



Heat-induced interactions between microfluidized hemp protein particles and caseins or whey proteins

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ABSTRACT

The rising demand for sustainable proteins leads to increased interest in plant proteins like hemp protein (HP). However, commercial HP's poor functionality, including heat aggregation, limit its use. This study explored the heat-induced interactions of hemp protein particles (HPPs) with milk proteins, specifically whey proteins and caseins. Using various analysis techniques—static light scattering, TEM, SDS electrophoresis, surface hydrophobicity, and free sulfhydryl content—results showed that co-heating HPPs with whey protein isolate (WPI) or sodium caseinate (NaCN) at 95 °C for 20 min reduced HPPs aggregation. HPPs/WPI particles had a $d_{4,3}$ of ~3.8 μm, while HPPs/NaCN were ~1.9 μm, compared to ~27.5 μm for HPPs alone. SDS-PAGE indicated that whey proteins irreversibly bound to HPPs, through disulfide bonds, whereas casein bound reversibly, possibly involving the chaperone-like property of casein. This study proposes possible mechanisms by which HPPs interact with milk proteins and impact protein aggregation. This may provide opportunities for developing hybrid protein microparticles.

1. Introduction

In the last decade, the application of plant proteins in food products has increased considerably because of a need to balance and diversify overall protein resources and feed a growing world population within planetary boundaries (Akharume et al., 2021). However, the inferior functional properties of most plant proteins have limited their wider implementation as food ingredients (Day, 2013; Hadnadev et al., 2018). To overcome this challenge and meet the requirements of functionality, plant proteins can be combined with dairy proteins under specific conditions to create hybrid protein ingredients (Boland et al., 2013). For example, it has been reported that hemp globulin and sodium caseinate complexes exhibited good emulsification properties, forming stable oil-in-water emulsions (Chuang et al., 2020). Similarly, pea and whey protein mixtures exhibited improved gelling properties in terms of storage modulus and gel hardness compared with pea protein alone (Wong et al., 2013).

Microparticulation of proteins is a technique that produces uniform spherical particles, with sizes often between 0.1 and 10.0 μm. The functional properties of microparticulated proteins depend on their morphology, size, surface and internal features of the particles (Beran et al., 2018). This technique was initially proposed to modify whey

proteins to create protein ingredients with unique properties, including fat-mimetic ability, increased heat stability and protein fortification, without affecting the product quality (Ipsen, 2017). More recently, plant proteins, such as soy and zein, have been explored in this context and proposed as fat replacers for plant-based food products (Nourmohammadi et al., 2023). However, a gap that exists in the literature is the potential of hybrid microparticles produced by combinations of plant and milk proteins, which could extend milk protein applications while facilitating the use of plant proteins in food formulations.

Hempseeds are a good source of high-quality protein with low allergenicity (Callaway, 2004; Wang, Jiang, & Xiong, 2018). Hemp protein (HP) contains about 25 % albumin and 67–75 % globulin (Aluko, 2017). The 11S globulin (edestin) is a homohexamer containing six subunits (52 kDa) linked by non-covalent interactions. Each subunit has a basic subunit (around 18 and 20 kDa) and an acid subunit (around 34 kDa) connected covalently (Potin & Saurel, 2020; Wang, Jin, & Xiong, 2018) and five cysteine residues are found in each subunit. Two cysteine residues form a disulphide bond linking the basic subunit and the acid subunit. Within the acid subunit, two cysteine residues form the intra-chain disulphide bond. The other cysteine residue remains as a free thiol group, which makes HP prone to aggregation under specific conditions

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(Chuang et al., 2019; Docimo et al., 2014; Tang et al., 2006). HP forms large aggregates upon heating above its denaturation temperature (92 °C). This has been attributed to the relatively high free thiol content of edestin, which can undergo thiol-disulphide interchange reactions, resulting in aggregation (Tang et al., 2006). Thus, maintaining the thermal stability of HP during food processing remains a challenge.

Milk proteins contain about 20 % whey proteins and 80 % caseins (Goulding et al., 2020; Singh & Havea, 2003). Whey proteins contain two main fractions, β -lactoglobulin (β -Lg; 54 %) and α -lactalbumin (α -La; 21 %) (Hussain et al., 2012). Whey proteins in their native state are globular proteins with good solubility. However, they unfold and expose buried hydrophobic groups and sulphhydryl groups upon heating above 75 °C. Through the sulphhydryl-disulphide interchange and hydrophobic interactions, unfolded whey proteins link together forming aggregates (Anema, 2020; Dissanayake & Vasiljevic, 2009). In contrast, caseins (containing about 10 % α _{S1}-casein, 40 % α _{S2}-casein, 40 % β -casein and 10 % κ -casein) show excellent heat stability because of their flexible secondary and tertiary structures (Broyard & Gaucheron, 2015). Moreover, caseins can act as chaperones and inhibit the aggregation of globular proteins under stressful environments, such as high temperatures (Yong & Foegeding, 2010). The hydrophobic amino acid side chains of caseins can interact with unfolded whey proteins and the negatively charged group can create repulsion to keep the folded chain flexible (Akbari et al., 2018; Koudelka et al., 2009). Most of this research has been carried out in milk protein systems where caseins (particularly β -casein) have been shown to inhibit heat-induced aggregation of whey proteins (Akbari et al., 2018; Guyomarc'h et al., 2009; Kehoe & Foegeding, 2011, 2014; Koudelka et al., 2009; Liyanaarachchi et al., 2015).

Previous studies reported that the combination of plant proteins with whey proteins may reduce the heat-induced aggregation of plant proteins (Ma et al., 2024). For instance, co-heating soy 11S globulin with β -Lg (Anuradha & Prakash, 2009) and pea globulin with β -Lg (Chihl et al., 2016) led to the formation of smaller aggregates compared with individually heated soy and pea globulin. However, only one study has reported that the addition of sodium caseinate inhibits heat-induced aggregation of molecular HP globulin and increase protein solubility in a high-salt environment (Chuang et al., 2019). From these studies, it appears that both whey proteins and caseins can restrict the heat-induced aggregation of the above-mentioned plant proteins via different mechanisms.

However, the main limitation of the previous studies is that they described heat-induced interactions using relatively pure isolated protein fractions. Particularly, for HP/milk protein mixtures, there is no information on protein-protein interactions using commercial protein ingredients. The processing conditions used in the production of commercial protein ingredients, particularly plant proteins, often cause extensive denaturation and aggregation of proteins. As a result, the commercial HP ingredients have poor solubility in water. In this study, therefore HP dispersions were first microfluidized to generate hemp protein particles (HPPs) and then their heat-induced interactions with milk proteins (whey protein isolate, WPI or sodium caseinate, NaCN) were studied. The aim of the study was to provide new knowledge that can be used to produce new functional HP/milk protein microparticles for food applications.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) containing 92.0 % protein, 0.9 % fat, 1.6 % ash and 5.2 % moisture and sodium caseinate (NaCN) containing 92.3 % protein, 0.6 % fat, 4.0 % ash and 4.8 % moisture were purchased from Fonterra Co-operative Group Limited, Auckland, New Zealand. The hempseed protein (HP) concentrate powder was purchased from a local supermarket (Davis Trading Company Ltd., Palmerston North, New Zealand). All chemicals were purchased from Sigma-Aldrich Ltd. (St.

Louis, MO, USA), and the reagents were made up in Milli-Q water (Milli-Q apparatus; Millipore Corp., Bedford, MA, USA).

2.2. Proximate analysis

The proximate composition of HP was analysed as follows: protein content was determined using the Kjeldahl method (AOAC 991.20, nitrogen factor 5.21); fat, ash and moisture content were determined according to AOAC 922.06, AOAC 942.05 and AOAC 925.10, respectively; and carbohydrate content was calculated by subtracting the sum of the protein, ash and fat from the total solid. The HP powder contained 59.8 % protein, 2.4 % fat, 10.7 % ash, 6.8 % moisture and 20.2 % carbohydrate.

2.3. Preparation of hemp protein particles

A dispersion of HP was prepared by mixing HP powder in Milli-Q water at 1 % (w/w) protein concentration and stirring for 2 h at 20 °C. The pH was adjusted to 11, using 1 M NaOH, followed by 2 h stirring. The HP dispersion was centrifuged (3000g, 30 min) to remove insoluble material (such as insoluble fibre). The resulting supernatant (mostly containing HP) was collected and adjusted to pH 7 using 1 M HCl. The dispersion composed by the supernatant was processed by microfluidization (M-110P, Microfluidics, Newton, MA, USA) at 200 MPa and 2 passes to produce HP particles (HPPs) that were used in the mixtures with milk proteins.

2.4. Preparation of HPPs/WPI and HPPs/NaCN mixtures

Stock solutions of WPI or NaCN were prepared by dissolving 1 % (w/w) protein in Milli-Q water with magnetic stirring for 2 h. HPPs dispersion was mixed with WPI or NaCN to achieve a final protein concentration in the mixture at 0.25 % HP and 0.25 % WPI or NaCN. The pH of the protein mixtures was adjusted to pH 7 using 1 M NaOH, followed by heating at 95 °C for 20 min in the water bath. The heating condition was selected based on our previous study that suggested the most interactions between HP and β -Lg happened in the first 20 min. After the heat treatment, the samples were cooled down in ice to 20 °C immediately. Sodium azide (0.02 %, w/w) was added to inhibit microbial growth.

2.5. Particle size analysis

The particle size of unheated HPPs and heated HPPs/WPI and HPPs/NaCN mixtures was measured by static light scattering using a Mastersizer 2000 and a Hydro MU unit (Malvern Instruments, Worcestershire, UK). The refractive indices of hemp protein and water were 1.45 and 1.33, respectively. The data was reported in volume-weighted mean diameter $d_{(4,3)}$, calculated as the average of triplicate measurements.

2.6. Transmission electron microscopy

Sample preparation and negative staining for transmission electron microscopy (TEM) were performed as described by Vincekovic et al. (2014). An aliquot of 80 μ L of protein solution was placed on a formvar/carbon-coated 200 mesh copper grid for 4 min. The excess sample was removed by filter paper. 80 μ L of uranyl acetate (2 %, w/w) was placed on the grid for another 4 min and the excess staining solution was removed with filter paper. The stained sample was imaged at 6000 \times , 16500 \times and 20,500 \times magnification by a transmission electron microscope (Philips CM10) (Eindhoven, the Netherlands) at 100 kV.

2.7. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protein composition was analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a Tris-

glycine gel under non-reducing and reducing conditions as per the protocol described by Dave et al. (2019) and Manderson et al. (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. Ten microlitre samples 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 by the molecular imager Gel Doc XR (Bio-Rad Laboratories, Richmond, CA, USA) and analysed by ImageLab software.

2.8. Free sulphhydryl content

Free sulphhydryl (SH) contents of unheated and heated HPPs, WPI, NaCN and their mixtures were measured using Ellman's reagent [5,5'-dithiobis (2-nitrobenzoic acid); DTNB] as described by Xu et al. (2022) with slight modifications. Protein samples (1 mg/mL) were mixed with Tris-glycine buffer (pH 8) containing 86 mM Tris, 90 mM glycine, 40 mM EDTA, and 8 M urea. Ellman's reagent (4 mg/mL) was also made in Tris-glycine buffer. A 1.5 mL sample was mixed with 20 µL Ellman's reagent followed by 30 min incubation. The absorbance of resulting 5-nitro-2-thiobenzoic acid (TNB) chromogen was measured by a spectrophotometer (Genesys 10-S; Thermo Fisher Scientific Inc., USA) at a wavelength of 412 nm. The Tris-glycine buffer with Ellman's reagent was used as blank.

Free SH content was calculated using Eq. (1):

$$\text{SH } (\mu\text{mol/g}) = (10^6 \times A \times D) / (1.36 \times 10^4 \times C) \quad (1)$$

where A is the absorbance value, C is the protein concentration, D is the dilution factor, 1.36×10^4 is the molar absorptivity of TNB and 10^6 is the conversion from molar basis to µmol/mL basis.

2.9. Surface hydrophobicity (H_0)

The surface hydrophobicity (H_0) of unheated and heated HPPs, WPI, NaCN and their mixtures was measured using 8-aniline-1-naphthalene sulfonic acid (ANS) as a fluorescent probe (Chihi et al., 2016). The protein sample was diluted in 10 mM sodium phosphate buffer (pH 7) to obtain protein concentrations ranging from 0.0004 to 0.02 %. Then 20 µL ANS solution (8 mM) was added to a 4 mL protein sample and incubated for 15 min in the dark. The fluorescence intensity was measured using a spectrofluorometer (FP-6500, JASCO, Tokyo, Japan) with an excitation wavelength of 390 nm and an emission wavelength of 470 nm.

The H_0 was calculated as the initial slope of fluorescence intensity versus protein concentration by linear regression.

2.10. Heat stability of the HPPs/WPI and HPPs/NaCN mixtures

45 mL heated HPPs/WPI and HPPs/NaCN mixtures as described in Section 2.4 were centrifuged (20,000g, 15 min) to collect true hybrid microparticles and remove the unbonded free protein. To test the heat stability of the HPPs/WPI and HPPs/NaCN particles and eliminate the impact of supernatant protein, the pellet was re-dispersed in Milli-Q water to reach the same volume as before centrifugation. These dispersions were reheated for a second cycle at 95 °C for 20 min using a water bath and the particle size was measured, as described in Section 2.4.

2.11. Data 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 between two groups, and one-way analysis of variance (ANOVA) for multiple comparisons, using Tukey's test with the level of significance set at $p < 0.05$.

3. Results and discussion

3.1. Particle size distributions of HPPs/milk protein dispersions

The effect of heat treatment on the particle size of different protein mixtures was investigated and the particle size distributions and volume-weighted mean diameter ($d_{4,3}$) are shown in Fig. 1. As can be seen from the size distributions (Fig. 1A), the unheated HPPs dispersion had a bimodal particle size distribution characterised by two peaks at 0.1–1 µm and 1–10 µm indicating the heterogeneity of the HPPs even after microfluidization. The $d_{4,3}$ value of HPPs was about 2.0 µm (Fig. 1B).

Heat treatment at 95 °C resulted in the formation of large-size HPPs aggregates as evidenced by a shift of the peaks to the right and a large population of particles located in the 1–100 µm particle size range (Fig. 1A), with a $d_{4,3}$ of 27.5 µm (Fig. 1B). In native HP, heat-induced aggregation was expected because of the large percentage of globulin in its composition (60–80 %), which is a group of proteins sensitive to heat (Wang & Xiong, 2019). In particular, it has been reported that HP heated at temperatures ≥ 80 °C results in extensive aggregation (Raikos et al., 2015) due to the high content of cysteine residues (Wang & Xiong, 2019) and disulphide bond formation. In our study, although the manufacturing process for hemp protein powder and the microfluidization process used for creating HPPs may have changed the protein structure, we hypothesise that the heat treatment was still able to modify the surface structure of HPPs and expose the free thiol groups and hydrophobic groups for further particle-particle interactions.

Interestingly, the heated HPP/WPI dispersion had a greater population of particles located between 0.1 and 10 µm (Fig. 1A) and the $d_{4,3}$ was much smaller (3.8 µm) (Fig. 1B) than the heated HPP. The relatively low degree of aggregation observed in heated mixtures compared with heated HPPs dispersions suggests that whey proteins could restrict aggregation of HPP (Ma et al., 2024). In other plant protein/whey protein systems, similar phenomena have been observed (Anuradha & Prakash, 2009; Chihi et al., 2016).

On the contrary, heating of HPPs in the presence of NaCN resulted in a particle size distribution similar to that of unheated HPPs (Fig. 1A) with $d_{4,3}$ of 1.9 µm (Fig. 1B). This indicates that the caseins were also able to inhibit HPPs aggregation during heating. A similar effect has been seen in recent studies using soluble pea proteins and casein micelles combinations, and HP globulin/NaCN mixtures (Beghdadi et al., 2022; Chuang et al., 2019). Chuang et al. (2019) reported the addition of 0.05 % NaCN in 0.1 % molecular HP globulins at a high salt concentration (0.5 M ionic strength) could form small intermediate aggregates and delay the formation of large aggregates. It has also been shown that NaCN restricts heat-induced aggregation of whey proteins (Morgan et al., 2005). For instance, mixing α_{S1} -casein with whey protein (1: 1) reduced whey protein aggregation during thermal treatments and this is due to casein's chaperone effect (Bhattacharyya & Das, 1999). Liyanaarachchi et al. (2015) also reported that casein can govern the aggregation of whey protein and prevent the denaturation of whey protein during heat treatment, owing to its chaperone-like activity. Therefore, it is likely that NaCN could have restricted the aggregation of HPPs via a chaperone-like effect. This will be discussed in detail in Section 3.3.

3.2. Morphology of HPPs and HPPs/milk protein dispersions

The morphology of HPPs, WPI, NaCN and their mixtures (HPPs/WPI and HP/NaCN) was analysed by transmission electron microscopy (TEM) (Fig. 2). TEM images of unheated HPPs showed the presence of large aggregates with an irregular shape that seems to be composed of

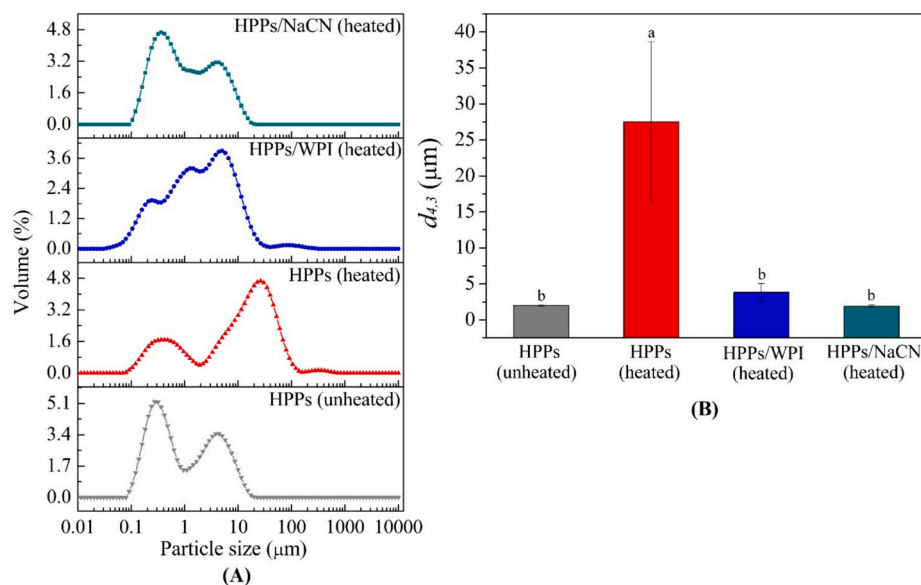


Fig. 1. Particle size distributions (A) of hemp protein particles (HPPs) (unheated and heated, 95 °C for 20 min), HPPs/whey protein isolate (WPI) (heated) and HPPs/sodium caseinate (NaCN) (heated) dispersions and (B) their corresponding volume-weighted mean diameter ($d_{4,3}$, μm). Different lowercase letters indicate significant differences ($p < 0.05$).

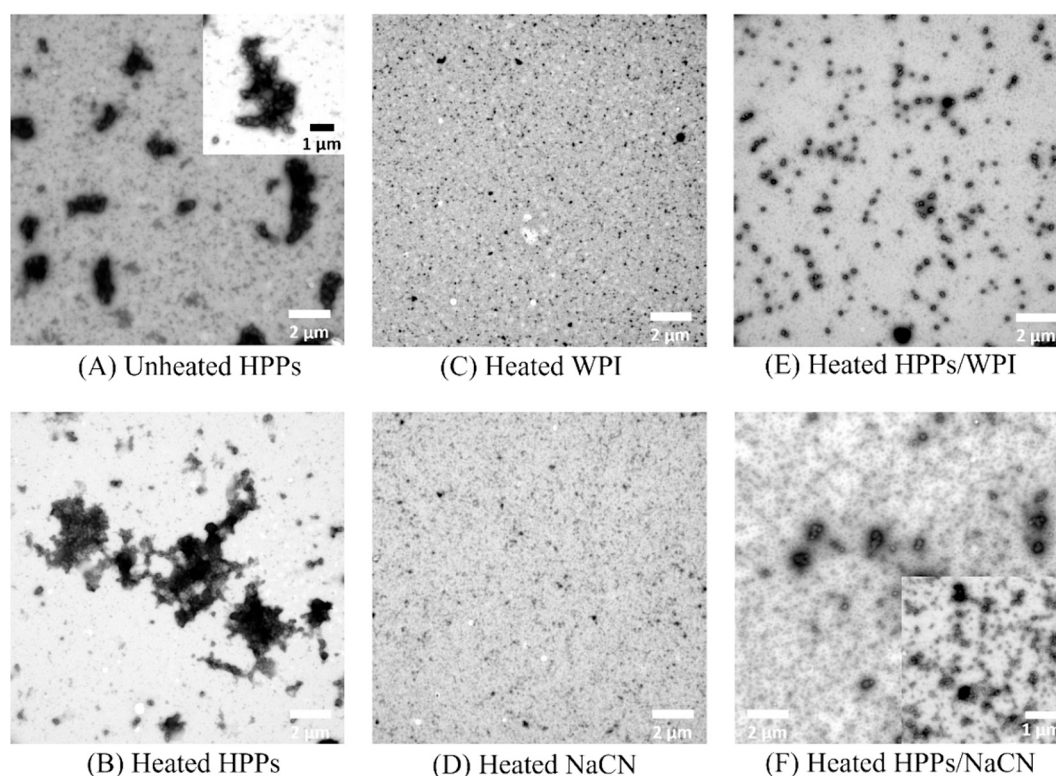


Fig. 2. Transmission electron microscopy of unheated/ heated hemp protein particles (HPPs) and heated whey protein isolate (WPI), sodium caseinate (NaCN) and their mixture with HPPs.

smaller protein particles, as seen in Fig. 2A. Heat treatment led to the formation of even larger and amorphous HPPs aggregates (Fig. 2B), which is consistent with particle size analysis showing that heat treatment induced a considerable increase in the volume-weighted mean diameter ($d_{4,3} = 27.5$ μm).

The heated WPI and NaCN on the other hand showed well-dispersed small particles (Fig. 2C, D). Co-heating HPPs and WPI resulted in spherical particles (Fig. 2E), which appear to be smaller than those

observed in heated HPPs but much larger than the particles in the heated WPI, which could indicate that both proteins could have participated in the microparticles formation. On the contrary, there were two main types of aggregates/particles observed in the heated HPPs/NaCN mixture (Fig. 2F); large aggregates that resemble HPPs-rich particles and small aggregates that could be casein-rich particles. This observation will be discussed further in the following sections.

3.3. Protein composition of HPPs and HPPs/milk protein dispersions

To analyse the protein interactions in HPPs/WPI and HPPs/NaCN dispersions before and after the heat treatment, SDS-PAGE was conducted on the dispersions and their corresponding supernatant and sediment fractions obtained by centrifugation. Fig. 3 shows the SDS-PAGE protein patterns obtained from HPPs/WPI dispersions before and after heating under non-reducing conditions (Fig. 3A) and reducing conditions (Fig. 3B).

Under non-reducing conditions (Fig. 3A), the composition of the unheated HPPs/WPI (lane 2) was characterised by the presence of a thick band at 52 kDa that corresponded to 11S globulins, a predominant protein in HP that is known to have six non-covalently linked subunits (Shen et al., 2021). Other predominant bands identified below 20 kDa were β -Lg and α -La, the two main proteins in WPI (Singh, 2009) (lane 1, Fig. 3A). In the unheated mixture, proteins from HPPs and WPI could be separated upon centrifugation, observing that the whey proteins remained in the supernatant (lane 3, Fig. 3A), while HPPs migrated into the sediment. No clear bands were seen from HP in the gel, probably

because the sediment was not fully solubilized in the non-reducing buffer (lane 4, Fig. 3A). After heating, the total and supernatant fractions had a similar composition (lanes 5 and 6, Fig. 3A) mainly consisting of whey proteins (β -Lg and α -La) and albumins from HP (~10 kDa). It is well studied that heat (above 70 °C) can promote the formation of β -lg dimers (Boland, 2011; Wijayanti et al., 2014). Hence, it was also possible to observe β -lg dimers (~35 kDa) that are formed within this temperature/time settings (Schokker et al., 1999).

Under reducing conditions (Fig. 3B), there were a significantly larger number of bands in the total, supernatant and sediment before and after heating. Under reducing conditions, disulphide bonds are reduced due to the presence of β -mercaptoethanol, and SDS breaks down the hydrophobic interactions. Therefore, all protein aggregates are separated into their individual monomeric protein forms. For instance, the total fraction of the unheated HPPs/WPI dispersion showed 5 main predominant bands (lane 2, Fig. 3B); three from HPPs and two from WPI. The band at 34 kDa corresponded to the acidic subunit of 11S globulin in HP (edestin), and two bands at 18 and 21 kDa were linked to their basic subunits. It is known that the disruption of disulphide bonds of edestin

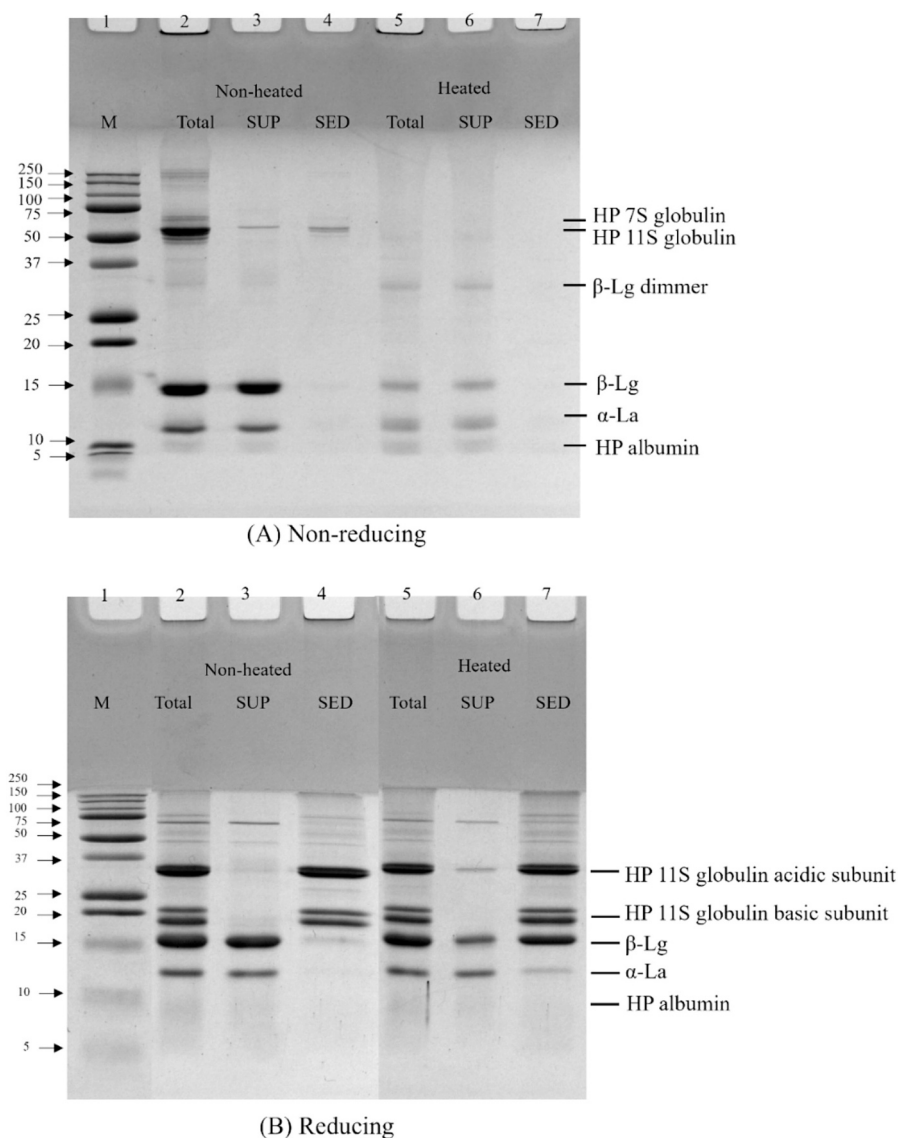


Fig. 3. SDS-PAGE under (A) non-reducing conditions and (B) reducing conditions of heated (95 °C for 20 min) and unheated hemp protein particles/whey protein isolate (HPPs/WPI) dispersion and their supernatant and sediment. Lanes are: 1, marker; 2, unheated HPPs/WPI dispersion; 3, supernatant from unheated HPPs/WPI dispersion; 4, sediment from unheated HPPs/WPI dispersion; 5, heated HPPs/WPI dispersion; 6, supernatant from heated HPPs/WPI dispersion; 7, sediment from heated HPPs/WPI dispersion.

cause the separation of their elementary basic and acid units (Potin & Saurel, 2020; Wang, Jin, & Xiong, 2018). In addition, β -Lg and α -La bands (18 and 14 kDa, respectively) were also observed in the total fraction (lane 2, Fig. 3B). After centrifugation, the supernatant (lane 3, Fig. 3B) was mainly composed of whey proteins (β -Lg and α -La), whereas the sediment fraction (lane 4, Fig. 3B) was rich in HP globulins.

When the HPPs/WPI mixture was heated, the intensity of both β -Lg and α -La bands in the supernatant (lane 6, Fig. 3B) was considerably reduced compared with the total fraction (lane 5), suggesting possible interactions between HPPs and WPI. However, a proportion of β -Lg and α -La also remained in supernatant (lane 6, Fig. 3B). In contrast, the sediment fraction (lane 7, Fig. 3B) from heated HPPs/WPI showed bands related to 11S globulin (acid and basic units) β -Lg and a faint α -La band. This result suggests that heat-induced interactions preferentially occur between HPPs and β -Lg and to a lesser degree between HPPs and α -La, as indicated by the great proportion of α -La remaining in the supernatant fraction.

Whey proteins are globular proteins in their native state, the sulphhydryl groups are buried within the tertiary structure (Anema, 2020; Hoffmann & van Mil, 1997). Heat treatment ruptures the forces that

hold the globular structure and exposes the sulphhydryl groups, resulting in thiol oxidation and thiol/disulphide exchange to form disulphide bridges between different protein molecules and eventually resulting in large aggregates (Anema, 2020; Singh & Havea, 2003). In the presence of HPPs, denatured whey proteins may either self-aggregate or interact with the disulphide bonds on the surface of the HPPs to generate HPPs/WPI hybrid particles. This interaction could also explain the smaller particle size in the mixture compared with heated HPPs. It is possible that the reactive groups on the surface of HPPs were occupied by denatured whey proteins, which led to fewer sites for HPPs to self-aggregate. In a milk system, Corredig and Dalgleish (1999) found that the amount of β -Lg binding with the casein micelles during heat treatment of milk was limited by the available active sites on the micelles. In addition, the improved thermal stability of microparticulated whey proteins was revealed because there were little or no reactive sites available for aggregation after heating (Ryan et al., 2012; Shi et al., 2021). In this study, the binding of denatured whey protein on the surface of HPPs may have acted as a barrier limiting the growth of HPPs, but the location of whey proteins in the particles needs to be confirmed with further studies.

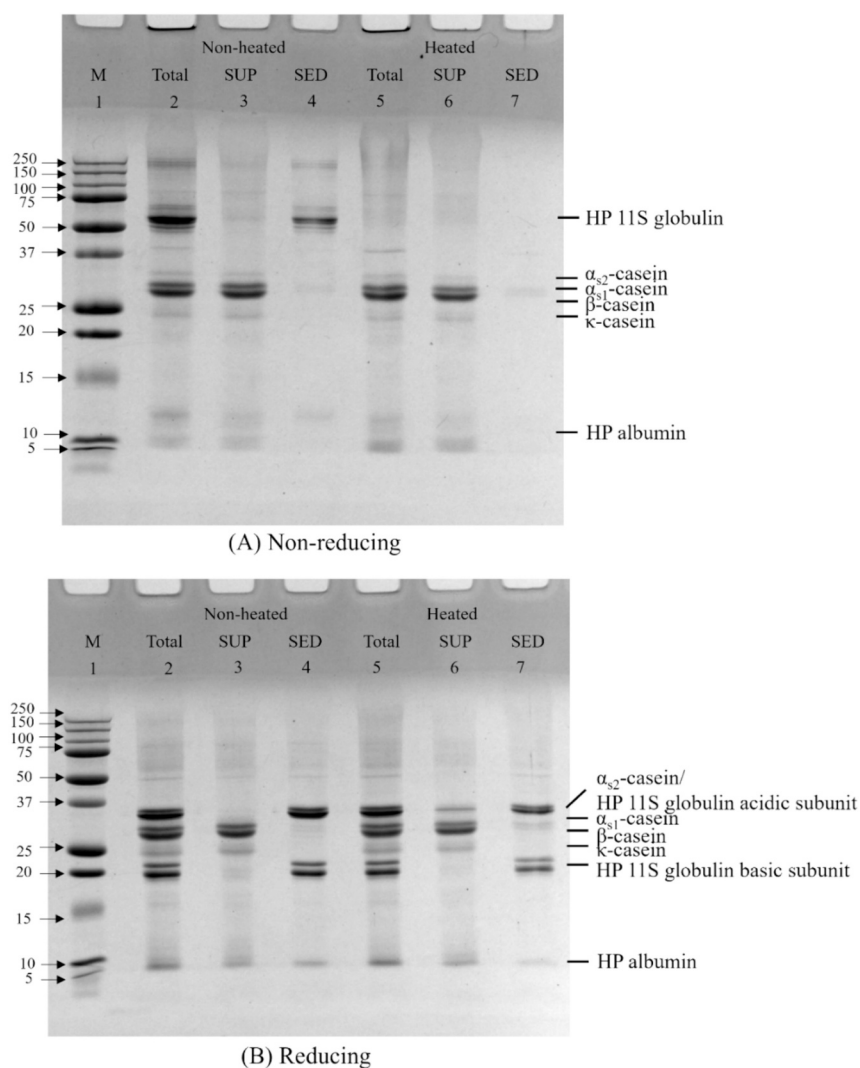


Fig. 4. SDS-PAGE under (A) non-reducing conditions and (B) reducing conditions of heated (95 °C for 20 min) and unheated hemp protein particles/sodium caseinate (HPPs/NaCN) dispersions, the total, supernatant and sediment fractions. Lanes are: 1, marker; 2, unheated HPPs/NaCN dispersion; 3, supernatant from unheated HPPs/NaCN dispersion; 4, sediment from unheated HPPs/NaCN dispersion; 5, heated HPPs/NaCN dispersion; 6, supernatant from heated HPPs/NaCN dispersion; 7, sediment from heated HPPs/NaCN dispersion.

It is worth noting that although α -La interacted with HPPs, the proportion of interacted α -La was less than the proportion of interacted β -Lg. α -La is not able to form aggregates when heated alone since it lacks free thiol groups (Wijayanti et al., 2014). In heated milk, α -La firstly forms aggregates with β -Lg, then associates with casein micelles (Anema & Li, 2003). A similar scenario may occur in heated HPPs/WPI mixtures, although this association behaviour may vary with heating conditions (such as heating rate, temperature and pH) (Anema, 2021).

Fig. 4 shows the SDS-PAGE patterns obtained from HPPs/NaCN dispersions before and after heating and under non-reducing conditions (Fig. 4A) and reducing conditions (Fig. 4B). Under non-reducing conditions, α _{S1}- and β -caseins were observed as the two most predominant protein bands in NaCN (lane 1, Fig. 4A), while α _{S2}- and κ -caseins presented as two faint bands. Similar to WPI and HPPs mixtures, the unheated NaCN and HPPs could be separated upon centrifugation, with NaCN in the supernatant and HPPs in the sediment (lanes 2 and 3, Fig. 4). After the heat treatment, the casein bands remained in the supernatant (lane 6, Fig. 4A), while the HP bands could not be seen in the sediment under non-reducing conditions (lane 7, Fig. 4A). This indicates that HP particles formed aggregates with very large molecular weights that could not enter the stacking gel.

However, under the reducing conditions (Fig. 4), the HPPs bands were clearly visible in the sediment after heating, indicating inter-HPPs aggregation involved new disulphide linkages (lane 7, Fig. 4B). Interestingly, in contrast to whey proteins (Fig. 3), no casein protein was observed in the sediment of the heated HPPs/NaCN mixture (lane 7, Fig. 4B), suggesting caseins did not form stable complexes with HPPs during heat treatment.

In contrast to HPPs/WPI complexation, the mechanism of restricting the aggregation of HPPs in the presence of NaCN could be attributed to the molecular chaperone-like properties of caseins (especially α _{S1}- and β -caseins) that could prevent protein aggregation under unfavourable conditions (Kehoe & Foegeding, 2011; Yong & Foegeding, 2010). Akbari et al. (2018) suggested that hydrophobic interactions are the driving force for casein self-assembling and the first interaction between caseins and their substrate proteins. The high number of proline residues in both α _{S1}- and β -caseins gives them flexible, open and disordered structures that facilitate the binding with other target proteins (Trewick et al., 2011). The steric hindrance provided by caseins could prevent further protein aggregation (Chuang et al., 2019). Chuang et al. (2019) proposed the hydrophobic groups from α _{S1}- and β -casein interacted with other HP globulin molecules through hydrophobic interaction. In terms of the caseins and HPPs interactions in this study, it is possible that heat treatment altered the surface structure of HPPs to expose more hydrophobic groups which could interact with caseins, hence preventing the aggregation of HPPs.

However, it is widely accepted that as temperature decreases, the magnitude of hydrophobic interactions would be decreased (Dias et al., 2010; McClements & Keogh, 1995; Scheraga et al., 1962). Sun et al. (2022) also highlighted that temperature effects the hydrophobic interactions, which in turn influences protein stability. It was found that in NaCN, caseins would aggregate through hydrophobic interactions during heating, but would dissociate upon cooling (Chuang et al., 2019; Hadjsadok et al., 2008). Kehoe and Foegeding (2014) found that there was no evidence to prove that stable β -caseins/ β -Lg complex was formed after cooling. For this reason, in the HPPs/casein mixture, caseins are likely to dissociate from the HPPs during cooling.

Based on the particle size results and SDS-PAGE analysis, it could be inferred that both WPI and NaCN had the ability to decrease the extent of heat-induced aggregation of HPPs, but this effect may be achieved through different mechanisms. WPI would bind with the HPPs and form stable complexes, involving the formation of new disulphide bonds. However, the covalent interactions were absent in the HPPs/caseins interactions. It appears that hydrophobic interactions occurred at high temperatures to prevent HPPs aggregation, but these interactions were diminished at low temperatures. Hence, no stable complexes were found

when the mixture was cooled to room temperature.

3.4. Free sulphhydryl groups and surface hydrophobicity

The changes in free sulphhydryl (SH) groups of individual protein ingredients and their mixtures before and after heating are shown in Fig. 5A. Measuring the changes in free SH groups could provide us with useful information about the conversion of free SH to disulphide linkages during heating.

The HPPs dispersion contained 19 μ mol/g free SH groups which after heating decreased to 7 μ mol/g, suggesting either intra-particle or inter-particle disulphide bonds might have been formed. In the milk protein dispersions, the free SH content of unheated WPI was 41 μ mol/g in agreement with the value previously reported for WPI (37 μ mol/g) (Xu et al., 2022). A reduction in free SH groups after heating was observed; this could be attributed to the denaturation of whey proteins in which thiol groups were exposed and interacted to form new disulphide bonds (Boland, 2011). In contrast, unheated NaCN had 1 μ mol/g free SH groups and there was no significant change after heating. This was expected because the main casein molecules in NaCN (α _{S1}- and β -casein) do not contain free SH groups (Holt et al., 2013).

In HPPs/WPI dispersions, heat treatment led to a decrease in free SH groups (Fig. 5A). This is because of either thiol-disulphide exchange/sulphydryl oxidation between HPPs and unfolded whey proteins, or the intra- or inter-self-interactions in HPPs or whey proteins. A similar reduction in -SH groups was also reported in other protein mixtures of co-heated whey proteins and other plant proteins. For instance, Chihi et al. (2016) observed a decrease in the free SH group in a heated pea globulin/ β -Lg mixture indicating the disulphide bond formation.

The HPPs/NaCN dispersions also showed a decrease in free SH groups after heating (Fig. 5A). This would be mainly contributed by the intra- or inter-interaction of HPPs since no covalent link was formed between HPPs and caseins (Fig. 4). Both free SH groups of HPPs/WPI and HPPs/NaCN after heat treatment were very close to their calculated theoretical values, which are the averages of individually heated proteins. This may suggest that the amount of available and reacted free SH groups in individual heated protein or heated protein mixtures were similar. It is possible that during the heat treatment of mixed protein, the exposed free SH groups of the individual proteins also participated in either self-interactions or interacted with other proteins.

The surface hydrophobicity values of HPPs, WPI, NaCN and their mixtures are shown in Fig. 5B. The determination of surface hydrophobicity could offer some additional information on the change of protein structure during heating and indicate possible hydrophobic interactions. The surface hydrophobicity of HPPs increased significantly after heating in accordance with the increased surface hydrophobicity in heated HP globulins. The surface hydrophobicity of whey proteins increased from 2800 to 4316 after heating while that of NaCN remained steady at 1600–1700 (Fig. 5B). This trend was consistent with other studies on the surface hydrophobicity of NaCN (Chuang et al., 2019) and whey proteins (Hussain et al., 2012; Ryan et al., 2012) before and after heating. It is expected that some of the buried hydrophobic groups would be exposed during the unfolding of the native globular structure of whey proteins under heat treatment and could contribute to the increase of surface hydrophobicity (Comfort & Howell, 2002; Ryan et al., 2013). As caseins have no well-defined tertiary structure there would be no change expected in surface hydrophobicity after heat treatment (Bryant & McClements, 1998).

The surface hydrophobicity of the heated HPPs/WPI mixture also increased, but the values were lower than the calculated theoretical value of the heated HPPs/WPI mixture possibly because of the lack of interactions. The lower actual surface hydrophobicity value suggests that HPPs and WPI interacted with each other via hydrophobic interactions rather than binding with fluorescence probes (ANS). Chihi et al. (2016) reported an increased surface hydrophobicity value of the pea protein/ β -Lg mixture after heating and they proposed both covalent

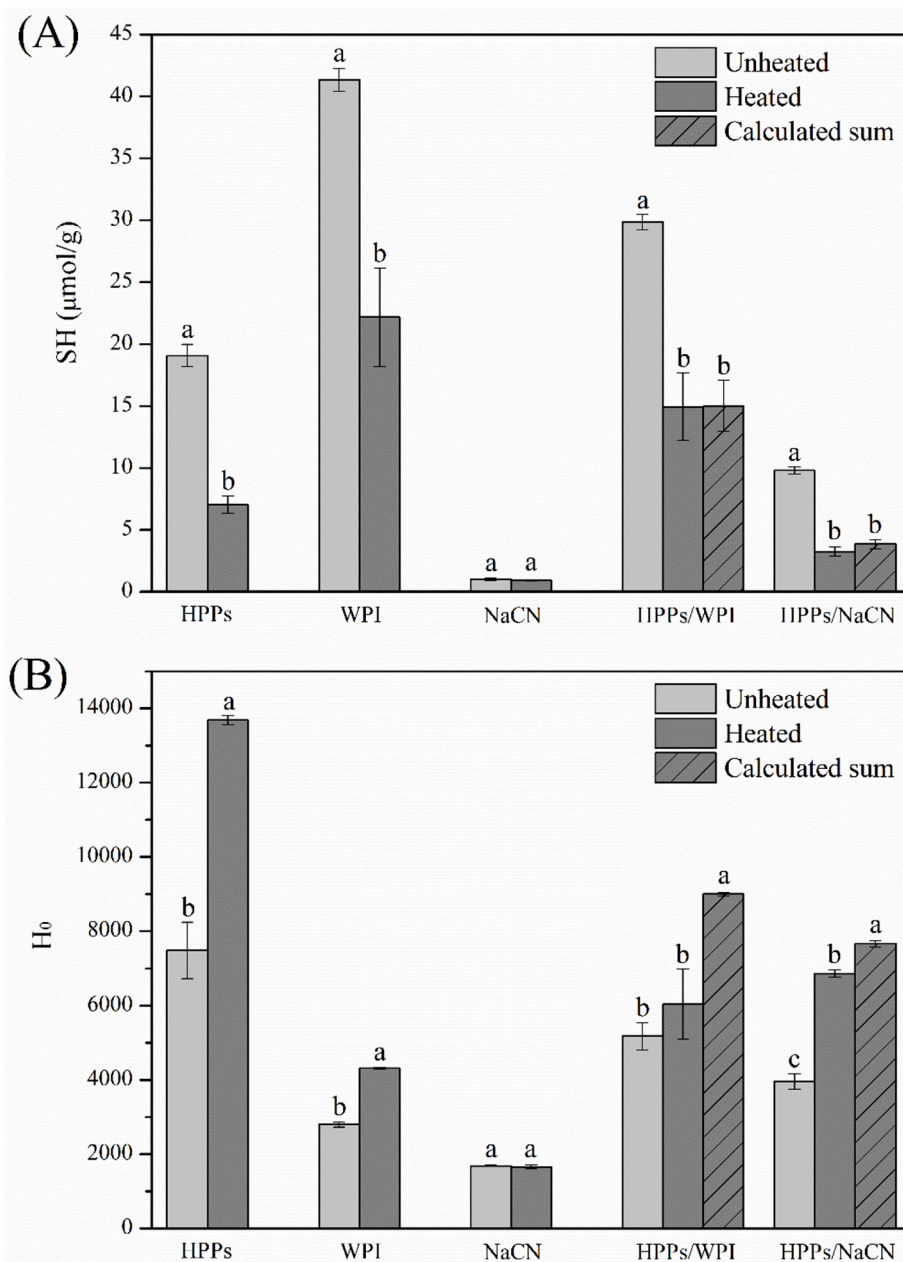


Fig. 5. Free sulphhydryl (SH) groups (A) and surface hydrophobicity (H_0) (B) of unheated (light grey bar) and heated (dark grey bar) hemp protein particles (HPPs), whey protein isolate (WPI), sodium caseinate (NaCN), HPPs/WPI and HPPs/NaCN dispersions, and the calculated theoretical value (dark grey bar with stripes pattern) of heated HPPs/WPI and HPPs/NaCN mixtures, assuming no interactions. Different lowercase letters indicate significant differences of each sample before and after heating ($p < 0.05$).

and non-covalent interactions helped the formation of protein aggregates. Gong et al. (2019) suggested that an increase in the surface hydrophobic groups may facilitate the formation of disulphide bonds during aggregation. Hence, hydrophobic interactions could be another driving force for creating the HPPs/WPI aggregates.

On the other hand, considering the low surface hydrophobicity of NaCN, the value of the HPPs/NaCN mixture had a greater extent of increase after heating compared with HPPs/WPI. In contrast to HPPs and WPI mixture, the increase of surface hydrophobicity was mainly contributed by HPPs. Compared with the calculated sum of HPPs/NaCN after heating, there was a slight decrease in actual surface hydrophobicity, and the extent of reduction was less than that of HPPs/WPI. The reduction of surface hydrophobicity may be attributed to some interaction between HPPs. This explanation could be supported by a finding

from a study of the β -casein/ β -Lg system in which β -Lg dominated the increase of surface hydrophobicity. The surface hydrophobicity of heated β -casein/ β -Lg was only slightly lower than the expected value (Kehoe & Foegeding, 2014). They suggested that β -casein bound with β -Lg to improve the stability during heating and the complex might dissociate after cooling (Kehoe & Foegeding, 2011, 2014). A similar phenomenon would be expected to occur in HP and NaCN mixtures if we assume temperature-dependent reversible hydrophobic interactions between these proteins.

3.5. Thermal stability of HPPs/milk protein hybrid particles

The heated HPPs/WPI and HPPs/NaCN mixtures resulting in hybrid particles were cooled to room temperature and then reheated (95 °C for

20 min) to assess their thermal stability. As can be seen in Fig. 6A, the particle size distributions of HPPs/WPI and HPPs/NaCN particles after heating were very similar to the original size distribution of the original protein mixtures, suggesting that these particle protein systems were heat-stable under the conditions tested.

The protein particles and soluble proteins of heated HPPs/WPI and HPPs/NaCN mixtures were separated by centrifugation. The dispersion of sediment was then re-heated to assess the heat stability of the hybrid particles themselves. The particle size distribution of re-dispersed sedimentable fractions from HPPs/WPI and HPPs/NaCN dispersions are shown in Fig. 6B. Firstly, results showed that re-dispersed particles from

heated HPPs/WPI and HPPs/NaCN dispersions were slightly larger compared with the dispersions prior to centrifugation (Fig. 6A), which was expected because centrifugation can induce particle aggregation. Secondly, the particle size distribution of the re-dispersed HPPs/WPI did not change significantly after re-heating, which could be interpreted as if the proteins in the supernatant did not contribute to the thermal stability of the HPPs/WPI microparticles.

Microparticulated whey protein exhibits enhanced heat stability since the reduction of free thiol groups led to limited interactions among whey protein microparticles (Dissanayake & Vasiljevic, 2009). In this study, during the denaturation of whey proteins and the formation of

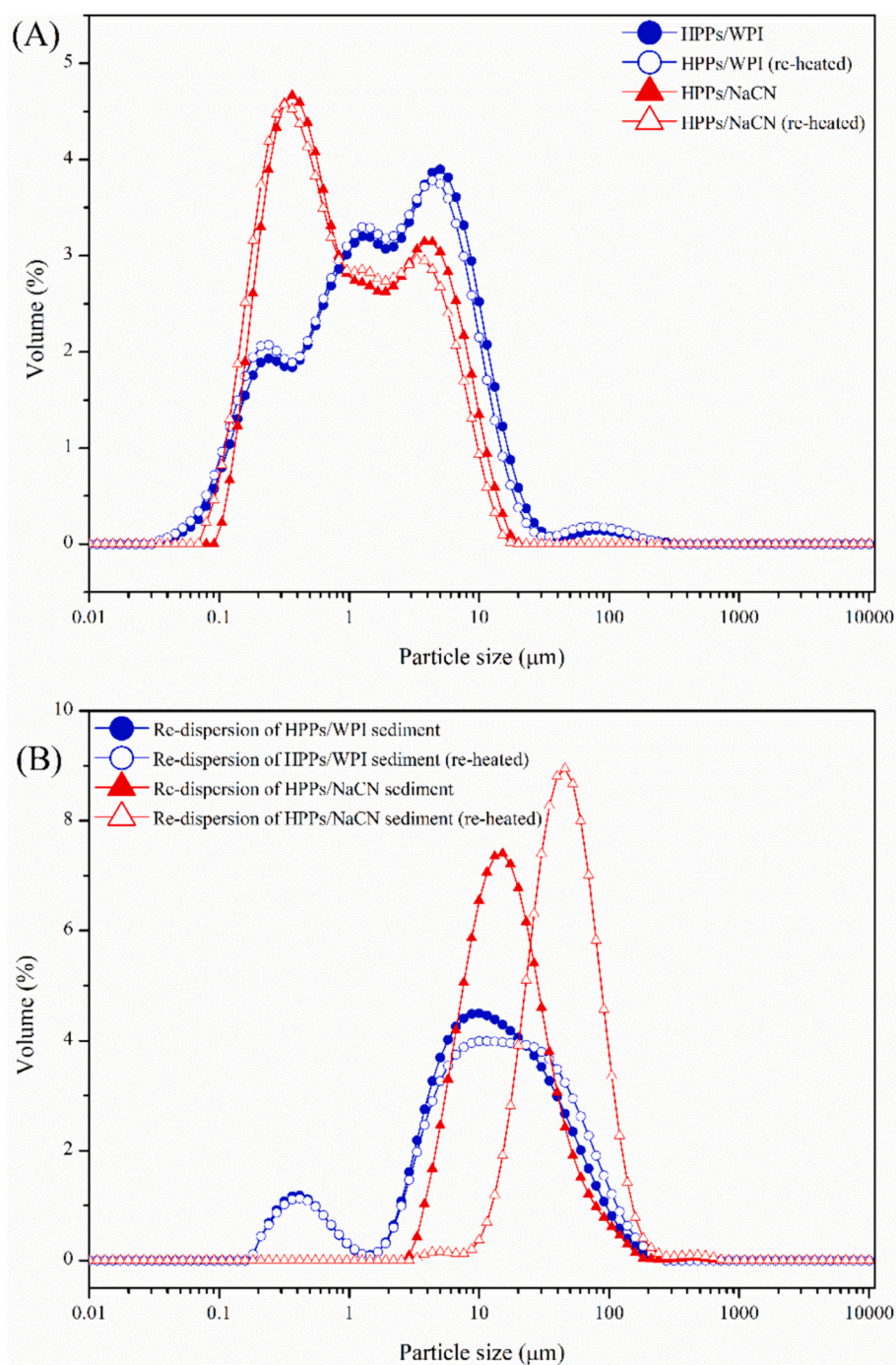


Fig. 6. Particle size distributions of (A) heated hemp protein particles/whey protein isolate (HPPs/WPI) and heated hemp protein particles/sodium caseinate (HPPs/NaCN) dispersions and their re-heated (95 °C for 20 min) dispersions; and of (B) the re-dispersion of the sediment collected from heated HPPs/WPI and HPPs/NaCN, and their re-heated (95 °C for 20 min) dispersions.

irreversible HPPs/WPI microparticles, it was expected that most of the exposed reactive groups would have already interacted with each other, which resulted in fewer groups available during the subsequent heat treatment.

Interestingly, the particle size distribution of the re-dispersed sedimentable fraction obtained from HPPs/NaCN after re-heating showed significantly larger particle populations from $\sim 20\ \mu\text{m}$ to $\sim 60\ \mu\text{m}$. We could speculate that the HPPs/NaCN particles were reversible because the hydrophobic interactions were reduced at low temperatures (Chuang et al., 2021; Sun et al., 2022). The dissociated HPPs were susceptible to heating and self-aggregated without the chaperone-like action provided by caseins after removing soluble NaCN.

3.6. Hypothetical mechanism of heat-induced interactions of HPPs/WPI and HPPs/NaCN

According to all the results above, a possible mechanism of heat-induced interactions of each HP/milk protein mixture could be depicted (Fig. 7). Heat treatment exposes the free SH groups and hydrophobic groups on the surface of hemp protein particles. Meanwhile, heat treatment leads to the unfolding of $\beta\text{-lg}$ in WPI to expose its free SH groups (Anema, 2020; Singh & Havea, 2003).

When the HPPs and WPI are co-heated (Fig. 7A), the denatured whey proteins may either self-aggregate or interact with the surface of the HPPs. This interaction may involve disulphide bond formation between hemp and whey protein involving sulphhydryl-disulphide interchange reactions. The association of WPI on the surface of HPPs could restrict the further aggregation of HPPs since denatured WPI could occupy some of the reactive sites on the surface of HPPs. The resulting HPPs/WPI

particles are stable after cooling because of the irreversible nature of the disulphide bonding between HPPs and WPI. After a second heating cycle, HPPs/WPI particles show heat stability as most of exposed reactive groups have already interacted when forming the HPPs/WPI particles and the fewer reactive sites remain for further aggregation.

In the case of HPPs/NaCN mixtures, when the HPPs are co-heated with NaCN (Fig. 7B), the exposed hydrophobic groups at the surface of HPPs preferentially interacted with caseins. The chaperone-like property of casein molecules may provide steric hindrance to prevent the further aggregation of HPPs during heating. However, the association of caseins onto the surface of HPP was reversible upon cooling. With the decrease in temperature, the hydrophobic interactions would decrease, which could lead to the dissociation of caseins from HPPs and formation of large HPPs aggregates.

4. Conclusions

This study revealed different possible mechanisms of heat-induced aggregation of HPPs in the presence of WPI or NaCN. It was found that the whey proteins associate irreversibly via disulphide bonds with HPPs upon heating. The modification of HPPs through this binding of whey proteins restricts further aggregation of HPPs upon continued heating. In addition, casein interacted with HPPs reversibly, possibly involving the chaperone-like property of casein molecules. This mechanism could be applied to enhance the heat stability of HPPs for various food applications. This study is pioneering in describing possible mechanisms of heat-induced interactions between hemp protein (HP) and milk proteins. However, future research should explore other factors affecting heat-induced aggregation, such as a range of protein

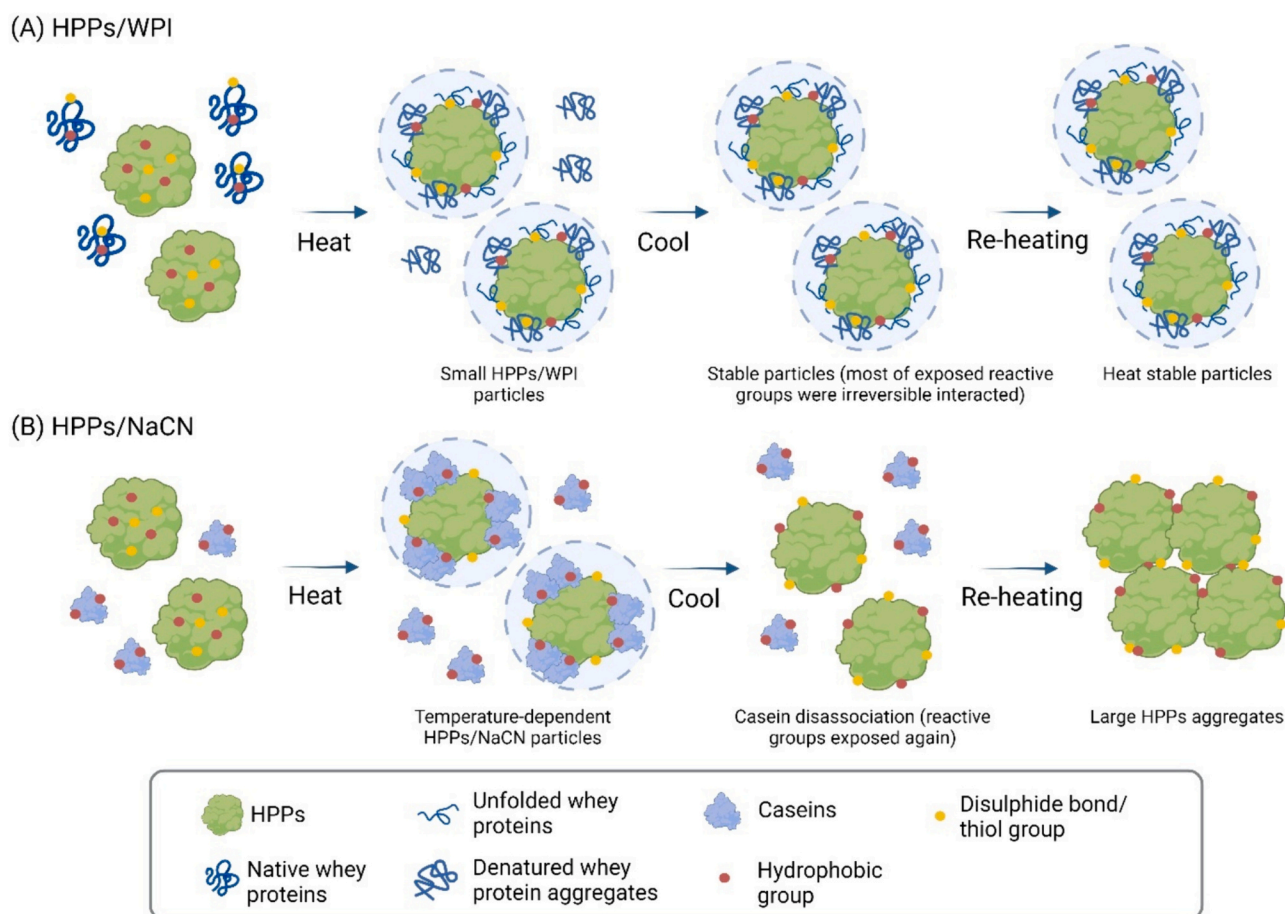


Fig. 7. Schematic representation of possible mechanisms by which (A) hemp protein particles/whey protein isolate (HPPs/WPI) and (B) hemp protein particles/sodium caseinate (HPPs/NaCN) interact during heat treatment (95 °C, 20 min) and cooling down.

concentrations and HP to milk protein ratios to gain a more comprehensive understanding of protein aggregation behaviour. In summary, this study explored a feasible strategy to develop more functional plant and dairy based food products by combining hemp proteins with milk proteins.

CRedit authorship contribution statement

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

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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