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Analysis of heat-induced BSA aggregates by scattering methods

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The structure of aggregates formed by heating dilute BSA (bovine serum albumin) solution was analyzed using a laser light scattering method. The fractal structure was observed on the scale length from decades to hundreds nanometers. With increase in salt concentration of the solution, the value of fractal dimension D_f for the aggregates tended to increase, suggesting that the aggregates were reconstructed by salt addition.

The aggregate structure in BSA gels prepared from concentrated solution was also investigated using a small angle X-ray scattering method on the scale length from several to decades nanometers. It was suggested that the aggregates in the gels were formed through the process different from the conventional cluster-cluster aggregation model mentioned above.

1. INTRODUCTION

The protein aggregates in a gel would considerably influence the physical properties of the gel. We have shown that the elastic behavior of the heat-induced BSA (bovine serum albumin) gels was a reflection of the fractal structure of the protein aggregates on the scale length of micrometers [1]. In addition, we have observed that the value of the fractal dimension D_f for heat-induced BSA gels varied according to the condition of the solvent used in preparation of gels. However, the method for controlling the fractal dimension D_f is not satisfactorily known. To control the fractal dimension of aggregates, it is necessary to elucidate the mechanism for the development of fractal structure of aggregates.

Scattering methods are very effective to analyze colloidal structure below hundred nanometers. The analysis of aggregate structure on such scale using the scattering methods would help us to guess the mechanism of development of the fractal structure in protein aggregate gels. Light scattering gives information on dilute colloidal structure on the scale length from decades to hundreds nanometers. The investigation on the dilute system is very important because dilute colloidal system has been theoretically examined on the basis of the cluster-cluster aggregation model. In a previous study, we have already analyzed the fractal structure of aggregates formed by heating dilute bovine serum albumin (BSA) solution with the static light scattering (SLS) method [2]. The results showed that controlling the pH of

the solution, the value of D_f had almost the same value as predicted by the cluster-cluster aggregation model. However, the effect of salt on D_f in the dilute system has not been examined. On the other hand, small angle X-ray scattering (SAXS) gives information on the scale length from several to decades nanometers.

In this study, by using laser light scattering, the structure of aggregates formed by heating BSA dilute solutions at various salt concentrations was investigated. The aggregate structure in BSA gels prepared from concentrated solution was also analyzed using SAXS method

2. MATERIALS AND METHODS

2.1. SLS

BSA was obtained from Boehringer Mannheim GmbH (Mannheim, Germany; ref.238040). All other chemicals were of reagent grade.

BSA aggregate suspensions (BSA concentration; 0.1wt%) were prepared by almost the same method as that described in the previous work [2]. BSA was dissolved in 50 mM HEPES buffer (pH 7.0). The ionic strength of the buffer I_s was varied from 0.2 to 1.0M by the addition of NaCl to the buffers. The solutions were kept at 25℃ for about 20min and then heated at 95℃. The samples were used for SLS measurement. The aggregates prepared by heating BSA solutions with $CaCl_2$ were also prepared. BSA was dissolved in 50 mM HEPES buffer (pH 7.0) containing 5mM $CaCl_2$; the solution was heated in the same way as mentioned before and used for SLS measurement.

The fractal dimension D_f of the aggregates was evaluated using the following equation [2]:

$$I(q) \propto q^{-D_f} \qquad (1 < D_f < 3) \tag{1}$$

where I(q) is the total scattered intensity and q is the length of scattering vector defined by

$$q=(4\pi n_s/\lambda)\sin(\theta/2) \tag{2}$$

where n_s, λ, and θ are the refractive index of the solvent, the wavelength of the light source in vacuum, and the scattering angle, respectively. The scale length d can be calculated using q as follows [6]:

$$d=2\pi/q \tag{3}$$

SLS measurements were done similarly to that described in the previous work [2] on a System 4700 (Malvern);the light source was a 30mW He-Ne laser(wavelength λ= 632.8nm; NEC Co., Ltd.). The value of q was varied from 5×10^6 to 3×10^7 m^{-1} (scale length d; 2.1×10^{-7} to 1.3×10^{-6} m). From Eq. (2), D_f was evaluated from the slope of the double logarithmic plot of I(q) *vs.* q.

2.2. SAXS

BSA were dissolved in three kinds of buffer: (A) 50 mM acetate buffer (pH 5.1, I_s =0.1 M);

(B) 50mM HEPES buffer (pH 7.0, I_s=0.2 M); (C) 50mM HEPES buffer (pH 7.0, I_s=0.012 M). The ionic strength of the buffer I_s was adjusted by the addition of NaCl to the buffers The solutions were degassed under vacuum for 3 min to remove air. The pH of the solutions was then adjusted to the initial pH of the buffers, using NaOH and HCl solution. The final BSA concentrations were (A) 16.6%; (B) 17.4%; (C) 17.5%, respectively. After preheated at 50℃ for 60min, the solution was gelled by heating at 95℃ for 10 min in 0.7 mm diameter sample capillary tube (Mark-Rhörchen, Berlin, Germany).

The SAXS measurements were done with a Kratky Compact Camera System (Anton Parr GmbH, Austria). The X-ray source was CuK_α (wavelength λ = 0.154 nm, Phillips Co., the Netherlands). The scattered intensities I were detected with a proportional counter as a function of q defined by Eq.(2); value of n_s can be taken as unity in case of SAXS [6]. The value of q was varied from 8.2×10^7 to 5.6×10^9 m^{-1} ($1.1\times10^{-9} < d < 7.7\times10^{-8}$ m)

The effect of the slit collimation system from a Kratky camera was corrected by using the method of Guinier and Fournet [7] after subtraction of the blank scattering from the capillary tube.

3. RESULTS

3.1. SLS

Figure 1 shows the double logarithmic plots of I(q) *vs.* q for the sample prepared from a BSA solution containing NaCl. Both plots were linear, indicating that aggregates prepared from BSA solutions containing NaCl were fractal. The values of D_f for (A) and (B) were evaluated to be 2.19 and 2.84, respectively.

Table 1 summarizes the values of D_f of aggregates prepared from BSA solutions containing NaCl at various I_s, including the result of the previous work (I_s=0.1 M) [2]. The values of D_f were larger for longer heating time at an identical value of I_s and tended to increase with increasing I_s. The values of D_f were larger than the maximum of D_f, 2.1 predicted by the conventional "cluster-cluster aggregation model" mentioned before [3-5].

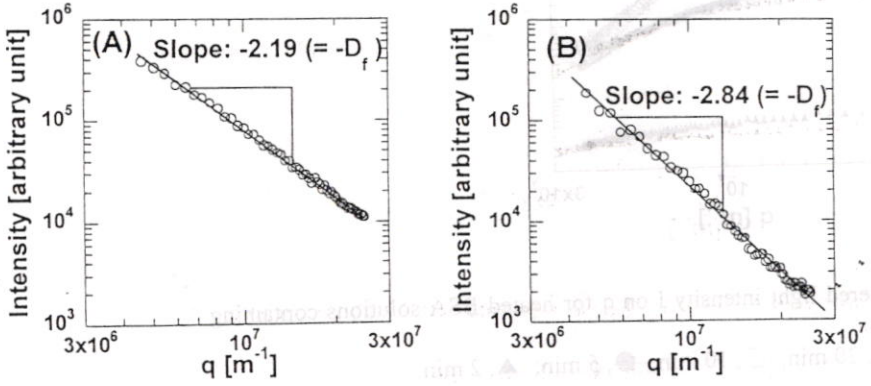

Figure 1 Dependence of scattered light intensity I for heated BSA solutions on q. (A) I_s=0.2 M; heating time was 90 min.(B) I_s=0.6; heating time was 13 min.

Table 1 Fractal dimension of BSA aggregates containing NaCl.

Ionic strength I_s [M]	NaCl concentration [M]	Heating time [mim]	Fractal dimension, D_f
0.1	0.0868	90	2.11 [2]
0.2	0.186	40	2.08
0.2	0.186	90	2.19
0.2	0.186	120	2.27
0.3	0.286	17	2.08
0.3	0.286	25	2.39
0.4	0.386	20	2.53
0.5	0.486	16	2.50
0.6	0.586	13	2.84
1.0	0.986	10	2.12
1.0	0.986	11	2.71

In Figure 2, I *vs.* q plots for aggregates containing $CaCl_2$ are shown. The linear relationship was not observed, indicating that the aggregates in these samples were not fractal. However, the aggregates in these samples may be fractal, considering that aggregates in BSA gels containing $CaCl_2$ were fractal over the range of about 0.2-10 micrometers from image analysis [1]. Another experimental methods that can clarify the aggregates structure at the scale length larger than that by SLS used in this study, should be developed to confirm that the aggregates formed with $CaCl_2$ are fractal.

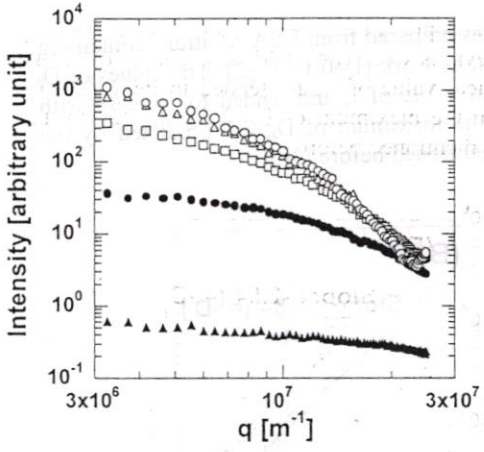

Figure 2 Dependence of scattered light intensity I on q for heated BSA solutions containing $CaCl_2$ at various heating times.
Heating Tome: ○, 45 min;△, 30 min; □, 10 min; ●, 5 min; ▲, 2 min.

3.2. SAXS

Figure 3 shows the double logarithmic plots of I(q) vs. q for the sample prepared using the

buffer (A) 50 mM acetate buffer (I_s = 0.1 M); (B) 50mM HEPES buffer (I_s=0.2 M); (C) 50mM HEPES buffer (I_s=0.012 M). All of the three plots were not single straight lines, indicating that these gels were not fractal predicted by Eq.(1).

The scattering pattern for the gels prepared at pH5.1, shows a peak at $q \fallingdotseq 2.5 \times 10^8 m^{-1}$. From the value of q at the peak top, the corresponding scale length d were evaluated to be about 25 nm, using Eq.(3). At $q > 2.5 \times 10^8$ m^{-1} (scale length d < 25 nm), the plot had a linear region, whose slope value was close to –4; $I \propto q^{-4}$, which corresponds to the well-known Porod's law for the scattering profile characteristic to a smooth surface object [7,8]. The scattering profile of the gels prepared with 50 mM HEPES buffer had no peak.

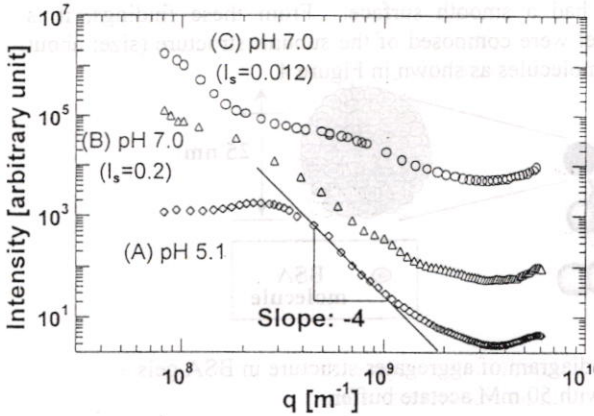

Figure 3 Small angle X-ray Scattering curves for the BSA gels.
Buffer: (A) 50 mM acetate buffer (pH5.1);
(B) 50mM HEPES buffer (pH 7.0, I_s=0.2 M);
(C) 50mM HEPES buffer (pH 7.0, I_s=0.012 M).

3.4. DISCUSSION

Scattering methods (light, X-ray) have no need for processing samples before observation such as in electron microscopy, and are useful techniques for the characterization of the native gel microstructure, however, there have been only few studies investigating the structure of BSA aggregates by scattering methods.

The fractal structure of aggregates in the dilute system has been examined by the cluster-cluster aggregation model [3,4]; diffusing particles or aggregates in certain medium stick to each other at a probability P on contact. According to the computer simulation of the cluster-cluster aggregation model, the values of the fractal dimension D_f for the aggregates are 1.8 at P=1 (the diffusion-limited cluster-cluster aggregation; DLCCA) and 2.1 at P<<1 (the reaction-limited cluster-cluster aggregation; RLCCA)[3]. As stated before, we have already analyzed the fractal structure of aggregates formed by heating dilute BSA solution at the condition of I_s=0.1 M (NaCl concentration was 0.0868 M)[2]; the value of D_f varied from 1.8 to 2.1 by controlling solution pH. On the other hand, increasing the salt concentration as shown in Table, the values of D_f could be larger than the maximum of D_f, 2.1 predicted by the

conventional "cluster-cluster aggregation model"[3-5]. According to Meakin *et. al.*[9], the value of D_f can be larger than 2.1 by "restructuring" of aggregates during aggregation. The restructuring of aggregate structure by NaCl addition would cause the larger values of D_f as shown in Table. In the preceding study, the values of D_f for BSA gels with salt addition were approximately 2.6-2.8[1], while those of D_f for the gels prepared without salt addition were about 2. Salt addition would also cause restructuring protein aggregates in the gels and result in a higher value of D_f.

The peak of the SAXS profile for the gels at pH 5.1 suggested that the gels had a periodic structure[6,7] whose size was about 25 nm.; it is larger than that of one BSA molecule (about 3 nm). In addition, the existence of region of Porod's law at the scale length below 25 nm, indicated that the periodic structure had a smooth surface. From these findings, it is suggested that the aggregates in this gel were composed of the subunit structure (size; about 25nm) packed tightly with the protein molecules as shown in Figure 4.

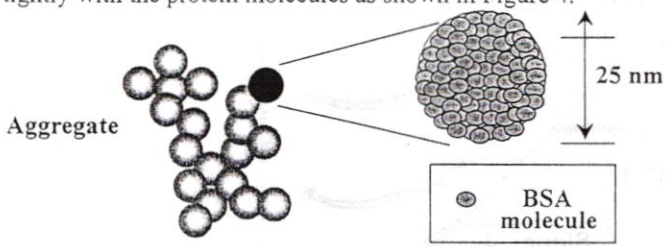

Figure 4 Schematic diagram of aggregates structure in BSA gels prepared with 50 mM acetate buffer.

For all of three gels examined in this study, the fractal structure was not observed on the scale length accessible by the SAXS measurement, indicating that the aggregates in these gels were formed through the process different from the conventional cluster-cluster aggregation model mentioned above.

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