IIII Original Paper IIII

Dependence of Convection Flow Rates on Gravity around Growing Hen Egg-White Lysozyme Crystals

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Abstract

Convection flows induced by formation of solute depletion zone around growing protein crystals are visualized to confirm whether growth of crystals at a ceiling position (top wall of a growth container) can really suppress this convection flow effectively or not. First, we observed the convection flows around the crystal at the ceiling position at 1.0 G. Second, parabolic flight experiments revealed that flow rates of polystyrene particles (as marker particles, 500 nm in diameter) around a crystal at the ceiling position did not indicate zero at high-gravity conditions, and the particles almost stop at zero gravity. Thus, stable microgravity experiments are still indispensable to attain complete convection-free conditions.

Keyword(s): Convection flow, Flow rates, Solutal convection, Ceiling position
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1. Introduction

Growth of high quality protein crystals (Max resolution < 1.5 Å) is a prerequisite for structure-based drug design (SBDD) which is famous for anti-influenza or anti-HIV drugs[1]. Since the success in development of new important drugs yields huge sales (several tenth ~ hundredth billion dollars), many drug companies go head-to-head all around the world.

On the other hand, growth of such high-quality protein crystals is devilishly hard. Actually, only 11,135 out of 126,405 structures (by 6/13/2018) registered in the protein data bank[2] show diffraction resolution higher than 1.5 Å at the present stage.

In such a situation, a lot of crystallization experiments are conducted in the international space station (ISS), since NASA reported that about 20 % of space grown protein crystals show higher diffraction resolution than that of best crystals grown on the earth[3]. Additionally, an attempt argued that more complete requirement studies on growth kinetics of protein crystals before space experiments resulted in the improvement in diffraction resolution of about 70 % of space grown crystals[4].

Although many studies have been done in space thus far[5], we have not answered the following essential question "why the quality of protein crystals improves in space?" completely. To solve the problem, Tsukamoto et al. conducted the NanoStep project as an ISS Kibo experiment in 2012[6]. They directly measured face growth rates R and apparent step velocities V of tetragonal hen egg-white lysozyme (HEWL) crystals as functions of supersaturation σ ($\sigma = \ln(C / C_e)$) under microgravity condition for the first time. R and V with σ in space were faster than those on the ground contrary to conventional expectations[7]; many people had believed that crystal growth rates in space would be slower than those on the ground, since mass transportation in solution is limited to diffusion under a microgravity condition, whereas convection flows are also induced around the growing crystals on the ground.

Main reason of the opposite results is speculated as follows. The main impurity of the system was known to be covalently bound dimer of HEWL molecules. The dimer molecules in solutions are transported on the growth interface of HEWL crystals by convection flows on the ground at the same time with monomer molecules. The dimers are known to suppress the advancements of molecular steps on the surface[8]. On the other hand, under microgravity conditions, mass transportation is limited to diffusion processes. In this case, flux of the dimers onto the surface becomes smaller than that of the monomers, since diffusion constant of the dimer is significantly less than that of the monomer. The smaller flux of impurity molecules would result in the weaker suppression of the step advancements; R and V with σ in space become faster than those on the ground.

If the above speculation is truth, an essential point of improvement of crystal quality in space with larger impurities is due to suppression of convection flows. This shows that convection suppression experiments on the ground would achieve the improvement of crystal quality effectively.

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Although three representative methods of ground-based convection-free experiments conducted thus far are growth-in-gel[9], magnetic levitation[10], and ceiling (upside-down) methods[11], gel itself is known to have impurity effects on growth processes, and strong magnetic fields themselves affects growth rates as strong external fields. A ceiling method is conventionally illustrated as shown in **Fig. 1**, and only this method of the above three seemed to work. Adawy et al. reported that the quality of the crystals grown at the ceiling position improved, and they concluded that the improvement is due to slower growth rates as a result of the suppression of convection flows at the ceiling position [11]. However, they did not measure the rate of convection flows directly.

In this study, we tried to confirm whether convective flows are induced around growing HEWL crystals even at ceiling position or not. In practice, first, convection flows induced by growing crystals were observed at 1.0 G in our laboratory. Second, to confirm the inhibition of convection at 0.0 G and to amplify the effects of gravity at 1.5 and 2.0 G, we also measured the macroscopic flow rates around the crystals with parabolic flight experiments.

2. Experimental

2.1 Sample Preparation

Polystyrene particles (Thermo Fischer Scientific, 5050A (diameter: 500 nm) for parabolic flight experiments and 5065A (diameter: 650 nm) for 1.0 G experiments in laboratory) were mixed into growth solution (volume fraction $\varphi = 10^{-5}$ for

parabolic flight experiments and 10^{-4} for 1.0 G experiments) and used as marker particles for monitoring convection flows.

Although lysozyme molecules could adsorb onto the surface of polystyrene particles to some extent, we believe that there are not serious problems for the usage as marker particles, since there are no specific adsorption sites for lysozyme on the surface, and growth of lysozyme crystals normally proceeds in the presence of particles. Growth solution was prepared as follows. 30 mg・mL^{-1} HEWL (Seikagaku-kogyo Co. Ltd., six times recrystallized) and 25 mg・mL^{-1} NaCl are dissolved into 0.05 M sodium acetate buffer (pH = 4.5). A seed crystal nucleated and grown in the growth solution without marker particles was chemically cross-linked using glutaraldehyde solution, and then the fixed seed crystal was again set into a growth container (O-ring cell holder) with the growth solution and marker particles[12]. Epitaxial regrowth occurred onto the chemically fixed seed crystals.

2.2 Apparatus

In situ observation of convection flows around growing crystals at 1.0 G in our laboratory was conducted using an inverted phase-contrast microscope (Nikon, TE2000U) with 20× objective (Nikon, ELWD Plan Fluor 20×/0.45).

Gravity conditions (including about 20 second microgravity conditions) were well controlled during parabolic flight experiments. Parabolic flight experiments were conducted using facilities on board a turbojet aircraft (Mitsubishi Heavy Industries Inc., MU-300 type) in cooperation with Diamond Air Service Inc. (DAS). Growth surface of the crystals were observed using an inverted phase-contrast microscope (Olympus, IMT) with 20× objective (Olympus, LWDCDPlan20PL) set on a vibration control device which was fixed on duralumin rack (**Fig. 2**).
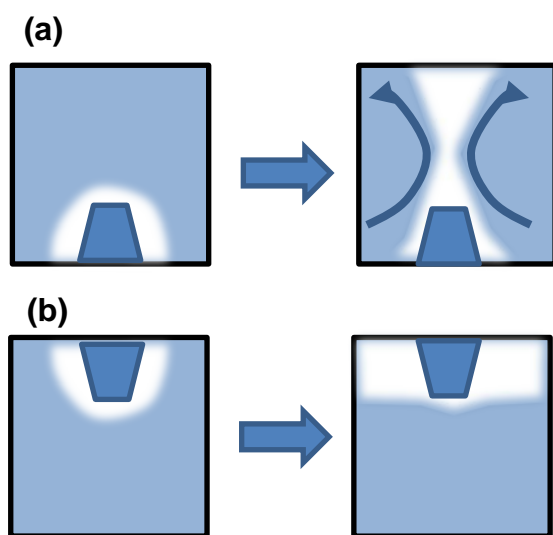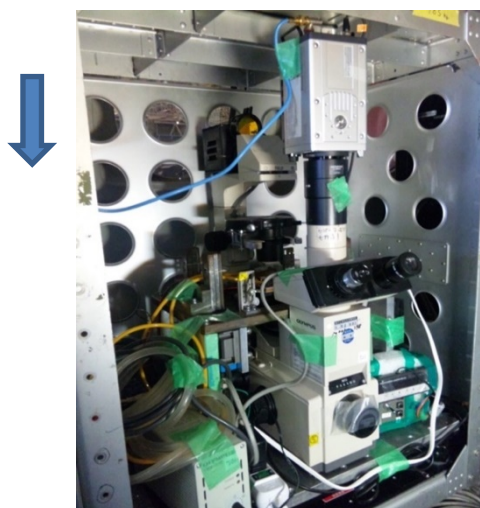

(a)

(b)

Fig. 1 Schematic illustrations of developments of concentration distributions around growing crystals at the bottom (a) and ceiling (b) positions of growth containers. Color density in each cell schematically indicates solute density. In (a), buoyancy driven convection flows occur, while it is conventionally believed that no convection flows are induced in (b).

Fig. 2 Experimental setup in a MU-300 rack. An inverted microscope is fixed on a vibration control device. Precise focus adjustments during parabolic flights are achieved by using a piezo stage. The blue bold arrow shows the direction of gravity.

Precise focus adjustments during parabolic flights are achieved by using a piezo stage (NANO CONTROL, NC 1000 series).

3. Results and Discussion

3.1 Convection Flows Induced by Growing Crystals at 1.0 G in Laboratory

Using the same crystal, we observed solution flows around the growing crystal both at the bottom and ceiling position at 20 ℃. At the bottom position, we confirmed convection flows around the growing crystal as shown in **Fig. 3(a)**. A marker particle moved toward the bottom crystal, since the upper streams of convection flows were probably induced by the formation of solute depletion zone around the bottom crystal, followed by macroscopic flows from the periphery to the central crystal as shown in **Fig. 1(a)**. Contrary to the expectation from **Fig. 1(b)**,
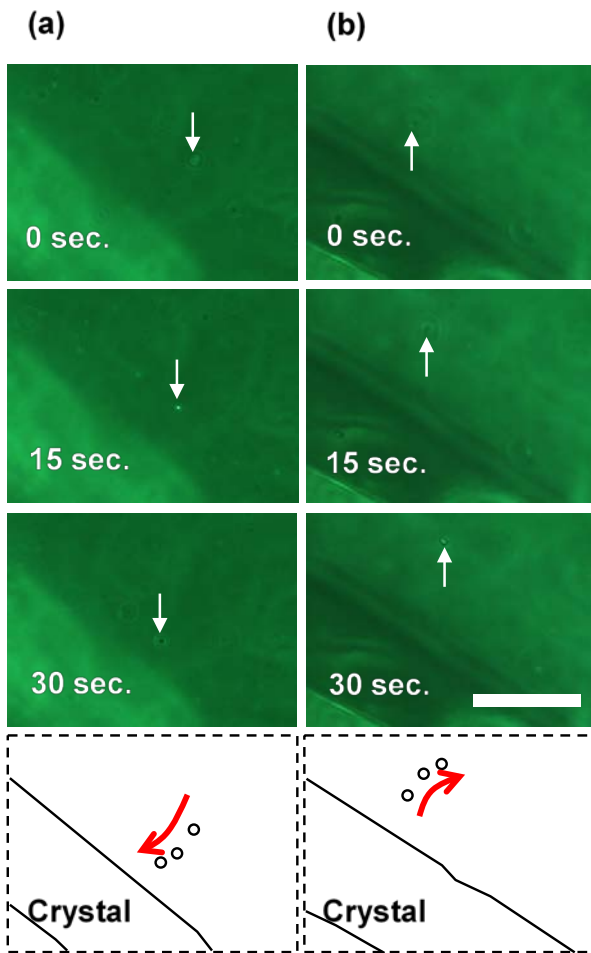

Fig. 3 Visualization of convection flows around a growing crystal at the (a) bottom and (b) ceiling position with time at 1.0 G. A marker particle indicated by white arrows moves toward the crystal at the bottom position (a), whereas that moves away from the crystal at the ceiling position (b) along the bold red arrows as shown in bottom schematic illustrations. Scale bar represents 50 μm.
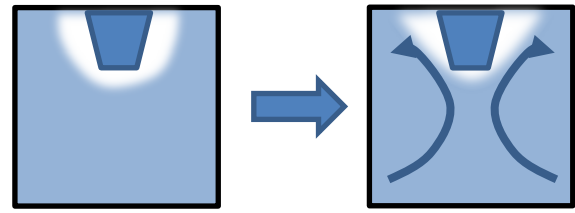

Fig. 4 Schematic illustrations of developments of concentration distributions around growing crystals at the ceiling position of growth container which we reconsidered after the experiments. Buoyancy driven convection flows similar to those shown in **Fig. 1(a)** are probably induced even around ceiling crystals.

we also confirmed convection flows away from the crystal at the ceiling position (ceiling crystal) as shown in **Fig. 3(b)**. This is probably due to the upper streams toward the ceiling crystal induced by the formation of the depletion zone, followed by macroscopic flows from the center of the crystal to the periphery as shown in **Fig. 4**. We consider the reason why such macroscopic flows were induced by the ceiling crystal as follows.

1. As long as the supersaturation condition continues, the upper streams around the crystal continues, since the solute depletion zone around the crystal continuously forms.
2. The upper streams pull a part of supersaturated solution below the crystal upward as well as displace a part of solution lateral to the ceiling crystal to the periphery.
3. The part of solution displaced to the periphery pushes the other part of the solution near side wall of the container downwards.
4. Repetition of the processes 2 and 3 continuously generates solutal convection flows as shown in **Fig. 4**.

To confirm the inhibition of convection at 0.0 G and to amplify the effects of gravity at 1.5 and 2.0 G, we measured the macroscopic flow rates around the crystals with parabolic flight experiments.

3.2 Experimental Concerns of Parabolic Flight Experiments

First experimental concern of parabolic flight experiments was the possibility of crystals adhering to the ceiling position peeling off in the hyper gravity state (around 2.0 G) before becoming microgravity. The hyper gravity state is inevitable in the parabolic flight. Fortunately, the crystals did not peel off even under hyper gravity on 15 parabolic flights.

Second experimental concern was the possibility of generation of thermal convection, since in the presence of thermal convection flows, we cannot measure the rates of solutal convection flows in detail. Room temperature in MU-300 was controlled around 20 ℃ during the experiments, while the bottom of the O-ring cell holder was directly contact with the copper jacket of thermo-controlling unit, and the jacket was controlled around 15.0 ~ 16.5 ℃ during the experiments. Owing to this setup, we believed that thermal convection was effectively suppressed,
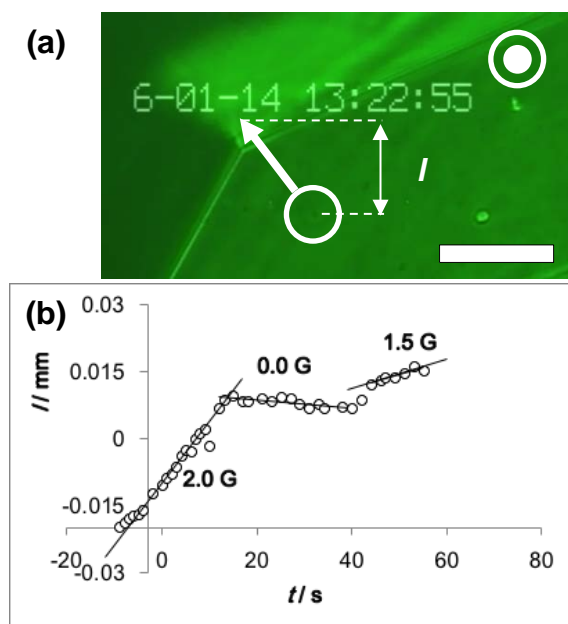
(a)

(b)

Fig. 5 Visualization of convection flows around a growing crystal at a ceiling position. (a) A marker particle (in a white circle) moves along the bold white arrow. The circled dot indicates the direction of gravity out of the page. Scale bar represents 30 μm. (b) The particle displacement *l* shown in (a) (mm) with time (s). Flow rates of the marker change with gravity.

Fig. 6 Flow rates *F* of marker particles around the same crystal at ceiling (■) and bottom (□) positions with gravitational acceleration values *G*.

since the density of cooler solution at the bottom of the container was larger than that of warmer solution at the upper part.

3.3　Rates of Convection Flows

The transmission phase contrast image of the growth interface of a crystal at the ceiling position (ceiling crystal) from the bottom of the cell are shown below (**Fig. 5(a)**). The circled dot indicates the direction of gravity out of the page. Particles flew just below the growth interface from the center of the interface to the periphery along the white arrow. The particle displacement *l* is shown as a double-headed arrow in **Fig. 5(b)**.

As the temperature at this time was 15.2 to 15.5 ℃, supersaturation σ of the growth solution was sufficiently large, and solute depletion zone was probably generated around the crystal. As can be seen from the graph, even around the ceiling crystal, solutal convection definitely occurred during growth.

Moreover, in the case of solutal convection around the growing crystal at the bottom position (bottom crystal), a flow toward the crystal was induced from the periphery of the crystal, whereas in the case of that around the ceiling crystal, a flow going outward from the crystal was induced.

Flow rates *F* are defined as the in-plane displacement of marker particles along the flow directions (for instance, bold white arrow shown in **Fig. 5(a)**) per second, and *F* under microgravity and high-gravity conditions are shown in **Fig. 6**. ■
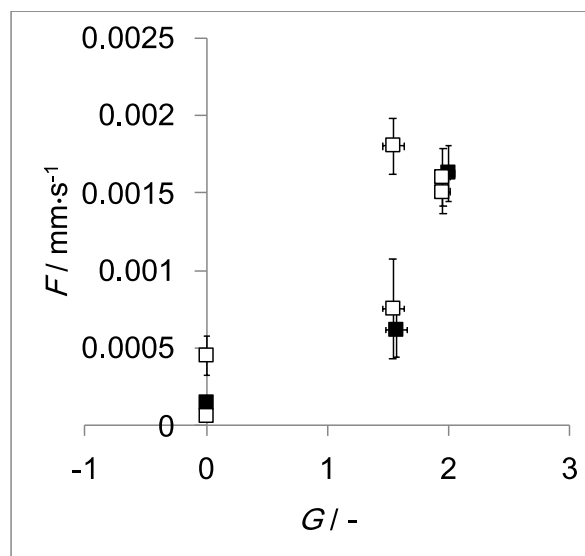
indicates the velocities around the ceiling crystal, and □ indicates that around the bottom crystal. Vertical error bars are the errors on the slope calculated from the results of least square fitting of straight lines as shown in **Fig. 5(b)**. *F* of both ceiling and bottom crystals increased with the increase of gravity. Although *F* approached 0 mms^{-1} at 0 G, the marker particles did not completely stop, and flow which was, for instance, due to G-jitter still remained slightly. Thus, especially for protein crystals, the international space station (ISS) experiments are indispensable for ideal convection-free experiments.

Although there would be small errors which are related to the slight difference in the distance from the crystal surface or that in temperatures (■: 15.4 ± 0.1 ℃, □: 16.3 ± 0.1 ℃), following results are obtained,

(1) Only at 0.0 G, *F* of both ceiling and bottom crystals approached zero (not completely stopped).

(2) Convection flows around the growing crystals are induced even at the ceiling position.

(3) *F* of both ceiling and bottom crystals increased with the increase of gravitational acceleration.

Additionally, *F* of the ceiling crystals steadily reproduced throughout the 15 times parabolic flights (about a one-hour experiment); the convection flows were not temporally induced, and the concentration distribution around the crystal was probably kept to have similar shape throughout the experiment, since the total inner volume of the cell (about 5.5 mm in diameter × 1.5 mm in height) was sufficiently larger than the volume of the crystal (several hundred μm in size), and thus supersaturation σ of the growth solution around the crystal was kept almost constant during the experiments (**Fig. 4**).

Simultaneously, there should be another reason why the quality of protein crystals grown at the ceiling position in Adawy's paper becomes better than that at the bottom position[11]. Formation of macroscopically stable concentration distribution in a growth container due to its elongated configuration in vertical direction (Eppendorf tube: inner diameter ~ 7 mm, depth ~ 28 mm) is the most probable candidate, whereas significant convection flows around a crystal should occur continuously during the crystallization at the ceiling position from our results. In their case, the supersaturation value at the ceiling position probably becomes lower than that at the bottom position. Lower supersaturation around ceiling crystals owing to the macroscopic concentration distribution essentially results in better crystal quality as previously reported[13]. Effects of the lower supersaturation on the crystal quality would be dominant in the experimental setup of Adawy et al.[11], since they showed that tetragonal HEWL crystals grown at the ceiling position are elongated along the c-axis of the crystals, while those grown at the bottom position indicate rather block-like shape. The elongation is due to the anisotropy of the dependence of growth rates on supersaturation; a low supersaturation condition is known to result in the elongation along the c-axis of tetragonal HEWL crystals [14].

4. Conclusions

Convection flows induced around growing tetragonal hen egg-white lysozyme (HEWL) crystal were characterized using polystyrene particles (500 nm and 650 nm in diameter) as marker particles at 1.0 G in laboratory and at 0.0, 1.5 and 2.0 G on board a turbojet aircraft. The key findings obtained in this study are summarized as follows:

(1) Flow rates around the growing crystal both at bottom and ceiling positions similarly increased with the increase of gravity.

(2) The directions of the convection flows induced around the HEWL crystal growing at the bottom and ceiling position were reversed. Convection flows around the crystal at the bottom position were induced from the crystal periphery toward the center of the crystal, while that around the crystal at the ceiling position was induced from the crystal center toward the crystal periphery.

(3) In order to achieve ideal convection-free experiments, microgravity conditions are still necessary, whereas even parabolic flight experiments were one step away. Thus, especially for the improvements of the quality of protein crystals, long-term and stable microgravity conditions such as ISS experiments are indispensable[15].

Acknowledgement

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