Effect of Flow Field (Stirring) on the Heat-Induced Fibrillogenesis of β-Lactoglobulin in the Presence of Glucose at Neutral pH

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ABSTRACT: *The effect of stirring on the amyloid fibrillogenesis of β-Lactoglobulin (βLG) at pH 7 was studied in the presence of glucose (Glu). Fibrillogenesis was carried out by heating the 0.30 mM βLG solution at pH 7 with and without glucose (37.5 mM) for 24 hrs at ≥80°C under stirring (250 and 474 rpm) conditions. For control samples, βLG solutions with and without glucose were incubated under unstirred conditions. The flow-induced birefringence method was used to characterize the fibrillogenesis, revealing the coil-stretch transition and pointing out the existence of worm-like flexible fibrils in all samples. Atomic Force Microscopy (AFM) was used as a morphology that clearly showed the flexible fibrils in all samples and also revealed that the fibril lengths shortened on increasing the stirring rate. This shortening in lengths might be possible due to weak hydrophobic interaction at pH 7 resulting in fragmentation of fibrils over stirring. Glucose inhibited the fibrillogenesis of βLG even on stirring.*

KEYWORDS: *β-Lactoglobulin (βLG); Fibrillogenesis, Glucose (Glu); Flow-induced birefringence; Elongational flow field.*

INTRODUCTION

β-Lactoglobulin (βLG), a globular whey protein belongs to the lipocalin family of proteins [1]. It is found especially in cow and sheep's milk and other mammalian species except for human milk [2]. Its single polypeptide chain is composed of 162 amino acids and contains two disulfide bridges and a single free cysteine (Cys121) with a molecular weight of 18,400 Da [2, 3]. βLG monomer consists of nine anti-parallel β-strands and one α-helix [4]. Physiologically, βLG exists as a dimer but dissociates into monomers below about pH 3 with precise its native state [5].

βLG has been widely studied for the formation of fibrils because of its abundance and easy availability. βLG fibrils have a significant role in the food industries (to change the textural properties of the food products). Moreover, fibrils obtained *in vitro* also have an analogous structure to the amyloid fibrils formed *in vivo* during several neurodegenerative diseases (Alzheimer's disease,

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Prion disease, Parkinson's disease, Huntington's disease, etc.) [6, 7]. Fibrillogenesis of βLG has been studied under various conditions such as temperature [8], pressure [9], and chemical denaturants [10, 11]. Generally, heat-induced fibrillogenesis of βLG plays a very significant role in increasing the functionality of food products in the food industries and has been studied under various conditions (pH, concentration, and ionic strength) providing different sizes and morphology [12, 13].

Earlier, fibril formation of βLG and WPI (whey protein isolate) have been studied most frequently [14-17]. The kinetic studies of the heat-induced fibrillogenesis of different proteins including βLG and WPI (whey protein isolate) were performed with various processes (stirring, shearing, shaking, or mechanical agitation) using different techniques and demonstrated that the rate of fibrillogenesis enhanced in all processes [18-20]. In our previous study, we have also shown that the fibrillogenesis of βLG at pH 2 enhanced with the increasing stirring rate (0, 250, and 474 rpm) [21] but fibril length slightly decreased at a higher rate (474 rpm) due to the fragmentation. In the presence of reducing sugars, the structural effect of βLG has been studied due to its use to change the aroma, taste, and color of the food products in the food industries [22]. Reducing sugars interact to the amino acid via a well-known Maillard reaction gave an unstable Schiff's base. This Schiff's base condenses and oxidizes via Amadori rearrangement to produce brown nitrogenous polymers and copolymers [23-25] have investigated that glucose inhibited the heat-induced fibrillogenesis of βLG at neutral pH. As it is already discussed [19] we have also shown that the stirring enhanced the heat-induced fibrillogenesis of βLG at pH 2 [21] but at pH 7 and in the presence of glucose (Glu), the effect of stirring on the heat-induced fibrillogenesis of βLG is still unknown.

In this research article, the object of the study was to determine the effect of the flow field (stirring) on the fibrillogenesis of βLG at pH 7 in the presence of glucose (Glu). An elongational flow field birefringence study, a prominent technique was performed by using a Four-Roller Mill (FRM) to analyze the fibrillogenesis of βLG. The rotational diffusion coefficient, D_r, demonstrates the rigid-rod-like while coil-stretched transition for the worm-like flexible molecules can be elucidated easily by this birefringence study using FRM which gives the length of the grown fibrils.

EXPERIMENTAL SECTION

Materials

β-Lactoglobulin, (≥90%, lyophilized powder) was purchased from Sigma (product reference L0130, batch code SLBC2933L), D-(+)-Glucose anhydrous (batch code M3R2875) from Nacalai Tesque, Inc, Kyoto, Japan, Sodium dihydrogenphosphate dehydrate (code 317-18) from Nacalai Tesque, Inc, Kyoto, Japan and Disodium hydrogenphosphate (Lot KSP2903) from Wako Pure Chemical Industries, Ltd, Japan.

Glycation process and fibril formation:

An aqueous βLG protein solution was dialyzed against Milli-Q (pH 2) overnight to eliminate the cations and then freeze-dried. Glycation was performed by mixing 37.5mM glucose with 0.30mM βLG protein in 20 mL of 0.1 M, pH 7 sodium phosphate buffer solution. The glycated solution was then incubated at a high temperature (≥80°C) in a glass vessel for 24 hours. To show the effect of stirring on the fibrillogenesis of βLG in the presence of glucose, samples were incubated under rest and stirred conditions. Stirring of two different rates (250 and 474 rpm) was applied using a magnetic stirrer. At the same, control samples were also prepared without glucose under the same conditions. After heating, all samples were cooled at 4°C for 24 hours for measuring spanking birefringence.

Flow-induced birefringence measurements

Taylor's Four-Roller Mill (FRM) was set up as mentioned in our previous study [21] to measure flow-induced birefringence. All birefringence measurements were performed at room temperature and repeated several times to diminish the errors.

Atomic Force Microscopy (AFM)

AFM images were taken by using an MFP-3D-BIO™-AFM (Asylum Research UK Ltd. Oxford, UK). A sharp and V-shaped silicon nitride cantilever (Olympus Optical Co., Ltd., Japan) having a spring constant of 0.04 N/m was used. Samples for AFM imaging were prepared by diluting the fibril solutions with solvent (0.1mM, pH 7 Sodium phosphate buffer) as 1 in 100. Approximate 80μL of diluted samples were taken out using a micropipette suffused on the mica plate (10x10 mm) and kept at room temperature for 10 min to adsorb the fibrils. The Mica plate

was then rinsed out with a small amount of Milli-Q and left for 24 hours before imaging.

X-Ray Diffraction (XRD)

Powder forms of the samples were achieved by casting on the Taflon plate. X-ray data were collected by using Cu K_α radiation from a Micromax 007HF X-ray generator (Rigaku, Tokyo) at 40 kV and 20 mA. The diffraction images were recorded on an R-AXIS 4++ (Rigaku) imaging plate area detector. All diffraction data were collected by CrystalClear (Rigaku) and analyzed by FIT 2D (Andy Hammersley) software.

Thioflavin T (ThT) assay

Thioflavin T (ThT) has a characteristic property to diagnose the amyloid fibrils. To prepare the stock solution of 50 mL, 0.8mg/mL of ThT was mixed in sodium phosphate buffer (10 mM, pH 7.0) with 150 mM NaCl and filtered using a 0.22 μm syringe filter. This stock solution was stored in dark to stable for one week. 1 mL of stock solution was diluted with 50 mL of the same buffer and used as a working solution on the day of the analysis. An approximate 50 μL protein sample was mixed with 5 mL of working solution to analyze fluorescence spectra using a Fluorescence spectrophotometer (F-2500, Hitachi, Japan). Fluorescence spectra were measured at the excitation wavelength (λ_{ex}) of 450 nm and emission wavelength (λ_{em}) of 480 nm.

RESULTS AND DISCUSSION

The Secondary structure of βLG with and without glucose during heat-induced fibrillogenesis

X-Ray Diffraction (XRD)

Wide-Angle X-ray Diffraction (WAXD) data were obtained for the native and incubated βLG with and without glucose shown in Fig. 1. Two sharp and strong diffraction peaks were obtained at ~4.7 Å (meridional reflection) and ~10 Å (equatorial reflection) for all samples shown in Fig. 1(a) indicate the β-strands perpendicular to the fiber axis separated by 4.7-4.8 Å and β-sheets parallel to the fiber axis separated by 10-11 Å respectively. These diffraction peaks define the basic cross-β structure of the amyloid fibrils. On the other hand, native βLG also revealed similar diffraction peaks (Fig. 1(b)) to those obtained for incubated βLG and βLG+Glu indicating an exclusive secondary structure having few regions of β-sheet [8]. Native βLG showed higher diffraction intensity due to the highly dense sample than the incubated one.

Thioflavin T (ThT) assay:

Thioflavin T (ThT) is a benzothiazole dye that interacts with the amyloid fibrils and forms a very highly fluorescent complex. It was very difficult to distinguish between native and incubated samples using X-ray diffraction (XRD). Therefore, we have also monitored the β-Lactoglobulin fibrils with and without glucose using the ThT assay shown in Fig. 2. High fluorescence intensity was found for all incubated samples to those obtained for un-incubated ones and free ThT. This revealed that the ThT dye bound to the grooves formed by side chains of amino acids of the β-sheets in the amyloid fibrils resulting higher fluorescence intensity. However, in free ThT benzylamine and benzathiole rings rotate freely around their shared C-C bond which quenched the excited states causing low fluorescence intensity [26]. ThT does not interact with the native globular proteins.

Flow-induced birefringence measurements of stirred and unstirred βLG and βLG+Glu:

Our earlier study [21] has shown that a non-localized birefringence (evidence of the rigid-rod like molecules) was observed for the 4 wt % βLG incubated at ≥80°C, pH 2 under stirred and unstirred conditions. In the present study, a localized birefringence pattern along a streamline [See Video AVI βLG and AVI βLG+Glu] passing through the stagnation point towards the outlet direction was observed for the βLG with and without glucose heated at ≥80°C, pH 7 under stirred and unstirred conditions. This localized birefringence is attributed to the coil-stretch transition for the 'worm-like' flexible polymers in which birefringence intensity Δn starts to increase at a critical strain rate $\dot{\varepsilon}_c$ [27]. This implies that the 'worm-like' flexible fibril molecules were observed in the samples that were incubated at ≥80°C, pH 7 with and without glucose. Fig. 3 shows the birefringence intensity, Δn for the βLG and βLG+Glu under the stirred and unstirred conditions as a function of strain rate $\dot{\varepsilon}$. We found a very small (approximate negligible) critical strain rate $\dot{\varepsilon}_c$ for all samples. Therefore, it was very difficult to distinguish the degree of fibrillogenesis between all samples because critical strain rate $\dot{\varepsilon}_c$ is

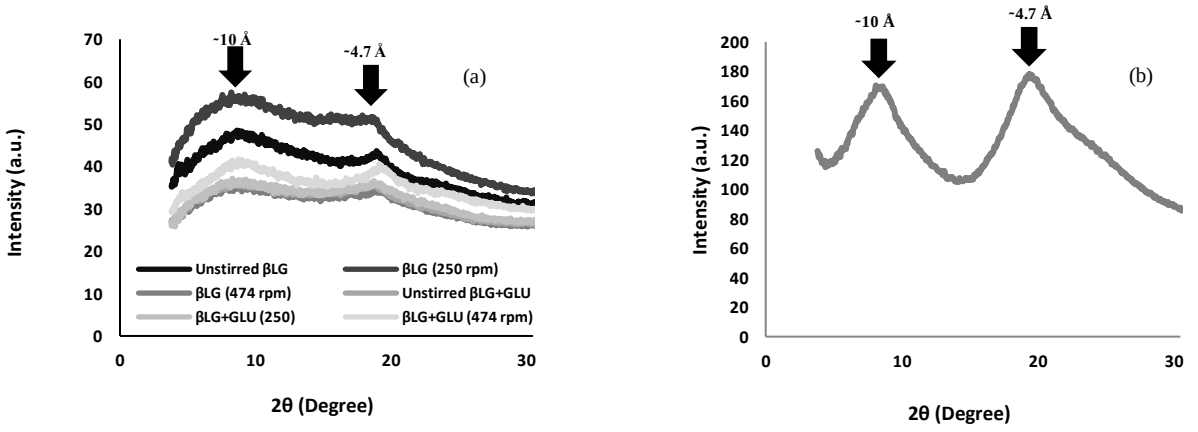

Fig. 1: Wide-angle X-ray diffraction (WAXD) data of (a) Incubated βLG with and without glucose under stirred and unstirred condition, and (b) Native βLG.

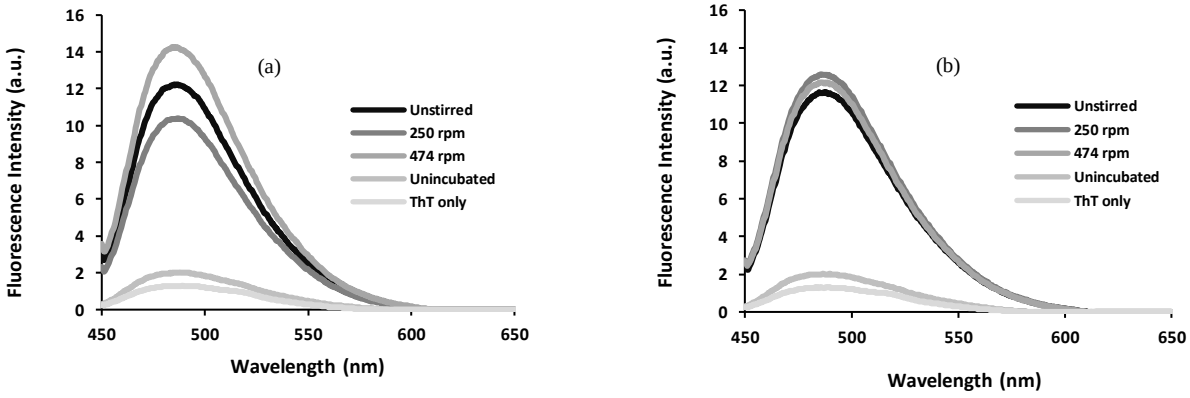

Fig. 2: Thioflavin T (ThT) data; (a) βLG, and (b) βLG+Glucose.

considered to be inversely proportional to the degree of fibril formation. This small critical strain rate $\dot{\varepsilon}_c$ might be possible due to the presence of small aggregated particles of protein, gave the non-localized birefringence (slightly uniform brightened area enclosed by four-roller) resulting in obstruction of critical strain rate $\dot{\varepsilon}_c$

Morphology of heat-induced βLG fibrils using Atomic Force Microscopy (AFM):

A morphological study of the fibrils obtained after heating the βLG with and without glucose at ≥80°C, pH 7 for 24 hrs was carried out which adhered to the birefringence study shown in Fig. 4. The AFM images clearly showed that the longer fibrils were obtained in the samples which were incubated without glucose. This suggests that glucose inhibits the fibrillogenesis of βLG already discussed in an earlier study [24]. Some aggregated particles were also found in all samples. In this study, our main focus was to study the effect of stirring on the βLG with and without glucose. AFM image [Fig. 4(a)] shows that the unstirred βLG sample heated at ≥80°C, pH 7 for 24 h has longer fibrils than those obtained in stirred [250 and 474 rpm] conditions [Fig. 4(b) ~ (c)]. In the presence of glucose, we also got the same result where the unstirred sample had longer fibrils [Fig. 4(d)] than stirred samples [Fig. 4(e) ~ (f)]. Histogram, a graphical representation [Fig. 5] was made to distinctly study the effect of stirring on the fibrillogenesis of βLG with and without glucose. In the case of βLG without glucose, the longest fibrils of the length 0.22~0.24 μm (frequency; f=2) for unstirred, 0.2~0.22 μm (f=1) for 250 rpm and 0.16~0.18 μm (f=1) for 474 rpm

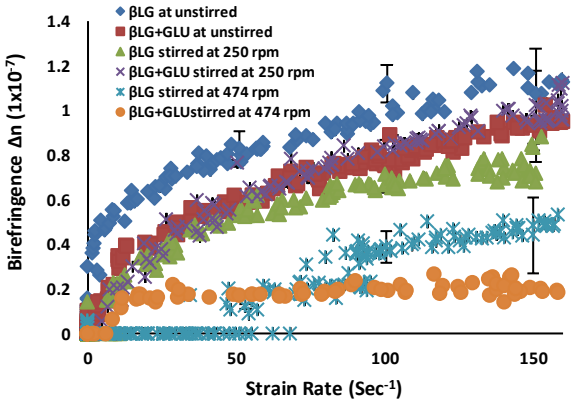

Fig. 3: Flow-induced birefringence (Δn) as a function of strain rate for the stirred and unstirred βLG and βLG+Glu solution at pH 7 incubated at ≥80°C for 24 h.

[Fig. 5(a)] were observed, whereas shortest fibrils having the length of 0.0~0.02 μm (f=5) for unstirred, 0.02~0.04 μm (f=3) for 250 rpm and 0.02~0.04 μm (f=9) for 474 rpm [Fig. 5(a)] were observed. Fibrils having the lengths 0.04~0.08 μm (f=85) for unstirred, 0.04~0.06 μm (f=37) for 250 rpm and 0.04~0.08 μm (f=86) for 474 rpm were most commonly observed. On the other hand, in the presence of glucose, fibrils had a length of 0.2~0.22 μm (f=1) for unstirred, 0.16~0.18 μm (f=3) for 250 rpm and 0.12~0.14 μm (f=2) for 474 rpm [Fig. 5(b)] were longest. The shortest fibrils were found in the range of 0.02~0.04 μm for all the conditions; unstirred (f=8), stirred at 250 rpm (f=40), and stirred at 474 rpm (f=7) [Fig. 5(b)]. Most of the fibrils were observed at 0.04~0.06 μm; unstirred (f=40), stirred at 250 rpm (f=145), and stirred at 474 rpm (f=48).

It has been observed from Fig. 5 (a) that the amyloid fibrils length decreased when we stirred the βLG solution at pH 7 which was incubated ≥80°C for 24 h. The order of the length of the fibrils was 474 rpm < 250 rpm < 0 rpm. The fragmentation of the fibrils on increasing the stirring rate was due to the weak hydrophobic interaction. Hydrophobic interaction plays an important role in the formation of amyloid fibrils but due to high pH this interaction weakens therefore at pH 7 we observed worm-like flexible fibrils. These flexible fibrils can fragment easily over stirring even at a small rate (250 rpm) and cannot be elongated to each other because of weak interaction resulting in shortening fibrils length on increasing stirring rate. On the other hand, it was also observed that the amyloid fibrils growth was inhibited for βLG solution at pH 7 which was incubated ≥80°C for 24 h in the presence of glucose [Fig (5b)]. Less number of amyloid fibrils were obtained for the βLG solution incubated in the presence of glucose as compared to the βLG solution incubated without glucose. Fig. 6 (a)~(c) clearly shows that the glucose inhibited the fibrillogenesis of βLG solution at pH 7 which was incubated ≥80°C for 24 h. In Fig. 6 (a), an abundance of long fibrils was obtained in unstirred βLG samples than those obtained in the presence of glucose. Similar results were obtained for those samples which were stirred at 250 rpm and 474 rpm [Fig. 6 (b)~(c)]. The inhibition of amyloid fibrils growth was due to the presence of Maillard reaction during the glycation of βLG solution [28]. The effect of stirring on the βLG solution at pH 7 in the presence of glucose was similar to those obtained for βLG solution without glucose and the order of the fibril length was 474 rpm < 250 rpm < 0 rpm however the fragmentation of fibrils during stirring of sample in the presence of glucose was slightly higher than those obtained for the samples without glucose showing in Fig. 6 (b)~(c).

CONCLUSIONS

In this study, we have investigated the effect of stirring on the glycated β-lactoglobulin solution at pH 7 incubated over ≥80°C for 24 hrs. The *Coil-stretch* transition was obtained from the birefringence measurement for the βLG solution with and without glucose at pH 7 incubated over ≥80°C for 24 hrs in stirred (250 and 474 rpm) and unstirred conditions, revealing that the worm-like flexible fibrils formed in all the samples which were also confirmed by the AFM study. The effect of stirring on the βLG solution with and without glucose at pH 7 incubated over ≥80°C for 24 h was analyzed and found that fibrils in all samples were fragmented over stirring in the order of 0<250 rpm<474 rpm due to the weak hydrophobic interaction between the fibril molecules at pH 7. We have also found that the fragmentation of fibrils during stirring of βLG solution in the presence of glucose was slightly higher than those obtained in the absence of glucose Maillard reaction between glucose and βLG did not play a crucial role in the effect of stirring but inhibited the fibrillogenesis of βLG even on stirring, confirmed by AFM images. β-lactoglobulin and other whey proteins are widely used as functional ingredients therefore this study can be useful in the food industries to change the textural properties through fixing the rate of the flow field.

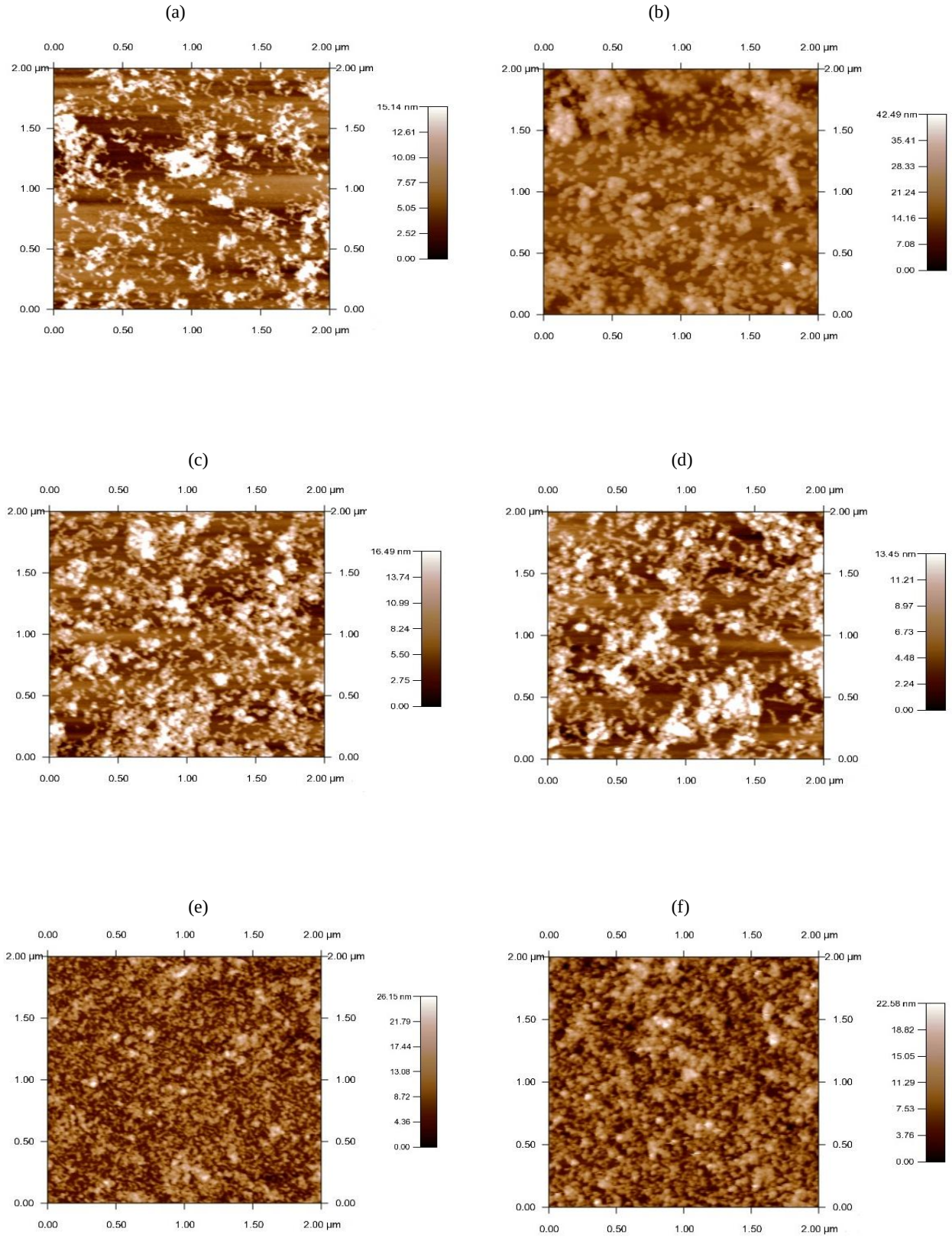

Fig. 4: AFM images of (a) Unstirred βLG, (b) Stirred βLG (250 rpm), (c) Stirred βLG (474 rpm), (d) Unstirred βLG+Glu, (e) Stirred βLG+Glu (250 rpm), and (f) Stirred βLG+Glu (474 rpm) at pH 7 incubated at ≥80°C for 24 h.

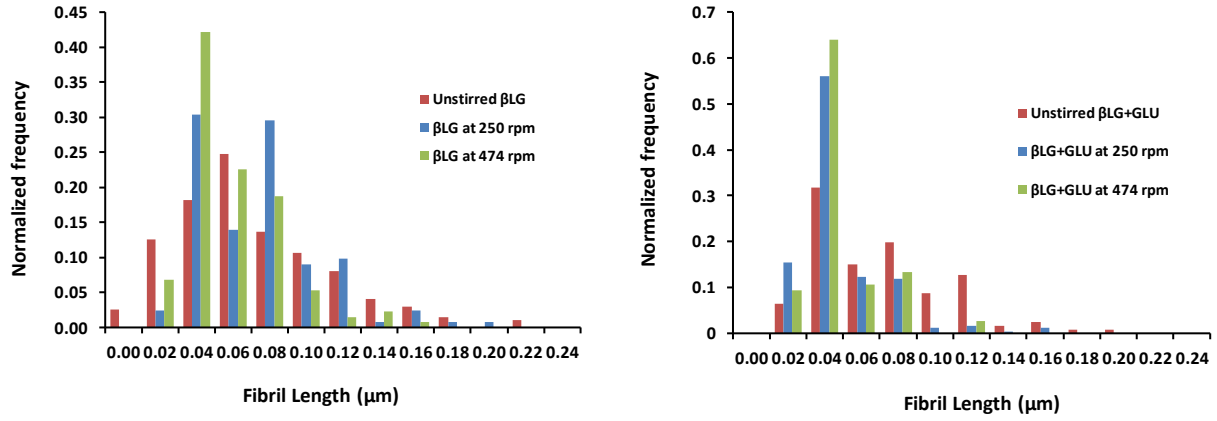

Fig. 5: Histograms of fibrils length from AFM images of stirred and unstirred (a) βLG, and (b) βLG+Glu solution at pH 7 incubated at ≥80°C for 24 h.

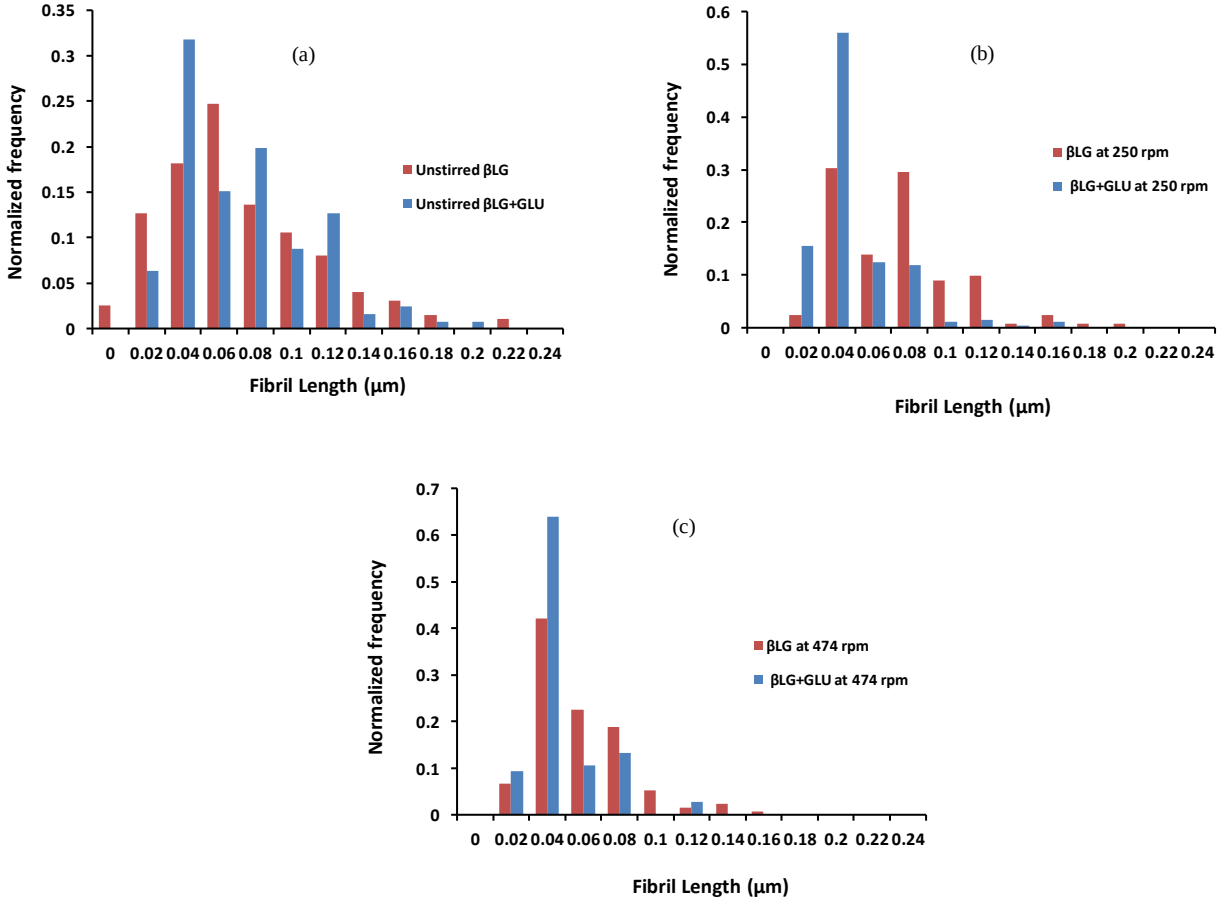

Fig. 6: Histograms of fibrils length from AFM images of βLG and βLG+Glu (a) Unstirred (b) Stirred (250 rpm) and (c) Stirred (474 rpm) solution at pH 7 incubated at ≥80°C for 24 h.

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