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Effect of Superfine Grinding on the Structural and Physicochemical Properties of Whey Protein and Applications for Microparticulated Proteins

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Abstract Effects of superfine grinding on structural and physicochemical properties of whey protein concentrate (WPC) and applications for microparticulated whey protein (MWP) were investigated. WPC and MWP particle sizes significantly (p<0.05) decreased after superfine grinding from 62 and 15 μm to 15 and 9 μm, respectively, compared with controls. A basis for exploration of WPC applications as a fat replacer because of WPC and MWP particle size reductions due to superfine grinding is established. The effect of superfine grinding on molecular configuration, thermal stability, and physicochemical properties of WPC were also investigated. Amounts of free sulfhydryl groups decreased significantly (p<0.05) from 18.16 to 14.49 μmol/L, and thermodynamic properties, including transition temperature, were changed after superfine grinding. The whey protein solubility, protein surface hydrophobicity, oil binding capacity, foaming capacity, and foaming stability of WPC were all improved after superfine grinding.

Keywords: whey protein concentrate, superfine grinding, physicochemical propertie, structural propertie, microparticulated whey protein

Introduction

Whey proteins are widely used in food formulations to provide high nutritional quality and unique functional properties, and are applied in the pharmaceutical and biomedical fields due to potent biological activities (1). Important commercial whey protein products are whey protein concentrates (WPCs) with up to 85% protein. WPCs have been used as nutritional and functional ingredients in different food products due to low fat and lactose contents and high protein levels (2).

There is growing demand for low-fat products in the dairy industry. In order to improve the sensory properties and nutritional value of dairy products (3), microparticulated whey protein (MWP) has been used as a replacement for fat in ice cream (4), yogurt (5), and cheeses (6). In addition to enhanced heat stability, MWP exhibited functionalities different from native whey proteins, such as improved emulsifying activities and gelling properties (7). MWP consists of small 0.1-10.0 μm spheroidal particles after shearing of coagulating proteins. This diameter is too small for the human tongue to distinguish as individual particles. Thus, MWP is perceived as a creamy, smooth fluid (8).

Production of MWP involves application of a heat treatment and a high-shearing technology simultaneously to whey proteins (8). The equipment used to accomplish the synchro function of heat and high-shearing requires scrupulous design and customization, leading to an increase in production costs, but good product quality with a particle size of 0.5-3.0 μm (9). In addition, MWP can also be obtained from whey protein based on successive thermocoagulation and microparticulation (10). Sizes of whey protein particles produced in this way are difficult to reduce to the size of homogenized milk fat globules (0.5-3.0 μm). Alternative processes can be used instead, such as extrusion cooking under an acid pH or dynamic high pressure shearing, known as microfluidization (5). MWP is generally produced using these methods at low whey protein (WP) concentrations of (2-6%).

Superfine grinding technology has shown potential for production of nutraceuticals and functional foods (11). Some studies reported that superfine powders exhibited higher fluidity and protein solubility values, electric conductivity. and water holding capacities (12) and, thus, the quality of food products produced using superfine grinding was improved (13). Nevertheless, little information is available regarding the structural and physicochemical properties of superfine grinding-treated WPC-80 (whey protein concentrate containing 80% protein) (sWPC-80) and associated microparticulated protein particles (sMWP).

The objective of this study was to investigate the effect of

superfine grinding on the particle size and morphology of a whey protein concentrate (WPC and sWPC) and associated microparticulated protein particles (MWP and sMWP). The molecular configuration (protein distribution, conformation, and free sulfhydryl and disulfide linkage group contents), thermal stability and physicochemical properties (solubility, surface hydrophobicity index, water holding, and oil binding capacity, emulsification properties, and foaming properties) of WPC and sWPC were also determined to provide a basis for exploration of applications as food ingredients.

Materials and Methods

Materials Commercial WPC-80 (whey protein concentrate containing 80% protein) was purchased from Fonterra Commercial Trading Co., Ltd. (Shanghai, China). The composition of WPC-80 was 80.16% protein (dry basis), 3.60% lactose monohydrate, 3.28% starch, 3.87% fat, 5.09% moisture, and less than 4.00% ash. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) and 8-anilino-1-naphthalene sulfonic acid (ANS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Commercial soybean oil was purchased from a local market in Tianjin City, China. All other chemicals used were of analytical grade.

Superfine grinding treatment of whey protein Dried WPC-80 powder was ball-milled in a CJM-SY-B type high-energy nano-mill (Taiji Ring Nano Products Co., Ltd., Qinhuangdao, China) for 8 h to obtain sWPC-80 powder (14).

Microparticulation of whey protein A 0.12 g/mL WPC-80 and a 0.12 g/mL sWPC-80 solution were prepared based on dispersal of powder in deionised water with adjustment of the pH to 4.5 using 0.1 M HCl. After heating in a XMTD-204 water bath kettle (Tianjin Honour Instrument Co., Ltd., Tianjin, China) at 85°C for 35 min, MWP and sMWP dispersions were obtained after homogenization in an Ultra-turrax T18 High-Speed Homogenizer (IKA, Staufen, Germany) for 5 min at 10,000 rpm.

Particle size distribution Particle size distributions of WPC-80 and sWPC-80 were determined using a BT-2001 Laser analyzer (Dandong Bettersize Instruments Ltd., Dandong, China) and air was used as a dispersion medium. Particle size distributions of MWP and sMWP dispersions were determined using a BT-9300S Laser analyzer (Dandong Bettersize Instruments Ltd.) with ultrapure water used as a dispersion medium. Laser analyzers function on the principle of dynamic light scattering.

Scanning electron microscopy Samples of the proteins were prepared for scanning electron microscopy (SEM) based on mounting onto a copper holder and coating with 10 nm of sputtered gold using a sputter-coater (Hummer XP, Anatech, CA, USA). SEM images were obtained using a Hitachi SU-1510 SEM (Hitachi, Tokyo, Japan) at an acceleration voltage of 10 kV.

Structural characterization

***Gel electrophoresis*:** SDS-PAGE was carried out for visualization of the protein distribution in WPC-80 and sWPC-80 samples according to the method of Chicón *et al*. (15) with slight modification. The protein distribution was identified using a pre-stained protein maker (Sigma-Aldrich). Aliquots (10 μL) of WPC samples and Mw markers were loaded on hand-cast 12% separating gel (pH 8.8) and 5% stacking gel (pH 6.8) followed by electrophoresis using a Mini-Protean II electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) at 200 V for 60-80 min, then stained using 0.1% Coomassie Brilliant Blue (R-250) for 15 min, followed by de-staining as outlined by Laemmli (16).

Circular dichroism spectra Circular dichroism (CD) experiments were performed using a Jasco J-810 CD spectrophotometer (Jasco, Tokyo, Japan) equipped with a temperature-controlled holder (Jasco). An average of 3 scans was recorded at 200 nm/min. WPC-80 and sWPC-80 spectra were obtained at 25°C using a protein concentration of 2 mg/mL in a 10 mM phosphate buffer (pH 7.0). The path length of the cylindrical cuvette used for analysis was 0.2 cm. The baseline spectrum of the buffer was subtracted from the spectrum of each WPC sample and CD spectra were recorded based on residue ellipticity (deg·cm^2/dmol). Percentages of the protein secondary structure (α-helix, β-sheet, β-turn, and random coil) were calculated using spectra software (17).

Free sulfhydryl and total sulfhydryl and disulfide linkage group contents Free sulfhydryl (SH_F) and total sulfhydryl (SH_T) and disulfide linkage group (SS) contents were determined according to Janatova *et al.* (18) using DTNB, also known as Ellman's reagent. The absorbance at 412 nm (A_{412}) was measured by TU-1810 Ultraviolet spectrophotometer (Persee General Instrument Co., Ltd., Beijing, China) and the SH_F content was calculated as:

$$SH_F\ (\mu mol/L)=\frac{73.53\times A_{412}\times D}{C} \quad (1)$$

where D is the dilution factor=3.02 and C is the protein concentration =5 mg/mL.

For determination of SH_T and SS contents, 2 mL of Tris buffer (pH 8.0) containing 480 mg/mL of urea and 0.02 mL of β-mercaptoethanol were added to 1 mL of a WPC sample (5 mg/mL) and kept at 25°C for 1 h. Then, 10 mL of 12% trichloroacetic acid was added and kept again at 25°C for 1 h. Subsequently, WPC samples were separated using a TDZ5-WS centrifuge (Xiangyi Centrifuge Instrument Co., Ltd., Changsha, China) at 3,000×*g* for 15 min, then dissolved in 6 mL of Tris buffer, followed by addition of 0.04 mL of Ellman's reagent. The absorbance at 412 nm (A_{412}) was measured by TU-1810 Ultraviolet spectrophotometer (Persee General Instrument Co., Ltd.) and SH_T and SS contents were calculated as:

$$SH_T\ (\mu mol/L)=\frac{73.53\times A_{412}\times D}{C} \quad (2)$$

$$SS\ (\mu mol/L)=\frac{SH_T-SH_F}{2} \quad (3)$$

where D is the dilution factor=6.04 and C is the protein concentration =5 mg/mL.

Thermal stability Differential scanning calorimetry analysis using a DSC-60A instrument (Shimadzu Co., Ltd., Kyoto, Japan) was conducted to determine temperatures of denaturation and enthalpy of WPC-80 and sWPC-80 samples, which were allowed to equilibrate for 24 h at 35°C in an electric blast drying oven prior to measurement. Temperature scans were conducted from 20 to 150°C at a heating rate of 5°C/min. Thermogravimetric (TG) measurement was carried out on a Shimadzu TGA-50 thermogravimetric analyzer. Non-isothermal experiments were performed in a temperature range 25-600°C with heating rates of 10°C/min for each sample. The average sample size was 5 mg and the flow rate of nitrogen was 50 cm^3/min.

Physicochemical properties

***Protein solubility*:** Protein solubility was determined following the method of BS ISO15323:2002 (19). As an index of solubility, the Nitrogen Solubility Index (NSI) (19) was calculated as:

$$NSI\ (\%)=\frac{\text{protein conc. in supernatant (mg/mL) 100}}{\text{protein conc. in initial supernatant (mg/mL)}} \quad (4)$$

***Protein surface hydrophobicity index*:** The protein surface hydrophobicity index (PSH) was determined based on a fluorescence spectroscopy method using an ANS probe following the method of Alizadeh-Pasdar and Li-Chan (20) with some modification. A 0.5 g/kg protein stock solution and an 8 mM ANS solution were prepared using a 0.01 M phosphate buffer at pH 7.0. Then, 4 mL of a protein solution with a different concentration from 0.05 to 0.25 g/kg was mixed with 20 μL of the ANS probe solution and kept in the dark for 25 min before fluorescence measurements. The fluorescence intensity was recorded at 340 nm for excitation and 500 nm for emission using a FL-2500 fluorescence spectrophotometer (Hitachi). The PSH value was determined from the initial slope of the plot of fluorescence intensity vs. protein concentration. Within the protein concentration range used in these experiments, linear relationships were obtained (R^2=0.98-0.99).

***Water holding and oil binding capacities*:** The water holding capacity (WHC) and oil binding capacity (OBC) of WPC-80 and sWPC-80 samples were determined using the methods of Beuchat (21) and Chakraborty (22), respectively. A dried 0.1 g sample was mixed with 10 mL of distilled water/soybean oil in a 50 mL pre-weighted centrifuged tube and agitated thoroughly using a vortex mixer for 5 min. Samples were kept at room temperature of 25°C for 30 min, then centrifuged using a TDZ5-WS centrifuge (Xiangyi Centrifuge Instrument Co., Ltd.) at 3,000×g for 20 min. The supernatant was decanted and the centrifugal tube containing the sediment was weighed. WHC and OBC values were defined as:

$$WHC/OBC\ (g/g)=\frac{\text{sediment weight}-\text{dry weight}}{\text{dry weight}} \quad (5)$$

Foaming properties: The foaming capacity (FC) and the foaming stability (FS) were determined according to the method of Makri *et al.* (23) with slight modification. A total of 25 mL (V_{liquid}) of 0.2% WPC-80 and sWPC-80 were prepared in distilled water and pH adjusted to 7.0 using 1.0 M NaOH. WPC solutions were foamed by whipping using a high-speed blender at room temperature for 2 min. Whipped WPC samples were immediately transferred to a 50 mL graduated cylinder. The total volume of foam was recorded immediately (V_{foam0}) and again after 30 min (V_{foam30}). FC and FS values were calculated as:

$$FC\ (\%)=\frac{V_{foam0}}{V_{liquid}} \quad (6)$$

$$FS\ (\%)=\frac{V_{foam30}}{V_{liquid}} \quad (7)$$

Statistical analysis All analyses were performed in triplicate. Manuscript figures represent one parallel experiment with results presented as a mean±standard deviation. Data were evaluated using a one-way analysis of variance (ANOVA), followed by Student's t-test of Origin8.0 (OriginLab Corporation, Northampton, MA, USA). Differences were considered statistically significant at p<0.05.

Results and Discussion

Particle size distributions of WPC and MWP Particle distributions of WPC-80 powers and MWP dispersions were unimodal (Fig. 1). After superfine grinding, the peak particle size value of WPC-80 powder decreased from 62±3 to 15±1 μm, indicating that superfine grinding reduced the mean particle size of whey protein (Fig. 1). The trend of particle size change was identical to reports for other superfine grinding-treated powders (13). Accordingly, changes in sizes of WPC particles probably affected particle sizes of MWP samples. The peak particle size value of MWP dispersions decreased from 15±1 to 9±1 μm, which is a desirable result for practical production and application of microparticulated proteins (Fig. 1). According to Chung *et al.* (7), distributions of non-homogenized MWP became monomodal for both non-homogenized and homogenized systems, with a large mean particle diameter of >70 μm after heat treatment. In comparison with MWP produced using either extrusion cooking under an acidic pH or dynamic high pressure shearing (microfluidization), sMWP dispersions at relatively high concentrations of whey protein were perceived as a creamy, smooth fluid and could be directly used as a high-fat food mimetic ingredient.

Morphologies of WPC and MWP SEM was used to observe changes in size and morphology during superfine grinding and

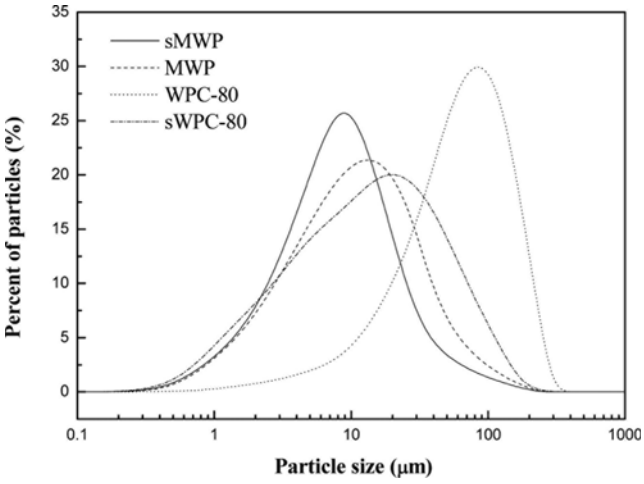

Fig. 1. Particle size distribution of protein powers and MPPs.

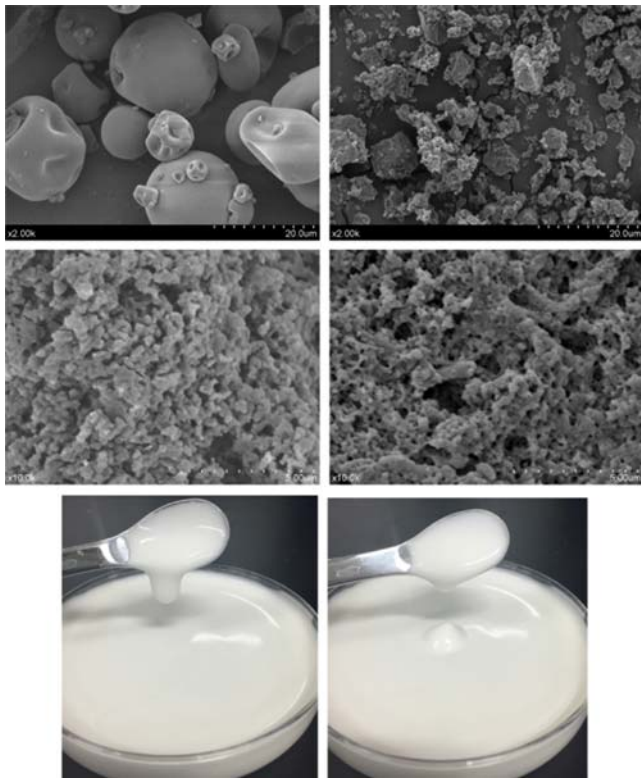

Fig. 2. SEM images of (A) WPC-80, (B) sWPC-80, (C) MWP, (D) sMWP, and pictures of (E) MWP, (F) sMWP.

microparticulation. Consistent with the particle size distribution, SEM images of WPC-80 particles showed hollow microspheres with smooth surfaces (Fig. 2A). After superfine grinding, the size of sWPC-80 powder significantly decreased, and coarse particles with fine fractions were formed (Fig. 2B) due to mechanical milling. During this process, a combination of flattening, aggregation, and fracture effects resulted in different shapes of sWPC-80 particles, which have an impact on physicochemical and functional properties of whey proteins. Furthermore, sMWP exhibited a smaller size, but a higher

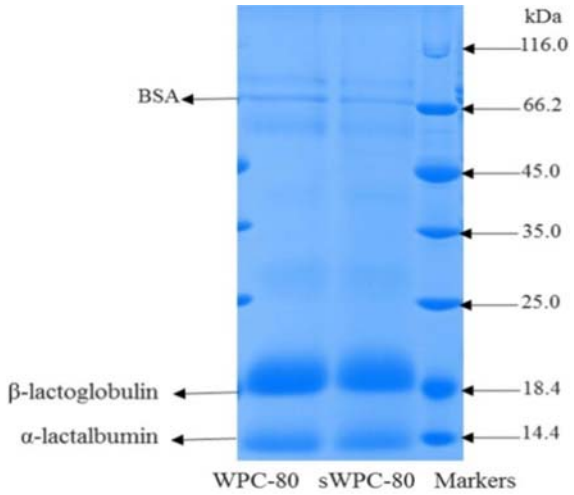

Fig. 3. SDS-PAGE image of WPC-80 and sWPC-80.

degree of crosslinking than MWP (Fig. 2C and 2D). The sMWP dispersion exhibited a higher viscosity and lower diffusion than the MWP dispersion, (Fig. 2E and 2F). Further studies should focus on the effect of superfine grinding on structural, thermodynamic, and physicochemical WPC properties.

Effect of superfine grinding on WPC structural properties SDS-PAGE analytical results of WPC-80, sWPC-80, and Mw protein standards are shown in Fig. 3. WPC-80 contained α-lactalbumin (14.4 kDa), β-lactoglobulin (18.4 kDa), and BSA (66.4 kDa). In comparison, sWPC-80 exhibited a similar SDS-PAGE profile with individual subunits of α-lactalbumin, β-lactoglobulin, and BSA, which indicated that superfine grinding did not influence the protein distribution of WPC. Furthermore, addition of β-mercaptoethanol broke existing intramolecular disulfide linkages and prevented formation of new intermolecular disulfide bonds, restricting protein aggregation to only non-covalent linkages (24). SDS-PAGE results explained that superfine grinding could not induce non-covalent associations of WPC molecules, but could not indicate the formation of intermolecular disulfide bonds.

The secondary structure of WPC was investigated using CD measurements (Fig. 4A). The CD spectra of both native and superfine grinding-treated WPC-80 showed a negative π-π* transition at 216 nm, indicating the existence of a β-sheet structure. A slight increase in α-helix (from 5.9 to 6.3%) and β-sheet contents (from 45.9 to 46.5%), and a decrease in β-turn (from 9.1 to 9.0%) and random coil contents (from 39.0 to 38.2%) were evident. The secondary structure of proteins depends on both the local sequence of amino acids and interactions between different groups of protein molecules. Thus, superfine grinding significantly (p<0.05) disrupted intramolecular WPC interactions without denaturation of whey proteins, compared with controls, in agreement with observed increases in disulfide bond contents and hydrophobic interactions after superfine grinding, which were responsible for aggregation of proteins.

Free sulfhydryl and total sulfhydryl and disulfide linkage group

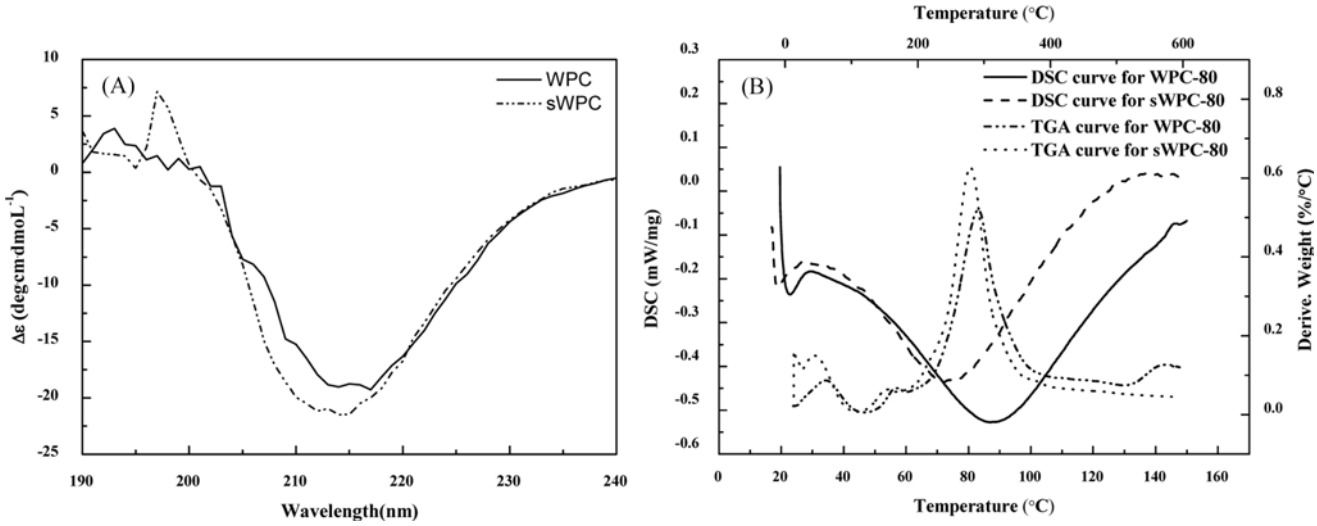

Fig. 4. Far-UV CD spectra (A), DSC thermograms and TGA (B) of WPC-80 and sWPC-80.

contents in WPC are important parameters for influencing the functional properties of WPC products if rearrangements occur due to superfine grinding. Average SH_F, SH_T, and SS WPC-80 values were 18.16, 185.34, and 83.59 μmol/L, respectively (Table 1). After superfine grinding, the amount of total sulfhydryl groups was not significantly (p>0.05) different from untreated WPC with an average SH_T value of 185.95 μmol/L. The SH_F value significantly (p<0.05) decreased to 14.49±0.02 μmol/L and the SS value significantly (p<0.05) increased to 85.68 μmol/L, partly due to exposure of buried WPC sulfhydryl groups at the protein surface and increased contact with air or cavitation-generated hydrogen peroxide due to turbulent flow, high pressure, and shear force, and partly due to breakage of disulfide bonds between subunits and re-formation in the interior of subunits. Thus, formation of disulfide linkages during superfine grinding was improved.

Effect of superfine grinding on WPC thermodynamic properties

Differential scanning calorimetry (DSC) is used to characterize physical and chemical events based on changes in either the enthalpy or heat capacity (25). DSC studies were carried out to identify WPC thermodynamic properties (Fig. 4B). DSC spectrograms revealed changes of heat enthalpy that accompanied breakage of chemical bonds in heated protein denaturation. According to Aguilera and Rojas (26), the denaturation temperature of α-lactalbumin is 61-61.5°C, and β-lactoglobulin and bovine serum albumin (BSA) are 75.9-81.2 and 71.9-74°C, respectively. However, DSC thermograms in this study showed that WPC-80 had a single endothermic peak with onset, peak, and endset temperatures of 40.52, 87.78, and 122.39°C. sWPC-80 had a narrower endothermic peak with onset, peak, and endset temperatures of 46.08, 71.72, and 104.03°C. Endothermic transitions of whey protein fractions overlapped, in particular for α-lactalbumin and β-lactoglobulin (27). The average peak difference between WPC-80 (83.88±4.49°C) and sWPC-80 (74.29±1.38°C) was significant (p<0.05), indicating that superfine grinding had an important effect on the thermodynamic properties of WPC.

Thermogravimetric analysis (TGA) is a method of thermal analysis in which changes in physical and chemical properties of materials are measured based on mass loss with increasing temperature at a constant heating rate (28). TGA curves for WPC-80 and sWPC-80 are shown in Fig. 4B. Both systems showed similar behaviors with 3 main stages of mass loss. The first stage, observed at 55.93°C for sWPC-80 and at 57.80°C for WPC-80, was related to loss of free water (Fig. 4B). The second stage, observed up to 180°C, was related to loss of adsorbed and bound water and was not considered for determination of kinetic parameters (29). The peak temperature of TGA curves of sWPC-80 significantly (p<0.05) increased from 158.41 to 184.91°C, compared with controls. The third stage, which was associated with protein degradation, was analyzed in greater detail to determine the kinetic parameters of proteins. The degradation peak temperatures of WPC-80 and sWPC-80 were 287.43 and 290.93°C, respectively, and the difference was not significant (p>0.05). Transformation and aggregation resulting from superfine grinding probably did not cause degradation.

Effect of superfine grinding on WPC physicochemical properties

Changes in particle size and conformation probably affected WPC physicochemical properties. Therefore, protein solubility, PHS, WHC/OBC, and foaming properties (FC/FS) were measured for WPC-80 and sWPC-80 to evaluate the effect of superfine grinding on the physicochemical properties of a WPC product. The protein solubility of WPC-80 was improved significantly (p<0.05) from 75.54 to 78.26%, compared with controls, which indicated that the protein solubility of WPC-80 in water was increased as the particle size of WPC-80 powder decreased after superfine grinding (Table 2). The PSH value for WPC-80 was significantly (p<0.05) lower than for sWPC-80,

Table 1. SH_F, SH_T, and SS contents of WPC-80 and sWPC-80

Sample	SH_F (μmol/L)	SH_T (μmol/L)	SS (μmol/L)
WPC-80	18.16±0.61[a1)]	185.34±1.74[a]	83.59±0.74[a]
sWPC-80	14.59±0.02[b]	185.95±0.31[a]	85.68±0.15[b]

[1)]Values that do not bear the same letter in the same column are significantly different (p<0.05).

Table 2. Physicochemical properties of WPC-80 and sWPC-80.

Physicochemical properties	WPC-80	sWPC-80
Solubility (%)	75.54±1.88[b]	78.26±0.39[a]
Protein Surface Hydrophobicity	1194.2±107.5[b]	1453.3±145.9[a]
Water Holding Capacity (g/g)	1.89±0.05[a1)]	1.71±0.09[b]
Oil Binding Capacity (g/g)	2.99±0.43[b]	4.40±0.46[a]
Foaming Capacity (%)	40.00±3.33[b]	57.78±1.92[a]
Foaming Stability (%)	28.89±1.92[b]	41.11±1.92[a]

[1)]Values that do not bear the same letter in the same row are significantly different (p<0.05).

indicating that superfine grinding improved exposure of hydrophobic groups in proteins and, thus, increased the surface hydrophobicity of WPC-80 powder. The PSH value of WPC-80 had the same trend of change as protein solubility, in agreement with the report of Wagner *et al.* (30). In contrast, Nakai and Li-Chan (31) reported that ANS hydrophobicity was inversely related to the solubility of soy protein. This conflicting observation may be attributed to differences in the extent of intermolecular interactions between hydrophobic groups on the surface of protein molecules. Therefore, the increase in solubility of sWPC-80 with increased surface hydrophobicity was an indication of decreased intermolecular interactions and smaller particle sizes of sWPC-80.

Solubility and PSH are important physical properties due to influences on functional properties of proteins, such as WHC/OBC and foaming properties (9). Increases in protein solubility and PSH values caused a reduction in WHC values and an increase in OBC values (Table 2). Superfine grinding of whey protein had a significantly (p<0.05) negative effect on the water-holding capacity of WPC-80 products, but a significantly (p<0.05) positive effect on the oil-binding capacity, compared with controls. According to the study of Sodini *et al.* (32), both heat treatment and a more acidic pH of whey contribute to lower WHC values, and a more open structure of the gel network was obtained due to denaturing of whey proteins during heating at an acidic pH, making removal of water during centrifugation easier. Superfine grinding improved exposure of hydrophobic groups in proteins and resulted in easy integration with oil, which finally led to a decreased water-holding capacity and an increased oil-binding capacity.

Foaming capacity and foaming stability values of WPC-80 and sWPC-80, representing WPC samples foamed at 0 min and after equilibration for 30 min, respectively, are shown in Table 2. WPC-80 was not a good foaming agent with an FC value at 0 min of only 40%. Therefore, WPC products may not be suitable for foaming in food systems such as cake and ice cream. The foamability of sWPC-80 increased significantly (p<0.05) after superfine grinding with an FC value at 0 min of 57.78%. In addition, although the foaming stability of both WPC-80 and sWPC-80 samples decreased with increasing time, sWPC-80 had a higher FS value (41.11 min) than WPC-80 (28.89 min), indicating that the foaming stability of WPC also significantly (p<0.05) increased after superfine grinding, perhaps due to an increase in solubility and molecular flexibility of proteins, leading to protein orientation, interaction, and spreading at the interface to form thicker and more viscous films (33).

In conclusion, molecular configuration, thermodynamic properties, and physicochemical properties of WPC and sWPC were investigated. Superfine grinding improved protein solubility, protein surface hydrophobicity, the oil binding capacity, and foaming properties of WPC as the particle size decreased and the disulfide bond content increased. A sMWP dispersion exhibited a smaller particle size than MWP. Thus, sMWP, perceived as a creamy, smooth fluid, can be directly used in high-fat foods as fat mimetic. Exploration of WPC fat replacement applications is indicated because of a WPC particle size reduction and associated microparticulated proteins.

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