Atomic force microscopy and Raman scattering spectroscopy studies on heat-induced fibrous aggregates of β -lactoglobulin

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Abstract. Nanometer-thick fibrous aggregates of β -lactoglobulin alone and its mixture with other globular proteins were formed by heating aqueous solutions at pH 2 with maintaining an effective level of electrostatic repulsion among denatured protein molecules. In atomic force microscopy (AFM) images, these fibrous aggregates appeared to be fairly uniform in width and height and composed of strings of globular elements. Fibrous aggregates formed in β -lactoglobulin individual systems were only slightly thicker than the size of the native β -lactoglobulin monomer, while those formed in the presence of other globular proteins were more than twice thicker, suggesting that different species of globular proteins were incorporated into each individual fibrous aggregate in the mixed systems. At neutral pH, aggregates were generally composed of ellipsoidal primary particles much larger than the size of the monomer, suggesting that aggregation proceeds in two steps at neutral pH. Molecular structural changes probed by Raman scattering spectroscopy revealed that considerable fractions of β -sheet structures remained to be folded during the formation of fibrous aggregates but α -helix structures were partially lost. It was also suggested that a limited extent of hydrophobic interactions among heat-denatured protein molecules is required for the fibrous aggregation.

1. Introduction

The formation of fibrous aggregates from heat-denatured globular proteins is not only an important subject in food science [1–5] but also potentially relevant to the so-called amyloidosis such as Alzheimer's and prion diseases [2,6]. When heated in an aqueous solution, globular protein molecules unfold at least partially and aggregate via hydrophobic interactions among the exposed hydrophobic core that is inaccessible to the solvent in the native state. Aggregates are usually particulate in shape with a diameter in the order of micrometers [1]. A white opaque gel, like boiled egg white, is formed if the protein concentration exceeds a given critical concentration [1]. However, if pH is shifted far away from the isoelectric point of the protein and the ionic strength is maintained at a sufficiently low level, suppressed electrostatic screening results in slow aggregation and eventually the formation of finely stranded aggregates, the thickness of which is in the order of nanometers [1,5]. The heated solution in this case may form a macroscopic gel but remains to be translucent or even transparent.

It is considered that various globular proteins without structural similarities can form fibrous aggregates when protein molecules are only partially denatured and aggregate slowly [6]. However, exact mechanisms of the fibrous aggregation of globular proteins have not yet been elucidated. Predominantly

β -sheet secondary structures are known to be a common conformational feature in fibrously aggregated proteins and peptides [6,7], while relatively little information is available regarding intermolecular crosslinking sites. The aggregation rate is likely to be regulated predominantly by the balance between electrostatic repulsive and van der Waals attractive forces but a possibility of involvements of other intermolecular forces cannot be excluded [3,8].

β -Lactoglobulin, the major globular protein in bovine milk, is often used as a model globular protein in branches of biomedical and biophysical fields due to its abundant supply and ease of purification. β -Lactoglobulin is known to be relatively heat-stable: heating at 80°C causes only a ca. 10% increase in radius of gyration [9]. In the food industry, enormous amounts of protein ingredients containing β -lactoglobulin are continuously produced as byproducts from cheese manufacture. Effective utilization of such ingredients is thus a vital issue of the industry. So-called whey protein isolates (WPIs) are highly purified protein ingredients, containing normally ca. 70% w/w of β -lactoglobulin, with other minor globular proteins in milk, such as α -lactalbumin, bovine serum albumin, and immunoglobulins, and only ignorable amounts of non-protein components. The goal of this study was to gain insights into fibrous aggregation phenomena of β -lactoglobulin in individual or mixed globular protein systems. Structures of heat-induced aggregates formed in β -lactoglobulin and WPI solutions were visualized using atomic force microscopy (AFM). Molecular structural changes during heat-induced aggregation of β -lactoglobulin were investigated by Raman scattering spectroscopy.

2. Materials and methods

2.1. Materials

Three times crystallized β -lactoglobulin (a mixture of variant A and B, product no. L-0130) was purchased from Sigma Chemicals (St Louis, MO). WPI was a Bi-Pro grade product supplied by Davisco Foods International (LeSuer, MN). Other chemicals were of reagent grade quality. Protein samples were dissolved in distilled water or 0.1 or 0.3 mol/dm³ NaCl aqueous solutions. The protein solutions were adjusted to pH 7, 5.4, or 2 by adding small amounts of hydrochloric acid or sodium hydroxide.

2.2. Atomic force microscopy

Two to eleven percent protein solutions were heated in sealed Pyrex tubes immersed in a hot water bath preset at 80°C for pre-specified times and diluted to protein concentrations of ca. 40–44 μ g/ml. Aliquots (2 μ l) were taken immediately, spread onto freshly cleaved mica sheets, and imaged under butanol. Imaging under butanol was preferable in order to avoid unfavorable interactions between the probe and sample. AFM images were produced using an East Coast Scientific (Cambridge, UK) manufactured atomic force microscope located at the Institute of Food Research (Norwich, UK). Direct current contact mode was employed for imaging: topographical images were generated from vertical movements of the sample during scanning necessary to maintain the preset cantilever deflection and complementary error signal mode images were generated based on the slight fluctuations of the cantilever deflection around the preset value.

2.3. Raman scattering spectroscopy

Fifteen percent β -lactoglobulin solutions were heated in sealed containers in a water bath preset at 80°C for 60 min to form gels, cooled to room temperature, and kept in a cold room (5°C) overnight. Raman scattering spectra were recorded on a Raman microscope (System 1000, Renishaw plc, Old Town, UK) with excitation from the 782 nm line of a titanium sapphire crystal laser (Mira model 900-P, Coherent Inc., Santa Clara, CA). A drop (50 μ l) of the unheated sample solution or a gel sample that was cut into a disk (3 mm in diameter and 2 mm in height) was placed on a quartz plate. The laser was focused through a $\times 50$ objective at a power of 50 mW on the sample surface. Back-scattering was then collected with the same objective and captured using a Peltier cooled charge-coupled-device (CCD) array detector. The accuracy of the wavenumber was checked daily using the 520 cm^{-1} band of silicon. Each spectrum was smoothed with the 15 points fifth degree Savitsky–Golay function using the Grams/386 software (Galactic Industries Corporation, Salem, NH).

3. Results and discussion

3.1. Atomic force microscopy

Nanometer scale structures of fibrous aggregates of globular proteins have been investigated traditionally using transmission electron microscopy [1]. However, electron microscopy images can be influenced by inevitable elaborate preparation procedures. AFM imaging is normally conducted in atmospheric pressure, thus providing an alternative microscopy method suitable for investigating normally hydrated biopolymer samples [5,10,11]. Samples to be imaged by AFM are generally examined on a flat and rigid substrate [10]. Biopolymer samples are usually deposited onto freshly cleaved mica surfaces [10].

Heat-treated protein solutions were diluted to 40–44 $\mu\text{g/ml}$ before spread onto mica in this study. These concentrations were found to be optimal for observing heat-induced aggregates in preliminary tests. Figure 1 is an image of unheated 40 $\mu\text{g/ml}$ β -lactoglobulin spread onto mica. Height profiles in the images revealed a multilayer coverage of β -lactoglobulin molecules on the mica surface at this concentration, while there was no indication of the existence of large aggregates.

Figure 2 represents fibrous aggregates formed in a β -lactoglobulin aqueous solution at pH 2 by heating at 80°C for 90 min in the presence of 0.1 mol/dm^3 NaCl. Addition of 0.1 mol/dm^3 NaCl was necessary at this pH in order to reasonably accelerate the aggregation rate and avoid partial precipitation of aggregates. The width and height of these strands are fairly uniform although the observed widths are almost an order

Fig. 1. Topographical AFM image (2 $\mu\text{m} \times 2 \mu\text{m}$) of native β -lactoglobulin spread onto mica.

Fig. 2. Topographical (a) and equivalent error signal mode AFM image ($1.2\ \mu\text{m} \times 1.2\ \mu\text{m}$) (b) of β -lactoglobulin aggregates formed in a 2% w/w aqueous solution at pH 2 by heating at 80°C for 90 min in the presence of $0.1\ \text{mol/dm}^3$ NaCl.

Fig. 3. Topographical (a) and equivalent error signal mode AFM image ($5\ \mu\text{m} \times 5\ \mu\text{m}$) (b) of WPI aggregates formed in a 2% w/w aqueous solution at pH 2 by heating at 80°C for 180 min in the presence of $0.1\ \text{mol/dm}^3$ NaCl.

of magnitude larger than the measured heights. Such effects are well-known as probe broadening effects, the extent of broadening being determined by the shape and curvature of the probe tip and objects [10]. The measured heights of fibrous aggregates in Fig. 2 were ca. 4 nm, only slightly thicker than the diameter of the monomer (3.7 nm) [12]. Therefore, these fibrous aggregates are likely to be composed of strings of partially unfolded β -lactoglobulin monomers.

WPI was also found to form fibrous aggregates at pH 2 by heating at 80°C in the presence of $0.1\ \text{mol/dm}^3$ NaCl (Fig. 3). The width and height of the fibrous aggregates were once again fairly uniform. However, the measured heights were ca. 10 nm, suggesting that these strands were composed not only of β -lactoglobulin but also other globular proteins and/or that the presence of the other proteins modified the aggregation mechanisms of β -lactoglobulin. Previous studies in the literature suggest that β -lactoglobulin can be coupled with α -lactalbumin, the second major protein in WPI, to form heat-induced aggregates [13]. Further investigation using a modified surface probe microscopy would be useful for validating this hypothesis. AFM with antibody derivatized probes or scanning near-field optical microscopy with fluorescently labeled proteins may be used to differentiate protein species coupled in an aggregated fiber.

At neutral pH without added salt, β -lactoglobulin forms a translucent gel if the protein concentration is sufficiently high. Electron microscopy images have revealed such a gel is composed of monomer/dimer-thick fine strands [14]. On the other hand, scattering studies have proposed that heat-induced aggregation of β -lactoglobulin at neutral pH is a two-step process, consisting of the formation of primary globular aggregates, designated as globules, and the subsequent aggregation of the globules [15]. The present

Fig. 4. Topographical (a) and equivalent error signal mode AFM image ($5\ \mu\text{m} \times 5\ \mu\text{m}$) (b) of β -lactoglobulin aggregates formed in a 2% w/w aqueous solution at pH 7 by heating at 80°C for 60 min in the absence of added salt.

Fig. 5. Topographical (a) and equivalent error signal mode AFM image ($7\ \mu\text{m} \times 7\ \mu\text{m}$) (b) of WPI aggregates formed in an 11% w/w aqueous solution at pH 7 by heating at 80°C for 60 min in the absence of added salt.

AFM images of heat-induced β -lactoglobulin aggregates formed at pH 7 without added salt revealed polydisperse granular aggregates together with the secondary aggregates of those granules (Fig. 4). The non-aggregated primary granules are believed to be equivalent to globules in the previously proposed two-step aggregation model. However, the heights of individual globules were ca. 18 nm, more than four times of the diameter of the native β -lactoglobulin monomer. Therefore, the formation of fine-stranded gel networks of β -lactoglobulin at neutral pH can be explained if the growth of the primary aggregates is forced to be terminated by the secondary aggregation that should occur more frequently at a higher protein concentration sufficient to cause gelation. The formation of heat-induced aggregates of WPI at neutral pH without added salt was then conducted at a much higher protein concentration (11% w/w). Visualized aggregates are much larger but generally composed of fairly regular globular aggregates (Fig. 5), suggesting that the two-step aggregation model is also applicable to WPI. The measured heights of the globules were ca. 11 nm, shorter than β -lactoglobulin individual systems, indicating that heat-induced aggregation of β -lactoglobulin was obstructed by the presence of other whey proteins and/or that accelerated secondary aggregation among primary globules limited the growth of the globules.

3.2. Raman scattering spectroscopy

Raman scattering spectroscopy and infrared spectroscopy are two of rare spectroscopy methods that are capable of investigating protein molecular structures at high protein concentrations sufficient to cause macroscopic gelation. However, infrared spectroscopy is difficult to be performed for aqueous samples due to strong absorption by water. Additionally, β -lactoglobulin in deuterium oxide exhibits a

Fig. 6. Raman scattering spectra of 15% w/v β -lactoglobulin at pH 2 without added salt. (a) Unheated solution. (b) Gel formed by heating at 80°C for 60 min.

Fig. 7. Raman scattering spectra of 15% w/v β -lactoglobulin at pH 5.4 without added salt. (a) Unheated solution. (b) Gel formed by heating at 80°C for 60 min.

Table 1

Relative Raman scattering intensities and peak wavenumbers of selected bands of β -lactoglobulin

Conditions	I_{760}/I_{1005}^*	I_{855}/I_{830}^*	I_{940}/I_{1005}^*	1240 cm^{-1} *	1665 cm^{-1} *
pH 2, 0 mol/dm^3 NaCl	0.40 (0.45)	0.78 (0.63)	0.11 (0.09)	1242 (1238)	1666 (1670)
pH 7, 0 mol/dm^3 NaCl	0.39 (0.45)	0.86 (0.73)	0.30 (0.24)	1241 (1242)	1666 (1671)
pH 7, 0.1 mol/dm^3 NaCl	0.39 (0.44)	0.82 (0.63)	0.32 (0.28)	1241 (1241)	1667 (1672)
pH 5.4, 0 mol/dm^3 NaCl	0.38 (0.35)	0.78 (0.58)	0.29 (0.29)	1241 (1242)	1666 (1670)
pH 7, 0.3 mol/dm^3 NaCl	0.39 (0.35)	0.82 (0.53)	0.32 (0.31)	1241 (1241)	1666 (1670)

*Numbers in parenthesis are results for heat-induced gels.

broad infrared spectrum with relatively few features [16]. Raman scattering spectra, on the other hand, give distinct intensity bands (e.g., Figs 6 and 7), the assignments of which can be found in the literature [17,18]. Therefore, comparisons of major Raman scattering bands were made between translucent β -lactoglobulin gels, formed at pH 2 and at pH 7 without added salt, and opaque gels, formed at pH 5.4 and at pH 7 in the presence of 0.3 mol/dm^3 NaCl (Table 1). Opaque gels were also formed at pH 7 in the presence of 0.1 mol/dm^3 NaCl but their opacity appeared to be less than other two types of opaque gels.

A distinct peak at 760 cm^{-1} is known to be sensitive to the environment polarity of tryptophan residues: a more intense band arises from more buried residues into apolar environments [18]. The normalized intensity of this band, using the band intensity at 1005 cm^{-1} according to conventional procedures [18], tended to slightly increase in the formation of translucent gels but slightly decrease in the formation of opaque gels (Table 1).

The relative intensity of doublet bands at 830 and 855 cm^{-1} reflects the environment of the tyrosine side chain: a decrease in the relative intensity of the higher wavenumber band indicates increased buriedness of tyrosine residues [17]. A decrease in the relative intensity of the higher wavenumber band appeared to be more pronounced in the formation of opaque gels (Table 1).

The normalized intensity of the band around 940 cm^{-1} is considered to be proportional to the α -helix content [17,18]. Relatively weak intensities of this band are consistent with the low α -helix content in this protein [19]. Even lower intensities at pH 2 than at pH 5.4 or 7 seem to indicate inefficient Raman scattering from the α -helix at pH 2 due to protonation of carboxyl side groups and ionization of amino side groups since the secondary structures of β -lactoglobulin is known to be exceptionally stable against acid [19]. On heating, the α -helix band intensity decreased more pronouncedly on the formation of translucent gels than opaque gels, while the band was not totally lost. The α -helix structure is the most heat-labile secondary structure in β -lactoglobulin that is totally unfolded at ca. 70°C at least in a dilute solution [20]. Thus, the results summarized in Table 1 may indicate that the heat-stability of the α -helix structure is improved at a higher concentration. Additionally, non-aggregated protein molecules during heating are likely to renature on cooling and contribute to the band intensity.

The peak position of the band around 1240 cm^{-1} is a measure of the β -sheet structure content and is known to shift to higher wavenumbers with unfolding β -sheets [17,18]. Unchanged peak positions (Table 1) due to heat-induced gelation, except for the case at pH 2, are an indicative of insignificant overall unfolding of β -sheets. The peak shift to a lower wavenumber at pH 2 suggests that the β -sheets are strongly hydrogen bonded [16].

The peak position of the band around $1665\text{--}1672\text{ cm}^{-1}$ is determined by the balance between contributions from α -helix bands having a peak around 1660 cm^{-1} and those from β -sheet and disordered structure bands having a peak around 1670 cm^{-1} [21]. The general peak shifts from ca. 1666 cm^{-1} to $1670\text{--}72\text{ cm}^{-1}$ due to heat-induced gelation (Table 1) suggest decreases in α -helical structure contents and increases in disordered and/or β -sheet structure contents.

It is worth noting that opaque gels generally showed an intense and broad band centered around 1345 cm^{-1} (Fig. 7). Since this band is considered to reflect environments around hydrophobic aliphatic and aromatic side chains [18], significant hydrophobic interactions are likely to result in the formation of opaque gels. Raman scattering spectroscopy was thus confirmed to be capable of differentiating fibrously aggregated translucent gel networks from particulate aggregated opaque gels based on the environment around certain aromatic amino acid side chains and the secondary structure contents. The only noticeable difference between the formation of translucent gels at pH 7 and that at pH 2 was more strongly hydrogen bonded β -sheets at pH 2.

4. Conclusion

AFM has been confirmed to be a versatile method for investigating the formation of fibrous aggregates of heat-denatured globular proteins. Finely stranded aggregates of β -lactoglobulin formed at pH 2 appeared to be strings of monomers, while the structural unit of aggregates formed at neutral pH itself was

confirmed to be granular aggregates. It was suggested that, in the presence of other species of globular proteins in milk whey, β -lactoglobulin was coupled with other proteins to form fibrous aggregates. The formation of fibrous aggregates was found to accompany a loss of α -helix structures generally, while further investigation is needed to clarify the molecular origin of the shift from the two-step aggregation at neutral pH to the fibrous aggregation at acidic pH.

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References

- [1] A.H. Clark, in: *Functional Properties of Food Macromolecules*, 2nd edn, S.E. Hill, D.A. Ledward and J.R. Mitchell, eds, Aspen Publishers, Inc., Gaithersburg, 1998, pp. 77–142.
- [2] W.S. Gosal and S.B. Ross-Murphy, *Curr. Opin. Colloid Interface Sci.* **5** (2000), 188–194.
- [3] S. Ikeda, E.A. Foegeding and T. Hagiwara, *Langmuir* **15** (1999), 8584–8589.
- [4] S. Ikeda, K. Nishinari and E.A. Foegeding, *Biopolymers* **56** (2001), 109–119.
- [5] S. Ikeda and V.J. Morris, *Biomacromolecules* **3** (2002), 382–389.
- [6] J.I. Guijarro, M. Sunde, J.A. Jones, I.D. Campbell and C.M. Dobson, *Proc. Natl. Acad. Sci. USA* **95** (1998), 4224–4228.
- [7] Y. Takahashi, A. Ueno and H. Mihara, *Structure* **8** (2000), 915–925.
- [8] S. Ikeda and K. Nishinari, *Biomacromolecules* **1** (2000), 757–763.
- [9] G. Panick, R. Malessa and R. Winter, *Biochem.* **38** (1999), 6512–6519.
- [10] V.J. Morris, A.R. Kirby and A.P. Gunning, *Atomic Force Microscopy for Biologists*, Imperial College Press, London, 1999.
- [11] S. Ikeda, V.J. Morris and K. Nishinari, *Biomacromolecules* **2** (2001), 1331–1337.
- [12] P. Aymard, T. Nicolai, D. Durand and A. Clark, *Macromolecules* **32** (1999), 2542–2552.
- [13] G.M. Kavanagh, A.H. Clark and S.B. Ross-Murphy, *Macromolecules* **33** (2000), 7029–7037.
- [14] M. Langton and A.-M. Hermansson, *Food Hydrocolloids* **5** (1992), 523–529.
- [15] P. Aymard, J.C. Nicolai and D. Durand, *J. Chim. Phys.* **93** (1996), 987–997.
- [16] T. Lefèvre and M. Subirade, *Biopolymers* **54** (2000), 578–586.
- [17] B.G. Frushour and J.L. Koenig, *Biopolymers* **14** (1975), 649–662.
- [18] E.C.Y. Li-Chan, *Trends Food Sci. Technol.* **7** (1996), 361–370.
- [19] F. Fogolari, L. Ragona, L. Zetta, S. Romagnoli, K.G. De Kruijff and H. Molinari, *FEBS Lett.* **436** (1998), 149–154.
- [20] X.L. Qi, C. Holt, D. McNulty, D.T. Clarke, S. Brownlow and G.R. Jones, *Biochem. J.* **324** (1997), 341–346.
- [21] E.B. Carew, H.E. Stanley, J.C. Seidel and J. Gergely, *Biophys. J.* **44** (1983), 219–224.