STRONG MAGNETIC FIELD EFFECT ON THE DISSOLUTION PROCESS OF TETRAGONAL LYSOZYME CRYSTALS

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ABSTRACT

Either a homogeneous or inhomogeneous magnetic field has been known to dampen the protein crystal growth. To date the mechanism is not clear. However, it was generally proposed that the magnetic field may dampen the convection in the solution, resulting in a reduced crystal growth rate and possibly a good crystal quality, similar to the case of protein crystal growth in space. To understand the mechanism of the magnetic field effect on protein crystal growth, further explorations on the magnetic field effect on protein solution, on the processes of crystal growth and dissolution, and on different crystallization (solution) systems, should be valuable. In this paper we present our recent efforts to study magnetic field effects on the dissolution processes of tetragonal lysozyme crystals under a strong magnetic field. A layer of oriented tetragonal lysozyme crystals was prepared under a temperature gradient and magnetic field, after that the crystals were dissolved by increasing the temperature of the solution. The lysozyme molecules will diffuse upwards due to the steep concentration gradient at the lower side of the cell caused by the dissolution. The evolution of the concentration in the solution was measured in-situ using a Mach-Zehnder interferometer. The results confirmed that the dissolution process of the crystals was slowed by the magnetic field. Judging from the concentration evolution versus time at different positions in the solution, we concluded that the apparent diffusion coefficient of lysozyme molecules was decreased by the magnetic field. The results were discussed using a suspended crystal model in the initial dissolution stage.

INTRODUCTION

Recently, the study of crystallization of biological macromolecules has become a key technology for modern molecular biology due to the importance of sufficient large and perfect crystals to XRD structural analysis. In the numerous approaches to grow crystals of high quality and large size, the utilization of a magnetic field has been considered as a beneficial growth environment to improve protein crystal quality. Attempts to grow better protein crystals in a magnetic field (either homogeneous or inhomogeneous) have been initiated recently and positive results have been obtained (Lin *et al.*, 2000; Sato *et al.*, 2000). The most likely mechanisms for the quality improvement are attributed to the magnetic field damping of convection in the solution (for example, in the inhomogeneous magnetic field convection can be reduced by the balance between an upward magnetization force and a downward gravity (Wakayama *et al.*, 1997)). An orientation effect may reduce the inhomogeneity between the mosaic structures (Lin *et al.*, 2000; Sato *et al.*, 2000). The former (reduced convection) is similar to the case of protein crystal growth in space. It will thus be meaningful to investigate the possibility of using low cost magnetic fields to simulate or even replace the microgravity conditions to some extent for the growth of high quality protein crystals (Brooks et al., 2000), although the magnetic field can not create a physically real microgravity condition. Studies concerning the physical effects of magnetic field on the bulk protein solution, on the processes (crystal growth and dissolution), and on the behaviors of the various species in the solution will be valuable for the clarification of the mechanism of the protein crystal growth under a magnetic field.

Recently we reported our discovery of the magnetic field damping effect on the process of protein crystal growth (Yin *et al.*, 2001). Yanagiya *et al.* (2000) also reported similar results in a different system. Although

convection was proposed as one crucial factor in altering the growth rate of lysozyme crystals, we still can not be certain if other reasons, like growth kinetics or diffusion governed mass transport, are really negligible during growth, especially when all reported dampened growths were observed in convection restricted conditions. The purpose of this paper is to check if the magnetic field shows any significant effects on the diffusion process. For this we studied the dissolution process of a crystal layer. First a layer of tetragonal crystals at the bottom of a cell was prepared under a temperature control program; then the crystals were dissolved in a strong magnetic field. The lysozyme molecules will diffuse upwards due to the large concentration difference. By measuring the concentration of the protein in the solution *in-situ* using a Mach-Zehnder interferometer, the diffusion process can be inferred. We have already observed a magnetic field damping effect on the dissolution of lysozyme crystals in a thin solution (thickness 1mm) (Yin, et al., 2002) using the above method. It was found that the magnetic field can significantly decrease the apparent diffusion coefficient of lysozyme in a supersaturated solution. In this paper we present our more recent further study on this subject. We found that in a cell with thicker inner size (*i.e.*, solution thickness 3mm) the concentration variation during the dissolution was slightly different from that previous reported using a thinner cell. At the same time, a new phenomenon, *i.e.*, the formation of orthorhombic crystals, occurred at the cell bottom.

EXPERIMENTAL

The experimental setup was described elsewhere (Yin et al., 2001). In the current study, the sample holder was improved by mounting water-cooling jackets on both the upper and lower sides, so that the temperatures on both sides can be controlled from 0ºC to 60ºC. The configuration of the sample holder is shown in Figure 1. The temperatures at the upper and lower sides of the cell were controlled by Peltier devices. Either temperature gradient or isothermal conditions can be realized with high accuracy (to ±0.1ºC) using this sample holder.

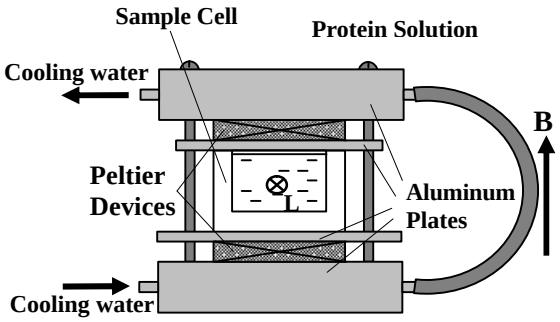


Fig. 1. Schematic illustration of the sample holder. The temperature configuration of the cell was controlled by the two Peltier devices. "**L**" gives the direction of the light beam. "**B**" gives the direction of the magnetic field.

In this experiment, the sample cell was placed at the center of a superconducting magnet (JMTD-10T100M, maximum field strength: 10T; made by Japan Magnet Technology, Inc.) so that a highly homogeneous magnetic field can be applied to the sample. The inner dimensions of the quartz cell were 3mm thick, 10mm in height, and 15mm in width.

The concentrations of the starting aqueous protein solution were as follows: hen egg-white lysozyme (HEWL, Seikagaku kogyo, six times recrystallized) 45 mg/ml, NaCl 40mg/ml, and the pH of the solution was adjusted to 4.60 using 1N HCl solution.

The concentration of lysozyme in the solution was measured in-situ based on the relationship of the refractive index and the protein concentration in the solution under different temperatures at a certain point *(x,y)*:

$$\left\{\left[\frac{\partial n}{\partial C}\right]_T \left[C(x,y)-C_0\right]+\left[\frac{\partial n}{\partial T}\right]_C \left[T(x,y)-T_0\right]\right\}d=\frac{l}{2p}\Delta f(x,y) \qquad (1)$$

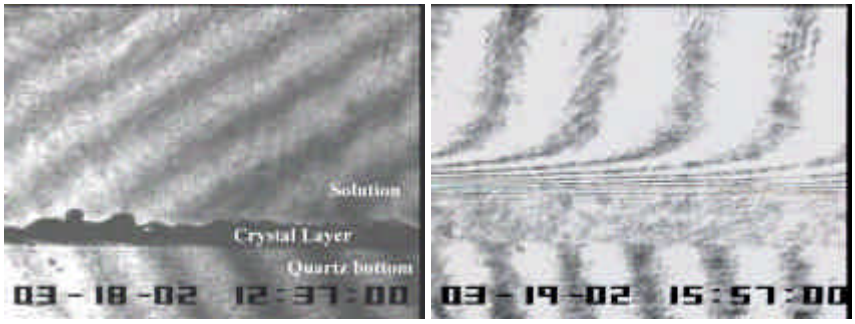
where n: refractive index; *C*: concentration of the solution; *C(x, y)*: concentration at point *(x, y)*; C_0: initial concentration; *T(x, y)*: temperature at point *(x, y)*; T_0: initial temperature; *d*: thickness of the solution in the direction of the optical path, here *d*=1mm; l: wavelength of the optical light source (l=780nm); $\Delta f(x, y)$: phase

difference between the initial stage and studied stage at point *(x, y)*. From the above equation, the concentration of the solution at any time and any region *C(x, y)* can be calculated after we obtained the interference fringe shift data, which shows the phase differences over time.

RESULTS

Growth of crystal Layer and dissolution of the layer

To make the dissolution process simple and easy to study, a layer of tetragonal lysozyme crystals should be prepared before the dissolution started. To grow a crystal layer we need a temperature gradient to make the temperature at the cell bottom suitable for the crystal nucleation and growth. If the solution is kept at an isothermal condition, nucleation will occur everywhere in the cell. In some solution and cell size conditions we probably need a carefully screened temperature program (varying with time!) so that the crystal will grow from the bottom only, since a possible largest temperature gradient might not be (large) enough for the growth of the layer only at the cell bottom, nucleation and growth at places near the cell bottom might also occur if the supersaturation at that region is suitable for the nucleation and growth. Furthermore we must consider the possibility of the formation of the orthorhombic crystals at the upper part of the cell due to higher temperatures, the well known temperature corresponding the phase change of lysozyme crystals. As growth of orthorhombic crystals will not stop during the subsequent dissolution, their nucleation in the initial stage should be better avoided. In the present study we succeeded in growing a tetragonal crystal layer at a temperature gradient 2.93°C/mm by setting the temperature control at 50°C and 6°C at the upper and lower sides of the cell, respectively. To make the comparison conditions the same we tried to grow all crystal layers in strong magnetic field (10T) so that the layered crystals can be oriented with their c-axis aligned along the magnetic field. Figure 2 shows the fringe images during the layer growth and dissolution. Figure 2 (a) shows the picture captured during the layer growth under 10T. At the cell bottom we can observe the crystal layer. As the layered crystals are highly oriented, we may get a good chance to study the growth process of different crystallographic faces. For example, in the current case as shown in the figure, the growth of the {101} faces of the crystals can be studied.



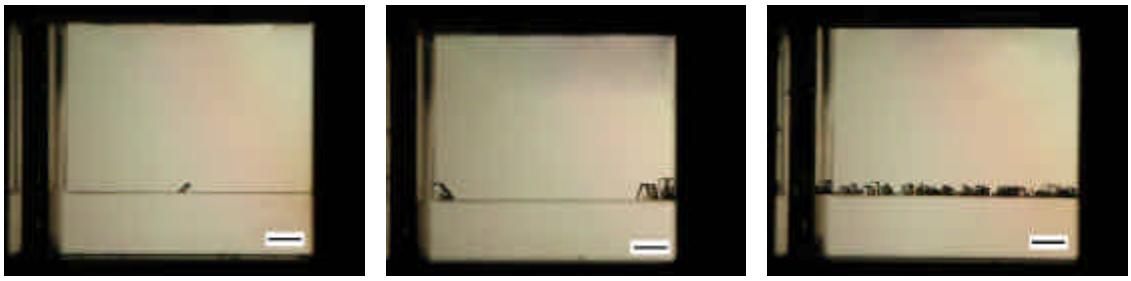
(a) (b)

Fig. 2. Fringe images during the layer growth and dissolution. (a) crystal layer growth (10T); (b) crystal dissolution (0T). The size of the fringe images is: 6.4×4.8mm^2.

After the crystal layer has been prepared, the temperature program was changed from 50°C to 55°C at the upper side and from 6°C to 50°C at the lower side of the cell within 30min, respectively. The tetragonal crystals will be dissolved at this moment. During the dissolution process, the concentration in the solution near the crystals will change drastically. Figure 2 (b) shows the fringe image during the dissolution of the crystal layer (captured at 0T). According to the spatial shift of the fringes we can obtain the concentration distribution in the observed field. From Figure 2 (b) we notice that the fringes curved sharply at one end near the crystals. This represents the existence of a steep concentration gradient in that region. Whereas in Figure 2 (a) the fringes are straighter and more evenly distributed, indicating that the concentration in the solution is relatively more homogeneous, without any sharp change of the concentration. In the current conditions the mass transport process during the process of dissolution was basically a diffusion process, thus we can get information about the solute diffusion through observing the variation of the concentration.

Effect of Magnetic field strength on dissolution

As observed previously in a cell with solution thickness of 1 mm, height 15 mm and width 26 mm, we found that the crystal can be completely dissolved and the result obtained that the apparent diffusion coefficient of lysozyme is decreased by the magnetic field (6T) (Yin *et al.*, 2002). Here we used a cell with different dimensions (thickness 3 mm, height 10 mm, and width 15 mm), different phenomenon was observed. Figure 3 shows the cell at different magnetic field strengths after 24 hours dissolution. Figure 3 (a) shows the case at 0T. We can see that a small crystal was formed at the cell bottom. The crystal should be an orthorhombic crystal because at such high temperature the crystal didn't dissolve, instead, it grew. At 6T, orthorhombic crystals were still observed, however, the size and number of the crystals were obviously larger than at 0T. At 10T (Figure 3 (c)), such tendency became clearer: we can see many crystals formed at the cell bottom and were highly oriented. This indicates that the crystals were nucleated and grew under a strong magnetic field and their orientation was affected by the magnetic field. Such phenomenon was not observed in the previous study using the cell with inner thickness of 1 mm, in which conditions no crystals were found in the cell at both 0T and 6T. The formation of orthorhombic crystals was probably caused by the high concentration at the bottom region of the cell during the dissolution. When the dissolution occurred, the concentration near the cell bottom would be very large and should be favorable for the nucleation and growth of the orthorhombic crystals. This is clear because we didn't find any crystals formed at other locations. In the case of the 1 mm cell, orthorhombic crystals were not observed in the studied time period (about 24 hours). Keeping the sample at high temperature for longer time will of course finally lead to the formation of orthorhombic crystals, but in such case the crystals will not form only at the cell bottom, in stead they may be randomly distributed in the cell.



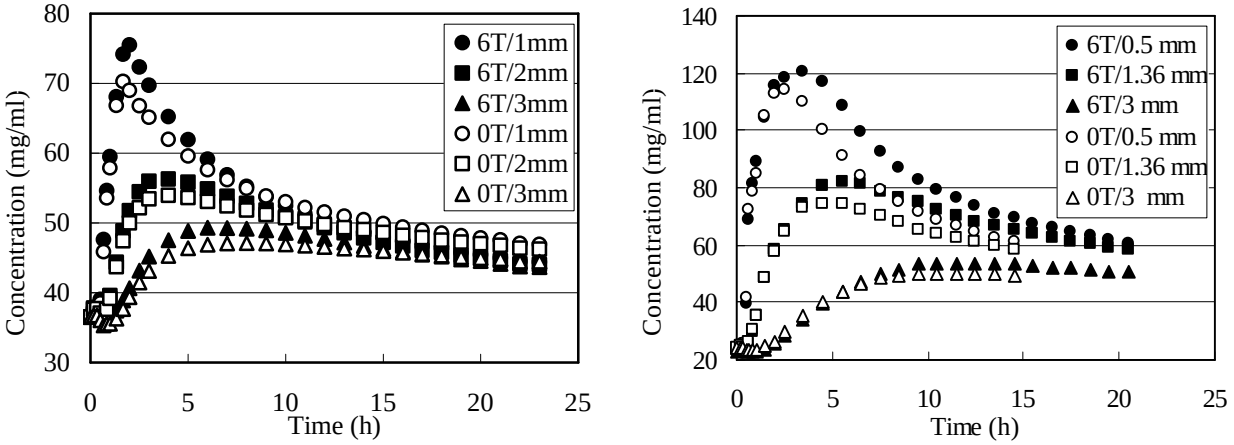
(a) (b) (c)

Fig. 3. Orthorhombic crystals grown after the dissolution process of tetragonal lysozyme crystals started for 24 hours. (a) 0T, (b) 6T, (c) 10T. The scale bars in the pictures represent 2mm.

Time dependence of concentration during the dissolution

The concentration distribution of the solution was monitored throughout the whole experimental process starting from the growth to the dissolution processes. Here we present the results during the dissolution. Figure 4 (a) and (b) show the concentration evolution at different positions in the cell during the crystal dissolution for 3 mm cell and 1 mm cell, respectively. All measured points are at the vertically central line of the cell. We can see that at both cases (1mm and 3mm) the concentration at any point will reach their maximum levels, after that the concentration will decrease. This is easy to understand by taking account of the two factors governing the concentration level at a specific point near the dissolving crystals: one is the dissolution process which supplies lysozyme molecules to this point, which will cause the concentration to increase; Another factor is the transport of lysozyme to lower concentration region which will decrease the concentration at the point. If the dissolution of crystals finishes, the supply of lysozyme molecules will stop, thus the increase of the concentration will stop. Due to the continuous transport of lysozyme to lower concentration region, the concentration at the studied point will decrease. From the figure we can see that the time needed for reaching the maximum values are different depending on their distance to the cell bottom. At regions nearer to the crystals, the time needed for reaching a maximum value was shorter. This can be understood as the result of competition between the above two factors. It is reasonable to imagine that not all regions in the solution will undergo the same concentration evolution route as shown in the figure. The concentration at the upper part of the cell will only experience an increase and finally reach its equilibrium level.

From both Figure 4(a) and (b) we can see that the maximum levels of the concentration at 6T were obviously higher than their counterparts at 0T. From this we conclude that the mass transport process was faster at 0T than at 6T, because at 0T more lysozyme molecules were transported to further distance so that the accumulated lysozyme in a specific region (near the dissolving crystals) will not reach a higher level than at 6T.



(a) (b)

Fig. 4. The evolution of lysozyme concentration at three points versus time in the absence and presence of the magnetic field. The legend "6T/1mm" means the measurement result for the point which is 1mm above the cell bottom and under magnetic field 6T. (a) Inner cell dimensions: 3mm thick, 10mm height, and 15mm width; (b) Inner cell dimensions: 1mm thick, 15mm height, and 26mm width.

In Figure 4 (a) we noticed that the concentration under the magnetic field decreased to lower concentration since the dissolution for more than 10 hours. Considering this phenomenon with the observed size and number of orthorhombic crystals formed at the cell bottom as shown in Figure 3 (a) and (b), we can understand that the growth of orthorhombic crystals at the cell bottom consume more lysozyme molecules so that the concentration decreased to lower level than at 0T. In the case of 1mm cell, as there were no orthorhombic crystals observed at the cell bottom, it is understandable that at the same time period the concentration at 6T is still higher than in the case at 0T. In the case when under stronger magnetic field at 10T (for 3 mm cell), the concentration will decrease faster due to the formation of large amount of orthorhombic crystals, so that we could not expect higher maximum concentration at 10T than at 0T.

DISCUSSIONS

In the present experiment, although orthorhombic crystals formed at the cell bottom during the dissolution, the whole dissolution process of the tetragonal lysozyme crystals was still a diffusion-dominated process. As the solution is highly supersaturated, it will be convenient to characterize the diffusive transport of the lysozyme molecules by an apparent bulk diffusion coefficient D_{app}, which can be related with the bulk diffusion flux j in the solution with lysozyme concentration C according to Fick's law of diffusion:

$$j = -D_{app} \frac{\Delta C}{\Delta z} \tag{2}$$

Judging from the comparison between 0T and 6T, we can obtain the conclusion that the depressed dissolution process implies that the apparent diffusion coefficient D_{app} of the molecules in the solution was decreased by the magnetic field. We will present elsewhere the measured diffusion coefficient of lysozyme in different magnetic fields, which was also in good agreement with the current result. Here we will discuss the possible mechanism for the diffusion decrease under a strong magnetic field.

By using the orientation effect of a strong magnetic field on lysozyme crystal growth, Ataka et al (1996) and Wakayama (1997) found that the crystals will nucleate first and remain suspending during the subsequent growth in the solution before they reach a critical size and sink. In the suspended state crystals larger than a critical size will be magnetically oriented due to their anisotropy in magnetic susceptibility. Here we propose that in the solution there exists many suspended crystals. Part of these crystals whose size is large enough will be magnetically oriented in the solution. Figure 5 schematically shows the state of the suspended crystals in the solution in the absence (a) and in the presence of magnetic field (b). Now let's consider the diffusion of a lysozyme molecule M. When the magnetic field is not applied (Figure 5(a)), the suspending crystals have no preferable orientation (there is no torque exerted on the crystals). The diffusing molecule will migrate from a

higher to lower concentration region. If a crystal blocks the way, the molecule can migrate by rotating the crystal. While when the magnetic field is applied (Figure 5 (b)), a torque will be exerted on the crystals so that

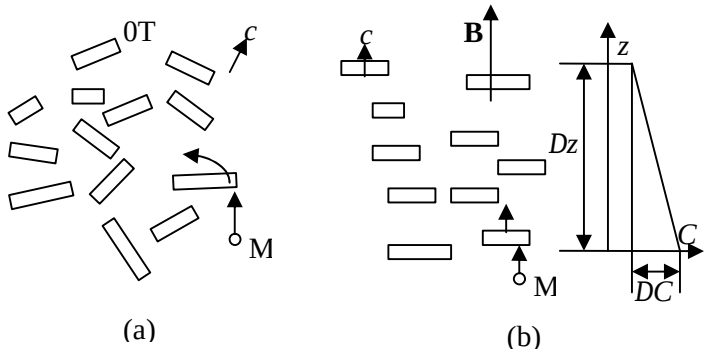


Fig. 5. Schematic illustration of the suspending crystal model. (a) In the absence of magnetic field; (b) in the presence of magnetic field. **B:** the direction of the magnetic field; *c*: the direction of the *c* axis of the crystal, *C*: the concentration; Z: the diffusion distance along the concentration gradient; M: diffusing molecule.

the crystals will be oriented to the magnetic field direction. If many small suspending crystals are oriented along one direction, we can imagine that a fraction of the solution has ordered structure. The orientation of the ordered solution fraction should be relatively stable if the magnetic field is maintained. In this case the diffusing molecule has to migrate by pushing the oriented crystal if the latter blocks the way, or by changing the migration directions, which will result in a longer diffusing distance as compared with the case without magnetic field. Apparently the oriented suspending crystals will act as stronger barriers for diffusing molecules than the non-oriented crystals. With increasing temperature in the solution, the suspending crystals will also be dissolved (first decrease size, then lose orientation, and finally disappear). Seemingly the environment for the diffusing molecules will soon be the same in the two cases with and without magnetic field. However, the initial stage of the magnetic field damping of the diffusion will cause the subsequent damping of the diffusion even if all of the suspending crystals are dissolved. Because the previous damping will cause a little larger concentration at the region near the dissolving crystals, such larger concentration is one major reason for the significant decrease of the apparent diffusion coefficient. Support for this idea can be found in the early study by Myerson and his coworkers (Sorell & Myerson, 1982; Chang & Myerson, 1986) on the diffusion coefficient of the solute molecules in a supersaturated solution.

CONCLUSIONS

In this paper, we conducted a further investigation based on the previous study on the magnetic field effects on the dissolution process of tetragonal lysozyme crystals. The following conclusions were obtained:

(1) The apparent diffusion coefficient of lysozyme molecules in the solution was decreased by a magnetic field.
(2) Due to the damping of the dissolution process, orthorhombic crystal might be formed at the cell bottom. The amount of orthorhombic crystals varied significantly with the magnetic field strength.

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