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FTIR Spectroscopic Study of Heat-Induced β-Lactoglobulin Solution under Flow Field

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Effect of stirring on the secondary structure of amyloid fibrillogenesis of β-lactoglobulin (βLG) at pH 2 and at pH 7 with and without glucose was studied. Fibrillogenesis at pH 2 was carried out by heating the 4w % β-lactoglobulin and at pH 7 by heating the 0.30 mM β-lactoglobulin in 0.1 M, pH 7 sodium phosphate buffer solution with and without glucose (37.5 mM) for 24 h under stirring (250 and 474 rpm) conditions. For control samples, β-lactoglobulin solutions were incubated under unstirred condition at pH 2 and pH 7. The secondary structure of the amyloid fibrils which corresponds the β-sheet structure was studied by using Fourier transform infrared (FTIR) spectroscopy revealed that the stirring does not affect the secondary structure of the β-lactoglobulin fibrils at pH 2 as well as pH 7 with and without glucose.

Keywords: β-Lactoglobulin, Fibrillogenesis, Glucose, FTIR.

INTRODUCTION

Amyloids are insoluble fibrous protein aggregates sharing specific structural traits. Amyloid is known to be made up of proteins which have lost their native structure and turned into insoluble β-sheet polymers [1]. The fibrils can be created *in vitro* from a range of different proteins and peptides. Globular proteins generally function in the form of monomers or small oligomers in aqueous solution both *in vivo* and *in vitro* and are mainly stabilized by electrostatic and/or hydrophobic interactions. There are approximately 20 known amyloid diseases have been reported in patients with neurodegenerative diseases including Alzheimer's disease, Prion disease, Parkinson's disease and Huntington's disease [2-4]. Disease-related protein fibrils were found to be identical to those obtained *in vitro*, with an identical fibrillar structure independent of the amino acid sequence.

β-Lactoglobulin (βLG) is a globular whey protein widely used in food industries. It is a major component (about 60 %) of the whey protein isolate (WPI) and belongs to lipocalin family of proteins [5]. It is found especially in cow and sheep's milk and other mammalian species except for human milk [6]. Its single polypeptide chain is composed of 162 amino acids with anti-parallel β-strands and one α-helix [7]. It contains two disulphide bridges and a single free cysteine (Cys121) with a molecular weight of 18,400 Da [1,8]. β-Lactoglobulin contains two major variants (A and B) in bovine milk differing by two amino acids aspartic acid (Asp) and valine (Val) in variant A which replaced by glycine (Gly) and alanine (Ala) in variant B at two positions 64 and 118 [6].

Physiologically, β-lactoglobulin exists as a dimer but dissociates into monomers below about pH 3.0 without disturbing its native state [9]. Its isoelectric point is near about ~ 5.1 pH. At neutral pH, it exists as a dimer while at below pH 3.0 and above pH 8.0 exists as a monomer [10,11]. β-Lactoglobulin can form fibrils upon heating above its denaturation temperature (75 °C) under acidic condition (pH 2) and low ionic strength [12,13] or by adding denaturants [14,15]. Heat-induced fibrils from β-lactoglobulin were more extended, smoother and markedly longer in length than water-alcohol-induced fibrils at pH 2 [14]. Fibril formation upon heating dominates by the interchange of disulphide bonds and hydrophobic interactions. Fibrillogenesis of the β-lactoglobulin proceeds through breaking the disulphide bridges and followed by reacting the free sulphydryl groups with each other [16]. Heating under neutral pH, β-lactoglobulin forms short rod-like particles [14]. Fibrillogenesis of β-lactoglobulin also depends on the protein concentration. Arnaudov *et al.* [13] reported that the fibril formation of β-lactoglobulin occurs at a critical concentration (2.5 wt %). However, fibrils also observed at low concentration [13]. β-Lactoglobulin converts in to fibrils with increasing concentration and most commonly observed

at 4 wt % while β-lactoglobulin forms gel when protein concentration is sufficient high [17,18].

The fibrillogenesis of β-lactoglobulin has generally been attributed to a nucleation growth mechanism [13,19]; nuclei are initially formed in the lag phase and this process is commonly assumed to be the rate-determining step of fibril formation. The nucleation phase is followed by the growth phase, in which nuclei are elongated and start to grow the fibrils by attaching monomers. The nucleation growth mechanism of fibrillogenesis can be influenced by several factors such as seeding [20], stirring [21], shearing [22] and sonication [23]. Various techniques have been used earlier to analyze the effect of flow field on the fibrillogenesis of β-lactoglobulin and WPI (whey protein isolate) [24-26]. We have analyzed the effect of flow field (stirring) on the fibrillogenesis of β-lactoglobulin at pH 2 [27] and also pH 7 with and without glucose [28] incubated at ≥ 80 °C for 24 h.

The present work is a continuation of our previous work in which we are characterizing the secondary structure of β-lactoglobulin fibrils by using Fourier transform infrared (FTIR) spectroscopy. Structural study of β-lactoglobulin and its fibrils has been done by many researchers [29]. However, FTIR study of the β-lactoglobulin at pH 2 and pH 7 with and without glucose incubated at ≥ 80 °C for 24 h under flow field still needed more attention for the wide-spread application of fibrils.

EXPERIMENTAL

β-Lactoglobulin fibril formation: β-Lactoglobulin, from bovine milk (≥ 90 %, Lyophilized powder) was purchased from Sigma (product reference L0130, batch code 030M7025V). β-Lactoglobulin protein was dissolved in Milli-Q and dialyzed with Milli-Q (pH 2 HCl) for 24 h at room temperature to remove the contaminants. The dialyzed protein solution then freeze dried by using liquid nitrogen and operated vacuum pump for overnight. To prepare the stock solution (20mL of 4 % w/w at pH 2), β-lactoglobulin protein (freeze dried) dissolved in 20 mL Milli-Q (pH 2 HCl) and stirred about 30 min until dissolve completely. This solution was heated in a glass vessel at high temperature (≥ 80 °C) for 24 h using metal heating plate attached with a magnetic stirrer. On the other hand, 20 mL of 0.30 mM β-lactoglobulin sample was prepared at pH 7 by using 0.1 M, pH 7 sodium phosphate buffer solution and also glycated with 37.5mM glucose and heated at ≥ 80 °C for 24 h. All samples were prepared under stirred and unstirred condition. The rate of the stirring was fixed at about 250 and 474 rpm. After heating, samples were cooled at 4 °C.

Atomic force microscopy (AFM): Atomic force microscopy 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 spring constant of 0.04 N/m was used. Samples for AFM imaging were prepared by diluting the fibril solutions with their respective solvents as 1 in 100. Approximate 80 µL of diluted samples were taken out using a micro pipette and suffuse on the mica plate (10 × 10 mm) and kept at room temperature for 10 min to adsorb the fibrils. Mica plate was then rinsed out with a little amount of Milli-Q and left to dry in the desiccator for 24 h before imaging.

Fourier transform infrared (FTIR) spectroscopy: FTIR measurements were carried out by using the polyethylene substrates (Type 61 polyethylene 3M IR card) at which β-lactoglobulin fibril solutions (stirred and unstirred) were applied and waited for completely dry. FTIR 700 (JASCO, Tokyo, Japan) spectrometer was used for FTIR analysis.

RESULTS AND DISCUSSION

Secondary structure of heat-induced fibrillogenesis of β-lactoglobulin solution at pH 2: Effects of stirring on the heat-induced fibrillogenesis of β-lactoglobulin at pH 2 have been studied in previous study [27] and shown that the fibrillogenesis enhanced with stirring rate but slightly decreased at higher stirring rate due to the fragmentation of fibrils. Rigid-rod like fibrils were observed after heating the β-lactoglobulin sample at ≥ 80 °C for 24 h at pH 2 as shown in Fig. 1(a). The secondary structure of proteins associated with the "β-sheet" which is the common structure of the amyloid fibrils. Generally, FTIR spectra of the proteins give the strong absorption band in the 1700-1600 cm^{-1} region (amide I) indicate the C=O stretching mode of the peptide chain [30-32]. In this amide I region (1700-1600 cm^{-1}), the band appears at 1660-1640 cm^{-1} for α-helix and random coils and also band at 1640-1620 cm^{-1} appears for β-sheet. These bands in the FTIR spectra have also explained the secondary structure of the β-lactoglobulin [33-36]. Fig. 2 shows the FTIR spectra of stirred (250 and 474 rpm) and unstirred 4 % w/w β-lactoglobulin solution at pH 2, heated at ≥ 80 °C for 24 h. One sharp and strong absorption band appeared in the amide I region at1636 cm^{-1} for unstirred, 1633 cm^{-1} for stirred (250 rpm) and 1636 cm^{-1} for stirred (474 rpm) β-lactoglobulin solution which corresponds to the formation of the β-sheet. Moreover, two sharp bands also appeared at 1540 and 1233 cm^{-1} for unstirred solution which describe the N-H bending coupled with the C-N stretching (amide II, about 1550 cm^{-1}) and C-N stretching coupled with the N-H bending (amide III, 1250 cm^{-1}) of the peptide chain, respectively. Similarly for stirred (250 rpm) solution at 1538 and 1233 cm^{-1} and for stirred (474 rpm) at 1542 and 1236 cm^{-1}. These bands (amide II and amide III) also indicate the secondary structure of the proteins [30,37,38]. It is clear from the FTIR results that the spectra for stirred (250 and 474 rpm) and unstirred solutions nearly similar, existing β-sheet structure, therefore, there was no effect of stirring on the secondary structure of 4 % w/w β-lactoglobulin solution at pH 2 that was heated at ≥ 80 °C for 24 h. FTIR bands for the native β-lactoglobulin also show the similar bands to those incubated because native β-lactoglobulin has β-sheets already.

Secondary structure of heat-induced fibrillogenesis of β-lactoglobulin solution at pH 7 with and without glucose: Worm-like flexible fibrils were observed for the β-lactoglobulin solutions with [Fig. 1(c)] and without glucose [Fig. 1(b)] at pH 7 incubated at ≥ 80 °C for 24 h under flow fields also discussed in previous study [28] showed that the fibrils length were decreased with stirring rate due to the weak hydrophobic interaction between fibrils. However, glucose slightly inhibited fibrillogenesis of β-lactoglobulin while it did not play any role on the effect of stirring over fibrillogenesis. FTIR spectroscopic studies were also done to describe the secondary

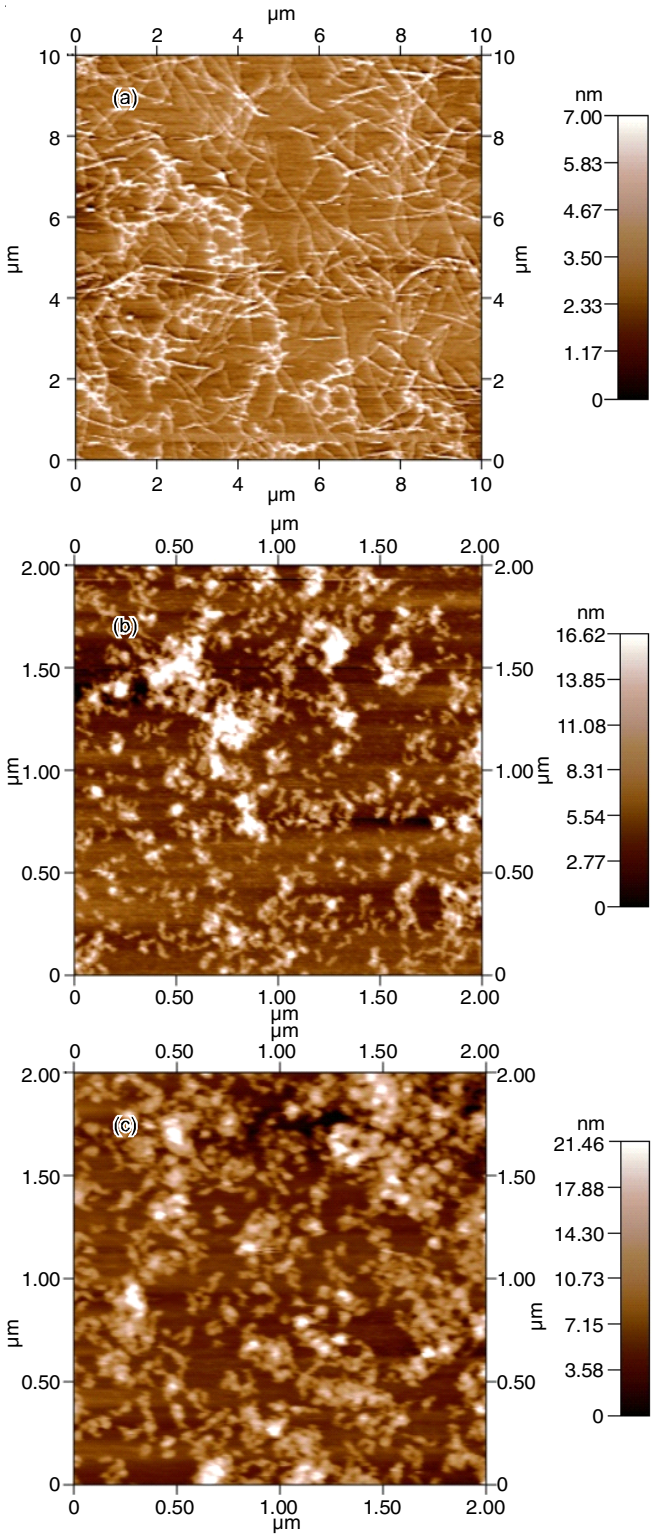

Fig. 1. AFM images of β-lactoglobulin (βLG) amyloid fibrils; (a) at pH 2 (b) at pH 7and(c) at pH 7 with glucose incubated at ≥ 80 °C for 24 h

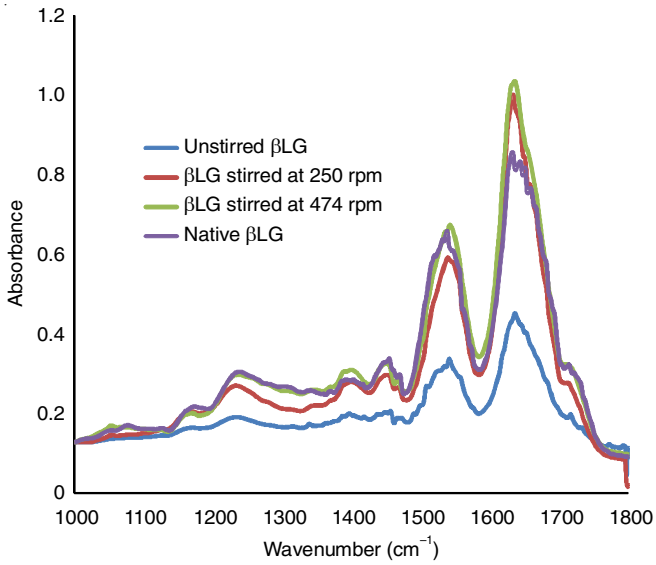

Fig. 2. FTIR spectra of β-lactoglobulin (βLG) solution at pH 2 incubated at ≥ 80 °C for 24 h under stirring conditions (0, 250 rpm and 474 rpm)

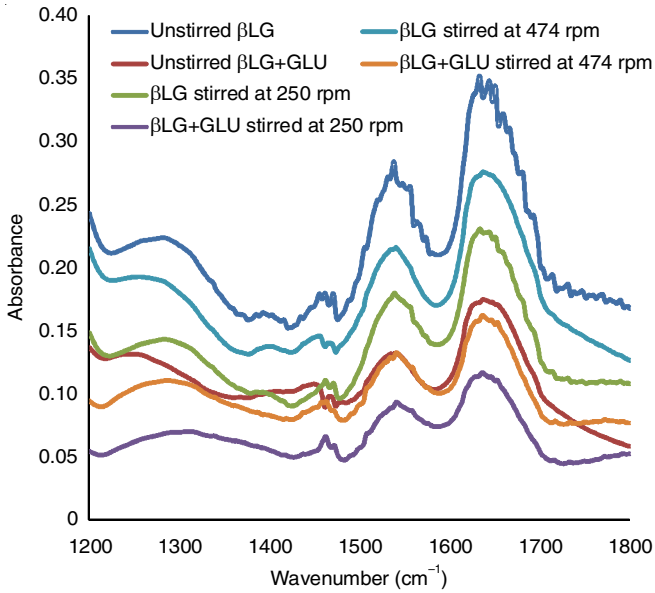

Fig. 3. FTIR spectra of β-lactoglobulin (βLG) solution at pH 7 incubated at ≥ 80 °C for 24 h under stirring conditions (0, 250 rpm and 474 rpm) with and without glucose

structure of the β-lactoglobulin fibrillogenesis at pH 7 incubated at ≥ 80 °C for 24 h under flow fields with and without glucose shown in Fig. 3. For the samples without glucose, a strong absorption bands of 1633 cm^{-1} for unstirred, 1634 cm^{-1} for stirred (250 rpm) and 1637 cm^{-1} for stirred (474 rpm) were found in the amide I region which corresponds the C=O stretching mode of the peptide chain. Amide I bands are the main bands to describe the secondary structure of the fibrils. Two more bands which are also described the secondary structures were found in the amide II and amide III regions. Absorption bands 1537 cm^{-1} in the amide II and 1281 cm^{-1} in the amide III regions corresponds to the N-H bending coupled with the C-N stretching and C-N stretching coupled with the N-H bending of the peptide chain respectively for the unstirred case. 1538 and 1283 cm^{-1} for the samples stirred at 250 rpm while 1540 and 1255 cm^{-1} for the samples which were stirred at 474 rpm were also observed in the amide II and amide III regions. On the other hand, β-lactoglobulin samples which were heated with glucose under flow fields also gave the similar bands as without glucose showing in Fig. 3. One strong band in the amide I region was observed at 1637 cm^{-1} for unstirred, 1636 cm^{-1} for stirred at 250 rpm and also 1636 cm^{-1} for stirred

at 474 rpm. Similar to the without glucose samples, samples containing also corresponds the bands in amide II and amide III regions. 1540 and 1248 cm^{-1} for unstirred, 1540 and 1312 cm^{-1} for the sample stirred at 250 rpm and 1540 and 1287 cm^{-1} for the stirred at 474 rpm.

Amide I bands are mostly similar for the sample which were stirred at a different stirring rate in both conditions (pH 2 and 7). Absorption bands for the β-lactoglobulin samples incubated at pH 7 with and without glucose are broadened and weaker than those observed at pH 2. This broadening and weakening in absorption bands show that samples at pH 7 contain immature fibrils with aggregation of the protein. It mostly affects in the amide III region corresponds to the C-N stretching coupled with the N-H bending (amide III, ~ 1250 cm^{-1}) of the peptide chain.

Conclusion

Rigid rod-like fibrils were observed for the β-lactoglobulin incubated at ≥ 80 °C for 24 h at pH 2, while worm-like at pH 7 with and without glucose. FTIR study shows no effect of stirring on the secondary structure of β-lactoglobulin fibrils at pH 2 as well as pH 7 with and without glucose which is described by the absorption bands in the amide I region corresponds strongly the existence of β-sheets in the fibrils.

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