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Original article

Synergistic effects of alkaline and heat treatments on structural and functional properties of mung bean protein isolate: improving physicochemical stability of plant-based emulsions

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Summary Plant-based meat alternatives often require fat replacers to mimic the texture of traditional products. This study aimed to develop plant-based emulsion gels using mung bean protein isolate (MBPI) as a potential fat substitute. However, creating these gels via heat setting requires a high protein concentration, which demands modification of the MBPI structure to enhance emulsifying properties. This study investigated synergistic effects of alkaline treatment (0.3 or 3.5% Na_2CO_3) and heat treatments (40 or 70 °C) on the functional properties of MBPI at high protein levels, for potential application as a plant-based emulsion. The combined treatments reduced the zeta potential of protein suspensions from −9 to −19 mV and altered the protein conformation to form smaller particles (from 426 to 166 μm) with increased β-sheet content. These treatments improved dispersibility of 8% MBPI suspension (58 to 86%), emulsifying activity index (6.34–10.89 m^2 g^{-1}), and stability coefficient (43 to 96%). Notably, MBPI samples treated with 0.3% Na_2CO_3 at 40 and 70 °C exhibited excellent emulsifying properties, forming stable monolayers at the oil–water interface, likely due to the increased surface activity of MBPI. Increasing protein concentration to 11% facilitated heat-set gel formation; however, addition of 3.5%-Na_2CO_3 induced premature gelation, limiting its application in emulsions. At 0.3%-Na_2CO_3, increasing the protein content from 8% to 11% and the oil content from 10% to 30% further reduced emulsion droplet size, especially for MBPI treated with 0.3% Na_2CO_3 at 70 °C (MB-0.3%-70 °C) from 5.10 to 2.61 μm, likely due to decreased coalescence. This treatment yielded superior MBPI-stabilised emulsion gels with enhanced penetration, fluid retention, and stability by possibly reducing protein aggregation. These findings demonstrate the potential of MBPI modified by combined addition of 0.3% Na_2CO_3 and heat treatment, particularly MB-0.3%-70 °C, as a promising ingredient for producing plant-based emulsions.

Keywords Alkaline heating, alternative protein, mung bean, plant-based emulsion.

Novelty Impact Statement

- Synergistic effects of 0.3% Na_2CO_3 addition and heat treatments on MBPI were shown to be a potential technique to enhance MBPI's functional properties at high protein levels, for developing plant-based emulsion gels as fat replacers.
- These treatments impacted the interfacial behaviour of MBPI by alteration of protein conformation and hydrophobic interactions, along with decreasing particle sizes.
- MB-0.3%-40 °C and MB-0.3%-70 °C formed monolayers at the interface and likely stabilised the emulsion by reducing protein aggregation.
- Heat-set emulsion gel from MB-0.3%-70 °C at 11% protein showed a more stable gel due to improving technological properties compared to untreated MBPI.

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Introduction

The rise of plant-based meat alternatives has driven research into protein-enriched plant-based products that functionally replace meat for sustainability

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(Kyriakopoulou *et al*., 2021). Legume storage proteins such as those from peas, faba beans, and mung beans serve effectively as alternative proteins in emulsion-based products due to their water-binding and emulsion/gel stabilisation abilities (Kyriakopoulou *et al*., 2021). However, native legume proteins exhibit poor solubility/dispersibility in aqueous solutions due to tight subunit interactions via noncovalent and covalent interactions. This limited solubility hinders their functional utility, particularly their emulsifying properties (Liu *et al*., 2015). Therefore, modifying legume protein structure to enhance solubility is crucial (Jiang *et al*., 2018). The amount of soluble protein is important in emulsification, and a key functionality for emulsion-based products, since it facilitates the adsorption onto oil–water interfaces and stabilisation of an emulsion (Lee *et al*., 2021). Among legume proteins, mung bean protein isolate (MBPI) has gained prominence as a promising ingredient for plant-based meat alternatives due to its amino acid profile, foaming capacity, and gelling behaviour, comparable to commonly used soy protein (Schlangen *et al*., 2022; Shrestha *et al*., 2022). Previous studies have shown that the solubility and emulsifying properties of 8S globulin, the main protein fraction in MBPI, were highly pH-dependent. A higher solubility was observed in alkaline environments above pH 7 (Liu *et al*., 2015).

Heat treatment, a common food processing technique, also can modify a protein's functionality by altering its structure. However, the heat treatment alone caused a minor increase or no change in emulsion activity index (EAI) of plant proteins, as reported by Wang *et al*. (2018) and Nivala *et al*. (2021). Some researchers have shown that combining alkaline treatment with heating can improve plant protein functionalities. For instance, Wang *et al*. (2018) reported that alkaline pH treatment with heating disrupted protein subunits of hemp seed protein isolate, unfolded the tertiary structure, and increased hydrophobic groups, leading to enhanced solubility and emulsifying activity. Similarly, Wang *et al*. (2020) observed a synergistic effect of alkaline treatment (pH 10) with heat treatment at 40 °C on peanut protein isolate functionalities, including solubility and gelling properties, compared to heat treatment alone. Therefore, combining alkaline treatment and heat treatment has great potential to improve the functional properties, particularly solubility and emulsifying properties of MBPI.

Plant-based emulsion gels stabilised by proteins have recently emerged as promising fat replacers in plant-based meat products. These gels are incorporated with extruded proteins to formulate products like patties and vegan sausages. The gels effectively mimic the texture and water-holding capacity of pork backfat, a key component in traditional meat products (Janardhanan *et al*., 2022). Formulating protein-stabilised emulsion gel by heat setting requires high protein concentrations (8–11.5%) in the continuous phase as reported by Baune *et al*. (2021). While existing research explored the impact of pH-shifting (alkaline treatment and followed neutralisation) and heating at low protein concentrations (1.5–5% (w/v) protein contents) of plant proteins (Wang *et al*., 2018, 2020; Alavi *et al*., 2021), the combined effects of alkaline treatment and heat treatment on protein functionalities at higher concentrations relevant to plant-based meat analogs remain unreported. This study aimed to address this gap by investigating the synergistic effects of these factors on MBPI functionalities at high protein concentration, particularly solubility and emulsifying properties, relevant for formulating plant-based meat products. Addition of sodium carbonate (Na_2CO_3) concomitant with heat treatment was used to alter the MBPI structure in this research. A previous study reported that using Na_2CO_3 in the range of 0.05–4% improved the solubility of pea protein isolate by increasing the pH (Reinkensmeier *et al*., 2015). Moreover, Na_2CO_3, a generally recognised as safe (GRAS) alkaline salt used in food production such as noodles, is a suitable choice for such applications. This study provides a promising way to improve the solubility/dispersibility and emulsifying properties of MBPI, which may lead to MBPI-based products of higher quality and expand the utilisation of MBPI in the future.

Materials and methods

Materials and chemical reagents

Commercial mung bean protein isolate (MBPI) was purchased from Shuangta Food Co., Ltd (Yantai, China) and was stored at −18 °C. MBPI, analysed by using AOAC (2016), contained 81.98 ± 0.02% protein, 8.27 ± 0.02% carbohydrate, 5.46 ± 0.00% moisture content, 0.01 ± 0.00% fat, 3.64 ± 0.00% ash, and 0.65 ± 0.03% fibre. Sodium carbonate at AR grade and refined pure coconut oil were procured from Kemaus (Cherrybrook, New South Wales, Australia), and Siam Union Sahamitr Co., Ltd. (Thailand), respectively. Sodium dodecyl sulfate (SDS) was purchased from Kemaus. N,N-Dimethyl-6-propionyl-2-naphthylamine (PRODAN) was purchased from Sigma-Aldrich (Prague, Czech Republic).

Preparation of MBPI suspension treated with various sodium carbonate levels and heat treatments

MBPI powder was dissolved in either distilled deionised (DDI) water or sodium carbonate (Na_2CO_3) solution at 0.3, or 3.5% (w/v) to obtain MBPI suspensions with 8% protein (w/v). The MBPI suspensions

with heat treatment were then prepared by stirring at various temperatures (25, 40, and 70 °C) for 30 min. After cooling in water for 15 min, the pH values of MBPI suspensions were then measured at 25 °C using a digital pH meter (FiveEasy, Mettler Toledo, USA). MBPI suspensions treated without Na_2CO_3 at 25, 40, and 70 °C are referred to as a control, MB-0%-40 °C, MB-0%-70 °C, respectively. MBPI suspensions treated with 0.3% Na_2CO_3 at 25, 40, and 70 °C are defined as MB-0.3%-25 °C, MB-0.3%-40 °C, MB-0.3%-70 °C, respectively. Whereas MBPI suspensions treated with 3.5% Na_2CO_3 at 25, 40, and 70 °C are named as MB-3.5%-25 °C, MB-3.5%-40 °C, and MB-3.5%-70 °C, respectively. These samples were prepared prior to the performance of the analyses described below.

Characterisation of MBPI treated with various Na_2CO_3 levels and heat treatment

Particle size distribution and zeta potential

The particle size distribution and electrical charge (zeta [ζ]-potential) of MBPI suspension were determined at 25 °C, following the method of Kaewmungkun & Limpisophon (2023) with some modifications using a particle sizer and zeta potential analyser (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). After centrifugation of 8% MBPI suspension at 2500 ***g*** for 10 min, to avoid multiple scattering, all supernatants were diluted to obtain 0.1 mg mL^{-1} protein concentration by using various solutions. These buffers included phosphate buffer pH 7 for the control, MB-0%-40 °C, and MB-0%-70 °C, glycine-NaOH buffer pH 9.5 for MB-0.3%-25 °C, MB-0.3%-40 °C, and MB-0.3%-70 °C, and glycine-NaOH buffer pH 10.5 for MB-3.5%-25 °C, MB-3.5%-40 °C, and MB-3.5%-70 °C. A refractive index of water equal to 1.33 was used to calculate the particle size distribution.

Secondary structure by attenuated total reflectance–Fourier transform infrared (ATR-FTIR)

The ATR-FTIR spectra of MBPI suspensions with various Na_2CO_3 levels and heat treatment were analysed using an Alpha-E Bruker FTIR spectrophotometer (Bruker Optik GmbH, Ettlingen, Germany), equipped with a universal Zn-Se ATR accessory. The FTIR spectra over a 4000–500 cm^{-1} range were recorded by 64 scans with a spectral resolution of 4 cm^{-1}. A second-derivative analysis of the amide I region (1700–1600 cm^{-1}) by Gaussian curve-fitting was used to obtain quantitative analysis of the secondary structural components of MBPI suspensions using an Origin2018 software (OriginLab Corporation, Northampton, MA, USA). Gaussian peaks were assigned to their corresponding structure based on their centre. The components centred in the regions of 1615–1640 cm^{-1}, 1641–1649 cm^{-1}, 1650–1659 cm^{-1}, 1660–1680 cm^{-1}, and 1680–1688 cm^{-1} have been assigned to β-sheet structure, random coil, α-helix, β-turn, and β-antiparallel, respectively (Shevkani *et al.*, 2019; Brishti *et al.*, 2020). Determination of the area under each component peak was then used to calculate the percentage of each band.

Surface hydrophobicity

PRODAN was used as a probe to determine surface hydrophobicity of protein at various pH since it is a hydrophobic and uncharged fluorescence probe binding to hydrophobic patches on the surface of proteins, resulting in an increasing fluorescence intensity. Surface hydrophobicity measurements were based on a fluorescence method from Moll *et al.* (2022) with some modifications. MBPI suspensions (8%, w/v) were centrifuged at 2500 ***g*** for 10 min to obtain supernatant. To keep the original pH of MBPI samples after addition of Na_2CO_3, the samples were then diluted in the range of 0–2.50 mg mL^{-1} protein by using various buffers as described above (Section 'Particle size distribution and zeta potential'). The protein sample (180 μL) was mixed with 20 μL of PRODAN (1.41 mM in methanol), then incubated for 15 min in the dark. A solution of 180 μL of protein sample with 20 μL of each buffer was used as a blank for each protein sample. Relative fluorescence intensity (RFI) was determined at 25 °C using a Tecan Spark multimode plate reader (Tecan, Zurich, Switzerland) in a 96-well microplate. The excitation and emission wavelengths were set to 365 and 465 nm, respectively. The slope of the net relative fluorescence intensity versus concentration was used as a value for surface hydrophobicity (H_0). The coefficient of determination (R^2) was at least 0.99.

Intrinsic fluorescence

The fluorescence spectrum of proteins is used as an indicator to characterise the structural conformation of proteins with varied environmental hydrophobicity. Emission spectra of most proteins are typically dominated by the contribution of tryptophan which can be induced by excitation of the sample at 290 nm (Gao *et al.*, 2022). The intrinsic fluorescence spectroscopy was determined following the method of Gao *et al.* (2022). MBPI suspension after centrifugation at 2500 ***g*** for 10 min was diluted with various buffers as described above (Section 'Particle size distribution and zeta potential') to obtain 10 mg mL^{-1} protein concentration. The fluorescence emission spectra (295–500 nm) were recorded using a Duetta fluorescence and absorbance spectrometer (Horiba Scientific, Kyoto, Japan) at a fixed excitation wavelength of 290 nm. A constant slit of excitation and emission was set at 5 nm. The integration time was 1 s.

Adsorption behaviour and interfacial dilatational rheology
The adsorption behaviour and interfacial dilatational rheology of the MBPI samples treated with alkali and heating were determined by an automatic drop tensiometer (ADT, Teclis, France). For the ADT measurement, a pendant droplet with an area of 20 m^2 was created at the tip of a straight G18 needle with 8% MBPI (w/w) suspensions in the pendent droplet and coconut oil in the bulk phase. The droplet shape was fitted with the Young-Laplace equation to calculate the interfacial tension (mN m^{-1}). The pendant droplet was initially equilibrated for 3 h, during which the time evolution of the interfacial tension was recorded. Equilibration was then followed by a frequency sweep and amplitude sweep. The frequency sweep was conducted at a fixed amplitude of 3% with a frequency increasing from 0.005 to 0.1 Hz, while the amplitude sweep was performed at a fixed frequency of 0.02 Hz with an increasing amplitude from 1 to 30%.

Functional properties of MBPI suspension from 8% MBPI treated with Na_2CO_3 and heat treatment

MBPI suspensions at 8% protein treated with Na_2CO_3 and heat treatment were prepared as described in Section 'Preparation of MBPI suspension treated with various sodium carbonate levels and heat treatments'.

Dispersibility
The MBPI suspension (25 mL) was centrifuged at 7000 ***g*** for 30 min at 4 °C using a high speed refrigerated centrifuge (CR22N; Eppendorf Himac Technologies Co., Ibaraki, Japan) to obtain a supernatant. The supernatant was placed in an aluminium pan and then dried in an oven (Memmert UM 400; Memmert GmbH + Co.KG, Schwabach, Germany) at 105 °C. The dried sample from the supernatant was weighted. Dispersibility of the MBPI suspension was calculated according to Ferreira *et al.* (2022) using an equation as follows:

$$\text{Dispersibility}(\%) = \left(1 - \frac{SC_0(g) - SC_S(g)}{SC_0(g)}\right) \times 100$$

where SC_0 is the solid content in the 25 mL suspension before centrifugation and SC_S is the solid content in the supernatant.

Characterisation of emulsions prepared from 8% MBPI treated with Na_2CO_3 and heat treatment
MBPI suspensions at 8% protein (w/v) in various concentrations of Na_2CO_3 (0, 0.3, 3.5% (w/v)) were prepared at various temperatures (25, 40, 70 °C) as described above. The MBPI suspensions were homogenised with coconut oil at a ratio of 9:1 (v/v) at 13 500 rpm for 2 min, followed by 24 000 rpm for 2 min at 30 °C using a high-speed homogeniser (Ultra Turrax T25; IKA Co., Braun, Germany) to obtain protein-stabilised emulsions. The emulsions prepared from MBPI treated without Na_2CO_3 at 25, 40, and 70 °C are referred to as E-control, E-0%-40 °C, and E-0%-70 °C, respectively. The emulsions prepared from MB-0.3%-25 °C, MB-0.3%-40 °C, and MB-0.3%-70 °C are defined as E-0.3%-25 °C, E-0.3%-40 °C, and E-0.3%-70 °C, respectively. Whereas The emulsions prepared from MB-3.5%-25 °C, MB-3.5%-40 °C, and MB-3.5%-70 °C are named E-3.5%-25 °C, E-3.5%-40 °C, and E-3.5%-70 °C, respectively.

Emulsifying properties. Emulsifying properties including emulsion activity index (EAI) and stability coefficient of the MBPI emulsions were determined. The fresh emulsion was diluted 250 times with 0.1% SDS solution and then vortexed immediately. The absorbance of the diluted emulsion was evaluated at 500 nm using a UV–visible spectrophotometer (G10S UV–Vis, Thermo Fisher Scientific Inc., Waltham, MA, USA). EAI was calculated following the method of Ge *et al.* (2021) using the following equation:

$$\text{EAI}\left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times A_0 \times D}{C \times \theta \times 10^4}$$

where A_0 is the absorbance at 500 nm, D is the dilution factor (250), C is the initial protein concentration (g mL^{-1}), and θ is the oil volume fraction of the emulsion (0.1).

For stability coefficient of MBPI emulsions, the fresh emulsion was immediately diluted 100 times with DDI water to determine the absorbance of the diluted emulsion at 750 nm using a UV–visible spectrophotometer. Another fresh emulsion was immediately centrifuged at 2000 ***g*** for 15 min at 25 °C to destabilise the emulsion system. The supernatant from the centrifuged emulsion was then diluted with DDI water by 100 times to determine the absorbance at 750 nm. The stability coefficient was calculated using the following equation of Li *et al.* (2019):

$$\text{Stability coefficient}(\%) = \frac{A_2}{A_1} \times 100$$

where A_1 is the absorbance value of the original emulsion at 750 nm and A_2 is the absorbance value of the supernatant at 750 nm.

Droplet size of emulsions. The droplet size of MBPI emulsions was measured following the method of Baune *et al.* (2021). MBPI emulsions were diluted using DDI water in ratio of 1 to 10, then stirred at 350 rpm for 1 h. After centrifugation at 3070 ***g*** for 5 min, the cream phase was used to analyse droplet

size distribution at 25 °C using a laser light scattering instrument (Mastersizer 2000; Malvern Instruments, Worcestershire, UK). Refractive indexes of 1.33 for the water phase and 1.459 for the oil phase were used. The density of emulsion evaluated using a pycnometer (Superior Marienfeld, Lauda-Königshofen, Germany) was used to convert the unit of SSA from $m^2\ g^{-1}$ to $m^2\ mL^{-1}$. The cream was dispersed in a wet dispersion unit (Hydro 2000s; Malvern Instruments) using DDI water until a laser obscuration value of about 12%. The volume mean diameter (D [4, 3]) in μm, surface mean diameter (D [3, 2]) in μm, and specific surface area (SSA) in $m^2\ mL^{-1}$ were calculated.

Interfacial protein concentration. Surface protein concentration (SPC) of oil droplets was used to quantify the protein concentration at the oil–water interface following the method and calculation of Fernandez-Avila & Trujillo (2016). After centrifugation of MBPI emulsions at 4430 ***g*** for 30 min at 25 °C, adsorbed-protein content in the cream phase was determined by using the Kjeldahl procedure (AOAC, 2016). The crude protein content was calculated as nitrogen content × 6.25. SPC was calculated using the following equation:

$$\mathrm{SPC}\left(\mathrm{mg\ m^{-2}}\right) = \frac{\text{Adsorbed protein (mg)/Emulsion (mL)}}{\mathrm{SSA}\left(\mathrm{m^2\ mL^{-1}}\right)}$$

Determination of emulsions and heat-set emulsion gels from 11% MBPI treated with alkaline and heat treatments at various oil contents

To produce a heat-set emulsion gel using MBPI suspensions, a higher protein concentration was necessary compared to the previous study (8% protein). Therefore, both MB-0.3%-40 °C and MB-0.3%-70 °C suspensions were prepared at 11% protein content. These suspensions were then homogenised with varying coconut oil contents (10–30% w/w emulsion) following the above procedure to obtain E-0.3%-40 °C and E-0.3%-70 °C, respectively. The emulsion from 11% MBPI treated without Na_2CO_3 at 25 °C (E-control) was used as a control.

Droplet size distribution

The droplet size values of the protein-stabilised emulsions from various oil contents were determined including D [4, 3], D [3, 2], and SSA, as described in Section 'Droplet size of emulsions'.

Technological properties of heat-set emulsion gels

Penetration force. The penetration force of heat-set emulsion gels was determined following the method of Câmara *et al*. (2020) with some modifications. Emulsions were placed into a stainless-steel tube (30 mm in diameter and 25 mm in height), then heated at 90 °C for 1 h, followed by cooling down to 4 °C for 10 min. Prior to determination, the heat-set emulsion gel was kept at 4 °C for 24 h. Penetration tests were performed at 25 °C using a TA-XT2 texture analyser (Stable Micro System, Surrey, UK) with a 5-kg load cell equipped with a cylindrical stainless plunger (6 mm in diameter). Analysis was carried out at a test speed of 0.80 mm s^{-1}. The penetration force in gram force (g) unit was derived from the maximum force at the point of gel rupture.

Total expressible fluid. Total expressible fluid (TEF) was used to determine stability of heat-set emulsion gels using a modified method of Kamani *et al*. (2019). Protein-stabilised emulsions (10 g) were placed into a tube and centrifuged at 10 000 ***g*** for 15 min at 25 °C. Subsequently, the heat-set emulsion gels were set in a water bath at 90 °C for 60 min. The emulsion gels were then left to stand upside-down for 45 min to release the exudates, kept at 4 °C overnight, and then weighed. The measurements were carried out in three replicates. The TEF was calculated using the following equation of Kamani *et al*. (2019):

$$\mathrm{TEF}(\%) = \frac{\mathrm{M_{sample}(g) - M_{pellet}(g)}}{\mathrm{M_{sample}(g)}} \times 100$$

where M_{sample} is the weight of original emulsion and M_{pellet} is the weight of the heat-set emulsion after it was kept at 4 °C overnight.

Time sweep rheology

Both E-0.3%-40 °C and E-0.3%-70 °C at 11% protein with 30% coconut oil were prepared as described above. Rheological properties of the emulsion were determined in the linear viscoelastic regime and compared with emulsion from 11% MBPI without Na_2CO_3 and heat treatment at 30% oil (E-control) using small-amplitude oscillatory shear (SAOS) measurements, with an Anton Paar Physica MCR 301 (Anton Paar Co., Ltd., Graz, Austria). SAOS tests were conducted using a parallel plate geometry (50 mm in diameter, 1 mm gap). The emulsion samples (approximately 3 g) were transferred onto the bottom plate with a spatula and then covered with a thin layer of paraffin oil around the edge of the sample to prevent water evaporation. The samples were equilibrated for 10 min at 25 °C before conducting a time sweep measurement. The measurement was conducted at 1% strain and 1 Hz frequency for 3 h at 25 °C. Storage modulus (G′) and loss modulus (G″) of three emulsions were recorded as a function of time each 10 min during the tests, to compare the rheological behaviour of the emulsions, prior to heat-set gelling.

Statistical analysis

The experimental design was completely randomised. One-way analyses of variance were conducted, and mean comparisons were performed using Duncan's multiple range tests. The test level of significance was set at $P < 0.05$. Data analysis was conducted using IBM SPSS Statistics version 28.0 (Thaisoftup Co., Ltd., Bangkok, Thailand).

Results and discussion

Characteristics of MBPI treated with various Na_2CO_3 levels and heat treatment

pH, particle size, and ζ-potential of MBPI suspension

Table 1 shows a significant increase in pH for MBPI suspensions treated with Na_2CO_3. The suspensions with 0.3 and 3.5% Na_2CO_3 exhibited a rise in pH from 7.13–7.14 to 9.39–9.43 and 10.56–10.57, respectively. However, heat treatment did not affect the pH of MBPI suspensions. In Table 1, the average particle sizes (166–274 nm) of MBPI samples treated with either 0.3 or 3.5% Na_2CO_3 at heat treatments (40 and 70 °C) were smaller than those of the control at 426 nm (MBPI without Na_2CO_3 at 25 °C). Our study found that MBPI suspensions treated with 0.3% Na_2CO_3, resulting in a MBPI suspension pH of 9.4, had an average particle size of 166–219 nm. This was similar to the particle size of MBPI prepared at pH 9 (194 nm) as a report of Liu *et al.* (2021). These observations suggested that the alkaline pH condition possibly influences the particle size of MBPI suspensions. At alkaline pH conditions, the particle size was likely smaller, since these pH values (9.4–10.5) are far from the pI of MBPI (4.5–4.6) as reported by Brishti *et al.* (2020) and Liu *et al.* (2021), leading to stronger electrostatic repulsion between the protein molecules. This may lead to the disruption of protein aggregates, and even changes in MBPI structure (Sun *et al.*, 2023). A smaller particle size of soybean protein isolate (SPI) in the range of 193–500 nm was also found after combined treatment of pH-shifting (pH 9–12) with heat treatment at 50 °C for 1 h, as compared with those without heat treatment at pH 7 (1283 nm) (Sun *et al.*, 2023).

Effect of alkalinity on particle size of MBPI was supported by ζ-potential values, as shown in Table 1. The ζ-potential values of MBPI treated with 0, 0.3, and 3.5% Na_2CO_3, corresponding to pH 7.1, 9.4, and 10.5 were approximately −9.3, −19.2, −19.5 respectively. It indicated that MBPI with a higher amount of Na_2CO_3 had more negative charge on the protein surface, regardless of the heating effect. In general, a value of the ζ-potential far away from zero indicates more electrochemical stability of the system. Increasing net negative charge by alkaline environment would improve more electrochemical stability of protein particles via electrostatic repulsion and favour dissolving and dispersion of MBPI (Liu *et al.*, 2021; Kaewmungkun & Limpisophon, 2023). The MBPI samples treated with 0.3–3.5% under heat treatment in this study showed ζ-potential values around −19 mV, which is significantly less negative than that of MBPI at pH 9 (−42 mV) from another report (Liu *et al.*, 2021). Protein isolates of red lentils from various sources at the same pH also showed significantly different ζ-potential values, because genetic variations and differences in

Table 1 pH, Particle size, ζ-potential, and initial interfacial tension (after 2 s) of MBPI treated with various Na_2CO_3 levels and heat treatments

MB-Na_2CO_3(%)-Temperature (°C)	pH	Average particle size (nm)	ζ-potential (mV)	Interfacial tension (mN m^{-1}) (after 2 s)
Control	$7.14^{c} \pm 0.01$	$426.20^{a} \pm 13.45$	$-9.26^{a} \pm 0.09$	$10.42^{ab} \pm 0.12$
MB-0%-40 °C	$7.13^{c} \pm 0.03$	$345.17^{b} \pm 23.23$	$-9.36^{a} \pm 0.12$	$11.75^{a} \pm 0.31$
MB-0%-70 °C	$7.13^{c} \pm 0.03$	$219.83^{d} \pm 0.40$	$-9.31^{a} \pm 0.11$	$11.62^{a} \pm 0.16$
MB-0.3%-25 °C	$9.39^{b} \pm 0.03$	$218.90^{d} \pm 5.80$	$-19.10^{b} \pm 0.20$	$9.16^{bc} \pm 0.11$
MB-0.3%-40 °C	$9.43^{b} \pm 0.05$	$209.43^{d} \pm 2.10$	$-19.23^{b} \pm 0.15$	$8.84^{c} \pm 0.22$
MB-0.3%-70 °C	$9.43^{b} \pm 0.06$	$165.97^{e} \pm 0.70$	$-19.20^{b} \pm 0.17$	$6.74^{d} \pm 0.95$
MB-3.5%-25 °C	$10.56^{a} \pm 0.06$	$334.70^{b} \pm 2.66$	$-19.53^{c} \pm 0.15$	≤1[†]
MB-3.5%-40 °C	$10.57^{a} \pm 0.05$	$273.63^{c} \pm 7.03$	$-19.57^{c} \pm 0.12$	≤1[†]
MB-3.5%-70 °C	$10.57^{a} \pm 0.02$	$177.13^{e} \pm 0.55$	$-19.53^{c} \pm 0.12$	≤1[†]

The values are expressed as mean ± SD ($n = 3$). Different superscripts within the same parameter indicate significant differences ($P < 0.05$). Control means MBPI suspension treated without Na_2CO_3 at 25 °C. MB-0%-40 °C and MB-0%-70 °C mean MBPI suspensions without Na_2CO_3 treated at 40 and 70 °C, respectively. MB-0.3%-25 °C, MB-0.3%-40 °C, and MB-0.3%-70 °C mean MBPI suspensions treated with 0.3% Na_2CO_3 at 25, 40, and 70 °C, respectively. MB-3.5%-25 °C, MB-3.5%-40 °C, and MB-3.5%-70 °C mean MBPI suspensions treated with 3.5% Na_2CO_3 at 25, 40, and 70 °C, respectively.

[†]The surface tension was immediately reduced to lower than 1 mN m^{-1} after droplet formation, and could not be continuously measured by the ADT, because of noise and droplet loss.

environmental growth conditions influenced amino acid profile and surface charge (Lee *et al.*, 2021). Here, the extraction and drying process of our MBPI source may also have significantly affected the ζ-potential.

Secondary structure

Treatment with alkaline Na_2CO_3 solution under heating contributed to an increase in electrostatic repulsion, which can result in a weakening of the secondary and tertiary structure of protein molecules; therefore, FTIR was used to provide information on the secondary structure of proteins. β-sheet content increased from 32% to 36% in MBPI treated with increasing Na_2CO_3 content under heat treatment (40 and 70 °C), as shown in Fig. 1. The highest content (36%) was observed for MB-3.5%-70 °C condition. Among the remaining conditions, MB-0.3%-70 °C, MB-3.5%-25 °C, and MB-3.5%-40 °C exhibited the next highest β-sheet contents around 35%. Heat treatment alone did not affect a change in β-sheet content (32%) of MBPI ($P \geq 0.05$). Effects of alkaline pH and heating resulting in increasing content of β-sheet was also found in peanut protein isolate at pH 10 under 40 °C heating (Wang *et al.*, 2020). Higher content of β-sheet in plant proteins is usually related with improvement of food functionalities like better gel properties, or stabilisation of oil–water interfaces (Wang *et al.*, 2020; Khan *et al.*, 2023). Contents of random coil and α-helix were decreased after MBPI was treated with 0.3 and 3.5% Na_2CO_3 under heat treatment (40–70 °C). Among the samples, MB-3.5%-70 °C exhibited the lowest content of random coil and α-helix concomitant with the highest content of β-sheet, as compared with the control. This could indicate the conversion of random coil and α-helix to β-sheet. The conversion of random coil to β-sheet would provide more ordered secondary structure (Brishti *et al.*, 2020; Wang *et al.*, 2020). A decrease in α-helix content suggests the unfolding, dissociation, and rearrangement of protein molecules (Li *et al.*, 2020). Moreover, low content of α-helix in soybean protein provided greater flexibility of its structure, resulting in good functionality properties like emulsifying and foaming properties (Yan *et al.*, 2021b). Increasing temperature from 25 to 70 °C increased the β-turn content of MBPI treated with the same Na_2CO_3 concentration. The content of β-turn structure, a product of the unfolding of any higher-order structures, was also increased after 70–90 °C heating of 5% SPI for longer time (Wang *et al.*, 2014). In general, β-structures including parallel and antiparallel β-sheets and β-turns are major secondary structure components in pulse proteins. Higher content of β-structures is related with higher thermal stability as compared to proteins with higher content of α-helix (Shevkani *et al.*, 2019). In our study, heat treatment significantly increased the β-structure content of Na_2CO_3-treated MBPI, rising from 54.90% to 59.55% for 3.5% Na_2CO_3, and from 50.51% to 55.55% for 0.3% Na_2CO_3. In contrast, the increase was less pronounced for MBPI without Na_2CO_3, rising only from 50.34% to 52.48%. These findings indicated that the combined effect of alkaline and heat treatments synergistically altered the secondary structure of MBPI.

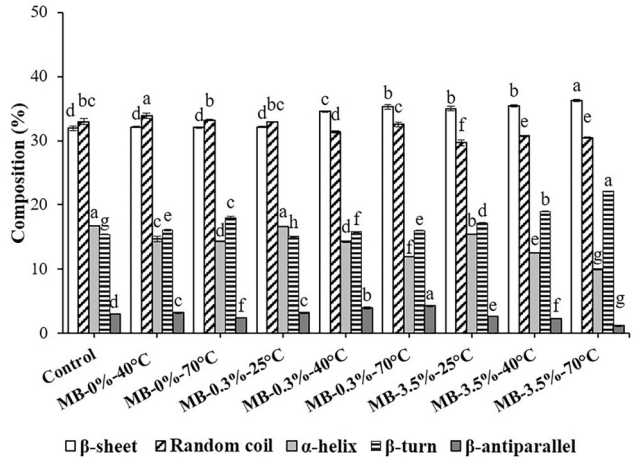

Figure 1 The secondary structure compositions of MBPI suspension treated with various Na_2CO_3 levels and heat treatment. Different letters for the same structure indicate significant differences ($P < 0.05$). The values are expressed as mean ± SD ($n = 3$); control means MBPI suspension treated without Na_2CO_3 at 25 °C. MB-0%-40 °C and MB-0%-70 °C mean MBPI suspensions without Na_2CO_3 treated at 40 °C and 70 °C, respectively; MB-0.3%-25 °C, MB-0.3%-40 °C, and MB-0.3%-70 °C mean MBPI suspensions treated with 0.3% Na_2CO_3 at 25, 40, and 70 °C, respectively; MB-3.5%-25 °C, MB-3.5%-40 °C, and MB-3.5%-70 °C mean MBPI suspensions treated with 3.5% Na_2CO_3 at 25, 40, and 70 °C, respectively.

Surface hydrophobicity and intrinsic fluorescence intensity

A remarkable increase in surface hydrophobicity (H_0 index) was found by combined treatment at low Na_2CO_3 concentration (0.3%) and under heat treatment at 40–70 °C, as compared to those without Na_2CO_3 (Fig. 2a). Increasing H_0 index corresponds to exposure of hydrophobic groups within the protein, indicating a change in tertiary structure of the protein. Intrinsic fluorescence is also used to determine conformational changes, since fluorescence intensity exhibits the changes of fluorescence quantum yield of aromatic amino acid residues, especially tryptophan with excitation at 290 nm (Gao *et al.*, 2022). In Fig. 2b, three MBPI samples treated with 0.3% Na_2CO_3 (MB-0.3%-70 °C, following MB-0.3%-40 °C, and MB-0.3%-25 °C) had high fluorescence intensities, followed by the samples treated with heat treatment alone (40–70 °C). Whereas the lowest fluorescence intensities were found in MBPI treated with 3.5% Na_2CO_3 with and without heat treatment,

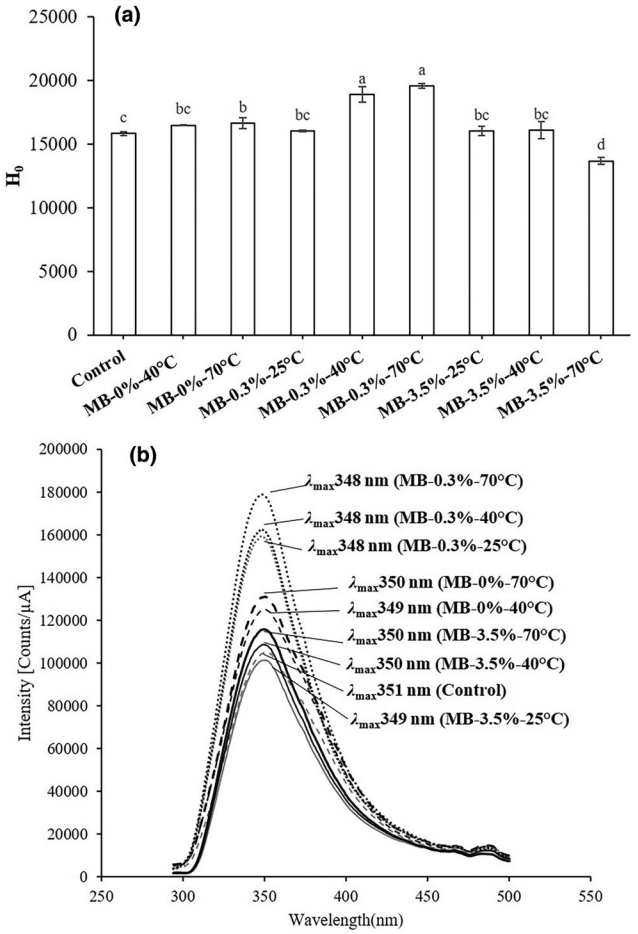

Figure 2 Surface hydrophobicity (H_0) of MBPI suspension treated with various Na_2CO_3 levels and heat treatment (a) and their intrinsic fluorescence emission spectra at 290 nm excitation (b). Sample abbreviations are defined in Fig. 1. The values are expressed as mean ($n = 3$). Bars marked with a different letter in (a) are significantly different ($P < 0.05$).

and the control. MB-0.3%-70 °C and MB-0.3%-40 °C with relatively high fluorescence intensity (i.e., enhanced exposure of the chromophores) demonstrated the highest H_0 value. Increasing H_0 value, caused by exposing hydrophobic groups, was also found in alkaline pH-shifting-treated SPI at pH 12 as compared with untreated pH-7 SPI (Yan *et al.*, 2021a). However, MB-3.5%-70 °C condition at 10.5 pH had low H_0 index, concomitant with relatively small fluorescence intensity. This might be because hydrophobic regions of MBPI under combined treatment of high concentration (3.5% Na_2CO_3) with heat treatment at 70 °C were more exposed, and subsequently aggregated through hydrophobic interactions. Lower H_0 index in quinoa protein isolate treated at pH 10.5 with heating was also found as compared with those treated at pH 8.5 (Mäkinen *et al.*, 2015).

Peak values (λ_{max}) of fluorescence intensity in all MBPI samples were greater than 330 nm as shown in Fig. 2b. It indicated that a chromophoric group, particularly tryptophan, is in a polar environment. Regardless of heat treatment, MBPI samples treated with 0.3% Na_2CO_3 (MB-0.3%-25 °C, MB-0.3%-40 °C, and MB-0.3%-70 °C) had lower λ_{max} at 348 nm than the control sample (351 nm). Decreasing λ_{max} suggested that more chromophoric groups are exposed to polar environments (Gao *et al.*, 2022). According to the increasing fluorescence intensity with decreasing λ_{max}, conformational changes in MBPI samples with 0.3% Na_2CO_3 addition might facilitate more available binding sites for the PRODAN probe in surface hydrophobicity measurements, which resulted in greater H_0 value.

Oil–water interfacial properties of 8% MBPI

Interfacial adsorption behaviour. To see how the observed changes in particle size, ζ-potential, secondary structure, and hydrophobicity affect the oil–water interfacial properties, the adsorption behaviour of MBPI at different conditions (control, MB-0%-40 °C, MB-0%-70 °C, MB-0.3%-25 °C, MB-0.3%-40 °C, and MB-0.3%-70 °C) was studied using a drop tensiometer, as shown in Fig. 3a. Although in emulsification, there is a substantial contribution of transport towards the interface by convection, tensiometry under quiescent conditions (where the transport is purely diffusive), can still provide information on the effects of the combined treatment on surface activity and on the viscoelastic properties of the films the proteins form. In the initial stages of adsorption (up to ~2 s), the MBPI treated with 0.3% Na_2CO_3 has a lower interfacial tension in the range of 6.74–9.16 mN m^{-1}, than those without Na_2CO_3 treatment (10.42–11.75 mN m^{-1}), indicating the MBPI with Na_2CO_3 treatment adsorbed faster to the oil–water interface. This is in line with the smaller particle size of MBPI with Na_2CO_3 treatment (Table 1), which would allow for faster diffusion towards the interface. Besides, the MBPI treated with 0.3% Na_2CO_3 shows slightly higher surface hydrophobicity (Fig. 2) than those without Na_2CO_3 treatment, indicating the Na_2CO_3 treatment exposed more hydrophobic groups of MBPI. The increased hydrophobicity may reduce the energy barrier which needs to be overcome upon adsorption to the oil–water interface, which may also have increased the adsorption rates of MBPI with Na_2CO_3 treatment. For the samples treated with 3.5% Na_2CO_3 the surface tension immediately dropped to values below 1 mN m^{-1} (Table 1) upon creation of a droplet, making it impossible to determine the time evolution of the surface tension, due to noise and droplet loss. These samples had only a slightly smaller particle size than the samples treated at 0.3%

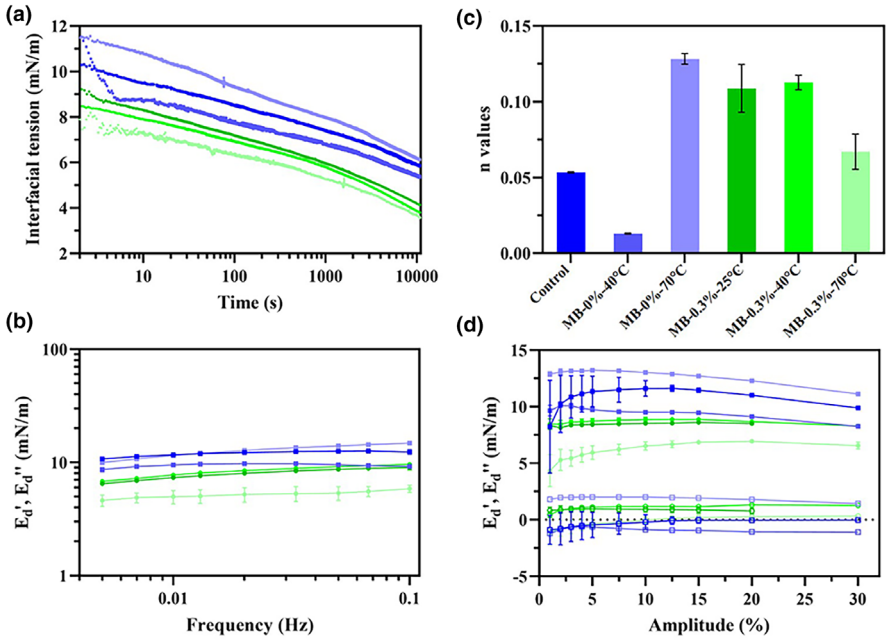

Figure 3 Oil–water interfacial properties of 8% MBPI suspensions treated with various Na_2CO_3 and heat treatment. (a) Interfacial tension (mN m^{-1}) as a function of time of control (■), MB-0%-40 °C (■), MB-0%-70 °C (■), MB-0.3%-25 °C (●), MB-0.3%-40 °C (●), and MB-0.3%-70 °C (●). (b) Interfacial dilatational storage modulus (E_d') as a function of frequency for control (■), MB-0%-40 °C (■), MB-0%-70 °C (■), MB-0.3%-25 °C (●), MB-0.3%-40 °C (●), and MB-0.3%-70 °C (●). (c) The values of power-law exponent n obtained from the interfacial dilatational frequency sweeps at a fixed 3% amplitude for control (■), MB-0%-40 °C (■), MB-0%-70 °C (■), MB-0.3%-25 °C (■), MB-0.3%-40 °C (■), and MB-0.3%-70 °C (■). (d) Interfacial dilatational storage modulus (E_d') and loss modulus (E_d'') as a function of amplitude (%), at frequency of 0.02 Hz, for control (— and —), MB-0%-40 °C (— and —), MB-0%-70 °C (— and —), MB-0.3%-25 °C (— and —), MB-0.3%-40 °C (— and —), and MB-0.3%-70 °C (— and —).

Na_2CO_3 and had a somewhat lower exposed hydrophobicity. This would not explain the significant drop in surface tension. A possible reason could be interactions with polyphenols, since the pH of these samples was high (~10.5), which can lead to oxidation of the polyphenols, and interactions with the proteins. For whey protein, these interactions were shown to lead to a lower surface tension (Yang *et al.*, 2021).

Interfacial dilatational rheology. After 3 h of adsorption, the oil–water interface for MB-0%-40 °C, MB-0%-70 °C, MB-0.3%-25 °C, MB-0.3%-40 °C, and MB-0.3%-70 °C was subjected to frequency sweeps and amplitude sweeps as compared to the control. In the frequency sweeps (Fig. 3b), the MBPI with and without Na_2CO_3 treatment showed a low-frequency dependence. The data from the frequency sweep were fitted with a power law model ($E_d' = \omega^n$) to obtain the exponent n. An exponent of 0.5 indicates that the response of the interface is mainly dominated by the exchange between bulk and interface (Lucassen & Van Den Tempel, 1972). The MBPI samples showed exponents ranging from 0.01 to 0.11 (Fig. 3c) and were clearly lower than 0.5, indicating limited exchange between bulk and interface during oscillation. Together with the fact that the dilatational storage modulus is larger than the dilatational loss modulus, E_d'', this low exponent indicated the proteins formed soft disordered viscoelastic solid structures at the oil–water interface (i.e., a soft 2d gel or glass-like phase), irrespective of the treatment.

In the amplitude sweeps (Fig. 3d), the storage modulus of all interfaces shows only a mild decrease for increasing amplitudes, indicating limited disruption of the interfacial microstructure, and hence a stretchable oil–water interface in response to large dilatational deformations. The MBPI treated with 0.3% Na_2CO_3 shows a slightly lower E_d' at all amplitudes than those without Na_2CO_3 treatment, indicating the Na_2CO_3 treatment to MBPI clearly reduces the interfacial stiffness. As shown in Table 1, the MBPI with 0.3% Na_2CO_3 shows more negative charges than those without Na_2CO_3 treatment. The more negatively charged MBPI with 0.3% Na_2CO_3 led to greater electrostatic repulsion between the protein molecules at the oil–water interface, possibly leading to more expanded and less densely packed protein layers at the interface. As a result, the MBPI with 0.3% Na_2CO_3 formed a

less stiff and relatively more mobile oil–water interface. The effect of heat treatment on the dilatational moduli was relatively mild.

Heat treatment under alkaline conditions (induced by Na_2CO_3 addition) could significantly alter protein structure. This treatment likely created a semi-dissociated state, allowing solvent molecules to access newly exposed regions within the protein, as observed in the secondary structure analysis. This possibly enhances accessibility and promotes hydration and solubilisation of the MBPI (O′ Flynn *et al*., 2021). The connection between these structural changes at the protein level and the resulting improvement in functional properties regarding protein-stabilised emulsion characteristics was explored in the next section.

Functional properties of MBPI after combined treatment with Na_2CO_3 and heat treatment

Dispersibility

In Table 2, the effect of heat treatment only increased dispersibility of MB-0%-70 °C from 59 to 78% as compared with the control. Heat treatment would generally modify protein functionalities through a change in protein structure including unfolding of native tertiary structures, and exposure of hydrophobic regions and free sulfhydryl (-SH) groups. Heat-denatured molecules, possibly leading to the formation of aggregates, can cause changes in protein functionalities such as protein solubility/dispersibility and emulsion activities (Alavi *et al*., 2021).

Investigating the effect of Na_2CO_3 concentration, dispersibility values of both MB-0.3%-25 °C and MB-3.5%-25 °C increased from 59 to 64–66% as compared to the control.

While we used solid content determination after centrifugation and drying to assess dispersibility, this method may not be the most accurate for measuring pure protein solubility due to the presence of residual salts like Na_2CO_3. However, the observed increase in dispersibility compared to the control suggested a potential increase in soluble proteins. This is because even though the MB-3.5%-25 °C sample had a higher Na_2CO_3 concentration (potentially contributing to higher solid content), it showed similar dispersibility to the MB-0.3%-25 °C sample. This indicated more soluble proteins in both samples as compared to the control. Furthermore, the addition of Na_2CO_3 at both 0.3% and 3.5% resulted in an increase in suspension pH to 9.4–10.5 (Table 1). Increased pH is known to enhance protein solubility, which may explain the observed increase in dispersibility despite the presence of residual salts.

At alkaline pH far, the protein molecules had more negative charges on the surface. Therefore, the hydrophobic interaction between protein molecules within protein aggregates could be overcome by electrostatic repulsion, and hydration, which can promote solubility (Ladjal-Ettoumi *et al*., 2016). These results concurred with others who found that at alkaline pH (above 8 to 10), the solubility of protein isolates from various pulses such as pea, chickpea, lentil, and mung bean were increased as compared to pH 7 (Ladjal-Ettoumi *et al*., 2016; Liu *et al*., 2021).

At the same Na_2CO_3 concentrations (0.3% and 3.5%), increasing the temperature of heat treatment from 25 to 70 °C significantly increased dispersibility values of MBPI suspensions from 64 to 86% ($P < 0.05$) as shown in Table 2. The combined positive effects of pH adjustment to 9–12 and heating (80–90 °C) on higher protein dispersibility were also found in other protein isolates from soybean and hemp (Wang *et al*., 2018; O′ Flynn *et al*., 2021). Heat denaturation of proteins could generate different structures such as flexible strands, nano, and microparticles depending on protein concentration, heating temperature, more importantly, pH and ionic strength (Alavi *et al*., 2021).

Table 2 Functional properties of MBPI treated with various Na_2CO_3 levels and heat treatments

MB-Na_2CO_3 (%)-Temperature (°C)	Dispersibility (%)	EAI ($m^2\ g^{-1}$)	Stability coefficient (%)
Control	$58.87^{e} \pm 1.08$	$6.34^{d} \pm 0.30$	$43.15^{d} \pm 1.13$
MB-0%-40 °C	$58.69^{e} \pm 0.15$	$6.49^{d} \pm 0.14$	$43.85^{d} \pm 0.94$
MB-0%-70 °C	$77.51^{b} \pm 0.70$	$6.84^{d} \pm 0.19$	$38.84^{e} \pm 0.66$
MB-0.3%-25 °C	$65.57^{d} \pm 0.56$	$8.54^{c} \pm 0.19$	$40.10^{e} \pm 1.64$
MB-0.3%-40 °C	$68.56^{c} \pm 0.78$	$9.60^{b} \pm 0.39$	$46.38^{c} \pm 0.98$
MB-0.3%-70 °C	$78.30^{b} \pm 0.76$	$10.62^{a} \pm 0.16$	$48.04^{c} \pm 1.26$
MB-3.5%-25 °C	$63.86^{d} \pm 2.45$	$6.72^{d} \pm 0.57$	$96.31^{a} \pm 0.98$
MB-3.5%-40 °C	$69.67^{c} \pm 0.37$	$8.54^{c} \pm 0.42$	$94.06^{b} \pm 1.38$
MB-3.5%-70 °C	$86.43^{a} \pm 0.69$	$10.89^{a} \pm 0.55$	$33.86^{f} \pm 1.24$

The values are expressed as mean ± SD ($n = 3$). Different superscripts within the same parameter indicate significant differences ($P < 0.05$). Sample abbreviations are defined in Table 1.

Characterisation of emulsion from 8% MBPI treated with Na_2CO_3 and heat treatment

Emulsifying properties of MBPI (EAI and stability coefficient). Proteins act as emulsifiers via adsorption to the oil–water interface, where they lower the surface tension, and can form a viscoelastic film which provides resistance against coalescence (Zhang *et al*., 2022). The homogenisation process can influence protein adsorption to the oil–water interface, by reducing particle size (i.e., breaking up of aggregates), and by an additional convective contribution to transport towards the interface, on top of a diffusive contribution. Faster protein migration to the oil–water interface corresponds to a higher EAI. In Table 2, combined treatment with Na_2CO_3 and heat treatment increased EAI values of MBPI suspensions from 6.34 to 8.54–10.89 $m^2\ g^{-1}$ as

compared with those without Na_2CO_3 at 25 °C (control). At the same Na_2CO_3 level, increasing heating temperature could significantly increase EAI values ($P < 0.05$). MBPI samples treated with either 0.3% or 3.5% Na_2CO_3 gave high EAI values, and this is most likely related to their higher dispersibility (Table 2). Others reported that the pH-dependent EAI of red lentil protein isolates was also closely related to their solubility/dispersibility, implying that high soluble protein content was essential for good emulsifying ability (Lee *et al.*, 2021). The highest value of EAI at 10.62–10.89 $m^2\ g^{-1}$ was found in MBPI treated with both Na_2CO_3 concentrations at 70 °C, indicating that heat treatment under alkaline treatment by addition of Na_2CO_3 would improve emulsifying activity of MBPI. This might be because the protein structure partially unfolded from this combined treatment, exposing more hydrophobic sites, resulting in faster adsorption, and greater surface activity (Wang *et al.*, 2018; Lee *et al.*, 2021). Partial unfolding by pH-adjustment to 11.5 at 80 °C also resulted in improvement of emulsifying activity in kidney bean protein concentrate (Choe *et al.*, 2022). Wang *et al.* (2018) also reported that hemp seed protein isolate modified by heat treatment at 60 °C with shifting to pH 12 provided a maximum EAI of 7.39 $m^2\ g^{-1}$ as compared with heating at other temperatures between 20 and 80 °C. However, the heat treatment alone (without Na_2CO_3 addition) did not improve the emulsifying activity of MBPI, as evidenced by the unchanged EAI values in MBPI under control, MB-0%-40 °C, and MB-0%-70 °C conditions ($P \geq 0.05$). Consistent with our findings, previous studies also reported minimal effects of heat treatment alone on EAI. Wang *et al.* (2018) observed no change in EAI of hemp seed protein isolate, and Nivala *et al.* (2021) reported a minor increase in EAI of faba bean protein isolate after heat treatment.

As mentioned above, proteins form viscoelastic films around oil droplets to form a stable emulsion (Zhang *et al.*, 2022). A higher value of the stability coefficient indicates greater emulsion stability (Li *et al.*, 2019). The stability coefficient in MBPI samples treated under alkaline conditions (MB-0.3%-40 °C, MB-0.3%-70 °C, MB-3.5%-25 °C, and MB-3.5%-40 °C) was improved compared with those without Na_2CO_3 at 25 °C (control) as illustrated in Table 2. A possible explanation for this could be that the increase of pH to alkaline conditions by addition of Na_2CO_3 increased the charge of the adsorbed proteins and therefore the electrostatic repulsion between oil droplets (Lee *et al.*, 2021). Therefore, flocculation and coalescence of the oil droplets would be delayed, improving emulsion stability. This agrees with the findings of Liu *et al.* (2021) that an alkaline pH of 9–10 improved solubility as well as emulsion stability of MBPI, compared to pH 7, by enhancement of the repulsive interaction between the oil–water interfacial layers. However, MBPI treated with 3.5% Na_2CO_3 at 70 °C showed the lowest value of the stability coefficient, even though it could rapidly adsorb at the interfacial layer, as indicated by the highest EAI value. As explained in Section 'Surface hydrophobicity and intrinsic fluorescence intensity', this phenomenon may be related to changes in intrinsic properties and surface hydrophobicity.

Droplet size distribution of emulsion at 8% MBPI. Volume mean diameter (D [4, 3]), surface mean diameter (D [3, 2]), and specific surface area (SSA) were used to characterise the droplet size distribution of emulsions stabilised by 8% MBPI (Table 3). The emulsions prepared with increasing Na_2CO_3 levels and concomitant heat treatment exhibited progressively smaller droplets (*D* [4, 3]: 3.84–5.70 μm; *D* [3, 2]: 2.43–3.51 μm) as compared to the control (E-control) at pH 7 (*D* [4,3]: 6.89 μm; *D* [3, 2]: 4.45 μm). This aligns with previous findings by Zhang *et al.* (2021) who reported smaller *D* [4, 3] values for protein-stabilised emulsions prepared with soy protein isolate-whey protein isolate blends under alkaline

Table 3 Droplet size and SPC of emulsion from MBPI at 8% protein treated with various Na_2CO_3 levels and heat treatments as compared to those from untreated MBPI

Emulsion	*D* [4, 3] (μm)	*D* [3, 2] (μm)	SSA ($m^2\ mL^{-1}$)	SPC ($mg\ m^{-2}$)
E-control	$6.89^{a} \pm 0.38$	$4.45^{a} \pm 0.40$	$1.36^{e} \pm 0.13$	$3.80^{bc} \pm 0.15$
E-0.3%-40 °C	$5.64^{b} \pm 0.02$	$3.10^{c} \pm 0.01$	$1.94^{c} \pm 0.01$	$2.18^{d} \pm 0.07$
E-0.3%-70 °C	$5.10^{c} \pm 0.34$	$2.97^{c} \pm 0.03$	$2.01^{c} \pm 0.01$	$1.54^{e} \pm 0.14$
E-3.5%-25 °C	$5.70^{b} \pm 0.14$	$3.51^{b} \pm 0.03$	$1.73^{d} \pm 0.01$	$5.29^{a} \pm 0.31$
E-3.5%-40 °C	$4.43^{d} \pm 0.36$	$2.65^{d} \pm 0.00$	$2.18^{b} \pm 0.15$	$4.22^{b} \pm 0.05$
E-3.5%-70 °C	$3.84^{e} \pm 0.13$	$2.43^{d} \pm 0.07$	$2.42^{a} \pm 0.09$	$3.40^{c} \pm 0.50$

SPC, Surface protein concentration; SSA, specific surface area.

The values are expressed as mean ± SD ($n = 3$). Different superscripts within the same parameter indicate significant differences ($P < 0.05$).

E-control means protein-stabilised emulsion from MBPI treated without Na_2CO_3 at 25 °C. E-0.3%-40 °C and E-0.3%-70 °C mean protein-stabilised emulsions from MBPI treated with 0.3% Na_2CO_3 at 40 and 70 °C, respectively. E-3.5%-25 °C, E-3.5%-40 °C and E-3.5%-70 °C mean protein-stabilised emulsions from MBPI treated with 3.5% Na_2CO_3 at 25, 40, and 70 °C, respectively.

conditions (pH 9–11) as compared to pH 7. Alavi *et al.* (2021) also reported that faba bean protein treated with alkaline pH 11 and heating at 90 °C exhibited increased solubility. This likely contributed to prolonged emulsion stability due to the formation of smaller droplets, as compared to those without alkaline treatment and heating. Besides, the increasing Na_2CO_3 level from 0 to 3.5% also dramatically reduces the initial interfacial tension (Table 1) and increases the adsorption rates of MBPI to the oil–water interface, which reduces the energy required to form new droplets and can prevent the emulsion droplets from coalescence during emulsification, creating emulsions with smaller particle size.

The increasing Na_2CO_3 levels and heat treatments also led to higher SSA values as compared to the control ($P < 0.05$) (Table 3). Higher SSA indicates a larger total droplet surface area stabilised by the protein molecules (Lin *et al.*, 2020). In this study, the emulsions prepared from MBPI treated under alkaline and heat treatment conditions generally exhibited better emulsion stability due to smaller droplet sizes, as evidenced by the stability coefficient in Table 2 (except MB-3.5%-70 °C). The exception observed with MB-3.5%-70 °C sample might be explained by the low surface hydrophobicity (H_0) index measured for this condition (Fig. 2a). As discussed in the surface hydrophobicity section, exposing a large number of hydrophobic regions under this treatment likely lead to subsequent protein aggregation. Aggregated proteins exhibited reduced flexibility, potentially hindering effective interaction with the oil–water interface, and contributing to poor emulsion stability. Furthermore, higher numbers of smaller droplets can also lead to unstable emulsions (Damodaran, 2017), since more proteins may be needed to adequately coat the increased interfacial area and stabilise the emulsion. These observations highlight the importance of considering both droplet size and intrinsic protein properties, particularly surface hydrophobicity, for achieving effective emulsification with protein-based stabilisers.

Surface protein concentration. The surface protein concentration (SPC) is an attribute to characterise protein adsorption at the oil–water interface of protein-stabilised emulsion. After adsorption of proteins at the interface, they may adopt conformations different from their native structure in aqueous solution (Zhang *et al.*, 2022), although the degree to which this happens depends strongly on protein source, oil type, and environmental conditions. The value of SPC is often used as an indicator of how the protein is adsorbed. For instance, a low SPC of approximately 1 mg m^{-2}, often indicates that the protein molecules are significantly unfolded. An SPC in the range of 1–3 mg m^{-2}, is an indication that a monolayer of globular proteins may be present. Larger values might be yielded from multilayer of proteins or aggregated proteins (Damodaran, 2017; Hebishy *et al.*, 2017). Analysis of the SPC values in Table 3 reveals significant variation ($P < 0.05$) despite a constant protein concentration (8%) in the emulsions treated with different Na_2CO_3 levels and heat treatment conditions. Notably, the SPC values for E-0.3%-40 °C and E-0.3%-70 °C (2.18 and 1.54 mg m^{-2}, respectively) suggest these treatments influenced the protein behaviour at the interface, potentially leading to monolayer formation. Whereas SPC values for other conditions ranged from 3.40–5.29 mg m^{-2}, indicating a possible multilayer protein structure at the interface or the presence of larger aggregates. The lower SPC values in E-0.3%-40 °C and E-0.3%-70 °C could be attributed to faster protein spreading during homogenisation and rearrangement of adsorbed protein molecules at the interface, as suggested by Fernandez-Avila & Trujillo (2016). Furthermore, they reported a potential positive correlation between low SPC values and high EAI values, which aligns with our observations in E-0.3%-40 °C and E-0.3%-70 °C.

To summarise, combined treatment of alkaline and heat treatments improved functional properties of commercial MBPI samples. Five samples (MB-0.3%-40 °C, MB-0.3%-70 °C, MB-3.5%-25 °C, MB-3.5%-40 °C, and MB-3.5%-70 °C) exhibited improved dispersibility, and emulsifying properties by enhancing protein flexibility concomitant with high zeta potential and smaller particle sizes. These properties contributed to enhanced emulsion stability by affecting the microstructure formed at the oil–water interface. These findings suggest the potential of these five samples as emulsifiers for stabilising heat-set emulsion gels in plant-based meat products. Our previous study found that formulating an emulsion gel via heat setting required increasing the MBPI concentration from 8% to 11%. Other researchers (Baune *et al.*, 2021; Janardhanan *et al.*, 2022) also suggested a protein concentration range of 8–16% for gel stabilisation, depending on the plant protein type. However, MBPI suspensions at 11% protein treated with 3.5% Na_2CO_3 at all temperatures (25, 40, and 70 °C) formed gels before homogenisation with oil, as shown in Fig. S1. This behaviour aligns with previous studies (Liu *et al.*, 2021) demonstrating gel formation of MBPI at high protein content (13%) under alkaline conditions. We recognise that the preparation process of the MBPI might have an impact on its functionality, and thereby on the effects the combined alkali and heat treatments have on that functionality. For example, MBPI produced using spray-drying, is more commonly used in meat emulsion products, while MBPI produced by freeze-drying is better for meat extenders (Brishti *et al.*, 2020). Future research could explore different sources of MBPI, with different drying processing histories, to establish how these processes would influence the original MBPI sample and the impact of the combined effects of alkaline and heat treatments.

Additionally, varying oil contents are expected to influence the characteristics of both emulsions and heat-set emulsion gels. To optimise MBPI as a fat replacer in plant-based meat alternatives, tailoring its functionality to different fat levels is crucial. Therefore, only MB-0.3%-40 °C and MB-0.3%-70 °C at 11% protein were chosen for further investigation. These samples were used to prepare emulsions, designated as E-0.3%-40 °C and E-0.3%-70 °C, respectively. The characteristics of these protein-stabilised emulsions and their corresponding heat-set emulsion gels were evaluated with varying oil contents (10–30%). These results were compared to a control prepared without Na_2CO_3 at 25 °C as a control.

Characteristics of emulsions and heat-set emulsion gels from 11% MBPI treated with alkaline and heat treatments at various oil contents

Droplet size distribution of emulsions from 11% MBPI at various oil contents

In Table 4, droplet size, serving as an indicator of emulsion stability (Fernandez-Avila & Trujillo, 2016), was evaluated in protein-stabilised emulsions prepared with enriched MBPI (11%) and varying oil contents (10–30%). Emulsions formulated with 0.3% (w/v) Na_2CO_3 at 70 °C (E-0.3%-70 °C) consistently displayed the smallest droplet size with the highest SSA value, followed by those from E-0.3%-40 °C, indicating greater emulsion stability (Fernandez-Avila & Trujillo, 2016). Remarkably, protein content (8% vs. 11%) did not significantly affect the observed trends in droplet size across different treatments (Tables 4 and 5). However, the increasing protein content from 8 to 11% resulted in smaller droplets in the emulsions with the same 10% oil content. This result was also found in a study of Krstonošić *et al.* (2020). MBPI samples (protein) acted as emulsifiers, adsorbing at the oil–water interface and forming a protective layer around oil droplets. This layer prevented coalescence and improved emulsion stability (Krstonošić *et al.*, 2020). The observed effect of protein content at 10% oil might be due to the availability of more protein molecules for interfacial adsorption at this specific concentration, leading to a denser layer and reduced interfacial tension (Liu *et al.*, 2024). These findings suggest that MBPI treated with a combination of Na_2CO_3 addition and heat treatment can effectively regulate droplet size, leading to stable emulsions with potential applications in food product development.

Increasing coconut oil content (10–30%) led to emulsions with smaller particles for all three MBPI samples (Table 4). This observation aligned with previous findings reported by Liu & Tang (2013). Their study showed that increasing oil volume (20–60%) in enriched protein-stabilised emulsions led to smaller oil droplet size. This effect was attributed to the formation of thicker interfacial layers around the droplets, hindering destabilisation of the emulsion. Two possible explanations can be attributed to this phenomenon. Firstly, the increased coconut oil content likely resulted in a more viscous emulsion. Secondly, the inherent amphiphilic nature of MBPI, possessing both hydrophilic and lipophilic groups, allowed it to function as both a thickener and emulsifier within the oil–water system. This increased viscosity might have hindered oil droplet collision and coalescence, contributing to a smaller average droplet size (Liu *et al.*, 2024).

Technological properties of heat-set emulsion gels

Increasing oil content (from 10% to 30%) resulted in a small increase in penetration forces for heat-set emulsion gels prepared from E-control and E-0.3%-40 °C (Table 5). The highest penetration force was observed in the emulsion gel prepared from E-0.3%-70 °C. The penetration force remained unchanged ($P \geq 0.05$) for the emulsion gels prepared from E-0.3%-70 °C with varying oil contents. The superior performance of the emulsion gel prepared from E-0.3%-70 °C, with its higher penetration force, can likely be attributed to the effects of the alkaline treatment and heat treatment on the protein structure. As shown in Tables 1 and 4, this treatment resulted in

Table 4 Effect of oil content on droplet size and specific surface area (SSA) of protein-stabilised emulsion from MBPI at 11% protein treated with various Na_2CO_3 levels and heat treatments as compared to those from untreated MBPI

	***D* [4, 3] (μm)**			***D* [3, 2] (μm)**			**SSA (m^2 mL^{-1})**		
	Oil (% w/w emulsion)			**Oil (% w/w emulsion)**			**Oil (% w/w emulsion)**		
Emulsion	**10**	**20**	**30**	**10**	**20**	**30**	**10**	**20**	**30**
E-control	$3.86^{a} \pm 0.01$	$3.86^{a} \pm 0.01$	$3.39^{c} \pm 0.01$	$2.57^{a} \pm 0.00$	$2.51^{b} \pm 0.01$	$2.15^{e} \pm 0.02$	$2.33^{i} \pm 0.01$	$2.39^{h} \pm 0.01$	$2.80^{e} \pm 0.02$
E-0.3%-40 °C	$3.41^{bc} \pm 0.06$	$3.44^{b} \pm 0.01$	$2.95^{d} \pm 0.01$	$2.19^{d} \pm 0.00$	$2.28^{c} \pm 0.02$	$1.96^{f} \pm 0.02$	$2.74^{f} \pm 0.00$	$2.64^{g} \pm 0.02$	$3.05^{d} \pm 0.03$
E-0.3%-70 °C	$2.75^{e} \pm 0.02$	$2.63^{f} \pm 0.01$	$2.61^{f} \pm 0.01$	$1.87^{g} \pm 0.01$	$1.80^{i} \pm 0.01$	$1.82^{h} \pm 0.00$	$3.21^{c} \pm 0.02$	$3.35^{a} \pm 0.01$	$3.30^{b} \pm 0.01$

The values are expressed as mean ± SD ($n = 3$). Different superscripts within the same parameter indicate significant differences ($P < 0.05$). Sample abbreviations are defined in Table 3.

Table 5 Effect of oil content on technological properties of heat-set emulsion gels from MBPI at 11% protein treated with various Na_2CO_3 levels and heat treatments as compared to those from untreated MBPI

	Penetration force (g)			TEF (%)		
	Oil (% w/w emulsion)			Oil (% w/w emulsion)		
Heat-set emulsion gel condition	10	20	30	10	20	30
E-control	$7.57^{d} \pm 0.60$	$8.25^{bc} \pm 0.22$	$8.60^{b} \pm 0.34$	$4.26^{a} \pm 0.09$	$3.77^{b} \pm 0.12$	$3.39^{c} \pm 0.19$
E-0.3%-40 °C	$7.89^{cd} \pm 0.10$	$8.20^{bc} \pm 0.20$	$8.51^{b} \pm 0.39$	$3.77^{b} \pm 0.08$	$3.44^{c} \pm 0.06$	$2.95^{d} \pm 0.15$
E-0.3%-70 °C	$9.34^{a} \pm 0.15$	$9.56^{a} \pm 0.29$	$9.53^{a} \pm 0.74$	$3.55^{c} \pm 0.03$	$3.48^{c} \pm 0.07$	$2.53^{e} \pm 0.06$

TEF, total expressible fluid.

The values are expressed as mean ± SD (penetration force; $n = 7$, TEF; $n = 3$). Different superscripts within the same parameter indicate significant differences ($P < 0.05$). Sample abbreviations are defined in Table 3.

smaller average particle size for the MBPI and smaller emulsion droplets, and this may have led to a more homogeneous gel. According to Fig. 2, this sample also had more exposed hydrophobic regions, and conformational changes in the protein structure, as indicated by surface hydrophobicity and intrinsic fluorescence intensity results. Similar findings were reported for emulsion gels prepared from soybean protein after alkaline treatment (pH 9) with microwave heating (Xu *et al*., 2022). Smaller emulsion droplets provided more potential binding sites per unit volume during gel formation. This contributed to a more compact and uniform internal structure within the emulsion gel, consequently improving textural properties like hardness.

Lower total expressible fluid (TEF) in the emulsion gel indicates a greater ability to retain fluids (water and oil) and reflects the structural stability of the gel. As shown in Table 5, the TEF values of emulsion gels from both E-0.3%-40 °C and E-0.3%-70 °C were lower as compared to those from E-control. The emulsion gel containing 10% (w/w) coconut oil (E–control) exhibited the highest amount of liquid release (4.26%), indicating poor fluid-holding capacity. This is likely because the protein, after treatment with appropriate heat treatment and alkaline conditions, could expose hydrophobic regions that interact with the oil phase at the interface. Increasing the coconut oil concentration from 10 to 30% (w/w) in both E-0.3%-40 °C and E-0.3%-70 °C conditions resulted in a significant decrease in the amount of fluid released to 2.95% and 2.53%, respectively ($P < 0.05$). The remarkable improvement in fluid-holding capacity observed with the increased penetration force in these emulsion gels, particularly under E-0.3%-70 °C conditions, might be due to the formation of more cross-links during heat-induced gelation, triggered by the smaller droplet size (Table 4) as described by Xu *et al*. (2022). Therefore, a synergistic effect between alkaline treatment and heat treatment, particularly under E-0.3%-70 °C conditions, led to an enhanced emulsion gel network structure. Based on the smaller droplet size and superior technological properties, three emulsions (E-control, E-0.3%-40 °C, and E-0.3%-70 °C) prepared from 11% MBPI suspension mixed with 30% oil were selected for further investigation of emulsion stability by a time-dependent rheology.

Time-dependent viscoelastic properties of protein-stabilised emulsion

To examine the rheological behaviour of the emulsions prior to heat-set treatment, a time sweep test was used to monitor how the viscoelastic properties of protein-stabilised emulsions changed over time at constant strain, frequency, and temperature. In the protein-stabilised emulsion prepared from MBPI without Na_2CO_3 addition and heat treatment (E-control) at 30% oil content, the storage modulus (G′) was

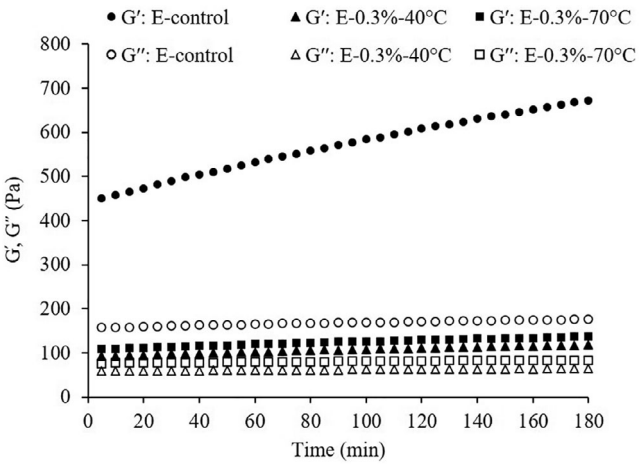

Figure 4 Time dependence (180 min) of storage modulus (G′) and loss modulus (G″) at 25 °C for protein-stabilised emulsions at 11% MBPI (E-0.3%-40 °C and E-0.3%-70 °C) with 30% oil as compared with a control (E-control). Frequency was equal to 1 Hz, and amplitude was 1%. The data are expressed as means from duplicate measurements. Sample abbreviations are defined in Table 3.

significantly higher than the loss modulus (G″) from the start of the measurement (Fig. 4). This indicates the development of an emulsion gel network even before heat-induced gelation. The value for G′ was continuously increasing during the 3 h time interval. This might be due to protein aggregation occurring over time (Kornet *et al.*, 2022). The untreated sample had a low ζ-potential which may result in low stability against aggregation. In contrast, no significant changes in G′ and G″ were observed for the E-0.3%-40 °C and E-0.3%-70 °C samples during the 3-h measurement period, but also here the value for G′ was slightly higher than the value of G″, although the loss tangent was still close to 1. This suggests that modification of MBPI by alkaline and heat treatment could reduce protein aggregation. This reduction in aggregation led to improved dispersion of protein particles within the protein-enriched system. Enhanced dispersion was likely due to increased electrostatic repulsion caused by the higher pH and modified protein conformation (Sankowski *et al.*, 2023). The greater stability of the protein-stabilised emulsion at a pH far from the protein's pI observed in this study aligns with findings from other reports (Kornet *et al.*, 2022). These findings indicate that MBPI modification using 0.3% Na_2CO_3 and heat treatment at 40 °C or 70 °C is a promising approach to stabilising protein-enriched emulsion systems and makes them more suitable for processing into heat-set gels. While heat-set emulsion gels of both E-0.3%-40 °C and E-0.3%-70 °C exhibited enhanced fluid-holding capacity, the E-0.3%-70 °C gel demonstrated slightly greater hardness (Table 5). This variation in texture could influence the application of these gels as fat replacers in plant-based meat products. Incorporating these emulsions into model plant-based meat systems should be explored in future studies. Furthermore, to achieve a wider range of textural properties in plant-based emulsions, future studies could explore the incorporation of various polysaccharides or hydrocolloids. This would allow for the fabrication and characterisation of heat-set emulsion gels with tailored textures, ultimately contributing to the development of more versatile plant-based meat alternatives.

Conclusion

Plant-based emulsion gels prepared from MBPI have the potential to be promising fat replacers in plant-based meat products. However, creating these gels via heat setting requires a high protein concentration, which demands modification of MBPI's structure to enhance emulsifying properties. This study investigated combined effects of Na_2CO_3 concentration (0.3% and 3.5%) and heat treatment (40 and 70 °C) on functional properties of commercial MBPI for improving physicochemical stability of plant-based emulsions at high protein concentration. Treatment with all Na_2CO_3 concentrations and heat treatments demonstrated a synergistic effect, improving MBPI dispersibility and emulsifying properties at 8% protein, suggesting the potential for more stable protein-stabilised emulsions. These treatments influenced protein conformation and a decrease in particle size, with higher β-sheet content and potential hydrophobic interactions impacting interfacial behaviour. To create heat-set emulsion gels, a higher MBPI concentration at 11% was necessary. However, the combined effect of 3.5% Na_2CO_3 with heat treatment at 11% protein resulted in gels before emulsion preparation, so only the lower Na_2CO_3 concentration of 0.3% could be used at this protein content. Increasing protein content (from 8% to 11%) and oil content (10–30%) also led to smaller droplets in the emulsion, possibly due to hindered coalescence. Notably, the combined treatment at 11% protein was effective particularly for the MB-0.3%-70 °C sample, with the MB-0.3%-40 °C sample also showing positive effects. This resulted in superior outcomes, with smaller droplets and enhanced technological properties of the resulting emulsion gels (penetration force and TEF values). Furthermore, this treatment might reduce protein aggregation, leading to more stable gels from protein-stabilised emulsions as indicated by time-dependent rheology results. Overall, this study demonstrates the synergistic impact of alkaline treatment with 0.3% Na_2CO_3 and heat treatment on MBPI modification, leading to enhanced functionality in plant-based emulsion systems.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Kanokrat Limpisophon: Conceptualization; methodology; validation; writing – review and editing; writing – original draft; supervision; funding acquisition; visualization; resources; data curation; investigation. **Xingfa Ma:** Investigation; validation; writing – original draft; software. **Leonard M. C. Sagis:** Conceptualization; writing – original draft; writing – review and editing; validation; software; visualization; methodology. **Athiya Nonthakaew:** Investigation; validation; writing – original draft; software; formal analysis. **Pattariga Hirunrattana:** Investigation; formal analysis; validation; writing – original draft; data curation; software.

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Data availability statement

Research data are not shared.

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Supporting Information

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

Figure S1. Appearance of MBPI suspensions treated with alkaline and heat treatments at 11% protein after leaving for 30 s.