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Influence of micro-impurity on protein crystal growth studied by the etch figure method

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ABSTRACT

The investigation of the effect of micro impurity on crystal growth by optical microscopy has been validated. The results showed that the growth rate of a lysozyme crystal was affected even if the concentration of impurity of fluorescent-labeled lysozyme (abbreviation, F-lysozyme) was very small. Different concentrations of F-lysozyme had different effects on crystal growth rate. The growth rate decreased much more as F-lysozyme concentration increased. The density of incorporated F-lysozyme on different grown layers of a lysozyme crystal during crystal growth was obtained from the results of flat-bottomed etch pits density.

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1. Introduction

Protein crystallization is the first step of structure analysis of protein crystals by X-ray or neutron diffraction. It is necessary to purify protein before its crystallization, because the impurities adsorbed on a crystal surface strongly affect the surface morphology [1], nucleation and growth kinetics [2,3], diffraction [4] and crystal quality [5,6]. However, even if a protein solution has been purified, such as in the case of a commercial protein solution, the concentrations of impurities in protein solution are still high compared with a purified inorganic material [7]. Moreover, the impurity molecules in a protein solution are difficult to be removed [8]. For example, in a protein solution, there still exists a small amount of protein molecules even after careful purification of protein solution. They are usually protein oligomer or homologous protein molecules with a similar structure of protein molecules. We call these molecules micro-impurity. Micro-impurity molecules affect protein crystal quality although their concentration in protein solution is low. For example, the point defects caused by the adsorption of micro-impurities on crystal surface strongly affect the crystal quality [9,10]. However, until now only a very small number of works focus on micro-impurity in protein crystallization. In order to obtain a high-quality crystal for analyzing its structure, we have to know how micro-impurities affect the crystallization process.

The methods of investigating micro-impurity are, to some degree, different with popular ones of studying impurities with high concentrations. HPLC [2], electrophoresis [11,12], and fluorescence intensity [13] are the main methods. However, they have some limitations. For example, HPLC and electrophoresis are invasive methods and can only obtain qualitative results. The fluorescence intensity method can obtain quantitative results, but it is a time-consuming method and requires fluorescent concentration. The etch figure method, which is usually applied in inorganic material studies, has been applied in protein crystallization research recently. In 2004, Hondoh et al. [9,10] studied the surface morphology of lysozyme crystals after etching by AFM. They found three types of etch pits: flat-bottomed type, deep flat-bottomed type, and point-bottomed type. Their results showed that in the case of lysozyme crystal grown from purified solution, the origin of flat-bottomed etch pits on a crystal surface was vacancies. However, in the case of crystals grown from commercial lysozyme solution, the flat-bottomed etch pits were caused by the adsorption of impurity molecules. They analyzed the adsorption kinetics of impurities from the results of shape and etch pits density (EPD).

We have tried to observe and determine flat-bottomed etch pits by the etch figure method using phase contrast microscopy. By the optic method, we have some advantages when compared with AFM, such as precise temperature control and non-invasive observation. According to the sequence of the three types of etch pits, we can find the flat-bottomed-type etch pits. Then we can

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obtain the density of impurities incorporated with a crystal according to the EPD of flat-bottomed etch pits. We should point out that the etch figure method is in particular suitable for micro-impurity study, since etch pits usually merge when the concentration of impurity is high.

In this paper, we adopted fluorescent-labeled lysozyme (F-lysozyme) as a model impurity molecule. We added F-lysozyme into specially purified lysozyme solution, where a seed lysozyme crystal started to grow. We etched the newly grown layers of a crystal and studied the effect of micro-impurity on crystal growth process and the amount of incorporated impurity on different layers of a crystal.

2. Materials and methods

2.1. Materials

The solution used for the experiments contained 1.01 mM HEWL (99.99% purity, Maruwa Food Industries, Inc., Japan), F-lysozyme (with different concentrations in different experiments), 25 mg/ml NaCl, and 50 mM sodium acetate (pH 4.5). F-lysozyme was labeled with a fluorescent label, tetramethylrhodamin-5-isotiocyanate, according to the method described in Ref. [8]. All of the agents used in the experiments were analytically pure and filtered with 0.22 μm Millipore filter membrane before use. An inverted phase contrast microscope (Olympus, IX71) was used. Its vertical and lateral resolution is 0.005 and 0.5 μm, respectively. The temperature inside the observation cell is $\pm 0.1\,^{\circ}C$. F-lysozyme concentrations used in experiments were 0, 280, 330, 380, 430, and 480 nM.

2.2. Methods

Lysozyme seed crystals were obtained by the batch method at 20 °C. After the size of a seed crystal reached 200–300 μm, it was transferred to an observation cell. Height of the newly grown layers was several μm. We observed the {1 1 0} face of a tetragonal lysozyme crystal. Temperature inside the observation cell was 20 and 33 °C under crystal growth and etching conditions, respectively. The seed crystal in the observation cell grew for 5–6 h, and the etching process continued for 40–50 min. The morphologies of the {1 1 0} face of a lysozyme crystal were taken by a CCD camera automatically for every minute during the etching process.

We analyzed etching figures by the Image-Pro Express software. We chose an area ($241 \times 156\,\mu m^2$) for analysis, inside which there were no micro-crystals, adsorbed particles, or big deep flat-bottomed etch pits on a crystal surface. The chosen area for observation was far away from the corner and edge of the crystal. We distinguished flat-bottomed etch pits according to the sequence of appearance of etch pits [14] and their gray values. We measured the number of flat-bottomed etch pits.

3. Results and discussion

3.1. Observation of etch pits by the optical method

We observed the formation process of etch pits *in situ* by the phase contrast microscopy. The phase contrast microscope used in our experiments has a vertical resolution of 5 nm, which was almost the same as the height of one elementary step of a lysozyme crystal. Therefore, we can easily find etch pits on a crystal surface. Thanks to the large enough observation area, the error of statistical results in our experiments was small. Moreover, the optic method used in this work can avoid the problems in AFM observations, such as the disturbance of tips of the cantilever, evaporation of protein solution, etc.

The impurity effect of F-lysozyme was obvious [8]. After comparison with the crystals grown from commercial lysozyme solution (6× re-crystallization, containing 1.5% impurity molecules), purified lysozyme solution, and purified lysozyme solution containing micro-impurities (the maximum impurity concentration was 0.024%), we found that the EPD were different, which are shown in Fig. 1(a)–(c), respectively. The EPD in Fig. 1(a) was 10 times more than that in Fig. 1(c). Moreover, etch pits easily merged under the former conditions than under the latter one. This demonstrated that the etch figure method could not be used under the condition of solution containing high-concentration impurities.

The etch pits appeared in the observation area 5–10 min after the etching process started. The etch pits elongated in the $[\bar{1}\,1\,0]$ direction with the shape similar to 2D islands on a crystal surface. In Fig. 1(c), two types of etch pits were found in the observation area: one was flat-bottomed etch pits, and the other was deep flat-bottomed etch pits. Few of the point-bottomed etch pits were found. We found that the positions of flat-bottomed and deep flat-bottomed etch pits were independent under our etching conditions. In the etching process, the first appeared etch pits were deep flat-bottomed ones, the number of which kept constant in the whole etching process. This result was the same as that of Hondoh et al. [9,10], showing that the observation of etch pits caused by micro-impurities using phase contrast microscopy was feasible. Moreover, we found that the number of flat-bottomed etch pits gradually increased with the etching time within 40 min. However, there was suddenly an order of magnitude increase in the number of flat-bottomed etch pits after an etching time of 40 min, indicating that the top surface of the seed crystal before growth started to be etched. Therefore, the number and positions of flat-bottomed etch pits at the etching time 40 min showed the adsorption results of F-lysozyme at the beginning of the crystal growth process in solution containing F-lysozyme.

We also observed the morphologies of the {1 1 0} surface of the same lysozyme crystal after etching and growing for several times. We etched a lysozyme crystal at 33 °C for 40 min, then grew the crystal for 80 min at 20 °C in order to let the newly appeared

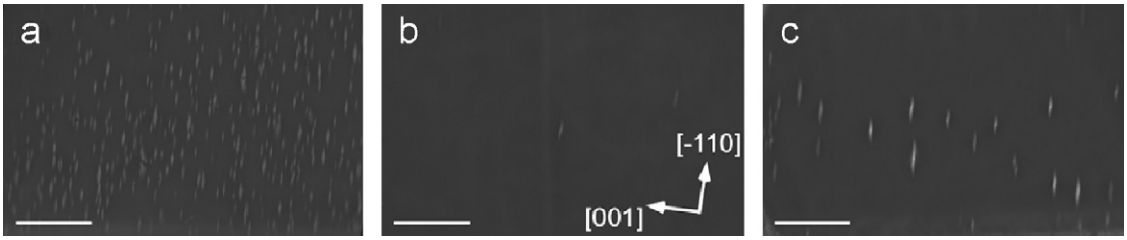

Fig. 1. Morphologies of the {1 1 0} surface of the same lysozyme crystal grown from the different solutions after etching for 15 min. (a) Commercial lysozyme solution, (b) purified lysozyme solution, and (c) purified lysozyme solution containing 430 nM F-lysozyme. The scale bar represents 25 μm.

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