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A mild hybrid liquid separation to obtain functional mungbean protein

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ABSTRACT

A dry fractionation combined with an aqueous phase separation approach is developed as a mild and sustainable mungbean protein extraction process. Mungbean seeds were dehulled or intactly milled and air classified to produce fine fractions which are enriched in proteins. Subsequently, aqueous phase separation was employed to yield a 4 layer system with increased protein content in some of the layers. The protein content of different layers was found to depend especially on pH. The highest protein yield for Layer 1 was 80.9% at pH 8. Variations in air classification speed, stirring time, and salt addition did not lead to significantly increased protein yield. Finally, shear viscosity and oscillatory measurements were performed to investigate the rheological behaviour of the mungbean proteins obtained by the hybrid method. The viscosity of the mild extracted mungbean proteins was found lower than commercial mungbean protein concentrates at a comparable protein concentration, whereas the mild process did not have a significant influence on heat-set gelation.

1. Introduction

Plant proteins are drawing increasing interest as food ingredients. The global plant protein market is expected to increase from 10.3 billion dollars in 2020 to 15.6 billion dollars by 2026 (MarketsandMarkets, 2021). This increase is a consequence of the continuous growth of the world population and the shift of people's dietary pattern, towards a more sustainable, healthy and balanced diet. Therefore, plant proteins have been studied as a potential replacer for the typical animal-derived protein-rich products, such as cheese, milk, and meat (Mattice & Marangoni, 2020; Rinaldoni et al., 2014; Waschatko et al., 2012; Wang et al., 2019; Kumar et al., 2016).

At this moment, the protein-rich fraction is extracted from plants by so-called wet fractionation. This yields relatively pure protein isolates, but requires large amounts of water and often leads to a loss of the native structure of the proteins (Pelgrom, Boom, & Schutyser, 2015; Schutyser & van der Goot, 2011). In turn, this leads to poor solubility, relatively high viscosity and compromised functionalities for emulsification, gelation etc.

For applications such as plant-based beverages, presenting a pleasant viscosity while possessing a desired protein concentration is especially problematic. For example, a challenge is the relatively high protein concentration of minimal 6 (v/v) % that is demanded. This often results in a drink with a high viscosity, which is usually undesirable for high protein drinks (de Kort et al., 2011). Additionally, the preferred viscosity depends on the consumer group. For instance, Streimikytė et al. (2020) reported that the geriatric consumers with swallowing difficulties probably prefer to products with 'honey' – level (350–1750 mPa.s) viscosity. Therefore, plant protein ingredients that have a good solubility with low viscosity would be favoured for plant-based beverages.

Except for high protein content, the undesired high viscosity can also be generated by thermal preservation processes due to protein structure alteration even denaturation. Several approaches such as vacuum packing, freeze drying, irradiation, *etc*. have been carried out to eliminate this disagreeable influence. Moreover, milder fractionation procedures help to preserve the native structure of plant proteins, leading to lower viscosities than at comparable protein concentration, when compared with proteins obtained via other fractionation procedures (Purwanti et al., 2011). An example is dry fractionation, which is both more sustainable and leaves protein structures intact. In this procedure, milling is followed by air classification to separate components based on the difference in density or size. Eventually, this gives a fine fraction rich in native proteins and a coarse fraction rich in starch.

Previous research has shown that peas, lupine, and beans can all be processed using dry fractionation (Pelgrom et al., 2013; Pelgrom, Wang, et al., 2015; Schutyser & van der Goot, 2011; Simons et al., 2017; Wu &

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Nichols, 2005). Schutyser et al. (2015) reported that after air classification protein concentration can be increased from 23.8 to 58.8 g/100g dry matter for yellow pea, 40.4–59.4g/100g dry matter for lupine. The method has also been applied for soybean. Xing et al. (2018) found that for this case, protein can be enriched from 37 g/100g dry matter to 45 g/100g dry matter (for defatted soybean).

So far, the crucial relation between plant protein functionality and the type of processes used, has only been addressed for the (currently) most common commercial crops such as soybeans and yellow pea. For other potentially important crops, this relation has not yet been investigated. Here we focus on an attractive proteinaceous material, viz. mungbean (*Vigna radiata (L.)*), which possesses a well-balanced amino acid composition and is cultured diffusely all over the world. Its high protein- and low lipid content make it suitable for dry fractionation. Additionally, mungbean is known for its possible health benefits such as lowering of plasma cholesterol, prevention of diabetes (Du et al., 2018; Kudre et al., 2013). Commercial mungbean proteins are mostly extracted by wet fractionation, which causes protein denaturation during both the protein precipitation and drying steps, typically leading to loss of proteins native structure. Thus, research on native mungbean protein would be interesting and promising. However, research on the functionality of mildly extracted mungbean proteins is still lacking.

Here we consider a mild mungbean purification using milling and air-classification to obtain a protein enriched fine fraction (MBFF) and a dehulled protein enriched fine fraction (DMBFF), followed by dissolution and centrifugation (at 2500 g) to increase protein content. Different types of particles swell to different extents in the dispersions such that after (low-speed) centrifugation, one typically obtains a number of distinct layers which are separated based on Stokes law (dictated by a factor $\Delta\rho.a^2$ where $\Delta\rho$ is the density and a is the radius of the particles, provided $Pe \gg 1$ such that diffusion does not dominate the transport). The total number of layers depends on the protein source used. For instance, pea flour was reported to form 4 distinct layers (Pelgrom, Boom, & Schutyser, 2015), while quinoa flour exhibited 3 layers (Avila Ruiz et al., 2016). Starches, proteins, and cell wall materials typically separate into different layers, with the top layers being protein-rich and the bottom layer being starch-rich (Pelgrom, Boom, & Schutyser, 2015). In earlier work, this hybrid extraction method was shown to be promising for extracting native pea protein: protein yields were found to be 66.9 g/100 g dry matter in the top phases. The hybrid separation used considerably less water and energy as compared to conventional separation methods (Pelgrom, Boom, & Schutyser, 2015; Schutyser, 2015).

For the hybrid Mungbean protein purification described above we study the influence of basic process parameters such as milling wheel speed, classification rotation speed, dehulling, stirring time, pH, and salt addition on the protein extraction and resulting functionality of the proteins. In particular, we focus on measuring and understanding differences in the viscosity between the mildly purified mungbean proteins and commercial mungbean protein concentrates at comparable protein concentrations.

2. Materials and methods

2.1. Materials

Dried mungbean (*Vigna radiata)* used for the experiments was purchased from a local Asian store. The specification for the dried mungbean with 6.4% moisture content was protein 24 g/100 g, fat 2 g/100 g, carbohydrate 60 g/100 g (all based on dry weight). All beans still contained their hulls and had not been heated. As the reference, a commercial mungbean protein concentrates M65 was obtained from Barentz B.V. (Hoofddorp, Netherlands). The characteristics of M65 were protein 67.5g/100g, fat 0.10g/100g, carbohydrate 20.98g/100g and moisture content 6.88 g/100g. All other chemicals (Analytical grade) were obtained from Sigma Aldrich (St. Louis, Missouri, United States).

2.2. Methods

2.2.1. Dehulling

Since polyphenols may interact with proteins both reversibly and irreversibly, we removed the seed coat before milling to produce dehulled mungbean fine fraction (DMBFF) and dehulled mungbean coarse fraction (DMBCF). The schematic overview of the mungbean hybrid separation method is shown in Fig. 1. Dehulling was carried out by soaking the seeds in excess water for 8 h at room temperature. Afterward, the seed coat was removed by manual abrasion. After dehulling, beans were dried using a Venticell Laboratory Oven LSISB2V/VC111 (MMM Medcenter Einrichtungen GmbH, Planegg, Germany). The final moisture content was lower than 9% as required to perform the subsequent dry fractionation step at 50 °C without denaturing the proteins.

2.2.2. Dry fractionation

For both mungbean fine fraction (MBFF) and DMBFF, beans were milled using a Multimill Hosokawa System (Hosokawa Micron B·V., Doetinchem, the Netherlands) equipped with a ZPS50 (Hosokawa Micron B·V., Ausburg, Germany) impact mill. Wheel speed was set to 8000 rpm and 4000 rpm for MBFF and DMBFF, respectively. Mill feeding was carried out by a screw feeder at a rate 20 rpm and a batch size was used of 400 g at the airflow 52 m^3/h. After milling, the Multimill Hosokawa was equipped with an ATP50 (Hosokawa Micron B·V., Ausburg, Germany) air classifier. Subsequently, the impact milled flour was further air classified at different classifier wheel speeds (1000 rpm, 2500 rpm and 4000 rpm for MBFF, 8000 rpm for DMBFF), as indicated, at constant feed rate (20 rpm) and airflow (52 m^3/h). The final protein-rich Mungbean fine fractions (MBFF) and starch-rich coarse fractions were obtained, the MBFF was used for experiments.

2.2.3. Scanning electron microscope

MBFF produced at different classification speeds were fixed on sample holders with Carbon adhesive discs, the excess of flour was removed using an air gun. Afterward, the samples were sputter coated with 2 nm Tungsten (MED 020, Leica, Vienna, Austria) and investigated with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands). All images were recorded at a working distance of 5 mm with SE detection mode at 2 kV at room temperature.

2.2.4. Layer formation in centrifugal field

For layer formation in the centrifugal field, 20 g of MBFF was dissolved in 80 g Milli-Q water (PURELAB Ultra, United Kingdom) at room temperature. Stirring times were 10, 30, and 120 min, as indicated. After stirring, the samples were transferred into 50 mL blue cap centrifuge tubes and centrifuged at 2500 g for 30 min by a Hermle Z-383K centrifuge (HERMLE Labortechnik GmbH, Wehingen, Germany), resulting in layer formation.

Layer formation experiments were also performed at different ionic strengths and pH values (as indicated, respectively). For these experiments, MBFF was first dissolved in Mili-Q water. Next, the pH was set by the addition of small amounts of 1M HCl or NaOH solutions, and the ionic strength was adjusted by the addition of solid NaCl.

Layers are numbered from the top of the tube, with the centrifugal field pointing downwards. The top layer (Layer 1) was collected and prepared as a concentrated stock solution (referred to as stock) by Amicon Stirred Cells Ultrafiltration system (Merck KGaA, Darmstadt, Germany) using a 10 kDa filter membrane. The stirred cell was pressurized to 3.8 atm until the desired protein concentration (approximately 20%) was reached. The protein content of the stock solution was determined by Dumas (Nitrogen analyser, Flash EA 1112 series, Thermo Scientific, Breda, the Netherlands) with a nitrogen conversion factor of 6.25 (Mariotti et al., 2008). Before measurements, stock solutions were stored in the fridge at 4 °C for no longer than 5 days.

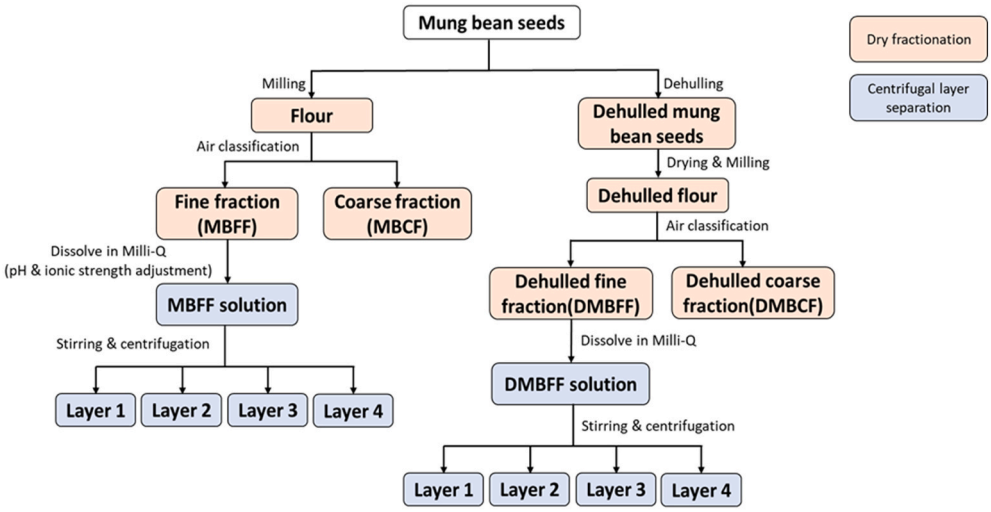

Fig. 1. Overview of dry fractionation to obtain mung bean fractions (left) and dehulled mung bean fractions (right) and mildly hybrid liquid separation process to produce mung bean proteins. Mung bean seeds were dehulled (for dehulled fine fraction) then milled or directly milled (for fine fraction) to liberate proteins from the seeds. Subsequently, fine fractions were dissolved in ultrapure water and stirred at different conditions to extract proteins. The fine fraction solution was centrifuged at 2500 RCF to separate proteins in Layer 1 from other components.

2.2.5. Particle size distribution

Particle size distribution of the MBFF powders were analysed using a laser diffraction Mastersizer 3000 equipped with an Aero S dry dispersion unit (Malvern Instruments Ltd., United Kingdom), the pressure was set at 200 kPa. For the layers obtained after centrifugation, the particle size distribution measurements were conducted by a laser diffraction particle size analyser Mastersizer 2000 (Malvern Instruments Ltd., United Kingdom), the particle refractive index was set at 1.48 for all samples.

2.2.6. SDS-PAGE

Non-reducing SDS-PAGE were conducted to analyse the protein composition. An Invitrogen 4–12% gradient Bis-Tris gel and 20x MES SDS running buffer (Thermo fisher scientific Inc., Waltham, Massachusetts, United States) were used. Samples were taken from the different layers obtained (1–4) after centrifugation of dissolved MBFF. Subsequently, samples were diluted with deionized water to reach a protein concentration range suitable for SDS-PAGE analysis. The gel electrophoresis was performed for 30 min at a constant voltage (200 V) using an XCell Surelock Mini-Cell electrophoresis system (Thermo fisher scientific Inc., Waltham, Massachusetts, United States). Afterward, the gels were stained using Simply Blue Safe Stain and imaged using a Biorad GS900 gel scanner (Bio-Rad, Hercules, California, United States).

2.2.7. Protein content

Dry matter content (g/g) of the different samples were determined by drying the samples overnight in a Venticell Laboratory oven at 105 °C. To determine the protein content, the Dumas analysis was used with a nitrogen conversion factor of 6.25 as introduced in section 2.2.4. Protein content of layers and MBFF was calculated by Eq. 1

$$Protein\ content\ (dm\ \%) = \frac{protein\ from\ Dumas\ [g]}{total\ mass\ [g]} \times 100\% \quad (1)$$

Protein yield was calculated for each layer by Eq. 2

$$Protein\ yield\ (\%) = \frac{protein\ in\ layer\ from\ Dumas\ (g)}{total\ protein\ in\ sample\ from\ Dumas\ (g)} \times 100\% \quad (2)$$

2.2.8. Mineral content analysis

The mineral composition was determined using inductively coupled plasma atomic emission spectroscopy (ICP-OES) (iCAP 6300, Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States).

2.2.9. Rheology

2.2.9.1. Flow curves before heat treatment. A stock solution with a protein concentration 22% was prepared by concentrating MBFF Layer 1 obtained at pH 8 (the maximum protein yield pH condition for the aqueous phase separation) using ultrafiltration. This stock solution was used to make a concentration series (5.3%–16.5%) by diluting with Milli-Q water. M65 reference samples were made similarly with concentrations 5%–11%, where the upper limit was chosen in view of the observed M65 solubility of 11.0 wt%. The shear viscosity was measured over a shear rate range from 1 s^{-1} to 1000 s^{-1} using an MCR 502 rheometer (Anton Paar, Graz, Austria), which was equipped with a sand-blasted concentric cylinder (CC17) geometry (Anton Paar, Graz, Austria). Data were analysed using the Power-law model to acquire the flow index (n) and consistency index (K). A volume of 4.7 ml of each sample was loaded in the cup of the geometry and a solvent trap was used to prevent solvent evaporation. The flow curves were measured in duplicate.

2.2.9.2. Small-strain dynamic rheology during heat treatment. After the flow curve measurements, small-strain dynamical rheological measurements were performed during heat treatment. The temperature was increased from 23 °C to 90 °C at a constant rate of 3 °C/min, kept at 90 °C for 30 min and finally decreased again at a rate of 3 °C/min. During the heat treatment, values for the storage modulus (G′) and loss modulus (G″) were recorded at a strain of 1% and a frequency of 1Hz. The loss tangent (tan δ = G‘‘/G’) at the end of the heat treatment was also calculated. These measurements were conducted in duplicate.

2.2.10. Statistical analysis

All measurements were performed at least in triplicate except for indicated otherwise. The results are presented as the mean ± standard deviation of replicates. Statistic analysis was conducted by IBM SPSS Statistics 25 (IBM SPSS Inc., Chicago, USA) with a one way ANOVA using the post-hoc method Tukey at $P < 0.05$ level.

3. Results and discussion

3.1. Dry fractionation

The particle size distributions of the fine fractions of the Mungbean flours (MBFF) after air classification at different speeds were analysed using static light scattering. The resulting particle size distributions are shown in Fig. 2A. For all three speeds used (1000 rpm, 2500 rpm, 4000 rpm classifier wheel speed) there are two main peaks, a smaller one at

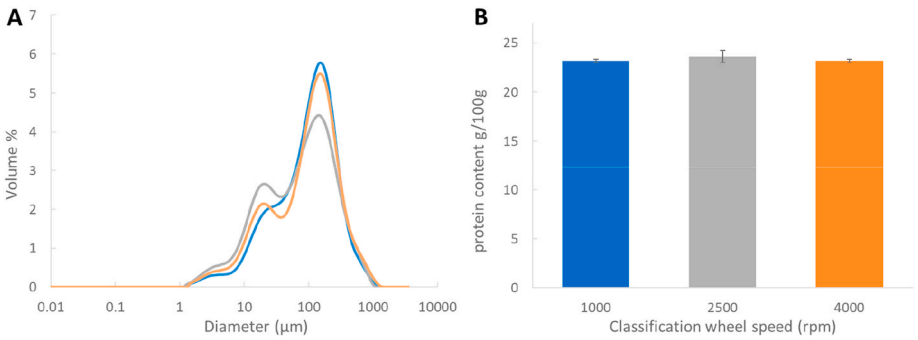

Fig. 2. The relative volume as a function of particle diameter distribution (A) and protein content (B) of mung bean powder produced at 1000, 2500 and 4000 rpm classifier wheel speed. All measurements were carried out in triplicate.

around 20 μm and a larger one at around 150 μm. There is little difference between the particle size distributions at these different air classification speeds. Next, the protein contents were determined for the MBFF. Results are shown in Fig. 2B. There is little variation in protein content, all powders have a protein content in the range of 23–24 g/100 g.

To determine the nature of the two particle populations in the powders, we turn to scanning electron microscopy. A number of typical images are shown in Fig. 3. Despite the similarity in particle size distribution obtained from light scattering, a comparison of Fig. 3A–C seems to indicate that at 1000 rpm air classification is insufficient and large fragments still exist in the fine fraction. In general, after dry fractionation, for fractured mungbean seed cells, a considerable amount of oval starch granules with a typical size of 20 μm and several protein bodies with a much smaller size than starch granules can be seen to be liberated. The protein bodies are much smaller in size, they can be better recognized in the image taken at a higher magnification shown in Fig. 3D. Only dry fractionation does not separate all protein bodies from all starch granules: many protein bodies are associated with (fragments of) starch granules. Hence, if higher concentrations of native Mungbean proteins are required, a further purification step will be necessary, for which we here use (layer-based) centrifugal separation of (rather concentrated) aqueous dispersions of MBFF.

3.2. Layer formation after centrifugation

After dispersing the MBFF in ultrapure water, and after centrifugation, the MBFF has separated into 4 clearly distinguishable layers, as shown in Fig. 4A. Layers are numbered from top to bottom, with the centrifugal field pointing downwards. The compositional analysis of the layers will be discussed later, we start by noting some general features. For these layer-based separations of plant proteins, it is found that the top layers contain most of the protein, whereas the bottom layers contain most of the carbohydrates (Pelgrom, Boom, & Schutyser, 2015). In our case, the volume of Layer 1 was by far the largest. Layer 1 was a low viscosity liquid. Layer 2 had a soft gel-like consistency, whereas Layer 3 and 4 had a solid-like consistency, with Layer 4 being the hardest. From here on, we mostly focus on Layers 1 and 2, which contain most of the Mungbean protein.

First, for Layers 1 and 2, a stock solution and a commercial Mungbean protein sample (denoted by M65) we analysed the size distribution of the particles in the dispersions, using static light scattering. Results are shown in Fig. 4B. Layer 1 showed a highly polydisperse distribution with a peak at 0.2 μm and aggregates size around 100 μm, Layer 1

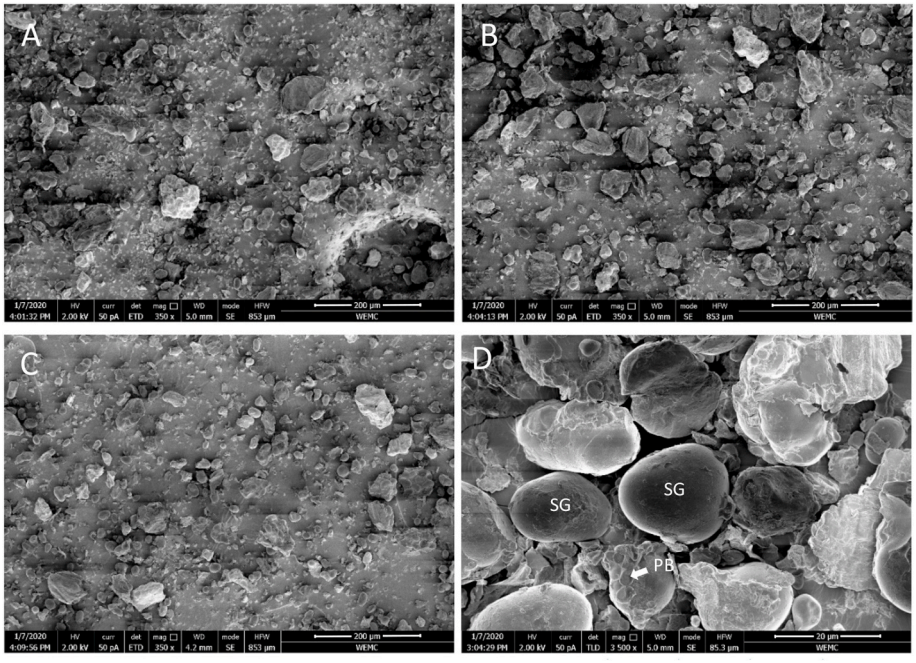

Fig. 3. *Morphology of mung bean flours produced at a classifier speed of A) 1000, B) 2500, C) 4000, D)* 2500 rpm*, showing unground fragments and individual starch granules associated with protein bodies. PB: protein body, SG: starch granule. Scale bars correspond to* 200 μm *(A, B, C), and* 20 μm *(D), respectively.*

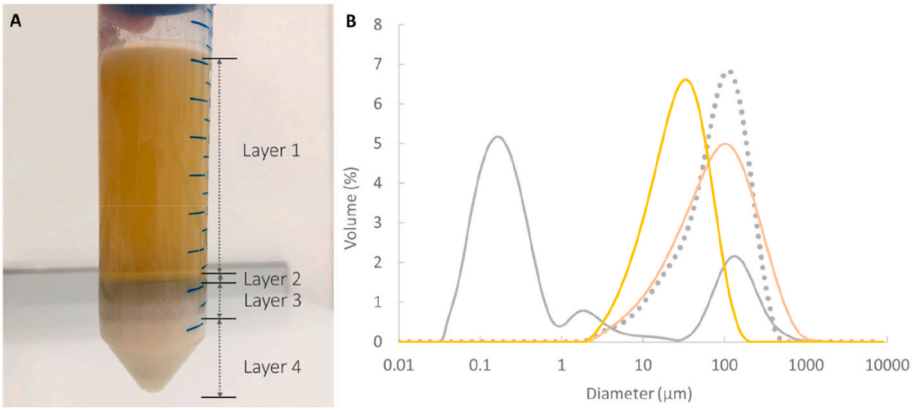

Fig. 4. Aqueous phase separation mung bean A) after 120 min of stirring and 30 min centrifugation at 4500 *g*; B) particle size distribution of Layer 1 (grey solid line), Layer 2 (yellow solid line), concentrated stock solution (dark yellow solid line) and commercial product M65 (grey dashed line). All measurements were carried out in triplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

concentrate, Layer 2 and M65 presented a peak around 100 μm, 35 μm and 120 μm, respectively. Since Layer 1 was a low viscosity liquid layer, it was believed to mostly contain free protein particles. And Layer 2 could be considered as more dense protein particles separated by the centrifugal force. Although a similar particle size was confirmed for Layer 1 concentrate and M65 at the same protein concentration, rheological behaviour such as viscosity of these two samples were found different. The rheological properties will be investigated and discussed in detail later.

Next an SDS-PAGE analysis under nonreducing condition was carried out on Layers 1 and 2, to determine whether some Mungbean proteins preferentially partition in one of the layers or not. Results are shown in Fig. 5. Major bands are similar in (relative) intensity for Layers 1 and 2: bands around 66 KDa, 50 KDa, 31 KDa, and 26 KDa can be attributed to the most abundant 8S globulins (Vicilin) in Mungbeans. Bands around 40 KDa and 20 KDa can be considered as 11S globulins (Legumin) (Tang & Sun, 2010). Except for some bands related to smaller proteins present in phase 2, SDS-PAGE shows no noticeable difference between the protein compositions of Layers 1 and 2. Layer 2 does show more protein material that has not migrated into the gel, signifying that it contains more protein in an aggregated state, as was also found using light scattering. In short, the conclusion is that with respect to protein composition, SDS-PAGE does not show much difference between the layers, the only difference is in the total concentration and the aggregation state of the proteins.

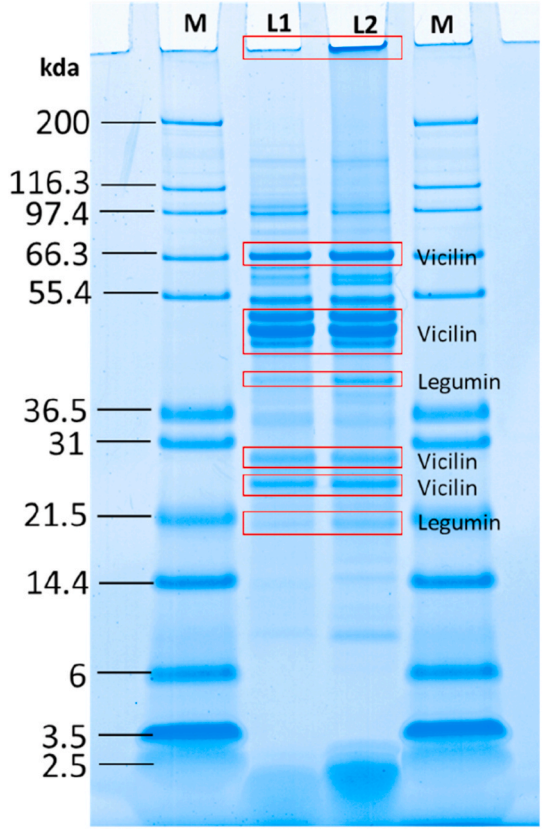

Fig. 5. *The non-reducing SDS-PAGE pattern of Layer 1 and Layer 2 of MBFF generated by aqueous phase separation at* pH *8 without salt addition. M, L1, and L2 represent Marker 12, Layer 1, and Layer 2, respectively.*

3.3. Protein content and yield

Subsequently, we analysed the protein content on a dry matter basis (g/g dm) and as a yield for each of the 4 layers, for several variations of the fractionation and purification process parameters. Parameters that were varied were: the wheel speed for the air-classification, stirring time, pH and concentration of added NaCl for the solvent to disperse the MBFF. Results are shown in Table 2. First, irrespective of process parameters, and as expected, we find that protein yield decreases with layer number. By far, most of the protein ends up in Layer 1. The protein concentration of the Layer 2 is higher, but since its volume is much smaller, the protein weight is lower than Layer 1 (3.188 g and 0.352 g for Layer 1 and Layer 2, respectively), leading to the protein yield of Layer 2 is lower. When optimizing the fractionation and purification process, we focus on the combined protein yield for Layers 1 and 2, to which we simply refer as 'protein yield'. Likewise, when investigating the functionality of the isolated Mungbean proteins, we will do so for the combined Layers 1 and 2.

We find that many of the varied processing parameters do not strongly affect the protein yield of layer. First, consider the speed used for air-classification. When increasing the wheel speed from 1000 rpm to 4000 rpm (keeping the stirring time for the resulting MBFF at 10 min), we find the protein yield increases moderately from 78.9% to 84.4% (See Supplementary Fig. SI. 2). The difference between the yields for 2500 rpm and 4000 rpm was not large, thus, 2500 rpm was used for the remainder of the work. The effect of stir time when dispersing the MBFF, was even less pronounced (See Supplementary Fig. SI. 1), and we use the shortest stirring time (10 min) for the remainder of the work. Likewise, we find that added NaCl only has a minor influence on the protein yield of the layer (See Supplementary Fig. SI. 3), hence for the remainder of the work no NaCl was added.

The only process parameter we investigated that significantly affects

Table 1
Elements distribution upon different fractions and treatments.

Elements	Unit	MBCF	MBFF	Layer 1	Layer 2	Layer 3	Layer 4	STOCK	M65
Ca	g/kg	1.92	0.51	0.83	2.55	0.57	n/a*	0.88	4.10
Cu		0.010	0.011	0.028	0.021	0.009	0.002	0.037	0.016
Fe		0.104	0.102	0.124	0.164	0.101	n/a	0.133	0.180
K		12.3	12.8	30.0	20.5	12.7	2.7	15.8	3.7
Mg		1.97	1.54	3.36	7.32	1.25	0.24	2.60	1.13
Mn		0.014	0.014	0.027	0.093	0.013	n/a	0.031	0.034
Na		n/a	n/a	4.970	2.973	2.051	0.395	2.610	4.334
P		4.14	4.83	11.20	18.7	4.0	0.88	9.53	7.44
S		1.92	2.21	5.22	4.01	2.07	0.37	4.26	3.24
Zn		0.028	0.031	n/a	0.074	0.025	n/a	0.084	0.057
Cl		0.40	0.55	1.67	n/a	0.36	0.08	1.04	0.29

*n/a represents results lower than the detection limit. The detection limit for Ca: 0.15 g/kg; Fe: 60 mg/kg; Mn: 0.05 g/kg; Na: 15 mg/kg; Zn: 5 mg/kg.

the protein yield is the pH of the MBFF dispersion used for the centrifugal layer separation. Results for the protein contents of the layers, and the protein yield as a function of pH (taken from Table 1), are shown in Fig. 6. When dispersed in Milli-Q water, the pH of the MBFF dispersions was around 6.5. Acid and base were added to the dispersions to bring the pH to values of pH 6 and pH 8 respectively. We find that adding acid strongly reduces protein yield, whereas adding base, leads to a small increase of the yield. The highest yield, obtained for air classification at 2500 rpm and 10 min stirring at pH 8, with no added salt, is 85.1%. While in practice it may not be worthwhile to perform the additional pH adjustment, for investigating the functionality of the Mungbean proteins, we here continue with the Layer 1 proteins obtained at the highest yield conditions (air classification at 2500 rpm, 10 min stirring at pH 8, no added salt).

Finally, we also investigated the effect of dehulling on protein yield (data not in Table 1). Dehulled mungbean seeds were milled at 4000 rpm, and subsequently air-classified at a wheel speed of 8000 rpm, resulting in Dehulled Mungbean fine fractions (DMBFF). We find that DMBFF has a much higher protein content (49 g/100 g dm, at 4000 rpm wheel speed) than MBFF (highest value is 24g/100g dry matter, at 8000 rpm wheel speed). This result is not unexpected, since dehulling leads to a significant reduction of non-protein material. Afterward, we continued by performing centrifugal layer separation on the DMBFF. Surprisingly, here we find that the dehulling reduces the overall protein yield (Supplementary Fig. SI. 4A), the total protein yield of DMBFF Layer 1 and Layer 2 was 72.8% whereas the total yield of MBFF was 79.8% (neutral pH, no salt added). It is believed that the aqueous system applied during centrifugal separation has a limited protein dissolve capacity. Since DMBFF brings more proteins than MBFF into the system and a part of the excessive proteins cannot be dissolved in the aqueous system, unable to be maintained in Layer 1 or Layer 2 after centrifugation. The extra proteins added but cannot be dissolved could lower the protein yield. Wheel speed and air classification speed employed to produce DMBFF could also influence the separation efficiency. The setting we applied in the present study (mill speed 4000 rpm, classification speed 8000 rpm) probably break the seeds insufficiently, therefore, it cannot liberate maximum protein bodies from seeds. However, further research should be performed to determine the optimum settings for DMBFF dry fractionation.

3.4. Mineral analysis

Mineral content is a key attribute of food ingredients that may (partly) depend on fractionation and purification processes. Therefore, we have analysed the mineral content for the MBFF and the MBCF, as obtained from the air-classification. Similarly, we analysed the mineral content of the Layers 1–4 obtained of the MBFF dispersions, a stock solution obtained by ultrafiltration of Layer 1, and a reference commercial Mungbean protein powder (labelled M65). Except for powder samples, other liquid or semi-solid samples are freeze dried before analysis. Results are shown in Table 1.

Results were obtained using a flow injection analyser for Cl and ICP-OES for all other elements. The coarse and fine flour fractions MBCF and MBFF present comparable mineral contents except for Ca^{2+}. This is probably caused by the higher fraction of Calcium-enriched hull (Lombardi-Boccia et al., 1998) in the coarse fraction. In general, the total mineral content of the stock is 36.99 g/kg which is higher than M65 (24.51 g/kg), the difference between these samples is largely contributed by K content which came from the raw material. Whereas mungbean protein samples possess higher total amount of ions than commercial whey proteins. The Ca content of Layer 1 and stock (0.9 g/kg) is 10-fold higher than whey proteins (0.09 g/kg) (Cornacchia et al.,2014). This property makes the stock a promising protein ingredient that can be employed in calcium fortification protein drinks.

For the layers, we find that Ca, Fe, Mg, Mn, P, Zn ions are enriched in Layer 2, presumably because they are more strongly associated with proteins. This result is partly aligning with the previous research. According to Posch et al. (1995) and Wiiliams & Silva (2000), Mn and Zn as transition metal ions, interacting strongly with proteins via electrostatic forces. The presence of Mg- and Ca-ions probably can be explained by previous observation for soybean seed (Sussulini et al., 2006). The content of Ca and Mg depends on applied extraction approaches since they were found to possess moderate interactions with proteins. Due to mildly extraction method employed in the present study, Ca and Mg were allowed to maintain their coordination with proteins, therefore, these ions were preserved.

On the other hand, for Cu, K, Na, S, and Cl the amounts correspond to water content in all layers, more amount of elements were detected in the layer which has higher water content. It is suggesting that they are mostly not associated with proteins or polysaccharides. While K, as a monovalent ion, tended to be lost during processing since it binds to protein weakly (Sussulini et al., 2006). This can also explain the considerable reduction in Na and K content that we observe after ultrafiltration to obtain the stock solution from Layer 1 (from 4.970 to 2.610 g/kg and 30.0 to 15.8 g/kg, respectively). Amounts of Sodium in Layer 1 are similar to those in the M65 and lower than M65 after ultrafiltration. Unfortunately, this value is still significantly higher than commercial whey proteins, it is undesirable when considering the low sodium diet recommendation. A further filtration process can be applied to remove the extra sodium from the stock solution to qualify it as a commercial plant protein ingredient if necessary. Note that no significant amounts of sodium were present in the dry fractions. This implies that the high Sodium content is due to additions during processing, in particular during pH adjustment.

3.5. Rheology

First, we consider the viscosity of the mildly purified Mungbean proteins before thermal treatment. Results for the flow curves of the MBFF Layer 1 protein and the M65 protein isolate at different

Table 2
Protein content and protein yield of aqueous phase separation systems under different conditions.

Rotation speed (Classification) RPM	Stirring time	pH	Salt addition	L1			L2			L3			L4		
				Protein yield (%)	protein content (g)	dry matter (g)	Protein yield (%)	protein content (g)	dry matter (g)	Protein yield (%)	protein content (g)	dry matter (g)	Protein yield (%)	protein content (g)	dry matter (g)
1000	**10 min**	**6.5**	**0 M**	69.94	3.11	5.52	7.75	0.34	0.56	10.64	0.47	3.02	13.31	0.59	7.82
2500		**6**		28.83	1.28	3.27	23.85	1.06	1.88	24.66	1.10	1.97	13.40	0.60	10.49
		6.5		71.81	3.19	5.42	7.92	0.35	0.57	15.61	0.69	5.74	7.51	0.33	5.59
			0.25 M	76.94	3.42	6.85	7.65	0.34	0.55	6.47	0.29	1.52	14.88	0.66	8.91
			0.5 M	75.62	3.36	7.76	4.66	0.21	0.33	11.01	0.49	4.03	9.15	0.41	7.28
		8	**0 M**	80.90	3.59	6.17	3.92	0.17	0.65	7.83	0.35	3.43	8.46	0.38	6.37
	30 min	**6.5**		74.02	3.29	5.64	8.36	0.37	0.57	10.64	0.47	1.95	15.45	0.69	7.81
	120 min			75.62	3.36	5.72	6.71	0.30	0.42	12.25	0.54	2.88	13.48	0.60	7.46
4000	**10 min**			76.06	3.38	5.80	8.09	0.36	0.54	8.88	0.40	2.23	14.15	0.63	7.69

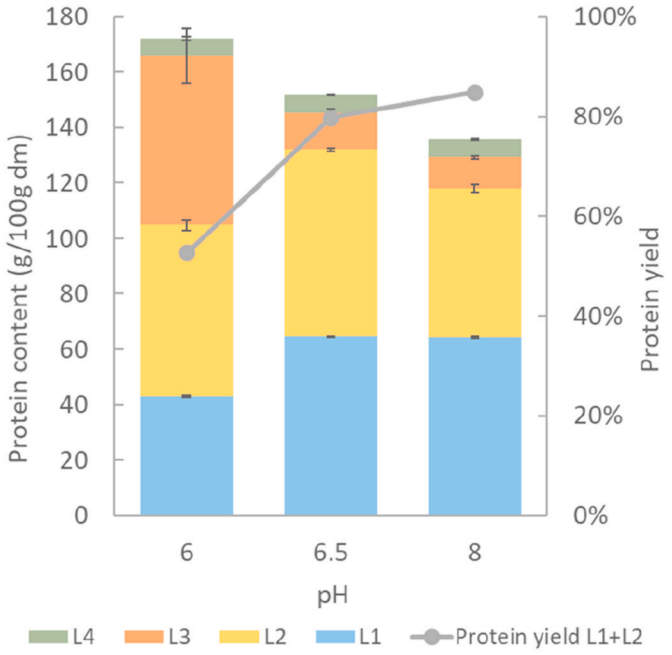

Fig. 6. Protein content and yield of 2500 rpm mung bean fine fraction at pH 6, pH 6.5 (neutral) and pH 8 aqueous phase separation with 10 min stirring. All measurements were carried out in triplicate.

concentrations are shown in Fig. 7. All samples showed shear-thinning behaviour. At comparable protein concentration, M65 was found to have significantly higher viscosities than the MBFF Layer 1 samples. The highest viscosities were found for M65 at a concentration of 11%, while at a concentration of 16.5%, the MBFF Layer 1 proteins still exhibit a viscosity appreciably less than the M65 proteins at 11%. A powder – law model was used to characterise the rheological behaviour of protein solutions since it fitted the viscosity versus shear rate data well. Table 3 showed that all samples are typical pseudoplastic fluids with flow indices $n < 1$. Protein solutions exhibited significant decreases ($P < 0.05$) in flow index n with the increase in protein concentration (indicating increased pseudoplasticity), whereas the consistency index K increased upon the increasing the protein concentration. Hence,

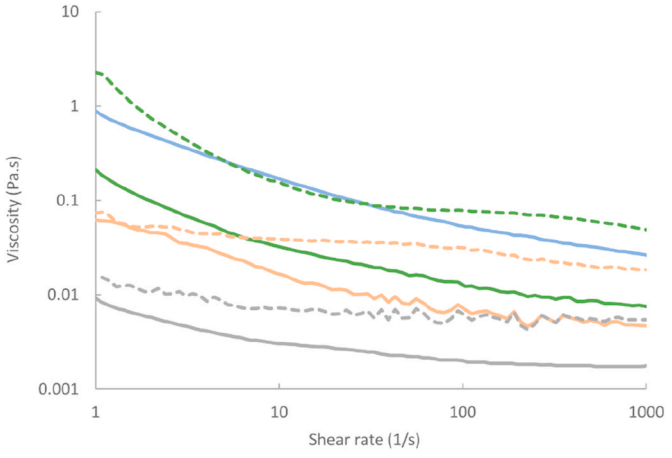

Fig. 7. Viscosities of MBFF aqueous phase separation samples (solid lines) at protein concentrations 5.3% (grey), 8.3% (orange), 11.0% (green), 16.5% (blue) and commercial mung bean protein concentrates M65 (dashed lines) at 5.3% (grey), 8.3% (orange) and 11.0% (green). MBFF samples were prepared by diluting the 22.0% concentred protein stock solution. All measurements were carried out in duplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Rheological characteristics of M65 and MBFF protein solutions.

Sample	Protein concentration	Flow index n	Consistency index K (Pa.s^n)	Determination coefficient R^2
M65	5.3%	0.88 ± 0.01^a	0.01 ± 0.00*^{b}	0.99 ± 0.00*
	8.3%	0.83 ± 0.01^{ab}	0.06 ± 0.01^b	1.00 ± 0.00*
	11.0%	0.55 ± 0.02^c	0.69 ± 0.00*^{a}	0.97 ± 0.01
MBFF	5.3%	0.79 ± 0.02^{ab}	0.01 ± 0.00*^{b}	0.99 ± 0.00*
	8.3%	0.63 ± 0.09^{bc}	0.05 ± 0.03^b	0.97 ± 0.02
	11.0%	0.56 ± 0.01^c	0.11 ± 0.00*^{b}	0.97 ± 0.01
	16.5%	0.52 ± 0.06^c	0.60 ± 0.23^a	0.98 ± 0.01

*Represents the standard deviation is lower than 1%.
Values are presented as mean ± standard deviation. Letters indicate significant difference between protein samples at $P < 0.05$.

viscosity was much higher and and shear thinning behaviours was more pronounced at higher protein concentrations (Dissanayake et al., 2013). Remarkably, we find that K increased sharply (from 0.06 to 0.69) for M65 when protein concentration increased from 8.3% to 11.0% while for MBFF it only increased from 0.05 to 0.11. This finding correlated with the research of Bains and Pal (2019), who showed a similar trend for emulsions. The differences observed for the consistency factor K increase could be related to the water-binding capacity of the protein. Štreimikytė et al. (2020) reported that protein possessing good water-binding capacity, had a more pronounced shear-thinning behaviour in solution.

We also consider structure formation during heat treatment at higher protein concentrations (>5%). Small deformation rheology is used to monitor protein gelation during the thermal treatment, which consists of a heating ramp (23 °C–90 °C at 3 °C/min), followed by 30 min at 90 °C, and a cooling ramp (90 °C–23 °C, at 3 °C/min). The results are shown in Fig. 8. The minimal gelling concentration is around 10% for both the MBFF Layer 1 protein and the commercial M65 concentrates (Fig. 8C). As shown in Fig. 9, the final gel moduli (after the gels having been cooled down back to the room temperature) increase rapidly with increasing protein concentration, once above the critical concentration of around 10%, with not much difference between the MBFF Layer 1 proteins and the M65 protein isolate.

4. Conclusion

We have shown how air-classification and centrifugal layer separation can be combined to mildly purify Mungbean proteins, minimizing the level of denaturation. In particular, we found that after performing centrifugal layer separation on the MBFF at either its natural pH of 6.5 or at pH 8, both the yield (71.61% and 80.92%, respectively) and protein content (64.57% and 64.22%, respectively) of the Layer 1 fraction are very high.

In general, one expects protein denaturation and aggregation during ingredients preparation to have a deleterious effect on the resulting ingredient functionality (Schutyser & van der Goot, 2011). For example, for beverages, one requires high solubility and low viscosity. Indeed, we find that solubility is lower, and viscosity is higher for the reference

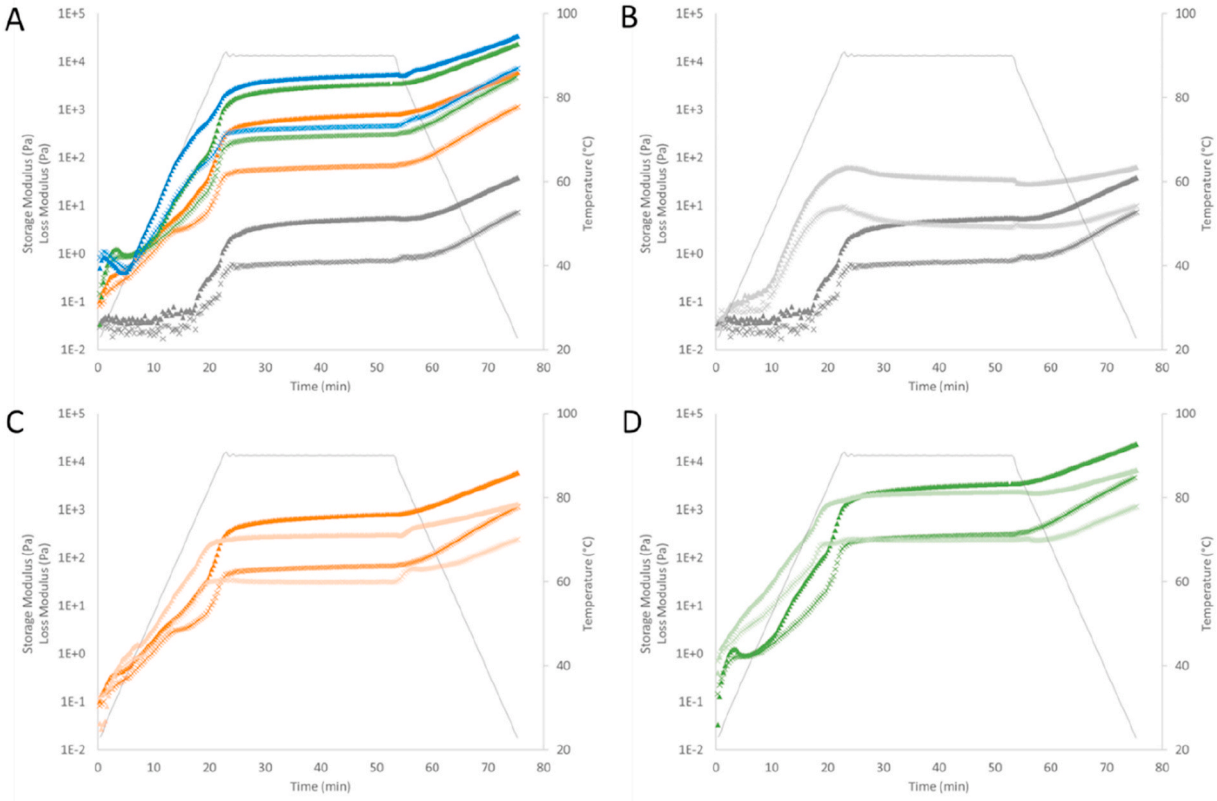

Fig. 8. The temporal evolution of the storage modulus G' (triangles) and loss modulus G''(crosses) of A) MBFF protein dilutions with 5.3% (grey), 8.3% (orange), 11.0% (green) and 16.5% (blue) protein concentration; B) MBFF protein dilution (grey) and M65 solution (light grey) with a total protein concentration of 5.3%; C) MBFF protein dilution (orange) and M65 solution (light orange) with a total protein concentration of 8.3%; D) MBFF protein solution (green) and M65 solution (light green) with a total protein concentration of 11.0%. The thermal treatment is shown by the dashed line. All measurements were conducted at a constant frequency of 1Hz and a constant strain of 1%. All measurements were carried out in duplicate. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

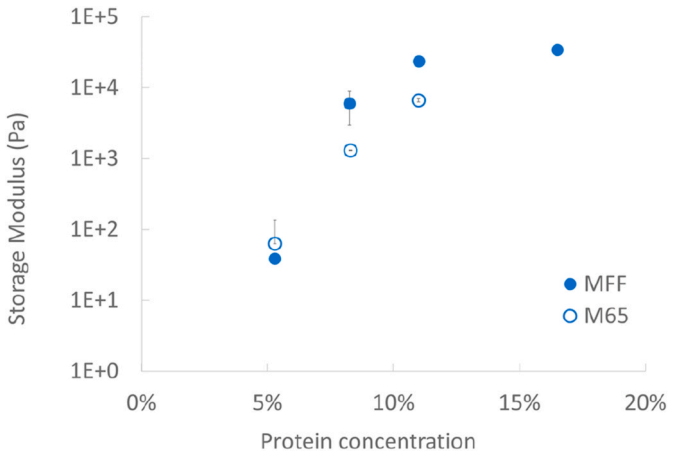

Fig. 9. Gel properties of MBFF (solid symbols) and M65 (open symbols) heated at 90 °C: A) storage modulus after thermal treatment as a function of protein concentration. All measurements were carried out in duplicate.

commercial M65 Mungbean protein concentrates, as compared to the MBFF Layer 1 proteins.

Both differences can probably be attributed to the formation of protein aggregates during ingredient preparation in the case of the commercial isolate M65. Indeed, from light scattering we found (Fig. 4B) that the average particle diameters in dispersions of MBFF Layer 1, concentrated MBFF Layer 1 and M65 were, respectively, 0.2, 100, and 120 μm.

Our results are consistent with what has been reported for other sources, such as for pea. Kornet et al. (2020) found that for more mildly processed pea protein fractions, the viscosity increased less rapidly with increasing protein concentrations than pea protein fractions that had experienced more extreme pH and temperature values. It should be noted that except for conditions applied to produce protein ingredients, unavoidable subsequent heat treatments such as Pasteurisation could have an influence on the viscosity of final products as well. Remarkably, we find that at least for this case, the purification process and the associated degree of protein denaturation and aggregation, does not seem to have a strong impact on heat-set gelation.

A crucial final step will be to develop a drying process for the MBFF Layer 1 dispersions that likewise maintains the native state of the proteins, such that these mildly purified Mungbean proteins can be applied in, for example, plant protein beverages.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors have declared that no known competing interests could influence the work studied in this publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112784.

References

Avila Ruiz, G., Arts, A., Minor, M., & Schutyser, M. (2016). A hybrid dry and aqueous fractionation method to obtain protein-rich fractions from quinoa (Chenopodium quinoa willd). *Food and Bioprocess Technology, 9*(9), 1502–1510. https://doi.org/10.1007/s11947-016-1731-0

Bains, U., & Pal, R. (2019). In-situ continuous monitoring of the viscosity of surfactant-stabilized and nanoparticles-stabilized pickering emulsions. *Applied Sciences, 9*(19), 4044. https://doi.org/10.3390/app9194044

Cornacchia, L., Forquenot de la Fortelle, C., & Venema, P. (2014). Heat-induced aggregation of whey proteins in aqueous solutions below their isoelectric point. *Journal of Agricultural and Food Chemistry, 62*(3), 733–741. https://doi.org/10.1021/jf404456q

Dissanayake, M., Ramchandran, L., & Vasiljevic, T. (2013). Influence of pH and protein concentration on rheological properties of whey protein dispersions. *Int.Food Res.J, 20*(5), 2167.

Du, M., Xie, J., Gong, B., Xu, X., Tang, W., Li, X., & Xie, M. (2018). Extraction, physicochemical characteristics and functional properties of Mungbean protein. *Food Hydrocolloids, 76*, 131–140. https://doi.org/10.1016/j.foodhyd.2017.01.003

Kornet, C., Venema, P., Nijsse, J., van der Linden, E., van der Goot, A. J., & Meinders, M. (2020). Yellow pea aqueous fractionation increases the specific volume fraction and viscosity of its dispersions. *Food Hydrocolloids, 99*, 105332. https://doi.org/10.1016/j.foodhyd.2019.105332

de Kort, E., Minor, M., Snoeren, T., van Hooijdonk, T., & van der Linden, E. (2011). Effect of calcium chelators on physical changes in casein micelles in concentrated micellar casein solutions. *International Dairy Journal, 21*(12), 907–913. https://doi.org/10.1016/j.idairyj.2011.06.007

Kudre, T. G., Benjakul, S., & Kishimura, H. (2013). Comparative study on chemical compositions and properties of protein isolates from mungbean, black bean and Bambara groundnut. *Journal of the Science of Food and Agriculture, 93*(10), 2429–2436. https://doi.org/10.1002/jsfa.6052

Kumar, P., Chatli, M. K., Mehta, N., Singh, P., Malav, O. P., & Verma, A. K. (2016). Meat analogues: Health promising sustainable meat substitutes. *Critical Reviews in Food Science and Nutrition, 57*(5), 923–932. https://doi.org/10.1080/10408398.2014.939739

Lombardi-Boccia, G., Lucarini, M., Di Lullo, G., Del Puppo, E., Ferrari, A., & Carnovale, E. (1998). Dialysable, soluble and fermentable calcium from beans (Phaseolus vulgaris L.) as model for in vitro assessment of the potential calcium availability. *Food Chemistry, 61*(1–2), 167–171. https://doi.org/10.1016/s0308-8146(97)00040-x

Mariotti, F., Tomé, D., & Mirand, P. P. (2008). Converting nitrogen into protein—beyond 6.25 and jones' factors. *Critical Reviews in Food Science and Nutrition, 48*(2), 177–184. https://doi.org/10.1080/10408390701279749

MarketsandMarkets. (2021). *Plant-based protein market worth $15.6 billion by 2026*. Retrieved from https://www.marketsandmarkets.com/PressReleases/plant-based-protein.asp. (Accessed 8 April 2021) Accessed.

Mattice, K. D., & Marangoni, A. G. (2020). Physical properties of plant-based cheese products produced with zein. *Food Hydrocolloids, 105*, 105746. https://doi.org/10.1016/j.foodhyd.2020.105746

Pelgrom, P. J. M., Boom, R. M., & Schutyser, M. A. I. (2015a). Functional analysis of mildly refined fractions from yellow pea. *Food Hydrocolloids, 44*, 12–22. https://doi.org/10.1016/j.foodhyd.2014.09.001

Pelgrom, P. J. M., Vissers, A. M., Boom, R. M., & Schutyser, M. A. I. (2013). Dry fractionation for production of functional pea protein concentrates. *Food Research International, 53*(1), 232–239. https://doi.org/10.1016/j.foodres.2013.05.004

Pelgrom, P. J. M., Wang, J., Boom, R. M., & Schutyser, M. A. I. (2015b). Pre- and post-treatment enhance the protein enrichment from milling and air classification of legumes. *Journal of Food Engineering, 155*, 53–61. https://doi.org/10.1016/j.jfoodeng.2015.01.005

Purwanti, N., Smiddy, M., Jan van der Goot, A., de Vries, R., Alting, A., & Boom, R. (2011). Modulation of rheological properties by heat-induced aggregation of whey protein solution. *Food Hydrocolloids, 25*(6), 1482–1489. https://doi.org/10.1016/j.foodhyd.2011.02.027

Rinaldoni, A. N., Palatnik, D. R., Zaritzky, N., & Campderrós, M. E. (2014). Soft cheese-like product development enriched with soy protein concentrates. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology, 55*(1), 139–147. https://doi.org/10.1016/j.lwt.2013.09.003

Schutyser, M. A. I., Pelgrom, P. J. M., van der Goot, A. J., & Boom, R. M. (2015). Dry fractionation for sustainable production of functional legume protein concentrates. *Trends in Food Science & Technology, 45*(2), 327–335. https://doi.org/10.1016/j.tifs.2015.04.013

Schutyser, M. A. I., & van der Goot, A. J. (2011). The potential of dry fractionation processes for sustainable plant protein production. *Trends in Food Science & Technology, 22*(4), 154–164. https://doi.org/10.1016/j.tifs.2010.11.006

Simons, C., Hall, C., & Biswas, A. (2017). Characterization of pinto bean high-starch fraction after air classification and extrusion. *Journal of Food Processing and Preservation, 41*(6). https://doi.org/10.1111/jfpp.13254

Štreimikytė, P., Keršienė, M., Eisinaitė, V., Jasutienė, I., Lesauskaitė, V., Damulevičienė, G., Knašienė, J., & Leskauskaitė, D. (2020). Formulating protein-based beverages for the dysphagia diets of the elderly: Viscosity, protein quality, in vitro digestion, and consumers acceptability. *Journal of the Science of Food and Agriculture, 100*(10), 3895–3901. https://doi.org/10.1002/jsfa.10431

Sussulini, A., Garcia, J. S., Mesko, M. F., Moraes, D. P., Flores, É. M., Pérez, C. A., & Arruda, M. A. (2006). Evaluation of soybean seed protein extraction focusing on metalloprotein analysis. *Microchimica Acta, 158*(1–2), 173–180. https://doi.org/10.1007/s00604-006-0678-7

Tang, C.-H., & Sun, X. (2010). Physicochemical and structural properties of 8S and/or 11S globulins from mungbean [vigna radiata(L.) wilczek] with various polypeptide constituents. *Journal of Agricultural and Food Chemistry, 58*(10), 6395–6402. https://doi.org/10.1021/jf904254f

Wang, R., Jin, X., Su, S., Lu, Y., & Guo, S. (2019). Soymilk gelation: The determinant roles of incubation time and gelation rate. *Food Hydrocolloids, 97*, 105230. https://doi.org/10.1016/j.foodhyd.2019.105230

Waschatko, G., Junghans, A., & Vilgis, T. A. (2012). Soy milk oleosome behaviour at the air–water interface. *Faraday Discussions, 158*, 157. https://doi.org/10.1039/c2fd20036h

Wu, Y. V., & Nichols, N. N. (2005). Fine grinding and air classification of field pea. *Cereal Chemistry Journal, 82*(3), 341–344. https://doi.org/10.1094/cc-82-0341

Xing, Q., de Wit, M., Kyriakopoulou, K., Boom, R. M., & Schutyser, M. A. I. (2018). Protein enrichment of defatted soybean flour by fine milling and electrostatic separation. *Innovative Food Science & Emerging Technologies, 50*, 42–49. https://doi.org/10.1016/j.ifset.2018.08.014