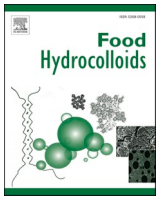
Food Hydrocolloids 171 (2026) 111833

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd



Emulsifying properties of hemp and whey protein complexes achieved by microparticulation

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ARTICLE INFO

Keywords:
Hemp protein
Whey protein
Microparticles
Hybrid protein
Pickering emulsion

ABSTRACT

Hemp is a sustainable source of protein. However, the utilisation of commercial hemp protein (HP) is limited due to its poor functionality. This study provided a microparticulation method to produce hybrid microparticles by complexing HP and whey protein isolate (WPI), and investigated their emulsifying potential. The emulsions, composed of 10 % oil and 0.25–1.8 % protein (non-microparticulated or microparticulated HP/WPI), were produced and the impact of microparticulation on the emulsifying ability of HP/WPI was explored using static light scattering, CLSM, TEM and SDS electrophoresis analysis. The results showed that non-microparticulated HP/WPI stabilised emulsions exhibited preferential whey protein adsorption at the oil-water interface, leading to sufficient protein coverage at most protein concentrations (0.25–1.8 %) with relatively small droplet size (~0.5 μm) and minimal flocculation. In contrast, in the 'emulsifier-poor' regime (0.25–1 %), microparticulated HP/WPI stabilised emulsions displayed larger droplet size with clear signs of bridging flocculation. However, when the protein concentration was sufficient (≥1.5 % protein), it reached a similar droplet size as that of non-microparticulated HP/WPI emulsion with minimal flocculation. Microparticulation increased HP loading at the interface, while emulsions stabilised by non-microparticulated HP/WPI showed less HP adsorption. Transmission electron microscopy further confirmed the microparticle coverage. Moreover, the heat stability of microparticulated HP/WPI stabilised emulsions increased, compared with non-microparticulated HP/WPI. These findings highlight the potential of microparticulated HP/WPI systems in the application of emulsification and enhance HP applications in the food industry.

1. Introduction

An oil-in-water emulsion is a colloidal dispersion consisting of dispersed oil droplets within an aqueous phase (McClements & Jafari, 2018). Emulsifiers play an important role in stabilising oil droplets and improving mouthfeel and rheological properties (Aloo et al., 2024). Protein is widely used as an emulsifier in emulsion formulations and stabilisation due to its amphiphilic nature (Lam & Nickerson, 2013). Beyond isolated proteins, protein nanoparticles and microparticles are increasingly explored in food colloids to build novel emulsion structures (Amagliani & Schmitt, 2017; Dickinson, 2015; Nicolai, 2016).

Hemp (*Cannabis sativa* L.) protein has garnered increasing attention for its high nutritional value, excellent digestibility, and balanced amino acid profile (Wang & Xiong, 2019). However, its use in the food application, particularly as an emulsifier, is still limited (Ajibola & Aluko, 2022; Kahraman et al., 2022; Liu et al., 2024). One major limitation is its low water solubility, with only ~10 % solubility at pH 7, which is significantly lower than that of other plant proteins such as soy protein (Hadnađev et al., 2018). Additionally, HP has a strong tendency to form large, dense aggregates at neutral pH, mainly due to its high free sulfhydryl content, inducing extensive disulphide bond formation (Dapčević-Hadnađev et al., 2019; El-Sohaimy et al., 2022; Tang et al., 2006). These structural characteristics also hinder its functionality, such as emulsification. For instance, the presence of large protein aggregates and limited solubility also reduces its ability to rapidly and effectively adsorb to the oil–water interface, resulting in poor emulsifying properties. Studies have consistently shown that HP has inferior emulsifying activity compared to soy or canola proteins (Tang et al., 2006; Teh et al., 2014). Recent findings by Liu et al. (2023) also reported the low emulsifying activities and large standard deviations, with oil droplet sizes ranging from 40 to 90 μm in HP stabilised emulsions.

There have been only a limited number of studies done to improve

This article is part of a special issue entitled: 30Y IHC published in Food Hydrocolloids.

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https://doi.org/10.1016/j.foodhyd.2025.111833
Received 5 June 2025; Received in revised form 16 July 2025; Accepted 30 July 2025
Available online 31 July 2025

the emulsifying properties of hemp protein. Yin et al. (2008) used enzymatic hydrolysis treatment to improve the protein solubility. However, the emulsifying activity index was remarkably decreased. Other strategies have focused on complexing hemp protein with polysaccharides at low pH. Feng et al. (2021) complexed pectin with hemp protein at pH 3, and Gholivand et al. (2024) complexed hemp protein with carrageenan, alginate, gum arabic and pectin at pH 3 or pH 3.5 to improve the dispersibility of hemp protein. However, the complexation and emulsification were performed at a very acidic pH, which is not ideal for food applications. The neutralization of pH after processing may cause re-aggregation of hemp protein (Chuang et al., 2021). High alkaline-thermal treatments have also been explored. Wang et al. (2018) used alkaline conditions (pH 12) combined with thermal treatment to improve the solubility and emulsifying activity of hemp protein isolate. However, the toxic by-product, lysinoalanine, produced at high alkaline pH and high-temperature environment, needs to be carefully monitored. Therefore, a novel process that can reinforce the nutritional and functional properties of hemp protein is demanded.

One possible approach to improve plant protein emulsifying properties is by complexation with milk proteins (Alves & Tavares, 2019). The functional synergy effect of the mix of some other plant proteins and milk proteins has been reported in recent years (Hinderink et al., 2019; Jose et al., 2016; Kim et al., 2020; Roesch & Corredig, 2006; Wong et al., 2013; Yerramilli et al., 2017). Previous studies have shown that heat-induced interaction and complexation of soy protein with whey protein (Anuradha & Prakash, 2009; Roesch & Corredig, 2005) and pea protein with β-lactoglobulin (β-Lg) (Chihi et al., 2016) lead to hybrid aggregates that are smaller than individual plant protein aggregates. These plant/milk protein complexes may offer great potential for improving functionality, especially emulsifying properties in this study.

Little work has been conducted on the complexation of hemp protein with milk proteins and their emulsifying properties. Only one published study reported that pH-cycling complexation turned insoluble hemp globulin into hemp globulin/sodium caseinate particles that have emulsifying functionality (Chuang et al., 2020). However, due to the relatively high free thiol content in hemp edestin, large hemp protein aggregates formed when heated above the denaturation temperature (92 °C) (Tang et al., 2006). Maintaining the thermal stability of hemp protein during food processing remains a challenge.

On the other hand, heat-induced protein interactions undertaken under control conditions can lead to the formation of protein microparticles that may have enhanced thermal stability (Ma et al., 2024b). This process, known as microparticulation, involves thermal treatment, shear, or pH shifts to form protein microparticles (Ipsen, 2017; Shi et al., 2021) and could offer a solution to overcome the limitations abovementioned of hemp protein. Our previous work was the first to report that whey protein can form complexes with hemp protein particles, effectively reducing aggregation and improving thermal stability (Ma et al., 2024a; Ma et al., 2024b). Nevertheless, the emulsifying properties of the hemp/whey protein microparticle have not yet been explored.

In oil-in-water emulsions, the particles could irreversibly adsorb at the oil–water interface and form a mechanical barrier due to their high desorption energy, thereby providing mechanical and steric stabilisation against coalescence and flocculation (Dickinson, 2012; Tcholakova et al., 2008). This is because microparticles often exhibit higher surface hydrophobicity, which could promote protein interaction at the interface to form thick and rigid films providing steric stabilisation (Sun et al., 2015). Previously, microparticulated protein stabilised emulsions show greater thermal stability compared to those stabilised by isolated proteins, likely because fewer reactive sites remain available for heat-induced aggregation (Çakır-Fuller, 2015).

In this study, we provided a microparticulation method for creating hemp/whey protein microparticles by heat treatment at a controlled pH environment, followed by size reduction. The emulsifying properties of hybrid protein microparticles, the surface structure of these resulting emulsions and their heat stability were examined.

2. Materials and methods

2.1. Materials

Whey protein isolation (WPI) containing 92.0 % protein, 0.9 % fat, 1.6 % ash and 5.2 % moisture was 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). The HP powder contained 59.8 % protein, 2.4 % fat, 10.7 % ash, 6.8 % moisture and 20.2 % carbohydrate. The proximate composition of both protein ingredients was analysed as follows: protein content was determined using the Kjeldahl method (AOAC 991.20, nitrogen factor 5.21; AOAC 2023a); fat, ash and moisture content were determined according to AOAC 922.06, AOAC 942.05 and AOAC 925.10, respectively (AOAC 2023b,c,d; respectively); and carbohydrate content was calculated by subtracting the sum of the protein, ash and fat from the total solids. 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. Preparation of HP/WPI microparticles

A hemp protein (HP) dispersion was prepared by mixing powdered HP in Milli-Q water at 2 % (w/w) protein concentration. The mixture was stirred for 2 h at 20 °C. The pH was then adjusted to 11 with 1 M NaOH, followed by stirring for 2 h to increase protein solubility. The dispersion was centrifuged at 3000×*g* for 30 min to remove insoluble materials (e.g., fibres). The resulting supernatant was adjusted to pH 8 using 1 M HCl, and homogenised using a two-stage valve homogeniser (APV 1000, SPX, Silkeborg, Denmark) set at 300 bar (first stage) and 50 bar (second stage). The protein dispersion was passed twice through the homogeniser with no holding time between passes. The resulting dispersion was used in subsequent steps. Separately, a whey protein isolate (WPI) stock solution was prepared by dissolving WPI powder in Milli-Q water at 3 % (w/w) and stirring for 2 h. The pH of the protein dispersion was adjusted to 8 using 1 M NaOH.

To prepare the HP/WPI microparticles, HP and WPI dispersions were mixed to achieve a final protein concentration of 1 % HP and 1 % WPI (w/w) in the mixture. The pH was re-adjusted to 8. Then, the protein mixture was subjected to a heat treatment of 95 °C for 30 min in a water bath. After the treatment was completed, the protein dispersion was rapidly cooled down to 20 °C in an ice bath. Finally, the pH of the protein dispersion was adjusted to 7 (using 1 M HCl), and passed twice through a two-stage valve homogeniser using the same pressure conditions described to produce HP dispersions.

2.3. Preparation of HP/WPI emulsions

To prepare emulsions stabilised by either microparticulated or non-microparticulated HP/WPI, two types of coarse emulsions were first prepared. For microparticulated emulsions, preformed HP/WPI microparticles were dispersed in water containing 10 % (w/w) soybean oil to achieve final protein concentrations of 0.25 %, 0.5 %, 1.0 %, 1.5 %, or 1.8 % (w/w). For non-microparticulated emulsions, a dispersion of HP particles/WPI mixture (at the same protein ratios used in microparticle preparation) was directly dispersed at corresponding total protein concentrations and oil concentration. Coarse emulsions were prepared using a benchtop Ultra-Turrax mixer (IKA, Wilmington, NC, USA) for 30 s at room temperature. The resulting coarse emulsions were then passed 2 times through a two-stage homogeniser at 300 bar (first stage)/50 bar (second stage) to produce fine emulsions stabilised by either microparticulated or non-microparticulated HP/WPI. Sodium azide (0.02 %, w/w) was added to inhibit microbial growth.

2.4. Droplet size analysis

The droplet size of the HP/WPI emulsion was measured by static light scattering using a Mastersizer 2000 and a Hydro MU unit (Malvern Instruments, Worcestershire, UK). The refractive index was 1.45. The data were reported in volume-weighted mean diameter $d_{(4,3)}$, calculated as the average of triplicate measurements. The surface weighted mean diameter $d_{(3,2)}$ was also collected to calculate surface protein coverage in the following section.

2.5. Transmission electron microscopy

Transmission electron microscopy (TEM) was employed to observe the microstructure of HP/WPI microparticles and their stabilised emulsions as described by (Li et al., 2021). Samples were sealed in agarose tubes (3 % agarose) and placed into 3 % glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) for 24 h, followed by washing 3 times with 0.1 M sodium cacodylate buffer. The samples were then post-fixed with 1 % osmium tetroxide in 0.1 M sodium cacodylate for 1 h at room temperature, overnight at 4 °C and another 1 h at room temperature. The samples were washed 3 times again as described above and dehydrated with a graded acetone series (25, 50, 75, 95 and 100 % acetone) for 45 min each concentration. The dehydrated samples were first embedded with resin and acetone (1:1, v/v) overnight on a rotator, then the resin and acetone (1:1, v/v) was replaced with fresh 100 % resin for 8 h; this was repeated 4 times. After that, samples were embedded in moulds with 100 % resin and incubated in a 60 °C oven for 48 h. Thin sections were cut from the resin blocks on an ultramicrotome and then mounted on copper grids using a Coat-Quick "G" pen (Daido Sangyo, Tokyo, Japan). The grids were stained with saturated uranyl acetate and lead citrate with 50 % ethanol, respectively, followed by MilliQ water washing between each step. The stained sample was imaged by a transmission electron microscope (FEI Tecnai G2 Spirit BioTWIN, FEI Company, Prague, Czech Republic) paired with a Veleta TEM camera (Olympus SIS, Hamburg, Germany).

2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protein composition in the HP/WPI microparticle 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 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.

2.7. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM; Model Zeiss LSM900 with Airyscan 2, Carl Zeiss, Jena, Germany) was used to investigate the microstructure of HP/WPI emulsion using the staining protocols described by Gallier et al. (2012). Nile Red (1 mg/mL in acetone) and Fast Green FCF (1 mg/mL in Milli-Q water) were used to selectively stain neutral lipids and proteins, respectively. Each sample (100 μL) was mixed with Nile Red (2:100, v/v) and Fast Green FCF (6:100, v/v). The stained emulsion sample was placed on a concave microscope slide and covered by a coverslip (0.17 mm thick), avoiding an air bubble between the sample and the coverslip. The freshly prepared sample slide was immediately examined by CLSM with a 63× oil immersion objective lens.

2.8. Total protein coverage

The emulsions were centrifuged at 45,000×*g* for 40 min at 20 °C. The subnatant layer and sediment layer were carefully collected. The cream layer was dispersed in deionised water and re-centrifuged at 45,000×*g* for 40 min to obtain washed cream. The protein content in the subnatant and sediment was analysed separately using the Kjeldahl method.

Adsorbed protein (g) was calculated using equation (1):

$$\text{Adsorbed protein (g)} = \text{Total protein (g) used in emulsion} - [\text{protein (g) in the subantant} + \text{protein (g) in the sediment}] \quad (1)$$

Total surface protein (mg/m^2) was calculated using equation (2):

$$\text{Total surface protein } (\text{mg}/\text{m}^2) = \frac{[\text{Adsorbed protein (g)} \times \text{d}_{3,2}]}{[6 \times \text{V} \times \phi]} \times 10^{-2} \quad (2)$$

where V (mL) is the volume of emulsions, and ϕ is the volume fraction of the oil in emulsions (Chang et al., 2016).

2.9. Protein composition on the emulsion surface

The washed emulsion cream was spread and dried on a filter paper, then was analysed using SDS-PAGE under reducing conditions as described in the previous section to determine the composition of the adsorbed protein at the surface of the emulsion. The resulting gels were scanned using a Gel Doc XR (Bio-Rad Laboratories) and analysed by ImageLab software for densitometric analysis. The percentage composition of each sample was expressed as the individual protein intensity as a fraction of the sum total.

2.10. Heat stability of emulsions

The heat stability of emulsions stabilised by microparticulated HP/WPI and non-microparticulated HP/WPI at protein concentrations ≥1 % was evaluated. A 10 mL sample of emulsion was transferred into a glass tube and heated in a water bath at different temperatures (60, 70, 80 and 90 °C) for 20 min, followed by rapid cooling in ice to 20 °C. The droplet size of the heated emulsions was analysed to investigate their heat stability.

2.11. Data analysis

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 with the level of significance set at $p < 0.05$.

3. Results and discussion

3.1. Particle size and morphology of HP/WPI microparticles

Microparticulated proteins, particularly microparticulated whey proteins, are commonly produced using heating and shearing treatment. This processing can create protein particles within the 0.1–10.0 μm size range that are desired for different protein functionalities (Shi et al., 2021). In this study, HP/WPI microparticles were produced through heat treatment under slightly alkaline conditions, followed by size reduction. To evaluate the functional effects of microparticulation, emulsions were also prepared using a non-microparticulated HP/WPI mixture as a control in the following sections. This control mixture contained the same HP particles and WPI blend (same protein ratio and concentration), but without undergoing any heat treatment. Thus, it represents the unstructured protein mixture before microparticle

formation. Although both samples originate from the same components, the presence or absence of heat-induced interactions and particle formation may result in different functionality.

The heat treatment of protein particles at pH 8 was the initial step in creating the HP/WPI complexes. As shown by the appearance of heated HP/WPI dispersion (Fig. 1A–), this treatment produced a well-dispersed suspension of protein particles. In contrast, heating HP individually resulted in a strong coagulum (Figs. 1A-2). This is attributed to the high free thiol content in HP, which leads to the formation of large protein aggregates via disulphide bonds upon heating (Tang et al., 2006; Wang & Xiong, 2019). Consistent with our previous findings, WPI was observed to interact with HP particles, preventing excessive aggregation (Ma et al., 2024a). Because individual HP particles are not ideal for heat treatment, only HP/WPI microparticles were examined in this study.

The impact of microparticulation on the HP/WPI dispersion/complex was first assessed by particle size measurement (Fig. 1B). The size distribution of the non-microparticulated HP/WPI dispersion revealed a bimodal distribution, with two peaks at 0.1–1 μm and 1–10 μm. This indicates that the majority of particles before microparticulation present as large aggregates, which could potentially hinder their effectiveness as emulsifiers in stabilising emulsions (Li et al., 2022). In contrast, the distribution of the HP/WPI microparticles shifts significantly to the left, with a majority of particles concentrated around a main peak at approximately 0.1 μm. The size range (10–200 nm) is known to support the formation of stable Pickering emulsions (Gricius & Oye, 2023).

The inset bar graph further illustrates that the volume-weighted mean diameter ($d_{4,3}$) of HP/WPI decreased from 3 μm to 0.2 μm after microparticulation. This substantial reduction supports the hypothesis that the heat treatment introduced the complexation between HP and whey protein (Ma et al., 2024a), and the following homogenisation process effectively broke down the HP/WPI complex to a desired size for emulsification.

Transmission electron microscopy (TEM) illustrates the structural morphology of HP/WPI microparticles (Fig. 1C). In the untreated HP/WPI dispersion (Fig. 1C), the particles appeared as large aggregates with irregular shapes in several micrometres in size, which were most likely HP particles. In contrast, the HP/WPI microparticles (Figs. 1C-2) were significantly smaller and more uniformly dispersed, which is consistent with the particle size analysis shown in Fig. 1B. These findings suggest that microparticulation effectively breaks down aggregates and leads to more homogeneous size distribution.

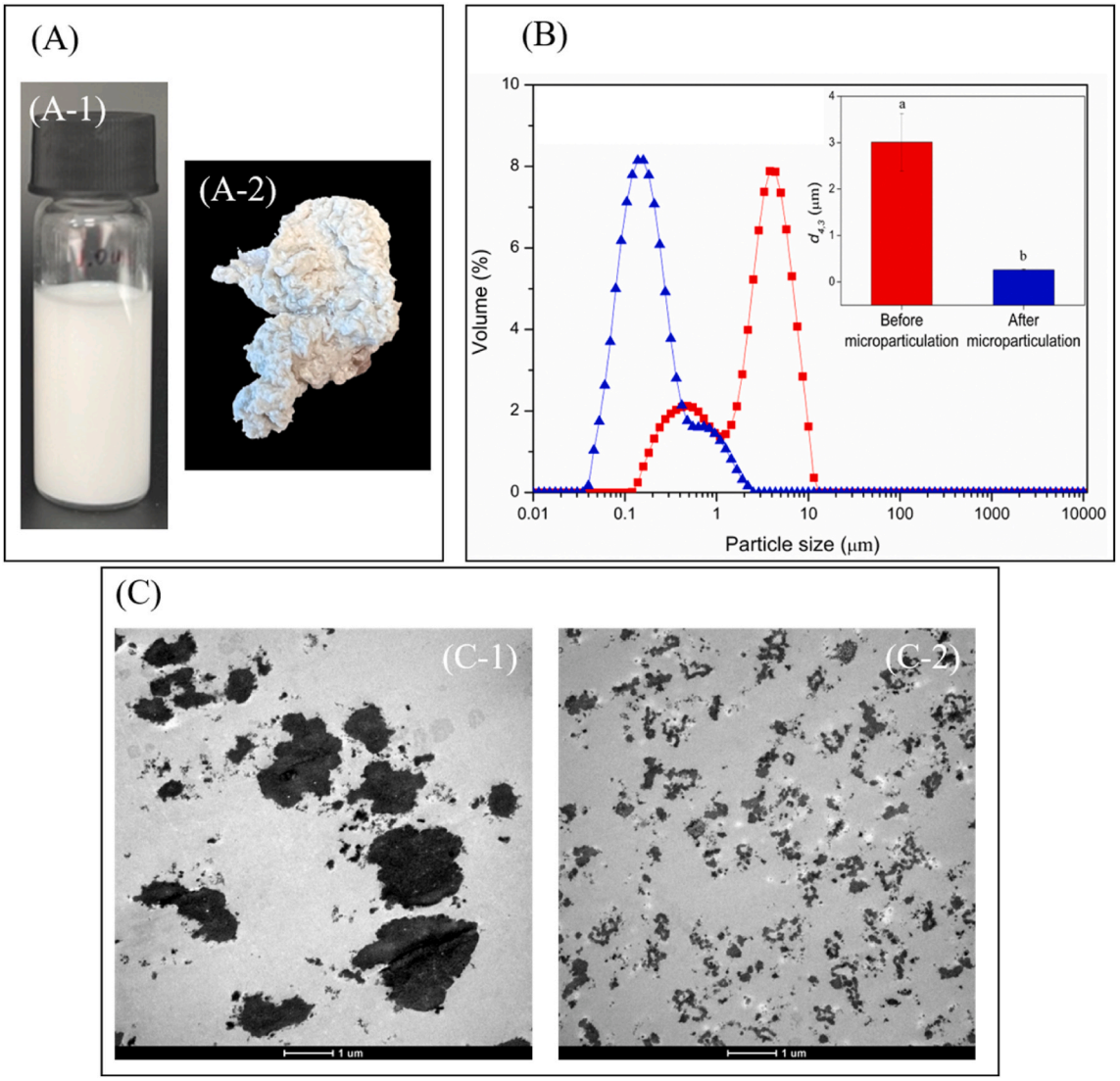

Fig. 1. Panel A: visual observations of heated HP with (A-1) the presence of WPI and (A-2) the absence of WPI at pH 8. Panel B: particle size distribution of HP/WPI before (■) and after (■) microparticulation; the inserted bar graph shows their corresponding volume-weighted mean diameters ($d_{4,3}$, μm), different lowercase letters indicate significant differences ($p < 0.05$). Panel C: transmission electron microscopy of HP/WPI particles before (C-1) and after (C-2) microparticulation.

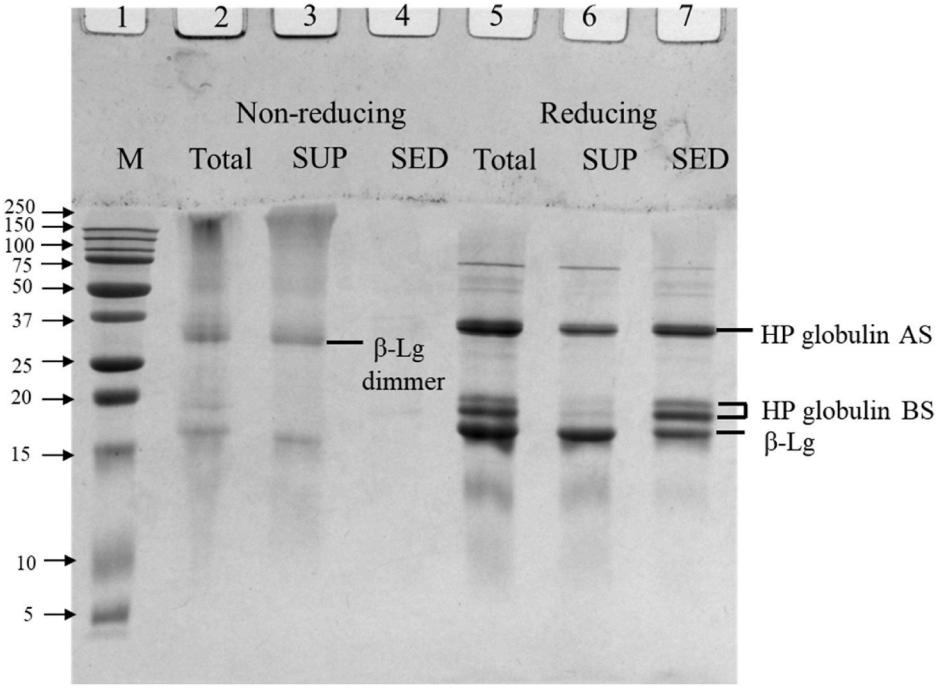

Fig. 2. SDS-PAGE under non-reducing (lanes 2–4) and reducing (lanes 5–7) conditions of HP/WPI microparticle dispersion (Total) and their supernatant (SUP) and sediment (SED). Lane 1, marker; lanes 2 and 5, HP/WPI dispersion; lanes 3 and 6, supernatant from HP/WPI; lanes 4 and 7, sediment from HP/WPI. AS, acidic subunit; BS, basic subunits; β-Lg, β-lactoglobulin.

3.2. Protein composition of HP/WPI microparticles

To analyse the protein interactions and compositions in HP/WPI microparticles, SDS-PAGE was conducted on the entire dispersion, as well as the corresponding supernatant and sediment fractions under both non-reducing and reducing conditions (Fig. 2).

Under reducing conditions, the disulphide bonds and hydrophobic interactions were disrupted with dithiothreitol (DTT) and SDS (Potin et al., 2022). Thus, the HP/WPI microparticles were dissociated into their monomeric forms. Therefore, the total fraction of HP/WPI showed 4 main predominant bands (lane 5, Fig. 2). The bands at 34 kDa, 21 kDa and 18 kDa correspond to the acidic subunit (AS) and two basic subunits (BS) of hemp globulin (Potin & Saurel, 2020; Wang & Xiong, 2019). On the other hand, the major whey protein, β-Lg, was also observed and marked (Singh, 2009).

Notably, both individually unheated and heated WPI at pH 8 were still soluble and remained in the supernatant fraction. On the other hand, both individually unheated and heated HP globulin were still relatively insoluble at pH 8 and could be collected in the sediment pellet (data not shown). As reported in our previous study, at unheated conditions or with no protein-protein interactions, the WPI and HP can be separated upon centrifugation at 20,000×*g* for 15 min (Ma et al., 2024b).

However, after co-heating with WPI and HP at pH 8, the band intensity of supernatant β-Lg (lane 6, Fig. 2) was reduced, and part of β-Lg was merged into the sediment fraction (lane 7, Fig. 2). This suggests the heat-induced interactions between HP and WPI. A plausible explanation could be that heat treatment unfolded the globular structures of whey proteins and exposed the sulphydryl groups of both HP and whey proteins (Anema, 2020; Singh & Havea, 2003), leading to thiol/disulphide exchange to form disulphide bridges between a proportion of WPI and HP and create HP/WPI hybrid particles. This observation aligns with the reported studies that WPI could bind with the HP via new disulphide bonds, thereby restricting the growth of HP aggregates (Ma et al., 2024a; Ma et al., 2024b).

Interestingly, a portion of the acidic subunit (AS) of HP was detected in the supernatant fraction after co-heating with WPI at pH 8 (lane 6, Fig. 2). This band was absent in the supernatants of individually heated HP at pH 8 or co-heated HP/WPI at pH 7 Ma et al., 2024a; Ma et al., 2024b), suggesting that the AS was present in the soluble phase only under specific co-heating conditions at pH 8. Moreover, under non-reducing conditions (lane 3, Fig. 2), high molecular weight protein bands exceeding 75 kDa and protein retained in the stacking gel were observed. These protein bands were absent in the co-heated HP/WPI at pH 7 (Ma et al., 2024b), indicating that disulphide cross-linking between the AS of HP and WPI proteins occurred only at pH 8. Upon subsequent reducing conditions, these high molecular weight aggregates dissociated into AS of HP and β-Lg, supporting the involvement of thiol/disulphide exchange.

The stability of HP is closely related to the pH conditions. One study reported that the ζ-potential of the HP protein body was strongly negatively charged (~−38 mV at pH 8), which led to strong internal electrostatic repulsion and the tendency of particle dissociation (Do et al., 2024). Wang et al. (2018) also noted that combining alkaline pH and heating dissociated the complex of acidic and basic subunits of HP due to the strong electrostatic repulsion and weakening hydrogen bonding. Makinen et al. (2016) reported that the disulphide bonds linking acidic and basic subunits of quinoa globulin were disrupted by heating at pH 8.5. These findings in this study suggest that synergistic effects of combining heat treatment with mild alkaline conditions caused dissociation of HP globulin sub-units (aided by electrostatic repulsion and weakening of AS–BS interactions), allowing the AS to become available for interactions with WPI, resulting in soluble HP AS–WPI aggregates. Although further experimental confirmation (e.g. LC-MS/MS identification or thiol-blocking assays) would strengthen this hypothesis, SDS-PAGE data provide compelling indirect evidence for such interactions at pH 8.

The combined data sets of SDS-PAGE, particle size analysis and TEM imaging (Section 3.1) support that the process described in this study successfully produced HP/WPI hybrid microparticles, with reduced mean size and improved dispersibility. Although additional characterisation could further reveal molecular-level interactions or surface

properties, they were out of the scope of this investigation.

3.3. Emulsifying ability of microparticulated versus non-microparticulated HP/WPI

The emulsifying ability of non-microparticulated and microparticulated HP/WPI was evaluated by determining the average droplet diameter ($d_{4,3}$) as a function of the protein concentration (Fig. 3A). For non-microparticulated HP/WPI stabilised emulsions (Fig. 3A: blue line with solid triangles), the droplet size decreased from ~1.8 μm to ~0.7 μm as protein concentration increased from 0.25 % to 0.5 %, with minimal change at higher concentrations (0.5 %–1.8 %). The "true" droplet size, measured after mixing the emulsions in SDS buffer (Fig. 3A: blue line with open triangles), only reduced from ~0.7 μm at 0.25 % protein concentration to ~0.5 μm at 0.5 % protein concentration and kept steady at higher protein concentrations. The small difference between the droplet sizes of SDS-treated and untreated emulsions indicates that the flocculation was not significant, particularly at 0.5 %–1.8 % protein concentrations.

In contrast, the emulsions made with microparticulated HP/WPI

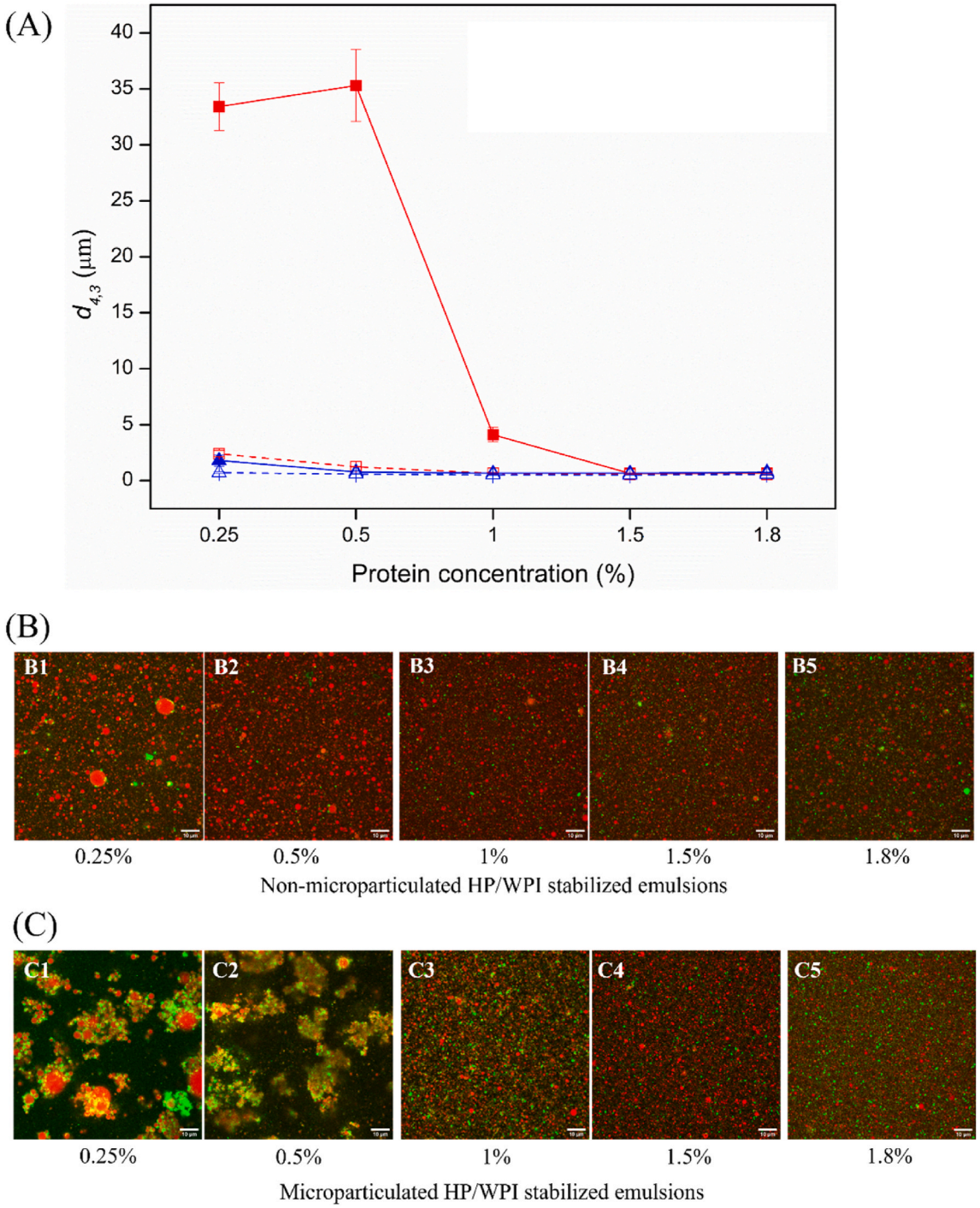

Fig. 3. Panel A, average droplet diameter ($d_{4,3}$) of non-microparticulated HP/WPI (▲,△) and microparticulated HP/WPI (■,□) stabilised emulsions as a function of the protein concentration, with (open symbols) and without (closed symbols) SDS. Panels B and C, CLSM images of non-microparticulated HP/WPI (B1–5) and microparticulated HP/WPI (C1–5) stabilised emulsions as a function of the protein concentration. The scale bar is 10 μm.

exhibited larger $d_{4,3}$ values (Fig. 3A: red line with solid squares). Droplet size initially decreased rapidly from 33.4 μm to 4.1 μm with an increase in protein concentration from 0.25 % to 1 %, then kept steady at 0.6 μm between 1.5 % and 1.8 % protein. Interestingly, although the microparticulated HP/WPI emulsion exhibited larger average sizes at low protein concentrations (0.25–0.5 %), the "true" droplet size (in SDS; Fig. 3A: red line with open squares) was much smaller, reducing from ~2.4 μm at 0.25 % protein concentration to ~1.2 μm at 0.5 % protein. Moreover, the gap in droplet size between microparticulated HP/WPI emulsion with SDS (0.7 μm) and without SDS (4.1 μm) was narrowed at 1 % protein. With further increasing protein concentrations (1.5–1.8 %), the droplet sizes of microparticulated and non-microparticulated HP/WPI emulsion (with or without SDS) were very close at ~0.5–0.6 μm.

Although hemp protein generally has inferior emulsifying properties (Tang et al., 2006), typically 6–15 μm emulsion droplet size (Chen et al., 2023), the non-microparticulated HP/WPI mixture exhibited relatively good emulsifying properties with small droplet size, particularly at low protein concentrations. This could be attributed to the excellent emulsifying properties of whey proteins (Fan et al., 2021; Schröder et al., 2017), which may work as the main emulsifying ingredients in the protein mixture due to their preferential adsorption. In a mixed emulsifier system, the interfacial composition of droplets depends on the relative adsorption rates of the different types of emulsifiers (Dickinson, 1992). When multiple proteins are present, such as the mixed whey/-other protein system, competition for adsorption sites can occur. For example, in pea/whey protein stabilised emulsions, a significant amount of pea protein was replaced by β-lactoglobulin (Hinderink et al., 2019, 2021). When both β-Lg and egg ovalbumin participated in emulsification, β-Lg dominated the interfacial composition, because of its higher interfacial activity (Dalgleish et al., 1991). In our study, whey proteins, due to their lower molecular mass compared with large HP particles, adsorbed more quickly than larger, more rigid hemp protein particles. This fast adsorption can lead to predomination in the interface, enhancing emulsion stability (McClements & Jafari, 2018).

The large differences between the emulsions with and without SDS suggest the existence of flocculation in microparticulated HP/WPI stabilised emulsions. The droplet size obtained is affected by two factors: droplet breakage during homogenisation and droplet coalescence after homogenisation (Schröder et al., 2017). In the 'emulsifier-poor' regime, where the microparticulated HP/WPI was insufficient to stabilise the newly created surface of emulsion droplets, and the final droplet size was strongly related to the droplet-droplet coalescence of initially formed small droplets (not completely covered by emulsifiers). Thus, resulting in large droplet size and strong flocculation (Schwenzfeier et al., 2013; Tcholakova et al., 2008).

Generally, depletion and bridging flocculation are two types of droplet-droplet interactions in biopolymer-stabilised emulsions (Dickinson, 2003). The protein concentration is critical because high concentrations could induce osmotic pressure imbalance and depletion flocculation (Hinderink et al., 2019). However, insufficient protein concentration in the system leads to bridging flocculation because the droplets' surface cannot be completely covered by proteins, and the same macromolecule adsorbs on multiple droplets, creating flocs (McClements, 2015). In our study, the flocculation phenomenon is likely driven by bridging flocculation. The extent of flocculation also depends on the particle size and flexibility; larger or more rigid particles (such as microparticulated HP/WPI) are less able to deform and wrap tightly around droplets, increasing the likelihood of droplets bridging.

However, at 1.5 % protein concentration and above, the droplet size became relatively independent of protein concentration, which indicates the 'emulsifier-rich' regime (Tcholakova et al., 2008). In this regime, microparticulated HP/WPI was sufficient to stabilise the small, broken-up droplets during homogenisation. At these higher concentrations, the surface of newly formed oil droplets becomes fully saturated with HP/WPI particles, minimising uncovered patches that would otherwise lead to coalescence or bridging flocculation. Additionally, increased surface coverage leads to greater steric hindrance, which contributes to enhanced droplet stability and reduced aggregation.

In comparison, due to the presence of unbound whey protein molecules in the non-microparticulated HP/WPI, better emulsifying ability was observed at low protein concentrations, with droplet size becoming independent of protein concentration at levels ≥0.5 %. This 'emulsifier-rich' behaviour at low protein concentration is consistent with reports showing that as little as 0.4 % WPI is sufficient to stabilise emulsions (Schwenzfeier et al., 2013). This can be attributed to the rapid interfacial adsorption of WPI molecules, which are small, flexible, and possess strong surface activity. These characteristics allow them to rapidly diffuse and rearrange at the oil–water interface, forming an interfacial layer even at relatively low concentrations.

However, although whey protein had excellent emulsifying ability, when the microparticulated HP/WPI was sufficient (≥1.5 % protein concentration), the emulsifying ability of microparticulated HP/WPI matched that of non-microparticulated HP/WPI, as droplet size was predominantly determined by the efficiency of droplet breakup during homogenisation (Tcholakova et al., 2008).

The microstructures of the emulsions stabilised by microparticulated and non-microparticulated HP/WPI were analysed by CLSM (Fig. 3B and C). For non-microparticulated HP/WPI stabilised emulsions (Fig. 3B1–5), as protein concentration increased, the droplet size became more uniform, averaging ~0.5 μm, which aligned with the particle size data. Similarly, in a mixed plant/milk protein system, Zhang et al. (2021) also reported that the addition of whey protein in soy protein facilitated the formation of emulsion with smaller and more uniform emulsion droplets. These CLSM images confirmed that in non-microparticulated HP/WPI stabilised emulsions, whey protein, being the main emulsifier with its low molecular weight and fast adsorption rate, exhibited a superior emulsifying ability. This allowed it to stabilise oil droplets effectively with minimal size variation once the protein concentration exceeded 0.5 %.

In contrast, the microparticulated HP/WPI stabilised emulsions at 'emulsifier-poor' regime (0.25 %–0.5 % protein concentrations) (Fig. 3C1 and C2) had larger droplet size and were extensively flocculated and formed clusters. This microstructure reflects a system undergoing bridging flocculation, where HP/WPI microparticles act as physical connectors across droplets due to incomplete surface coverage. As the available emulsifiers were insufficient to saturate the newly formed oil–water interface, droplets became interconnected via shared particles. It has been reported that bridging flocculation was observed in

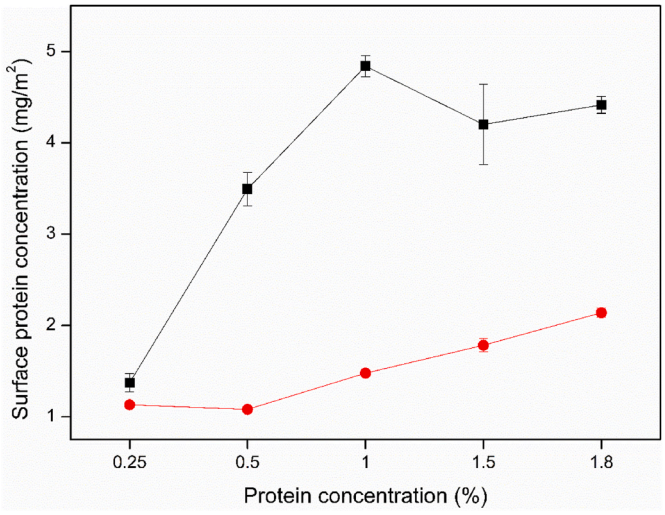

Fig. 4. Surface protein concentration of non-microparticulated (●) and microparticulated (■) HP/WPI stabilised emulsions as a function of the protein concentration.

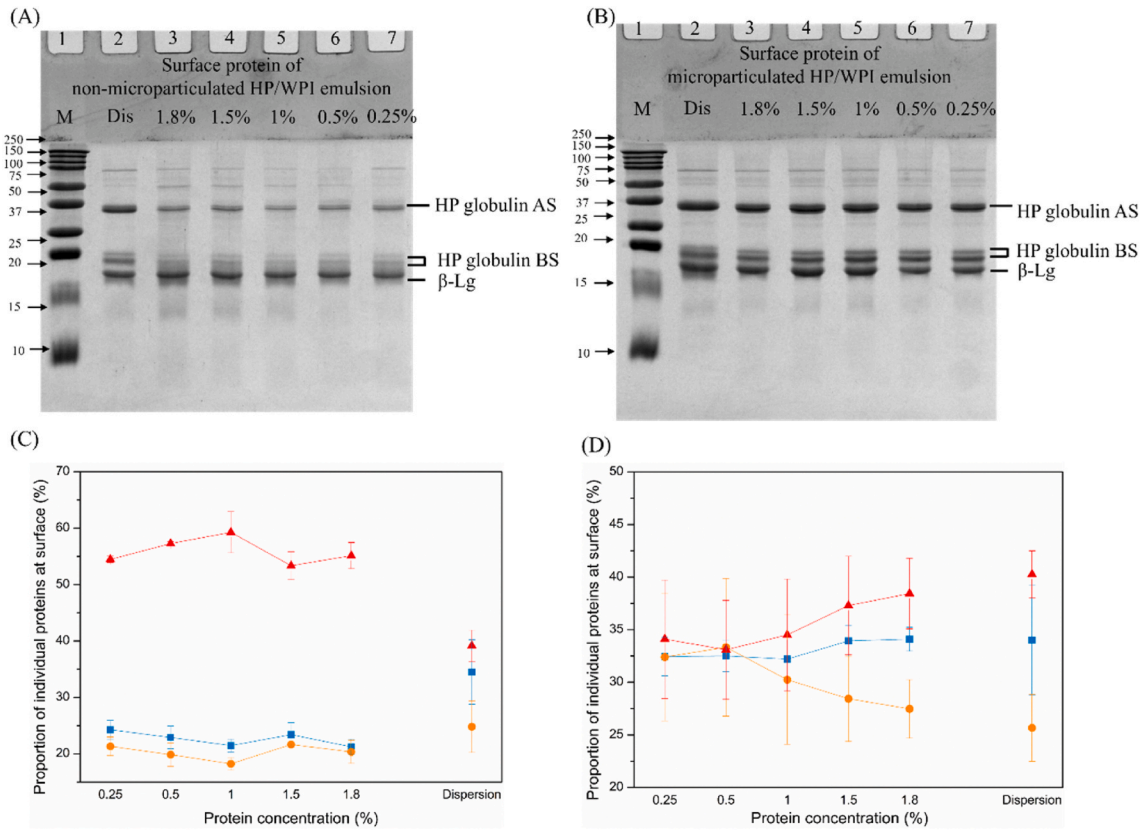

Fig. 5. SDS-PAGE under reducing conditions of surface proteins of (A) non-microparticulated HP/WPI and (B) microparticulated HP/WPI stabilised emulsions. Lane 1, marker; lane 2, HP/WPI dispersion (Dis); lanes 3–**7**, surface proteins of emulsions at 1.8–0.25 % protein concentrations (AS, acidic subunit; BS, basic subunits; β-Lg, β-lactoglobulin). Panels C and D show proportions (%) of HP acidic subunit (■), HP basic subunits (●) and β-lactoglobulin (▲) at the surface of non-microparticulated HP/WPI and microparticulated HP/WPI stabilised emulsions, respectively, as a function of the protein concentration.

low protein/oil ratio sodium caseinate stabilised emulsions by sharing of emulsifiers between droplets (Dickinson et al., 1997).

In contrast, at higher protein concentrations (≥1.5 %), sufficient microparticulated HP/WPI was available to saturate the droplet surfaces. As a result, CLSM images showed well-dispersed, individual droplets below 1 μm in diameter, consistent with the particle size measurements. This transition from flocculated to uniform emulsions with increasing protein concentration highlights the critical role of protein concentration and surface coverage in determining emulsion microstructure and stability.

3.4. Adsorbed protein on emulsion surface

To help reveal the interfacial properties of non-microparticulated and microparticulated HP/WPI stabilised emulsions, the surface protein concentrations were measured (Fig. 4). For all emulsions, surface protein load increased with total protein concentration. However, the extent of increase was much greater for microparticulated HP/WPI stabilised emulsions than for non-microparticulated ones. It has been reported that 1.5 mg/m^2 whey protein was sufficient to provide monolayer coverage for 20 % oil emulsion droplets (Hunt & Dalgleish, 1994). In this study, whey protein likely adsorbed preferentially onto the droplet surface, and the available whey protein was sufficient to cover the surface. As a result, the further increase in protein concentration only marginally increased the surface protein adsorption.

In contrast, microparticulated HP/WPI emulsions exhibited much higher surface protein load, rapidly increased from ~1 mg/m^2 to ~5 mg/m^2 at the 'emulsifier-poor' regime (0.25–1 % protein concentration), then slightly decreased to ~4.5 mg/m^2 at the 'emulsifier-rich' regime (Fig. 4). The large particles in microparticulated HP/WPI dispersion compared with protein molecules in non-microparticulated HP/WPI dispersion contributed to the higher surface protein load. In particle-stabilised interfaces, the surface load (mg/m^2) is generally much higher than in emulsions stabilised by conventional emulsifiers (Berton-Carabin & Schroen, 2015). The high surface load is likely to contribute to a protective shell that could help protect droplets from coalescence (Yan et al., 2020).

It should be noted that the surface protein concentration depends on both total adsorbed protein content and specific surface area (Zhao et al., 2015). In this study, the adsorbed protein kept increasing at 1.5 % and 1.8 % total protein concentration. Therefore, the slight decrease in the surface protein concentration may be attributed to a slight decrease in the droplet size, which means that more surface area was required to be covered.

3.5. Protein composition of the emulsion surface

To analyse the protein composition on the surface of emulsions, the SDS-PAGE (under reducing conditions) patterns of non-microparticulated HP/WPI and microparticulated HP/WPI dispersions and the adsorbed proteins in their corresponding emulsions were shown in Fig. 5A and B. As discussed in Section 3.2, the main predominant protein bands from HP and WPI, representing HP acidic subunit (AS), basic subunits (BS) and β-Lg were marked in Fig. 5.

As can be seen, all major proteins (both HP and whey protein) participated in stabilising emulsions for all emulsions. However, compared with their dispersion, the non-microparticulated HP/WPI had a lower proportion of HP on the droplet surface (Fig. 5A), while the surface protein composition of microparticulated HP/WPI stabilised emulsion was similar to its dispersion (Fig. 5B). This supports the

hypothesis that in non-microparticulated HP/WPI, the whey protein molecules were preferentially adsorbed at the interface, compared with large HP particles. The preference adsorption of whey protein has also been reported by Ye (2008) that whey proteins adsorbed in preference to caseins at protein concentrations below 3 %.

On the other hand, the proportion of interfacial HP in microparticulated HP/WPI was higher than that in non-microparticulated HP/WPI, suggesting that the adsorption of HP on emulsification was increased by the microparticulation process. These HP/WPI microparticles adsorbed at the interface as a hybrid protein complex, which eliminated the effect of preferential adsorption of whey proteins.

To obtain a better understanding of the adsorbed protein composition, the change of individual proteins was determined using densitometric analysis (Fig. 5C and D). The proportions of β-Lg, HP AS and HP BS in both non-microparticulated HP/WPI and microparticulated HP/WPI dispersion were similar, at about 40 %, 35 % and 25 %, respectively.

However, in non-microparticulated HP/WPI stabilised emulsions, the interface was dominated by β-Lg (50–60 %), which was higher than that in the original protein dispersion (Fig. 5C). Consequently, the proportions of HP AS and BS were lower than the original dispersion because of the preference adsorption of whey protein. Interestingly, the effect of total protein concentration on the surface protein composition was minor. A possible explanation could be that there was sufficient protein to fully cover the droplets at low protein concentrations, hence the adsorbed protein composition did not significantly change when the protein concentration was increased. This is supported by the surface loading did not markedly change across different protein concentrations (Fig. 4).

On the contrary, the interface of microparticulated HP/WPI stabilised emulsion was dominated by both β-Lg and HP (AS and BS) at all tested protein concentrations (Fig. 5D). The surface protein composition at the 'emulsifier-rich' regime (1.5–1.8 %) was similar to the major constituents in the starting HP/WPI microparticle dispersion. It is possible that the complexation of two proteins results in co-adsorption as a group, leading to the same ratio of protein on the surface as in the aqueous phase.

Overall, non-microparticulated HP/WPI and microparticulated HP/

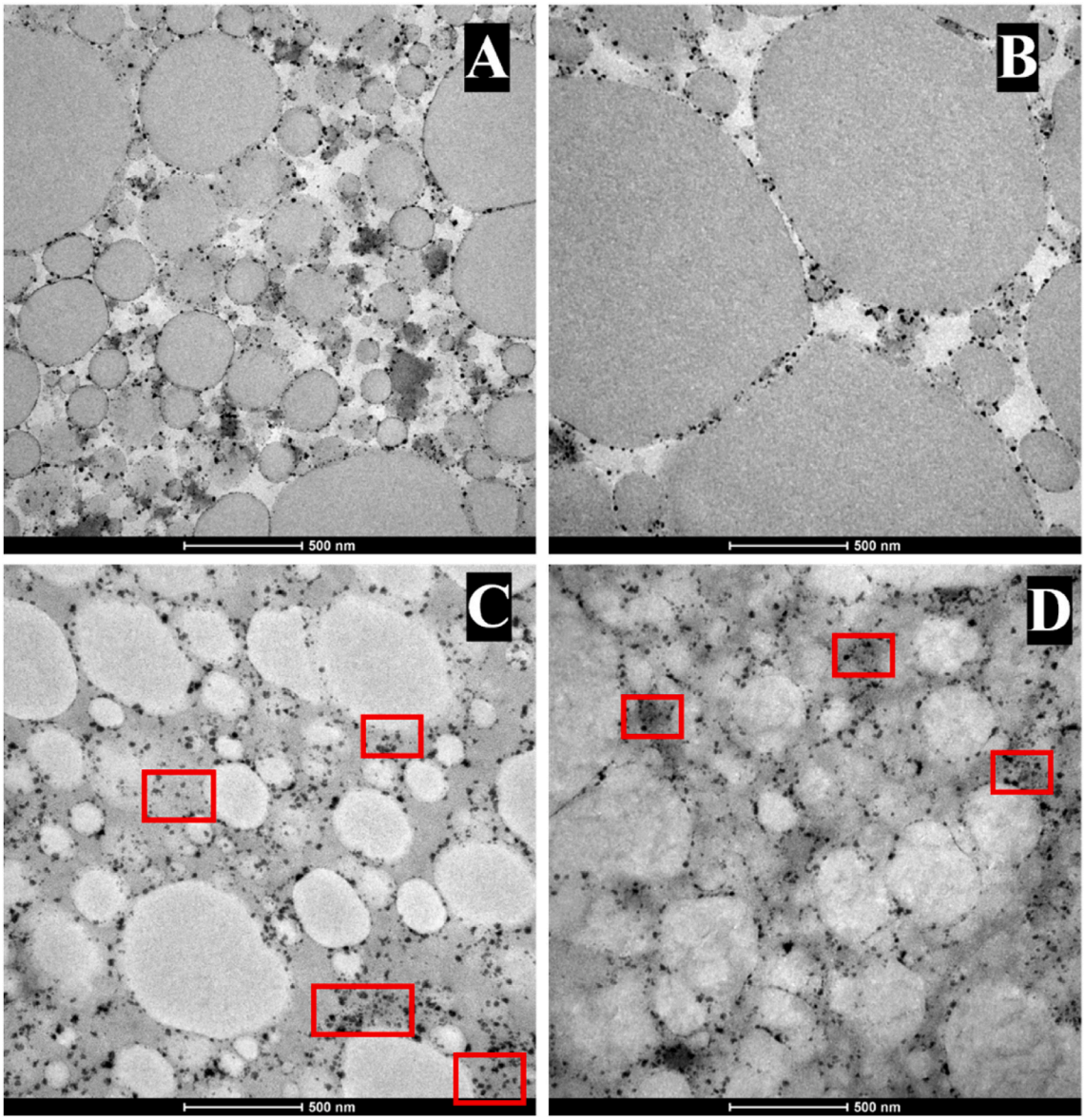

Fig. 6. Transmission electron microscopy of microparticulated HP/WPI stabilised emulsions at (A) 1.5 % and (B) 0.5 % protein concentrations and non-microparticulated HP/WPI stabilised emulsions at (C) 1.5 % and (D) 0.5 % protein concentrations. Red boxes highlight particles remaining in the continuous phase. The scale bar is 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

WPI exhibited different adsorption behaviour. The latter can increase the HP loading on the emulsion, which improves total protein loading and the utilisation of HP.

3.6. Microstructure of emulsion surface

The morphology of microparticulated HP/WPI and non-microparticulated HP/WPI stabilised emulsions was analysed by transmission electron microscopy (TEM) (Fig. 6). As can be seen, most HP/WPI microparticles were well dispersed around the surface of the droplet at both 'emulsifier-rich' regime (1.5 % protein) (Fig. 6A) and 'emulsifier-poor' regime (0.5 % protein) (Fig. 6B). However, the surface coverage by the particles seems to be somewhat incomplete. Sarkar et al. (2016) also reported the incomplete coverage of whey protein microgel particle stabilised Pickering emulsions. It is well known that it is not necessary for coverage by particles to be complete to produce stable Pickering emulsions, as long as the adsorbed particle layer forms a rigid interface (Sarkar et al., 2016; Yusoff & Murray, 2011).

However, a significant proportion of particles remained in the continuous phase (highlighted in red boxes, Fig. 6) of non-microparticulated HP/WPI stabilised emulsions at 1.5 % protein (Figs. 6C) and 0.5 % protein (Fig. 6D). This observation is in agreement with earlier discussion, where the preference adsorption of whey protein led to less loading of HP particles at the surface.

3.7. Heat stability of emulsions

The thermal stability of emulsions was evaluated by heating at different temperatures (60, 70, 80 and 90 °C) for 20 min. Only those emulsions with protein concentration ≥1 % were tested because microparticulated HP/WPI at these concentrations produced emulsions with droplet sizes comparable with those of non-microparticulated HP/WPI that were considered physically stable (i.e., minimal aggregation).

As shown in Fig. 7A, there was a minor change in droplet size of non-microparticulated HP/WPI stabilised emulsions when heated at 60 °C. At 1 % protein concentration, a negligible droplet size increase was observed when the temperature reached 90 °C. However, at higher protein concentrations (1.5 % and 1.8 %), droplet size slightly increased at 70 °C, followed by a substantial increase when the heating temperature reached 80 °C and 90 °C. The aggregation behaviour was visually confirmed in Fig. 7C, where a coagulation was observed for the 1.8 % protein emulsion heated at 90 °C, as evidenced by settling at the bottom of the test tube when inverted.

In contrast, microparticulated HP/WPI emulsions showed significantly improved heat stability. As seen in Fig. 7B, the droplet size remained stable across all tested temperatures. Moreover, the influence of protein concentration on heat-induced aggregation was negligible. This improvement was visually supported by Fig. 7D, where the emulsion treated at the highest protein concentration and temperature (1.8 % protein; 90 °C) retained fluid-like properties, still flowing when the test tube was inverted.

The droplet-droplet and protein-protein interactions would have an impact on the heat stability of emulsions (Liang et al., 2017). Without microparticulation, both HP and whey proteins remain heat-sensitive. Therefore, it is expected that non-microparticulated HP/WPI stabilised emulsions exhibited thermal instability. It has been previously described that irreversible β-Lg aggregates start being formed above 70 °C (Boland, 2011). Sava et al. (2005) demonstrated that β-Lg unfolds at 70 °C–75 °C and consequently aggregates at 78 °C–82.5 °C. In this study, a higher heating temperature (70 °C–80 °C) facilitated that adsorbed protein

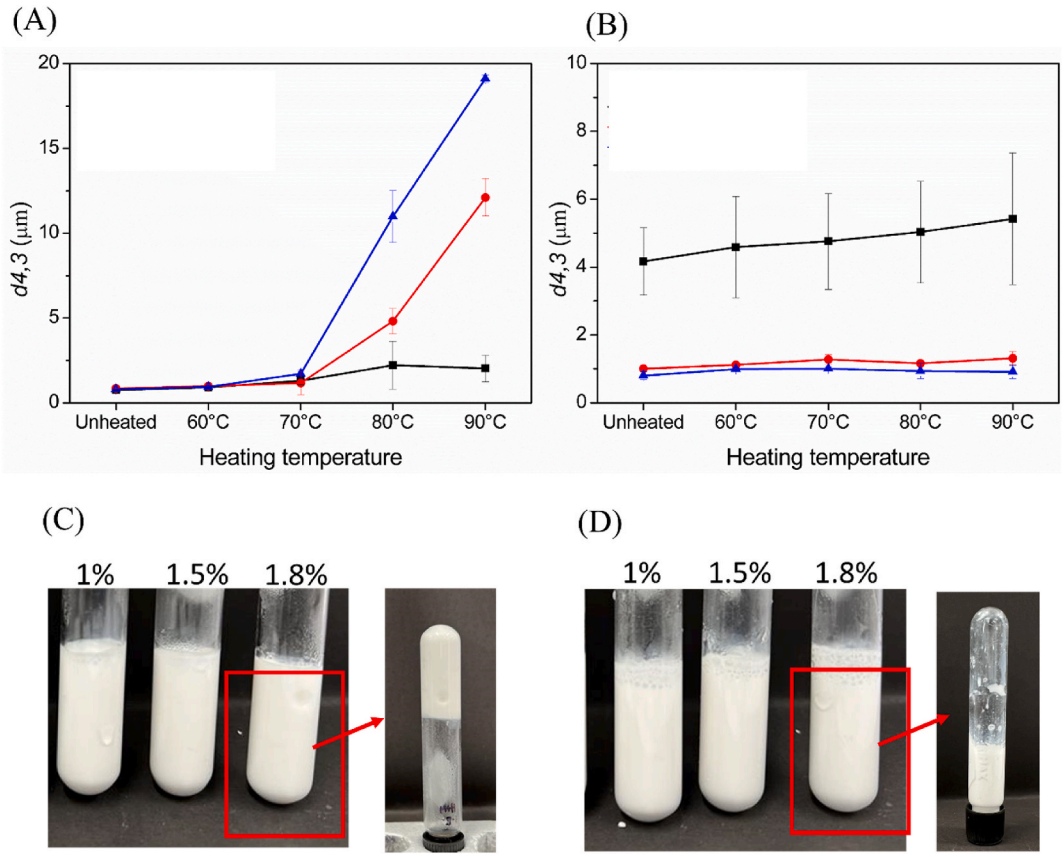

Fig. 7. Average droplet diameter ($d_{4,3}$) of (A) non-microparticulated HP/WPI and (B) microparticulated HP/WPI stabilised emulsions (■, 1 %; ●, 1.5 %; ▲, 1.8 %) before and after heating at 60, 70, 80 and 90 °C for 20 min. Panels C and D show the visual appearance of emulsions stabilised by non-microparticulated HP/WPI and microparticulated HP/WPI, respectively, after heating at 90 °C and sitting for 20 min.

interacted with other proteins, leading to the bridging of droplets.

Simultaneously, denaturation and structural rearrangement of non-adsorbed proteins resulted in exposure of the reactive groups, which could also induce their interactions with other non-adsorbed and adsorbed proteins in the aqueous phase (Allahdad et al., 2023). At 90 °C (close to the denaturation temperature of HP), HP also contributed to protein aggregation, which strengthened droplet and protein network formation. Similar temperature-dependent aggregation behaviour has been reported in soy protein stabilised emulsions, with more pronounced effects at 95 °C compared with 75 °C (Keerati-u-rai & Corredig, 2009).

Protein concentration also plays a crucial role in the heat-induced aggregation of emulsion. Heating hardly induced any aggregation when protein concentration was relatively low (1 %) due to less non-adsorbed protein in the continuous phase. It was found that whey protein stabilised emulsions exhibited more extensive aggregation at 3 % protein but showed no significant size change at 1.5 % between 55 °C and 95 °C heat treatment, because of the reduced level of non-adsorbed protein (Sliwinski et al., 2003).

On the other hand, the thermal resilience of emulsions stabilised by microparticulated HP/WPI highlights the protective role of hybrid protein microparticles. As shown in our previous study (Ma, Ye, et al., 2024), the complexation between HP and whey proteins promoted the interactions among the reactive groups, such as hydrophobic and free thiol groups, leading to thermally stable HP/WPI microparticles. Results obtained in the current study suggest that heat stable HP/WPI microparticles have the ability to form emulsions that are heat resistant. In addition, our results are in agreement with previous studies on microparticulated whey protein stabilised emulsions, where those formed with up to 8 % protein were found to be heat-stable (Çakır-Fuller, 2015). Preheating of proteins under controlled conditions can reduce the number of active sites available on protein particles to interact, inhibiting heat-induced aggregation (Ma et al., 2020).

4. Conclusions

This study demonstrates the significant influence of microparticulation on the stabilisation of HP/WPI emulsions, particularly under varying protein concentrations. Non-microparticulated HP/WPI stabilised emulsions exhibited small droplet size with good stability at low protein concentrations, where whey protein dominated the interfacial layer. However, HP adsorption was limited in non-microparticulated systems due to whey protein's preferential adsorption at the droplet interface. On the other hand, the microparticulation process improved HP adsorption at the interface, allowing the formation of protective particle layers that contributed to the integrity of the droplets. When the microparticulated HP/WPI was sufficient ($\geq$1.5 % protein), a stable emulsion could be made with a similar droplet size as that of non-microparticulated HP/WPI. Moreover, microparticulation of HP/WPI markedly enhanced the heat stability of protein-stabilised emulsions compared with their non-microparticulated counterparts. This improved thermal resistance is attributed to the prior interaction and structural rearrangement during microparticle formation, which minimises further aggregation upon heating. The findings suggest that the microparticulation of HP/WPI systems offers a promising strategy for enhancing HP utilisation in emulsions and thermally processed food systems requiring enhanced stability. Future research should focus on optimising the microparticulation process and further exploring the potential applications of these hybrid protein systems in food formulations.

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.

Acknowledgements

This research was funded by the Riddet Institute Centre of Research Excellence (CoRE) and the Tertiary Education Commission, New Zealand. Author Sihan Ma thanks the Riddet Institute for the PhD scholarship. The authors also acknowledge the Manawatu Microscopy and Imaging Centre (MMIC) of Massey University for providing support in microscopy data collection.

Data availability

Data will be made available on request.

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