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In-situ confocal Raman monitoring of evaporation process during protein crystallization

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ABSTRACT: We have introduced an in-situ Raman monitoring technique to investigate the crystallization process inside protein drops. In addition to a conventional vapour-diffusion process, a novel procedure which actively stimulates the evaporation from a protein drop during crystallization was also evaluated, with lysozyme as a model protein. In contrast to the conventional vapour-diffusion condition, the evaporation-stimulated growth of crystals was initiated in a simple dehydration scheme and completed within a significantly shorter time. To gain an understanding of crystallization behaviours under the conditions with and without such evaporation stimulation, confocal Raman spectroscopy combined with linear regression analysis was used to monitor both lysozyme and HEPES buffer concentrations in real time. The confocal measurements having a high spatial resolution and good linear response revealed areas of local inhomogeneity in protein concentration when the crystallization started. The acquired concentration profiles indicated that (1) the evaporation-stimulated crystallization proceeded with protein concentrations lower than those under conventional vapour diffusion, and (2) crystals under the evaporation-stimulated condition were noticeable within an early stage of crystallization before the protein concentration approached its maximum value. The HEPES concentration profiles, on the other hand, increased steadily towards the end of the process regardless of the conditions used for crystallization. In particular, the observed local inhomogeneities specific to protein distribution suggested an accumulation mechanism of protein molecules that initiates the nucleation of crystals.

KEYWORDS: biocrystallization, confocal Raman spectroscopy, hanging drop, evaporation stimulation, lysozyme

INTRODUCTION

In an attempt to understand the mechanism of protein crystal growth, various observation methods have been used, including dynamic light scattering[1], time-resolved fluorescence[2], and calorimetric techniques[3]. In particular, dynamic light scattering provides information about molecular aggregation of proteins on the basis of observed translational diffusion coefficients, which relates to the size of the aggregates. The dispersion of molecular weights estimated from the translational diffusion coefficient predicts the crystallizability of proteins[4]. The fluorescence technique, on the other hand, relies on the spontaneous emission of light by fluorophores covalently coupled to protein molecules. Differential scanning calorimetry in turn can be performed to determine the optimal nucleation temperature of proteins in solution over a range of temperatures (20–90 K)[3]. The technique has also been used to examine the transition between a native, biologically active protein conformation and a denatured, inactive conformation, which directly correlates to the thermal stability of such proteins.

Although those techniques have been widely used to study protein crystallization, none of them can fully determine the growth mechanism due to limitations either in the quantitative assessment or the identification of proteins. In recent years, Raman spectroscopy, structural information based on the vibrational energy of bonds, has become a valuable method to study proteins. The technique is capable of analysing individual constituents inside a protein drop during crystallization. To observe protein growth, the technique necessarily requires the capability for acquiring data from a small sampling volume with sufficient collection efficiency, regarding the limited size of a protein drop (of a few millimetres). In this respect, Raman monitoring has demonstrated a considerable potential for in-situ investigation of the compositional changes in a hanging drop throughout the crystallization process, which leads to insight into the degree of supersaturation and the crystallization

mechanism.[5–7]

It is also possible to use a confocal Raman microscope combined with an XY piezo-stage modified specifically to observe crystallization plates[8]. Such a combination permits the simultaneous measurement of both spectroscopic information and a microscopic image from a small local area within a drop. The major advantage of confocal Raman spectroscopy over the conventional Raman technique is the high spatial resolution achieved by spatially filtering the light coming from the out-of-focus regions. The confocality provides an extremely small sampling volume in the submicron range with a significant reduction in background signals coming from areas encircling the laser focus point. As a consequence, a confocal Raman spectrum of a protein crystal or even a microcrystal with a diameter less than 10 µm can be obtained at a high spectral signal-to-noise ratio within a minute[8].

In addition to the monitoring techniques, the development of the crystallization procedure itself is important to provide effective crystallization. Attempts to develop techniques for protein crystallization have been driven by increasing demands to understand its biological functions by X-ray crystallography. Unfortunately, performing crystallization experiments using screening methods in a conventional hanging drop is known to be a time-consuming task due to the wide variety of variables necessary for determining optimal crystallization conditions. Several methods have recently been developed to promote protein crystallization using a magnetic field[9–11], laser light[12,13], and zero gravity[14]. These developments have led to a quick and reliable crystallization procedure.

In the work described here, we have introduced an evaporation-stimulation procedure aimed to accelerate the rate of crystal growth. In this method, we progressively activated the evaporation from a protein drop by using water-adsorbing materials in a separate container. Although the dehydration from a protein drop is the key factor in inducing condensation of the drop and in initiating the nucleation process, an excess dehydration may aggregate nonprotein molecules. The erroneous recognition of such pseudo-crystal formation, however, can be avoided by using the spectral discrimination capability of real-time confocal Raman spectroscopy. To evaluate the feasibility of the method, lysozyme was crystallized and spectroscopically investigated under both conventional vapour-diffusion and evaporation-stimulated conditions. The Raman spectral features reveal changes in protein and buffer concentrations during the crystallization process independently, and present them as time-course profiles. Here we report observed specific features of concentration changes during crystal growth, which are presented in association with a characteristic rapid appearance of crystals under the evaporation-stimulated conditions.

MATERIALS AND METHODS

Protein sample preparation

Chicken egg white lysozyme was purchased from Wako Pure Chemical Industries, Ltd. and used without further purification. The crystallization of lysozyme was performed in a buffer solution containing 100 mM HEPES and 0.8 M NaCl at pH 7.5. The standard protein solutions were prepared in a concentration range of 10–100 mg/ml in triply distilled water. Crystallization experiments used polystyrene 24-well Linbro plates. Lysozyme was dissolved in triply distilled water to initial concentrations of 25.3 and 49.8 mg/ml. A 2-µl protein drop was then gently mixed with 2 µl of the NaCl/buffer solution and deposited on a siliconized cover slip, thereby reducing the actual protein concentration in a hanging drop to half of its original value. The cover slip glass was then inverted and placed over a crystallization well containing 500 µl of the NaCl/buffer reservoir for the vapour-diffusion procedure in the conventional setup. The top of each well in either technique was greased to ensure an airtight seal. The temperature for all experiments was set at 25 ± 2 °C.

Experimental design for evaporation stimulation

In the case of the evaporation-stimulation procedure, a crystallization well was modified and connected to a dehydration container through a tube, and was used with no reservoir incorporated. Fig. 1 shows a schematic diagram of the experimental setup used for the evaporation-stimulated crystallization in a hanging-drop configuration. Each well consisted of a channel connected to a silica-gel container through an on-off valve. The two containers of a protein drop and the water adsorber were intentionally separated to retard an excessive rate of water withdrawal from the protein drop. Because the quantity of silica gel in the container directly relates to the rate of evaporation, the weights of the oven-dried silica-gel granules used throughout the evaporation-stimulation experiments were restricted to be within the range of 0.25–0.28 g (∼10 tablets), in order to obtain an optimum evaporation rate and reproducible Raman results. The crystallization plate was then placed at the top of a translational piezo-stage to allow the drop position to be adjusted within the focal point of the

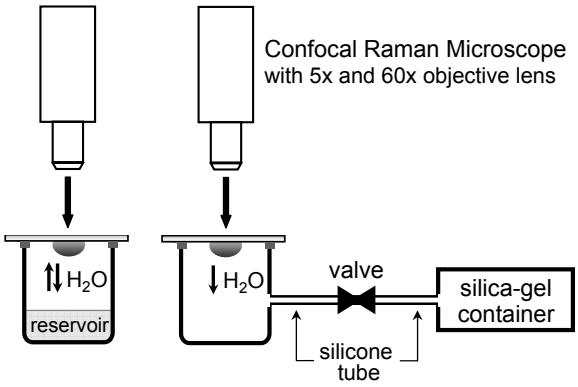

Fig. 1 Schematic diagram of conventional vapour-diffusion (*left*) and evaporation-stimulation (*right*) methods using hanging-drop well. For the latter technique, the well is connected to a silica-gel container through an on-off value and silicone tubes.

confocal laser beam. Note that excessive evaporation easily leads to over-dehydration of the protein drop and results in the appearance of solid materials on the cover slip. Even in such cases, Raman spectroscopic measurement can efficiently provide a correct spectral analysis of protein crystals from the solid materials embedded inside the drying drop.

Spectral data collection for Raman measurements

A confocal Raman crystal spectroscope[8] was developed from a basic model (Nanofinder 30, Tokyo Instruments) and equipped with a CCD detector (DU401-BR-DD, Andor Technology) and a 632.8-nm He-Ne laser (Melles Griot). This wavelength was chosen to avoid fluorescence and absorption from the protein. The incident laser beam with a power of 40 mW was positioned directly above a cover slip glass and focused into a protein drop through a 60× objective lens with an illumination of 10–15 mW, as measured by a laser power meter (Nova II, Ophir Japan Ltd.). Performing the crystallization in a hanging-drop scheme was thus most suitable to achieve an optimal optical efficiency and to provide convenience in locating various positions inside a drop. The spectral collection was acquired using a 10 s exposure time with 6 accumulations (otherwise mentioned specifically in the figure). Note that a longer exposure time under high illumination intensity might interrupt the growth process and tends to damage growing crystals. The Raman spectra of the individual lysozyme and HEPES standard solutions for 10 concentrations were measured to construct linear regression models for the individual components using the band areas at 1054 and 1557 cm^{-1}, which correspond to vibrations due to the phosphate moiety of the buffer solution and tryptophan in the protein, respectively. The peak-fitting method was applied to measure the band area using GRAMS/AI version 7.01 (Thermo Galactic). Lorentzian peak type and linear baseline were set as default acquisition parameters.

RESULTS

We first carried out an investigation to evaluate the potential of the evaporation-stimulation method using lysozyme drops at initial concentrations of 24.9 and 12.6 mg/ml in 24-well plates. As a reference, drops with the same concentrations were also used for the conventional vapour-diffusion technique. Fig. 2 presents the percentages of wells in which lysozyme crystals were successfully produced as a function of time. The vertical axis indicates the percentages of the number of wells showing crystals with respect to the total number of wells used in the experiment. Use of the lower initial protein concentration (12.6 mg/ml) retarded the crystallization process, resulting in twice as much time to have crystals produced in every well (i.e., 100% achieved) as that in the case of the higher protein concentration (24.9 mg/ml). By comparing the above two techniques, the rate produced by the evaporation-stimulation method rapidly approached 100% within 30 and 70 minutes for the high and low protein concentrations, respectively. These durations were approximately 17 and 14 times shorter than those of a conventional technique observed at

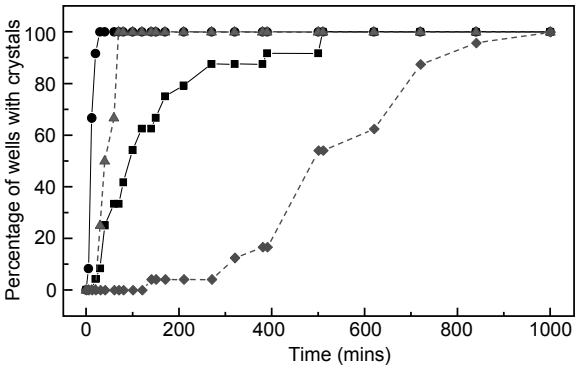

Fig. 2 Percentages of wells indicating presence of protein crystals as a function of time. Note that the total number of wells used for the crystallization experiments is 24. The percentages observed for lysozyme at 24.9 and 12.6 mg/ml under the evaporation-stimulated condition are represented by circles and triangles respectively, while those of the conventional vapour-diffusion condition are indicated by squares and diamonds for the respective concentrations.

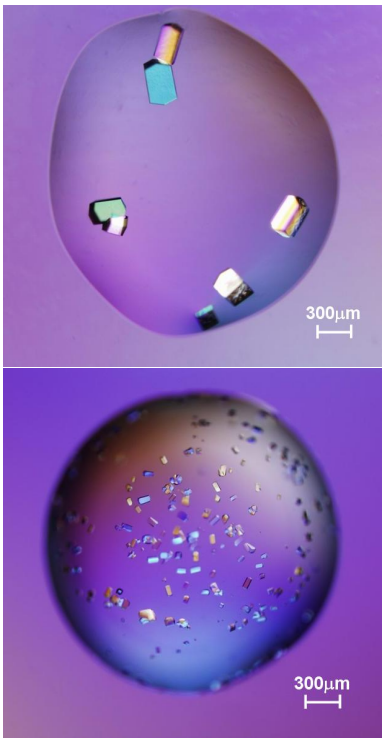

Fig. 3 Microscopic images of lysozyme crystals obtained by evaporation-stimulation (*top*) and conventional vapour-diffusion (*bottom*) methods using initial protein concentration of 12.6 mg/ml.

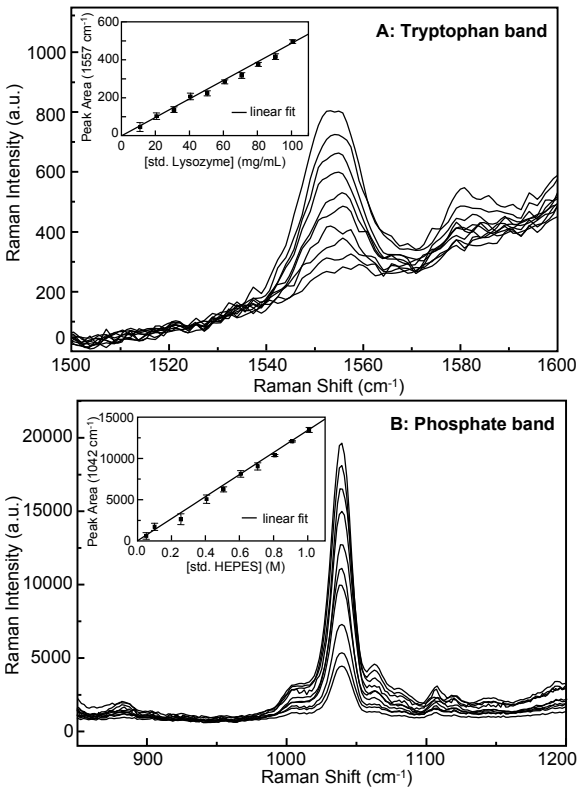

Fig. 4 Series of Raman bands at 1557 and 1042 cm^{-1} assigned to tryptophan moiety of lysozyme protein (A) and phosphate band of HEPES buffer (B), respectively. The spectra of the standard lysozyme and HEPES solutions were collected separately under the same conditions and with identical acquisition parameters. **Insets**: Plots of band area for corresponding bands as function of standard concentrations, which fit well with linear equations by through-zero linear fitting method.

the corresponding concentrations. The evaporation-stimulation method using a simple dehydration system consequently enables us to effectively accelerate the crystallization process of lysozyme and to expedite the appearance time of crystals in a drop.

The rate of vapour evaporation is known to affect the crystal quality. The quality of crystals produced under the stimulated condition, therefore, becomes an important factor, as well as the speed and probability of crystallization. Fig. 3 compares microscopic images of lysozyme crystals obtained using both methods at 12.6 mg/ml. Both photographs were taken using the same magnification (5× objective lens) with an identical viewing area. While many tiny crystals dominated the conventional vapour-diffusion experiments, the evaporation-stimulated crystallization instead yielded a smaller number of substantially larger crystals. Even though the crystals in both cases provided interpretable X-ray diffraction patterns, large crystals are in practice preferred for convenience in handling. However, note that the evaporation-stimulated crystallizations at higher protein concentrations (e.g., 24.9 mg/ml) instead yielded a number of tiny crystals with similar features (shape and size) to those obtained by a conventional technique.

To apply confocal Raman spectroscopy for quantitative measurements, we first investigated changes in spectral features as a function of protein concentration by collecting a spectral series of standard lysozyme solutions in the range of 10–100 mg/ml. The spectral features in Fig. 4A exhibit a gradual increase in the band intensity at 1557 cm^{-1} (vibrations due to the tryptophan moiety of lysozyme) with increasing protein concentration. In addition, the inset of Fig. 4A reveals a good linear relationship between peak areas of the tryptophan band and standard lysozyme concentrations. As shown, the plot was fitted to a linear equation of $y = 4.8739\,x$, with an optimized correlation coefficient of $R^2 = 0.9963$. The fitting illustrates a good linearity at least in the range of concentrations

used throughout this study. As in the tryptophan band, the band area observed for phosphate moiety at 1054 cm^{-1} in Fig. 4B also yielded a good linear correlation with the concentration of standard HEPES solutions. To ensure the certainty of spectral linearity, identical experiments were repeated with additional sets of standard lysozyme and HEPES solutions in the same concentration range. The linear calibrations reproduced insignificant differences in the slope value for both components (i.e., Δslope $<$ 3.5%). These results indicate the reliability and feasibility of applying a simple linear regression model to the collected confocal Raman spectra, in order to acquire real-time concentration profiles of protein and buffer during the crystallization process.

The concentration changes of both lysozyme and buffer in a hanging drop were measured by confocal Raman spectroscopy in real time. First, the buffer concentration was investigated using the band area of 1054 cm^{-1} as described above (Fig. 5A). In all cases, the HEPES concentration gradually and monotonically increased from the start. In particular, the curve corresponding to the conventional method at the lower initial protein concentration of 12.6 mg/ml tended to approach its plateau after approximately 1000 h, suggesting that the vapour diffusion reached an equilibrium stage at which the drop size remained unchanged. Such an equilibrium could also be observed even with the higher initial protein concentration if the measurement was not disrupted at 700 h. The evaporation-stimulation profiles, on the other hand, showed rapid and continuous increases of the buffer concentration throughout the process. This is because no equilibrium is obtained in such a system. In this figure, open circles and squares represent the time points at which the first crystal to appear was recognized. It should be noted that the appearance and growth of crystals did not affect the buffer concentration, and the evaporation from the drop proceeded continuously during crystal growth.

For further confirmation, a solution containing only 100 mM HEPES and 0.8 M NaCl (no protein included) was monitored for both conventional and evaporation-stimulation setups. The resultant HEPES profiles maintained similar temporal patterns to those in Fig. 5A (data not shown). Accordingly, the increase in HEPES concentration is due to a decrease in drop volume as a result of evaporation. As a consequence, the decrease in drop size during crystallization can be estimated from the HEPES concentration profile on the basis of the assumption that the total amount of HEPES inside the drop remains constant throughout the process. The calculated drop volumes shown in

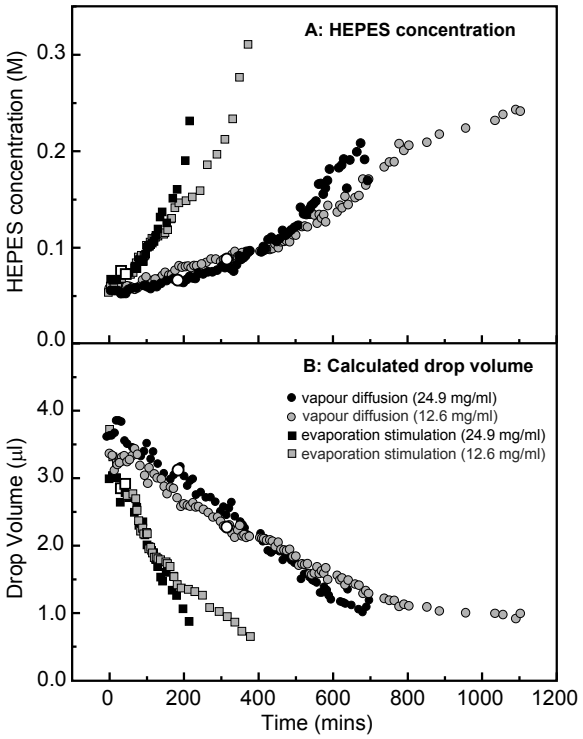

Fig. 5 (A) Concentration profiles of HEPES buffer obtained by vapour-diffusion and evaporation-stimulation methods at initial protein concentrations of 24.9 and 12.6 mg/ml. The profiles were transformed from the band area at 1042 cm^{-1} into the concentration scale using a linear regression model as shown in Fig. 4B. Open symbols indicate the points where the first crystals appeared. (B) Calculated drop volumes during crystallization of lysozyme under conditions corresponding to HEPES concentration profiles in (A).

Fig. 5B reveal that, in all cases, the volume started at $\sim$3.5 µl and ended at $\sim$1 µl, but the rate of change in volume depended on the technique. The evaporation-stimulation procedure exhibited $\sim$3 times higher rates of volume reduction, indicating higher evaporation rates. The conventional technique, on the other hand, used the vapour diffusion mechanism on the basis of differences in ionic strength between the drop and the reservoir, resulting in substantially lower rates of decrease in drop volume. However, the resulting lower evaporation rates and the measured spectral data were more reproducible with the closed-well setup of the vapour-diffusion technique.

The concentration profiles of lysozyme were monitored using the band at 1557 cm^{-1}. As shown in Fig. 6, the profiles represent changes in lysozyme concentration under conventional and evaporation-stimulated conditions at two different initial protein

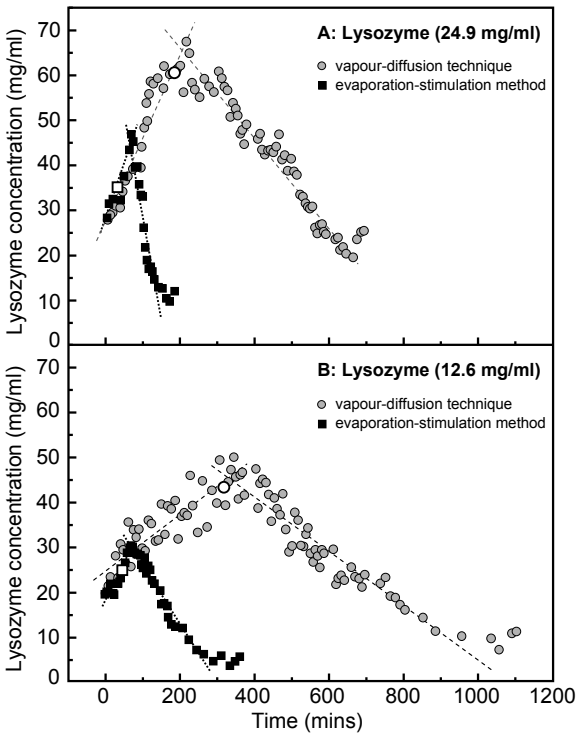

Fig. 6 Comparison of concentration profiles of lysozyme protein observed from vapour-diffusion and evaporation-stimulation measurements starting from initial protein concentrations of (A) 24.9 and (B) 12.6 mg/ml, respectively. The profiles were acquired on the basis of the band area at 1557 cm^{-1} using the individual linear regression model in Fig. 4A. Open symbols indicate the points where the first crystals appeared.

concentrations. In contrast to the monotonic increase in HEPES concentration, a time-course profile consisting of two phases was obtained for protein moiety in all cases. The initial phase is the steady increase in protein concentration, which is expected from the evaporation of water from a drop. Once the profile begins to level off, the second phase is then initiated by a gradual decrease in concentration, which was not observed in the buffer measurements. Under the conventional vapour-diffusion condition, the protein concentration at this stage is apparently affected by the appearance of crystals, which is attributed to the accumulation of protein molecules into crystals.

In the case of the evaporation-stimulation method, however, two critical differences in crystallization behaviour were found, although the two phases mentioned above existed. First, the evaporation-stimulation profiles clearly reveal that the concentration maxima were substantially lower and were reached within a much shorter time than those of the conventional technique. Second, crystals under the evaporation-stimulated condition were generated and observed at a point in time before the concentration maxima were reached, as indicated by the open symbols on the profiles. In contrast, in the conventional vapour-diffusion technique, crystals were produced when the protein concentration approached its peak level. Therefore, the evaporation-stimulated crystal growth and the concentration change of protein show an inconsistent correlation. Although the evaporation stimulation does not alter the overall profile pattern, these two critical differences imply that the crystallization behaviour is altered when evaporation within the drop is activated.

Finally, we investigated the local change or inhomogeneity of the concentrations inside a protein drop for both the setups of vapour diffusion and evaporation stimulation with the assistance of the high spatial resolution of the confocal system (i.e., 0.76/5.0 µm for lateral/axial resolutions, respectively)[8]. Confocal Raman spectra were acquired at various locations inside a protein drop using only a 10 s exposure time to ensure that the collection time was short enough to neglect the influence of crystallization process from changes in the observed spectral features. Regarding the tryptophan band at 1557 cm^{-1}, the spectral results present a noticeable difference in the peak area (Fig. 7A) indicating a nonuniformity of protein distribution inside the drop. Such a variation in intensity was also recognized even when a drop of protein and HEPES buffer were mixed homogeneously in an Eppendorf tube before being deposited onto a cover slide. However, the intensity fluctuation for the protein signal turned out to be indistinguishable in a solution containing only protein and HEPES buffer without salt, in which crystallization did not occur. In contrast, the results of the phosphate band at 1054 cm^{-1} (Fig. 7B) measured from the same spectral series as those in Fig. 7A indicate an indistinct variation in drops with or without salt. This suggests that HEPES molecules are distributed uniformly inside the drop, whereas the concentration of protein molecules is not uniform and molecules are dispersed differently depending on the location when crystallization takes place. Accordingly, these findings emphasize the effectiveness of using a confocal Raman microscope to study the molecular distribution of compositions inside a small inhomogeneous medium.

DISCUSSION

Regarding the relevant developments of the crystallization technique, silica gels were reportedly used

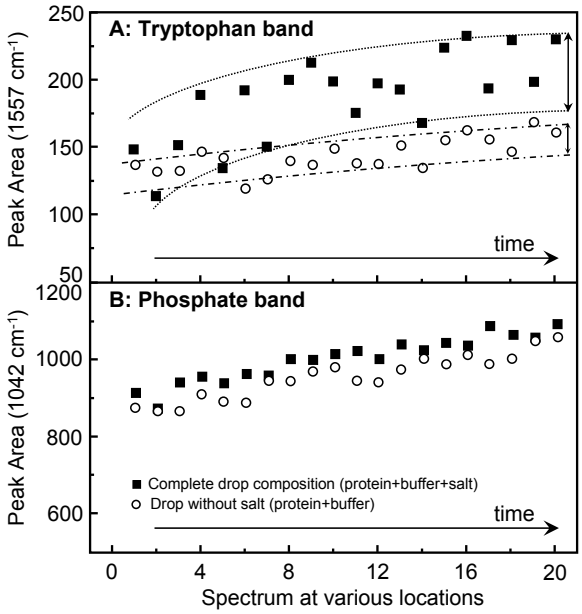

Fig. 7 Plots of band area for tryptophan (A) and phosphate (B) bands obtained from Raman spectral series, which were collected consecutively with time at different locations inside protein drop, using 10 s exposure time.

in crystal growth in the form of silicate gels[15,16]. However, its application to evaporation techniques has been very limited, probably owing to difficulties in controlling the process required to avoid over-dehydration of solution and a dried drop. Our evaporation-stimulation method using a simple dehydration system provides the advantage of a convenient procedure aimed at activating the vapour evaporation from protein drops, and was shown to enhance crystallization rates, thereby producing crystals in a much shorter time. The faster crystal appearance together with the increased probability of crystal formation at lower protein concentrations is beneficial in the screening process of buffer selection for new proteins to be crystallized. In this paper, the evaporation rate was controlled by simply adjusting the amount of silica gels. However, a more precise regulation of evaporation rate is certainly needed to grow various crystals.

Results from the confocal Raman investigations provide some insight into the crystallization mechanism. By comparison, increases in protein concentration towards the concentration peak in Fig. 6 were observed to proceed at faster rates than those estimated from the rate of decrease in the drop volume in Fig. 5B. In particular, under the evaporation-stimulated condition, the calculated concentrations at the peak maxima are 40.36 and 31.48 mg/ml for high and low initial protein concentrations, respectively. However, the peak concentrations obtained by Raman monitoring are 17% and 8% higher than these respective calculated values. Such a deviation of the peak concentration was also observed for the conventional vapour-diffusion process with significantly larger differences (i.e., 39% and 30% for high and low initial protein concentrations, respectively). This finding suggests that the enrichment of the protein concentration inside the drop is caused not only by the decrease in drop volume, but also by localized protein concentration variations due to interaction in a supersaturated solution. The inhomogeneity of only the protein concentration (and not the HEPES buffer concentration) is also confirmed by the analysis as shown in Fig. 7, where a significant fluctuation of the protein signal is observed with the system undergoing the crystallization process. Such a localized enrichment induces an accumulation of protein molecules, thereby forming nuclei prior to the growth stage at which crystals are observable by eye through a microscope. We have also speculated that, as illustrated in Fig. 6, the significant deviation in protein concentration at the starting point ($t = 0$) with respect to the values of the prepared concentration is attributed to the local inhomogeneity of the protein molecules inside the drop and partially due to the time lag between the protein-drop preparation process and the start of Raman spectral acquisition. The HEPES buffer, on the other hand, gave rise to a slight concentration shift at the zero-time point as well as an insignificant variation in the band area observed from various locations inside the drop, indicating a uniform dispersion independent of the protein distribution.

Confocal Raman observation reveals that the crystal growth is not initiated by a certain threshold concentration. Even with the vapour-diffusion process which proceeds in a quasi-equilibrium state, the concentrations at which crystals noticeably appeared have significant differences beyond the local variation of concentration in each drop. Such a local inhomogeneity of concentrations was recognized only when salt was added to the protein solution. Without salt, neither crystallization nor a local change in protein concentration occurs. Therefore, the fluctuation of local protein concentration is a particular phenomenon exhibited by protein molecules that may occur under supersaturated conditions, and the evaporation further induces such fluctuation and starts nucleation leading to crystal growth. It is difficult to explicitly define the saturation concentration for each protein drop because protein, buffer, and salt conditions differ slightly.

However, the fact that the lowest concentration at which the crystal growth was successfully observed was 10 mg/ml in our experience indicates that the concentrations used here correspond to supersaturated conditions. Not the enrichment of protein, but the triggering effect of rapid evaporation stimulates nucleation and results in the rapid growth of crystals. Moreover, the activated vapour evaporation produces higher rates of protein concentration change for both the initial phase of nucleation and the following growth stage, leading to a shorter crystallization process. As a consequence, the evaporation-stimulation method exhibits two beneficial outcomes, namely, an advanced nucleation of crystals and a higher growth rate than that in the conventional process.

Our finding of local concentration variation in a drop is in good agreement with the previously reported results based on other approaches[17,18]. In particular, Pullara et al[17] related dynamic light-scattering intensity fluctuations to spinodal temperatures at which anomalies in scattering intensity and photocorrelation lifetime were observed. Their observations showed that such fluctuations were an indication of the nucleation of protein crystals and indeed shortened crystal induction time. In practice, the technique would enable us to find the protein concentrations and NaCl composition of a crystallizing solution optimal for crystal growth. On the other hand, Tanaka et al presented microscopic images of droplets and sponge phase formation, which were found in the crystallization process of lysozyme solutions[18]. They used a solution with a high protein concentration of, for example, 100 mg/ml, but their finding may correspond to our observations of local concentration change because the spatial resolution of the present confocal system is as large as the size of the droplets. Using the confocal Raman technique, the acquired time-course protein profiles in Fig. 6, particularly in the case of vapour-diffusion experiments, show that the concentration fluctuation started from the beginning of crystallization and became significantly larger around its peak concentration. On the contrary, the degree of such fluctuation turned out to be less considerable afterwards and, as seen, the decrease in concentration was rather monotonic at the end of the process. Our observations using confocal Raman microscopy with a practical hanging-drop configuration therefore provide time-dependent quantitative discrimination of local changes, which relate to the phase-separation phenomenon leading to deeper and quantitative insight into the effect of such concentration fluctuation on the induction of nucleation and the formation of crystals.

CONCLUSIONS

We have applied in-situ confocal Raman spectroscopy to simultaneously trace concentration changes of both lysozyme protein and HEPES buffer within a hanging drop during crystallizations under conventional and evaporation-stimulated conditions. In contrast to the monotonic increase in HEPES concentration, protein-concentration profiles indicate distinguishable crystallization behaviours between the two setups. The use of the evaporation-stimulation method results in (1) lower overall concentrations throughout the process, and (2) advanced nucleation of crystals with a substantially higher growth rate leading to a shorter crystallization process. The observations of the calculated drop volume and the protein inhomogeneity acquired by confocal Raman investigation suggest the existence of protein accumulation in addition to the increase in protein concentration due to vapour evaporation. This study necessitates a further detailed study of the mechanism of protein crystallization in which evaporation is activated.

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REFERENCES

1. Boyer M, Roy M-O, Jullien M (1996) Dynamic light scattering study of precrystallizing ribonuclease solutions. *J Cryst Growth* **167**, 212–20.
2. Pan BL, Berglund KA (1997) Time-resolved fluorescence and anisotropy of covalently coupled 1-pyrenebutyric acid for monitoring the crystallization conditions of lysozyme. *J Cryst Growth* **171**, 226–35.
3. Darcy PA, Weincek JM (1999) Identifying nucleation temperatures for lysozyme via differential scanning calorimetry. *J Cryst Growth* **196**, 243–9.
4. Ferré-D'Amaré AR, Burley SK (1997) Dynamic light scattering in evaluating crystallizability of macromolecules. *Meth Enzymol* **276**, 157–66.
5. Schwartz AM, Berglund KA (1999) The use of Raman spectroscopy for in situ monitoring of lysozyme concentration during crystallization in a hanging drop. *J Cryst Growth* **203**, 599–603.
6. Schwartz AM, Berglund KA (2000) In situ monitoring and control of lysozyme concentration during crystallization in a hanging drop. *J Cryst Growth* **210**, 753–60.
7. Schwartz AM, Berglund KA (2001) Comparison of supersaturation profiles employed on lysozyme crystallization from a hanging drop. *Cryst Growth Des* **1**, 81–5.

8. Noda K, Sato H, Watanabe S, Yokoyama S, Tashiro H (2007) Efficient characterization for protein crystals using confocal Raman spectroscopy. *Appl Spectros* **61**, 11–8.
9. Ataka M, Katoh E, Wakayama NI (1997) Magnetic orientation as a tool to study the initial stage of crystallization of lysozyme. *J Cryst Growth* **173**, 592–6.
10. Zhong CW, Wang LB, Wakayama NI (2001) Effect of a high magnetic field on protein crystal growth—magnetic field induced order in aqueous protein solutions. *J Cryst Growth* **233**, 561–6.
11. Sazaki G, Yoshida E, Komatsu H, Nakada T, Miyashita S, Watanabe K (1997) Effects of a magnetic field on the nucleation and growth of protein crystals. *J Cryst Growth* **173**, 231–4.
12. Adachi H, Takano K, Hosokawa Y, Inoue T, Mori Y, Matsumara H, Yoshimura M, Tsunaka Y, Morikawa M, Kanaya S, Masuhara H, Kai Y, Sasaki T (2003) Laser irradiated growth of protein crystal *Jpn J Appl Phys* **42**, L798–800.
13. Watanabe S, Nagasaka S, Noda K, Tashiro H (2004) Protein crystallization using a high-repetitive low-power laser beam under tightly focused condition. *Jpn J Appl Phys* **43**, L941–3.
14. Miele AE, Federici L, Sciara G, Draghi F, Brunori M, Vallone B (2003) Analysis of the effect of microgravity on protein crystal quality: the case of a myoglobin triple mutant. *Acta Cryst* **D59**, 982–8.
15. Cudney B, Patel S, McPherson A (1994) Crystallization of macromolecules in silica gels. *Acta Cryst* **D50**, 479–83.
16. Ban T, Mitaku H, Suzuki C, Matsuba J, Ohya Y, Takahashi Y (2005) Crystallization and crystal morphology of silicalite-1 prepared from silica gel using different amines as a base. *J Cryst Growth* **274**, 594–602.
17. Pullara F, Emanuele A, Palma-Vittorelli MB, Palma MU (2005) Lysozyme crystallization rates controlled by anomalous fluctuations. *J Cryst Growth* **274**, 536–44.
18. Tanaka S, Ataka M, Ito K (2002) Pattern formation and coarsening during metastable phase separation in lysozyme. *Phys Rev E* **65**, 051804.