Protein crystallization by using porous glass substrate

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We investigated effects of a commercially available porous glass substrate (Corning Porous Glass No.7930) on the heterogeneous nucleation of proteins (hen egg white lysozyme (HEWL), thaumatin and apoferritin) in order to develop an improved method to facilitate the nucleation of protein crystals. We found that the porous glass substrate could promote the nucleation at lower supersaturations. The induction time for nucleation decreased, and the crystals obtained from porous glass substrates were larger than those from normal glass substrates. Many pores and channels with 10 - 100 nm in diameter were observed on the porous glass surface by atomic force microscopy (AFM). We believe that these pores and channels are crucial for facilitating the nucleation process in this work.

Keywords: protein crystallization, porous glass, nucleation.

1. Introduction

A high-quality protein single crystal is vital to the three-dimensional structural analysis of protein molecules. Nucleation is the first step necessary in the crystallization process. In spite of numerous trials, the control of the nucleation is still beyond our ability. It is difficult to control nucleation and obtain suitable quality of protein crystals for X-ray diffraction studies.

Nucleation is usually termed homogeneous when it occurs in the bulk solution, and termed heterogeneous on solid surfaces. In general, heterogeneous nucleation seems to occur dominantly on the immersed solid material surface, which decreases the activation energy for nucleation through the reduced interfacial free energy between the nucleus and the wetted solid surface. Thus, a lower supersaturation is required for nucleation when dealing with heterogeneous rather than homogeneous nucleation. It should be possible to favourably influence the nucleation rate by selectively varying the nature of the liquid-solid interface.

It was reported that protein crystals grew epitaxially on the surfaces of minerals (McPherson *et al.*, 1988), and a Poly-L-Lysine (PLL) modified glass substrate was used to control the heterogeneous nucleation of lysozyme crystals (Rong *et al*., 2001, Rong *et al*., 2002).

It was also reported (Chayen *et al*., 2001) that specially made porous silicon was used as a nucleant for protein crystallization. The porosity distribution between 5 and 10 nm was believed to be a crucial advantage in providing a substrate appropriate for the shape of the initial aggregates that it forms.

Furthermore, it was reported that protein crystals grown in lower supersaturations have better crystal qualities (Yoshizaki *et al*., 2001). If protein crystal nucleation could occur at lower supersaturation, higher-quality crystals may be expected.

Using a commercially available porous glass substrate (Corning Co., No 7930) with nano-pores and nano-channels, we examined the nucleation of various molecular weight (MW) proteins. The present work suggested that porous glasses entrap the protein molecules and/or their aggregates and encourage them to nucleate and form crystals.

The porous glass was evaluated as a substrate for crystallizing lysozyme, thaumatin and apoferritin in this work. Protein concentrations were used in a lower range than that of normal crystallization. Crystallization on the porous glass substrate was compared to that on a normal glass substrate for the same cell and same condition.

2. Experiments

Lysozyme was purchased from Seikagaku Kogyo; tetragonal lysozyme crystal was used as a model protein crystal. Thaumatin was from Wako Pure Chemical Industries, LTD.; and apoferritin was from Sigma. The crystallizing condition of lysozyme was lysozyme 55 mg/ml (78mg/ml on normal glass), NaCl 25 mg/ml in pH 4.5, 0.05 M NaAC buffer. The crystallizing condition of thaumatin was thaumatin 20mg/ml (40mg/ml on normal glass), 0.4M Rochell salt in pH 6.8, 0.1M PIPES buffer. The crystallizing condition of apoferritin was apoferritin 0.5mg/ml (1.0mg/ml on normal glass), 3% cadmium sulfate in pH 5, 0.2M NaAC buffer. The three protein crystallization temperatures were 20 degrees. The period of crystallization for lysozyme was 3 days, 2 weeks for thaumatin and 4 weeks for apoferritin.

According to our experiments the least protein concentrations for nucleation on normal glass vs. porous glass are as follows:

78mg/ml vs. 55mg/ml for lysozyme

40mg/ml vs. 20mg/ml for thaumatin and

1.0mg/ml vs. 0.5mg/ml for apoferritin

The concentrations for the nucleation were dramatically decreased when porous glass substrate was applied.

The surface of the porous glass substrate was investigated by atomic force microscope (AFM). The AFM system used in this work was a commercially available SPA400, SPI 3800N instrument (Seiko Instruments Inc., Japan). The scanner size was 20×20 μm, and 100μm-long cantilevers were used. The Zeta potential of the porous glass substrate was measured by ELS-6000 (Otsuka Electronics Co., Ltd).

The crystallization experiment was carried out by batch method in the crystallization cell (shown in Fig. 1). The O-ring was held

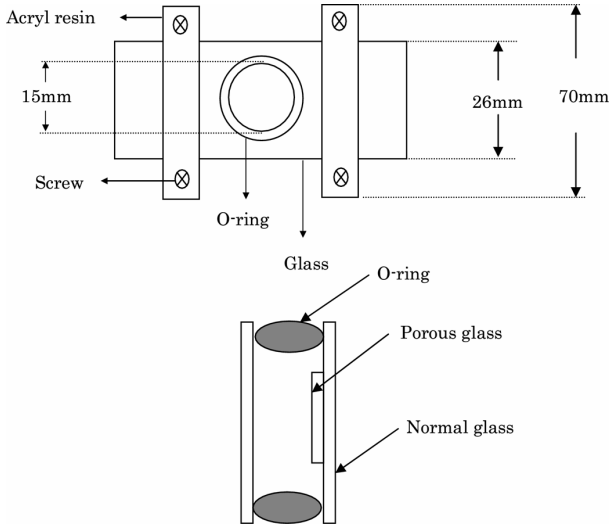

Figure 1 Schematic drawings (plan above, section below) of the crystallization cell..

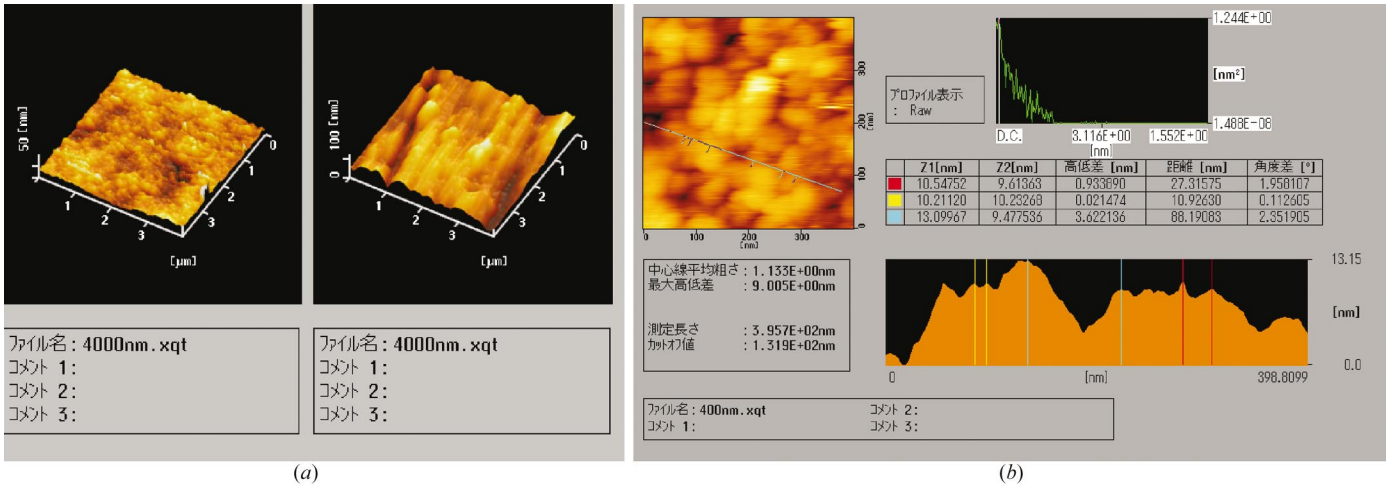

Figure 2 (a) Surface (left) and side surface (right) of porous glass (AFM observation). (b) Cross-section of the porous glass surface (AFM observation).

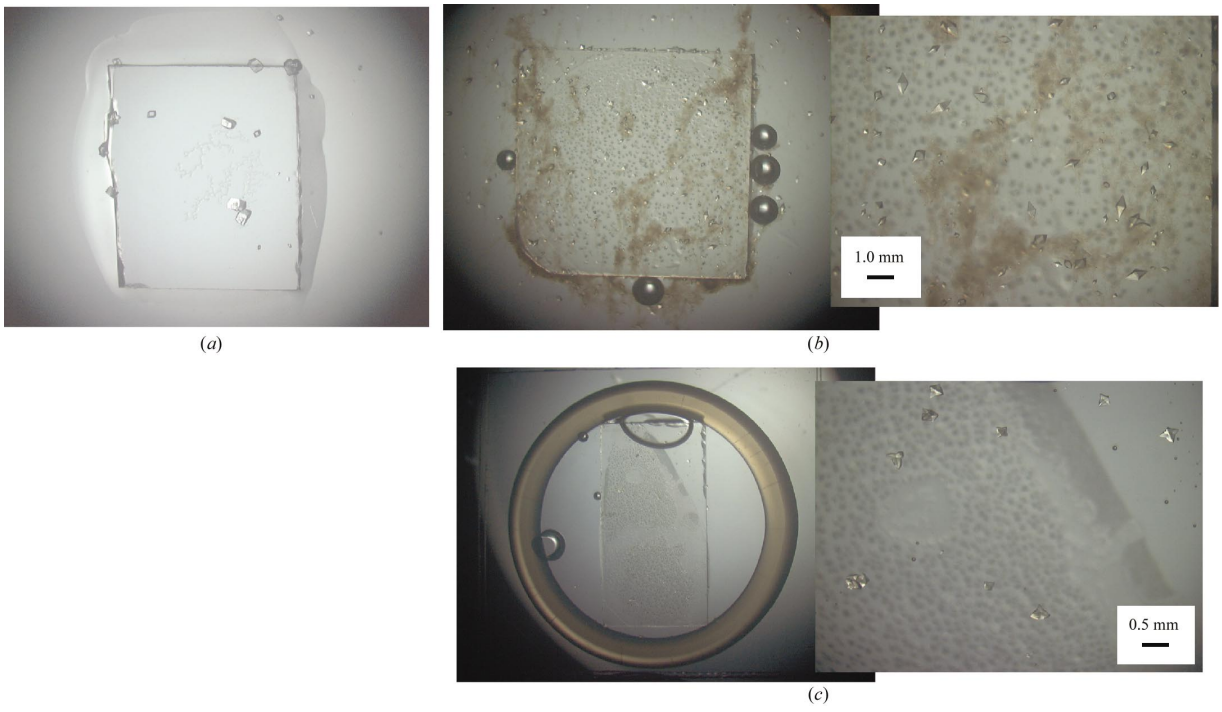

Figure 3 (a) Image of typical crystallization behavior observed for lysozyme on porous glass and normal glass substrates. (b) Images of typical crystallization behavior observed for thaumatin on porous glass with enlarged view and normal glass substrates. (c) Images of typical crystallization behavior observed for apoferritin on porous glass with enlarged view and normal glass substrates.

between normal glass substrates and porous glass was fixed on one side of the normal glass substrate by silicone sealant (Toray Dow Corning Silicone Co., SE9157). To avoid the nuclei or clusters settling from on the substrate by gravity, the cell was hung vertically in an incubator. The O-ring is 15 mm in diameter, and the cell capacity is about 300 µl.

3. Results and discussion

3.1. *Surface of porous glass substrate*

Typical images of the surface (left) and side surface (right) of porous glass substrate are shown in Fig. 2a, the glass is white and the pores and channels are dark in the AFM images. It was found that there are a lot of nano-pores and nano-channels in the porous glass substrate.

A cross section of the surface of porous glass substrate is shown in Fig. 2b. The distribution of pore size was found to be 10 -100 nm.

3.2. *Zeta potential of porous glass substrate*

The Zeta potential of the porous glass substrate was 0.1 ± 1mV while the normal glass substrate was -54.0 ± 4mV and PLL modified glass substrate was 45.0 ± 4mV. The charge of porous glass surface was nearly zero and a very weak electrostatic interaction between the protein molecules and the porous glass substrate is expected. Thus another feature of substrate structure (nano-pores and nano-

channels) is considered to facilitate the crystal nucleation process in this work.

3.3. *Protein crystallizations*

Porous glass successfully induced nucleation at lower lysozyme concentrations where no or almost no nucleation was observed on normal glass. Porous glass also led to the growth of larger single crystals as shown in Fig. 3(a). Many more tetragonal bipyramidal crystals of thaumatin were observed on the porous glass substrate than on normal glass, as shown in Fig. 3(b). For apoferritin, the effect of porous glass was also remarkable as shown in Fig. 3(c). Many skeletal crystals (between the octahedral and dendrite shape) were observed, while almost no crystals were observed on the normal glass.

The molecular weights of these proteins are lysozyme 14,000, thaumatin 22,200 (Iyengar *et al*., 1979) and apoferritin 444,000 (Hempstead *et al*., 1997). If we take account of the size of molecules from 3 nm of lysozyme (Li *et al*., 1999) to 13nm of apoferritin [7] and 10-100 nm of pores and channels with the stronger hydrophilicity and capillary phenomena of porous glass substrate, it is evident that porous glass is very suitable for adsorbing these protein molecules and/or their aggregates and encourage them to nucleate and form crystals.

4. Summary

The results presented in this work demonstrate that heterogeneous nucleation of proteins on porous glass can occur preferentially at lower supersaturations and the porous glass substrate markedly affects nucleation rates both in smaller and larger proteins. Porous glass, which allows faster crystal nucleation, should be very useful for controlling (promoting) the nucleation and growth of protein crystals in lower supersaturations.

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