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Original article

Enhancement of emulsion stability and functional properties of hemp protein-based dairy alternative by different treatments after cellulase hydrolysis

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Summary Hemp milk is a plant-based beverage emerging as a sustainable dairy alternative and it is essential to improve the emulsification properties and stability of hemp protein for increasing acceptability. This study demonstrates the preparation of protein-rich hemp milk with enhanced emulsion stability and functional properties through cellulase hydrolysis of fibre components in hemp protein. The emulsions were prepared using the hemp dispersions obtained after cellulase treatment followed by mechanical treatment (ME) and in combination with either enzymatic (trypsin) (EE) or mild chemical (pH-shift) treatment (PE). The influences of these treatments on the particle size distribution, zeta potential, flow behaviour, microstructure, flocculation, and coalescence properties of the emulsions are investigated. The emulsions (EE/PE) prepared with dispersions processed through tryptic or pH shift treatments showed improved stability as indicated by reduced flocculation and coalescence indices (8.9% and 10.8%, for EE and 11.8% and 13.2% for PE) as compared to only mechanically treated emulsion (ME) (37.5% and 15.3%). Furthermore, the enhanced stability of EE and PE samples is attributed to a reduction in particle size and enhanced zeta potential (EE: 45.1 mV > PE: 38.7 mV > ME: 34.1 mV).

Keywords Emulsion stability, fibre hydrolysis, hemp protein, plant-based emulsion, tryptic hydrolysis.

Introduction

Driven by the need for more sustainable and nutritious food to feed the growing population, a shift towards "sustainable diets" is encouraged. In this scenario, the preparation of plant-based milk (PBM) products has gained rapid attention worldwide. PBM is described as aqueous extracts derived from vegetable matter breakdown of legumes (soymilk), nuts (almond milk), cereals (oat milk) and other plant materials (Penha *et al.*, 2021). Plant-based proteins are finding applications in a variety of foods as functional ingredients. Moreover, proteins play a crucial role in stabilising colloidal systems due to amphiphilic nature and excellent interfacial properties (Rawal et al., 2023; Tarahi & Ahmed, 2023). One such budding plant-based protein is industrial hemp. Industrial hemp, scientifically known as *Cannabis sativa* L is emerging as a potential commercial crop due to its carbon-sequestering property, high biomass production and several useful by-products with a growing market size (compound annual growth rate of 16.8% through 2030) (Grand View Research, 2021; Ahmed *et al.*, 2022).

Hemp protein comprises high nutritional traits, typically containing 25–30% lipids, 20–25% proteins and rich in essential amino acids (Farinon *et al.*, 2020). Recently, there is an increasing interest in developing hemp-based foods, investigating allergenicity and health benefits (Rehman *et al.*, 2021). However, very limited research is done on developing hemp-based milk. This may be because, in comparison to other plant-based proteins (such as soy), hemp protein exhibits poor solubility, less emulsifying stability and water-holding capacity (Leonard *et al.*, 2019). Also, it often tends to flocculate and coalesce. The reason behind poor stability could be its complex structure or the presence of a significant amount of non-digestible fibre content (approximately 22.2% cellulose) in hemp seed, which decreases solubility, absorption and functional properties of the protein (Hernández &

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Gil, 2016; Garcia, 2017). Hence, this research is aimed to improve the functional properties of hemp protein milk, through various combinations of trypsin, mild-chemical, and mechanical treatments.

The mechanical treatment through high-shear homogenisation usually enhances the functional characteristics of plant-based proteins that generally have complex structures, by physically breaking down the particles, increasing surface area and protein solubility eventually resulting in uniformly distributed emulsion droplets (Wang *et al*., 2018; Dapčević-Hadnađev *et al*., 2019). The efficacy of such mechanical treatments has also been already demonstrated for soy protein and whey protein emulsions (Song *et al*., 2013; Yan *et al*., 2017).

Proteolytic hydrolysis is also used to enhance the solubility and modify the functional properties of plant protein isolates (soy, whey, and hemp) (Yin *et al*., 2008; Ghosh *et al*., 2017; Conti *et al*., 2019). Enhanced solubility and functional properties of hemp protein isolate after controlled tryptic hydrolysis (pH 8.0, 37 °C for 500 min) were reported. However, the emulsion stability was not well accomplished.

The other route to improve protein solubility and modify the functional properties of proteins is chemical treatment. Particularly, the pH-shift treatment is a simple method that is commonly adaptable in food industries. It involves initially adjusting the protein solution to an extremely acidic or alkaline pH value and then adjusting to neutral to allow the proteins to undergo a progressive partial unfolding and refolding. To date, some studies have reported enhanced functional properties of soy protein, pea protein and hemp protein (Jiang *et al*., 2009, 2017; Wang *et al*., 2018). A combination of pH-shift treatment with high-pressure homogenisation (HPH) or trypsin treatment with HPH has been employed to prepare hemp milk earlier (Wang *et al*., 2018). However, the protein content was still low in the final product, ranging from 0.25% to 1.5%. Moreover, less attention has been given to the amount of fibre content in hemp protein powder which affects protein solubility, which is also another pathway. According to a study on flaxseed protein isolate, hydrolysis of the fibre part could improve the protein solubility (with increased soluble protein content of 23%) (Udenigwe *et al*., 2009). Therefore, it is also worthwhile to investigate the fibre hydrolysis of hemp protein powder. Considering these research gaps and potential of each treatment, this study aimed to investigate the influence of fibre hydrolysis using cellulolytic enzyme for enabling protein solubility in dispersions, and the combination of mechanical treatment with proteolytic enzyme hydrolysis or mild-chemical treatment. By studying the protein solubility, surface tension and SDS-PAGE results of dispersions, followed by the functional characteristics of emulsions (based on particle size distribution, microstructure, flow behaviour, zeta potential, flocculation and coalescence index), this study establishes a sustainable approach for preparing plant-based dairy alternative using hemp protein.

Materials and methods

Materials

In this study, organic hemp protein powder (Bulk Nutrients, Australia) was used as plant protein. It consists of 50% protein, 20.5% fibre, 5% sugar and 11% total fat. Cellulase (MC23.66E) and trypsin (MC23.1M, Activity: 273 U mg^{-1}) enzymes (Southern Biological, Australia) were used for hydrolysis. The chemicals (sodium hydroxide, hydrochloric acid and SDS) were used as obtained from Sigma-Aldrich (Australia). Soy lecithin liquid and xanthan gum were purchased from the Melbourne Food Ingredient Depot (Australia).

Preparation of hemp protein dispersion (HPD)

Hemp protein powder (4% w w^{-1} protein) was dispersed in deionised water by mixing using an overhead stirrer for 3 h at 10 000 rpm at room temperature. This dispersion was referred as HPD.

Cellulolytic hydrolysis

Hydrolysis was performed by heating the dispersion to 37 °C, adjusting pH to 5.0 and treating with 1% (w w^{-1}) of cellulase enzyme for 3 h. The extent of fibre hydrolysis was 5.4% as determined by the means of the spectrophotometric method (Galant *et al*., 2015). The enzyme was inactivated by altering the pH to 10.0 using 2 N NaOH solution (37 °C, 1 h) (Andreaus *et al*., 1999). Finally, the pH of the dispersion was brought to 7.0 and then divided into three parts for further treatments. This dispersion (referred as cellulase-treated HPD) was then divided into three parts.

Enzymatic treatment (tryptic hydrolysis)

For the first part, the pH of the HPD was adjusted to 8.0 with 2 N NaOH and incubated at 37 °C for 15 min. Trypsin enzyme (enzyme activity: 273 U mg^{-1}, optimum temperature and pH conditions: 37 °C and 8.0–10.0, respectively) was added at an enzyme-to-substrate ratio of 1:200 (w w^{-1}). The dispersion was incubated at 37 °C to start the tryptic hydrolysis reaction and same pH and temperature conditions were maintained. After 3 h, the trypsin enzyme was inactivated by adjusting the pH to 2.0. The degree of hydrolysis was 8.24% as determined using the method given by Nielsen *et al*. (2006). Finally, the dispersion was then neutralised to a pH of 7.0.

pH-shift treatment
For the second part, the pH of the HPD was adjusted to 12.0 using 2 N NaOH at room temperature. After an hour, HPD was neutralised to pH 7.0 by the addition of 2 N HCl (Wang *et al.*, 2018).

Mechanical treatment
Third part was used directly for emulsion preparation through the mechanical treatment described below. These samples were labelled as mechanically treated and compared as the control for other treatments.

Homogenisation and emulsion preparation

The emulsions based on all the HPDs were prepared same homogenisation conditions. Each dispersion was pre-homogenised using Ultra-Turrax at 12 000 rpm for 5 min and then recirculated in a colloid mill LM 150 (Probst & Class GmbH & Co.KG, Germany) for 45 min and filtered through five layers of cheesecloth. Then, the filtrate was passed twice through a GEA Twin Panda 400homogeniser (GEA Italy) at 100 bar (2nd stage only). Finally, the oil-in-water emulsions were prepared by mixing each hemp protein solution (HPS) with 2% sunflower oil, 0.2% lecithin and 0.1% xanthan gum using Ultra-Turrax at 12 000 rpm for 5 min. A schematic representation of the methodology is provided in the supplementary (Figure S1). These samples were labelled as enzymatically (trypsin) treated emulsion (EE), pH-shift-treated emulsion (PE) and mechanically treated emulsion (ME).

Analysis of hemp protein dispersions (HPD)

All the characterisations including protein solubility, SDS-PAGE and surface tension were done using the standard protocols which are explained in the Data S1.

Characterisation of hemp protein emulsions (HPEs)

All the characterisations including particle size distribution, emulsion stability, zeta potential, microstructure and flow behaviour were done using the standard protocols which are explained in the Data S1.

Statistical analysis

The results of three replicates were analysed and are presented as mean ± standard deviation using Minitab® 19 software. The data obtained were assessed using one-way analysis of variance (ANOVA) using a confidence level of 95%. Pairwise comparisons were determined using Tukey's test with a 5% level of significance. *P*-values <0.05 were considered to be statistically significant.

Results and discussions

Characteristics of hemp protein dispersions

Protein solubility
The solubility of proteins is an important physicochemical property that affects the texture, colour, emulsifying and gel-forming properties of food products (Adebiyi & Aluko, 2011). As shown in Table 1, the lowest protein solubility (8.63%) was observed for only mechanically treated HPD, as compared to trypsin-treated HPD (18.98%) or pH-shift-treated HPD (11.60%). Only mechanically treated HPD exhibited the lowest solubility (8.63%) due to inherent folded protein structure and more hydrophobic interactions, as previously reported for hazelnut milk upon HPH treatment (Saricaoglu *et al.*, 2018). Combined with the mechanical treatment, the pH- shift treatment significantly increased the protein content (33.28%). The pH-shift treatment is a global treatment, modifying whole tertiary structure of protein which induces strong repulsive force inside the protein molecules, resulting in the unfolding of protein structures and more available charged particles to interact with the media (Jiang *et al.*, 2022). A similar trend was earlier observed with soy protein upon pH shift due to the extensive protein structural unfolding and cleavage of disulphide bonds of the proteins (Jiang *et al.*, 2010). Similarly, when enzymatically treated HPD was further treated mechanically, the protein solubility increased significantly to 30.82%. This could be explained by the action of trypsin which cleaves the peptide bond between the carboxyl group of arginine or lysine and the amino group of the adjacent amino acid. This increases the number of exposed ionisable amino and carboxyl groups (Simpson, 2006; Yin *et al.*, 2008).

Table 1 Effect of treatments on the protein solubility and surface of the hemp protein dispersions

Samples	Protein solubility (%)	Surface tension (mN m^{-1})
Hemp protein dispersion (HPD)	10.19 ± 0.07^{d}	40.226 ± 0.77^{d}
Cellulase-treated HPD	14.85 ± 0.06^{c}	46.764 ± 0.28^{c}
Trypsin-treated HPD	18.98 ± 0.73^{b}	30.830 ± 0.26^{f}
pH-shift-treated HPD	11.60 ± 0.74^{d}	34.12 ± 0.79^{e}
Only mechanically treated HPD	8.63 ± 0.70^{d}	47.19 ± 1.10^{bc}
Trypsin and mechanically treated HPD	30.82 ± 0.73^{a}	49.53 ± 0.23^{ab}
pH-shift and mechanically treated HPD	33.28 ± 1.38^{a}	50.55 ± 0.59^{a}

Samples within a column that do not share a superscript letters are significantly different ($P < 0.05$).

Surface tension

The surface tension is an important parameter that affects emulsion stability (D'Apolito *et al.*, 2018). As seen in Table 1, chemically and mechanically treated HPD exhibited the highest surface tension (50.55 mN m^{-1}), followed by trypsin and mechanically treated HPD (49.53 mN m^{-1}). The increased surface tension for pH-shift-treated samples can be attributed to the unfolding and refolding of proteins upon pH change to 12.0 and 7, respectively, which expose the polypeptide backbone and the hydrophobic chains to the surrounding solvent (Wang *et al.*, 2018). Additionally, homogenisation provides mechanical energy to disrupt the oil droplets and form new interfaces leading to an increase in the surface tension of emulsion (Mukherjee *et al.*, 2005).

Structural changes using SDS-PAGE analysis

To study the protein structure and effect of various treatments on the structural properties of hemp protein, SDS-PAGE analysis was performed.

As observed from lane 1 and lane 2 (Fig. 1), HPD and cellulase-treated HPD, respectively, were characterised by diffused bands ranging from 10 to 75 kDa. The presence of 20 and 33 kDa protein bands is associated with the main edestin protein fraction in hemp protein (Malomo *et al.*, 2014). After treating the HPD with cellulase (lane 2), diffused protein bands were observed. This might explain how cellulase improved the protein solubility by releasing the insoluble fibres which were hindering the protein solubility in the solution. This was also supported by particle size data that decreased significantly for cellulase-treated HPD (62.31 μm) than HPD (70.78 μm). For only the pH-shift-treated sample (lane 4), a heavier polypeptide was detected at approximately 50 kDa which probably came from the higher molecular weight disulphide bonded proteins. As explained earlier, the pH-shift treatment leads to extensive structural unfolding and cleavage of disulphide bonds in proteins (Jiang *et al.*, 2010). For both chemically and mechanically treated samples (lane 7), the high molecular proteins are broken down into smaller subunits. Similarly, it was observed that a combination of trypsin hydrolysis with homogenisation (lane 6) led to the formation of more soluble and small peptide structures. This shows the remarkable improvement in protein solubility with tryptic hydrolysis treatment. Similar results were reported for tryptic hydrolysis in the case of hemp, pea, soy and other plant 11 S globulins (Plumb & Lambert, 1990; Kimura *et al.*, 2008; Yin *et al.*, 2008).

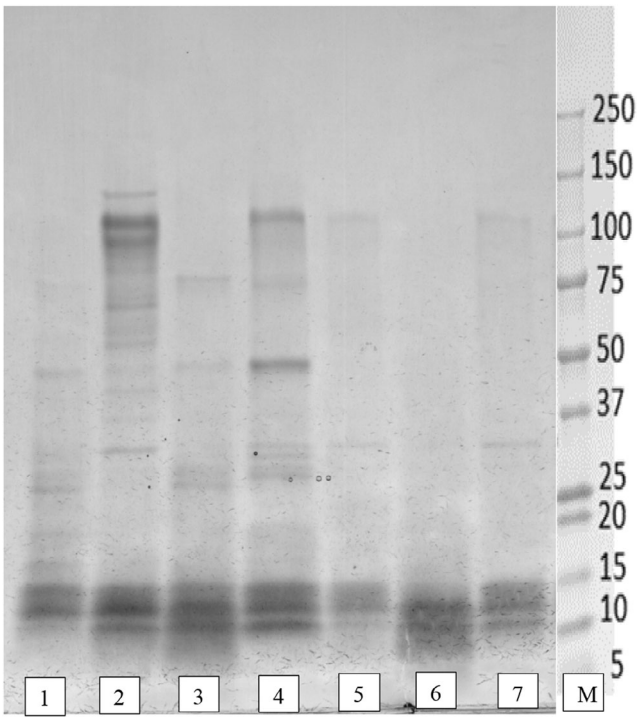

Figure 1 SDS-Page profiles of Hemp protein dispersion and solutions. Lane 1 represents the hemp protein dispersion; lane 2 represents cellulase-treated HPD; lane 3 represents enzymatically treated HPD; lane 4 represents pH-shift-treated HPD; lane 5 represents mechanically treated HPD; lane 6 represents enzymatic and mechanically treated HPS; lane 7 represents pH-shift and mechanically treated HPS and lane M represents the protein molecular weight marker (kDa).

Influence of treatment on the functional properties of emulsions

Particle size and microstructure

The particle size distributions of ME, PE and EE are presented in Table 2 and the variation of volume (%) vs. particle size of the emulsions is shown in Fig. 2.

All the emulsions showed a unimodal distribution (Fig. 2). The influence of treatments was seen as a decrease in $D_{4,3}$ values from 18.38 μm for ME to 17.10 μm for PE and 15.16 μm for EE (Table 2). For EE, 90% of the particles were below the particle diameter of 31.66 μm as compared to PE (38.03 μm) and ME (40.43 μm). It was observed that the combination of homogenisation along with either trypsin (EE) or pH-shift (PE) treatment is more effective in reducing the emulsion droplets' size than homogenisation alone (ME). As discussed earlier, during enzyme hydrolysis, trypsin enzyme cleaves peptide bonds leading to the formation of more soluble and small peptide structures (Andreaus *et al.*, 1999; Yin *et al.*, 2008), whereas pH shift treatment results in a more accessible polypeptide backbone, unfolded and refolded proteins with enhanced surface activity (Wang *et al.*, 2018). Upon homogenisation, these soluble proteins (in EE) or high surface tension (PE), allow the disruption of oil

Table 2 Effect of treatments on the particle size distribution, the flocculation index (FI) and coalescence index (CI) of the hemp protein emulsions

Parameters	ME	PE	EE
As prepared			
$D_{4,3}$ (μm)	18.38 ± 0.68^a	17.10 ± 0.09^a	15.16 ± 0.25^b
d (0.9) (μm)	40.43 ± 3.76^a	38.03 ± 0.31^a	31.66 ± 0.65^a
Stability test			
$D_{4,3}$ (μm) in water (0 h)	17.42 ± 0.07^a	18.82 ± 0.97^a	12.07 ± 1.13^b
$D_{4,3}$ (μm) in water (24 h)	17.35 ± 1.23^a	21.29 ± 0.83^a	13.39 ± 1.52^b
$D_{4,3}$ (μm) in SDS (0 h)	12.67 ± 0.37^b	16.82 ± 0.49^a	11.07 ± 0.92^b
$D_{4,3}$ (μm) in SDS (24 h)	11.43 ± 0.25^a	15.06 ± 0.84^a	12.41 ± 1.70^a
Flocculation index (FI) (%) (0 h)	37.50 ± 1.10^a	11.85 ± 2.56^b	8.94 ± 1.10^b
Flocculation index (FI) (%) (24 h)	51.70 ± 2.49^a	41.72 ± 13.53^a	8.06 ± 2.56^b
Coalescence index (CI) (%)	15.28 ± 0.61^a	13.19 ± 1.41^b	10.89 ± 2.14^b
Zeta potential (mV)	−34.05 ± 0.91^b	−38.70 ± 1.70^b	−45.05 ± 0.14^a

Samples within a column that do not share a superscript letters are significantly different ($P < 0.05$).

droplets and stabilisation of smaller droplets with enhanced efficiency of protein adsorption at the interface.

The size reduction was supported by the microstructure as evidenced from CLSM imaging. As microscopic methods can provide direct information about the particle size, flocculation and coalescence in emulsions (McClements, 2015), CLSM imaging was performed (Fig. 2b–d). The treatments had a profound effect on the microstructure of the emulsions as it can be observed how well protein was homogenised and dispersed with the fat giving an appearance of yellow droplets.

As observed in Fig. 2b (ME), the droplets were flocculated through protein bridges which could be due to the hydrophobic properties. It was also observed how the protein surrounded the fat globules (Fig. 2b, circled area). This is supported by the particle size data wherein ME exhibited the maximum $D_{4,3}$ (18.38 μm) as compared to EE (15.16 μm) and PE (17.10 μm) (Table 2). Similar microstructural properties were observed in a study wherein almond milk was prepared using HPH (Bernat *et al.*, 2014).

In the case of PE (Fig. 2c), there are aggregates or clusters of the oil droplets and a network of protein structures. This may be due to the combined effect of pH-shift treatment and homogenisation that unravels the protein structure that increases the surface hydrophobicity. The chemical treatment also leads to the adsorption of protein molecules onto the surface of the oil droplets which might stabilise the emulsion (Wang *et al.*, 2018). ME and PE showed similar microstructural properties (Fig. 2b,c) along with similar particle sizes (Table 2). The particles in both the emulsions are flocculated through protein bridges and some clusters have entrapped both protein and oil bodies. However, in the case of PE, the clusters are well spread out than ME and the fat globules are well surrounded by the proteins (Fig. 2c, circled area) which may be due to the mechanism of the pH-shift treatment.

In the case of EE, a striking difference between the confocal images can be observed (Fig. 2d). The results are in agreement with the least particle size of EE (15.16 μm) than ME and PE. Tryptic hydrolysis releases soluble peptides from the insoluble precipitates. It also increases the number of exposed ionisable amino groups which thereby increases protein solubility. Following that, when homogenisation is done, the fat and protein molecules are well interspersed with each other (Yin *et al.*, 2008). Similar results were obtained with trypsin hydrolysis and HPH on oyster protein isolates. Post-treatment, emulsion droplet size decreased, and the emulsion became more uniform due to an increase in soluble proteins and peptides with higher surface activity (Cha *et al.*, 2018).

Emulsion stability

Flocculation and coalescence occur during the storage of the emulsion due to attractive forces between the depleted surfactant ions. These interactions promote the formation of a liquid–solid phase transition state and lead to an increase in droplet size and a decrease in emulsion stability. Since the flocculation process affects the particle size, the flocculation index (FI) and coalescence index (CI) was measured using the $D_{4,3}$ value of the emulsions diluted in water as well as 2% SDS, to understand the influence of treatments on emulsions' stability (Table 2). Particle size distribution was measured for each treatment including its treated HPS, emulsion and emulsion treated with 2% SDS at 0 h (Fig. 3a–c).

At 0 h, the $D_{4,3}$ values for all the emulsions, namely, ME, PE and EE (17.42, 18.82, and 12.07 μm, respectively) were greater in water than that in 2% SDS (12.67, 16.82 and 11.07 μm, respectively) indicating the flocculation of oil droplets (Table 2) in water. Overall, the lower values in SDS are due to the fact it displaces the protein from the surface of the droplet as well as disrupts the flocs. According to Kong *et al.* (2017), the $D_{4,3}$ value of the deflocculated particles in the presence of SDS shows the ability of

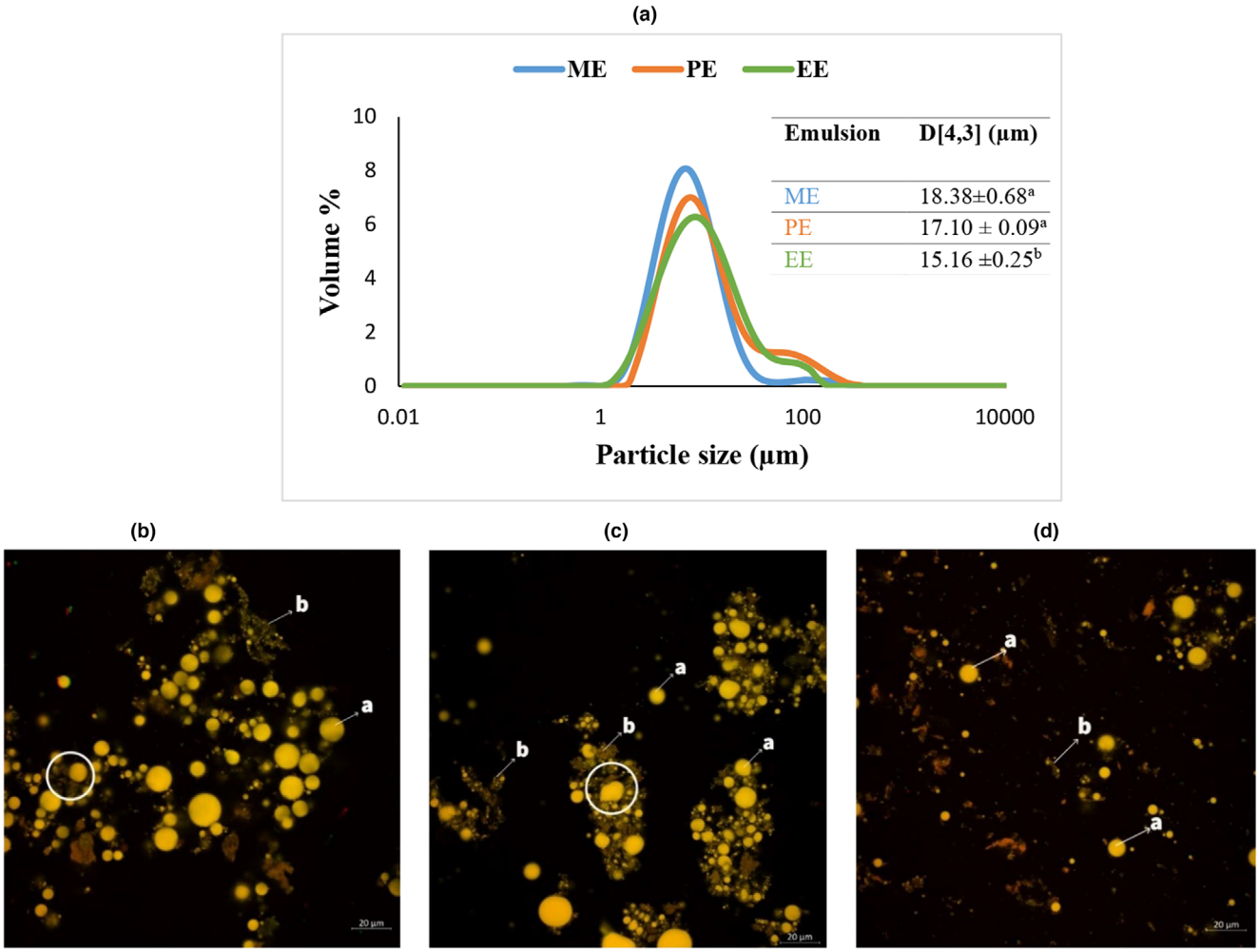

Figure 2 (a) Particle size distribution curves for ME, PE and EE; Confocal images of hemp protein emulsions prepared (b) mechanically, (c) chemically and (d) enzymatically. Images are representative of signals obtained from Nile Red (fat) and Rhodamine B (protein). Letters a and b represent the fat globule and proteins, respectively.

proteins in dispersing the oil phase very well into the aqueous medium. A similar trend was maintained even after 24 h.

The influences of additional treatments (EE, PE), can be seen as a decrease in FI and CI values, as compared to ME. For instance, at 0 h, the FI of EE (8.94%) and PE (11.85%) were significantly smaller as compared to ME (37.50%). Even after 24 h, a similar trend was maintained in FI (8.06% for EE, 41.72% for PE and 51.70% for ME). This indicated enhanced stability against flocculation for PE and EE than ME. The combination of tryptic or pH-shift treatment with homogenisation modifies the structure of the hemp protein adsorbed at the oil/water interface. As a consequence of pH-shift treatment, the exposed hydrophobic sites advance the interactions between adsorbed proteins to reduce the interfacial tension which enhances the emulsion stability (Wang *et al.*, 2018). In the case of EE, the treatment exposes charged domains from the core of the globular proteins present within the hydrophobic segments. This thereby increases the surface hydrophobicity and improves emulsion stability against flocculation (Tamm *et al.*, 2016).

A lower value of CI refers to the formation of a more stable emulsion since more coalescence of emulsion droplets means more destabilisation of the emulsion (Maphosa & Jideani, 2018). The CIs for PE (13.19%) and EE (10.89%) were significantly lower than ME (15.28%). The least CI in the case of EE attributes to the emulsification mechanism of hydrolysates which get adsorbed to the surface of the oil droplets during homogenisation and form a protective layer to prevent coalescence (Gbogouri *et al.*, 2004). For PE, the partially unfolded polypeptides easily get

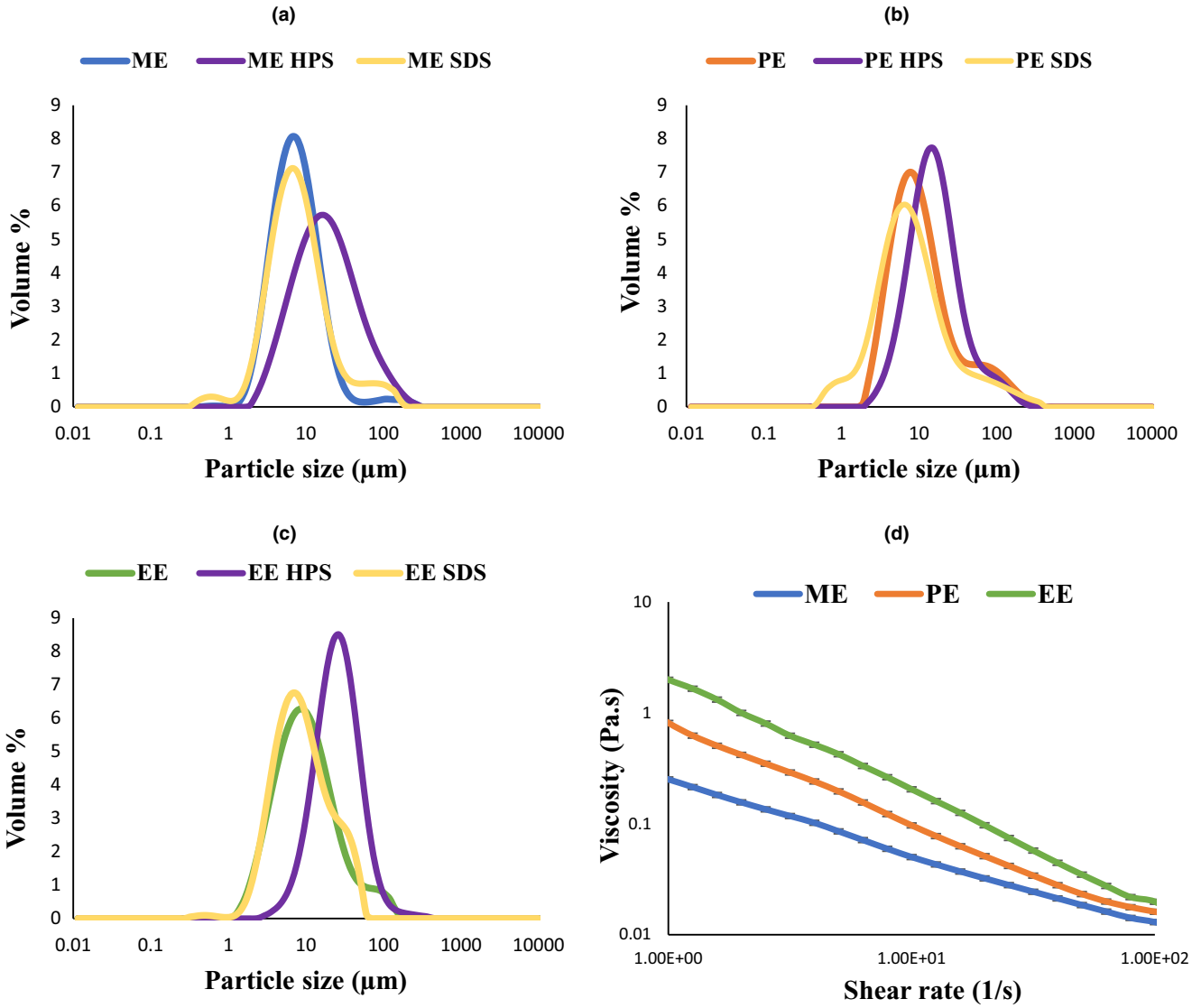

Figure 3 Particle size distribution curves for (a) ME, mechanically treated HPD and ME treated with 2% SDS; (b) PE, PE HPD and PE treated with 2% SDS; (c) EE, EE HPS and EE treated with 2% SDS; (d) Flow behaviour curves of the hemp protein emulsions (error bars represent the standard deviation of the duplicates).

adsorbed on the oil droplets' surfaces through hydrophobic interactions and form a multilayered thick protein membrane that withstands the coalescence of emulsion droplets. Overall, based on FI and CI results (Table 2), EE was the most stable emulsion (with the least FI and CI) followed by PE.

As observed from Fig. 3a–c, SDS did not have a significant effect in decreasing the particle size of the emulsion. It could be said that the emulsion droplets were not only formed by the aggregates of small droplets as the particle size does not significantly decrease with the addition of SDS which acts as a surfactant and leads to protein unfolding (Jafari *et al.*, 2018). It could be conferred that the increased particle size of the prepared HPEs was a result of the contribution of several materials such as carbohydrates, xanthan gum, and lecithin, present in the emulsion.

Zeta potential

Zeta potential gives an insight into the net charge on the particle surface and the degree of electrostatic repulsion between the emulsion droplets (Barba *et al.*, 2019). One of the major factors that affect the zeta potential is the pH of the medium. In this study, the

objective was to compare the stability of the HPEs prepared by different treatments. To have a common state of pH for all the emulsions, the zeta potential was studied at pH 7.0. Table 2 represents the data obtained from Zetasizer.

The negative values of the zeta potential (Table 2) suggest that the surface of the emulsion droplets possess a negative electric charge which arises from the dissociated polar groups of the adsorbed protein molecules (Wiacek & Chibowski, 2005). Generally, a specific value of zeta potential greater than 30 mV is desired to obtain an electrostatically stable emulsion since it will have high repulsive forces (Gumustas *et al*., 2017). In this study, it was observed that EE (45.05 mV) had the highest absolute zeta potential followed by PE (38.70 mV) and ME (34.05 mV) (Table 2), indicating that all the emulsions had high electrostatic repulsion, and this reason might support the physical stabilisation of the emulsions.

According to Lu & Gao (2010), the emulsion systems with a higher absolute value of zeta potential tend to form electrically stable emulsion systems as compared to the systems with low zeta potential which tend to exhibit poor physical stability. In the case of ME, exhibiting the least absolute zeta potential (34.05 mV), due to presence of less ionic surfaces (Cha *et al*., 2019). In the case of PE, the pH-shift treatment followed by homogenisation leads to the unfolding of the protein structure and enhances the exposure of hydrophobic surfaces which results in increased zeta potential than ME (38.70 mV) (Delahaije *et al*., 2013). For EE, the enhanced stability might be due to the presence of ionisable surface groups that were exposed after the tryptic hydrolysis. The hydrolysates act as surface-active agents and stabilise the emulsions due to the exposed hydrophobic and hydrophilic groups. Similar results were obtained in the case of tryptic hydrolysis of wheat gluten proteins (Cabrera-Chávez *et al*., 2010).

Flow behaviour

Flow behaviour properties give an insight into the interactions between the components of the emulsion system (Goodarzi & Zendehboudi, 2018). Figure 3d shows the flow behaviour plots of the HPEs.

Overall, all the samples showed a shear thinning behaviour with a reduction of viscosity when the shear rates increased (Fig. 3d). At 1 s^{-1}, EE showed the maximum viscosity (2.01 Pa.s) followed by PE (0.82 Pa.s) and ME (0.25 Pa.s). When the shear stress vs. shear rate graph was fitted into the Power Law model (Data not shown), the flow behaviour index (n) and consistency coefficient (K) values were: ME (0.67, 0.33 $Pa.s^n$), PE (0.60, 0.49 $Pa.s^n$) and EE (0.96, 2.18 $Pa.s^n$), respectively. The values for n for all the samples were <1, indicating shear thinning behaviour (REF). The K value which indicates the average viscosity of the system was the highest for EE, followed by PE and ME. This trend was also supported by the flow behaviour curves (Fig. 3d). The combination of trypsin treatment with homogenisation might lead to an increase in the viscosity of EE. The treatment leads to the formation of cohesive viscoelastic layers made up of hydrolysed proteins due to intermolecular interactions. This leads to the formation of a dense protein network and a high-viscosity product (Jeewanthi *et al*., 2015). Similar results were reported in a study on the tryptic hydrolysis of canola meal isolate that confirmed that the hydrolysates formed emulsions with a thick consistency, and smaller droplet sizes (Alashi *et al*., 2011).

In the case of PE, the combination of pH-shift treatment and homogenisation leads to an increase in the hydrodynamic volume size of the hemp protein molecules in the continuous phase. This might be caused by structural unfolding which enhanced the emulsion droplet interactions and thus increased the viscosity. Similar results were obtained by Wang *et al*. (2018) wherein the hemp milk prepared with the combination of pH-shift treatment and HPH was more viscous than the samples prepared from individual treatments.

Conclusion

In this study, the influence of mechanical, chemical (pH-shift) and enzymatic (trypsin) treatments on the emulsion stability and functional properties of hemp protein-based milk were investigated. A novel step of fibre hydrolysis of cellulose in hemp was employed to improve the solubility of the protein. A combination of trypsin/pH-shift treatment with mechanical treatment was more effective than the mechanical treatment alone in stabilising the emulsions in terms of particle size distribution, zeta potential, flocculation and coalescence. The results were in coordination with the microstructural changes and surface tension properties of dispersions. The trypsin and chemical treatments showed remarkable improvements in stabilising the emulsions by exposing the polypeptide chains and hydrophobic groups to the surrounding solvent.

As a novel processing method, a combination of these treatments might offer new opportunities to produce hemp milk.

Ethical guidelines

Ethics approval was not required for this research.

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Author contributions

Kirti Rawal: Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); writing – original draft (equal). **Pratheep Kumar Annamalai:** Writing – review and editing (equal). **Dian Widya Ningtyas:** Writing – review and editing (equal). **Sangeeta Prakash:** Conceptualization (equal); methodology (equal); project administration (equal); resources (equal).

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Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ijfs.16735.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methodology and characterisation.
Figure S1. Schematic diagram representing the preparation of hemp protein emulsions.

