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Impact of Stirring Speed on β-Lactoglobulin Fibril Formation

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Abstract β-Lactoglobulin (β-lg) can produce fibrils that have multi-functional properties. Impacts of different stirring speeds on characteristics of β-lg fibrils as a stable form in β-lg fibril solutions were investigated. Fibril concentration, fibril morphology, turbidity, particle size distribution, zeta potential, and rheological behavior of solutions were studied. Stirring enhanced fibril formation and stability of a fibril solution, in comparison with unstirred solutions. Increasing the stirring speed produced more turbidity and a greater distribution of particle sizes, higher viscosity values, but no differences in zeta potential values of β-lg fibril solutions. However, a high stirring speed is not feasible due to reduction of the fibril yield and changes in fibril morphology.

Keywords: fibril, β-lactoglobulin, stirring, zeta potential, morphology

Introduction

Whey proteins have been the subject of research due to nutritional and functional properties. Under appropriate conditions, whey proteins can self-assemble into long, semi-flexible structures known as amyloid fibrils (1) that may be useful as scaffolds for tissue engineering (2) or enzyme immobilization (3). In the food industry, fibrils act as a viscosity enhancer in gel formation at relatively low protein concentrations due to functional properties of emulsification, gelation, thickening, foaming, and water-binding (4-6). β-Lactoglobulin (β-lg) is the most abundant protein present at approximately 50% in whey protein with a secondary structure containing approximately 50% β-sheets, 9-12% α-helices, 8-10% turns, and 30-35% random coils (7). It is possible to isolate this protein using several methods. One of the most common methods for isolation of β-lg from whey proteins is precipitation at a low pH (8).

β-Lactoglobulin exhibits pH dependence in a monomer-dimer equilibrium. Primarily dimeric at a neutral pH, β-lactoglobulin dissociates into monomers at pH values above 8.0 and below pH 3.0 (9). Under high-temperature thermal treatment at a low pH, β-lg self-assembles into amyloid-like fibrils during heat-induced hydrolysis (10). Heating β-lg under acidic conditions leads to weak hydrophobic and hydrogen bond interactions of monomers that result in aggregation of β-lg, and the intramolecular β-sheet is shifted to an intermolecular β-sheet alignment to form fibrils (11). High temperatures decrease the stability of the native state and increase the unfolding state, which favors fibrillation of β-lg (12).

Assembly of β-lg into fibrils involves 3 stages of nucleation-dependent growth kinetics, including lag, growth, and stationary phases (13). The lag phase refers to time required for formation of nuclei during nucleation. After nuclei are formed, β-lg undergoes chemical-induced (14) or heat-induced unfolding (15), followed by alignment of the amyloidogenic regions in β-sheet-rich nuclei where nuclei are elongated to form fibrils via attachment of monomers during the growth phase. Eventually, the growth rate decreases and reaches zero to begin the stationary phase, indicating either a limitation in the monomer supply, or thermodynamic equilibrium (16).

The environmental factors of ionic strength, solution pH, temperature, and protein concentration have strong impacts on β-lg fibril formation (17). The nucleation time can be shortened by shearing during heating (18,19). In fact, high shear stress generated in a solution during rapid stirring can abolish the lag phase, which promotes secondary nucleation (20). However, no studies have been performed for investigation of the impact of different stirring speeds on the characteristics of β-lg fibrils formed after the fibrillation process has occurred. Thus, this study investigated the impact of different stirring speeds on characteristics of β-lg fibrils as a stable form in a β-lg fibril solution. Studied characteristics included the fibril concentration, fibril morphology, turbidity, and particle size distribution, in addition to zeta potentials and rheological behaviors.

Materials and Methods

Materials A BiPRO whey protein isolate (WPI) rich in β-lactoglobulin (approximately 70%) was obtained from Davisco Foods International, Inc. (Eden Prairie, MN, USA). All other chemicals were of analytical grade.

Preparation of β-lactoglobulin solutions and production of β-lactoglobulin fibrils β-Lg fibril solutions were prepared following a previous method with slight modification (21). In brief, 2.5% (w/w) WPI was dissolved in deionized water. For precipitation of denatured proteins, pH values of WPI solutions were adjusted to 4.6 using 6.0 M HCl. WPI solutions were centrifuged at 6,000x*g* for 30 min at 25°C (Heraeus Multifuge; Thermo Fisher Scientific, Waltham, MA, USA), and subsequently subjected to vacuum filtration unit (Sartorius, Gottingen, Germany) using 0.2 μm regenerated cellulose filter paper (Sartorius). Native β-lg solutions were formed via adjustment of WPI solutions to a pH of 2.0 using 6.0 M HCl. For initiation of fibrillation, native β-lg solutions were heated at 80°C in a water bath (Memmert WNB 14; Gemini BV, Apeldoorn, the Netherlands) for 20 h under stirring speeds of 400, 800, and 1,200 rpm (2Mag Magnetic Motion; Gemini BV) with and without stirring (0 rpm). Fibrillar β-lg solutions were rapidly cooled in an ice water bath for 30 min after 20 h of heating. β-Lg solution samples were diluted using pH 2.0 deionized water at a ratio of 1:1 (w/w) and used for physicochemical characterization comparisons. β-Lg solution samples were stored at 4°C in refrigerator (Sharp SJ313T; Sharp corp., Selangor, Malaysia) for subsequent use.

Thioflavin T (ThT) fluorescence assay A thioflavin T (ThT) fluorescence assay was used to measure β-lactoglobulin conversion into fibrils after heating of protein solutions following an established protocol (22). A 3.0 mM ThT stock solution was prepared by dissolution of 7.9 mg of ThT (Merck Chemicals Ltd., Nottingham, UK) in 8 mL of phosphate-NaCl buffer (10 mM phosphate and 150 mM NaCl at pH 7.0). This stock solution was filtered through a 0.2 μm filter paper (Schleicher & Schuell, Dassel, Germany). The ThT working solution was prepared via dilution of a stock solution 50x in a phosphate-NaCl buffer (10 mM phosphate and 150 mM NaCl at pH 7.0) before use to achieve a final ThT concentration of 60 μM.

For ThT fluorescence assays, 48 μL of a fibril sample was mixed with 4 mL of a ThT working solution. The mixture was vortexed briefly using vortex mixer (MaxiMix II; Thermo Fisher Scientific) and held at room temperature (24°C) for 1 min. Fluorescence values of fibril samples was measured using a fluorescence spectrometer (Perkin Elmer LS 55; Perkin Elmer, Waltham, MA, USA), at a scanning speed of 200 nm/min at excitation and emission wavelengths of 440 and 482 nm, respectively. The fluorescence value of an unheated protein solution was subtracted from all measurements. The ThT uorescence reading was recorded in arbitrary units (a.u.).

Negative stain transmission electron microscopy (TEM) Morphological characteristics of β-lg solutions were examined following an established protocol (23). In brief, a transmission electron microscopy (H-7100 TEM; Hitachi, Tokyo, Japan) was used at an accelerating voltage of 100 kV at different magnifications of 50,000x, 100,000x, and 150,000x for examination of β-lg monomer and fibril morphology. Fibril sample solutions were sonicated using sonicator (Ultrasonic bath SW3H; Thermo Fisher Scientific) for 60 s and diluted 100x using deionized water. A droplet of a diluted fibril solution was placed on a carbon-coated 300 mesh copper grid (Pro Sci Tech, Townsville, Australia), then removed using filter paper after 2 min. TEM sample staining was done by placing a droplet of a 3% uranyl acetate solution (Sigma-Aldrich, St. Louis, MO, USA) on the TEM grid, followed by removal using filter paper after 2 min, after which the grid was air-dried at room temperature (24°C) prior to insertion into the TEM for imaging.

Turbidity measurement Turbidity measurements were performed following the method of Mounsey *et al.* (6). Solution absorbance values were measured at a fixed wavelength of 600 nm using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) in 1 cm path length glass cuvettes against deionized water.

Particle size measurement Particle size measurements were performed following the method of Kehoe and Foegeding (24). Particle size distributions of β-lg solutions were determined using a Zetasizer Nano system (Malvern Instruments Inc., Malvern, UK). Refractive index values of fibril samples were set at 1.450 with water as a dispersant. Viscosity values of fibril samples were set at 0.8872 cP, which was assumed to be the value for water. For measurement, the temperature was set at 25°C with an equilibration time of 120 s. The measurement angle was set at 173° backscatter with a run time of 10 s for each measurement.

Zeta potential Zeta potential values of fibril samples were examined following the protocol of Kehoe and Foegeding (24). All fibril samples were diluted to 0.1% (v/v) using pH 2.0 deionized water before zeta potential measurements were made using a Zetasizer Nano system (Malvern Instruments). Refractive index values of fibril samples were set at 1.450 using water as a dispersant. The viscosity of fibril samples was set at 0.8872 cP, which was assumed to be the value for water. Measurements were made at 25°C with an equilibration time of 120 s. The Smoluchowski model (24) was applied for derivation of the zeta potential value. Measurement settings and voltages were set to auto to allow the software (Malvern Zetasizer software version 7.11) to determine the optimum voltage for the measurement and also for the number of runs performed per measurement.

Rheological measurements Rheological behaviors of β-lg solutions were determined following the protocol of Kehoe and Foegeding

(24). β-Lg solution samples were maintained at 25.00±0.01°C for analysis. A dynamic controlled stress rheometer (HAAKE Rheo Stress 600; Thermo Fisher Scientific) equipped with a stainless cone-plate at C20/1° with a diameter of 20 mm, a cone angle of 1°, and a truncation of 0.052 mm was used. A few droplets of β-lg sample solutions were placed on the Peltier plate of the rheometer, which was temperature-controlled using a thermostatic Universal Temperature Control (UTC).

β-Lg sample solutions were previously sheared at 50 1/s for 60 s. Then, a shear sweep was performed from 40 to 115 1/s and back to 40 1/s.

Statistical analysis All experiments were performed in duplicate and measurements were replicated three times. A one-way analysis of variance (ANOVA) was performed and mean values were compared using Turkey's multiple comparison test. Differences were considered significant at $p<0.05$. All statistical analyses were performed using Minitab 16 for Windows.

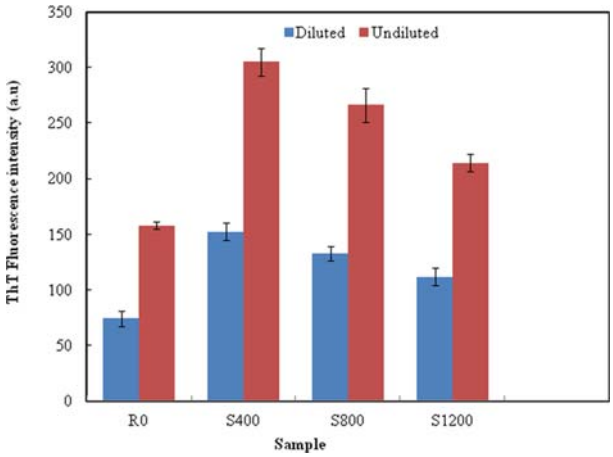

Fig. 1. ThT fluorescence intensity values of β-lactoglobulin fibril solutions as a function of stirring speed. Fibril solutions were heated at rest (series R0) or with constant stirring at 400 rpm (series S400), 800 rpm (series S800), and 1,200 rpm (series S1200). Diluted solutions were β-lactoglobulin fibril solutions diluted with pH 2.0 deionized water at a ratio of 1:1 (w/w).

Results and Discussion

ThT assay Thioflavin T (ThT) is a cationic benzothiazole dye with enhanced fluorescence upon binding with a protein assembly. Heating β-lg at a low pH causes fibrillation to occur, and ThT molecules will specifically bind to the β-sheet of fibrils to generate a fluorescent signal. Hence, the fluorescent signal generated is proportional to the fibril concentration (25). ThT fluorescence intensities of different fibril samples are shown in Fig. 1. Without thermal treatment, fibrillation does not occur. Hence, absence of fluorescence or marginal ThT fluorescence intensities of <10 a.u. were observed in unheated solutions, indicating an absence of fibrils in native β-lg solutions.

Stirring generates shear in β-lg solutions and flow of the solution upon heating enhances conversion of proteins into fibrils (19,26). Without stirring, the solution remains stagnant and, therefore, fewer proteins are converted into fibrils. Unstirred solutions in this study exhibited significantly ($p<0.05$) lower fluorescence intensity values than stirred solutions (R0). Heating without stirring of the solution at rest (R0) causes fewer spherulites to be converted into β-sheet fibrils (27). Nevertheless, fibril formation did not increase linearly with the stirring speed (Fig. 1). Thus, fibril formation was not dependent on the stirring speed.

ThT fluorescence development in S400 solutions stirred at 400 rpm suggested the greatest degree of fibril formation. There was a significant ($p<0.05$) decrease in fluorescence intensity values in S800 β-lg solutions of 266.23±15.21 a.u., compared with S400 solutions, and an even greater decrease in S1200 β-lg solutions of 214.25±7.98 a.u., compared with S400 and S800 solutions. In diluted fibril samples, ThT fluorescence intensities decreased by approximately two times, compared with undiluted fibril samples, because solutions were two-fold diluted using a 1:1 (w/w) ratio of water to solution. Loveday *et al.* (22) reported that the fluorescence intensity increases gradually during heating for up to 24 h, and that fibril yields do not change significantly at heating temperatures between 80 and 120°C, suggesting that increasing the temperature does not affect the balance between the fibrillation process and other reactions. A fibril solution heated at 80-85°C reportedly produced a higher fibril yield, compared with other heating temperatures (28), and heating at a higher temperature caused fibril hydrolysis and destroyed the β-sheet structure. Thus, 80°C was used as the heating temperature in this study.

TEM Change of β-lg monomers into fibrils upon heat treatment at pH 2 was confirmed using TEM images (Fig. 2). β-Lg appeared as a globular monomer before heat treatment (Fig. 2A-2C). However, heated solutions that contained β-lg both with and without stirring produced fibrillar aggregates (Fig. 2D-2O). The morphology of protein fibrils generally was long and oriented randomly with lengths of up to several μm. Without stirring, mixtures of long fibrils, short peptides, and small round structures were observed (Fig. 2D-2F). Oboroceanu *et al.* (29) suggested that the small round structures/aggregates consisted of disordered non-fibril peptides. With adequate stirring, β-lg produced long, unbranched, semi-flexible fibrils, similar to previous reports (15,22,27). Fibrils formed from S400 (Fig. 2G-2I) and S800 (Fig. 2J-2L) solutions were highly intertwined and did not differ in appearance. However, S400 solutions produced fibrils that were shorter in length and had a higher fibril density than S800 solution fibrils. A high stirring speed for S1200 solutions caused fragmentation and disruption of the fibrillar structure, which led to flocculation (Fig. 2M-2O).

TEM results in this study were in agreement with results from ThT

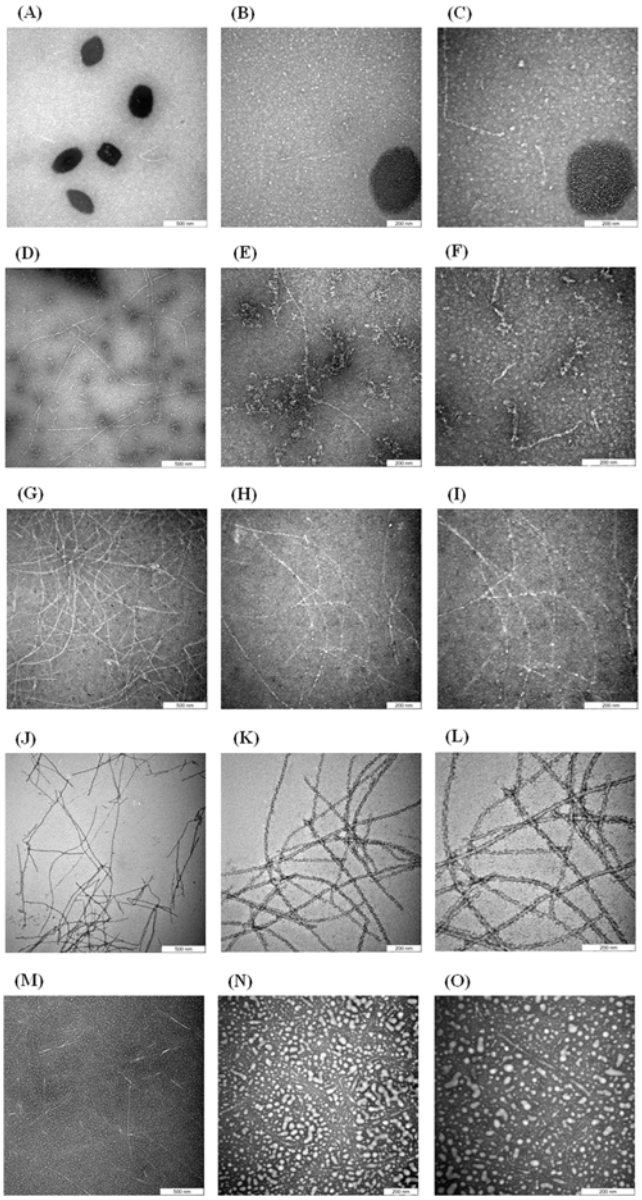

Fig. 2. TEM images of unheated β-lg solutions at different magnifications (A-C) and β-lg fibril solutions after heating for 20 h at rest (D-F), or with constant stirring at 400 rpm (G-I), 800 rpm (J-L), and 1,200 rpm (M-O). Magnifications from left to right were 50,000x, 100,000x, and 150,000x, respectively.

fluorescence assays. In unstirred solutions, lower ThT fluorescence values were not related to fibril destruction but were related to local gelation. Gel particles made fibril dispersion more difficult in the ThT reagent, resulting in lower ThT assay values (22). Heating of solutions at rest (R0) caused fewer spherulites to be converted into β-sheet fibrils (27), and stirring at 400 rpm in S400 solutions produced higher fibril densities, reflected in higher fluorescence intensity values obtained from ThT assays.

Turbidity Turbidity is a measurement used to quantify the degree to which light that is passing through a system is absorbed or scattered by suspended particles. Light scattering intensifies as the size and concentration of particles increases. Absorbance values of native β-lg solutions at 600 nm were 0.0025±0.0008, and there was a significant (p<0.05) increase in turbidity after heating of β-lg solutions as a function of stirring speed (Table 1). Heating of β-lg solutions reportedly caused formation of protein polymers and aggregates, contributing to light scattering and solution turbidity (30). Increasing the stirring speed resulted in a significant (p<0.05) increase in turbidity, compared with controls, indicating that formation of protein polymers and aggregates was promoted by higher stirring speeds. However, increased turbidity did not indicate higher solution fibril content. Increased turbidity could have been related to different species and different degrees of aggregation in protein solutions (31). Increased turbidity could have been due to either an increase in the size or an alteration in the relative size distribution of aggregates (24).

Table 1. Turbidity values of β-lactoglobulin fibril solutions

Sample[1)]	Undiluted	Diluted[2)]
R0	0.0330±0.0026$^{dA3)}$	0.0134±0.0004^{dB}
S400	0.0459±0.0030^{cA}	0.0270±0.0036^{cB}
S800	0.0935±0.0020^{bA}	0.0451±0.0030^{bB}
S1200	0.1290±0.0064^{aA}	0.0670±0.0013^{aB}

[1)]Solutions were heated at rest (series R0) or with constant stirring at 400 rpm (series S400), 800 rpm (series S800), and 1,200 rpm (series S1200).
[2)]Solutions were diluted using pH 2.0 deionized water at a ratio of 1:1 (w/w).
[3)]Mean±standard deviation (SD) (n=6) with different superscript letters (^{a,b,c,d}) indicate significant differences at p<0.05 within the same column. Mean±SD (n=6) with different superscript letters (^{A,B}) indicate significant differences at p<0.05 within the same row.

Not all protein monomers are converted into fibrils. Less than 40% of monomers are converted during the ibrillation process (32). β-Lg solutions contain a mixture of fibrils, aggregated spherulites, and non-aggregated peptides (32). Therefore, lower fluorescence intensity values in this study were observed in S1200 β-lg solutions than in S800 β-lg solutions. In addition, TEM images (Fig. 2M-2O) confirmed that β-lg fibril solutions contained a mixture of components rather than fibrils alone. Protein peptide clusters present in solutions disintegrated in water and, thus, decreased turbidity. In this study, the turbidity of β-lg solutions was decreased by 2x after dilution because β-lg solutions were diluted two-fold using pH 2.0 deionized water.

Particle size distribution The effect of stirring during heating of β-lg solutions on the particle size distribution was determined based on assays of particle size distributions at different stirring speeds. Intensity-based particle size distributions of β-lg solutions are shown in Fig. 3A. The wide size distribution of R0 solutions of approximately 20 to 1,000 nm indicates formation of irregularly sized fibrils. Two principal populations were observed in β-lg solutions that experienced stirring during heating. With stirring during heating at 80°C, the number of large β-lg particles increased (Fig. 3B). Therefore, stirring

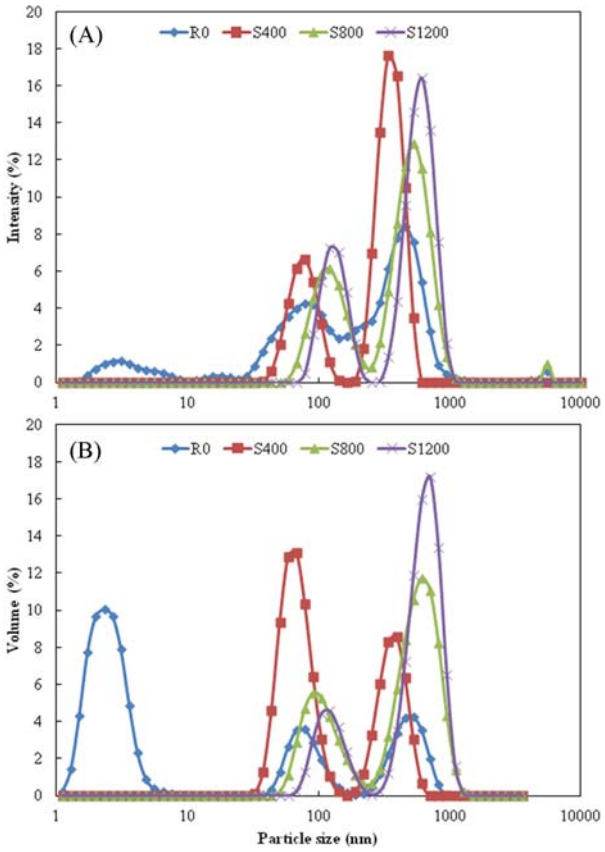

Fig. 3. Particle size distribution in β-lactoglobulin fibril solutions. (A) Particle size by intensity (B) particle size by volume of β-lactoglobulin fibril solutions heated at rest (diamond) and with constant stirring at 400 rpm (square), 800 rpm (triangle), and 1,200 rpm (cross).

induced formation of large β-lg aggregates during thermal treatment.

The presence of large particles was detected in S800 and S1200 β-lg solutions, indicating that a high stirring speed induced formation of aggregates or large particles. More large particles were formed as the stirring speed increased. Large particles formed in β-lg solutions increased the solution turbidity, which was consistent with turbidity results reported herein. A narrower distribution was evident in S400 solutions, indicating that fibrils, in addition to other aggregates in solutions, were relatively homogeneous in size at approximately 200 to 600 nm.

Zeta potential Zeta potential values of colloidal particles are used for stability studies of colloidal systems (33). All β-lg fibril solutions had positive zeta potential values ranging from +25.5 to +30.7 mV (Table 2), consistent with a recent report (34) in which β-lg solutions were shown to possess positive charges when heated at pH 2. Without heating, native β-lg solutions showed zeta potential values of +20.1±1.239 mV. There was a significant (p<0.05) increase in zeta potential values of β-lg solutions after thermal treatment. Zeta potential values of unstirred solutions (R0) were significantly (p<0.05) lower than for stirred solutions. However, different stirring speeds of 400, 800, and 1,200 rpm produced solutions with zeta potential values with no significant (p>0.05) differences from one another, indicating that stirring speed did not play an important role in regulation of the zeta potential of dispersion.

Table 2. Zeta potential values of β-lactoglobulin fibril solutions

Sample[1)]	Zeta potential (mV)	
	Undiluted	Diluted[2)]
R0	25.567±1.164[bA3)]	25.983±1.795[bA]
S400	30.000±1.193[aA]	30.800±1.584[aA]
S800	30.367±1.147[aA]	29.917±1.706[aA]
S1200	30.667±0.882[aA]	30.867±0.787[aA]

[1)]Solutions were heated at rest (series R0) or with constant stirring at 400 rpm (series S400), 800 rpm (series S800), and 1,200 rpm (series S1200).
[2)]Solutions were diluted using pH 2.0 deionized water at a ratio of 1:1 (w/w).
[3)]Mean±SD (n=6) with different superscript letters ([a,b]) indicate significant differences at p<0.05 within the same column. Mean±SD (n=6) with different superscript letters ([A]) indicate significant differences at p<0.05 within the same row.

Diluted fibril samples did not show any significant difference (p>0.05) in zeta potential values, similar to the non-diluted fibril samples. A positive or negative net charge on the protein surface provides a strong repulsive intermolecular interaction and prevents protein aggregation (34). Fibrils produced at pH 2.0 are in a repulsed state because the pH is far from the isoelectric point at approximately pH 5.0 (35). Particles with zeta potential values higher than +30 mV or lower than -30 mV are considered to be stable in colloidal systems. Therefore, stirring solutions during heating herein produced more stable dispersions.

Rheology Apparent viscosity values of undiluted and diluted β-lg fibril solutions, respectively, as a function of the shear rate are shown in Fig. 4A and 4B. Flow indices of all heated β-lg fibril solutions were similar and were categorized as non-Newtonian fluids with shear thinning specific to pseudoplastic fluids (36) over most of the covered shear rate range. β-Lg fibril solutions exhibited non-Newtonian behavior similar to unheated solutions (result not shown). The apparent viscosity was high at a low shear rate and decreased as the shear rate increased. Viscosity reflects the molecular movement of a viscoelastic body (37) and different molecular structures resulted in different viscosities. Long and heavily twisted molecular chains increased solution flow resistance and this change subsequently increased solution viscosity. The S1200 solution contained a larger volume of large aggregates (Fig. 3B) and, hence, the volume fraction of fibrils and the extent of fibril entanglement exerted effects on the viscosity of the fibril solution. Akkermans *et al.* (32) showed that viscosity values of WPI solutions increased upon addition of fibrils; however, this report should not be interpreted to indicate that more fibrils were present in S1200 solutions in this study. In fact, ThT fluorescence assay results herein demonstrated otherwise.

Different stirring speeds herein were shown to affect the characteristics of β-lg fibril solutions. Upon stirring, conversion of β-lg

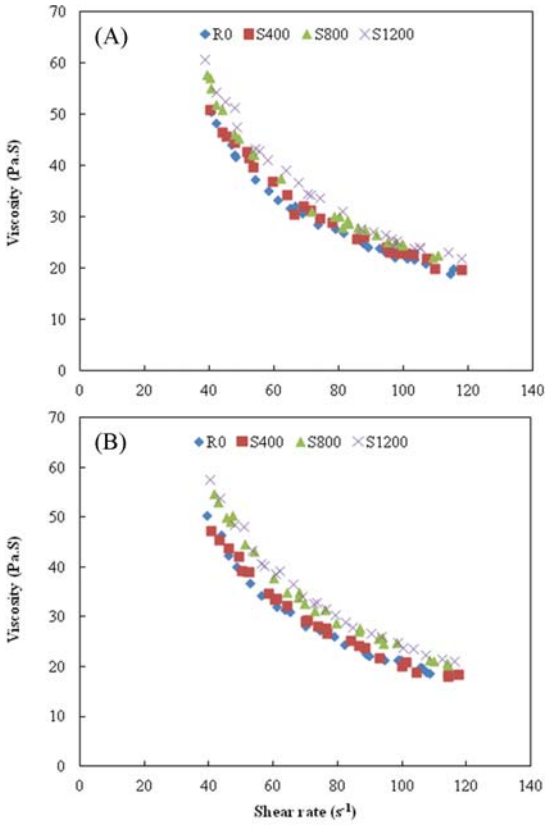

Fig. 4. Viscosity as a function of shear rate of (A) undiluted β-lactoglobulin fibril solutions heated at rest (diamond) and with constant stirring at 400 rpm (square), 800 rpm (triangle), and 1,200 rpm (cross). (B) represents viscosity as a function of shear rate of diluted β-lactoglobulin fibril solutions at a ratio of 1:1 (w/w), at pH 2.0 in deionized water.

into fibrils was enhanced and more stable and stronger β-lg solutions with repulsive forces were formed, compared with unstirred solutions. However, a high stirring speed is not feasible for producing fibril solution due to reduction of the fibril yield and changes fibril morphology. Future studies should focus on production of β-lg fibril solutions with consistent lengths and a predictable size distribution, in addition to a stronger repulsive state for β-lg solutions.

Disclosure The authors declare no conflict of interest.

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