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Abstract:

The influence of two heating protocols (protocol 1 and 2) on protein interactions and acid-induced gelation properties of casein micelle-pea protein mixture (CM-PP) was investigated and then compared to casein micelle-whey protein mixture (CM-WP). The CM:PP and CM:WP protein weight ratio for mixtures was 7.5:2.5, for a total protein content of 4% (pH 6.7). Protocol 1 consisted of a heat treatment (85 °C for 1h) of CM-PP and CM-WP mixtures, respectively. Regarding protocol 2, casein micelle, pea protein and whey protein stock dispersions were individually pretreated by heating (85°C for 1 h) before the mixtures were made and heated in the same conditions of protocol 1 (85 °C for 1 h). Heat pretreatment carried out in the protocol 2 significantly increased PP hydrophobicity and reinforced weak interactions of the initial pea protein particles. This pretreatment on protein stock dispersions led to twofold smaller pea protein particles compared to whey protein aggregates. The hydrophobic interactions between pea proteins and casein micelles promoted by the two heating protocols have greatly contributed to improve acid gelation functionalities of CM. Regardless of the heating protocol, acid-induced gelation of the CM-PP mixtures led to the formation of gel networks with a significant increase in stiffness and firmness compared to casein micelle or CM-WP mixtures gels.

Keywords: pea protein, whey protein, casein micelle, thermal processing, protein mixture, acid gelation, rheological properties.

Abbreviations: BSA, bovin serum albumin; CM, casein micelle; CM HT, heat-treated casein micelle; DB, dry basis; GDL, glucono-δ-lactone; MSH, 2-mercaptoethanol; M_w, molecular weight; NR, non-reducing conditions; P1, heat treatment protocol 1; P2, heat treatment protocol 2; PCN, native-like phosphocasein; PCS, photon correlation spectroscopy; pI, isoelectric point; PP, pea protein; PP HT, heat-treated pea protein; PPI, pea protein isolate; R, reducing conditions; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SH, thiol group; SMUF, simulated milk ultrafiltrate; WHC, water holding capacity; WP, whey protein; WP HT, heat-treated whey protein; WPI, whey protein isolate.

Introduction

A high proportion of food proteins consumed in developed countries are animal-based, for which production methods have detrimental impacts on the environment compared to plant-based proteins due to both higher inputs of natural resources and greater emissions of harmful pollutants (Nadathur, Wanasundara, & Scanlin, 2017; Sabaté & Soret, 2014). In high income countries, policy actions are now implemented to restore a healthy and sustainable balance in consumption concerning the ratio of plant to animal protein (De Boer & Aiking, 2019). In order to reduce the excess consumption of animal proteins with the aim of limiting environmental impacts, one of the solutions is to partially replace animal proteins by plant ones in processed foods and propose to consumers innovative food with nutritional and functional interest combining animal and plant proteins (Alves & Tavares, 2019; Guyomarc'h et al., 2021). However, the association of plant and animal proteins requires at present time better knowledge to understand the processing, the properties and the stability of these novel assemblies and optimize them.

Different proteins have been tested to develop these mixed systems as reviewed by Alves & Tavares (2019). Concerning the recurring animal proteins used in mixed systems, milk proteins have the advantage to be very well characterized by extensive research studies and the diversity of their physicochemical properties allows to explore varied association ways to plant proteins (Alves & Tavares, 2019). Bovine milk proteins consist in 80 % wt. caseins and 20 % wt. whey proteins with β-lactoglobulin, α-lactalbumin and bovine serum albumin as major fractions. The four fractions of caseins (αs_1-, αs_2-, β- and κ-caseins) are auto-associated to form casein micelles (CM) mainly through hydrophobic bonds and calcium phosphate nanoclusters bound to phosphoseryl residues of the casein chains (De Kruif et al., 2012; Walstra, Geurts, Noomen, Jellema, & Van Boekel, 1999). Casein micelles are spherical particles (~ 150 nm in average diameter) characterized by an hydrophilic surface made of the C-terminal part of κ-caseins (Walstra et al., 1999). This highly hydrated auto-assembly stabilized by a net negative charge is a remarkably stable colloidal structure not affected by aggregation phenomenon at milk pH and temperature lower than 100°C (Lucey & Singh, 2003). However, acidification (pI ~ 4.6) induces casein micelles coagulation (Horne, 1999). Heating above 70 °C CM in presence of whey proteins before acidification promotes whey protein denaturation but also their interactions with CM surface inducing strengthened acidified gels (Lucey & Singh, 1997; Lucey, Tamehana, Singh, & Munro, 1998).

Concerning plant proteins, proteins from pea (*Pisum sativum*) have gained great attention in food area over the last decade, particularly from a nutritional point of view since they contain all the essential amino acids and particularly a high content of lysine and branched chain amino (Lam, Can Karaca, Tyler, & Nickerson, 2018). Besides, the sustainable agronomic practices of pea crop, availability, low

cost and health benefits recognized to the extracted pea proteins represent interesting advantages over other plant proteins (Roy, Boye, & Simpson, 2010). Although pea off-flavor development is a weakness, pea proteins are characterized by their high digestibility and relatively fewer allergenic responses and negative health controversies compared to soy proteins (Fischer, Cachon, & Cayot, 2020). In contrast to traditional cereal proteins, the main pea proteins are globulins (~ 65-80 %) representing globular storage proteins classified according to their sedimentation coefficient. They consist of 7S vicilin (160 - 200 kDa), 7S convicilin (~ 280 kDa) and 11S legumin (360 - 400 kDa). The albumin fraction (20-35 % of total pea proteins) combines metabolic and enzymatic proteins (Owusu-Ansah & McCurdy, 1991). The proportion of these different proteins depends on genetic factors and, to a lesser extent, on the crop environment (Lam et al., 2018). Several interesting techno-functional properties were established for pea protein isolates such as solubility, water- and oil-holding capacity, emulsification, foaming and gelation (Lam et al., 2018). Pea protein gels have been well characterized and documented in the literature (Lu, He, Zhang, & Bing, 2019). However, the processing conditions applied to industrially obtain pea protein isolate may affect these functional properties, limiting their applications in the food industry (Barac, Pesic, Stanojevic, Kostic, & Cabrilo, 2015; Burger & Zhang, 2019).

An opportunity to partially replace animal-based proteins by plant-based proteins is to produce protein gels mixing milk and plant proteins. Particular attention has been given to the aggregation and gelation of soy proteins and their mixtures with milk proteins (Beliciu & Moraru, 2013; Martin, de los Reyes Jiménez & Pouvreau, 2016; Schmitt et al., 2019). Regarding pea/milk protein mixtures, several works have been published on heat-induced gelation or fermentation-induced gelation (Alves & Tavares, 2019 ; Ben-Harb et al., 2018; Chihi, Sok & Saurel, 2018; Silva et al., 2018; Silva et al., 2019; Yousseef et al., 2016), but few have been devoted to gels obtained from casein micelles and pea proteins mixtures by chemical acidification. Mession, Roustel, & Saurel (2017) characterized the glucono-δ-lactone acid gelation of non-fractionated pea protein extract or isolated fractions of 7S vicilin and 11S legumin combined with casein micelles (1:1 weight ratio, 3.6% total protein, initial pH 7.1). Before acidification, a heat treatment was applied directly to the pea protein-casein micelle mixtures or independently to the pea protein samples prior to mixing them with unheated casein micelles. The acidification of pre-heated non-fractionated pea proteins mixed with unheated casein micelles resulted in gels characterized by a rapid gelation, a higher final storage modulus and a higher gelation pH compared to single-protein gels. However, the acidification of the co-heated non-fractionated pea proteins-casein micelles mixture did not result in gel formation due to the presence of large and sedimentable pea protein aggregates.

Numerous studies have been devoted to the gelation of milk proteins, focusing on the impact of thermal denaturation of whey proteins and their covalent interaction through disulfide interaction

with the surface of casein micelles, increasing the strength of acid gels. But currently, little is known about the interactions between pea proteins and milk proteins, specifically between soluble pea proteins containing cysteine residues and casein micelles upon application of heat treatment and acidification process. Therefore, the purpose of the present study was to improve the multi-scale (molecular, microscopic, macroscopic) understanding of the acid CM-PP mixed gel structuring by comparing two different pre-gelation processes: (1) co-heating of untreated CM and PP (2) co-heating of previously independently heat-treated casein micelles and pea proteins. The acid gel structuring and properties obtained with CM-PP mixtures were compared to those obtained with casein micelles-whey proteins (CM-WP) mixtures subjected to the same pre-gelation protocols. Besides, the objective of this work was to functionalize casein micelles with a commercial pea protein isolate, at the same natural ratio observed for CM-WP, to evaluate its potential applications in food industry. Gelation kinetics, rheological and textural gel properties were compared and related to the physicochemical properties of the different mixed dispersions.

2. Materials and methods

2.1. Materials

Pea protein isolate (PPI) powder was kindly provided by Roquette Frères (Nutralys S85F, batch W278N, Lestrem, France), whey protein isolate (WPI) powder was obtained from Lactalis Ingredients (Prolacta 90, batch R51640, Bourgbarré, France) and native-like phosphocasein (PCN) powder was purchased from Ingredia Dairy Ingredients (Promilk 852B, batch 082156, Arras, France). PCN was industrially prepared by microfiltration, then diafiltration using the milk mineral soluble phase conferring a quasi-native state to the prepared casein micelles. The three commercial powders were stored at 4 °C away from moisture and light.

Tri-potassium citrate monohydrate, tri-sodium citrate dihydrate, KH_2PO_4, K_2SO_4 were purchased from Alfa Aesar (Karlsruhe, Germany), K_2CO_3 and $CaCl_2$, $2H_2O$ from Amresco (Solon, Ohio, USA), KCl, $MgCl_2$, TCA and KOH from VWR BDH Prolabo (Leuven, Belgium) and tris[hydroxymethyl] aminomethane (Tris), EDTA, glycine, sodium phosphate dibasic, sodium dodecyl sulfate, urea, 5,5'-dithiobis-(2-nitrobenzoic acid), 8-anilino-1-naphthalenesulfonic acid ammonium salt (ANS), glycerol, 2-mercaptoethanol, acrylamide/bis-acrylamide, ammonium persulfate, N, N, N', N'-tetramethyl ethylenediamine (TEMED) from Sigma (St. Louis, MO, USA). Coomassie brillant blue G250, D-(+)-gluconic acid δ-lactone were obtained from Fluka chemicals GmbH (Dublin, Ireland) and bromophenol Blue was and sodium dihydrogen phosphate monohydrate was purchased from Merck (Darmstadt, Germany). All solutions were prepared using Milli-Q ultrapure water (Millipore®).

2.2. Proximate composition analysis

Moisture and ash content of the commercial powders (PPI, WPI, PCN) were determined according to AOAC methods 925.10 and 923.0, respectively (AOAC, 2000). Protein content was determined using Kjeldahl method in a Büchi mineralization and distillation unit (KjelFlex K-360, Switzerland) with a nitrogen conversion factor of 6.25 and 6.38 for PPI and milk proteins, respectively (Shand, Ya, Pietrasik, & Wanasundara, 2007). Fat content was given by suppliers. Carbohydrate content was calculated by subtracting the sum of the protein, ash and fat contents from the total solid. The proximate composition analysis of the tree powders is summarized in Table 1.

2.3. Preparation of single protein dispersions

Pea (PPI) and whey (WPI) protein powders were suspended separately at 5 % (w/w) in Milli-Q ultrapure water, gently stirred at 300 rpm for 2 h at 20 °C and then overnight at 4 °C to allow complete hydration. The pH of the two dispersions was adjusted to pH 6.7 with 1 M NaOH or 1 M HCl without affecting their concentrations. The PPI dispersion was then centrifuged (ultracentrifuge Optima LE-80k, rotor 70 Ti, Beckman, California, USA) at 12000 × g for 20 min at 20 °C and the supernatant was collected as the protein soluble fraction (PP). The yield of the soluble fraction for the PP dispersions after the centrifugation was 34% with a PP protein content of 1.7% (w/w).

The PCN powder was suspended at 3.75 % (w/w) in lactose-free simulated milk ultrafiltrate (SMUF) according to Jenness & Koops (1962) to keep the mineral environment of native casein micelles. CM dispersion was stirred at 300 rpm for 30 min at 20 °C then the pH was adjusted to pH 6.7 with 1 M NaOH. CM dispersion was then stored at 4 °C overnight to improve the powder hydration and was then warmed for 1 hour at 40 °C and rapidly cooled to 20 °C just before experiments to ensure complete hydration.

2.4. Preparation of heat-treated protein mixtures

Mixture samples were prepared according to two different protocols as presented in Fig. 1.

Protocol 1. The CM stock dispersion was first mixed with PP or WP dispersion in a 7.5:2.5 protein ratio conducting to a total protein content of 4 % (w/w). This total protein content was selected based on the reference of the bovine milk protein concentration (Walstra et al., 1999). Mixtures were then stirred using a magnetic stirrer at 300 rpm for 30 min at 25 °C, placed in hermetically sealed 15 mL plastic conical tubes (Falcon, Corning, New York, USA) and equilibrated in a water bath at 40 °C for 2 min. Thereafter, sample temperature was increased to 85 °C at a rate of 1 °C/min, and then maintained at 85 °C for 1 h before a rapid cooling to 20 °C in ice. These mixtures were named (CM-

PP) P1 and (CM-WP) P1, respectively. Samples were then stored at 4 °C and equilibrated at 20 °C prior to analysis.

Protocol 2. The PP, WP and CM stock dispersions were placed in the hermetically sealed plastic conical tubes and then submerged in a water bath at 40 °C for 2 min for equilibration. Thereafter, sample temperature was increased to 85 °C at a rate of 1 °C/min, and then maintained at 85 °C for 1 h before cooling down to 20 °C using an iced water bath. The heated dispersions were named heat treated pea protein (PP HT), heat treated whey protein (WP HT) and heat-treated casein micelles (CM HT).

CM HT were mixed with PP HT or WP HT in a 7.5:2.5 protein ratio conducting to a total protein content of 4 % (w/w). After stirring using a magnetic stirrer at 300 rpm for 30 min at 25 °C, the same heat treatment procedure as described above was carried out to the mixed samples to obtain the heated mixtures (CM-PP) P2 and (CM-WP) P2.

2.5. Particle size distribution by photon correlation spectroscopy

The particle size distribution of the different dispersions was measured at 25 °C by dynamic light scattering using the Malvern NanoSizer (Zetasizer nano ZS, Malvern Instruments, Orsay, France) with a photodiode detector with 4 mW He–Ne laser operating at a wavelength of 633 nm and a fixed scattering angle of 173°. Prior to analysis, samples were diluted to 10 g/L with ultrapure water. Measurements were carried out in four-sided clear polystyrene cuvettes (Sarstedt, Numbrecht, Germany) with at least twelve runs per measurement. The dispersant viscosity and refractive index for water were 0.8875 mPa s and 1.330, respectively. The real refractive indexes was 1.36 for the milk protein (Regnault, Thiebaud, Dumay, & Cheftel, 2004) and 1.45 for pea protein isolate (Bogahawaththa et al., 2019). The minimal, maximal and mean diameters were determined from the light-intensity and the number frequency (%) distribution curves. Measurements were evaluated in quadruplicate.

2.6. Determination of sulfhydryl group content

The surface, free and total sulfhydryl (SH) groups of protein samples were determined using Ellman's method (Ellman, Courtney, Andres, & Featherstone, 1961) with some modifications. Protein stock dispersions were diluted to 7.5 mg/mL in tris-glycine buffer (86 mM Tris, 90 mM glycine and 4 mM EDTA, pH 8.0) for surface SH content measurement. For free SH content, protein stock dispersions were diluted to the same concentration in tris-glycine buffer containing 8 M urea then the solution was gently stirred overnight at 20 °C. To evaluate the total SH group content and calculate the disulfide bonds, 0.05 mL of 2-mercaptoethanol and 4 mL of tris-glycine buffer were added to 1 mL

of the protein stock dilution prepared in tris-glycine buffer containing 8 M urea then the mixture was incubated for 1 h at room temperature. Then 10 mL of 12 % (w/v) tri-chloroacetic acid (TCA) solution were added and the mixture was incubated for an additional hour. The precipitate after centrifugation (5000 × g for 10 min) was washed twice with 5 mL of TCA solution. After that, the precipitate was dissolved in 10 mL of tris-glycine buffer before adding 0.05 mL of Ellman's reagent (2 mM 5,5'-dithiobis-2-nitrobenzoic acid in the tris-glycine buffer) and incubating the mixture at room temperature for 20 min. The absorbance was measured at 412 nm using a spectrophotometer (UV 1900, Shimadzu UV-Vis, Japan).

The SH content was calculated according to Eq (1):

$$\mathrm{SH}(\mu\mathrm{mol/g}) = (73.53 * A * D)/C \qquad (1)$$

where A is the absorbance at 412 nm, C is the sample protein concentration (7.5 mg/mL), D is the dilution factor, the constant 73.53 is calculated as $10^6/(1.36 \times 10^4)$ (10^6 is the conversion from molar basis to µmol/mL basis and from mg to g solids; 1.36×10^4 is the molar absorptivity).

The SS content was calculated according to Eq (2):

$$\mathrm{SS}(\mu\mathrm{mol/g}) = (\mathrm{Total\ SH} - \mathrm{free\ SH})/2 \qquad (2)$$

All determinations were performed in triplicate.

2.7. Surface hydrophobicity

Surface hydrophobicity (Ho) was measured as reported by Kato & Nakai (1980) and Haskard & Li-Chan (1998) with minor modifications. A 8 mM ANS stock solution was prepared in phosphate buffer (0.01 M, pH7), ANS being used as the fluorescence probe. For each protein dispersion to analyze, dilutions were prepared in the same buffer to obtain 5 final protein concentrations ranging from 0.04 to 0.2 mg/mL. Then, 20 µL ANS stock solution were added to 4 mL of each protein concentration. The fluorescence intensity of the mixture was measured using a fluorescence spectrophotometer (Varian Cary Eclipse, Australia) at λ_{ex}= 365 nm and λ_{em}= 484 nm in quartz cuvette (101-QS, Hellma, Germany). The surface hydrophobicity of protein sample represents the initial slope of fluorescence intensity versus protein concentration calculated by linear regression. All determinations were performed in triplicate.

2.8. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis analysis was performed for all samples under native, non-reducing (NR) and reducing (R) conditions on a discontinuous buffer system according to Grácia-Juliá et al. (2008) .

2.8.1. Native-PAGE

Proteins were analyzed using minigels (80 × 80 × 1.5 mm) consisted in 3.5 % (v/v) acrylamide/bis-acrylamide (C = 37.5:1) stacking gel and 5 % (v/v) separating gel, prepared in 25 mM and 50 mM phosphate buffer (pH 7), respectively. Ammonium persulphate and TEMED were added at 1.8 and 2.8 mM, respectively. Protein samples were diluted at 5 mg/mL in 25 mM phosphate buffer (pH 7) in the presence of glycerol (150 mL/L) and bromophenol blue (0.0025 % (w/v)). Electrophoresis was carried out with a running 50 mM phosphate buffer at pH 7. Electrophoresis were carried out at a constant power of 12 W at 18 °C for 3 h. After completion, electrophoresis gels were stained with R-250 Coomassie blue and then washed.

2.8.2. SDS-PAGE

SDS-PAGE was conducted in 4 % stacking and 12 % separating gels. Samples were prepared in sample buffer containing 0.5 M Tris-HCl, pH 6.8, 2 % (w/v) SDS, 150 mL/L glycerol, 0.0025 % (w/v) bromophenol blue, and 5 % (v/v) 2-mercaptoethanol only in the case of reducing condition. Samples were then heated at 95 °C for 5 min and cooled at the room temperature. The migration was conducted with a tris-glycine running buffer (0.025 M Tris, 0.192 M glycine, 0.1 % (w/v) SDS) at pH 8.3. SDS-PAGE were run at a constant power of 9 W for 3 h at 18°C. Per well, 5 µL of sample containing 0.5 % (w/w) of proteins or 15 µL of molecular weight markers (Novex Sharp Pre-Stained Protein Standard, LC5800, Invitrogen, Carlsbad, USA) were loaded per well. After completion, electrophoresis gels were stained with R-250 Coomassie blue and then washed.

2.9. Rheological measurements

Rheological measurements of the different samples were carried out in a dynamic rheometer (MCR 300, Anton Paar, Graz, Austria) using a coaxial cylinder geometry (CC27). The evolution of the storage modulus (G') and loss modulus (G") was recorded for 5 h at a constant strain of 1 % and a frequency of 1 Hz. A sample volume of 20 mL was mixed with 2 % (w/w) gluconic acid δ-lactone (GDL) and poured into the rheometer cup maintained at 25 °C. The gel point was obtained when the storage modulus (G') was equal to the loss modulus (G"). Simultaneously, the kinetic of acidification at 25 °C was followed using pH-meter (C833, Consort, Turnhout, Belgium) equipped of a data acquisition system recording one measure each 10 seconds. The acidification kinetic and rheological analysis were carried out in triplicate.

2.10. Texture analysis of gels

The textural properties of gels formed by gelation at 25 °C with 2 % (w/w) GDL was determined using a TA-XT2i texture analyzer (Stable Microsystems, Godalming, England) equipped with a 5 kg

load cell. The gels were stored at 4 °C for 24 h prior to the texture profile analysis. The test was carried out with a back-extrusion cell (35 mm diameter disc, 5 mm thickness). The test speed was 1 mm/s and the probe moved down into the gel for a penetration depth of 20 mm. The curves of force (g) as a function of time (s) were recorded and characterized from two parameters: firmness (g) as maximum positive force peak during the compression and viscosity (g.s) as area under the curve of the return movement. All texture measurements were evaluated in triplicate.

2.11. Gel water-holding capacity

The water-holding capacity (WHC) of gels was determined according to Maltais, Remondetto, Gonzalez, & Subirade (2005) with minor modifications. Gels were prepared into 38 mL round centrifuge tubes (3117-0380, Thermo scientific, USA) from dispersions with addition of 2 % (w/w) GDL. Samples were incubated at 25 °C for 24 h for gelation, then stored at 4 °C for 24 h before being centrifuged at 5000 × g for 5 min at 20 °C (ultracentrifuge Optima LE-80k, rotor 70 Ti, Beckman, California, USA). Gels were weighed before and after centrifugation and WHC was calculated according to Eq (3):

$$WHC\ (\%) = (1 - Wr/Wt) * 100 \qquad (3)$$

where Wt is the total mass (g) of water in the sample and Wr is the mass (g) of water released by centrifugation from gels. Measurements were carried out in triplicate.

2.12. Statistical analysis

Results were expressed as the mean ± standard deviation. Statistical analyses of experimental data were performed by analysis of variance (ANOVA). Significant ($p < 0.05$) differences between samples were determined by Tukey's tests using the XLSTAT v2020 software. Linear regressions and related statistical parameters were obtained by Excel® software (2020.5.1.1040).

3. Results and discussion

3.1. Effect of the heat treatment protocols on single proteins and protein mixtures properties

The effect of the different heat treatment protocols was investigated on (i) the three individual protein dispersions (first step of protocol 2) and (ii) CM-PP and CM-WP mixtures (protocol 1 and second step of protocol 2) focusing on the size of the assemblies created and the type of interactions involved.

3.1.1. Effect of the heat treatment on single proteins

The size distribution curve in light intensity of soluble PP fraction obtained from the commercial

isolate showed a bimodal distribution characterized by a first population with a maximum located at 220 nm (peak 1) and a second one at 34 nm (Fig. 2a; Table 2). The size distribution curve in number frequency confirmed that the largest number of particles was characterized by a mean diameter of 28.8 ± 2.4 (Fig. 2b; Table 2). This indicated that the soluble PP from the commercial isolate are aggregated since the size of native legumin and vicilin oligomers was expected ~ 15 nm (Chihi, Mession, Sok, & Saurel, 2016). This aggregation phenomenon was confirmed from the native PAGE electrophoretic profile of the soluble PP fraction since a very intense band was present at the top of the stacking gel and also a strong one at the borderline between the stacking and separating gels (Fig. 3a – lane 1). These two bands revealed the presence of large protein complexes in the soluble PP dispersion with a molecular weight higher than 200 kDa as estimated compared to the molecular weight markers (Fig. 3a – lane M5). Chao & Aluko (2018) also observed by native PAGE similar pea protein aggregates in a commercial pea protein obtained by isoelectric pH precipitation. These constituents corresponded most probably to soluble pea protein aggregates, called native aggregates, generated during the industrial process since the pea protein isolate used in this study was obtained by isoelectric precipitation, purification then drying in a multi-stage spray dryer according to the supplier. The aggregation of globulins on the same commercial isolate was also observed by size exclusion chromatography with a single peak on the chromatogram indicating particle molecular weight of at least 2 700 kDa (Kornet et al., 2021). As SDS induced the dissociation of aggregates formed by non-covalent bonds, the SDS-PAGE pattern in absence of MSH of soluble pea proteins (Fig. 3b – lane 1) showed the different pea globulins bands: convicilin (72 kDa), legumin Lαβ (60 kDa) and four vicilin subunits (17, 30, 33, 50 kDa). However, some protein constituents did not enter in the stacking gel corresponding to aggregates not dissociated by SDS. These aggregates can be formed by intermolecular disulfide bonds involving cysteine thiol groups of legumin and convicilin. They were however not constituted with vicilin since this pea protein had not cysteine in its amino-acids sequence and consequently cannot be involved in intramolecular or intermolecular disulfide bonds leading the formation of such aggregates (Barac et al., 2010). On SDS-PAGE pattern in presence of MSH (Fig. 3c – lane 1), legumin was dissociated into legumin acidic subunit (Lα 38-40 kDa) and basic subunit (Lβ 19-22 kDa) linked by disulfide bond (Lam et al., 2018). Besides, no band of polypeptides were presented at the top of the stacking gel allowing to conclude that a part of native aggregates was associated via covalent bound. The heat treatment (85 °C for 1 h – step 1 protocol 2) of the soluble pea protein dispersion did not modify the size distribution curve profiles (Fig. 2c, d) and no difference was observed on the electrophoretic patterns between control (PP) and heated (PP HT) pea protein irrespective of the type of electrophoresis (Fig. 3a, b, c – lane 2). However, this processing induced a significant ($p < 0.05$) decrease in the mean diameter calculated from the distribution curves in intensity or in number frequency (Table 2). Besides, the surface thiols content

(Fig. 4a) and the hydrophobicity (Fig. 5) of PP was significantly ($p < 0.05$) increased by the heat treatment. These different changes suggested that the heat treatment of native pea aggregates has probably reinforced weak interactions between pea proteins involved in these aggregates.

The size distribution curve in intensity of untreated WP dispersion showed four different populations (Fig. 2a). The first one characterized by a maximum located at 3 nm corresponded to individual globular proteins such as α-La and β-Lg, the second one (14 nm) can be attributed to β-Lg dimers, the third one (72 nm) corresponded to BSA and immuno-globulins assemblies and the fourth one (462 nm) was probably due to some large aggregates (Fig. 2a, Table 2). The size distribution curve in number of WP displayed a bimodal population distribution with a mean diameter lower than 2 nm indicating that whey proteins from the isolate were mostly in their individual or dimeric form (Fig. 2b, Table 2). This was confirmed by the native (Fig. 3a line 7) and SDS-PAGE under non-reducing conditions (Fig. 3d line 7) electrophoresis patterns. The three major bands on SDS-PAGE profile at 13, 17 and > 160 kDa corresponded to α-lactalbumin, β-lactoglobulin and polypeptides subunits, respectively.

The heat treatment (step 1 of protocol 2) of WP dispersion induced the formation of large WP aggregates. The size distribution curves in light intensity and in number frequency (Fig. 2c, d) of WP HT displayed a monomodal distribution characterized by a mean diameter in number frequency of 40 nm (Table 2). The heated-induced aggregates were also observed from the native PAGE and SDS-PAGE electrophoretic profiles of WP HT in the form of a single intense band present at the top of the stacking gel (Fig. 3a line 8, Fig. 3d line 8). This band disappeared from the SDS-PAGE pattern in the presence of MSH (Fig. 3e line 8) demonstrating that WP heated-induced aggregates were created by disulfide bonds. As a result, the disulfide bond content of WP HT was significantly ($p < 0.05$) higher compared to that of untreated WP (Fig. 4b). The temperature applied during the heat treatment (85 °C), higher than the temperature of denaturation of α-lactalbumin, BSA and β-lactoglobulin (62 °C, 64 °C and 78 °C, respectively) induced the protein unfolding and the formation of intermolecular bonds, particularly disulfide bonds, leading to irreversible conformational and techno-functional changes to WP (De la Fuente, Singh, & Hemar, 2002). A significant ($p < 0.05$) increase in surface hydrophobicity of WP HT compared to WP was also observed due to the exposure of the hydrophobic clusters of unfolded proteins as a result of the heat treatment (Fig. 5).

The pre-heating (step 1 of protocol 2) of the soluble PP and WP led to protein aggregates characterized by specific physico-chemical properties. PP HT aggregates were twofold smaller compared to WP HT particles (Table 2) and the surface thiol groups of WP HT was significantly ($p < 0.05$) higher than those of the PP native aggregates and PP HT aggregates (Fig. 4a). Besides, although WP HT aggregates were characterized by a higher hydrophobicity compared to untreated WP, these aggregates remained less hydrophobic than PP HT particles.

Untreated and heat-treated (step 1 of protocol 2) casein micelles were characterized by a monomodal distribution in light intensity and in number frequency (Fig. 2a-d). A slight but significant decrease ($p < 0.05$) in the CM mean diameter from 285 ± 10 nm to 234 ± 4 nm (in light intensity) was noticed after the heat treatment (Table 2). This size reduction could be associated to an irreversible change of colloidal calcium phosphate and/or reassociation of κ-caseins on casein micellar surface (Le Ray, et al. 1998). In addition to the four casein fractions, α_{s1}- (23 kDa), α_{s2}- (25 kDa), β- (24 kDa), κ- (19 kDa) caseins, polypeptides of M_w in the range of 60 - 80 kDa were detected on SDS-PAGE profiles of CM and CM HT (Fig. 3b-e lanes 3-4) probably due to small amount of β-lactoglobulin dimers, lactoferrin, immunoglobulin G and bovine serum albumin, as already reported by Anema & Li (2000) and Beliciu & Moraru (2013).

3.1.2. Effect of heat treatments on CM-PP and CM-WP mixtures

The protocol 1 heat treatment was applied to the CM-PP and CM-WP mixtures prepared with untreated PP, WP and CM dispersions. The untreated CM-PP and CM-WP mixtures stood out particularly according to the size of the PP and WP soluble fractions mixed with CM. As previously described, PP fraction was composed of native aggregates formed by weak interactions and disulfide bonds while WP contained native individual whey proteins as particles with a diameter of a few nanometers. The comparison of the size distribution curves in light intensity of untreated CM-PP mixture (CM-PP) and CM-PP mixture treated with protocol 1 ((CM-PP) P1) showed a shift to lower size of the monomodal distribution resulting in a reduction of the mean diameter from 293 nm to 232 nm (Fig. 2e-f, Table 2). This size decrease induced by the heat treatment was also observed on the size distribution in number frequency with a reduction of the largest particles. The size distribution curves in light intensity and frequency number of (CM-PP) P1 mixture was finally very similar to those of the CM HT which resulted in not significantly different mean diameters for (CM-PP) P1 and CM HT (Fig. 2e-f, Table 2). No new bands were observed on non-reducing and reducing SDS-PAGE gels concerning (CM-PP) P1 (Fig. 3b-c lane 5), which indicated that no new complexes between pea protein and casein micelles were created. As (CM-PP) P1 mixture was characterized by a significantly ($p < 0.05$) higher hydrophobicity compared to untreated CM-PP mixture, it appeared that the protocol 1 generated an interactive potential via higher hydrophobic interactions that could be interesting in the gel process. The impact of the protocol 2 on the characteristics of the CM-PP mixture led to obtain particles larger than particles of CM HT and (CM-PP) P1 mixture (Fig. 2e-f, Table 2). The surface thiol groups and disulfide bonds of (CM-PP) P2 mixture were not significantly more numerous compared to those of the untreated CM-PP mixture indicating that the protocol 2 did not induce phenomena involving thiol groups such as disulfide bonds (Fig. 5).

The same technological route (protocol 1) applied to native CM-WP mixture induced a significant ($p < 0.05$) increase of the particle mean diameter from 254 ± 6 nm to 313 ± 4 nm in light intensity (Fig. 2g, Table 2) in accordance with Anema & Li (2003). Regarding the electrophoretic pattern of (CM-WP) P1 under non-reducing conditions, the bands corresponding to β-lactoglobulin and α-lactalbumin disappeared whereas large polypeptides (M_w > 260 kDa) was observed (Fig. 3d line 9). When reducing conditions were applied on SDS-PAGE, the band pattern was similar to that of the original fractions (Fig. 3e lane 9). In the same time for (CM-WP) P1, a higher amount of disulfide bonds associated to lower free surface thiols was observed compared to the untreated CM-WP mixture (Fig. 4). These heat-induced changes were attributed to direct interactions of β-lactoglobulin with κ-casein but also the formation of soluble aggregates created mainly by interactions between β-lactoglobulin and α-lactalbumin which then interacted with casein micelles via disulfide bonds and also hydrophobic and ionic interactions (Corredig & Dalgleish, 1999). Concerning (CM-WP) P2, the first heat treatment applied to WP alone led to whey protein aggregates involving disulfide bonds which reduced the direct interactions of β-lactoglobulin with κ-casein on casein micelle surface during the second heating stage. Consequently, the mean size of (CM-WP) P2 was significantly lower than that of (CM-WP) P1.

3.2. Acid gelation of heat-treated protein mixtures

3.2.1. Rheological behavior of heat-treated protein mixtures

The rheological behavior of the different heat-treated protein mixtures was compared with each other and also with the individual protein dispersions (PP, WP and CM) to evaluate the effect of the protein mixtures composition and of the heat treatment protocols on the formation of acid-induced gels. The rheological parameters during acidification of the different samples and the viscoelastic properties of the protein gels at the gel point and after 5 h of acidification are presented in Fig. 6 and Table 3, respectively. For all samples, a rapid decrease in pH was observed during the first 20 min of acidification characterized by a similar initial rate of acidification (Supplementary Fig. S1). The gel point ($\tan \delta = 1$, indicative parameter of the sol-gel transition) of (CM-PP) P1 and (CM-PP) P2 mixtures was reached almost three times faster compared to acid-induced gelation of CM HT dispersion and approximatively 1.5 times compared to (CM-WP) P1 and (CM-WP) P2 mixtures (Table 3). This more rapid sol-gel transition in the case of the (CM-PP) P1 and (CM-PP) P2 is probably associated to the influence of the PP aggregates pI allowing a gel point pH higher of 0.5 unit compared to CM HT gel point. Even if no significant difference was observed between the gel point parameters of (CM-PP) P1 and (CM-PP) P2, the storage modulus of (CM-PP) P1 at the final acidification point was significantly ($p < 0.05$) higher compared to (CM-PP) P2 (Table 3). As

expected, the gel point time of (CM-WP) P1 and (CM-WP) P2 mixtures was observed significantly ($p < 0.05$) earlier compared to CM HT (Table 3) since acid-induced gelation begins at a higher pH (~ pH 5.4) due to the presence of denatured whey proteins, particularly β-lactoglobulin and whey protein/k-casein aggregates (Anema, Lee, Lowe, & Klostermeyer, 2004; Donato, Alexander, & Dalgleish, 2007; Nicolai, 2019) and to the higher hydrophobicity of WP HT aggregates as also reported by Morand, Guyomarc'h, Legland, & Famelart (2012). As in the case of CM and PP mixtures, no significant difference was noticed concerning the pH and G' at the gelation point between (CM-WP) P1 and (CM-WP) P2 mixtures but G' after 5 h of gelation was significantly higher for (CM-WP) P2 compared to (CM-WP) P1. This difference observed in the case of the two types of mixtures, CM with PP or WP, could be attributed to the difference in surface free thiol groups before acidification, a higher level of surface free thiol groups favoring a higher storage modulus at the final acidification point. The tan δ evolution according to pH after gel point for (CM-PP) P1 and (CM-PP) P2 was characterized by a shoulder around pH 5. Mession, Roustel, & Saurel (2017) showed that the acid-induced gelation of the heated CM-fractionated vicilin or unheated CM-PP aggregates mixtures led to a higher final storage modulus compared to single protein gels, whereas, the presence of large and sedimentable aggregates in the case of the CM-PP and CM-legumin mixtures impaired gelation. This work revealed a net reinforcement of the gel network after addition of PP to the CM and a significant higher G' after 5 hours of acidification compared to the CM-WP mixtures. These gels characteristics may be due to differences in the size, number of aggregates or combination of both. These significant differences were also related to the type, nature and strength of interactions governing the gel structure.

3.2.2. Texture and water holding capacity of acid gels

The texture profile (gel firmness and viscosity index) and water holding capacity (WHC) of the acid-induced gels obtained from (CM-PP) and (CM-WP) mixtures were investigated and compared to those of CM HT gels as control (Fig. 7). Uniform gels obtained from (CM-PP) mixtures were characterized by a significant higher firmness and viscosity index than those obtained with CM control or heat-treated (CM-WP) mixtures. However, the greatest strength of the CM-PP gel protein network did not result in an improving of the water holding capacity compared to control gels. This suggested that the interactions established during the acidification and gelation of the heat-treated (CM-PP) mixtures allowed the coexistence of two independent gels stabilized by low-energy interactions that did not promote water retention in gel network after the centrifugation test. In contrast, the gels formed with heat-treated (CM-WP) mixture were more homogeneous and more stable, with a better affinity to water.

Conclusion

Heat treatment of PP induced a decrease in particle size and participated in an increase in surface hydrophobicity and thiols through the exposure of hydrophobic groups and sulfur-containing amino acid residues naturally present in native aggregates. On the other hand, heat treatment of the WP revealed an increase in particle size and surface hydrophobicity compared to the native proteins. These large aggregates were enhanced by the establishment of covalent bonds.

For the (CM-PP) Protocol 1 mixture, the application of heat treatment did not change the size of the micelles, presuming an absence of interaction between soluble pea proteins and micelles. In contrast, the same treatment applied to a mixture of pretreated proteins (CM-PP) Protocol 2 caused an increase in micelle size that can be justified by an increase in the surface hydrophobicity of the heat-treated pea protein aggregates that could interact with the casein micelles through hydrophobic bonds.

For both WP and CM mixtures, and as demonstrated by SDS-PAGE and size measurements, both protocols induced the association of these proteins through the establishment of hydrophobic and covalent interactions with κ-casein on the surface.

The properties of the casein gels obtained by acidification were strongly affected by the type of the soluble proteins (WP or PP) and their aggregated forms. Stiffer and firmer gels with faster gel setting times were obtained for PP and CM mixture, which seems to demonstrate that PP interacts with each other first to form a gel that is independent of the CM gel created at lower pH. These PP gels would likely be trapped in the milk gel, thus acting as a "space-filling gel" in contrast to the mixed WP and CM gels that are formed with complexes of these two proteins stabilized by covalent interactions. Thus, these results showed that pea proteins are interesting functional ingredients for food product formulations.

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Figure captions

Figure 1: Schema of the two protocols carried out to study the association of soluble pea (PP) or whey (WP) proteins to casein micelles (CM). Protocol 1: mixing of PP or WP with CM then mixture heating (85 °C for 1h). Protocol 2: pre-heating (85 °C for 1h) of PP, WP, CM dispersions separately before mixing PP or WP with CM then mixture heating (85 °C for 1h). For each protocol, the CM:PP or CM:WP protein ratio was 7.5:2.5 with a total protein content of 4% (w/w).

Figure 2: Particle size distribution curves in light intensity (a, c, e, g) or in particle number frequency

(b, d, f, h) of (a, b) untreated dispersions (PP: soluble pea protein dispersed in ultrapure water, WP: whey protein dispersed in ultrapure water, CM: casein micelle dispersed in SMUF at pH 6.7), (c, d) pre-heated dispersions (PP HT: pre-heated soluble pea proteins, WP HT: pre-heated whey proteins, CM HT: pre-heated casein micelles), (e, f) untreated and heated casein micelle-pea protein mixtures at 4% (w/w) total protein concentration and 7.5:2.5 protein ratio (CM-PP: untreated casein micelle-pea protein mixture, (CM-PP) P1: casein micelle-pea protein mixture protocol 1, (CM-PP) P2: casein micelle-pea protein mixture protocol 2) and (g, h) untreated and heated casein micelle-whey protein mixtures at 4% (w/w) total protein concentration and 7.5:2.5 protein ratio (CM-WP: untreated casein micelle-whey protein mixture, (CM-WP) P1: casein micelle-whey protein mixture protocol 1, (CM-WP) P2: casein micelle-whey protein mixture protocol 2). Protocol 1: mixing of PP or WP with CM then heating of the mixture (85 °C for 1h). Protocol 2: pre-heating (85 °C for 1h) of PP, WP, CM dispersions separately before mixing PP or WP with CM then heating of the mixture (85 °C for 1h). Measurements were carried out by photon correlation spectroscopy (PCS) at 25 °C. Mean curves were calculated from quadruplicate PCS measurements.

Figure 3: Electrophoretic patterns of (a) native PAGE, (b, d) SDS-PAGE in non-reducing condition and (c, e) SDS-PAGE in reducing condition of control and pre-heated PP (PP HT), WP (WP HT), CM (CM HT) dispersions (step 1 protocol 2) and mixtures of PP or WP with CM (2.5:7.5 protein ratio with 4 % (w/w) total protein concentration) treated according to protocol 1 ((CM-PP) P1 ; (CM-WP) P1) or protocol 2 ((CM-PP) P2 ; (CM-WP) P2). Control PP (lane 1), PP HT (lane 2), control CM (lane 3), CM HT (lane 4), (CM-PP) P1 (lane 5), (CM-PP) P2 (lane 6), control WP (lane 7), WP HT (lane 8), (CM-WP) P1 (lane 9), (CM-WP) P2 (lane 10). Makers for native PAGE: alpha-lactalbumin (lane M1), carbonic anhydrase (lane M2), albumin chicken egg white (lane M3), bovine serum albumin (lane M4), urease from jack bean (lane M5). Markers for SDS-PAGE (lane M): 12 protein bands ranging in molecular weight from 3.5 - 260 kDa. Cv: convicilin. Lαβ: legumin subunit, Lα and Lβ: acidic and basic legumin polypeptides, V: vicilin polypeptides, BSA: bovine serum albumin, α_{s1}, α_{s2}, β, κ: caseins, β-Lg: β -lactoglobulin, α-La: α -lactalbumin.

Figure 4: Surface thiol groups (a) and disulfide bonds (b) of untreated pea protein (PP), whey protein (WP) and casein micelle (CM), heated (85 °C for 1 h) pea protein (PP HT), whey protein (WP HT) and casein micelle (CM HT) and mixtures CM-PP and CM-WP treated by the two heat treatment protocols: protocol 1 (P1) and protocol 2 (P2). Means of triplicate plus standard deviation are shown. Bars with different letters are significantly different ($p < 0.05$).

Figure 5: Surface hydrophobicity of untreated pea protein (PP), whey protein (WP) and casein

micelle (CM), heated (85 °C for 1 h) pea protein (PP HT), whey protein (WP HT) and casein micelle (CM HT) and mixtures CM-PP and CM-WP treated by the two heat treatment protocols: protocol 1 (P1) and protocol 2 (P2). Bars with different letter significantly differ ($p < 0.05$). Means were of triplicate determinations.

Figure 6: Evolution of tan δ (G″/ G′) as a function of pH during acidification of the different samples. Acidification carried out with 2 % (w/w) GDL for 5 h at 25 °C.

Figure 7: Water holding capacity (a), gel firmness (b) and gel viscosity index (c) of acid-induced gels from heat treated casein micelle (CM HT), casein micelle-pea protein mixture (CM-PP) and casein micelle-whey protein mixture (CM-WP) treated by the two heat treatment protocols: protocol 1 (P1) and protocol 2 (P2). Acidification carried out with 2 % (w/w) GDL for 5 h at 25 °C. Bars with different letter significantly differ ($p < 0.05$).

Figure 1

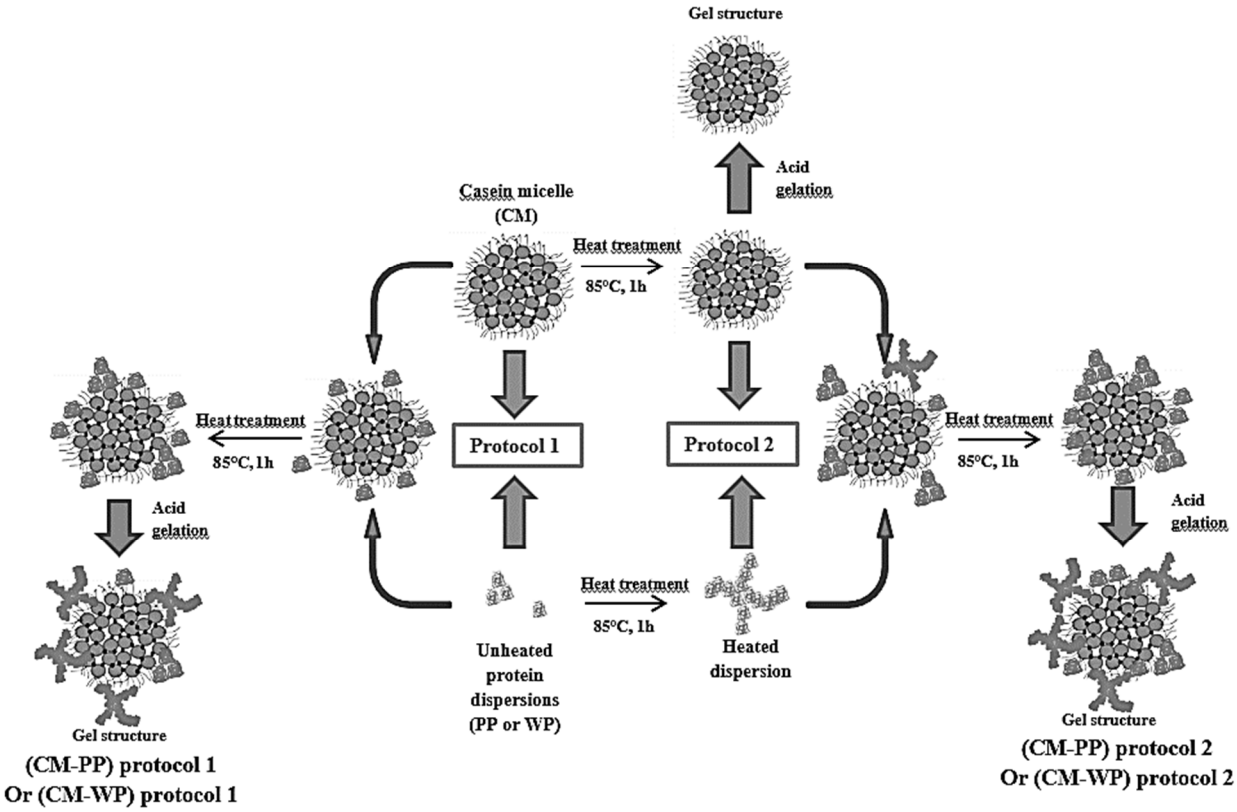
Gel structure
Acid gelation
Casein micelle (CM)
Heat treatment
85°C, 1h
Protocol 1
Protocol 2
Heat treatment
85°C, 1h
Heat treatment
85°C, 1h
Acid gelation
Acid gelation
Heat treatment
85°C, 1h
Unheated protein dispersions (PP or WP)
Heated dispersion
Gel structure
(CM-PP) protocol 1
Or (CM-WP) protocol 1
Gel structure
(CM-PP) protocol 2
Or (CM-WP) protocol 2

Figure 2

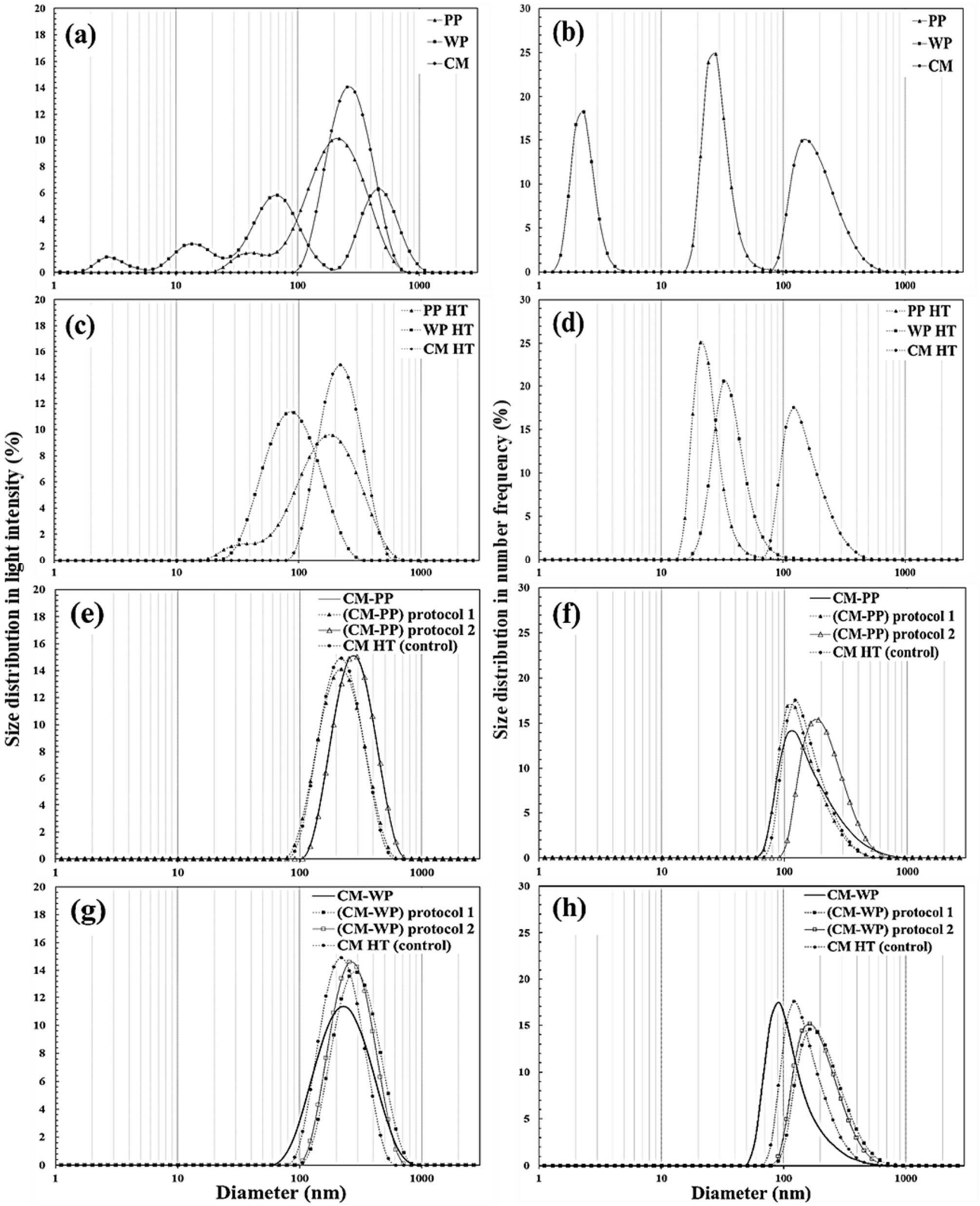
(a)
PP
WP
CM
(b)
PP
WP
CM
(c)
PP HT
WP HT
CM HT
(d)
PP HT
WP HT
CM HT
(e)
CM-PP
(CM-PP) protocol 1
(CM-PP) protocol 2
CM HT (control)
(f)
CM-PP
(CM-PP) protocol 1
(CM-PP) protocol 2
CM HT (control)
(g)
CM-WP
(CM-WP) protocol 1
(CM-WP) protocol 2
CM HT (control)
(h)
CM-WP
(CM-WP) protocol 1
(CM-WP) protocol 2
CM HT (control)
Size distribution in light intensity (%)
Size distribution in number frequency (%)
Diameter (nm)
Diameter (nm)

Figure 3

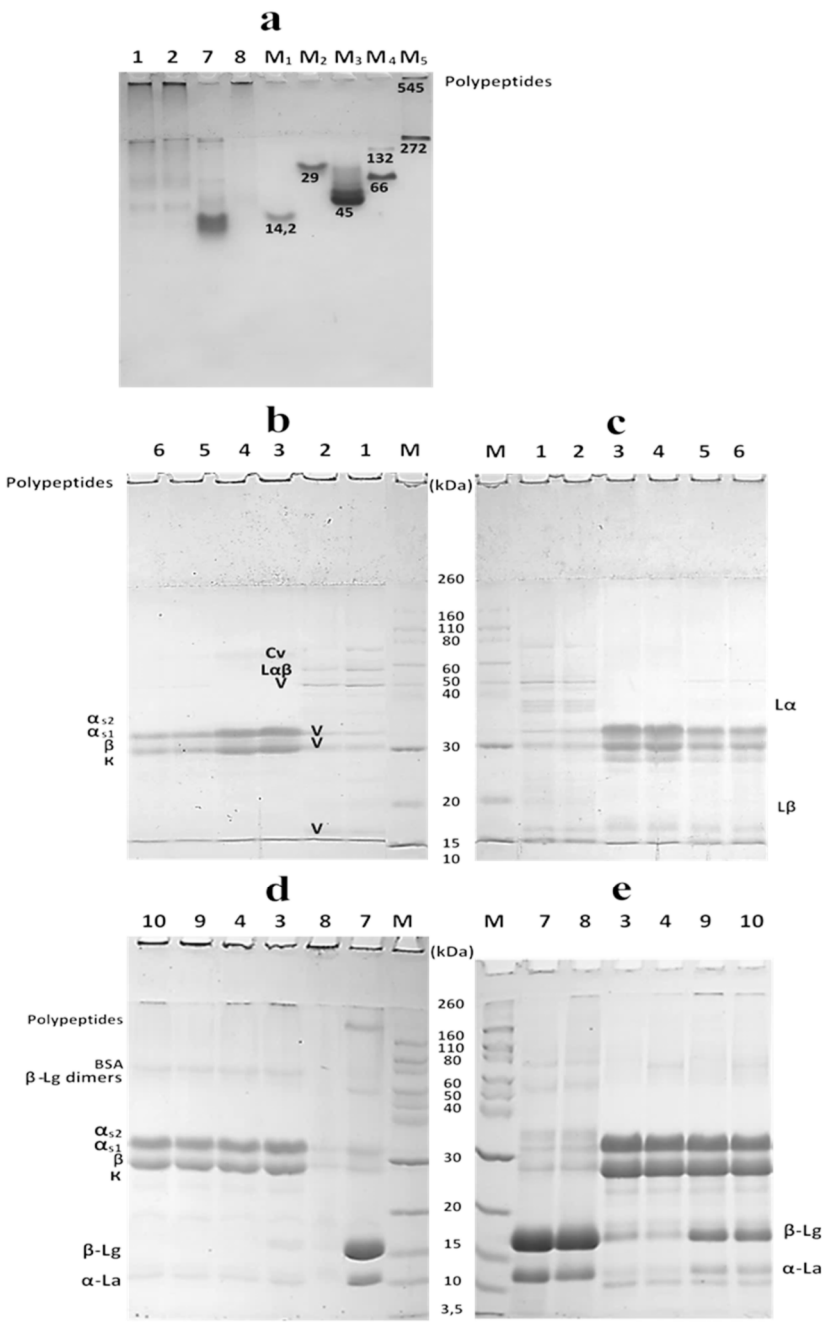
a
1 2 7 8 M1 M2 M3 M4 M5
Polypeptides
545
272
132
66
29
45
14,2
b
6 5 4 3 2 1 M
Polypeptides
(kDa)
Cv
Lαβ
V
αs2
αs1
β
κ
V
V
V
260
160
110
80
60
50
40
30
20
15
10
c
M 1 2 3 4 5 6
Lα
Lβ
d
10 9 4 3 8 7 M
(kDa)
Polypeptides
BSA
β-Lg dimers
αs2
αs1
β
κ
β-Lg
α-La
260
160
110
80
60
50
40
30
20
15
10
3,5
e
M 7 8 3 4 9 10
β-Lg
α-La

Figure 4

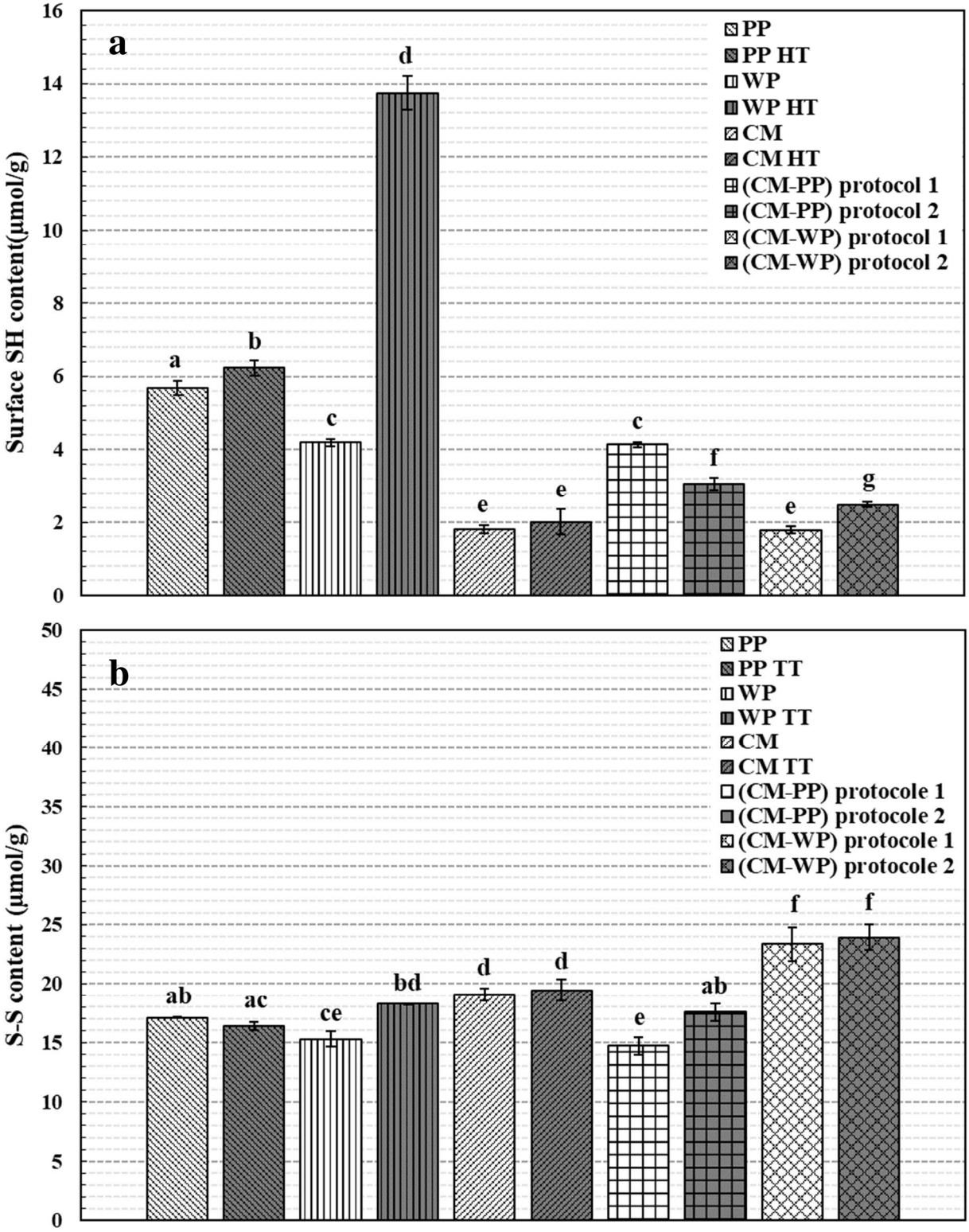
a
Surface SH content(µmol/g)
0
2
4
6
8
10
12
14
16
PP
PP HT
WP
WP HT
CM
CM HT
(CM-PP) protocol 1
(CM-PP) protocol 2
(CM-WP) protocol 1
(CM-WP) protocol 2
a
b
c
d
e
e
c
f
e
g
b
S-S content (µmol/g)
0
5
10
15
20
25
30
35
40
45
50
PP
PP TT
WP
WP TT
CM
CM TT
(CM-PP) protocole 1
(CM-PP) protocole 2
(CM-WP) protocole 1
(CM-WP) protocole 2
ab
ac
ce
bd
d
d
e
ab
f
f

Figure 5

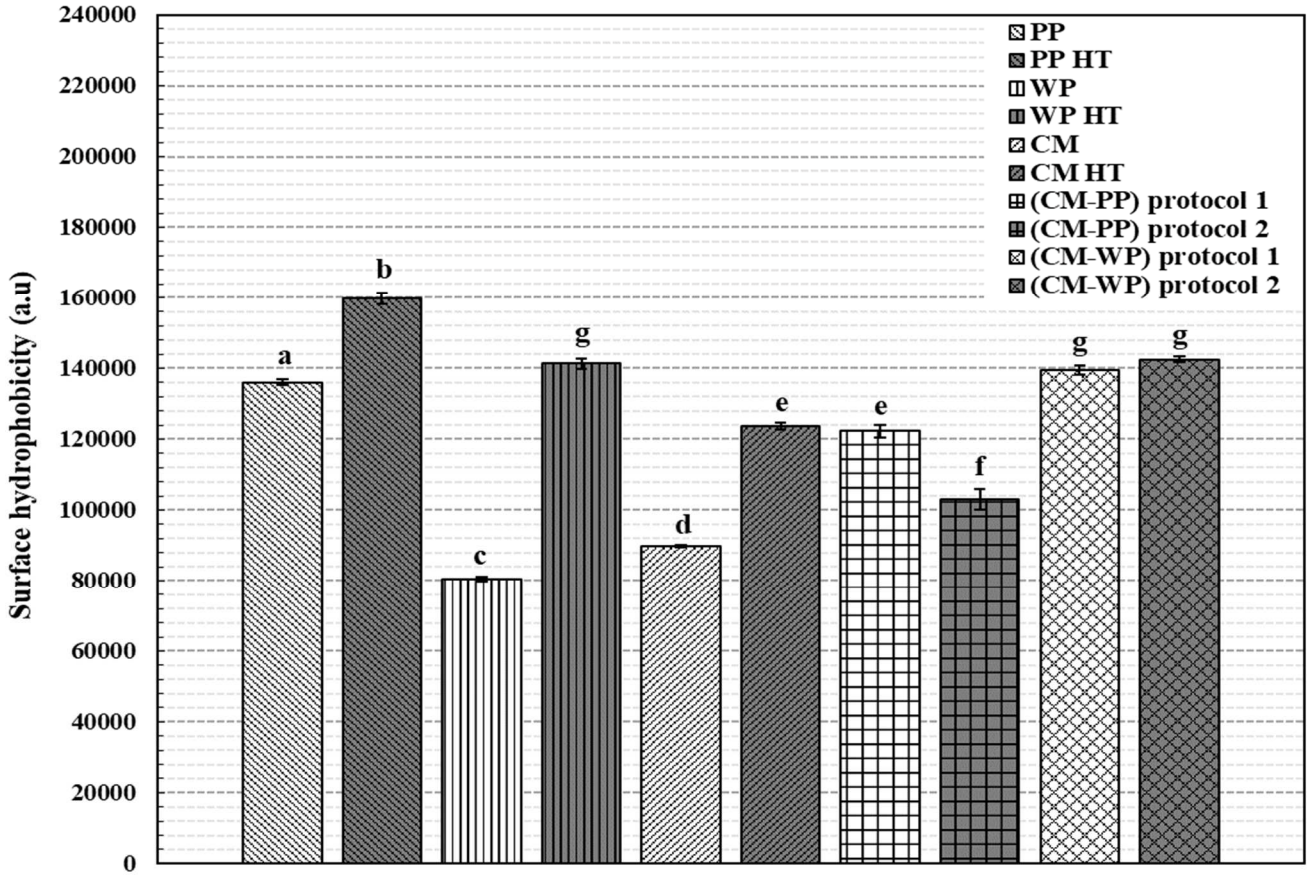
Surface hydrophobicity (a.u)
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
a
b
c
g
d
e
e
f
g
g
PP
PP HT
WP
WP HT
CM
CM HT
(CM-PP) protocol 1
(CM-PP) protocol 2
(CM-WP) protocol 1
(CM-WP) protocol 2

Figure 6

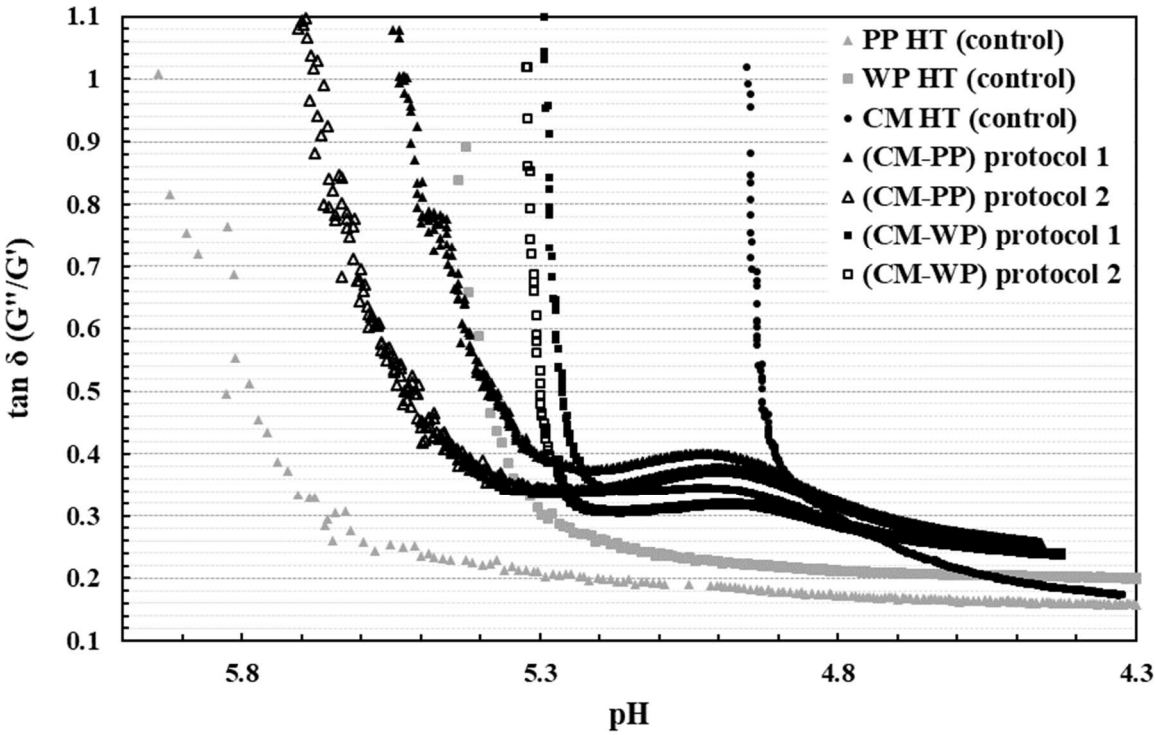
tan δ (G''/G')
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
5.8
5.3
4.8
4.3
pH
PP HT (control)
WP HT (control)
CM HT (control)
(CM-PP) protocol 1
(CM-PP) protocol 2
(CM-WP) protocol 1
(CM-WP) protocol 2

Figure 7

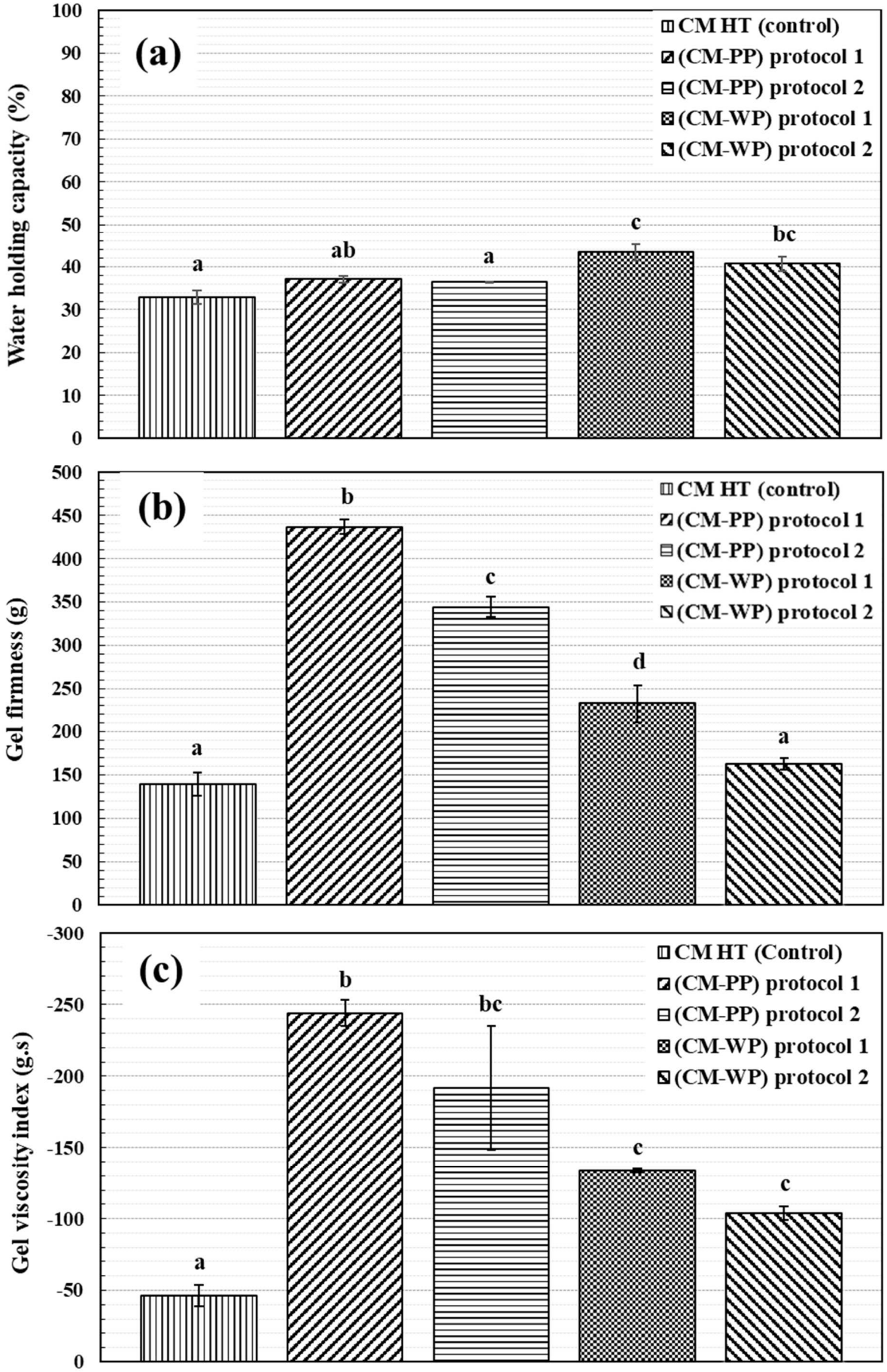
(a)
Water holding capacity (%)
100
90
80
70
60
50
40
30
20
10
0
a
ab
a
c
bc
CM HT (control)
(CM-PP) protocol 1
(CM-PP) protocol 2
(CM-WP) protocol 1
(CM-WP) protocol 2
(b)
Gel firmness (g)
500
450
400
350
300
250
200
150
100
50
0
a
b
c
d
a
CM HT (control)
(CM-PP) protocol 1
(CM-PP) protocol 2
(CM-WP) protocol 1
(CM-WP) protocol 2
(c)
Gel viscosity index (g.s)
-300
-250
-200
-150
-100
-50
0
a
b
bc
c
c
CM HT (Control)
(CM-PP) protocol 1
(CM-PP) protocol 2
(CM-WP) protocol 1
(CM-WP) protocol 2

Table 1

Proximate composition of commercial pea isolate (PPI), whey protein isolate (WPI) and native phosphocasein (PCN) powder.

Chemical composition	Pea protein isolate	Whey protein isolate	Native phosphocasein
Moisture (%)	6.10 ± 0.17[a]	6.19 ± 0.15[a]	6.55 ± 0.15[b]
Ash (% DB)	3.35 ± 0.83[a]	1.73 ± 0.07[b]	7.89 ± 0.07[c]
Protein (% DB)	83.23 ± 0.84[a]	93.97 ± 0.22[b]	83.16 ± 0.26[a]
Fat (% DB)*	0.16 ± 0.01[a]	1.85 ± 0.03[b]	1.95 ± 0.02[c]
Carbohydrate (% DB)**	13.27 ± 0.08[a]	2.45 ± 0.13[b]	7.00 ± 0.28[c]

Results expressed as mean ± standard deviation (n=3).

*: Supplier data.

**: Calculated by differences.

DB: Dry basis.

[a-c] Values followed by different letters within a line are significantly different ($p < 0.05$).

Table 2

Particle size characteristics of untreated pea protein (PP), whey protein (WP) and casein micelle (CM) dispersions, heated (85 °C for 1 h) pea protein (PP HT), whey protein (WP HT) and casein micelle (CM HT) dispersions, the untreated mixtures (CM-PP and CM-WP) and the mixtures obtained by the two heat treatment protocols: protocol 1 (P1) and protocol 2 (P2).

	Distribution in light intensity				Distribution in number frequency			
	Mean diameter (nm)	Minimal diameter (nm)	Value of the peak maximum (nm)	Maximal diameter (nm)	Mean diameter (nm)	Minimal diameter (nm)	Value of the peak maximum (nm)	Maximal diameter (nm)
PP	214 ± 3							
Peak 1		23	34 ± 3	59	29 ± 2	19	26 ± 2	111
Peak 2		59	220 ± 0	716	-	-	-	-
PP HT	167 ± 9							
Peak 1		19	30 ± 3	44	22 ± 2	16	21 ± 0	91
Peak 2		44	164 ± 0	615	-	-	-	-
WP	216 ± 18							
Peak 1		1.5	3 ± 0	5	2 ± 1	0.5	1 ± 0	1
Peak 2		5	14 ± 2	27	2 ± 1	1	2 ± 0	4
Peak 3		27	73 ± 17	151	-	-	-	-
Peak 4		151	462 ± 68	925	-	-	-	-
WP HT	98 ± 2	24	87 ± 7	295	40 ± 4	23	36 ± 5	135
CM	285 ± 10	106	255 ± 0	664	200 ± 2	91	153 ± 16	664
CM HT	234 ± 4	91	220 ± 0	531	159 ± 5	79	122 ± 0	507
CM-PP	294 ± 4	122	342 ± 0	647	173 ± 18	72	111 ± 10	868
(CM-PP)P1	232 ± 4	87	220 ± 0	531	151 ± 2	75	117 ± 10	531
(CM-PP)P2	298 ± 5	122	295 ± 0	680	221 ± 0	101	190 ± 0	680
CM-WP	254 ± 6	68	220 ± 0	787	123 ± 6	56	91 ± 0	615
(CM-WP)P1	313 ± 4	111	295 ± 0	787	219 ± 2	91	164 ± 0	787
(CM-WP)P2	287 ± 5	106	255 ± 0	712	203 ± 3	91	164 ± 0	647

Results expressed as mean ± standard deviation (n=3).

Values were calculated from the size distribution curves in light intensity and in number frequency as determined by photon correlation spectroscopy at 25 °C.

Table 3

Rheological parameters of acid-induced gelation (2 % (w/w) GDL at 25 °C) of heated casein micelle dispersion (CM HT, 3 %, (w/w)), (CM-PP) and (CM-WP) mixtures (4 % (w/w) total protein and 7.5:2.5 protein ratio) heat-treated according to the protocol 1 (P1) and protocol 2 (P2).

		CM HT	(CM-PP) P1	(CM-PP) P2	(CM-WP) P1	(CM-WP) P2
Gel point	Time (s)	5290 ± 131^{a}	1790 ± 85^{b}	1900 ± 82^{b}	3358 ± 46^{c}	2765 ± 26^{d}
	pH	4.94 ± 0.03^{a}	5.53 ± 0.05^{b}	5.46 ± 0.06^{b}	5.29 ± 0.06^{c}	5.32 ± 0.03^{c}
	G' (Pa)	0.27 ± 0.04^{a}	0.20 ± 0.01^{b}	0.21 ± 0.01^{b}	0.31 ± 0.02^{a}	0.30 ± 0.04^{a}
Final point (5 h of acidification)	pH	4.33 ± 0.08^{a}	4.47 ± 0.02^{b}	4.46 ± 0.05^{b}	4.43 ± 0.09^{ab}	4.43 ± 0.02^{ab}
	G' (Pa)	160 ± 7^{a}	726 ± 11^{b}	622 ± 60^{c}	429 ± 11^{d}	516 ± 16^{e}

Results expressed as mean ± standard deviation (n=3).

$^{(a\text{-}e)}$ Values followed by different letters within a line are significantly different ($p < 0.05$).

Graphical abstract