HP aggregates in 1 g/100g HP dispersions before and after microfluidization. Fig. 3A shows the presence of large, polydisperse amorphous aggregates in the untreated HP dispersion. However, in the microfluidised HP dispersion (Fig. 3B) the HP particles/aggregates appeared to disintegrate and were much smaller than in the original HP aggregates. This observation is consistent with the particle size distribution results (Fig. 2A). Interestingly, the HP particles that remained in the supernatant (Fig. 3C) appeared to be uniform and spherical with diameters in the range of 0.1–0.2 μm, which corroborated the $d_{3,2}$ value (0.19 ± 0.01 μm).

The protein composition of HP dispersions before and after microfluidization and their supernatants were analysed using SDS-PAGE under reducing conditions (Fig. 4A). The loading protein concentration for the supernatant of non-microfluidised HP was 10 times higher than that for the other samples. The actual intensity of bands without dilution would be much lighter than as it appears in Fig. 4A. HP consists of water-soluble albumins and salt-soluble globulins; some of these are marked as bands 1 to 7 in Fig. 4A. Globulins comprised approximately 7% 7S globulin of 48 kDa (band 1) and 93% 11S globulin consisting of an acid subunit of 34 kDa (band 2) and a basic subunit, shown as 2 bands of 20 kDa (band 3) and 18 kDa (band 4) (Potin & Saurel, 2020; Wang, Jiang, & Xiong, 2018). The albumin was composed of mainly 7 polypeptides of molecular mass below 35 kDa (some of which are indicated as bands 5–7) (Wang & Xiong, 2019).

The relative proportion of bands in each lane was estimated by the densitometric scanning of band intensity (Fig. 4B). Except for the supernatant obtained from non-microfluidised HP, the rest of the samples showed similar protein composition and relative proportions of four dominating bands; these were three heavy bands from 11S globulin subunits (bands 2–4) and one light band (band 7) from albumin. The other bands were either missing or had very low intensity. Compiling the band intensity data, the results showed that 11S globulin (including acid and basic subunits) was the major protein (80% of total protein) in untreated HP dispersion and microfluidised HP dispersion. This composition was consistent with other studies (Aluko, 2017; Tang et al., 2006). Due to the high solubility of hempseed albumin (Wang & Xiong, 2019), more albumin bands were observed in the untreated HP supernatant (above 55% of total protein).

The proportion of 11S globulin in the supernatant increased from 35% to about 80% after microfluidization. This indicates that microfluidization can effectively transfer about half of the "insoluble" globulin particles/aggregates into the supernatant. Moll et al. (2021) also reported the improvement of colloidal properties of insoluble pea protein by microfluidization. The solubility of the insoluble pea proteins increased from 23% to 86% at ≥125 MPa.

3.3. *Effect of heat treatment on HP/β-lg interactions*

The heat-induced interactions between HP and β-lg were determined. The particle size of unheated and heated protein mixtures was analysed and compared with individually heated HP supernatant (Fig. 5) to assess the extent of aggregation in these systems. The $d_{4,3}$ of 0.25HP/0.25β-lg mixture increased from 0.4 μm to 3.2 μm after heating, but the extent of increase was smaller than the individually heated 0.25HP, which reached 7.1 μm after heat treatment. This suggests that the presence of β-lg may have restricted the aggregation of HP particles during heating. In other plant protein and β-lg systems, Chihi et al. (2016) also reported the presence of β-lg in pea globulin formed globulin–β-lg aggregates and slowed the growth of aggregates during heat

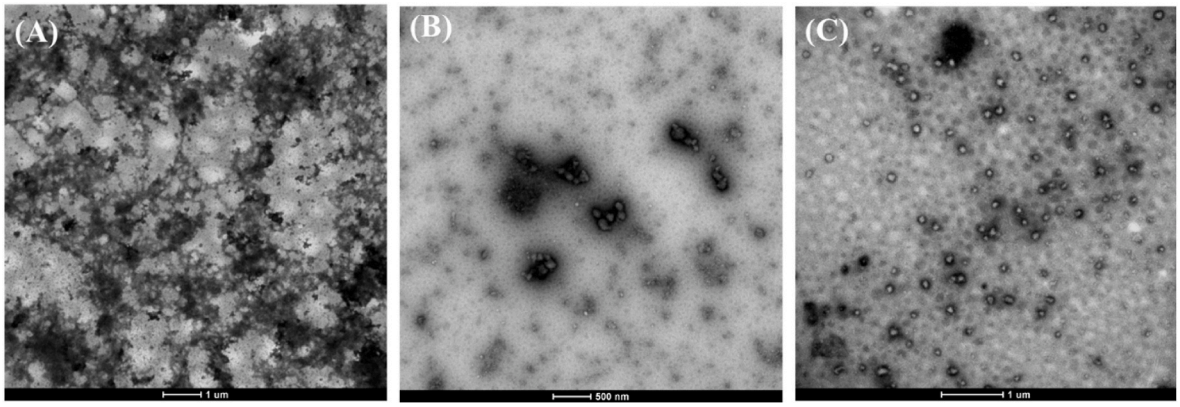

Fig. 3. Transmission electron micrographs of (A) 1 g/100g HP dispersion, (B) microfluidised (200 MPa, 4 passes) HP dispersion and (C) its supernatant. Images were captured at 9900x, 20500x and 16500x magnification, respectively.

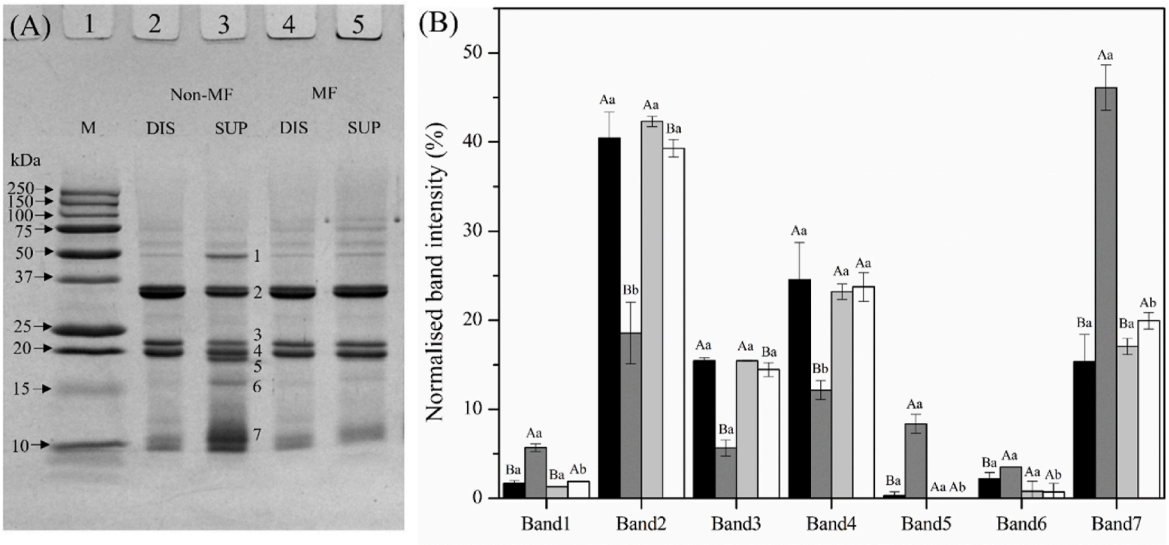

Fig. 4. Reducing SDS-PAGE (A) of non-microfluidised (Non-MF) and microfluidised (MF) HP dispersions (DIS) and their supernatants (SUP) (M, molecular mass marker) with (B) the normalised band intensity (%) analysis of a relative proportion of bands in each lane (■ and ■, non-microfluidised HP dispersion and supernatant, respectively; ■ and □, microfluidised HP dispersion and supernatant, respectively). In panel A, the protein bands labelled 1–7 are: 1, 7S globulin; 2–4, 11S globulin 34 kDa acid subunit (2) and 20 kDa (3) and 18 kDa (4) basic subunit; 5–7, albumin polypeptides. Different uppercase letters mean significant differences between Non-MF DIS and Non-MF SUP, or between MF DIS and MF SUP within each band ($p < 0.05$). Different lowercase letters indicate significant differences between Non-MF DIS and MF DIS, or between Non-MF SUP and MF SUP within each band ($p < 0.05$).

treatments.

TEM showed the presence of large HP aggregates in individually heated 0.25HP solution (Fig. 6A), suggesting HP particles are susceptible to aggregation by heat treatment. However, heated HP dispersion in the presence of β-lg showed relatively small particles with spherical shapes (Fig. 6B), confirming the particle size results (Fig. 5). A speculative hypothesis which could be confirmed by the following SDS-PAGE results is that the interactions between HP particles and β-lg may, to some extent, have prevented HP particles from aggregating. It is possible during heating that unfolded β-lg interacted with the surface of HP particles, and the association of β-lg with HP particle surface could have restricted the self-aggregation of HP particles. Some other studies also reported that plant globulins can interact with whey proteins to form aggregates through disulphide bonds and hydrophobic interactions under heat treatment, such as pea globulin/β-lg (Chihi et al., 2016) and soy protein/whey protein (Roesch & Corredig, 2005). In HP and other milk protein mixtures, Chuang et al. (2019) also found that hempseed globulin could form large protein aggregates when heated alone and interacted with sodium caseinate under thermal treatment and formed smaller aggregates.

To explore the interactions between HP and β-lg, the unheated and heated individual proteins (0.25HP or 0.25β-lg), or their mixtures (0.25HP/0.25β-lg) using SDS-PAGE (Fig. 7A). Unheated 0.25HP gave a major band at ~52 kDa (band A), which is the subunit of 11S globulin (Fig. 7A, lane 2). The hexamer of 11S globulin was probably disrupted under the non-reducing PAGE conditions (Hadnađev et al., 2018). Upon heat treatment, band A (heated 0.25HP) completely disappeared (Fig. 7A, lane 5), which suggests that 11S globulin aggregated via sulphydryl-disulphide exchange reactions leading to the formation of intermolecular disulphide bonds during the heat treatment.

This result is expected because hempseed globulin, similar to other plant globulin proteins, can be denatured by heating forming soluble and/or insoluble aggregates (Wang & Xiong, 2019) and the denaturation temperature for hempseed globulin is 91.9 °C (Wang et al., 2008). Commercial HP contains aggregated protein particles, possibly due to denaturation/aggregation during the protein powder manufacture. Such aggregates were broken down to some extent during the microfluidization process, but heat treatment may have modified the surface

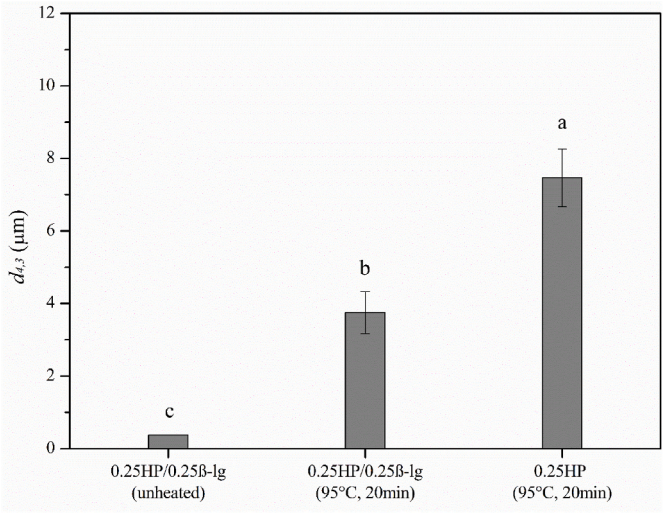

Fig. 5. Average particle size $d_{4,3}$ of unheated 0.25HP/0.25β-lg and of 0.25HP/0.25β-lg and 0.25HP after heat treatment at 95 °C for 20 min. Different lowercase letters indicate significant differences among samples ($p < 0.05$).

structure of HP particles, exposing the hydrophobic groups and thiol groups and so allowing further particle-particle interactions. Conversely, unheated 0.25β-lg showed only one band (band B) representing the monomer of β-lg (Fig. 7A, lane 3) but after heat treatment, two predominant bands could be observed: β-lg monomers (band B) and heat-induced dimers (band C) (Fig. 7A, lane 6). This is in agreement with previous studies that heat treatment above the denaturation temperature results in the formation of β-lg dimers and trimers that are linked via disulphide bonds (Schokker, Singh, Pinder, Norris, & Creamer, 1999).

In the protein mixtures, unheated 0.25 HP/0.25β-lg showed two distinct bands corresponding to HP 11S globulin and β-lg (Fig. 7A, lane 4). But after heating, the band corresponding to 11S globulin disappeared and the intensity of both β-lg monomers and dimers (Fig. 7A, lane 7) was reduced compared with that when β-lg solution was heated alone. This could be explained by the formation of disulphide bonds between HP particles and β-lg upon heating, which led to the formation of HP/β-lg aggregates with larger molecular weight protein aggregates that remained at the top of the stacking gel.

To further explore the protein-protein interactions, the unheated and heated protein mixtures (Total) and their supernatant (SUP) and sediment (SED) fractions were analysed using reducing SDS-PAGE (Fig. 7B). Under reducing conditions, the covelent inteactions formed between two proteins duing heating were further disrupted by breaking the

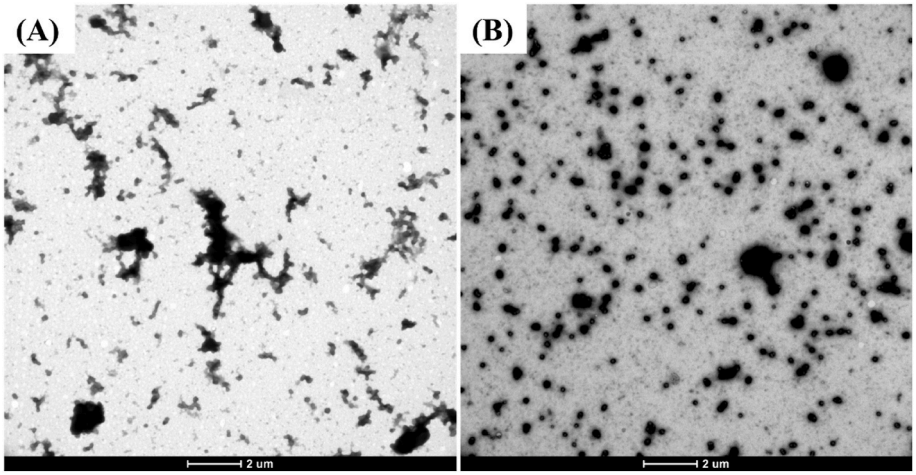

Fig. 6. Transmission electron micrographs of (A) 0.25HP and (B) 0.25HP/0.25β-lg after heating at 95 °C for 20 min. Images were captured at 6000x magnification.

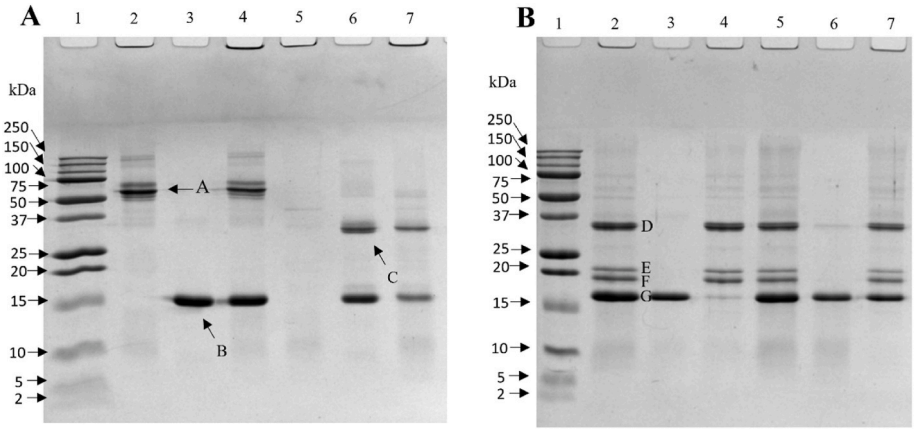

Fig. 7. Panel A: non-reducing SDS-PAGE of unheated (lanes 2–4) and heated (lanes 5–7; 95 °C, 20 min) dispersions of 0.25HP (lanes 2 and 5), 0.25β-lg (lanes 3 and 6) and 0.25HP/0.25β-lg (lanes 4 and 7). Lane 1, molecular mass marker. Panel B: reducing SDS-PAGE of unheated (lanes 2–4) and heated (lanes 5–7; 95 °C, 20 min) dispersions of 0.25HP/0.25β-lg (lanes 2 and 5; total) and the corresponding supernatants (lanes 3 and 6; SUP) and sediments (lanes 4 and 7; SED). Lane 1, molecular mass marker. Letters indicate: A, subunit of 11S globulin; B, β-lg monomers; C, β-lg dimers; D, HP globulin acid subunit; E, HP globulin basic subunit; F, HP globulin basic subunit; G, β-lg.

disulphide bonds using a reducing reagent (dithiothreitol).

Under reducing conditions, the total fraction of unheated 0.25HP/0.25β-lg mixture had three HP globulin bands (Fig. 7B, lane 2) consisting of band D, 34 kDa (acid subunit), band E (20 kDa) and band F (18 kDa) (two basic subunits) (Potin & Saurel, 2020; Wang, Jin, & Xiong, 2018). There was one band (band G) representing β-lg in the unheated protein mixture. After centrifugation of unheated 0.25HP/0.25β-lg mixture, all three HP bands were observed in the sediment (Fig. 7B, lanes 3 and 4), indicating that HP particles were completely sedimented at 20,000×*g*. As expected, no virtually β-lg band was found in the sediment of either heated 0.25% β-lg (data not shown) or unheated 0.25HP/0.25β-lg mixture, suggesting that native β-lg and heat-treated 0.25β-lg aggregates remain soluble under these conditions.

Interestingly, although the total fraction of 0.25HP/0.25β-lg mixture showed all protein bands corresponding to HP and β-lg after heat treatment, the sediment and supernatant fractions were remarkably different from their unheated counterparts. In fact, the sediment fraction of the heated 0.25HP/0.25β-lg mixture showed a β-lg monomer band (Fig. 7B, lane 7), which was absent in the sediment of the unheated mixture (Fig. 7B, lane 4). This suggests that β-lg interacted with HP particles possibly via sulphydryl-disulphide exchange reactions during heating, becoming associated with the sedimentable fraction. However, it should be noticed that the β-lg monomer was also seen in the supernatant of the heated mixture suggesting that only a proportion of β-lg interacted with the HP particles (Fig. 5B, lane 6).

3.4. Effect of heating time at 95 °C on HP/β-lg interactions

To understand the effect of heating time on the interaction between HP and β-lg, the supernatants from heated HP/β-lg mixtures at different times were analysed using SDS-PAGE under reducing conditions (Fig. 8A). Only β-lg monomer was observed in the SDS-PAGE gel at all heating times indicating that all HP particles were sedimented. The heated 0.25HP/0.25β-lg samples had lighter β-lg monomer bands (Fig. 8A, lanes 3–7) compared with the unheated sample (0 heating time; Fig. 8A, lane 2), which indicates that some of β-lg associated with the HP particles while a proportion of β-lg did not interact with HP particles and thus remained in the supernatant.

The band intensity (%) was estimated by the densitometric scanning of these gels to monitor the loss of β-lg in the supernatant due to its association with HP particles (Fig. 8B). The intensity of β-lg monomer was significantly reduced from 100% (0 min) to 68% and 66% after 10–20 min of heat treatment, but then remained steady until 60 min. This suggests that the rapid protein-protein interactions took place in the first 10–20 min and tended to plateau on prolonged heating.

Interestingly, Anema and Li (2003) reported similar association behaviour of β-lg with casein micelles in a heated milk system. Whey proteins were rapidly associated with casein micelles at high temperatures (90–100 °C) during the initial period of heating, but not all whey proteins were associated with the casein micelles. A proportion of denatured whey proteins remained in the milk serum as disulphide-bonded and hydrophobically associated aggregates. The proportions of denatured β-lg associated with the casein micelles varied from 50% to 80%, depending on the heating conditions, but not all β-lg associated with casein micelles (Anema & Li, 2003). A similar study reported that the extent of association of β-lg increased with increase in heating time and a maximum of 50% β-lg was associated with casein micelles (Oldfield, Singh, & Taylor, 1998; Oldfield, Taylor, et al., 2005).

To explore the role of disulphide bond formation in the association of β-lg with HP particles, the individually heated 0.25β-lg and heated protein mixture were analysed by non-reducing SDS-PAGE. When 0.25β-lg was heated alone, the band intensity of β-lg monomer (band A) decreased with an increase in heating time, while β-lg dimer (band B) increased with increasing heating time (Fig. 9A). Schokker et al. (1999) explained the formation of β-lg aggregates upon heating pure solution of β-lg. At low protein concentrations and short heating times, the free sulphydryl group and hydrophobic side chains of β-lg would be exposed but this unfolding of β-lg could be partly reversible. Upon prolonging heating time, the reactive β-lg monomers aggregated and formed certain dimers, trimers and oligomers through sulphydryl oxidation, thiol/disulphide exchange and non-covalent interactions.

A similar trend was observed in the co-heated 0.25HP/0.25β-lg mixture, but the band intensity of the β-lg dimer was much lower (Fig. 9B). The band intensity of β-lg monomer in 0.25β-lg and the protein mixture both decreased with heating time (Fig. 9C). However, the decrease in band intensity from the protein mixture was faster during the first 10 and 20 min, compared with 0.25β-lg heated alone. This indicates that during the first 20 min unfolded β-lg preferentially interacted with HP particles. It appears that once β-lg is unfolded, it tends to interact with HP particles or self-aggregate into dimers in the first 20 min. No percentage difference was seen at longer heating times (30–60 min), suggesting the amount of β-lg involved in these interactions was

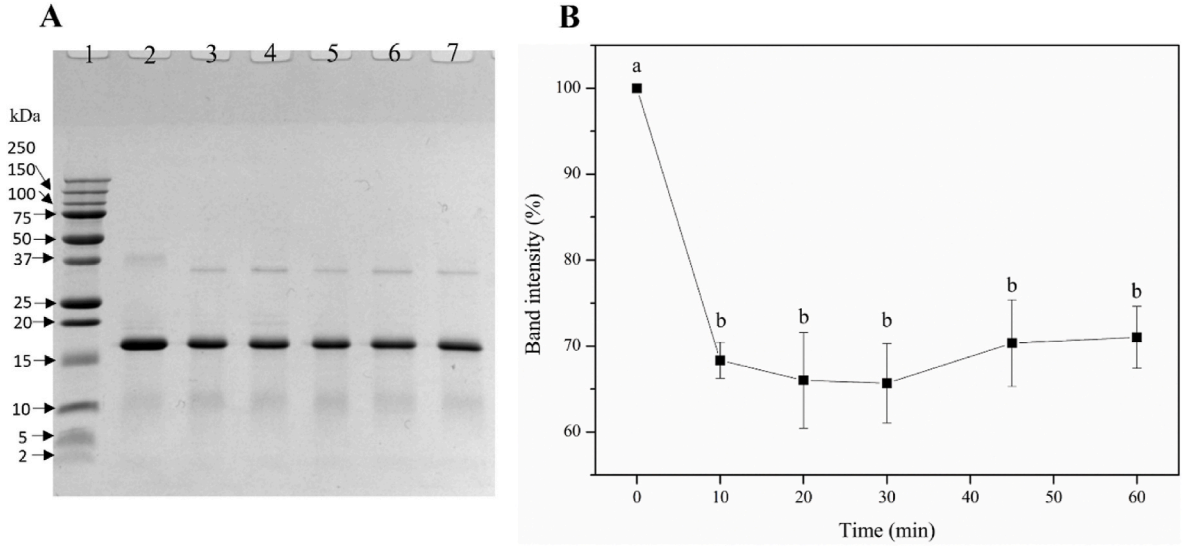

Fig. 8. Reducing SDS-PAGE (A) of the supernatant obtained from heating 0.25HP/0.25β-lg dispersions at 95 °C for (lanes 2–7, respectively) 0, 10, 20, 30, 45 and 60 min (lane 1, molecular mass marker) with changes in band intensity (%) of β-lg in the corresponding supernatants (B). Different lowercase letters indicate significant differences across heating times ($p < 0.05$).

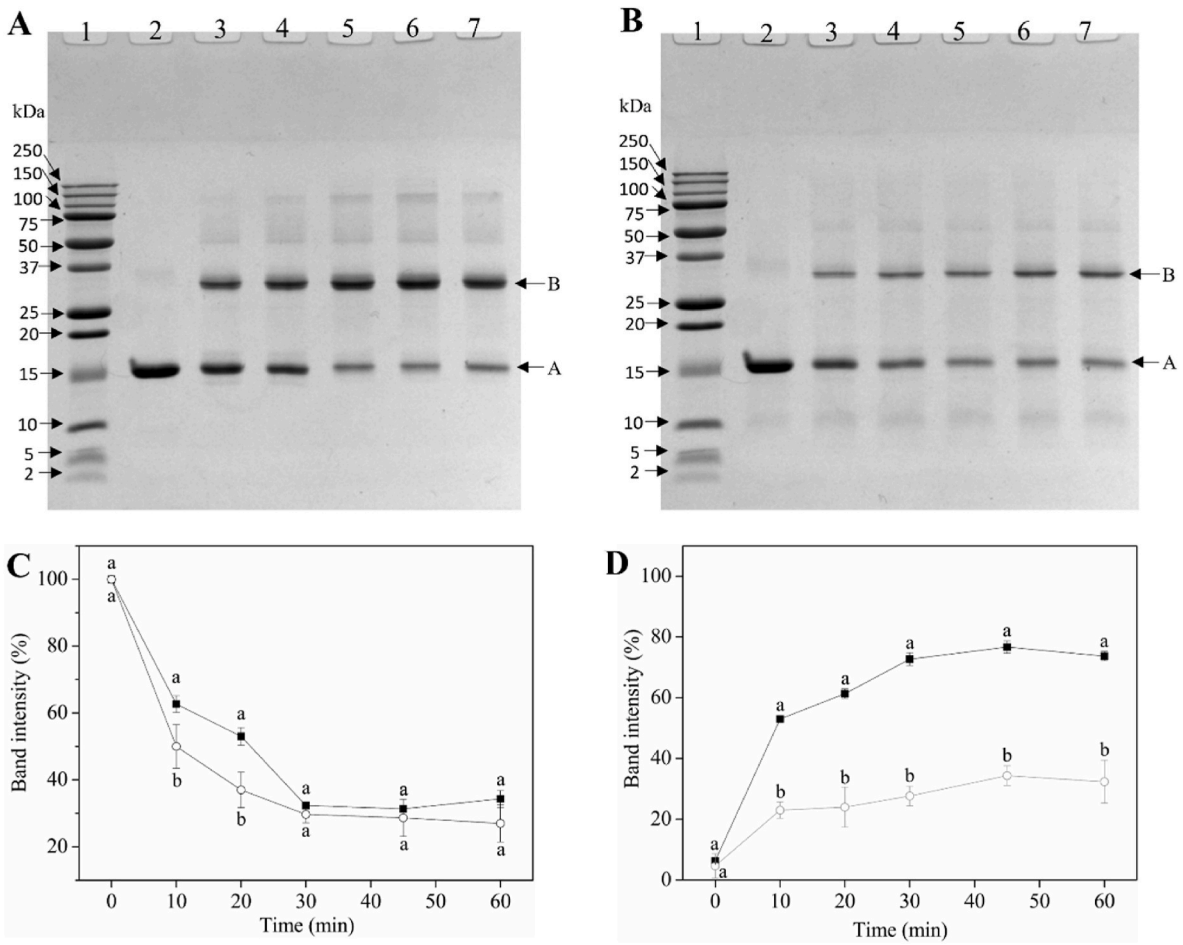

Fig. 9. Non-reducing SDS-PAGE of the supernatants obtained from heating (A) 0.25β-lg and (B) 0.25HP/0.25β-lg dispersions at 95 °C for (lanes 2–7, respectively) 0, 10, 20, 30, 45 and 60 min (lane 1, molecular mass marker; lettered arrows indicate: A, β-lg monomer; B, β-lg dimer) with changes in band intensity (%) of (C) β-lg monomer and of (D) β-lg dimer in the corresponding supernatants of the 0.25β-lg (■) and 0.25HP/0.25β-lg (◯) dispersions. Different lowercase letters indicate significant differences between 0.25β-lg and 0.25HP/0.25β-lg across heating time ($p < 0.05$).

the same in 0.25β-lg or 0.25HP/0.25β-lg.

Regarding the changes in β-lg dimer (Fig. 9D), the band intensity of β-lg dimer in 0.25β-lg increased with the increase in heating time from 7% to 70% at 30 min and remained steady thereafter. Most interestingly, β-lg dimer in the heated protein mixture showed relatively small increase in dimer formation. The percentage difference in dimer formation between the β-lg solution and the protein mixture increased to 45% in the first 30 min and stayed steady for the rest of the heating time. This suggests that there were fewer β-lg dimers remaining in the supernatant of the protein mixture after heating, possibly due to their association with HP particles.

One possible explanation is that once the β-lg monomer was denatured it preferentially interacted with HP, therefore there was less probability of β-lg forming dimers. Another possibility is that the β-lg dimers were rapidly formed before their interaction with the HP particles since the denaturation temperature of β-lg (74–76 °C) is lower than that of hempseed globulin (91.9 °C). In this scenario, β-lg dimers or higher polymers would be expected to associate with HP particles. These heat-induced molecule/particle interactions may be a similar case to that of heated milk containing whey proteins and casein micelle particles. The rate of whey protein denaturation was higher than the rate of whey-casein micelles association, and the β-lg aggregates were found to associate with casein micelles (Anema, 2007; Anema & Li, 2003; Oldfield, Singh, & Taylor, 2005).

3.5. *Effect of HP to β-lg ratio on heat-induced interactions*

In a milk system, Dalgleish, van Mourik, and Corredig (1997) showed that the association of β-lg with casein micelles was dependent on the β-lg:casein micelles ratio as there were only a certain number of sites on the surface of casein micelles that could interact with β-lg. The interaction between β-lg and casein micelles was limited when the β-lg:κ-casein ratio was above 0.6. However, the β-lg bound more efficiently to the casein micelles at this saturating level (i.e., β-lg:κ-casein <0.6).

Similar to that reported for casein micelle and β-lg association, it was thought that there would be a limited number of sites at the HP particle surface with which β-lg could interact. To investigate this, the association of β-lg with HP at higher HP:β-lg ratios (0.25HP/0.1β-lg and 0.25HP/0.05β-lg) was analysed using SDS-PAGE under reducing conditions and the change in the band intensity for β-lg (Fig. 10A, band A) was estimated by the densitometric scanning (Fig. 10B).

The intensity of the β-lg band decreased after heat treatment in each group (Fig. 10A) and the proportion of β-lg remaining in the supernatant decreased from about 70% at 0.25HP/0.25β-lg to about 20% at 0.25HP/0.05β-lg (Fig. 10B). Clearly, at higher HP:β-lg ratios, more reactive groups from the HP particles were available for interacting with β-lg. This percentage difference proved our hypothesis that the HP:β-lg ratio was an important factor affecting the extent of β-lg association.

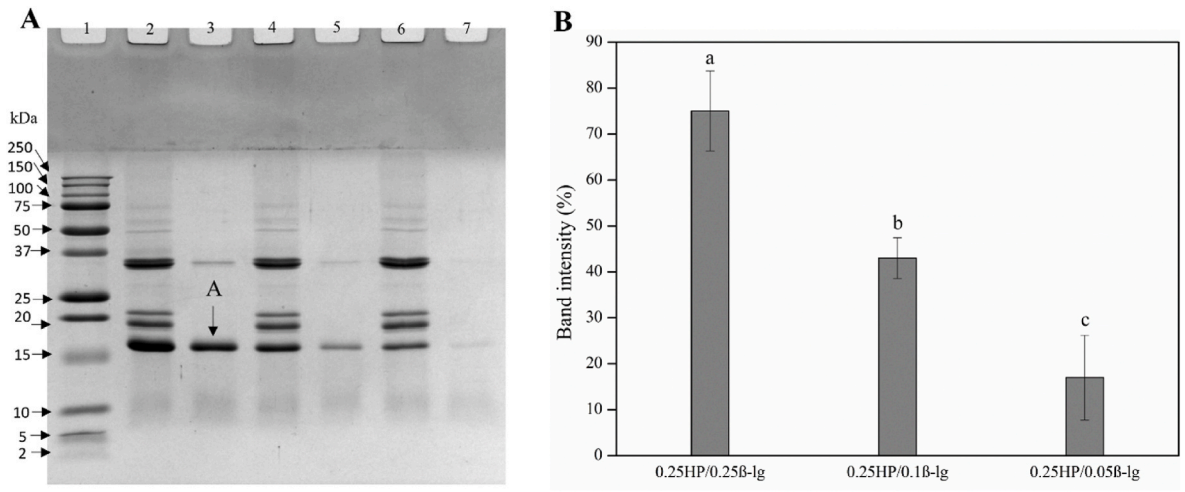

Fig. 10. Reducing SDS-PAGE (A) of dispersions of 0.25HP/0.25β-lg (lanes 2 and 3) 0.25HP/0.1β-lg (lanes 4 and 5) and 0.25HP/0.05β-lg (lanes 6 and 7) (lane 1, molecular mass marker) before heat treatment at 95 °C for 20 min (lanes 2, 4 and 6) and the corresponding supernatants after heat treatment (lanes 3, 5 and 7; β-lg band indicated by the letter A), with changes of band intensity (B; %) of β-lg in the supernatants. Different lowercase letters indicate significant differences between samples ($p < 0.05$).

3.6. Hypothetical mechanism of heat-induced interactions between HP particles and β-lg

According to all the results above, a possible mechanism by which HP/β-lg hybrid particles are produced after heat treatment is proposed (Fig. 11). When HP is heated individually (Fig. 11A), the surface of HP particles may be modified to expose the free thiol groups to allow thiol-disulphide interchange to form larger aggregates. The hydrophobic interactions may also contribute to the aggregation process.

Heat treatment causes unfolding of β-lg exposing free sulphydryl groups. The irreversible aggregates are formed by sulphydryl-disulphide interchange reactions and hydrophobic interactions (Brodkorb, Croguennec, Bouhallab, & Kehoe, 2016; Singh & Havea, 2003). When the HP particles and β-lg are heated together (Fig. 11B), the β-lg would first unfold at around 75 °C (Sava et al., 2005) and a proportion of the unfolded β-lg monomers may associate with HP particles. The free sulphydryl group of β-lg may be responsible for initiating this association via interaction with disulphide bonds present at the surface of HP particles.

Simultaneously, some β-lg monomers may self-aggregate into dimers or large aggregates. With increasing heat treatment to the denaturation temperature of HP (91.9 °C), more reactive groups, possibly thiols, of HP could be exposed on the surface of HP particles, allowing further association of β-lg with HP particle surfaces. Since the β-lg would occupy the reactive groups of HP particles, the aggregation of HP would be restricted, resulting in smaller aggregates compared with the individually heated HP particles.

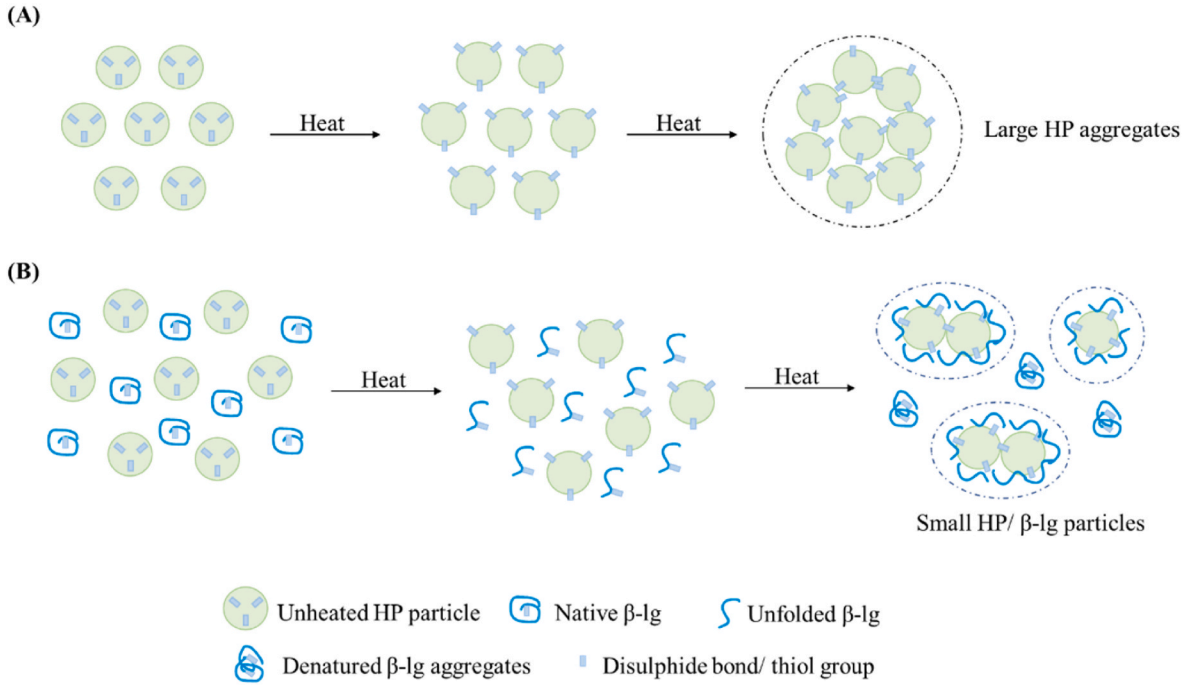

Fig. 11. Schematic representation of possible mechanisms by which large HP aggregates form in the absence of β-lg (A) and by which HP particles and β-lg interact (B) during heat treatment (95 °C, 20 min).

4. Conclusions

The HP aggregates were successfully disrupted by microfluidization. Although hempseed globulin is insoluble in water, a large amount of the HP globulin was transferred into the supernatant phase after microfluidization treatment. The HP particles and β-lg associated on heating above 90 °C because of the exposed reactive thiol groups of β-lg can interact with disulphide bonds on the surface of HP particles. The association of β-lg could limit the HP self-aggregation and could avoid the undesired large aggregates that may lead to inferior functionalities. Thus, HP/β-lg hybrid particles with uniform spherical shapes could be formed via heat treatment.

This is an exploratory study to offer an approach for improving the dispersibility of plant proteins and developing novel hybrid protein particles as food materials. Moreover, it is also interesting to understand the potential utilisation of these new hybrid particles in food applications such as emulsion, gelation and fat replacement.

Industrial relevance

The plant protein industry is rapidly growing, driven by consumers' preferences to reduce their consumption of animal protein due to environmental, health, and/or ethical reasons. Unfortunately, plant proteins pose various technological challenges that limit rapid food innovation. Nowadays, scientific advances achieved towards developing high-performance and eco-friendly protein ingredients are critically needed. One promising strategy is combining plant proteins with milk proteins to improve techno-functionality. However, to accomplish this outcome, fundamental knowledge on protein-protein interactions will contribute to advancing the generation of novel protein ingredients. This study particularly provides underlying insights that can be applied to develop microparticulated plant/milk protein ingredients for food applications.

CRediT authorship contribution statement

Sihan Ma: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Alejandra Acevedo-Fani:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Aiqian Ye:** Writing – review & editing, Supervision, Methodology. **Harjinder Singh:** Writing – review & editing, Supervision, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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