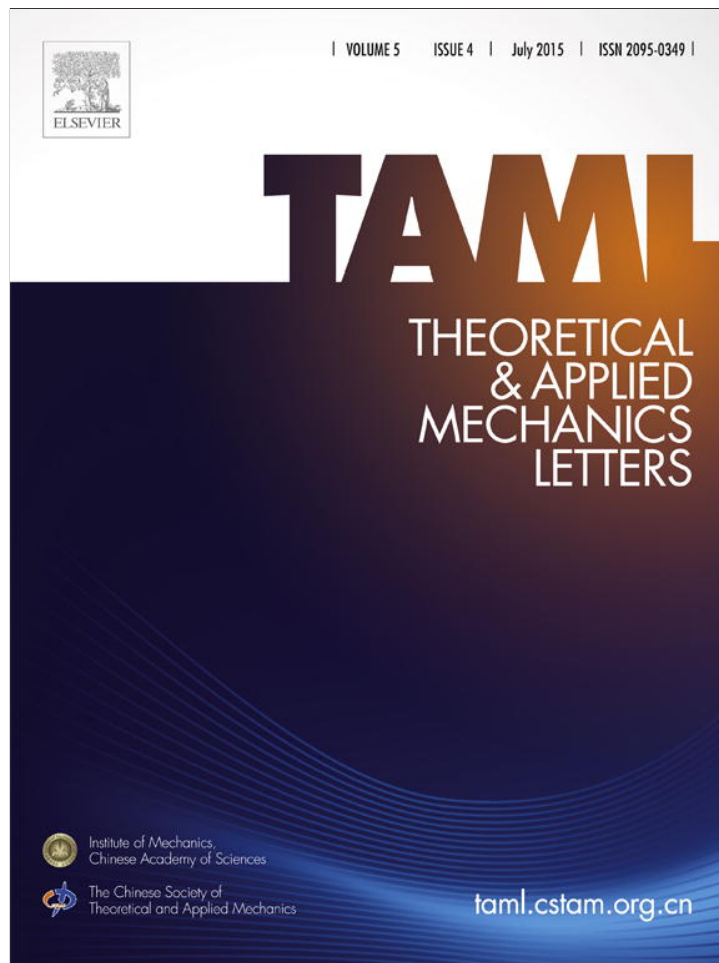


Provided for non-commercial research and education use.  
Not for reproduction, distribution or commercial use.



(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

**This is an open access article which appeared in a journal published by Elsevier. This article is free for everyone to access, download and read.**

**Any restrictions on use, including any restrictions on further reproduction and distribution, selling or licensing copies, or posting to personal, institutional or third party websites are defined by the user license specified on the article.**

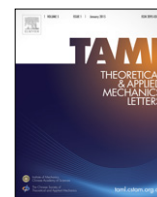
**For more information regarding Elsevier's open access licenses please visit:**

**<http://www.elsevier.com/openaccesslicenses>**



Contents lists available at ScienceDirect

## Theoretical and Applied Mechanics Letters

journal homepage: [www.elsevier.com/locate/taml](http://www.elsevier.com/locate/taml)

## Letter

## Direct observation of bunching of elementary steps on protein crystals under forced flow conditions

Gen Sazaki<sup>a,\*</sup>, Guoliang Dai<sup>b</sup><sup>a</sup> Institute of Low Temperature Science, Hokkaido University, Sapporo 060-0819, Japan<sup>b</sup> Key Laboratory of Microgravity, Institute of Mechanics, Chinese Academy of Sciences, Beijing 100190, China

## ARTICLE INFO

## Article history:

Received 20 January 2015

Accepted 12 May 2015

Available online 22 May 2015

\*This article belongs to the Biomechanics and Interdiscipline

## Keywords:

In situ observation

Step bunching

Step debunching

Elementary steps

Forced flow

Protein crystal

## ABSTRACT

Bunching of elementary steps by solution flow is still not yet clarified for protein crystals. Hence, in this study, we observed elementary steps on crystal surfaces of model protein hen egg-white lysozyme (HEWL) under forced flow conditions, by our advanced optical microscopy. We found that in the case of a HEWL solution of 99.99% purity, forced flow changed bunched steps into elementary ones (debunching) on tetragonal HEWL crystals. In contrast, in the case of a HEWL solution of 98.5% purity, forced flow significantly induced bunching of elementary steps. These results indicate that in the case of HEWL crystals, the mass transfer of impurities is more significantly enhanced by forced solution flow than that of solute HEWL molecules. We also showed that forced flow induced the incorporation of microcrystals into a mother crystal and the subsequent formation of screw dislocations and spiral growth hillocks.

© 2015 The Authors. Published by Elsevier Ltd on behalf of The Chinese Society of Theoretical and Applied Mechanics. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

For a crystal growing in a solution, buoyancy convection plays a significantly important role in the mass transfer of solute and impurities from a bulk solution to a crystal surface [1]. However, since atomic force microscopy cannot be used under flow conditions, it has been very difficult to visualize individual elementary steps, although lateral growth of steps on a crystal surface is the most fundamental elementary process during the layer-by-layer growth of a crystal. Relatively recently, Sazaki et al. [2] succeeded in visualizing individual elementary steps on a protein crystal surface by laser confocal microscopy combined with differential interference contrast microscopy (LCM–DIM). Maruyama et al. [3] observed, by LCM–DIM, elementary steps on a surface of a tetragonal crystal of model protein hen egg-white lysozyme (HEWL) under forced flow conditions, and studied effects of solution flow on step velocities. They found that solution flow enhances mass transfer of both solute and impurities.

Bunching of steps is one of the major effects caused by solution flow during the growth of crystals. In the case of inorganic crystals, such as ammonium dihydrogen phosphate (ADP) and potassium dihydrogen phosphate (KDP), there are detailed studies in which the formation of bunching steps by solution flow was macroscopically observed by optical microscopy [4,5]. Such studies revealed

that solution flow induces instability and then forms bunching steps on a downstream slope of a spiral growth hillock, where steps are moving in the same direction as the solution flows. In contrast, when the direction of solution flow is switched 180°, the bunching steps formed on the now up-stream slope disappear (debunching) and then bunching steps newly appear on the now down-stream steps. Such behavior is also modeled in detail [5].

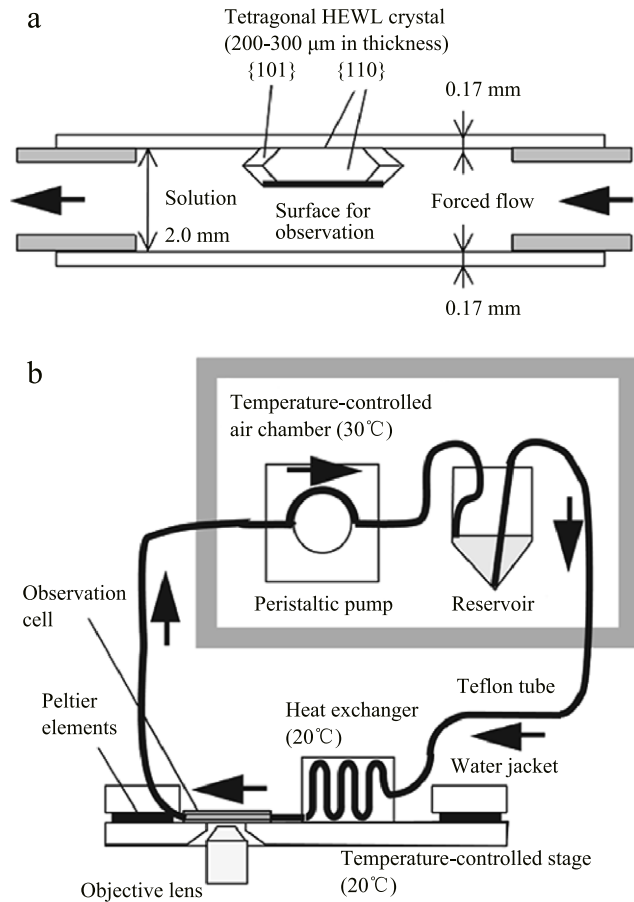
However, as far as we know, there is no study on the formation of bunching steps by solution flow on a protein crystal, which plays a crucially important role in the structural analyses of protein molecules by X-ray and neutron diffraction. Hence, in this study, we tried to directly visualize bunching processes of elementary steps, for the first time, on a tetragonal crystal of model protein HEWL. We discussed the effects of solution flow on the mass transfer of solute and impurity proteins, from the viewpoint of the bunching and debunching of steps.

HEWL of 99.99% and 98.5% purities were purchased from Maruwa Food Industries, Inc. and Seikagaku Co., respectively (99.99% HEWL was discontinued). Other chemicals were of the analytical grade. Seed tetragonal HEWL crystals were grown at  $(20.0 \pm 0.1)^\circ\text{C}$  from a solution containing 70 mg/mL HEWL of 98.5% purity, 25 mg/mL NaCl, and 50 mmol/L sodium acetate (pH 4.5).

An observation cell (Fig. 1(a)) was made of two glass plates of 0.17 mm thickness. The thickness of an HEWL solution between the two glass plates was 2.0 mm. The length and width of the cell were 20 mm and 5.0 mm, respectively. After the seed crystals

\* Corresponding author.

E-mail addresses: [sazaki@lowtem.hokudai.ac.jp](mailto:sazaki@lowtem.hokudai.ac.jp) (G. Sazaki), [dspr@imech.ac.cn](mailto:dspr@imech.ac.cn) (G. Dai).

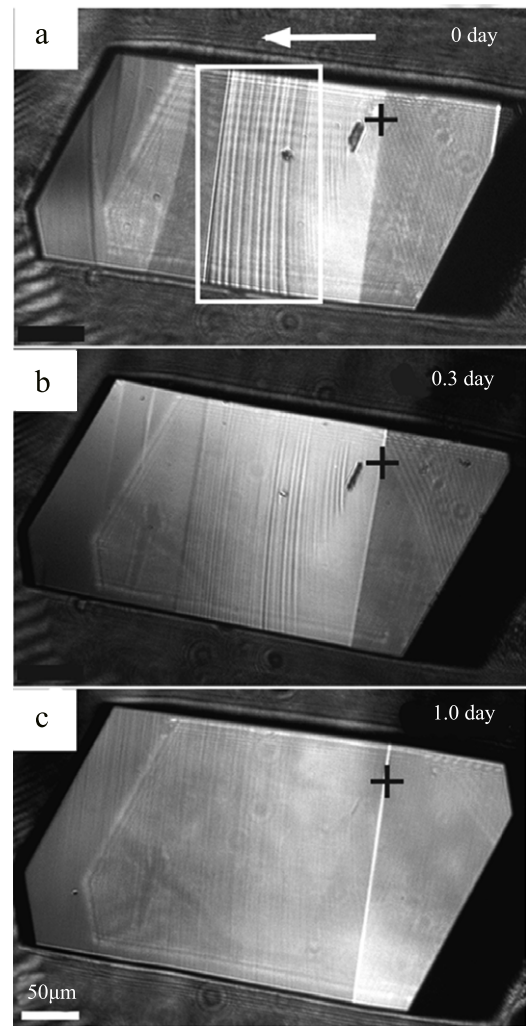


**Fig. 1.** Schematic illustrations of experimental setups: cross sectional views of (a) an observation cell and (b) a forced solution flow system.

had reached desirable size (0.2–0.3 mm), they were transferred to the observation cell filled with the solution of 98.5% HEWL. The observation cell was then placed upside-down for one day to attach the seed crystals on the ceiling of the cell.

Just before the observation, the cell was placed on a temperature-controlled stage in an upright way. Then, the solution inside the cell was replaced with a solution of 40 mg/mL HEWL of 99.99% purity using a flow system (Fig. 1(b)). The temperature of the cell was kept at  $(20.0 \pm 0.1)^\circ\text{C}$  all through the experiment, using Peltier elements. In contrast, the temperature of a peristaltic pump and a reservoir in a temperature-controlled air chamber was kept at  $(30.0 \pm 0.1)^\circ\text{C}$ . An unsaturated HEWL solution in the reservoir (at  $30.0^\circ\text{C}$ ) was pumped through a Teflon tube. Then after the temperature of the HEWL solution was cooled to  $20.0^\circ\text{C}$  by a heat exchanger, the supersaturated HEWL solution (at  $20.0^\circ\text{C}$ ) was pumped into the observation cell. The HEWL solution came out from the cell returned to the reservoir at  $30.0^\circ\text{C}$ . Then micro HEWL crystals formed in the cell and tubes were dissolved in the reservoir. Flow rates were determined volumetrically.

The free surface (the {110} face marked by a bold line in Fig. 1(a)) of the seed crystals, which were placed parallel to the solution flow, were observed by LCM–DIM at a certain flow rate. Details of the LCM–DIM system were explained in our previous work [2]. During the growth of a crystal, a solute-depleted zone is generally formed in the vicinity of a crystal. However, since our seed crystal was attached to the ceiling of the cell (Fig. 1(a)), a lightweight solution in the solute-depleted zone could not go down by gravity. Hence, even under very slow forced flows, our observation system could minimize the effects of buoyancy convection during the observation.

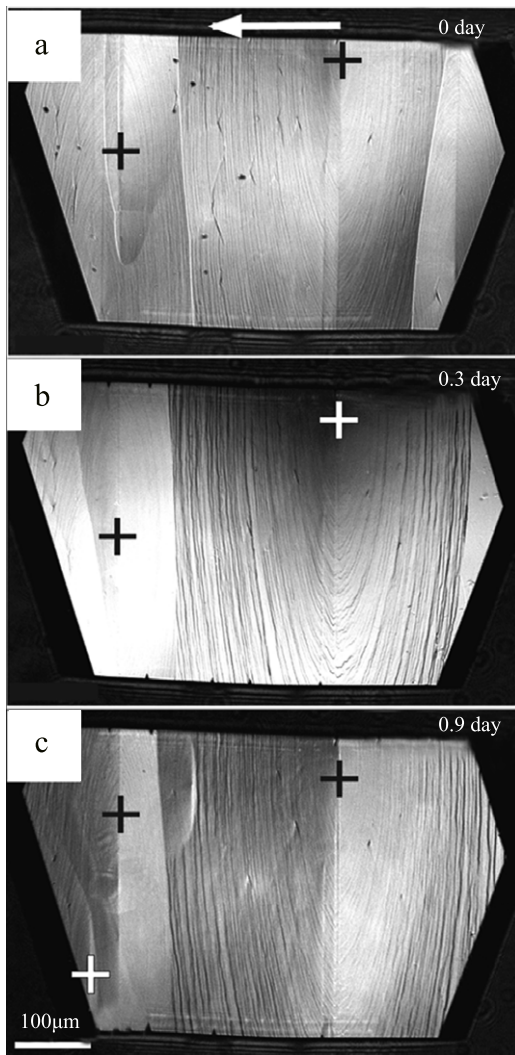


**Fig. 2.** A time course of a {110} face of a tetragonal HEWL crystal in a 99.99% purity HEWL solution at a flow rate of  $220 \mu\text{m/s}$ . (a) 0 day. (b) 0.3 day. (c) 1.0 day. A cross mark and an arrow indicate the center of a spiral growth hillock and the direction of forced flow, respectively. A white rectangle in plot (a) shows bunched steps.

We first observed a {110} face of a tetragonal HEWL crystal on which bunched steps were formed by chance during the sample preparation. Fig. 2 shows a time course of the {110} face in a 99.99% HEWL solution at a flow rate of  $220 \mu\text{m/s}$ . A cross mark indicates the center of a spiral growth hillock, which had a vicinal surface whose center exhibited the highest altitude. Since an arrow shows the direction of the forced flow, the right and left sides of the hillock corresponded to the up-stream and down-stream slopes of the hillock, respectively. At the beginning (Fig. 2(a)), the down-stream slope of the hillock exhibited bunched steps (marked by a white rectangle). Fig. 2 demonstrates that as time elapsed, the bunched steps disappeared. This result is opposite to those found on the spiral growth hillocks of ADP and KDP crystals [1]. We performed similar observations at the flow rates from  $55 \mu\text{m/s}$  to  $550 \mu\text{m/s}$ . However, at any flow rates, we could not find the formation of bunched steps by the forced flow on the downstream slope of the spiral growth hillock of the HEWL crystals. This result (Fig. 2) indicates that the coupling of bulk diffusion fields formed around adjacent steps on the HEWL crystal was weaker than in the cases of ADP and KDP crystals.

It is well known that a solution depletion zone, in which solute concentration becomes lower than in a bulk solution, is formed in the vicinity of a growing protein crystal. However, the growth rate

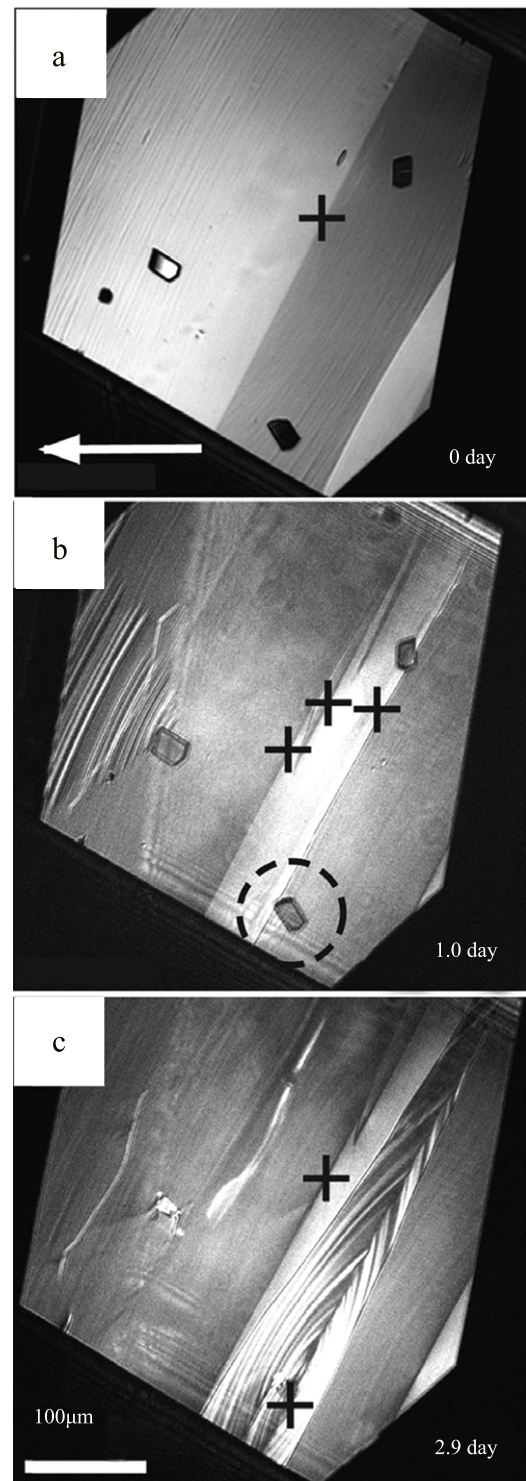




**Fig. 3.** A time course of a {110} face of a tetragonal HEWL crystal in a 98.5% purity HEWL solution at a flow rate of  $55 \mu\text{m/s}$ . (a) 0, (b) 0.3, and (c) 0.9 day. A cross mark and an arrow are the same as those in Fig. 1.

of HEWL crystals is about 50–500 times slower than those of ADP and KDP crystals [6]. Hence, the solute incorporation process on the HEWL crystal surface dominated the growth process rather than the preceding mass transfer process of solute HEWL molecules, resulting in the disappearance of bunched steps (debunching) by the solution flow. However, there exists a protein, such as insulin, whose crystal grows 10–100 times faster than HEWL crystals [7]. Therefore, the critical rate of a surface incorporation process, above which rate solution flow induces the step bunching, should be an interesting issue in the future.

In contrast to the case of the HEWL solution of 99.99% purity, when we gave forced flow of 98.5% purity HEWL solution to a spiral growth hillock, we obtained a different result. Fig. 3 presents a time course of a {110} face in a 98.5% HEWL solution at the minimum flow rate of  $55 \mu\text{m/s}$ . A cross mark and an arrow also show the center of a spiral growth hillock and the direction of the forced flow. As shown in Fig. 3, as time elapsed, bunched steps gradually formed on both the up-stream and down-stream slopes of the spiral growth hillock. This result indicates that the forced solution flow enhanced the mass transfer process of impurities more significantly than that of solute HEWL. The results shown in Figs. 2 and 3 demonstrate that the origin of the bunched steps on HEWL crystals is not the coupling of bulk diffusion fields of



**Fig. 4.** A time course of a {110} face of a tetragonal HEWL crystal in a 99.99% purity HEWL solution at a flow rate of  $55 \mu\text{m/s}$ . (a) 0, (b) 1.0, and (c) 2.9 day. A cross mark and an arrow are the same as those in Fig. 1. A dotted circle shows the microcrystal that induced the generation of a spiral growth hillock after the incorporation.

solute HEWL molecules but the inhibition of the growth of steps by impurities. However, Maruyama et al. [3] recently showed a flow rate at which solution flow might enhance the mass transfer of solute more significantly than that of impurities. Hence, it is necessary to study systematically the relation between a flow rate and purity of a protein sample.

We finally show a different effect of solution flow. Fig. 4 shows a time course of a {110} face in a 99.99% purity HEWL solution at a flow rate of 55  $\mu\text{m/s}$ . Microcrystals, which were formed in the observation cell, were adsorbed on a mother HEWL crystal surface. Then, as the mother crystal grew, the microcrystals were gradually incorporated into the mother crystal. After once the microcrystal (marked by a dotted circle in Fig. 4(b)) was fully buried beneath the mother crystal, the microcrystal (solid inclusion) generated strain, inducing the generation of screw dislocations and the subsequent formation of a spiral growth hillock. This result indicates that the strain generation mechanism reported so far on inorganic crystals [1] can be applied to protein crystals.

In this study, we observed the {110} surfaces of tetragonal HEWL crystals under forced flow conditions by LCM–DIM. In the case of the HEWL solution of 99.99% purity, the solution flow disintegrated the bunched steps into elementary steps. In contrast, solution flow of 98.5% purity HEWL induced the generation of the bunched steps. From these observations, we concluded that in the case of HEWL crystals, solution flow mainly enhanced the mass transfer of impurities rather than that of solute HEWL molecules. We also found that solution flow induced the adsorption of the microcrystals on the mother crystal surface, resulting in the formation of the screw dislocations and the spiral growth hillocks.

## Acknowledgments

The authors would like to thank Y. Saito and S. Kobayashi (Olympus Engineering Co., Ltd.) for their technical support of LCM–DIM. One of the authors G. Sazaki is grateful for Chinese Academy of Sciences visiting professorship for senior international scientists (2013T2J0060).

## References

- [1] A.A. Chernov, *Modern Crystallography III*, Springer-Verlag, Berlin, 1984.
- [2] G. Sazaki, T. Matsui, K. Tsukamoto, et al., In-situ observation of elementary growth steps on the surface of protein crystals by laser confocal microscopy, *J. Cryst. Growth* 262 (2004) 536–542.
- [3] M. Maruyama, H. Kawahara, G. Sazaki, et al., Effects of a forced solution flow on the step advancement on {110} faces of tetragonal lysozyme crystals: direct visualization of individual steps under a forced solution flow, *Cryst. Growth Des.* 12 (2012) 2856–2863.
- [4] A.A. Chernov, Yu.G. Kuznetsov, I.L. Smol'skii, et al., Hydrodynamic effects in growth of ADP crystals from aqueous solutions in the kinetic regime, *Sov. Phys. Crystallogr.* 31 (1987) 705–709.
- [5] A.A. Chernov, Notes on interface growth kinetics 50 years after Burton, Cabrera and Frank, *J. Cryst. Growth* 12 (2004) 499–518.
- [6] P.G. Vekilov, What determines the rate of growth of crystals from solution? *Cryst. Growth Des.* 12 (2007) 2796–2810.
- [7] I. Reviakine, D.K. Georgiou, P.G. Vekilov, Capillarity effects on crystallization kinetics: Insulin, *J. Am. Chem. Soc.* 125 (2003) 11684–11693.