

Highly purified glucose isomerase crystals under microgravity conditions grow as fast as those on the ground do

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Suppression of convection flows (solute transportation) and that of impurity incorporation into crystals seem to be the main reasons why the quality of protein crystals becomes better under microgravity conditions, whereas each precise mechanism has not been completely clarified yet. We tried to clarify the former reason by the in-situ observation of spiral growth hillocks on the $\{110\}$ faces of highly purified glucose isomerase (GI) crystals under microgravity conditions and on the ground. Lateral growth rates V_{lateral} of a spiral hillock on the $\{110\}$ face of a glucose isomerase crystal in situ under microgravity conditions and step velocities V_{step} of the same configuration on the ground took similar values as far as the maximum values are compared each other. This similarity indicates there are less influences of the convection flows on the growth rates of protein crystals contrary to conventional expectations.

1. Introduction

The second experiments of the Advanced Nano Step (AdNano) mission undertaken in the Japanese Experiment Module (KIBO) of the International Space Station (ISS) were conducted from 1 October to 5 November 2019, during the second flight of AdNano. We successfully

measured lateral growth rates V_{lateral} of a spiral hillock on the $\{110\}$ face of a glucose isomerase crystal in situ under microgravity conditions.

High quality protein crystals with their maximum resolution of diffraction higher than 1.5 Å are prerequisites for structure-based drug design.

Although crystallization under microgravity conditions is included as one of the most effective routes to obtain such high-quality protein crystals, precise mechanisms of the improvements of the quality under microgravity conditions have not been sufficiently studied due to the scarcity of highly purified protein samples.

Precise measurements of the advancements of molecular steps (step velocity V_{step}) on crystals with supersaturation using sufficient quantity of highly purified protein would enable us to clarify effects of gravity on elementary processes of crystal growth directly. In 2012, Tsukamoto conducted NanoStep mission in the KIBO of the ISS, and measured V_{lateral} in the $\langle 110 \rangle$ direction of spiral growth hillocks on the $\{110\}$ face of a highly purified (99.8% purity) tetragonal hen egg-white lysozyme (HEWL) crystal in situ under microgravity for the first time.^{2,3} V_{lateral} were almost equal to or slightly faster than step velocities V_{step} in the $\langle 110 \rangle$ direction of spiral growth hillocks measured on the earth.³ Although straight steps are preferable for further studies on the precise mechanisms of the improvements of crystal quality at the elementary step level owing to the anisotropy of the step properties,⁴ molecular steps of the HEWL crystals were basically curved and seemed difficult for the use as V_{lateral} in a particular direction to discuss more specific solute incorporation processes into kink sites.

In this study, advancements of straight interference fringes of two beam interferometer which reflect straight molecular steps on a spiral hillock on the $\{110\}$ face of 99.9% purity GI crystals

were measured as V_{lateral} in situ using the observation facilities (NanoStep hardware) onboard the KIBO of the ISS, and compared with V_{step} from ground-based experiments.

2. Materials and Methods

2.1. Sample preparation

We purified GI from *Streptomyces Rubiginosus* (purchased from Hampton Research corporation) up to 99.9% at Maruwa foods and biosciences, Inc. About 10 mgmL⁻¹ GI, 1 mM MgSO₄, and 0.91 M (NH₄)₂SO₄ were dissolved in 100 mM HEPES-NaOH buffer (pH = 7.0) as a starting solution. Beforehand, a GI crystal had been already grown using highly purified GI to some extent over **a seed GI crystal chemically fixed with 2.5% glutaraldehyde in 87.5 mM HEPES buffer**.⁵ The seed crystal was placed on the resin sample holder screwed into a quartz glass cell with two inlets. The cell was filled with the prepared solution.² For ground experiments, the solution contents were the same as those for the ISS experiments except for concentration (7.1 – 25.9 mg mL⁻¹).

2.2. In situ observation of growth of spiral hillocks

V_{step} were measured in situ by using a laser confocal microscope combined with a differential interference contrast microscope (LCM-DIM).⁶ The LCM-DIM system (Olympus Optical Co., Ltd.) was composed of a confocal unit FV300, an inverted optical microscope IX71, and a large shear length-type U-DICTHC Nomarski prism. To eliminate the interference fringes, a super luminescent diode (SLD) laser (Amonics Ltd., model ASLD68-050-B-FA: $\lambda = 680$ nm), whose coherence length is about 10 μm , was adopted as a light source.⁷

V_{lateral} were measured using the changes in several sets of interferograms on the $\{110\}$ surface of a glucose isomerase crystal on board the ISS. Details of the measurements and calculations have been described by Yoshizaki et al.²

3. Results and Discussion

3.1. Overview of the flight

For this second flight of AdNano, we prepared regrown crystal on the chemically fixed seed crystal on the ground before launch (Figure 1(a)). Packing of the samples with the regrown crystal was done similarly to that for the first flight of Advanced Nano Step mission at the end of August, 2019. Samples were launched from Tanegashima Space Center by the HTV8 flight on 25 September (JST), then transported to the ISS on 28 September (JST). The in-situ observation of growing crystal was successfully conducted from 1 October to 5 November. Samples were undocked from the ISS by the SpX-16 on 14 January (JST) and later splashed down in the Pacific Ocean on 14 January (JST). The bottom of the quartz glass cell with the sample was kept facing the direction of gravity during the descent and landing of Dragon capsule of SpX-16. The returned crystal was observed in situ at Tsukuba Space Center on 17 January (JST) (Figure 1(b)) and confirmed that it had been successfully and safely transported to the earth and Japan.

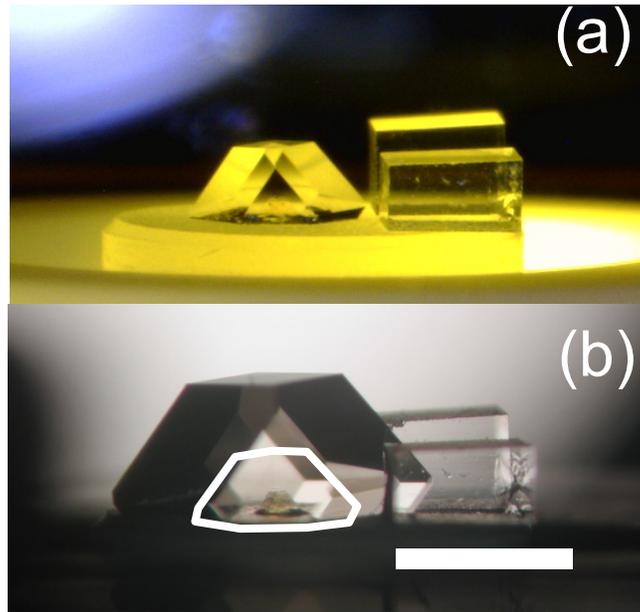


Figure 1. Stereomicroscopic images of the GI crystal used in the AdNano mission. (a) The crystal (left) and reference mirrors (right) before launch on 7 September (JST). (b) Returned crystal on 17 January. A small seed crystal is also seen inside of the crystal. White contour indicates outer shape of the crystal before launch. Scale bar represents 1 mm.

3.2. In situ observation of growing spiral growth hillocks

Both on the ground and under microgravity conditions, we could observe clear spiral hillocks with straight steps and sharp edges in situ (Figure 2). These spiral growth hillocks were probably induced by the slight lattice mismatch between GI seed crystals and regrown GI crystal parts, the surface roughness of the seed crystal, and resultant lattice distortions.⁸ From these micrograms, we analyzed V_{step} and V_{lateral} in the direction of arrows indicated in Figure 2 as the same crystallographic directions.



Figure 2. Micrograms of the $\{110\}$ faces of GI crystals used in a ground experiment (a) and the AdNano mission (b). (a): Spiral growth hillocks were clearly observed using an LCM-DIM. A white arrow indicates the direction in which we measured V_{step} in this study. Scale bar presents $20 \mu\text{m}$. (b): Closed parallelogram-shaped interference fringes on the parallelogram-shaped $\{110\}$ face indicate same altitudes of a spiral growth hillock. A black arrow indicates the direction in which we measured V_{lateral} . Scale bar represents $200 \mu\text{m}$.

3.3. Comparison between V_{step} and V_{lateral}

V_{step} and V_{lateral} were measured by changing temperature of samples and plotted against $C - C_e$ (C : concentration of GI, C_e : solubility of GI) (Figure 3). C of the ground experiments (for V_{step}) were determined by dilution ratios of mother liquors which had been prepared at Maruwa foods and biosciences, Inc. During the ground experiments, we also measured the equilibrium temperature T_e at each C by in-situ observation of growth and dissolution of elementary steps. C_e at each temperature for both ground and space experiments was calculated by using linear fitting function derived from van't Hoff plots of $\ln C$ vs. $1/T_e$. During the ISS experiments, we measured T_e by in-situ observation of fringe movements. Using the above linear fitting function, we calculated C at the T_e .

V_{step} and V_{lateral} took similar values from $C - C_e = 0$ to 6 mgmL^{-1} , while scatter of V_{lateral} at larger $C - C_e$ values were found. The results in these similar values are against the conventional hypothesis that crystal growth rates under microgravity conditions would be smaller than those on the ground due to the lack of convection flows around the growing crystals and the suppression of solute transportation to crystal growth interfaces. As comparison of both maximum values, V_{step} and V_{lateral} took similar values up to $C - C_e = 9 \text{ mgmL}^{-1}$, since the maximum values would reflect the most ideal values for each condition.³ For larger values of V_{lateral} from 9 mgmL^{-1} to larger $C - C_e$, two-dimensional nucleation started on the terraces of a spiral hillock, and resulted in larger lateral growth rates (apparent step velocities) in space than individual step velocities on the ground.³ In the case of lower values of V_{lateral} from $C - C_e = 6$ to 10 mgmL^{-1} would be due to the slow relaxation in saturated adsorption density of GI molecules with changing temperature. To clarify this assumption will be the future work.

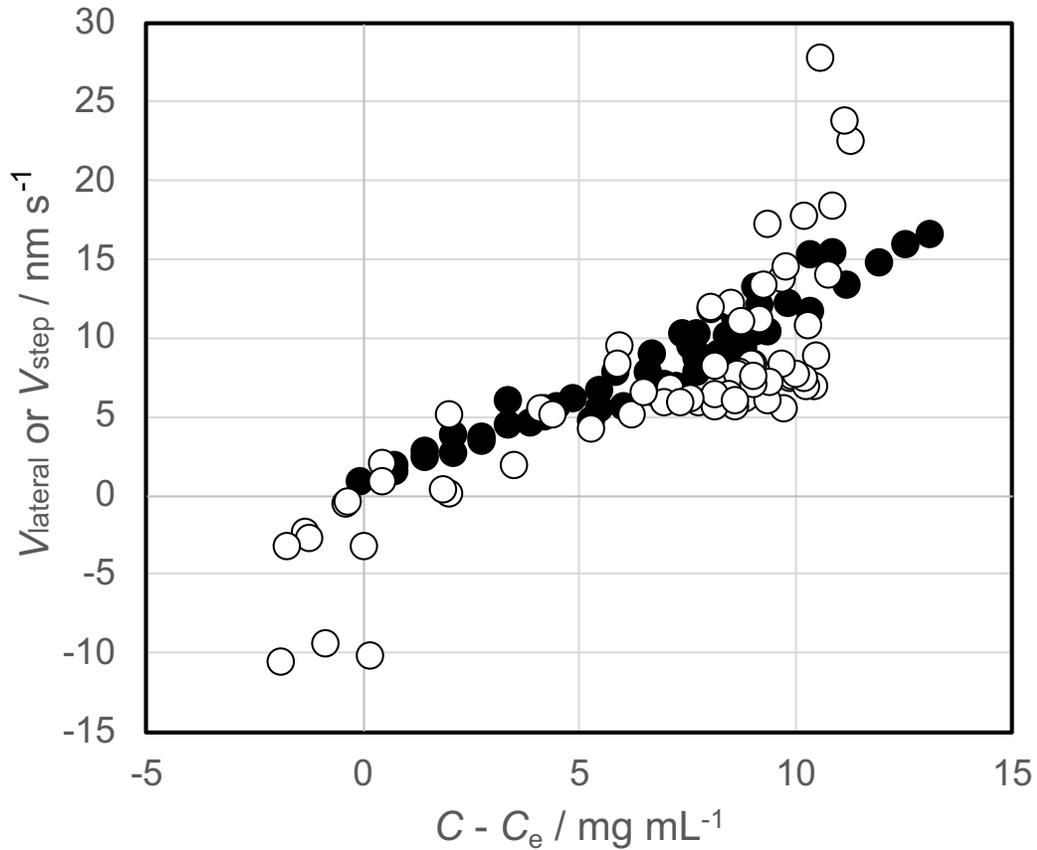


Figure 3. V_{step} measured on the ground (●) and V_{lateral} measured under microgravity conditions (○).

4. Conclusions

As a part of the Advanced Nano Step mission of the ISS KIBO experiments, we successfully measured V_{lateral} of a spiral hillock on the $\{110\}$ face of a highly purified glucose isomerase (GI) crystal with respect to $C - C_e$ in situ under microgravity conditions. Step velocities V_{step} of spiral hillocks on the $\{110\}$ face of highly purified GI crystals were also measured as ground-based control experiments. V_{lateral} and V_{step} took similar values contrary to conventional expectations. The key findings obtained in this study are summarized as follows:

1. Chemically fixed seed GI crystal was successfully functioned as a template of regrowth of highly purified GI crystal and a source of screw dislocations of spiral hillocks.
2. Spiral growth hillocks with straight elementary steps and sharp edges were successfully observed on the $\{110\}$ face of highly purified GI crystals using an LCM-DIM.
3. Parallelogram-shaped interference fringes with straight sides and sharp edges were successfully observed on the $\{110\}$ face of a highly purified GI crystal using the observation facility (NanoStep hardware) onboard the KIBO of the ISS.
4. V_{lateral} under microgravity conditions and V_{step} on the ground took similar values up to $C - C_c = 9 \text{ mg mL}^{-1}$ as far as the maximum values were selected.
5. V_{lateral} started to increase steeply around $C - C_c \approx 9 \text{ mg mL}^{-1}$. This indicates that the dominant growth mode changes from spiral to 2D nucleation growth at this critical concentration.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ISS, the international space station; GI, glucose isomerase; AdNano, Advanced Nano Step mission; LCM-DIM, a laser confocal microscope combined with a differential interference contrast microscope.

REFERENCES

- (1) Davis, A. M.; Teague, S. J.; Kleywegt, G. J. Application and limitations of X-ray crystallographic data in structure-based ligand and drug design. *Angew. Chem., Int. Ed.* 2003, 42, 2718–2736.
- (2) Yoshizaki, I.; Tsukamoto, K.; Yamazaki, T.; Murayama, K.; Oshi, K.; Fukuyama, S.; Shimaoka, T.; Suzuki, Y.; Tachibana, M. *Rev. Sci. Instrum.* 2013, 84, 103707–1–8.
- (3) Suzuki, Y.; Tsukamoto, K.; Yoshizaki, I.; Miura, H.; Fujiwara, T. *Cryst. Growth Des.* 2015, 15, 4787-4794.
- (4) Sleutel, M.; Willaert, R.; Gillespie, C.; Evrard, C.; Wyns, L.; Maes, D. *Cryst. Growth Des.* 2009, 9, 497-504.

(5) Iimura, Y.; Yoshizaki, I.; Rong, L.; Adachi, S.; Yoda, S.; Komatsu, H. *J. Cryst. Growth* 2005, 275, 554–560.

(6) Sazaki, G.; Matsui, T.; Tsukamoto, K.; Usami, N.; Ujihara, T.; Fujiwara, K.; Nakajima, K. *J. Cryst. Growth* 2004, 262, 536–542.

(7) Suzuki, Y.; Sazaki, G.; Matsumoto, M.; Nagasawa, M.; Nakajima, K.; Tamura, K. *Cryst. Growth Des.* 2009, 9, 4289–4295.

(8) Koizumi, H.; Tachibana, M.; Yoshizaki, I.; Fukuyama, S.; Tsukamoto, K.; Suzuki, Y.; Uda, S.; Kojima, K. *Cryst. Growth Des.* 2014, 14, 5111–5116.