Effect of Stirring and Static Heating on Fibrilization of Milk Whey Protein: A Processing Approach

G. Rathod and J. Amamcharla

Summary

When globular proteins such as whey proteins are converted to fibril structure, the functional properties, including viscosity and water holding capacity, are improved. Consequently, they can be used as functional ingredients in a variety of foods such as yogurt and bakery products. In this study, milk whey protein isolates were converted to fibrils by heating at low pH using two different approaches to reduce fibrilization time without compromising the quality of fibrils. Fibrils were made with an intervention of stirring to reduce fibrilization time. Continuous stirring improved the quality of fibrils and speed of the fibrilization.

Introduction

Food proteins are composed of amino acids (monomers) that link to form a peptide chain. The peptide chains develop various conformations, depending upon the makeup of amino acids, the charge groups, and the bonds between and within molecules. Most food proteins are found in the globular structure, and mostly found in tertiary and quaternary structures. Altering the native globular structure of a protein to a fibrillar structure can give new functional properties such as gelling, emulsification, and foaming capacity and surface activity. Structurally, fibrils are a few nanometers in diameter and a few microns in length and have a high aspect ratio, which can provide a space-filling ability to fibrils. These fibrils can give previously mentioned properties at a relatively lower protein content than the native proteins. The fibrilization of different plant-based food proteins such as soy protein, pea protein, maize protein, wheat protein, rice protein, and cottonseed protein have been extensively studied. Other than plant protein, animal proteins such as egg and milk proteins were also explored for fibril formation.

Milk proteins are composed of two principal proteins, casein and whey proteins, among which whey proteins, a globular protein, was extensively studied for fibril formation. There were several studies on the fibrilization of whey protein isolate (made from cheese whey) and a pure β-lactoglobulin fraction of whey proteins. Several researchers have tried different concentrations of whey protein (1–3%), with varying pH of 1.8 to 3 (below the isoelectric point of whey proteins) and different time-temperature combinations from 75–120°C (167-248°F) and 5–24 hours. Fibrils made by heating 2% whey

proteins solution at pH 2 at 80°C (176°F) for 20 hours were reported best among all other combinations in terms of fibrils morphology, length, and maturity of fibrils. However, 20 hours is a very time-consuming process and required a lot of energy. A faster method is required to make the process feasible for industry. During fibril formation, proteins are hydrolyzed into smaller molecular protein fractions (2–8 kD) due to heat and acid. Within this hydrolysate, some proteins act as nuclei and other protein fractions arrange themselves in a systematic order to form fibrils. Hence, hydrolysis of protein and subsequent nuclei growth in terms of fibrils is most important to make fibrilization develop fast. Most of the reported study was done using static heating in a glass container where the movement of the peptides was lower, hence the growth of nuclei took time to form fibrils with maturity. The stirring of acidified protein solution can increase the overall rate of reaction and accelerate the fibril growth by enhancing the material flow in the solution. Stirring can be used as an intervention to reduce the fibrilization time of whey proteins. Therefore, the study was designed to see the effect of stirring over static heating in the time frame of 20 hours.

Experimental Procedures

Methodology

Milk whey protein isolate (mWPI) powders (4.3% moisture, 91.6% true protein (87.6% whey proteins and 12.4% casein), 1.4% lactose, and 2.1% ash) were rehydrated to 2% (w/w) solution on a protein basis with distilled water and stored overnight at 4°C (39.2°F) to ensure complete hydration. The pH of mWPI solution was adjusted to 2 using 6 N hydrochloric acid and stored overnight at 4°C (39.2°F). The pH-adjusted mWPI solutions were heated to 80°C (176°F) for 20 h using two different approaches. In approach 1, pH adjusted mWPI solution was filled in the 20 heat-resistant screw cap test tubes (8 mL, 17 mm dia. × 63 mm height) (DWK Life Science, Millville, NJ) and placed in water bath (Fisher Scientific, Waltham, MA) maintained at 80±0.5°C (176±0.9°F). Every hour one tube was taken out without disturbing other tubes and immediately cooled with iced water to 4°C (39.2°F) and labeled with the respective hour. In approach 2, mWPI solution was heated in a glass container using a temperature-controlled circulatory water bath (Cole Parmer, Vernon Hills, IL) under gentle continuous stirring using a magnetic stirrer. The glass container was sealed to ensure no moisture loss during heating. A thermometer was used to continuously monitor and record the temperature during the heating process. Every hour a 10 mL representative sample was drawn using a disposable pipette and transferred in a glass tube, with subsequent cooling and labeling similar to approach 1. Samples were stored in a refrigerator at 4°C (39.2°F) until further analysis. All the samples were analyzed for thioflavin T fluorescence value, transmission electron microscopy, gel electrophoresis, rheology, and particle size.

Confirmation of the Presence of mWPI Fibrils

Thioflavin T Fluorescence Value

Phosphate-sodium chloride buffer (10 mM phosphate (Fisher Scientific, Waltham, MA) and 150 mM NaCl (Fisher Scientific, Waltham, MA), pH 7.0) was prepared and stored overnight. Thioflavin T fluorescence stock solution (3 mM) was prepared by dissolving Thioflavin T reagent (Acros Organics, Fisher Scientific, Waltham, MA) in the phosphate-NaCl buffer. The Thioflavin T fluorescence stock solution was filtered

through a 0.2 μm WhatmanTM syringe filter (GE Healthcare UK Limited, Buckinghamshire, UK) to remove any undissolved particles. The Thioflavin T fluorescence stock solution was stored at 4°C (39.2°F) in an amber-colored glass bottle covered with aluminum foil. The Thioflavin T fluorescence working solution was prepared by diluting the Thioflavin T fluorescence stock solution 50-fold in the phosphate-NaCl buffer (final Thioflavin T concentration 60 μM). For the assay, a 48 μL sample was added to 4 mL working solution; the mixture was vortexed using a mini vortex mixture (Fisher Scientific, Waltham, MA) and held at room temperature for 1 min before measuring in a spectrofluorometer (LS-55; Perkin Elmer, Waltham, MA). The excitation wavelength was fixed at 440 nm with a slit width of 10 nm and the emission wavelength was scanned from 460–500 nm with slit widths 5 nm. Fluorescence intensity recorded at 482 nm wavelength was taken as the Thioflavin T fluorescence value. The Thioflavin T fluorescence value of the Thioflavin T working solution was taken as blank and subtracted from all the measurements. For each sample, the triplicate analysis was carried out and the scans were averaged to improve the signal-to-noise ratio.

Transmission Electron Microscopy

Samples were prepared for transmission electron microscopy (TEM) by diluting the protein solutions to 0.05% w/w protein using deionized pH 2 water where pH of deionized water was adjusted with 6 N hydrochloric acid. Proteins (20 μL) were absorbed onto a Formvar/carbon-coated 200-mesh copper grids (Electron Microscopy Sciences, Fort Washington, PA) for 30–60 s. The excess solution was wicked off with filter paper, stained with 2% uranyl acetate aqueous solution (Ladd Research Industries, Inc., Burlington, VT) for 30–60 s and the excess stain was wicked off with filter paper. Grids were viewed by TEM at 100 kV (CM 100 TEM (FEI Company, Hillsboro, OR)), and images were captured using a digital camera (Hamamatsu model C8484, Bridgewater, NJ) and AMT software (Advanced Microscopy Techniques, Chazy, NY). The length and width of the mWPI fibrils were measured and analyzed using ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD).

Tris Tricine Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Samples for gel electrophoresis were diluted to 0.5% protein content and vortexed. Samples were diluted with tris-tricine sample buffer in a 1:2 ratio and vortexed. They were incubated at 37°C (98.6°F) for 15 minutes. Then, a 15 μl sample was loaded in the 16.5% precast Tris-Tricine gel (BioRad Laboratories, Hercules, CA), fitted into electrophoresis assembly (BioRad Laboratories, Hercules, CA) filled with 1X Tris-Tricine sample buffer (BioRad, USA) prepared from 10X Tris-Tricine buffer by mixing sample buffer, and double-distilled water in 1:9 ratio. The gel was run on 20 mA for 15 minutes for stacking. Then, gel was adjusted to 25 mA for 90 minutes. The gel was fixed in 10% trichloroacetic acid solution for one hour. The gel was removed and dipped into a tray filled with staining solution (Coomassie brilliant blue R-250, BioRad, USA) with continuous shaking for 24 hours. After that, staining solution was replaced with a destaining solution (10% methanol, 10% acetic acid, 80% double distilled water) and kept for 24 hours with continuous shaking at room temperature. Images were taken with a camera and analyzed with ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD).

Rheology

Continuous rotational flow data were collected at 20°C (68°F) using a stress-strain-controlled rheometer (MCR-92 Anton Paar, Vernon Hills, IL) fitted with a 50 mm diameter stainless steel cone with angle 1° and 101 μm gap at a varied shear rate from 0.01 s^{-1} to 200 s^{-1}. The sample was transferred to the center of the base plate with a disposable pipette and maintained at 20°C (68°F). Then the cone and plate probe were lowered so that gap between the probe and base plate remained 101 μm. The excess sample was trimmed and observed for even spread between the probe and base plate before starting the analysis. Along with viscosity, the consistency coefficient and flow behavior index were also calculated using the power-law model.

$$\tau = K\dot{Y}^{\eta}$$

Where τ = shear stress, $\dot{Y}$ = shear strain, K = consistency coefficient, and η = flow behavior index.

Statistical Analysis

Statistical analysis was performed by repeated measure ANOVA by SAS v. 9.4 software (SAS Inst., Cary, NC).

Results and Discussion

Thioflavin T Fluorescence Value

Thioflavin T fluorescence value of the static heating and stirring are shown in Figure 1. Thioflavin T fluorescence value of unheated mWPI solution at pH 2 was 35.28±4.05 and 32.63±3.78 for static and stirring heating samples, respectively. The Thioflavin T value increased with an increase in time for both the samples. At 10 h, the Thioflavin T fluorescence value of static heating was 277.88±58.8 AU, which was significantly ($P < 0.05$) lower than that of stirring heating (417.65±48.09 AU). Further, at 20 h, the Thioflavin T fluorescence value of the static heating samples was 404.27±25.05 AU, which was significantly lower than stirring heating samples (683.07±90.62 AU) and even lower than the Thioflavin T fluorescence value of the stirring heating sample at 10 h (417.65±48.09 AU). Both stirring and static heating samples were significantly ($P < 0.05$) different during the whole 20 h period (Table 1).

The Thioflavin T fluorescence value is an indicator of fibrilization, which increases with an increase in fibrils formation. The result showed that stirring heating had a higher Thioflavin T fluorescence value than static heating during the whole 20-h duration, which indicated that stirring heating produced a higher number of fibrils than static heating. Further, fibril growth have been divided in three phases, an initial slow increase in the lag phase, the rapid increase in the log phase, and little rise in the stationary phase in previous studies. A similar pattern was seen for static heating where an increase in Thioflavin T fluorescence value was slow (lag phase) until 3 hours, after that it increased (log phase) faster until 10 hours, and then a slow increase (stationary phase) was seen until 20 h. However, in the case of stirring heating, Thioflavin T value was increased (log phase) throughout the 20 h period. Fibrils are formed by the rearrangement of low molecular weight peptides, which are produced from protein due to heat acid hydrolysis. In the static condition, the movement of these peptides was limited, hence the rate

of fibril formation was slow. However, the formation of fibrils from the fibril nuclei will not be inhibited by the depletion of the peptide in the stirred solution, as would occur with static heating condition. Hence, stirring heating showed no stationary phase until 20 h.

Transmission Electron Microscopy

Transmission electron microscopy (TEM) images are shown in Figure 2. The TEM images show fibril formation from the native globular protein to fibrillar form in both the methods starting from 2 h. In static heating, very low fibrils can be seen at 2 h, and with an increase in heating time fibrils formation can be seen but that is not as intensive as the stirring heating. Large aggregates and fibrillar aggregates can be seen with an increase in time. In contrast to that, stirring heating showed a uniform distributed network of fibrils, and the number of visible fibrils was higher as compared to static heating. Further, aggregates were also less in stirring heating as compared to static heating. At 14 h, fibril from stirring heating was clear, intact, and mature, which can be seen in the image at lower magnification (Figure 2A) and higher magnification (Figure 2B) with very little aggregation. Further heating leads to a decrease in visible fibrils (Figure 2A and 2B).

In the case of stirring heating, continuous movement of the solution facilitated uniform heating of mWPI solution, and thereby uniform protein hydrolysis in peptides. Further, during fibril formation, stirring of solution provided enough hydrolyzed protein to fibril nuclei to form fibrils. Hence, fibrils formed in stirring heating were uniform and identical, showing less fibrillar aggregation as compared to static heating. In static heating, fibrils were formed based on the availability of hydrolyzed protein from the surroundings of fibril nuclei in solution, which cannot be uniform due to lack of movement of the solution that was produced by the stirring heating, hence fibrils formed are not uniform. Further, in a static condition, fibrils had enough time to cross-link, which led to aggregation which can be seen in the TEM images (Figure 2).

Gel Electrophoresis

Tris Tricine SDS-PAGE images are shown in Figure 3. Initially, both static and stirring heating showed a clear band of different whey protein fractions, among which β-Lg and α-La are the major whey protein fractions, however, there was no whey protein less than 10 kD. Upon heating at pH 2, the concentration of intact whey proteins, α-lactalbumin, and β-lactoglobulin, was decreasing with an increase in heating time, and the concentration of small molecular weight fractions below 10 kD was increasing with heating time. As compared to stirring heating (Figure 3B), static heating (Figure 3A) showed a faster conversion of whey protein into small protein fractions (2–10 kD) from the 2 hours itself and showed less intact whey protein at the end of 20 h.

In the fibril formation process, proteins were hydrolyzed into smaller sized protein fractions of 2–8 kD and the fractions rearranged themselves into fibrils. Under the action of SDS, fibrils which are made from small-sized protein fractions are dissociated again and can be seen as a separate gel band. Hence, the occurrence of a higher amount of small-sized protein could indicate a higher rate of hydrolysis which could enhance the formation of the fibril. But along with the number of small sized protein fractions, the systematic arrangement was needed for fibril formation. Stirring heating showed lower

conversion of small-sized protein fractions, but stirring may have enhanced rearrangement of these small protein fractions and thereby fibril formation.

Rheology

Table 1 shows rheological data for the stirring and static heating samples from 0–20 h. In the stirring heating sample, viscosity (at $100S^{-1}$) increased from 1.08±0.03 to 1.37±0.19 mPa.S within 2 h, and increased continuously until it reached 6.08±0.09 mPaS at 19 h. Unlike this continuous increase, the static heating sample showed a definite increase in viscosity, but this rise was irregularly reached to 6.48±1.10 at 19 h. A similar increase can be seen in the result of the consistency coefficient where stirring heating showed a steady increase compared to the discontinuous increase in static heating. On a similar pattern, the flow behavior index also decreased with time in both the methods but stirring heating showed a gradual decrease compared to a discontinuous decrease in static heating. The probable reason could be the method of manufacture of the fibril. In stirring heating, pH-adjusted protein solution was continuously stirred, which facilitated even distribution of hydrolyzed protein fractions for fibrils formation, hence the fibrils formed were aligned in similar orientation and remained parallel to each other in the solution. So, when they were analyzed for rheology, they may have aligned parallel to the plane of the probe. They showed lower viscosity at static heating at a lower shear rate. At a higher shear rate both the methods showed almost similar viscosity. However, static heating fibrils may have formed a three-dimensional network during the fibrils formation process. These networks were broken due to shear stress, and fibrils aligned with the probe at higher shear. Therefore, at higher shear, not much difference in viscosity was seen and a similar phenomenon was replicated in consistency coefficient and flow behavior index.

Conclusions

Milk whey proteins are globular in structure in their native state. The proteins can be converted to fibrils to harness the functionality of fibrils such as gelling, emulsification, and foaming. Fibrils are produced by heating milk whey protein solution at low pH, which hydrolyzes the protein into small-sized protein fractions, and the fractions are arranged systematically into fibrils. In conventional heating, the time required to produce fibrils is long, and the quality of fibrils is compromised. To expedite this process and improve the quality of fibrils, stirring was introduced as a processing aid, which can increase the rate of fibril formation and can produce uniform fibrils. Thioflavin T fluorescence value and transmission electron microscopic images showed that good quality fibrils could be produced by stirring while heating. Time could be reduced to 14 h rather than 20 h heating in the conventional process. Rheological studies showed a consistent rise in viscosity in stirring heating over static heating. Hence, it could be said that stirring heating can produce good quality fibrils in less time.

Table 1. Rheology of the stirring and static heating samples

	Apparent viscosity (m.Pa.S) $100S^{-1}$		Consistency coefficient (Pa.s^n)		Flow behavior index	
Time, h	Stirring heating	Static heating	Stirring heating	Static heating	Stirring heating	Static heating
0	1.08±0.03^{A}	1.09±0.03^{A}	0.0011±0.0002^{A}	0.0012±0.0001^{A}	0.99±0.03^{A}	0.99±0.01^{A}
2*	1.37±0.19^{A}	1.16±0.03^{B}	0.0022±0.0007^{A}	0.0012±0.0001^{B}	0.91±0.04^{B}	0.99±0.01^{A}
3	1.89±0.05^{A}	1.34±0.07^{B}	0.0046±0.0002^{A}	0.0019±0.0005^{B}	0.81±0.01^{B}	0.94±0.05^{A}
4	2.23±0.19^{A}	1.75±0.55^{A}	0.0068±0.0009^{A}	0.0063±0.0057^{A}	0.76±0.02^{A}	0.79±0.14^{A}
5	2.77±0.31^{A}	3.58±1.23^{A}	0.0098±0.0012^{A}	0.0386±0.0364^{A}	0.72±0.01^{A}	0.55±0.14^{B}
6	3.29±0.24^{A}	4.01±1.55^{A}	0.0133±0.0013^{A}	0.0631±0.0628^{A}	0.7±0.01^{A}	0.49±0.16^{B}
7	3.68±0.21^{A}	4.79±1.53^{A}	0.0165±0.0022^{B}	0.0922±0.0738^{A}	0.68±0.02^{A}	0.42±0.12^{B}
8	4.19±0.39^{A}	4.21±0.35^{A}	0.0180±0.0021^{B}	0.0580±0.0164^{A}	0.68±0.02^{A}	0.44±0.05^{B}
9	4.44±0.29^{A}	4.20±0.56^{A}	0.0219±0.0015^{B}	0.0619±0.0219^{A}	0.66±0.01^{A}	0.43±0.05^{B}
10	4.72±0.13^{A}	2.96±1.01^{B}	0.0227±0.0014^{A}	0.0328±0.0294^{A}	0.66±0.02^{A}	0.54±0.12^{B}
11	4.92±0.16^{A}	5.22±1.56^{A}	0.0246±0.0009^{B}	0.1011±0.0644^{A}	0.65±0.02^{A}	0.4±0.09^{B}
12	5.20±0.14^{A}	3.69±1.21^{A}	0.0279±0.0029^{A}	0.0428±0.0331^{A}	0.64±0.02^{A}	0.51±0.1^{B}
13	5.27±0.16^{A}	5.63±1.63^{A}	0.0272±0.0011^{B}	0.1331±0.0490^{A}	0.65±0.02^{A}	0.33±0.03^{B}
14	5.31±0.07^{A}	5.01±0.91^{A}	0.0277±0.0010^{B}	0.1089±0.0320^{A}	0.64±0.01^{A}	0.35±0.06^{B}
15	5.49±0.06^{A}	5.64±2.58^{A}	0.0294±0.0020^{A}	1.5642±2.9061^{A}	0.64±0.02^{A}	0.41±0.17^{A}
16	5.06±0.90^{B}	5.62±1.10^{A}	0.0288±0.0068^{B}	0.1426±0.0234^{A}	0.63±0.02^{A}	0.3±0.07^{B}
17	6.09±0.14^{A}	3.84±0.83^{B}	0.0337±0.0038^{A}	0.0687±0.0513^{A}	0.63±0.02^{A}	0.41±0.11^{B}
18	6.07±0.11^{A}	6.48±1.23^{A}	0.0368±0.0006^{B}	0.2418±0.1185^{A}	0.61±0.01^{A}	0.23±0.08^{B}
19	6.08±0.09^{A}	6.48±1.10^{A}	0.0330±0.0023^{B}	0.1611±0.0485^{A}	0.63±0.02^{A}	0.31±0.04^{B}
20	5.94±0.13^{A}	6.06±1.01^{A}	0.0323±0.0040^{B}	0.1287±0.0446^{A}	0.64±0.02^{A}	0.35±0.06^{B}

$^{A-B}$ Mean within a column (static heating and stirring heating) with different superscripts are different ($P < 0.05$) n = 4.
*Data were not collected for hour 1.

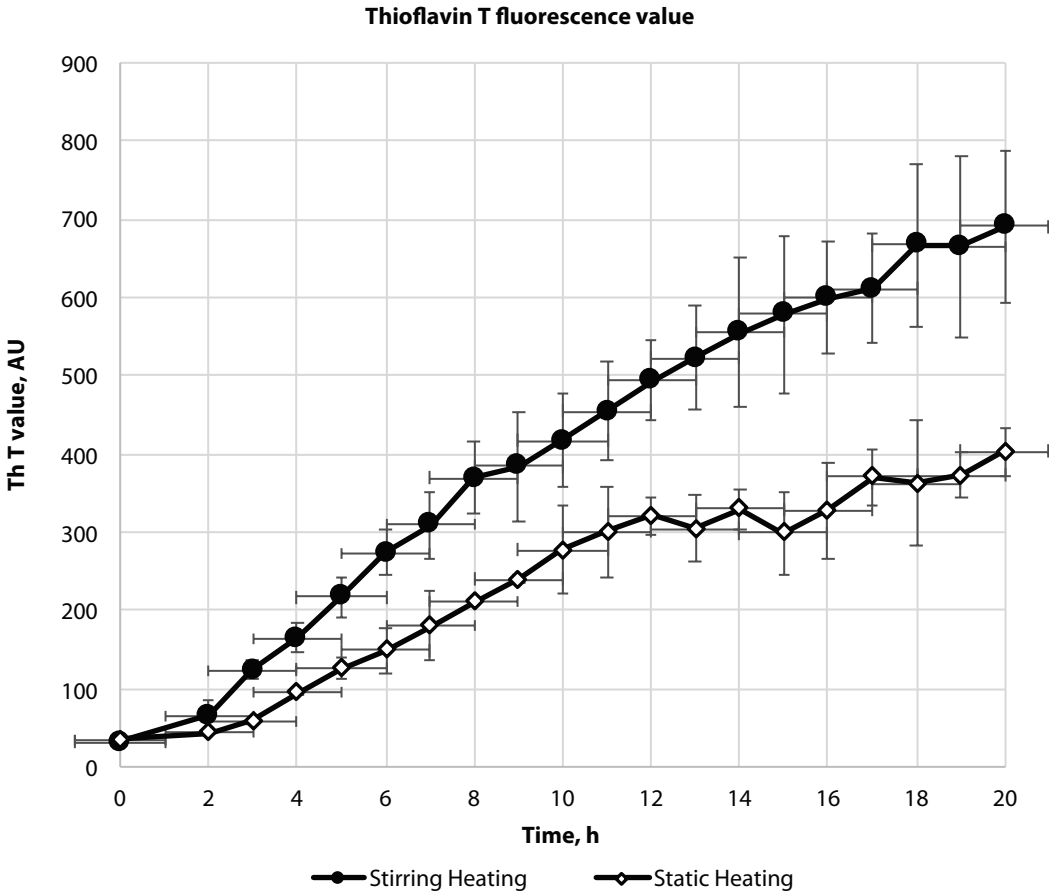

Figure 1. Thioflavin T value of stirring and static heating (0–20 h).

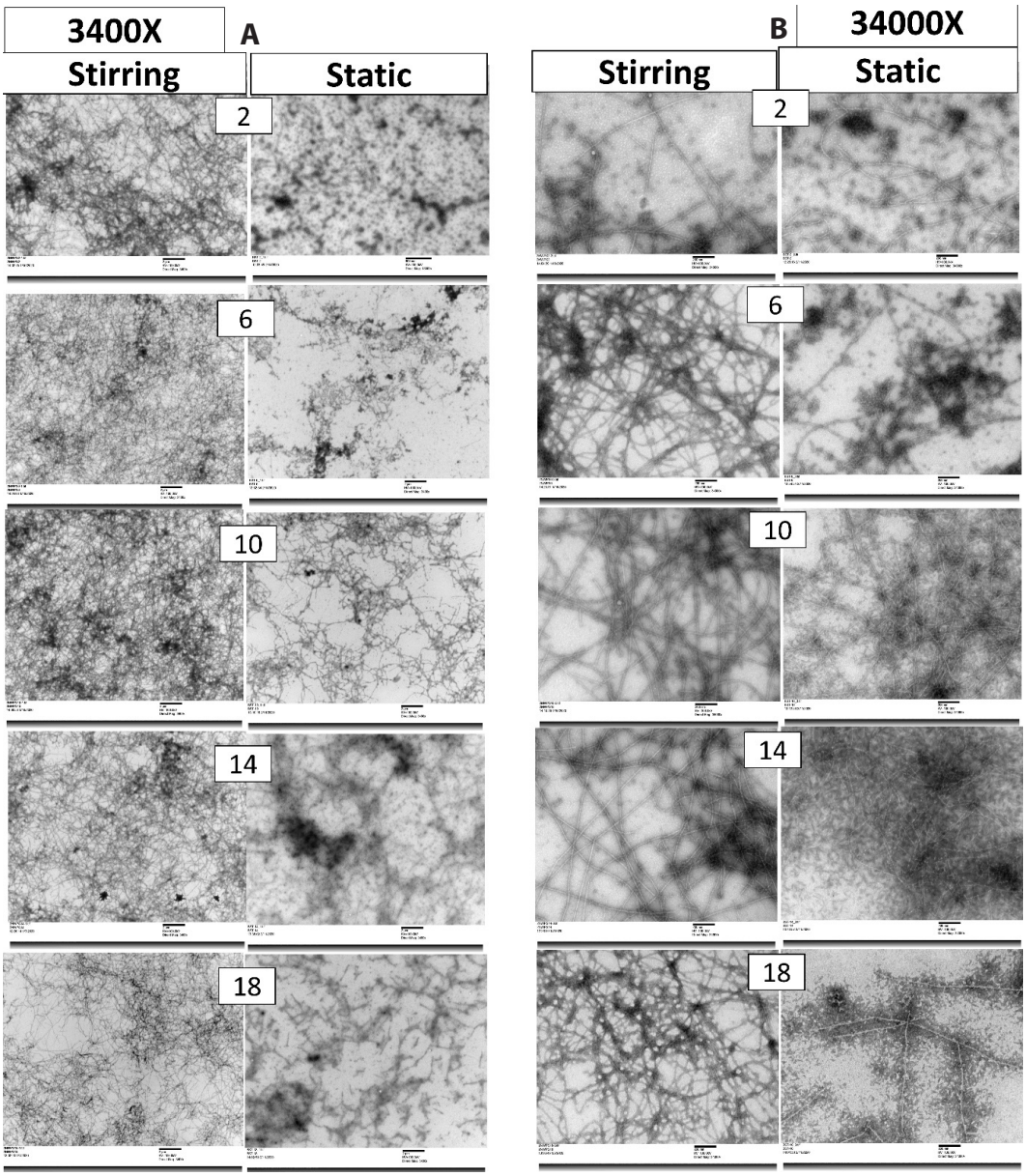

Figure 2. Transmission electron microscopy images of stirring and static heating samples at 80±0.5°C (176±0.9°F)at different hours. (A) Lower magnification. (B) Higher magnification.

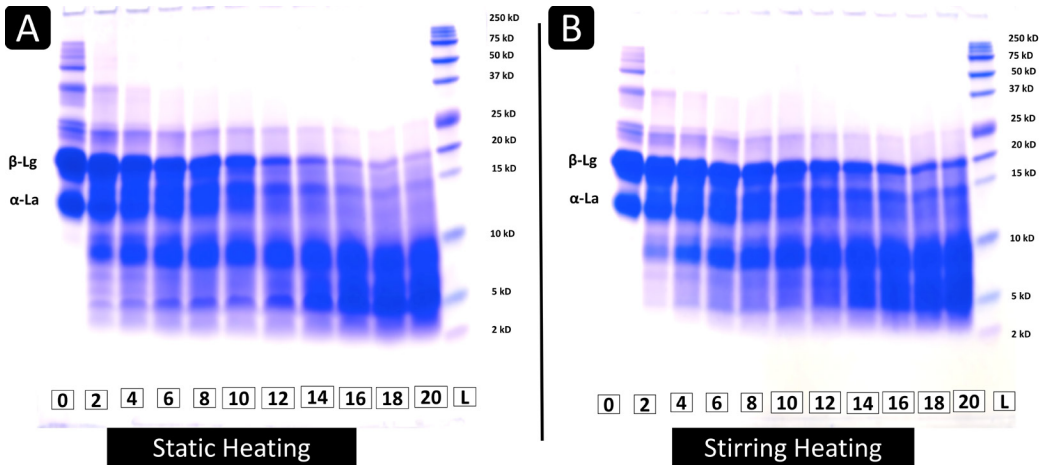

Figure 3. Tris-Tricine SDS PAGE of stirring heating sample (0–20 h, L-ladder). β-Lg = β-lactoglobulin. α-La = α-lactalbumin.