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Relation between the phase separation and the crystallization in protein solutions

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Liquid-liquid phase separation and crystallization (or solid-liquid phase separation) both occur in protein solutions. By adopting egg-white lysozyme for a model system, we compared two types of diagrams, a phase diagram of the liquid-liquid phase separation and a morphological diagram of protein crystals. By superimposing these diagrams, we distinguished two types of white precipitates, urchinlike spherulites arising from the crystallization and protein-rich droplets from the liquid-liquid phase separation. Furthermore, we observed a transformation from the protein-rich droplets to the spherulites, and simultaneously an unusual pattern evolution of the protein-rich phase unlike the conventional phase separation of typical binary mixtures. This is understood in terms of the competition between the crystallization and the liquid-liquid phase separation. [S1063-651X(97)50307-2]

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Synthesis of high-quality single crystals of proteins is indispensable for determining the structure of protein molecules by x-ray crystallography, but is difficult, in general, partly because the mechanism of protein crystallization has not been clarified in detail.

Constructing and utilizing a phase diagram of a protein solution is a basic strategy for protein crystallization. In protein solutions, it has been reported that solid-liquid phase separation (crystallization or amorphous precipitation) [1–3] and liquid-liquid phase separation [4–6] both occur, and that the liquid-liquid coexistence curve is located under the solubility or the solid-liquid coexistence curve in these phase diagrams [7–10]. The latter indicates that liquid-liquid phase separation occurs in metaequilibrium protein solutions [7–9].

In previous studies on protein solutions [4–9], the phase diagrams were constructed for the condition of crystallization occurring slowly enough not to affect the liquid-liquid phase separation. However, since the liquid-liquid coexistence phase is a metaequilibrium state as mentioned above, solid-liquid phase separation will finally occur and may interact with a domain coarsening process in the late stage of liquid-liquid phase separation. Thus, it is of interest to determine how liquid-liquid phase separation and solid-liquid phase separation are related to each other in protein solutions.

In this study, we investigated the morphology of lysozyme crystals or precipitates for various combinations of lysozyme and NaCl concentrations at *p*H 4.60 in the temperature range of 30.0–40.0 °C using an optical microscope with polarizers, in order to clarify the relationship between the solid-liquid phase separation and the liquid-liquid phase separation occurring in lysozyme solutions.

Hen egg-white lysozyme powder (Seikagaku Corporation, six times crystallized) was dissolved in deionized water (Organo Puric-*Z*), and the solution was passed through a 0.2 μm filter (Sartorius). The *p*H of the solution measured with a *p*H meter (Horiba F-7ss) was adjusted to 4.60 ± 0.05 with 1*N* HCl. We used no buffer so that we could define the ionic strength of the solution accurately, but due to the autobuffering effect of lysozyme, the *p*H remained almost constant for one month. We measured the liquid-liquid phase-separation temperature (the cloud point) T_{cloud} of supersaturated lysozyme solutions by use of a light scattering apparatus (Otsuka Electronics ELS-800) as described in Ref. [4]. We started the microscopic observation immediately after mixing the lysozyme and NaCl solutions. The crystallization or domain coarsening process of liquid-liquid phase separation was observed with an optical microscope (Olympus BX50-32). The temperature was controlled to within ± 0.1 °C by a temperature controller (Yamato-Komatsu CTE-22W).

The phase diagram of the liquid-liquid phase separation was obtained in previous works [4,5] at a fixed NaCl concentration of about 3 wt %, at which the liquid-liquid phase separation was much faster than the crystal growth. In this study, we carried out the light scattering measurement to obtain data for constructing phase diagrams for a variety of NaCl and lysozyme concentrations. The temperature of the solution was first kept at 50 °C and then lowered at a rate of about 3 °C/min. The scattered light intensity increased abruptly at a specific temperature for all the solutions studied. This temperature was defined as T_{cloud}. Figure 1(a) shows a three-dimensional phase diagram thus obtained, into which a NaCl concentration axis has been introduced. Figure 1(b) shows a cross section of the diagram in Fig. 1(a) for 35 °C as a guide to the eye. The results in Fig. 1(a) agree well with those in Ref. [4] for experimental conditions common to both. For example, for a protein concentration of 7.5 wt % and a NaCl concentration of 3.5 wt %, we determined T_{cloud} to be approximately 7 °C, which is comparable to the 8 °C reported by Ishimoto and Tanaka [4]. As a result of the extension of the NaCl concentration axis up to 11 wt %, the diagram includes the conditions under which both the liquid-liquid phase separation and the solid-liquid phase separation occur on closer time scales. Figure 1(a) and 1(b) show a curved surface and line, respectively, which represent the cloud point T_{cloud}. Below T_{cloud}, there coexist protein-rich

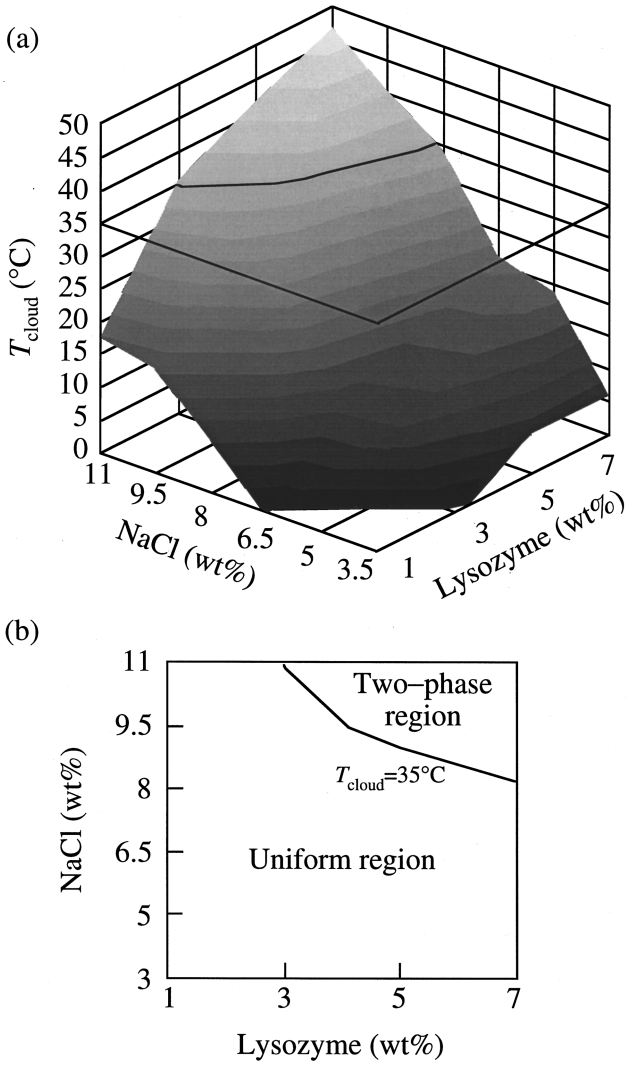

FIG. 1. (a) The phase diagram for the uniform solution (the uniform region) and the solution separated into protein-rich and protein-poor phases (the two-phase region) at *p*H of 4.60. The boundary surface (the T_{cloud} surface) was obtained from the temperature quenching phase separation by using light scattering apparatus. (b) The cross section of (a) at 35 °C.

and protein-poor solution phases (the two-phase region), while above T_{cloud} the solution remains uniform (the uniform region).

We previously reported on the morphodrom of lysozyme crystals or precipitates produced in the solutions [11], as shown schematically in Fig. 2. As the concentration of NaCl in the solution was increased with the lysozyme concentration fixed, orthorhombic rectangular crystals appeared in the solution after a few hours to a few weeks (region I), urchinlike spherulites precipitated after an induction time of about 2–12 h (region II), and random aggregates were observed immediately after mixing (region III). Although the precipitates in regions II and III both looked like white aggregates macroscopically, there was a clear boundary between regions II and III, since the precipitation occurred instantly in region III, while the solution after mixing was transparent for a while in regions I and II. Furthermore, random aggregates in region III disappeared after about half a day and instead, the urchinlike spherulites appeared and grew. This change in the

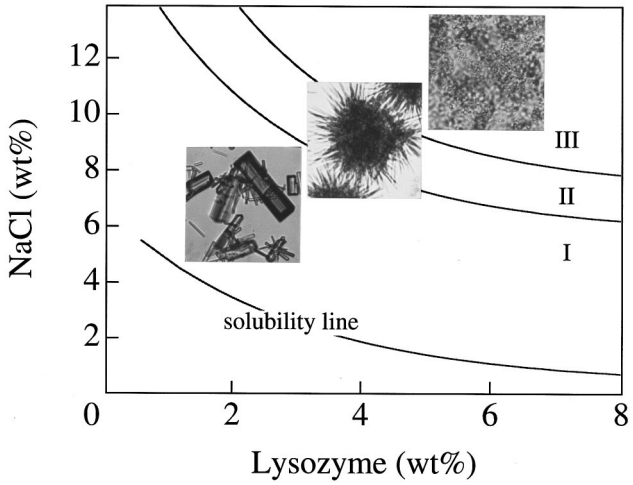

FIG. 2. The morphodrom of lysozyme crystals and precipitates with the solubility and liquid-liquid phase-separation lines. The random aggregates in region III disappeared and instead the spherulites were finally formed.

precipitates from the random aggregates to the spherulites is discussed below in more detail.

The boundary between the uniform and two-phase regions in Fig. 1(a) is in good agreement with boundary between regions II and III in Fig. 2. This indicates that region III in Fig. 2 corresponds to the two-phase region in Fig. 1, while regions I and II in Fig. 2 coincide with the uniform region in Fig. 1. Thus, it may be concluded that the random aggregates precipitated in region III arise from the protein-rich phase produced by the liquid-liquid phase separation. In other words, within the range of our experimental conditions, the random aggregates and crystals are produced by the two different phase separations, the liquid-liquid and solid-liquid ones. This means that the solid-liquid phase separation is equivalent to the crystallization in the lysozyme solution. We emphasize that the phase diagram in Fig. 1 is based on the results of the liquid-liquid phase separation experiment, while that in Fig. 2 is based on those of the morphological observation of crystal growth. A comparison has been made between these two different kinds of experiments for protein solutions and a clear relationship between the two has been demonstrated.

When the temperature T was lowered to a specific range below T_{cloud} rapidly enough, protein-rich droplets appeared due to the liquid-liquid phase separation as shown in the optical microscopic image in Fig. 3(a), and then they began to undergo a gradual change in shape. In this later period of liquid-liquid phase separation, we observed that crystalline spherulites appeared and grew to a size much larger than that of the protein-rich droplets, and then the droplets around the spherulites disappeared as seen at the center of Figs. 3(b) and 3(c). Figure 3(d) shows a polarized image of spherulites. This series of images indicates that the protein-rich phase, which produces random aggregates in region III in Fig. 2, is not in an equilibrium state but in a metastable state and the crystallization may occur subsequently. This result agrees well with the phase diagram constructed by Broide, Tomine, and Saxowsky [8], in which the liquid-liquid phase-separation curve exists under the solubility one of crystallization.

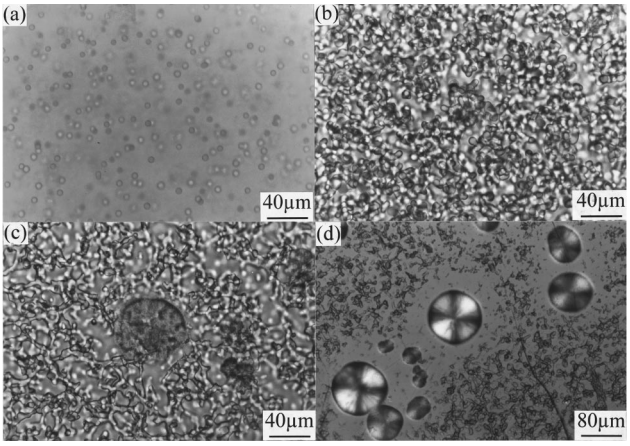

FIG. 3. The growth of a spherulite at the center of the picture and the time evolution of the protein-rich droplets around the spherulite. The concentrations of lysozyme and NaCl were 7 wt % and 8 wt %, respectively. The incubation temperature T_i was 31.2°C and the cloud point T_{cloud} was 33.8°C. (a) Immediately after liquid-liquid phase separation, the protein-rich droplets appeared. (b) 2.6 h later, the protein-rich droplets sedimented on the bottom of the observation cell and the spherulite was gradually formed. (c) 3.2 h later, the spongelike structure of the protein-rich phase appeared. (d) 4.8 h later, spherulites precipitated and the protein-rich phase disappeared. Only this picture was taken with polarizers. The magnifying scale was indicated in the pictures.

During the course of spherulite growth from a liquid-liquid phase-separated solution, we observed an unusual pattern evolution of the protein-rich phase in the parts surrounding the spherulites in Figs. 3(b)–3(d). In conventional phase separation of binary mixtures, the volume of each separated phase does not change, even if fusion or coarsening of the droplets takes place. Contrary to this, the total volume of the protein-rich droplets decreased and the droplets finally vanished. The initial liquid-liquid phase separation [Fig. 3(a)] proceeded to a later stage of coarsening after a few hours: the droplets changed into a spongelike structure by fusing with each other [Fig. 3(b)]. Then, the spongelike structure became thinner and decreased in volume [Figs. 3(c) and 3(d)]. If the crystallization did not occur, the protein-rich droplets coarsened and larger ones were formed by connection or fusion of smaller ones so that the area of interfaces between the protein-rich and protein-poor phases was reduced as in conventional phase separation. In the present case of phase separation, however, the protein crystallization interrupts the liquid-liquid phase separation at intermediate stages. Then, instead of fusion into larger droplets, the spongelike structure of the protein-rich phase disintegrates as the crystallization proceeds. Thus, the unusual pattern evolution of the protein-rich phase is ascribable to the competition between the phase separation and the crystallization in the protein solution.

In conclusion, to clarify the relationship between the solid-liquid phase separation and the liquid-liquid phase separation in lysozyme solutions, we compared two types of diagrams, a phase diagram of liquid-liquid phase separation and a morphodrom of crystals, and found a close relation between the two. We could distinguish two types of white precipitates, i.e., urchinlike spherulites and protein-rich droplets (random aggregates) produced by the liquid-liquid phase separation. We suggested that the random aggregates were formed due to the liquid-liquid phase separation and that the solid-liquid phase separation was equivalent to the crystallization in the lysozyme solution. Furthermore, the crystallization almost always occurred in the solution with the liquid-liquid phase separation, and we observed the complex pattern evolution due to the competition between the liquid-liquid phase separation and the crystallization.

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