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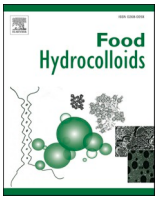

Soluble protein particles produced directly from mung bean flour by simple coacervation

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ABSTRACT

Poor solubility is a common characteristic of many plant protein ingredients which often hampers product formulation. We exploit simple coacervation, or liquid-liquid phase separation, of plant proteins in flours, to formulate plant proteins as powders consisting of submicron colloids with good solubility and dispersibility. We consider the specific case of mung bean flour, but the approach is more general. First, we study the influence of pH on the formation of submicron protein droplets ("coacervates") after alkaline protein extraction from mung bean flour. Next, the proteins in droplets were heat-set into colloidal protein microgels, and the morphology of the colloids was assessed by scanning electron microscopy. The mung bean protein colloids have an intrinsic viscosity much lower than typical food thickeners, indicating the dense nature of the particles. After spray-drying, they maintain a good dispersibility even close to the isoelectric point. Heat-induced gelation of redispersed protein particles resulted in gels with moduli much less than those of commercial mung bean protein concentrates at equivalent protein concentration. Hence, the main impact of the pretreatment is on dispersibility and heat stability, with relevance to formulations such as plant-protein based beverages.

1. Introduction

Concerns over sustainability of animal proteins in our diet have led to a shift in consumer preferences towards diets richer in plant proteins (Aydemir & Yemenicioğlu, 2013; Carbonaro, Maselli, & Nucara, 2015). This is accompanied by an intense interest in the food technology of processing plant proteins for novel food formulations (Aiking, 2011). It is generally acknowledged that many plant protein ingredients currently on the market suffer from problems with low solubility and low functionality, which is disadvantageous in formulating new food products using these ingredients. This behavior is probably caused by intrinsic properties of plant proteins and by processing steps used to isolate the proteins (Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). Various approaches have been studied to remedy the deficiencies of current plant protein ingredients. For example, dry fractionation (Assatory, Vitelli, Rajabzadeh, & Legge, 2019; Pelgrom, Vissers, Boom, & Schutyser, 2013) can be used to obtain powders that retain the native structure of the plant proteins, improving their functionality.

In applications such as beverages, it is often the solubility of plant proteins which is limiting formulations. Earlier, researchers have shown for whey proteins that a colloidal formulation is highly advantageous for formulating thermally stable dispersions high in protein (Sağ;lam, Venema, de Vries, Shi, & van der Linden, 2013; Saglam, Venema, de Vries, van Aelst, & van der Linden, 2012). Here we propose to investigate simple coacervation of plant proteins from flours as a mean to obtain dense, colloidally sized plant protein micro and nano gels, in order to formulate plant protein powders with high dispersibility.

It is well known that upon acidification, before becoming insoluble close to their iso-electric points, many plant storage proteins exhibit liquid-liquid phase separation, sometimes also called simple coacervation (Lazko, Popineau, & Legrand, 2004). Liquid-liquid phase separation of plant storage proteins has also been observed upon acidifying soybean fraction isolates (Nannan Chen, Zhao, Wang, & Dimova, 2020) or yellow pea flours (Lui, Litster, & White, 2007) initially dispersed at alkaline pH. For the case of flours (as opposed to the case of purified native plant storage proteins) macroscopic phase separation often does not occur, but rather micro phase separation is observed, in the form of the formation of small liquid, protein rich droplets which are typically of micron scale (Cochereau, Nicolai, Chassenieux, & Silva, 2019; Kornet et al., 2022; Li, Erni, Van Der Gucht, & De Vries, 2020; Lui et al., 2007).

For relatively pure plant storage proteins, a number of recent studies have highlighted that simple coacervation in combination with heat

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induced gelation can be used to create a wide variety of microstructures including thin-walled capsules (N. Chen, Zhao, Nicolai, & Chassenieux, 2017; Nannan Chen et al., 2020; Teng, Luo, & Wang, 2012; Zhao, Guo, Ding, Ye, & Liu, 2020). Microgels of plant storage proteins obtained directly from flours have not yet been studied extensively with respect to their functionality, as far as we are aware.

For the specific case of mung bean flour, our aim is therefore to identify conditions for simple coacervation, to obtain microgels by heat set gelation of the protein microdroplets, and to test whether these droplets can be spray dried or freeze dried while retaining their functional properties. We used dry fractionation to obtain ingredients that are somewhat higher in native proteins than whole mung bean flour.

As we will show, mung bean protein coacervates (MBPC) obtained from dry fractionated mung bean flour are much smaller (submicron) compared to the droplets previously found upon acidifying (defatted) soybean flour (Lui et al., 2007). We find the heat-gelled (sub)micron mung bean protein droplets can be spray dried and resuspended without affecting the colloidal nature of the particles, and we characterize the properties of the re-dispersed proteins using various physical-chemical approaches.

2. Materials and methods

2.1. Materials

Dried mung beans (Vigna radiata) were obtained from a local store. According to the supplier the composition of the dried mung beans was protein 24% (w/w), fat 2% (w/w) and carbohydrates 60% g (w/w). All beans still contained their hull and were unheated. Commercial mung bean protein concentrate (M65) containing 67.5 g/100 g protein, 0.10 g/100 g fat, and 20.98 g/100 g total carbohydrate was obtained from Barentz B.V. (Hoofddorp, The Netherlands). All other chemicals and reagents were analytical grade and purchased from Sigma Aldrich (Schnelldorf, Germany).

2.2. Methods

2.2.1. Dry fractionation

Mung bean seeds were milled at a wheel speed of 8000 rpm using an Alpine Multi-processing System (Hosokawa Micron B.V., Doetinchem, the Netherlands) equipped with a ZPS50 impact mill (Hosokawa Micron B·V., Ausburg, Germany). After the milling step, the Alpine Multi-processing system was equipped with an ATP50 air classifier to obtain a mung bean fine fraction (MBFF) at 2500 rpm classification speed. For both milling and air classification, the feed rate was set to 20 rpm, a batch size was used of 400 g, and the airflow was set to 52 m^3/h, continuously.

2.2.2. Protein colloid production and characterization

2.2.2.1. Protein colloid production. Sodium metabisulfite was dissolved at 15 mM in water. Next, 20% (w/w) MBFF was dispersed in this solution and stirred for 5 min. For alkaline protein extraction, the pH was adjusted to 8.5 using 1M NaOH and the dispersion was stirred for 1 h at room temperature. Next, the dispersion was centrifuged (Hermle Z-383K centrifuge, HERMLE Labortechnik GmbH, Wehingen, Germany) at 10000g for 30 min to remove the insoluble fraction, and the supernatant was filtered using a grade 1 filter paper (Whatman, Massachusetts, United States). Finally, the supernatant was adjusted to pH 7 using 1M HCl. This protein solution was stored at 4 °C until further use.

For protein colloid production, the protein solution was first allowed to equilibrate to room temperature. Next, the pH was slowly decreased by dropwise addition of 1M HCl. Samples were prepared with final pH values in the range of pH 5.8 to pH 6.8. Acidified mung bean protein samples for which micro-phase separation was established are referred to as "mung bean protein simple coacervates" (MBPSC). The micro-phase separation was examined under an Axioskop 50 Microscope (Zeiss, Vienna, Austria). MBPSC samples were heated at 80 °C for 20min to gel the submicron protein droplets, resulting in microgels are referred to as "mung bean protein colloids" (MBPC).

2.2.2.2. Scanning electron microscope (SEM). MBPC samples were CO_2 critical point dried (CPD) and investigated with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands). To prepare the CPD sample, a drop of 10% MBPC solution was transferred to a poly-L-lysine coated 12 mm diameter glass coverslip (Cellware, BioCoat, Discovery Labware Inc., USA) and fixed for 30 min. Afterwards, the coverslip was gently rinsed in water and dehydrated in a series of 30%, 50%, 70% and 100% (twice) acetone solutions for 10 min each. The sample was subsequently dried by CO_2 critical point dryer (CPD300, Leica, Germany). To make the sample conductive for SEM analysis, samples were sputter coated (SCD 500, Leica, Germany) with 2 nm tungsten. All images were recorded at a working distance of 5 mm with SE detection mode at a voltage of 2 kV at room temperature.

2.2.3. Drying techniques

2.2.3.1. Freeze drying. Mung bean Protein Colloids (MBPC) were freeze-dried using a Freeze dryer Alpha 2–4 LD plus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) to obtain freeze-dried mung bean protein colloids (FDMBPC).

2.2.3.2. Spray drying. Spray-dried mung bean protein colloid powder (SDMBPC) was obtained by drying MBPC samples (heat treated as described above) using a Buchi Mini spray dryer B-290 (BUCHI Corporation, New Castle, USA). The inlet and outlet temperature were 150 °C and 60 °C, respectively. The aspirator was set to 95% of its maximum aspirator rate, the pump speed was set to 15% of its maximum pump rate. Gas flow was set to 50 kg/h, and the nozzle cleaner was set on level 3 to prevent sample clogging.

2.2.4. Protein content

2.2.4.1. Dumas. The dry matter content of (possibly hydrated) MBFF was determined by weighing approximately 100 mg MBFF before and after drying. Samples were dried in an evaporation dish overnight in an oven at 105 °C. To determine the protein content, a Dumas analysis (Flash EA 1112 series, Thermo Scientific, Breda, the Netherlands) was performed, assuming a nitrogen conversion factor of 6.25 (Mariotti, Tome, & Mirand, 2008).

2.2.4.2. Internal protein concentration. The internal protein concentration $C_{internal}$ (kg/m^3) of the (heat-set) protein colloids in the FDMBPC was determined by the shear viscosity measurements. The protein colloids were harvested by centrifuging 10% (w/w) FDMBPC at 30000g for 30 min. The supernatant was removed and replaced by the same volume of a 20 mM sodium phosphate buffer (pH 7.0), in which the protein colloids were dispersed again at a weight concentration C (kg/m^3). The viscosity η of a dilution series of FDMBPC were determined as a function C using a capillary viscometer (Ubbelohde) placed in a water bath at 25 °C. For each C the viscosity η was measured in triplicate. The specific viscosity η_{sp} was obtained from:

$$\eta_{sp} = \frac{\eta - \eta_0}{\eta_0} \quad (1)$$

where η_0 is the viscosity of the continuous phase. The specific volume ν_{sp} (m^3/kg) of the FDMBPC can be obtained from a Huggins plot where the intrinsic viscosity $\frac{\eta_{sp}}{C}$ is plotted vs. C as:

$$\lim_{C\to 0}\frac{\eta_{sp}}{C}=\frac{5}{2}v_{sp} \tag{2}$$

Note that the intrinsic viscosity $[\eta]$ is defined as $[\eta] = \frac{\eta_{sp}}{C}$.

Here use has been made of Einstein's equation:

$$\eta_{sp}=\frac{5}{2}\varphi \tag{3}$$

and:

$$\varphi = v_{sp}\ C \tag{4}$$

Finally, the inverse of the specific volume v_{sp} gives the internal protein concentration $C_{w,internal}$ (kg/m^3) of the FDMBPC:

$$C_{internal}=\frac{1}{v_{sp}} \tag{5}$$

2.2.5. Colloidal stability

To investigate the colloidal stability of MBPSC, samples of 20 ml were loaded in a glass test tubes with a magnetic stirrer bar. The samples were heated at 80 °C in a water bath for 5, 10, 15 and 20 min. Immediately after the heat treatment, the samples were placed in an ice bath to prevent further aggregation. They were kept at 4 °C, together with an unheated reference sample, until further measurements. For all samples, the particle size distributions were measured using static light scattering (Mastersizer 2000, Malvern instruments, Malvern, United Kingdom), with a particle refractive index of 1.48.

2.2.6. Re-dispersibility and particle dissolution for dried protein particle powders

To investigate the dispersibility after drying, the SDMBPC or FDMBPC powders were dispersed in water. Particle size distributions after dispersion were determined using static light scattering (Mastersizer 2000, Malvern instruments, Malvern, United Kingdom). Both SDMBPC and FDMBPC powders were dispersed at 1% (w/w) in buffers at pH 4, 5, 6.75, 9 and 11 with 100 mM NaCl added. The dispersions were first stirred for 30 min at room temperature, before the pH values were adjusted by the dropwise addition of 1M NaOH or 1M HCl. Next, samples were stirred for 1 h and centrifuged (Hermle Z-383K centrifuge, HERMLE Labortechnik GmbH, Wehingen, Germany) at 17000g for 30 min. The protein concentration of supernatant was determined by DUMAS with a nitrogen conversion factor of 6.25, and the fraction of protein dissolved, $\Phi_{\text{dissolved}}$, was calculated from

$$\Phi_{dissolved}=\frac{C_{Supernatant}}{C_{Suspension}}\times 100\% \tag{6}$$

Where $C_{\text{Supernatant}}$ is the protein concentration of supernatant, and the $C_{\text{Suspension}}$ is the protein concentration of the suspension. To determine the effect of ionic strength on the dispersibility, 1% (w/w) FDMBPC and SDMBPC powders were also dispersed at 0 M, 0.1 M and 0.5M NaCl at pH 6. For these dispersions the same method and calculation were applied as above to determine the dispersibility.

2.2.7. Rheology

2.2.7.1. Shear viscosity. Shear viscosities of the samples were measured in triplicate, using a double gap cylinder (DG 26.7) geometry over a shear rate range of 1–100 s^{-1}. Measurements were conducted using a MCR 502 rheometer (Anton Paar, Graz, Austria). Flow curves were analyzed to obtain the flow behavior index (n) and consistency index (K) using a power-law model

$$\tau = K\dot{\gamma}^n \tag{7}$$

Where τ is the shear stress, K is the consistency index, $\dot{\gamma}$ is the shear rate, and n is the flow behavior index.

2.2.7.2. Oscillatory rheology. For oscillatory rheology, an MCR 502 rheometer combined with a sand-blasted concentric cylinder (CC17) geometry was used. Samples were monitored for the linear rheological behavior during their gelation induced by heating. Storage modulus (G′) and the loss modulus (G″) were recorded at a strain of 1% and a frequency of 1Hz, during a heat treatment consisting of an increase in temperature from 23 °C to 90 °C at a constant heating rate of 3 °C/min, a fixed temperature of 90 °C for 30 min, followed by cooling back to 23 °C at a rate of 3 °C/min. Measurements were conducted in duplicate.

2.2.8. Statistical analysis

Unless mentioned otherwise, for all measurements, results are presented as the mean ± standard deviation. Measurements were performed at least in duplicate. IBM SPSS Statistics 25 (IBM SPSS Inc., Chicago, USA) was used to conduct one way ANOVA analysis, using the post-hoc method Tukey at the $P < 0.05$ level.

3. Results and discussions

3.1. Mung bean protein simple coacervates (MBPSC)

Protein droplet formation upon acidifying flours dispersed in alkaline has been observed for especially soy and yellow pea (Lui et al., 2007). Here we sought to characterize the same phenomenon for alkaline extracted mung bean flour. To maintain full protein functionality, we first applied an air classification to separate the freshly milled mung beans, giving a mung mean fine fraction (MBFF) powder. A high concentration (20% wt) of MBFF was alkali extracted at pH 8.5. To check for liquid-liquid binary protein phase separation, something accompanied by droplet formation by the newly formed phase, we first performed optical microscopy on different samples obtained by acidifying the supernatant after alkaline extraction to various final pH values.

The results are shown in Fig. 1. We find that in the narrow pH window of pH 6.8 to 6.0, there was a progression from spherical, micron-sized droplets that first increased in number and then aggregated and acquired irregular shapes at pH 6. For further studies, we choose pH 6.75, since at this pH a large enough number of droplets appeared to be formed that do not aggregated. This optimum pH is higher than the value of pH 6.2–5.7 previously shown to induce liquid-liquid binary phase separation in alkaline extracted soy flour (Lui et al., 2007).

For a more precise assessment of the size distribution of the mung bean protein droplets, or MBPSC formed at pH 6.75, we used static light scattering to obtain the particle size distribution (PSD). Results are shown in Fig. 2a. We find a distinctly bimodal distribution with a large peak in the volume-weighted PSD at around 0.15 μm, and a smaller rather broad peak centered on 10 μm. Additionally, the large peak at 0.15 μm has a distinct shoulder at around 1 μm. This implies the droplets observed with the optical microscopy represented only a small fraction of the total particles that presented in the sample, and that most particles were in fact below the resolution of light microscopy.

Therefore, we also sought to use electron microscopy to visualize the many smaller particles presented in the MBSPC samples. Results of SEM imaging on the MBSPC samples is shown in Fig. 2b. This figure appears to be at least consistent with the PSD obtained from static light scattering in that it shows a few larger particles (indicated by a blue arrow in Fig. 2b), and many much smaller particles (indicated by yellow arrows in Fig. 2b), stuck to each other and to the larger particles.

None of the methods used above can distinguish protein-containing from non-protein containing particles. Therefore, we next used Confocal Laser Scanning Microscopy (CLSM). Freshly prepared MBSPC dispersions acidified down to pH 6.75 were stained with Rhodamine B and imaged using CLSM. Representative results are shown in Fig. 3. The CLSM-optical microscopy overlayed images clearly show that not all of the particles in the dispersions obtained from the mung bean flour were rich in protein: some could be small air bubbles, whereas others may be

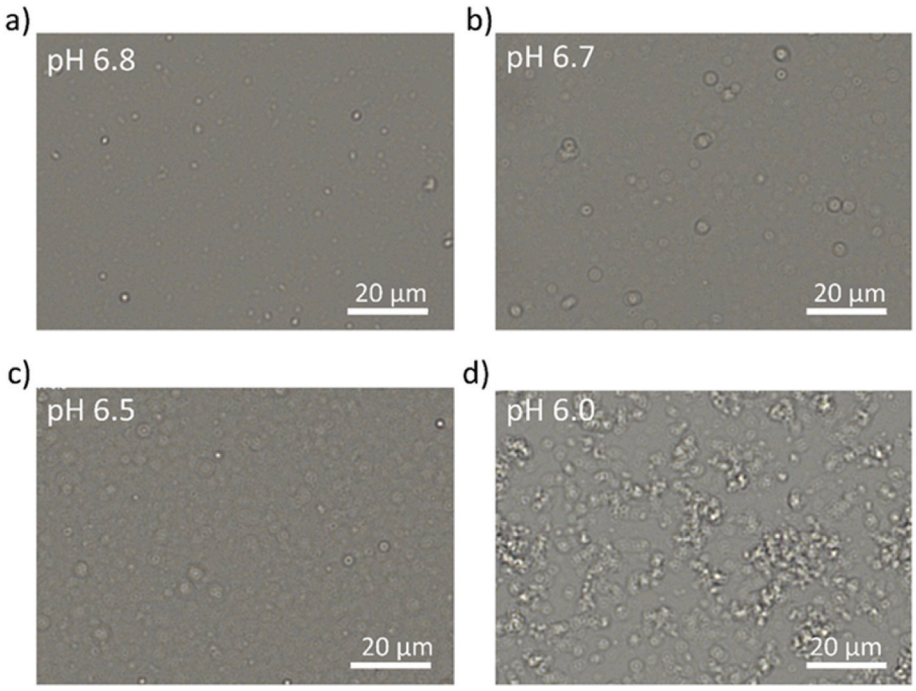

Fig. 1. Optical microscopy images of Mung Bean Protein Simple Coacervates (MBPSC) formed from air classified mung bean flour, by alkaline extraction of 20% (w/w) flour at pH 8.5 followed by acidification to the indicated pH values. Scale bar = 20 µm.

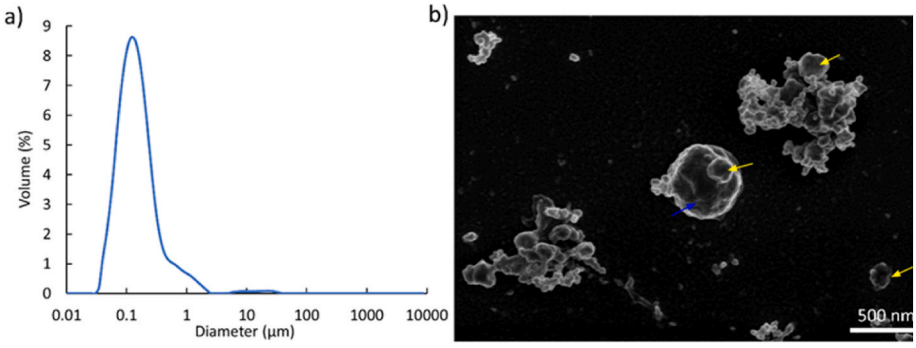

Fig. 2. a) Particle size distributions of mung bean protein simple coacervates, prepared by acidifying 20% wt of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75 as determined using static light scattering **b)** Scanning electron micrographs (SEM) of Mung bean protein colloids (critical point dried and Tungsten coated). Scale bar = 500 nm.

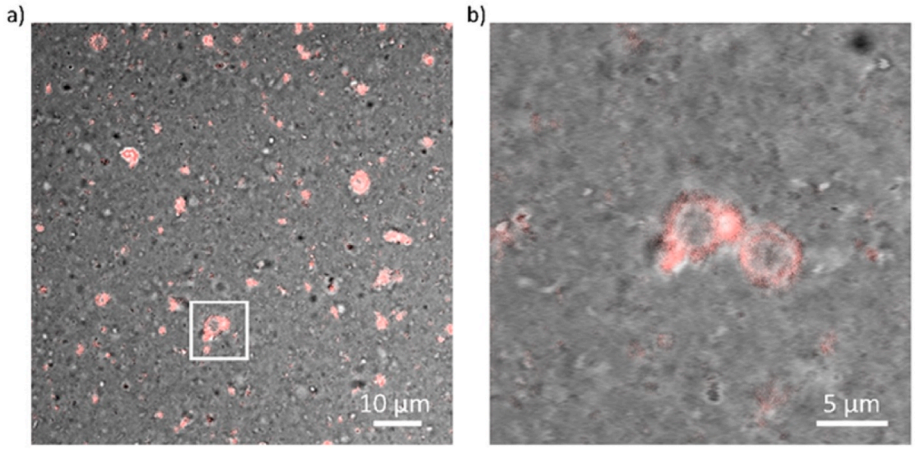

Fig. 3. Confocal laser scanning microscopy (CLSM) images of a) MBPSC prepared by acidifying 20% wt of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75, labeled with Rhodamine B. Scale bar = 10 µm. b) detail view of region indicated by the white square in Fig. 3a. Scale bar = 5 µm, respectively.

starch fragments not removed by the centrifugation step following the alkaline extraction. On the other hand, it is very clear that other particles do correspond to regions of high fluorescence and hence high protein concentration. Unfortunately, with CLSM we can only image the few very large particles that existed in the overall (volume weighted) distribution, which was dominated by the much smaller particles with a characteristic size of around 150 nm.

For the microscopically visible particles we can therefore conclude that they are oftentimes spherical and contain a high concentration of protein. The presence of these particles indicates that also for mung bean flour, it is possible to induce the formation of protein-rich simple coacervates, as was earlier also observed for soy flour. However, a

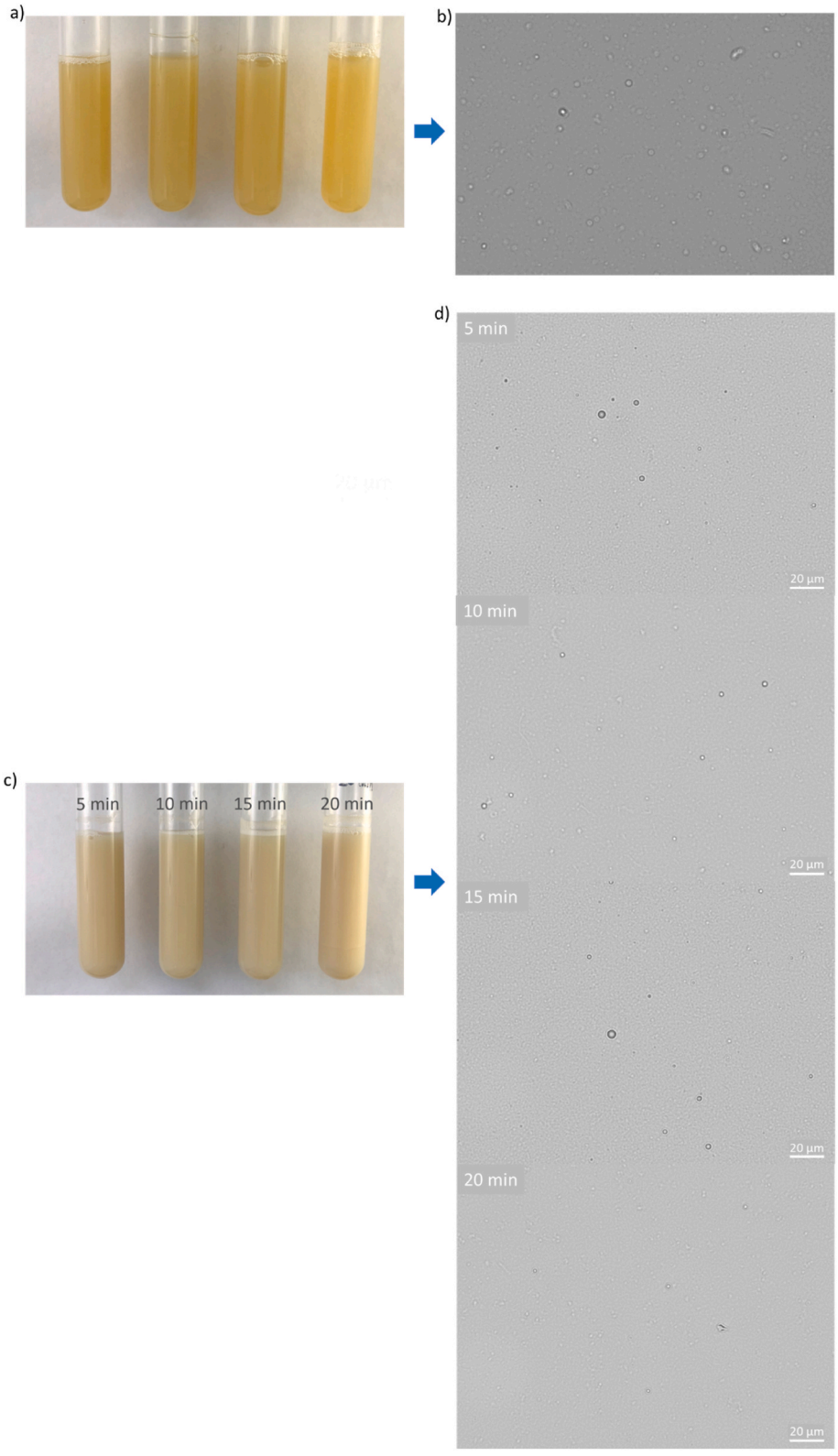

Fig. 4. Changes to macroscopic and microscopic visual appearance caused by heating MBPSC droplets, to form MBPC. Samples prepared by acidifying 20% wt of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75. a) Macroscopic appearance of unheated MBPSC, b) optical microscope images of MBPSC, c) Macroscopic appearance of MBPC (heated), and d) optical microscope images of MBPC (heated for 5 min, 10 min, 15 min, and 20 min at 80 °C, respectively). Scale bar = 20 µm.

characteristic difference appears to be that at least for the MBFF that we use here, these droplets for the majority, did not grow out to achieve sizes of many microns, as they did for soy flour, but instead, remained small, having submicron sizes.

3.2. Mung bean protein colloids (MBPC)

Since the simple coacervates are reversible structures formed by liquid-liquid phase separation, they may disappear, for example if the pH changes (Popello, Suchkov, Grinberg, & Tolstoguzov, 1992). A heating step is necessary to convert the mung bean protein simple coacervate (MBPSC) droplets into solidified mung mean protein colloids (MBPC) by gelling the protein coacervate droplets.

To investigate the stability of MBPC formed by heating MBPSC droplets, we first studied changes to macroscopic and microscopic visual appearance caused by heating. Results are shown in Fig. 4.

As is clear from Fig. 4a and c, at the macroscopic level, the heat treatment resulted in the dispersions becoming much opaquer, possibly indicating that denser, more strongly scattering particles generated upon heating. Consistently, optical microscopy, shown in Fig. 4b (MBPSC droplets, unheated) and Fig. 4d (MBPC, heated) suggests a higher optical contrast for the heated particles.

Even though in optical microscopy there were no indications of aggregation being caused by heating, this does not tell the whole story since the majority of the particles have submicron sizes and cannot be observed by optical microscopy. Therefore, particle size distributions before and after heating were obtained from static light scattering.

Results in Fig. 5a confirm that heating hardly influences the particle size distribution: no large aggregates were formed after heating. The volume averaged diameters of unheated (MBPSC droplets) at 5 min, 10 min, 15 min, and 20 min heated MBPC were centered around 0.15 μm, within the error of the measurement. Note that Fig. 5b shows volume averaged results, such that there were only a small number larger particles, with diameters between 1 μm and 10 μm, and most particles in the MBPC samples were submicron.

Achieving near complete preservation of the original size distributions upon heating is desired, but nontrivial. In the case of pea (Cochereau et al., 2019), structural changes were observed upon heating for simple protein coacervate droplets, and others including soy (Zhao et al., 2020), were changed into hollow microcapsules. Presumably, the fact that we use much less purified proteins and rapid heating may also have contributed to observed preservation of size distributions.

As a side note, we observed that the (unheated) MBPSC droplets undergo reversible self-association upon cooling to 4 °C. Even though at room temperature, the MBPSC dispersions were stable against sedimentation even at 10000g (for 30 min, Appendix Fig. SI. 1), storing them at 4 °C overnight led to the formation of a dense and a dilute layer (see Appendix Fig. SI. 2). Mild agitation at room temperature dispersed the droplets again. In contrast, the heated MBPC dispersions did not show this behavior.

3.3. Drying and redispersing MBPC

The MBPC can only be useful as a plant protein ingredient if we can actually dry them without loss of their functional properties and without loss of dispersibility. In particular drying methods can negatively affect the dispersibility of plant proteins (Pelgrom et al., 2013; Shen, Tang, & Li, 2021). Both freeze-drying and spray drying of MBPC dispersions were used to obtain, respectively FDMBPC and SDMBPC powders. They were dispersed at different pH and ionic strengths and their solubility/dispersibility were assayed using a centrifugation test. Results are shown in Fig. 6.

As shown in Fig. 6a, the FDMBPC and SDMBP were both more soluble at alkaline conditions than at acidic conditions. A minimum solubility was found around pH 5 as expected, since this was close to the isoelectric point of mung bean proteins (pH 4.6) (Du et al., 2018). Comparing with mung bean protein isolate, the FDMBPC had a much higher solubility even at the isoelectric point (approximately 20%). At pH 4, the FDMBPC and SDMBPC still showed remarkably high solubilities of around 47% and 30% respectively, while mung bean protein isolates typically have solubilities lower than 10% at this pH (Du et al., 2018; Kudre, Benjakul, & Kishimura, 2013). We attribute this good solubility close to the mild methods used during the MBPC production. Indeed, it is widely agreed that extensive purification can lead to protein denaturation and lower protein solubility (Du et al., 2018; Kudre et al., 2013; Pelgrom et al., 2013). Finally, the salt dependence of the FDMBPC and SDMBPC powders is shown in Fig. 6b. We found salt hardly affects the solubility of the FDMBPC and SDMBPC powders.

Next to knowing that the FDMBPC and SDMBPC powders have good re-dispersibility, we were interested in finding out whether particle sizes after redispersion will be very much affected by drying. Results are shown in Fig. 7. FDMBPC and SDMBPC powders were dispersed in water at a protein concentration (4% wt), identical to the protein concentration of the MBPC dispersions before drying. After re-dispersion, particle size distributions were determined, and compared to those of the original (unheated) MBPSC dispersions and (heated) MBPC dispersions. We found that both freeze drying and spray drying allow for the redispersion of the protein particles with sizes very close to those before drying. Unavoidably, there was a somewhat larger fraction of larger particles after drying, but this fraction remained small.

3.4. Viscosity and thermal stability of FDMBPC

We proceed with evaluating functional properties of the FDMBC powders for application in beverages. Desired functionalities are low viscosity and good thermal stability. A low viscosity arises if the MBPC are compact and non-swelling, or in other words, have a high internal concentration.

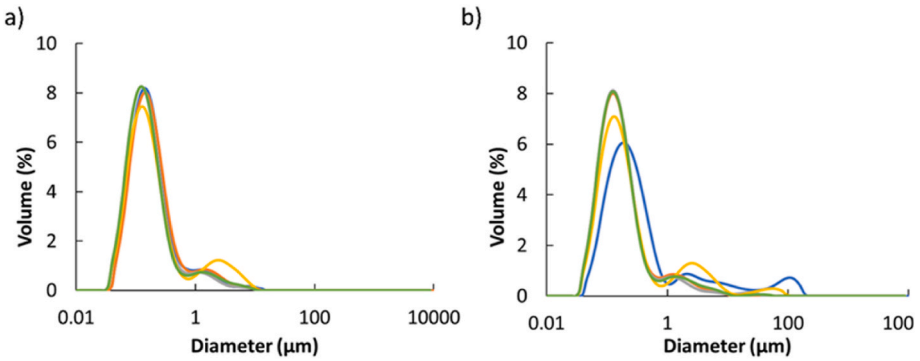

Fig. 5. Particle size distributions of unheated ( ) and 5 min ( ), 10 min ( ), 15 min ( ), 20 min ( ) heated MBPC, prepared by acidifying 20% wt of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75, on a) day 0 and b) day 5.

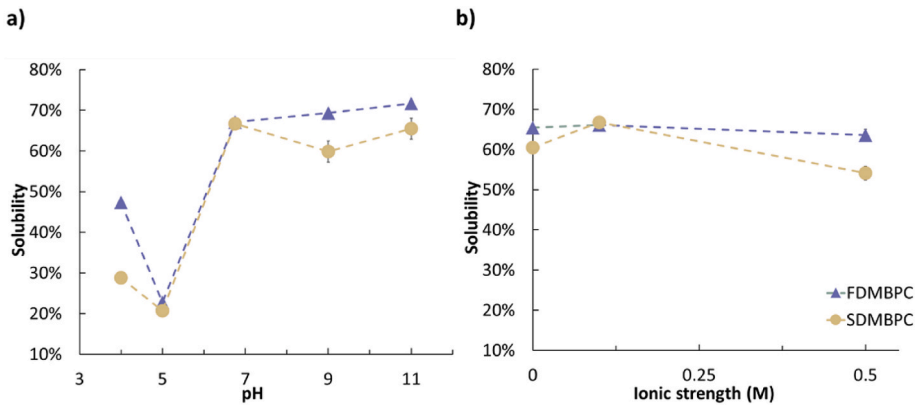

Fig. 6. Solubility curves (w/w) of freeze-dried MBPC (FDMBPC, triangles) and spray dried MBPC (SDMBPC, circles) as a function of **a)** pH (left) and **b)** ionic strengths (right). MBPC were prepared by acidifying 20% wt of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75.

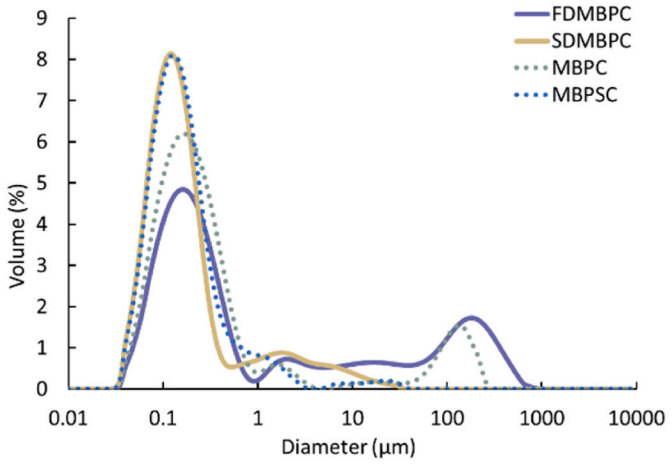

Fig. 7. Particle size distributions of unheated mung bean protein simple coacervates (MBPSC) dispersions (blue dash line), 20 min heated mung bean protein colloids (MBPC) dispersions (green dash line), re-dispersed freeze-dried mung bean protein colloids (FDMBPC) powder (purple line) and re-dispersed spray-dried mung bean protein colloids (SDMBPC) powder (yellow line). MBPSC were prepared by acidifying 20% wt of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75.

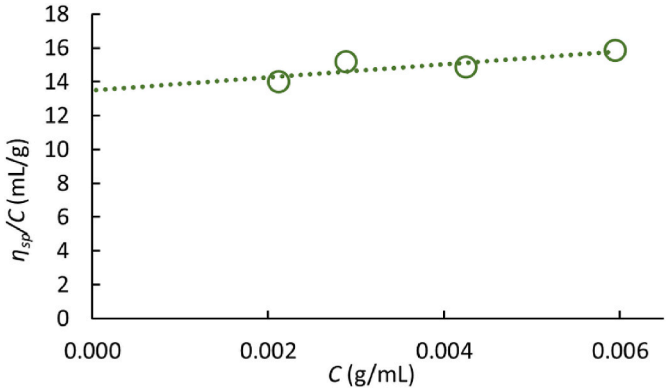

Fig. 8. Huggins plot for washed FDMBPC powder. η_{sp} is the specific viscosity measured using capillary viscometry. C is the weight concentration of washed FDMBPC, based on dry mass. The FDMBPC was prepared by acidifying 20% wt of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75 (MBPSC), heating for 20 min to form MBPC, and subsequent freeze drying to obtain the FDMBPC powder. The FDMBPC powder was washed once by preparing a 10% wt FDMBPC dispersion in PO_4 spinning down the protein colloids using an ultracentrifuge and resuspending the pellet to the desired concentration.

By using centrifugation to pellet pea protein coacervate dispersions, Kornet et al. (2022) estimated an internal protein content of the coacervate droplets around 45% wt via mass balance. Instead of that, we followed a more precise method of Saricay, Wierenga, and de Vries (2016) based on specific viscosity measurements (cf. section 2.2.4), and estimated the internal concentration for redispersed freeze-dried mung bean proteins colloids (FDMBPC). Assuming the MBPC are roughly spherical, we can estimate their specific volume from their intrinsic viscosity and the Einstein relation for the viscosity of hard spheres. Next, we can take the inverse of the specific volume as our estimate for the weight concentration (based on dry mass) inside the individual mung bean protein colloids.

A washing step was employed to ensure that dilution did not affect the viscosity of the continuous phase. Fig. 8 shows a Huggins plot for the specific viscosity (obtained using capillary viscometry) from which we can deduce the intrinsic viscosity by extrapolating to concentration zero. Results of the analysis of the Huggins plot are given in Table 1.

The average intrinsic viscosity $[\eta]$ for FDMBPC was 15 ± 1 mL/g. This is significantly lower than most food thickeners, which have intrinsic viscosities in the range of 30–150 mL/g (Saricay et al., 2016). Reported intrinsic viscosities for denatured whey protein isolates and concentrates (Daubert, Hudson, Foegeding, & Prabhasankar, 2006; Eissa et al., 2013) are also a factor two larger than those we found here. On the other hand, the internal concentration was on the lower end (approaching 20% wt) of the values of 20–45% wt reported previously for other simple legume protein coacervate droplets (Kornet et al., 2022; Li, Erni, Van Der Gucht, & De Vries, 2020; Lui et al., 2007). This may be related to the fact that we have additionally heated, dried and redispersed the protein colloids, and with the fact that the simple protein coacervates for mung bean flour appear to behave different from those in pea- and soy flour, where typical coacervate droplets sizes can be many microns, much larger than in the case of mung bean flour.

Table 1
Results of analysis of Huggins plot (Fig. 8) for washed FDMBPC powder.

Intrinsic viscosity $[\eta]$ (mL/g)	**15 ± 1**
Specific volume (mL/g)	6 ± 1
Internal concentration (g/mL)	0.17 ± 0.02

*All measurements were conducted at least in triplicate. Values are presented as mean ± standard deviation.

As mentioned, in the context of plant proteins for beverage applications we are looking for high dispersibility, low viscosity and basically no, or only very weak heat-induced gelation. While the fact that the

intrinsic viscosity of FDMBPC was low, is encouraging, we also would like to check the (shear-rate dependent) viscosity of redispersed FDMBPC powders at higher concentrations more representative of applications. This was performed for two representative pH values, pH 6.75 and pH 3.5. Results are shown in Fig. 9. Furthermore, we also investigated the recovery process of samples and there was no time dependency found for FDMBPC. The flow curves could be well represented by a power law model for which the fitting parameters (consistency index K and flow index n) are given in Table 2.

For pH 6.75, we found a nearly Newtonian behavior for concentrations of 6% and 8% wt. At higher concentrations, FDMBPC dispersions started exhibiting shear-thinning behavior as expected for concentrated dispersions of protein microgels (Saricay et al., 2016). For pH 3.5, it appeared that shear thinning already set in at lower concentrations, possibly indicating an increased swelling of the microgels due to increased electrostatic repulsion inside the microgels at low pH. In all cases the strong shear thinning implies that at the rather high shear rates of oral processing, viscosities of the FDMBPC dispersions will be experienced as low.

Finally, we turned to the rheological behavior of FDMBPC dispersions when heated. For beverage applications, ideally the plant protein dispersions should not be very sensitive to (re)heating, such as could occur e.g., during pasteurization or sterilization. To characterize changes to the rheology during heating, we used oscillatory rheology at a fixed frequency (1Hz) to determine the storage (G') and loss moduli (G'') of the FDMBPC dispersions during a heating and cooling ramp (hold time at 90 °C of 30 min). The ratio of these two modulus were described as loss factor tan δ (G''/G'). We did so for various concentrations and pH values. A commercial mung bean protein isolate (referred to as M65) was used as a benchmark to compare against. A representative measurement, for 12% FDMBPC at pH 6.75, is shown in Fig. 10. Final storage moduli and loss factor after cooling down to room temperature for all measurements are given in Table 3.

As shown in Table 3, final storage moduli (G′) seems independent of protein concentrations, while lower pH led significant increases in G' (a 3-fold and 2-fold increase, respectively). As a benchmark, 10% commercial mung bean protein M65 suspensions were prepared and measured at natural pH. For M65, a much higher G' was observed after cooling, indicating a less thermal stability possesses. This result is in line with loss factor results, where M65 samples showed a lower tan δ of 0.17 while FDMBPC samples all presented tan δ around 0.25. Indicating that after heating, M65 can be considered a stronger solid gel than FDMBPC. Hence, the commercial M65 protein concentrate gels much more readily than our FDMBPC powder, making the latter an attractive ingredient for formulating plant-based beverages.

Table 2
Rheological parameters (consistency index K and flow index n) and determination coefficients for flow curves of FDMBPC as shown in Fig. 9.

pH	Protein concentration	Flow index n	Consistency index K (Pa.s^n)	Determination coefficient R^2
Natural pH	6%	0.91 ± 0.01^{d}	0.01 ± 0.00^{a}	1.00 ± 0.00^{a}
	8%	0.88 ± 0.00^{d}	0.02 ± 0.00^{a}	1.00 ± 0.00^{a}
	10%	0.66 ± 0.05^{c}	0.52 ± 0.21^{ab}	1.00 ± 0.00^{a}
	12%	0.55 ± 0.01^{bc}	2.13 ± 0.20^{c}	1.00 ± 0.00^{a}
pH 3.5	6%	0.37 ± 0.01^{a}	0.09 ± 0.00^{a}	0.89 ± 0.01
	8%	0.36 ± 0.00^{a}	0.26 ± 0.01^{ab}	0.96 ± 0.00^{a}
	10%	0.37 ± 0.04^{a}	0.58 ± 0.05^{ab}	0.96 ± 0.01
	12%	0.42 ± 0.06^{ab}	0.71 ± 0.06^{b}	0.96 ± 0.02

All measurements were conducted at least in duplicate. Values are presented as mean ± standard deviation. Letters indicate significant difference at $P < 0.05$.
[a] Represents standard deviation is lower than 1%.

4. Conclusion

The mung bean protein colloids produced by simple coacervation were proven to be a promising vehicle to deliver proteins in beverage systems. This can be attributed to the relatively low intrinsic viscosity and good dispersibility of MBPC.

By employing an accurate approach to determine the internal protein content of MBPC, we found that the intrinsic viscosity of MBPC is approximately 15 ml/g, which is significantly lower than common protein-based food thickeners (30 ml/g - 150 ml/g). In the meanwhile, with an internal protein concentration of 0.17 g/ml MBPC has a good dispersibility both at acidic conditions and salt levels relevant to industry production practices. The drying method used did not significantly affect dispersibility of MBPC.

In general, the rheological behavior of a product is inseparably linked to the consumers acceptance. The viscosity of the FDMBPC was found in a range which is acceptable for consumers. As for the gelation behavior, the pH was found to have an influence on heat-set gelation of MBPC. The storage modulus of FDMBPC increased significantly after pH was decreased to 3.5.

In summary, this study shows that MBPC has a high dispersibility combined with a viscosity acceptable to consumers, which meets the main requirements for plant-based protein-based beverages. In addition,

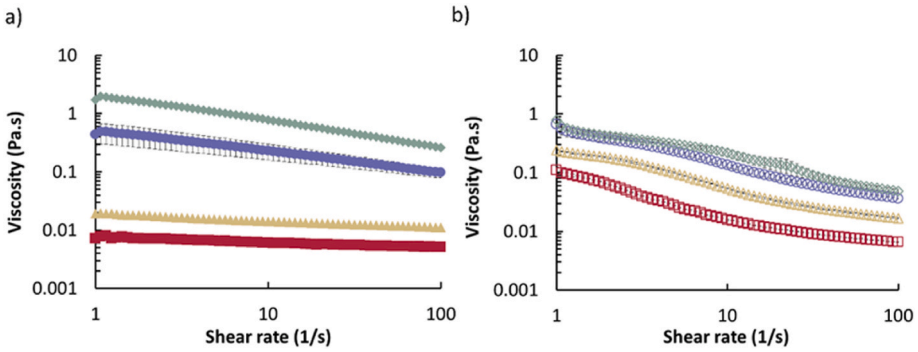

Fig. 9. *Viscosity of FDMBPC as a function of shear rate, for various concentrations, at both neutral and acidic* pH ***a)*** pH *6.75, protein concentrations: 6% (■),8% (▲),10% (●), and 12% (◆)* ***b)*** pH *3.5, protein concentrations: 6% (□),8% (△),10% (○), and 12% (◇). FDMBPC was prepared by acidifying 20% wt of alkaline extracted (*pH *8.5) air classified mung bean flour to* pH *6.75, heating and freeze drying. Error bars represent standard deviation of triplicate experiments.*

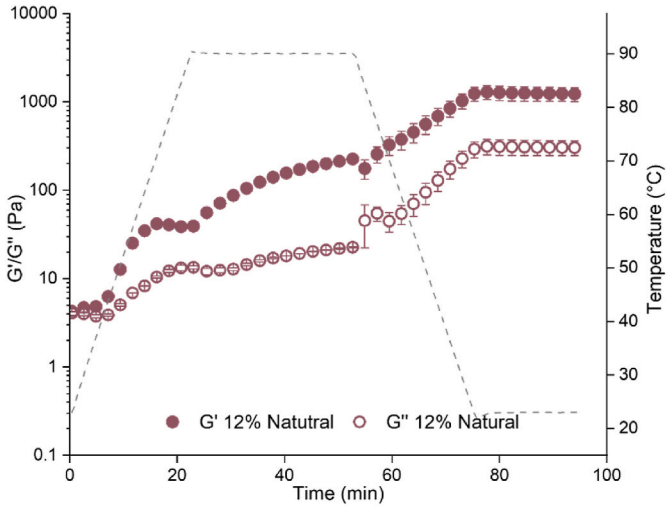

Fig. 10. Viscoelastic properties of FDMBC prepared by acidifying 20% wt. of alkaline extracted (pH 8.5) air classified mung bean flour to pH 6.75 and freeze drying. Storage modulus (solid symbols) and loss modulus (open symbols) versus time at 12% wt. protein concentration at a natural pH. The temperature profile is indicated by the dashed line. Error bars represent the standard deviation of the duplicates.

Table 3
The final average storage modulus (G') and loss factor (tan δ) of gelled FDMBPC heated at 90 °C and cooled to room temperature at Natural pH and pH 3.5. The commercial mung bean protein concentrate M65 was added as reference.

	Protein concentration	Storage modulus (G′, Pa)		Loss factor (tan δ)	
		Natural pH	pH 3.5	Natural pH	pH 3.5
M65	10%	6663 ± 254^{c}	/	0.174 ± 0.001^{a}	/
FDMBPC	10%	1006 ± 204^{a}	2979 ± 119^{b}	0.269 ± 0.008^{c}	0.225 ± 0.001^{b}
	12%	1231 ± 218^{a}	2532 ± 451^{ab}	0.246 ± 0.004^{bc}	0.266 ± 0.009^{c}

*All measurements were conducted in duplicate and values are shown as mean ± standard deviation. Letters present significant difference at a $P < 0.05$.

considering the remarkable resistance against sedimentation, coalescence, heat-induced gelation, MBPC can be an attractive building block for plant-protein based beverages.

Credit author statement

Qiuhuizi Yang: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Erik van der Linden:** Project administration, Supervision. **Paul Venema:** Supervision, Methodology, Conceptualization, Formal analysis, Writing – review & editing. **Renko de Vries:** Supervision, Conceptualization, Methodology, Formal analysis, Writing – review & editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2023.108541.

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