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The fluid phenomena in the crystallization of the protein crystal*

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This paper reports that an optical diagnostic system consisting of Mach–Zehnder interferometer with a phase shift device and image processor has been used for study of the kinetics of protein crystal growing process. The crystallization process of protein crystal by vapour diffusion is investigated. The interference fringes are observed in real time. The present experiment demonstrates that the diffusion and the sedimentation influence the crystallization of protein crystal which grows in solution, and the concentration capillary convection associated with surface tension occurs at the vicinity of free surface of the protein mother liquor, and directly affects on the outcome of protein crystallization. So far the detailed analysis and the important role of the fluid phenomena in protein crystallization have been discussed a little in both space- and ground-based crystal growth experiments. It is also found that these fluid phenomena affect the outcome of protein crystallization, regular growth, and crystal quality. This may explain the fact that many results of space-based investigation do not show overall improvement.

Keywords: convection, diffusion, sedimentation, protein crystal growth
PACC: 8740, 8110D

1. Introduction

Protein crystals must be grown from chemically complex aqueous solution that is restricted to rather narrow conditions.[1] To investigate these conditions, many variables must be sampled and examined. As a matter of fact, most of these factors are not recognized, and meanwhile the thermodynamic and kinetic process are not well understood yet, though considerable process of protein crystals growth has been studied. The various techniques of biological macromolecular crystal growth have been developed with the development of the rapid progress of the molecular biology, physics, chemistry and computer science. The processes of the protein crystal growth and the crystal characters have not been completely understood, because of the complication of the growing process. So far, the protein crystallization methods allow little or nearly no control to the crystallization process, and no prediction to the final outcome.

The solute convection influences crystal growth and crystal quality significantly. In order to minimize or eliminate the undesirable convection effects, many efforts have been made in both space- and ground-based protein crystallization experiment.[2−4] Nevertheless, the debating focus is whether the convection and sedimentation are important factors limiting the outcome of the protein crystal growth. If this is really the case, the protein crystal growth under microgravity (μg) conditions should undoubtedly result in marked improvement in both size and quality of resulting crystals. However, in the majority of cases, the crystals grown in space often show no improvement and even more inferior to those crystals that grown on earth. Understanding how μg affects crystal growth process must be preceded by understanding of the process on ground, and much careful experimental work has to be devoted on this topic.[5−8]

In order to understand how solute convection directly affects the outcome of crystal growth, the fluid dynamics has been applied to characterize the convection-diffusive supply fields in crystallizing solutions. On the other hand, due to the proteins are only rarely available in large quantities, recent advances in protein crystal growth have emphasized the utilization of micro scale techniques in which microlitre quantities of protein solution are used for vapour-diffusion, dialysis and liquid-liquid diffusion.

During investigations on the process of protein crystal growth, by using phase shift Mach–Zehnder

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interferometer, we observed the diffusion process, the sedimentation and the concentration capillary convection, and found these fluid phenomena seriously affect the crystal qualities. The interference fringes, related to the change of concentration, are visualized and measured. The present experiments demonstrate that there are major effects on the crystal growth: the sedimentation produced by gravity; the concentration capillary convection induced by surface tension; and the diffusion process. Carefully studying this fluid behaviour within the crystallizing micro-scale drop during the protein crystal growth could provide useful information to optimize crystal growth and hence to improve crystal quality. In this paper, the interesting observations and the detailed analyses are presented and discussed.

2. The materials and the experimental facility

The hen egg white lysozyme from Sigma (Cat, NO L6876, Lot NO 46F-80601) without any further purification before use was dissolved in the double-deionized water at 25 mg/mL which was centrifuged for 15 minutes at 8000 rpm. All other chemicals used in the present study were of analytical grade and were used without further purification, but all solutions were filtered through micro filters with pore size of 0.22 μL. The crystallization experiments were conducted by vapor diffusion configuration.

The protein trichosanthin (TCS) was used in this study. The TCS was a type I ribosome-inactivating protein (247a.a., Mr=27400Da), extracted from the plant root tuber of trichosanches kirilowill, which grows in south China. The dry TCS protein powder, at home purified, was dissolved in double-deionized water aqueous solution containing 5% sodium chloride at 30 mg/mL and stirred 2 min, and then centrifuged for 20 min at 10000 r/min. All other chemicals used in the present study were of analytical grade and used without further purification, but all solutions were subsequently filtered with a micro-filter of 0.22 μm before use.

The vapour diffusion method is usually used to protein crystal growth, performed by hanging drop method or sitting drop method. The precipitator in reservoir and the salt in the sample, or the organic solvent concentration, will change to reach to the equilibrium state by vapour diffusion. As the salt is used as precipitator, the precipitator concentration in protein sample must be smaller than that in reservoir. The water in protein sample droplet will vapourize and transfer into the solution in reservoir. The equilibrium will be obtained and the protein crystals are growing in the sample droplet.

The conventional vapour diffusion method usually used in laboratory is not convenient for optical measurement because of curving face of the solution drop. A modelling system for observing the crystal growth was designed to fit both optical interference measurement and vapour diffusion process as shown in Fig.1. It consisted of an optical glass system including a large cell for precipitator and a small cell 3 mm×2 mm×4 mm as sample volume. The construction of this growth chamber was almost the same as that of the conventional sitting drop, and the growth rate was slow in the present experimental method.

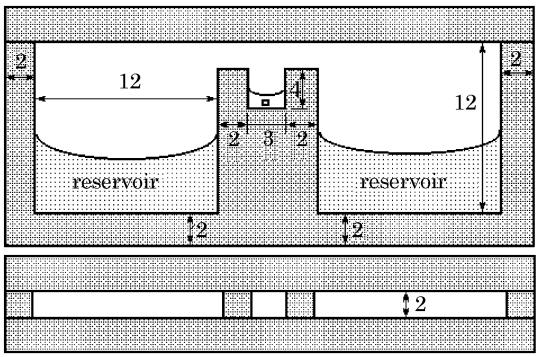
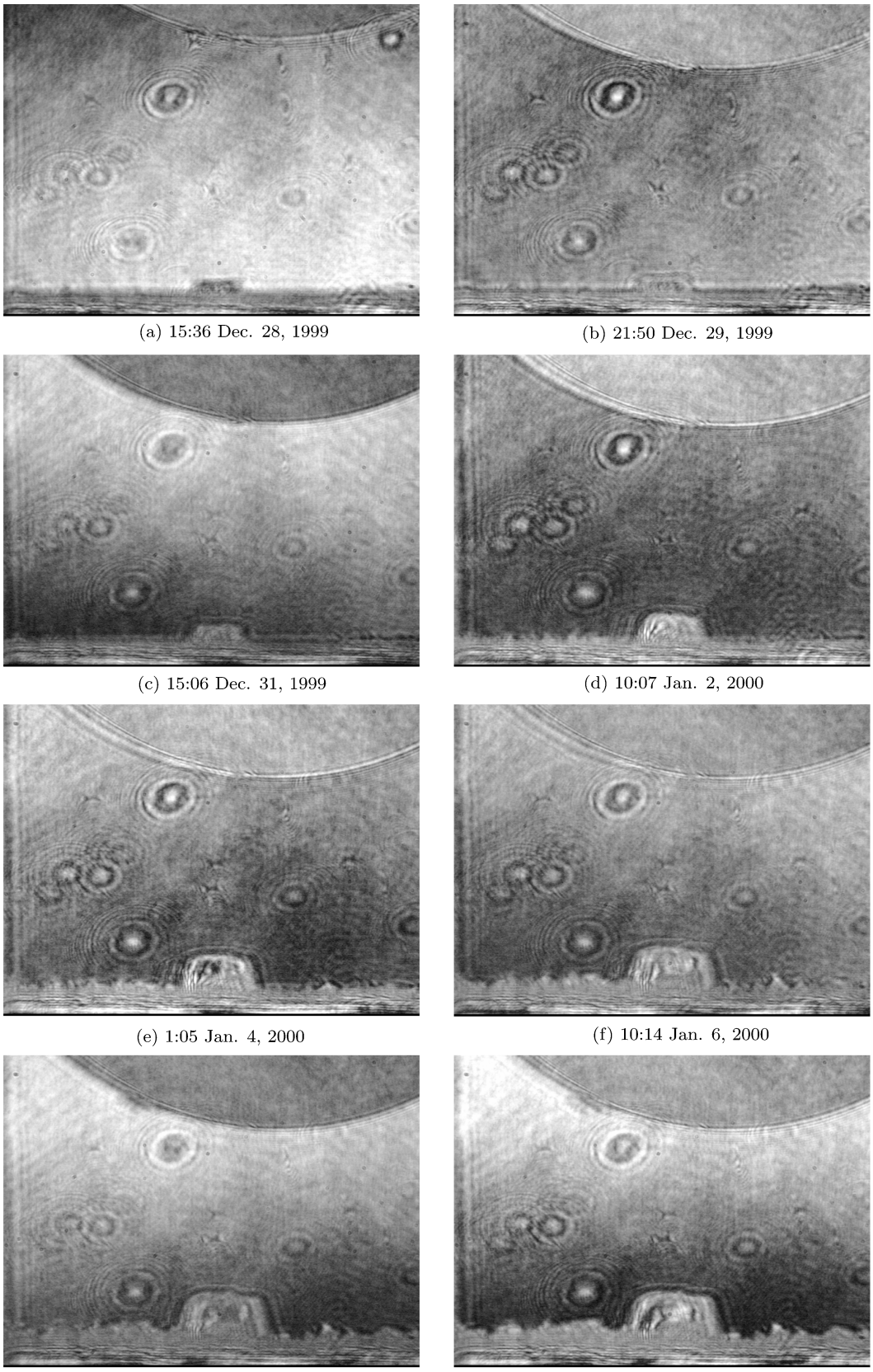


Fig.1. The modified cell of the vapour diffusion method.

A micro Mach–Zehnder interferometer and four-step phase shift technique had been used in the present experiment. The measured area of the interferometer was 2.0 mm×1.6 mm and the resolution was about 2 μm. The fringes carry the concentration gradient of the solution flow field in crystal growth cell. During the crystal growth, the environment temperature should be constant at 295 K, so, the interferometer with the protein crystal growth cell had been put in a biochemical incubator, in which the temperature was kept at constant value of 295 K.

3. The phenomena of diffusion and sedimentation

The present experiment started on 26 December 1999; over the initial two days, no crystal



(a) 15:36 Dec. 28, 1999 (b) 21:50 Dec. 29, 1999

(c) 15:06 Dec. 31, 1999 (d) 10:07 Jan. 2, 2000

(e) 1:05 Jan. 4, 2000 (f) 10:14 Jan. 6, 2000

(g) 9:11 Jan. 8, 2000 (h) 0:21 Jan. 9, 2000

Fig.2. The process of the lysozyme crystal growth on the bottom of the cell (2mm×1.6mm).

appeared. The growth cell with lysozyme protein crystal growth sample was put in the Mach–Zehnder interferometer, and the interferometer was modulated working in an infinity field, in which there was no interference fringe at this time. After about two days, some small lysozyme crystals grew in the growth cell, the interference fringes appeared, CCD camera recorded the process. The fringes directly inflected the concentration change during the crystal growth. The experimental process is shown in Fig.2. The fringe images of the micro Mach–Zehnder interferometer were captured at a regular interval of 3600 seconds. 259×4=1036 images had been recorded during the experimental period. A large crystal on the bottom of the growth cell was observed, its final size was about 0.5 mm. The face [110] and the four top faces [101] were grown much better than the four bottom faces [101]. In this experiment, some small crystals were nucleated inside the growth cell, and the interference fringe patterns were observed around these crystals respectively.

The four step phase shift interferograms had been recorded in the present experiment. Phase calculation and unwrapping had been operated.[9,10] The relation between the refractive index and the concentration had been obtained by using the WAY-15 ABBE REFRACTOMETER to measure the refractive index at different concentrations of the lysozyme solution. A formula relating the refractive index and the concentration may be written as n=1.3492–2.2660×$10^{-4}C$.

According to the technique of four step phase shift, the concentration gradient and the contour maps is given in Fig.3. X-axis stands for the length of the measured area. Y-axis stands for the width of the measured area. A crystal grew from 0 mm to 0.5 mm at the area where is the centre of X-axis and the top of Y-axis during the experimental process. The concentration difference in the diffusion layer must be confirmed by calculating the difference of ΔC inside the layer and outside the layer. According to the data of the concentration distribution, ΔC inside the layer was 5.7494 mg/mL, and ΔC outside the layer was 5.6399 mg/mL. The concentration difference induced by the crystal growth at the time 10:14 January 6 in the crystal growth process was about 0.1095 mg/mL.

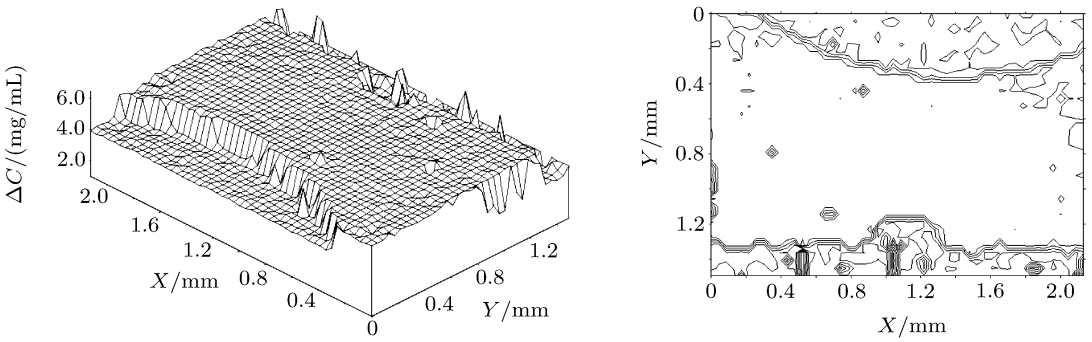


Fig.3. The concentration gradient and the counter map at 10:14 Jan. 6, 2000.

The concentration distribution with the change of the distance to the middle of the top crystal solid face at the time 10:14 January 6 is given in Fig.4. X-axis stands for the vertical distance to the centre of the up face of the crystal. And Y-axis stands for the ratio of the concentration C at current point to the concentration C_∞ far from the crystal. This curve clearly shows the diffusion boundary layer, which is estimated as $\delta \approx 22\,\mu$m in thickness. There was no change in concentration far from the growing crystal.

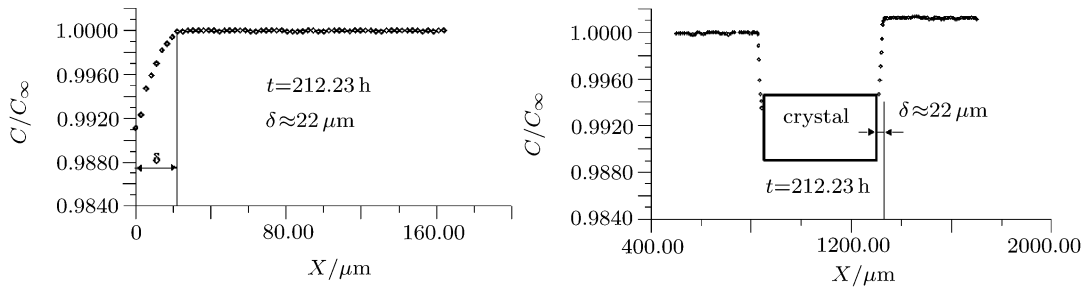


Fig.4. The concentration distribution around the crystal.

The experimental result shows that the crystal growth of the present model was dominated by diffusion process. The flux $\boldsymbol{J}$ may be given by the Fick law as

$$\boldsymbol{J} = -D \cdot \nabla C. \tag{1}$$

In the present experiment, D is the solute diffusion coefficient. According to the experimental data, the concentration difference was 0.1095 mg/mL= 0.1095 kg/m^3 in the diffusion layer which was 2.2×10^{-5} m in thickness. The diffusion coefficient of the system D=7×10^{-11} m^2/s,[9] the diffusion flux J is given as:

$$\begin{aligned}\boldsymbol{J} &= -7\times10^{-11}\times\frac{0.1095}{2.2\times10^{-5}}\\ &= -3.484\times10^{-7}\mathrm{kg/(m^2\cdot s)}\\ &= -0.3484\mathrm{mg/(m^2\cdot s)}.\end{aligned} \tag{2}$$

This value is very small, and relates to a slow growth rate of the protein crystal.

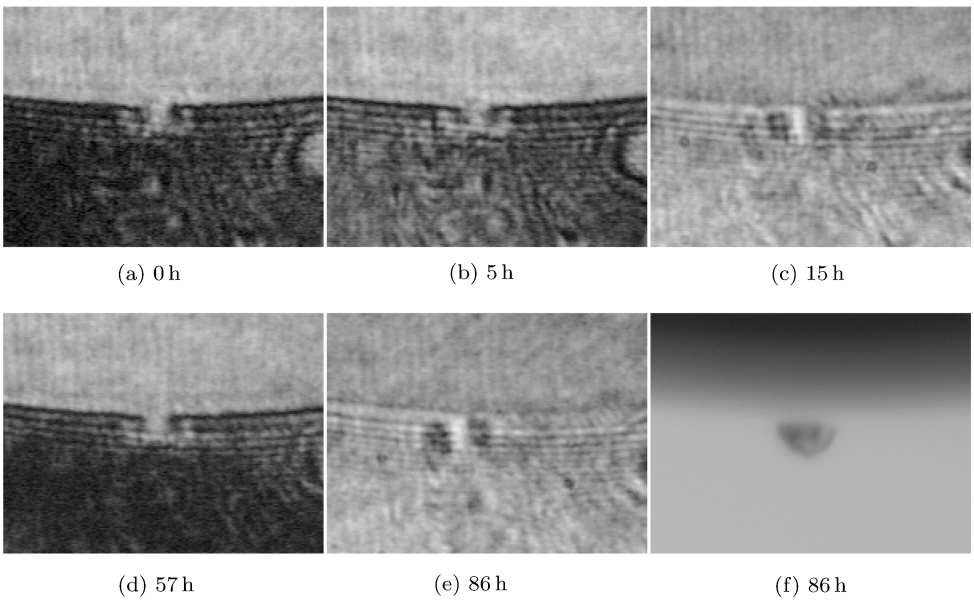
In the present experiment, a crystal grew on the bottom of the cell, several crystals grew inside the growth cell. This phenomenon demonstrates that the sedimentation exists in the crystallization of protein crystal. Because of the sedimentation effect, the protein crystals usually grew at the bottom of the growth cell. The bottom faces [101] of the crystal on the cell bottom were almost rejected because of the contact between the lower crystal faces and the cell boundary, faces [110] and the top faces [101] were growing well.

4. The phenomena of the concentration capillary convection

This experiment was set up as vapour-diffusion trials in a sitting drop arrangement in the cell. The TCS crystal growth cell was mounted at the sample stage of the micro Mach–Zehnder interferometer, then the whole system was put in an incubator, in which the temperature is 295 K. During the crystal growth, the fringe images were captured with an automated image acquisition CCD system.

To measure the protein concentration variation in the crystal growth cell during the experiment, the refractive index of each sampling drop was determined with a temperature controlled Abbe refractive meter, and the relationship between the protein concentration and refractive index was established, it was expressed as n=1.3583–2.1195×10^{-4}.

The concentration capillary convection around the growing protein crystals were observed and visualized by Mach-Zehnder interferometer during the course of the TCS crystal growth. Direct evidence of the presence of the concentration capillary convection in the crystal growth experiment is shown in Figs.5(a)–(5e), which are the images captured by the interferometer. The crystal on the liquid surface is shown in Fig.5(f), which is the picture taken by the microscope. The interference fringes on the liquid surface are rings on the left and right of this crystal. This



(a) 0 h (b) 5 h (c) 15 h

(d) 57 h (e) 86 h (f) 86 h

Fig.5. The fringe of the lysozyme crystal grown in liquid surface.

phenomenon demonstrates that the crystals growing along the vicinity of the liquid surface were correlated with the concentration capillary convection, and the post-nucleation growth rate was very small.

In conventional vapour-diffusion methods, the water vapourization is crucial. The equilibration of the vapour-diffusion experiment is driven by the difference between the chemical potential of the water in the droplet and that in the reservoir. In general arrangement, traditionally, the aqueous salt concentration in the well is double that in the droplet, thus the water will separate out from the droplet, where the chemical potential of water is lower, to the well reservoir where the chemical potential of water is higher. This departure of the water shrinks the overall volume of the droplet and diminishes the radius of curvature of the droplet, which serves to decrease its surface area (occurs where the change of surface tension occurs). On the other hand, the continuous transfer of the water molecules from the interior of the sitting droplet to the surface will condense and create a boundary layer rich in water, thus the concentration of the protein and crystallizing agent decrease, before final equilibrium is achieved. In addition, in conventional crystallizing methods, nucleation and post-nucleation growth proceed randomly in the protein mother liquor. It is therefore difficult to define where the nucleation position is. So, in the case that the nucleation occurs in the vicinity of the surface in the sitting droplet method, the post-nucleation growth rate should be small and unequal due to the regular growth sampled by the surface tension and unsteady concentration convection, and the lower concentration as well.

For the TCS crystal growth, the protein concentration gradient distribution obtained in the crystallization solution after two days of crystal growth are depicted in Fig.6, the concentration changes around the smaller crystal were much less pronounced due to the low growth rate at closely located solution surface. However, the concentration distribution on the line y=0.5979 mm, which across the crystal, was much more pronounced that the concentration capillary convection existed at the sides of the crystal if the crystal grew on the liquid surface, see Fig.7.

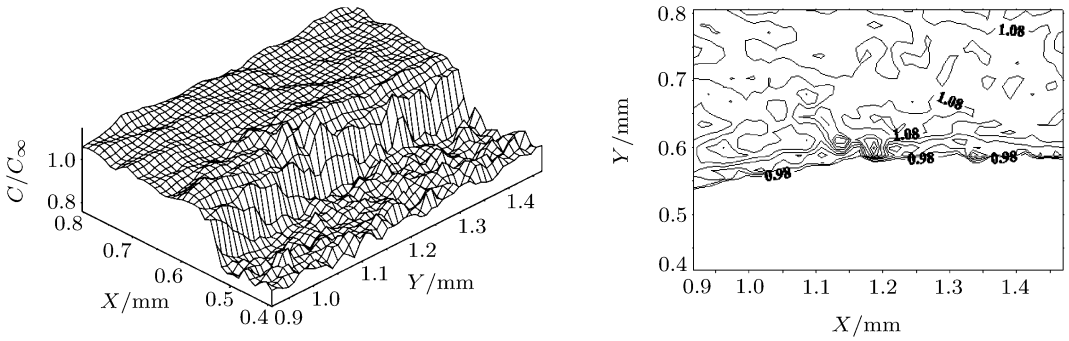


Fig.6. The concentration gradient and the counter map calculated from Fig.5(a).

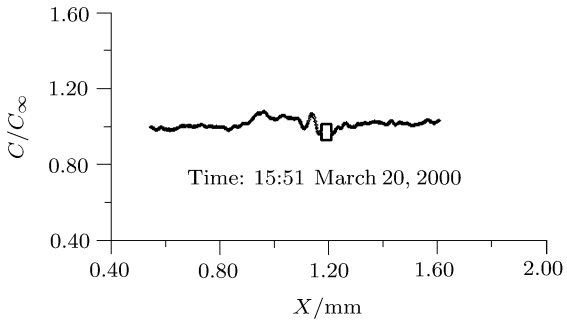


Fig.7. The concentration distribution on the line y=0.5979 mm.

5. Conclusion

The crystallization of protein crystal has been studied experimentally, the significant distribution of the concentration gradient has been calculated. The diffusion and sedimentation during the protein crystal growth has been visualized and analysed. When the crystal was growing, a thin diffusion layer existed only around the crystal, and almost no concentration changed outside the layer. The crystals usually grew on the bottom of the growth cell and usually grew many small crystals, because the solution concentration was large in the bottom of the cell in gravity, this caused the sedimentation phenomena.

The concentration capillary convection had been observed and visualized with micro-scale crystallizing sample of trichosantrin during their crystal growth under well-designed conditions. It seems that the concentration capillary convection arise from the combination of the surface tension and the convection in the crystallizing solution. Once the nucleation occurs at the vicinity of liquid surface of the crystallizing solution, the concentration capillary convection will interfere with the growth rate and the final crystal quality, which are the undesirable perturbation. To the author's knowledge, there is not any reports about the phenomenon of concentration capillary convection in protein crystal growth and the affection on the outcome of the growing crystals. Although somewhat preliminary, this observation is instructive.

The fluid phenomena studied in the present research work may be the reasons for the fact that the majority of the results of space-based investigations does not show improvement and even smaller crystal size and more imperfect structures. Understanding these dynamics should improve our ability to grow good quality protein crystals. So, the details of the phenomena described here should be pursued further.

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