Mechanism for Refunctionalizing Heat-Denatured Soy Protein by Alkaline Hydrothermal Cooking

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ABSTRACT: Using extrusion heat-denatured soy protein isolate (SPI) as a model, the mechanism for refunctionalizing heat-denatured soy protein by hydrothermal cooking (HTC) with alkali was studied. Heating causes soluble protein to form insoluble protein aggregates. Treating heat-denatured soy protein with alkali dispersion without HTC increased solubility and viscosity by dissolution of a portion of the protein aggregates and swelling of the large protein particles. This suspension was more stable to solid separation than that of the original untreated heat-denatured protein, but it was less stable than the protein suspensions that were refunctionalized by water dispersion with HTC or alkali dispersion with HTC. Water dispersion with HTC disrupted the large aggregates into smaller aggregates. The viscosity and total number of particles in the system also increased dramatically. The most significant effect was achieved with alkali dispersion (0.6 mmol NaOH/g) with HTC. The solubility increased from 4 to about 80% at neutral pH, and viscosity (at zero shear rate) increased by more than 1,000 times compared with extrusion heat-denatured SPI. Alkali dispersion (0.6 mmol NaOH/g) with HTC dissolved most of the protein particles, decreasing the particle number by a factor of almost 100. The suspensions of heat-denatured soy protein became much more stable after HTC as shown by particle settling velocities. The most effective treatment was alkali dispersion (0.6 mmol NaOH/g) with HTC, followed by water dispersion with HTC. The soy protein slurry refunctionalized by alkali dispersion (0.6 mmol NaOH/g) with HTC formed soft, translucent gels.

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KEY WORDS: Alkali dispersion, extrusion, heat-denatured protein, hydrothermal cooking, particle count, particle-size distribution, protein solubility profile, settling velocity, soy protein isolate, Stokes' law, viscosity.

Our previous work showed that hydrothermal cooking (HTC), especially when the protein was dispersed in alkali (0.2–0.8 mmol/g meal) prior to HTC, improved the functional properties, such as solids dispersibility, emulsification capacity, and foaming properties, of extruded-expelled (EE) soybean protein meals (1,2). The protein yield of soy protein isolate (SPI) after alkali dispersion with HTC (alkaline HTC) of EE soybean meal was improved by twofold (from 40 to 81%) compared with the original meal, whereas the protein yield after water dispersion with HTC (HTC without alkali) was 53%. We hypothesized that alkali dispersion with HTC increases the extractability of the protein and the stability of the resulting protein suspension (as SPI). The favorable increase in SPI properties could be due to increased protein solubility or dispersibility, decreased particle size of the denatured protein aggregates, increased viscosity, and/or reduced density differences between the dispersed and continuous phases. To eliminate interference from nonprotein components in EE meal, such as fiber and residual oil, we chose native SPI (with protein content >90%) as a model to study the mechanism of protein refunctionalization and improvement in SPI yield by alkali dispersion with HTC.

EXPERIMENTAL PROCEDURES

Materials. Native SPI (spray-dried without other intentional heat denaturation or additives) prepared at the Center for Crops Utilization Research, Iowa State University, was used as purified soy protein. A co-rotating laboratory-scale Leistritz Micro-18 twin-screw extruder (American Leistritz Corp., Sommerville, NJ), with the screw having a low shear configuration (18 mm diameter, 30 L/D ratio), was used to denature the protein in SPI. To make SPI extrudable, additional water was injected into the middle of the barrel. The maximal amount of water that could be added to native SPI before extrusion was only about 20%, which was less than required for trouble-free extrusion. The water feed rate was about 14 g/min. Screw rotation speed was set at 200 rpm. Moistened SPI was fed with a metering feeder (Accurate Inc., Whitewater, WI) at about 4 g/min. The internal temperature profile was 60, 90, 130, 130, and 130°C from the feed inlet to the die outlet. The extrudates were dried at ambient temperature for 2 d and then ground with a Magic Mill III Plus high-speed flour mill (Magic Mill, SSI Division, Salt Lake City, UT). The flour was then passed through a 40-mesh sieve and kept at ambient temperature to equilibrate the moisture content (5.1%). The protein dispersibility index of the heat-denatured SPI was 10.3 as measured by NP Analytical Laboratories (St. Louis, MO) by using AOCS official Method Ba 10-65 (3).

Treatments. Five treatments (Fig. 1), identified as "water-dispersed heat-denatured SPI without HTC," "alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC," "alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC," "water-dispersed heat-denatured SPI with HTC," and "alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC" were used. Water-dispersed heat-denatured SPI without

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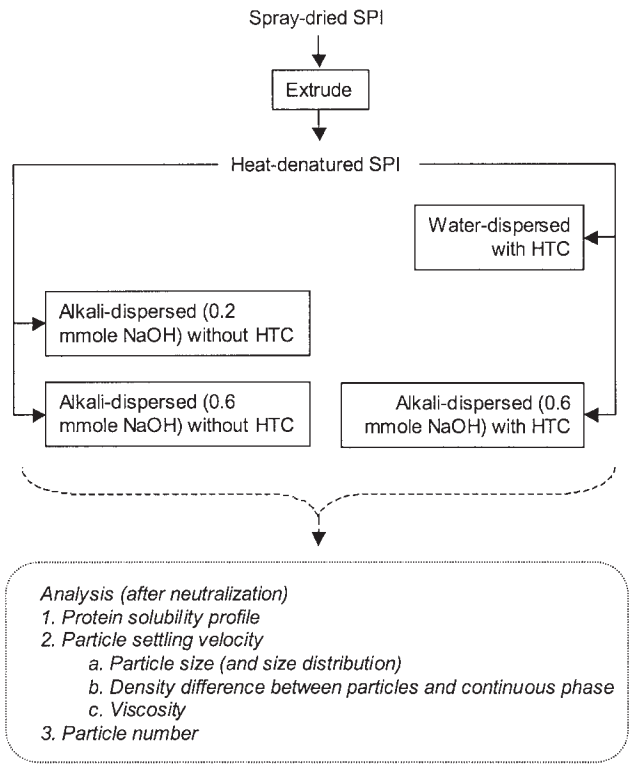

FIG. 1. Diagram of hydrothermal cooking (HTC) treatments for protein refunctionalization and analyses used to study the mechanism of the refunctionalization. SPI, soy protein isolate.

HTC was SPI after extrusion cooking to heat-denature the protein and dispersion in water at 10% (db: dry basis). Alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC was prepared by dispersing extrusion heat-denatured SPI as a 10% dry matter suspension, adjusting the pH to 8.5 with about 0.2 mmol NaOH/g SPI, and stirring at 60°C for 30 min as in conventional SPI extraction (4). Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC was prepared following the same procedure as alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC, except more alkali (0.6 mmol NaOH/g SPI) was added. This trial was designed to determine the effect of alkali since the same amount of alkali was used as in alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC. Water-dispersed heat-denatured SPI with HTC was HTC carried out on slurries of extrusion heat-denatured SPI without alkali addition. Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC was treated the same as water-dispersed heat-denatured SPI with HTC, except 0.6 mmol NaOH/g SPI was added before HTC. For all HTC treatments, a Moyno pump (2MI type SSQ; Robin and Myers, Inc., Springfield, OH) was connected with a hydroheater (size 300 type B; Hydrothermal Co., Milwaukee, WI) where culinary-grade steam (~90 psi pressure, 6.5 kg/cm^2) was infused to give the heat and shear treatment. The samples (20% slurry) were infused with steam in the hydroheater, then passed to a holding tube that provided a 42-s residence time at 154°C. The slurry was then released into the flash chamber. The cooking temperature was adjusted by a control valve located between the holding tube and the flash chamber; the temperature was monitored by a thermocouple (1). All HTC-treated SPI were cooled to ambient temperature, neutralized to pH 7, and stored at 5°C until analyzed.

Sample analyses. The protein solubility profile was determined as the percentage of solublized protein in the supernatant of 1% (db) dispersions in a series of pH values (2.5–10.5) after centrifugation. The pH was adjusted to specific levels with 2 N HCl or 2 N NaOH. After stirring for 1 h, the pH values of the suspensions were adjusted again, stirred for an additional 15 min, and centrifuged at 10,000 × *g* at 20°C for 10 min. The protein content of the supernatant was determined by using the biuret method (5) with BSA (Sigma, St. Louis, MO) as the standard. The initial protein content (N × 6.25) was determined by using a Rapid N III analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

The particle-size distribution and mean diameter of the dispersed particles of protein aggregates were measured by using a Mastersizer particle analyzer (Malvern Instruments Inc., Lombard, IL). The viscosity profiles of 10% sample (db) dispersions were measured at 25°C by using a HAAKE RheoStress viscometer RS150 (Gebrüder HAAKE GmbH, Karlsruhe, Germany).

To be able to use Stokes' law to explain our stability observations, we estimated "density difference," which is the difference between the density of dispersed particles and the density of the continuous phase. The method was derived from a centrifugation procedure, in which the density and mass of the supernatant under a series of centrifugation forces were measured and the data fitted with a linear model so as to extrapolate to an estimated density difference.

Our initial trial showed that the number of protein aggregates changed dramatically after alkali dispersion with HTC (alkaline HTC). The protein slurry was diluted to 1% in 20% aqueous glycerine and treated with 0.1% Coomassie Brilliant Blue G-250 to stain the protein. The suspension was spread onto a microscopic slide in a thin layer, and the slide was observed microscopically. Images were captured with a Zeiss Axioplan-2 microscope system (Carl Zeiss, Inc., Thornwood, NY). A magnification of 20× was selected because the range of particle sizes was easily observed. The particles were counted in a fixed area as seen on a computer screen. Dark-field microscopy was used to highlight the particles from the background. For each sample, three photos were randomly selected. The particle number report was the mean of the three counts. We assumed that the particles were uniformly distributed in the suspension, thus the particle number along any direction will be the same and the "particle number" may be used as a parameter across different dimensions. A simple mathematical function was used to convert particle number from count per area to count per volume (Fig. 2), and the particle concentrations were expressed as millions/mL in a 1% protein slurry (Table 1).

Experimental design and data analysis. All treatments were completely randomized with three replications. Statistical

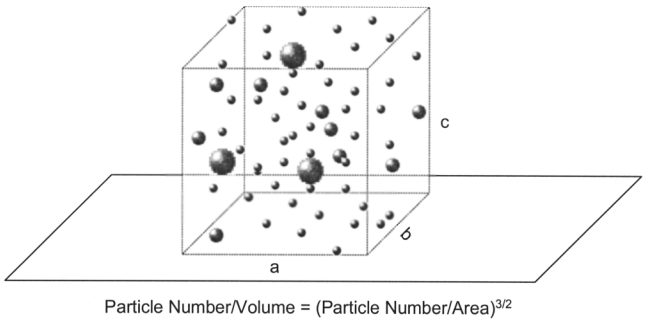

FIG. 2. Schematic figure showing how particle concentration was calculated.

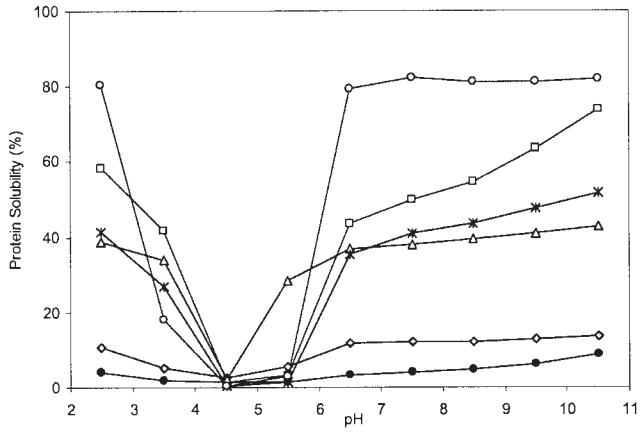

FIG. 3. Protein solubilities of heat-denatured SPI after different treatments. □, Water-dispersed spray-dried SPI; ●, water-dispersed denatured SPI without HTC; ◇, alkali-dispersed (0.2 mmol NaOH/g) denatured SPI without HTC; △, alkali-dispersed (0.6 mmol NaOH/g) denatured SPI without HTC; *, water-dispersed denatured SPI with HTC; ○, alkali-dispersed (0.6 mmol NaOH/g) denatured SPI with HTC. For abbreviations see Figure 1.

analysis was performed using General Linear Model procedures of SAS 8.02 (6).

RESULTS AND DISCUSSION

Protein solubility profiles. All samples had U-shaped solubility curves and isoelectric points (pI) of about pH 4.5, which is typical for soy protein (Fig. 3) As expected, extrusion heat denaturation dramatically reduced the solubility of spray-dried SPI. At pH values of 6.5 and 10.5, the solubilities of heat-denatured SPI decreased from 44 to 3% and 74 to 9%, respectively, compared with spray-dried SPI. Alkali dispersion without HTC increased the solubility of extrusion heat-denatured SPI. When heat-denatured SPI was treated by alkali dispersion (0.2 mmol NaOH/g) without HTC, the solubility increased only slightly compared with water-dispersed heat-denatured SPI without HTC. When treated with more alkali [alkali dispersion (0.6 mmol NaOH/g) without HTC], solubility increased much more. For example, at pH 6.5 the solubility of alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC increased from 12 to 37%. The increase in solubility suggests that, although the amount of alkali added was critical, mild heating (60°C) in alkali for a prolonged time (30 min), as in traditional SPI preparation, was not sufficient to solublize or disperse the heat-denatured protein. This was especially true for the low alkali addition (0.2 mmol NaOH/g SPI).

When treated with HTC at neutral pH (water dispersion with HTC), the solubility of heat-denatured SPI dramatically increased. For example, at pH 6.5, the solubility increased from 3 to 35%. Solubility was near that of spray-dried SPI without extrusion heat denaturation. The solubility steadily increased with increasing pH to 52% at pH 10.5 (water-dispersed with HTC). The solubility increase was greater in water-dispersed with HTC than that of alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC, which increased only from 37 to 43%. Thus, when comparing the solubilities resulting from the various treatments, HTC without alkali can refunctionalize heat-denatured soy protein considerably.

The most dramatic protein resolubilization occurred with alkali dispersion (0.6 mmol NaOH/g) with HTC. At pH 6.5, the solubility soared from 4% of that of water-dispersed heat-denatured SPI without HTC, to 80%. The solubility remained nearly constant as pH increased. Solubility of the alkali-dispersed HTC material was much higher than that of spray-dried SPI. The solubility profile of spray-dried SPI (before extrusion) was between that of water-dispersion with HTC and alkali-dispersion with HTC. Spray-drying and subsequent storage may have reduced protein solubility. At pH 5.5, alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC had considerable solubility (about 28%) compared with SPI with other treatments (all

TABLE 1
Particle Concentrations in SPI Slurries After Different Treatments[a]

	Without HTC			With HTC	
Treatment	Water-dispersed heat-denatured SPI	Alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI	Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI	Water-dispersed heat-denatured SPI	Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI
Particle concentration in 1% slurry (millions/mL)	14.3	76.2	5.2	2920	0.2

[a]SPI, soy protein isolate; HTC, hydrothermal cooking.

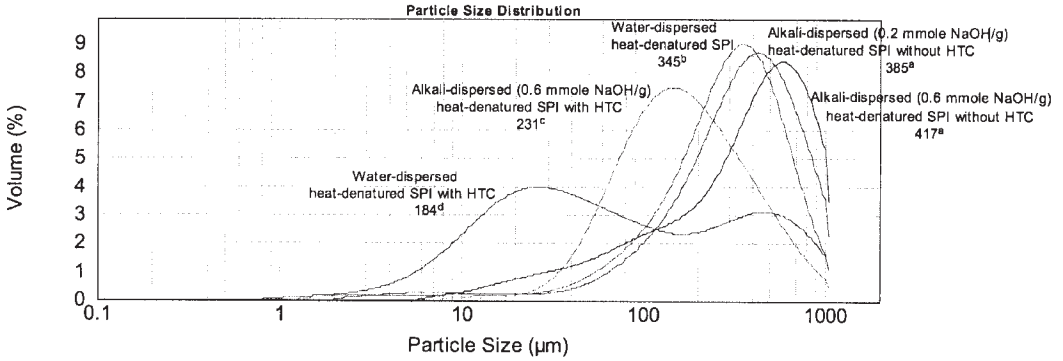

FIG. 4. Particle-size distributions and volumetric mean diameters of heat-denatured SPI after different alkali and HTC treatments. Means followed by the same letters are not significantly different ($P \leq 0.05$). For abbreviations see Figure 1.

below 6%). The reason is unknown. Apparently, high pH induced rapid solublization or dispersion of denatured SPI.

Settling velocity. Many liquid foods are suspensions of solid particles in liquid; these suspensions are termed sols. Maintaining a uniform dispersion of solid particles in the liquid continuous phase is critical to product quality. Over time, the solid particles will settle as a result of gravity. Estimates of the mean settling velocity of the particles are indicators of how stable the liquid suspension or food preparation will be during distribution and storage. Stokes' law can be used to estimate settling velocity of dispersed particles. Several factors influence settling velocity, including the size of particles (diameter), the density differences of the particles (dispersed or discontinuous phase), and the viscosity of the medium (continuous phase). We calculated settling velocities of the dispersed protein aggregates after various treatments based on the estimates of the parameters in Stokes' law.

The order of increasing mean particle diameters of the protein particles in the treated suspensions was as follows: water-dispersed heat-denatured SPI with HTC < alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC < water-dispersed heat-denatured SPI without HTC < alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC < alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC (Fig. 4) The mean particle diameter increased after alkali dispersion without HTC.

The particle size distributions were normal on a log scale for all treatments except for water-dispersed heat-denatured SPI with HTC, which was a bi-normal distribution (Fig. 4). After treatment with alkali alone (alkali dispersion without HTC), the peaks of the protein particle distribution shifted to the right, indicating larger size. More alkali addition (0.6 vs. 0.2 mmol NaOH/g SPI) resulted in larger particles (Fig. 4). Although alkali dispersion with mild heating may dissolve the small particles and partially dissolve the large particles of heat-denatured SPI, the effect of swelling may be more dominant. The net result was increased mean diameter of the dispersed particles. The more alkali was added, the more swelling there was, although more protein was also dissolved, as shown by the solubility data (Fig. 3).

Water-dispersed heat-denatured SPI with HTC caused a bi-normal distribution. One peak (at about 500 μm) appeared at the right side of the original heat-denatured SPI, the other (at 25 μm) showed up on the far left side. The peak at the right side suggests that while most of the large particles were disrupted into smaller particles by the high shear force, some resistant particles survived the treatment and swelled in the aqueous system even without alkali addition, because their mean diameters were about 500 μm, larger than that of the original heat-denatured SPI (345 μm). The peak at the far left side suggests the majority of disrupted aggregates had diameters around 25 μm. This observation supports our hypothesis that high shear during HTC disrupts the large aggregates of heat-denatured protein into smaller ones (2).

Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC reduced the mean particle size compared with the particles of water-dispersed heat-denatured SPI without HTC. The small particles may have been dissolved and the large particles disrupted and partially dissolved by the combination of alkali solublization and high shearing force during HTC. Although alkali dispersion (0.6 mmol NaOH/g) with HTC may cause protein aggregates to swell in the same fashion as alkali dispersion without HTC, the swollen particles must have been short-lived because the high shear force, high temperature, and alkali to which they were subjected during treatment also disrupted and solublized the particles. Therefore, the mean particle size was reduced.

It should be noted that particle size distribution and mean particle diameter were based on the remaining dispersed particles. The treatments changed not only the particle diameter or size distribution but also the total number of particles in ways such that the final products showed significantly different properties (Fig. 5). The protein particles in the slurry of heat-denatured SPI settled quickly, leaving a nearly clear solution in the upper portion. After alkali dispersion without HTC, the two layers did not clearly separate and the particles were better dispersed, although there were visible precipitates in the slurry. This was probably a result of more protein in the solution as well as the swelling of the protein particles. After water dispersion with HTC, the dispersion had a distinctive white milky color and a homogeneous

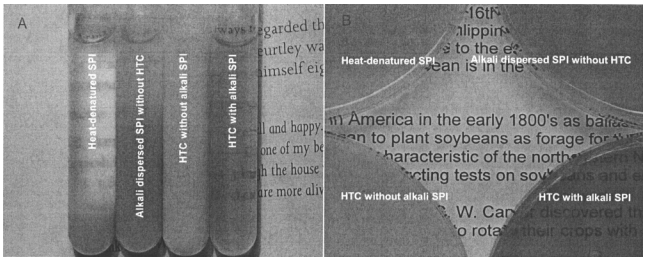

FIG. 5. Photographs of 10% dispersions of heat-denatured SPI after different treatments. For abbreviations see Figure 1.

viscous texture. The SPI suspension after alkali dispersion (0.6 mmol NaOH/g) with HTC formed translucent to transparent soft gels after neutralization (the print characters behind the gel are clearly visible in Fig. 5). These observations cannot be explained by mean particle size and size distribution alone.

The number of particles must be quantified in order to address the significant differences in sample properties. The results of particle number concentration are shown in Table 1. Alkali dispersion without HTC increased the particle number compared with water-dispersed heat-denatured SPI, possibly because of partial dissolution and physical disruption of the aggregates. The most dramatic changes occurred after HTC. The particle number concentration (millions/mL of 1% slurry) increased 200-fold after water dispersion with HTC, but decreased by almost 100-fold after alkali dispersion (0.6 mmol NaOH/g) with HTC compared with water-dispersed heat-denatured SPI without HTC. HTC disrupted particles, producing smaller ones, whereas alkali largely dissolved the proteins. The combination of alkali and shear at high temperature disrupted the aggregates and then dissolved the proteins more efficiently.

The viscosity profiles of the protein slurries after different treatments are shown in Figure 6. All samples exhibited typical non-Newtonian shear-thinning. Many models can describe viscosity behaviors of pseudoplastic materials of this type, such as the power law (Ostwald-de Waele), Carreau-Yasuda, Cross, Ellis, Meter, and the like (7). Usually the more comprehensive and better-fitting the model is, the more independent parameters are required. A simple logarithmic conversion was performed to describe the relationship between viscosity and the shear rate measured in our experiment (Fig. 7). All samples showed downward linear relationships between shear rates of 10 and 500 s^{-1} after log-log conversion. Even the slurry of water-dispersed heat-denatured SPI without HTC was a shear-thinning, non-Newtonian pseudoplastic suspension. The alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC, water-dispersed heat-denatured SPI with HTC, and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC exhibited almost perfect linear relationships (R^2 = 0.9995, 0.9982, 0.9989, and 0.9984, respectively). Therefore, the power law model was a good fit.

After heat denaturation, the viscosity of the spray-dried SPI dispersion decreased. Viscosities of all the HTC or alkali-dispersed heat-denatured SPI increased compared with untreated

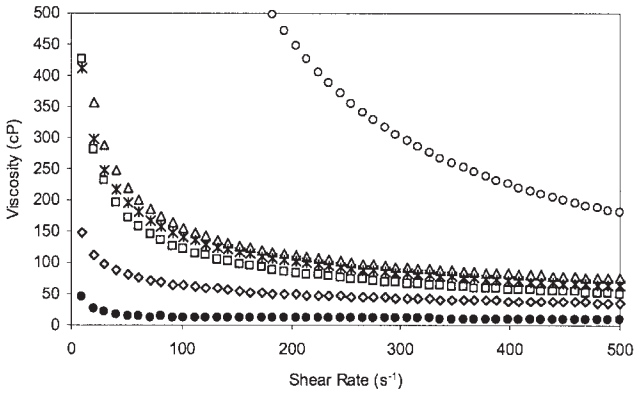

FIG. 6. Viscosity profiles of heat-denatured SPI after different treatments. □, Water-dispersed spray-dried SPI; ●, water-dispersed denatured SPI without HTC; ◇, alkali-dispersed (0.2 mmol NaOH/g) denatured SPI without HTC; △, alkali-dispersed (0.6 mmol NaOH/g) denatured SPI without HTC; *, water-dispersed denatured SPI with HTC; ○, alkali-dispersed (0.6 mmol NaOH/g) denatured SPI with HTC. For abbreviations see Figure 1.

heat-denatured SPI. The viscosity of alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC increased slightly. The increases were higher for the water-dispersed heat-denatured SPI with HTC and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC, where the viscosities were near that of spray-dried SPI dispersions. The viscosity increase of alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC was the greatest, followed by alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC,

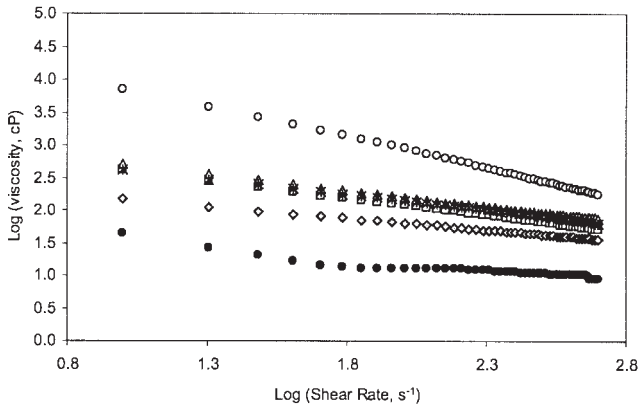

FIG. 7. Viscosity and shear rate relationships of heat-denatured SPI after different treatments. □, Water-dispersed spray-dried SPI; ●, water-dispersed denatured SPI without HTC; ◇, alkali-dispersed (0.2 mmol NaOH/g) denatured SPI without HTC; △, alkali-dispersed (0.6 mmol NaOH/g) denatured SPI without HTC; *, water-dispersed denatured SPI with HTC; ○, alkali-dispersed (0.6 mmol NaOH/g) denatured SPI with HTC. For abbreviations see Figure 1.

water-dispersed heat-denatured SPI with HTC, and alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC. Since the lines are above each other, there is little possibility that their relationship could change beyond the shear rate range

used in the analysis. It is well known that the power law model does not fit reality well at extreme shear rates (for example, zero and infinite shear rate). When a particle settles through a liquid phase under the force of gravity, there will be a near-zero shear rate. A viscosity value near zero-shear is needed for the Stokes' law particle settling velocity calculation. We regarded the shear rate of 1 s^{-1} as sufficiently low to represent the value at zero-shear. A zero shear-rate viscosity was extrapolated based on power law prediction from Figure 7.

The density difference in Stokes' law is defined as the mean density of particles (dispersed phase) minus the density of the continuous phase (we used the density of the supernatant after centrifugation at 15,000 × *g* for 15 min as the estimate for the density of the continuous phase) at ambient conditions. Because settling particles can precipitate under centrifugation, the mean density of the settling particles at different centrifugal speeds (rpm) were calculated by using the following formula, assuming the sum of the volume of precipitate and supernatant after centrifugation equals the volume before centrifugation:

$$\text{average density of settling particles} = \frac{\text{mass of precipitate}}{\text{volume of precipitate}} = \frac{\text{mass of precipitate}}{\text{total volume of slurry before centrifugation} - \text{volume of supernatant}} \quad [1]$$

The density difference between dispersed particles and continuous phase is centrifugation speed-specific when using this formula. An approach was needed to derive the density difference under gravity alone. We found a near-linear relationship between centrifugal speed (rpm) and density difference for heat-denatured SPI. We assumed that the relationship between density difference and centrifugation rpm is linear for our samples. Therefore, extrapolation was used to calculate the density difference at the centrifugal speed of zero (under gravity, when slurry is free to settle). Figure 8 shows one example of the density difference calculation. When speed = 0, density difference = 0.059 kg/m^3.

The accuracy of this method depends on the clear separation of precipitate and the measurement of supernatant volume. Considerable errors, especially for the viscous samples, were observed. The errors were so significant that sometimes no linear relationship was observed for certain samples. Another problem that may compromise the result is the small density difference between hydrated protein and continuous phase, especially considering the fact that many water molecules will hydrate the dispersed protein or indispersible aggregates or be physically trapped inside the protein matrix.

Nevertheless, this method did provide useful information. The approximations were acceptable on further consideration that the density difference in this experiment had a smaller impact on the settling velocity calculation than other parameters, either because of less relative change or the low power in the formula.

Stokes's law (settling velocity) calculations.

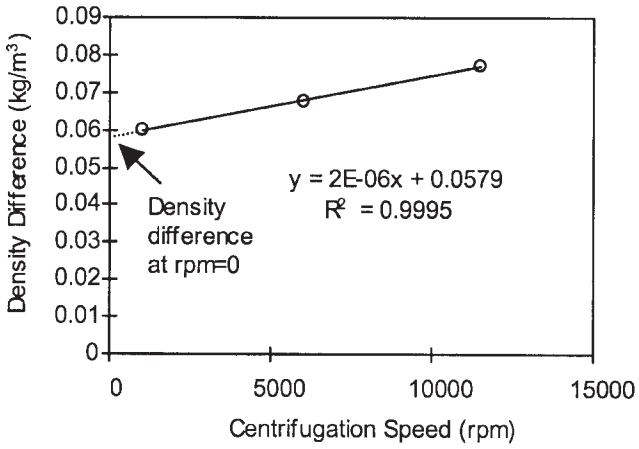

FIG. 8. Estimation of density difference under gravity using centrifugation method.

$$V = 2gd^2 \cdot \Delta\rho/(9\mu) \quad [2]$$

where V = settling velocity of protein particles, unit m/s; g = gravitational constant, 9.81 m/s^2; d = mean diameter of particles, m; $\Delta\rho$ = mean density difference between particles and water, kg/m^3; μ = viscosity of the suspension, kg/(m·s) (note: 1 N·s/m^2 = 0.1 kg/(m·s). The number of days it takes for the particles to settle a distance of 10 cm was also calculated to compare the relative stabilities of the dispersions after different treatments.

Table 2 shows that water-dispersed heat-denatured SPI with HTC and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC had dramatically reduced the settling velocities of the protein particles. The effect was achieved mostly by the increase in viscosity of the slurry. The settling velocity results generally agreed with observations of the dispersions. The particles of water-dispersed heat-denatured SPI without HTC settled most quickly, followed by particles in alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC, water-dispersed heat-denatured SPI with HTC, and alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC. The difference between alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC and water-dispersed heat-denatured SPI with HTC may be too small to be significant. It is very important to emphasize that for the alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC, not only a majority of the particles were solublized but also the solution formed soft translucent or transparent gels where few protein aggregates survived, thus no settling was observed.

It is worth noting that the viscosity term in Stokes equation is theoretically for the continuous phase or the medium. We used viscosity of the suspension for calculation, because it is difficult to define and justify a reasonable medium for all samples and treatments. Centrifugation could be used to remove the insoluble protein particles for measuring medium viscosity, but the speed to use was an unsettled question because of the different sizes of protein particles present in different samples.

TABLE 2
Settling Properties When Using Linear Extrapolation to Calculate Density Difference[a]

Treatment	Mean particle diameter (10^{-6} m)	Viscosity μ(shear rate = 0) (10^{-3} kg/ms)	Density difference (kg/m^{-3})	Settling velocity (10^{-9} m/s)	Time to settle 10 cm (d)
Water-dispersed heat-denatured SPI without HTC	345	52.3	0.050	247	4.70
Alkali-dispersed (0.2 mmol NaOH/g) heat-denatured SPI without HTC	385	327	0.028	27.9	41.4
Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI without HTC	417	1,480	0.021	5.5	211
Water-dispersed heat-denatured SPI with HTC	184	1,300	0.103	5.8	198
Alkali-dispersed (0.6 mmol NaOH/g) heat-denatured SPI with HTC	227	72,300	gel	gel	gel

[a]For abbreviations see Table 1.

We assumed that the contribution of the dissolved protein to viscosity is much greater than the contribution of the insoluble protein particles; therefore, our suspension viscosities may be a slight overestimation of the true viscosity of the medium. As a validation, the water-dispersed heat-denatured SPI without HTC had a much lower viscosity than the rest of the samples (Table 2), and such low viscosity was primarily produced by the insoluble protein particles.

Although water dispersion with HTC considerably refunctionalized heat-denatured soy protein and alkali dispersion without HTC partially refunctionalized heat-denatured soy protein, their individual effects were less significant than the combination of HTC and alkali dispersion, in which HTC and alkali work synergistically to refunctionalize heat-denatured soy protein. These effects were achieved by disrupting the large particles by high shear and high temperature and by dissolving the disrupted protein particles with alkali. The majority of the particles were dissolved and a translucent gel was formed, making soy protein stable and functional. The solubility of refunctionalized soy protein increased 20-fold, and the viscosity (at zero shear rate) was more than 1,000 times greater than that of extrusion heat-denatured SPI.

Another potential implication of alkaline refunctionalization of heat-denatured soy protein is that the treated protein product may have improved protein digestibility and bioavailability, therefore, a high-quality protein may be produced by this processing technique.

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