Effects of High Pressure on the Three-Dimensional Nucleation Rates of Glucose Isomerase Crystals

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Abstract. Three-dimensional nucleation rates $J$ and induction times $\tau$ of glucose isomerase (GI) crystals were measured by counting the number of observable micro crystals with time in situ under high pressure. We also determined the solubility $C_e$ under high pressure from the dependence of the growth and dissolution rates of the crystals on GI concentration $C$, and calculated supersaturation $\sigma (\sigma = \ln(C / C_e))$ under high pressure. Both $J$ and $\tau^{-1}$ increased with increasing pressure at the same $\sigma$. This indicates that the nucleation of the GI crystals was kinetically accelerated with pressure.

1. Introduction
Crystallization of protein is still a “bottleneck” for the three-dimensional (3D) structure analysis of a protein molecule. For instance, the success rate of crystallization is still less than 30% [1]. Without increasing the success rate, we cannot determine the 3D structures of many biologically important protein molecules.

Factors that accelerate the crystallization probably play an important role in improving the success rate. Visuri et al. reported, for the first time, that the crystallization of glucose isomerase (GI) crystals was significantly enhanced with increasing pressure [2]. However, they did not discuss further effects of pressure on the crystallization. Our group measured the solubility [3] and growth rate [4] of the GI crystal under high pressure. The solubility decreased with increasing pressure, and the growth rate increased with increasing pressure even at the same supersaturation condition. In addition, from the analysis of the growth rate, we concluded that two-dimensional nucleation on the crystal surface was enhanced under high pressure via the decrease in step ledge surface energy. However, no one has published the supersaturation dependency of the 3D nucleation rates $J$ of protein crystals under high pressure.

The mechanisms of high-pressure acceleration of 3D nucleation will play the most important role in the improvement of the success rate of crystallization, since the success rate of the 3D nucleation corresponds to that of the crystallization. The precise analyses of the supersaturation dependencies of $J$ will clarify the mechanisms.

In this study, we tried (1) to measure both $C_e$ and $J$ under high pressure, (2) to determine the supersaturation dependencies of $J$ under high pressure, and (3) to discuss the effects of pressure on $J$. 

2. Experimental

2.1. Sample preparation
Glucose isomerase from *Streptomyces rubiginosus* (Hampton Research, 5 times recrystallized) was used without further purification. The glucose isomerase solution for the crystallization contains 0.91 M ammonium sulfate, 1 mM magnesium sulfate, and these are dissolved in 6 mM tris hydrochloride buffer (pH = 7.0).

2.2. Experimental apparatus
A high-pressure vessel with transparent sapphire windows was used [5-6]. An inner cell (inner volume \(= 1 \times 6 \times 20 \text{ mm}^3\)) for *in situ* observation was made of glass slides, and equipped with soft silicone tubes for sample loading. The cell was set in the vessel, and crystals in the cell under high pressure were observed through the sapphire windows using a stereoscopic microscope (Nikon, SMZ800, objective: EDPlan×2 (N. A. = 0.2)). The solution and pressure medium were separated by soft silicone tubes of the cell. The solution around the crystals was pressurized via the tubes. The pressure in the vessel was well controlled automatically by a feedback system with a pressure sensor (accuracy of pressure: ± 0.5 MPa) and could be kept constant for a long time [6]. The temperature of the cell was directly controlled using a Cu jacket with a Peltier element. We could control the temperature from 15.0 to 35.0 °C with the accuracy of ± 0.2 °C.

2.3. Solubility measurements
To determine \(\sigma\) under high pressure, \(C_e\) was measured under high pressure. \(C_e\) was defined as the concentration at which the growth and dissolution rates \(R\) of crystal face became zero. To find the concentration, we fitted a linear function of the concentration to several values of \(R\). \(R\) was defined as the change in crystal size \(L\) over time \(t\). Each \(R\) was determined to be the slope of the tangent line of the \(t-L\) plots at \(t = 0\). \(L\) was defined as the distance between the point C and line A-B of a face of GI crystals as shown in Figure 1. Here \(L\) should be measured on crystallographically equivalent faces of GI crystals, since the GI crystals were surrounded by three crystallographically different faces. We calculated the face indices from the angles between adjacent faces.

Seed crystals were prepared at \(C = 37 \text{ mg mL}^{-1}\), \(T = 10.0 \text{ °C}\), and \(P = 0.1 \text{ MPa}\). After the crystals were grown for 20 hours, the solution in the cell was replaced with the fresh solution of a given GI concentration through the silicone tubes. It took no more than 10 minutes to change the solution and to apply pressure.

2.4. Nucleation rates \(J\) and induction time \(\tau\)
A supersaturated solution of a given GI concentration was transferred into an inner cell. The number of the observable crystals per unit volume \(N\) was counted with time \(t\) using a stereoscopic microscope. The nucleation rate \(J\) is defined as the slope of the tangent line of the \(t-N\) plots at the point of inflection. In practice, we fit Gompertz function, which is a sigmoid function, to the \(t-N\) plots, since Foubert et al. fit the Gompertz function to their data of released crystallization heat of fat crystals, and the fit of the Gompertz model seemed to be better than that of the mostly used Avrami model [7]. The Gompertz function we used is expressed as,

\[
N = a\exp\left[-\exp\left(-k(t-t_c)\right)\right],
\]

where \(t\) represents time, and \(a, k,\) and \(t_c\) are fitting parameters. We assume that \(J\) is defined as the slope of the tangent line of the Gompertz function at the point of inflection, since the slope provides the maximum value. From eq. (1), \(J\) is expressed as,

\[
J = \frac{ak}{e}.
\]

Induction time \(\tau\) is calculated by substituting \(N = 1\) into eq. (1), and expressed as,

\[
\tau = t_c - \frac{1}{k}\ln(-\ln(1/a))
\]
3. Results and Discussion

3.1. Solubility

Typical crystal faces observed in the cell were the {101} faces (Fig. 1). Figure 2 shows $R$ of the {101} faces with $C$ at $T = 20 \, ^\circ C$ and $P = 100 \, MPa$. From this figure, $C_e$ is determined to be $3.4 \pm 0.3 \, mgmL^{-1}$ at 100 MPa. The solubility at $T = 20 \, ^\circ C$ and $P = 0.1 \, MPa$ was also determined in the same way, and it was $4.4 \pm 0.7 \, mgmL^{-1}$. These results indicate that the solubility of GI crystals decreased under high pressure as described elsewhere [3-4].

![Figure 1](image1.png)

**Figure 1.** Determination of the size of a crystal face. Scale bar represents 0.1 mm.

![Figure 2](image2.png)

**Figure 2.** Changes in the growth rate $R$ at $20 \, ^\circ C$ under 100 MPa as a function of GI concentration $C$. Open squares are the data which are used for linear fitting. An open circle presents the solubility at this condition.

3.2. Nucleation rates

$N$ increased with time in a sigmoidal-like fashion (Figure 3). The Gompertz function fitted well all the $t - N$ plots. From the fitting parameters and eq. (2), $J$ was calculated and plotted against $\sigma$ (Figure 4). $J$ increased with increasing pressure at the same $\sigma$. We also determined $\tau^1$ using eq. (3). $\tau^1$ also increased with increasing pressure at the same $\sigma$ (Figure 4). The increase in $J$ and $\tau^1$ with pressure at the same $\sigma$ indicates that they are kinetically accelerated under high pressure.

Although nucleation of protein crystals under high pressure has been already studied by a few researchers [8-10], no one has succeeded in measuring $J$ directly and discussing the dependence of $J$ on $\sigma$. We previously measured $J$ of lysozyme under high pressure by *in situ* observation of the number of crystals using a diamond anvil cell [8]. $J$ decreased with increasing pressure at a constant concentration. However, since the solubility was not measured at that time, we could not separate the effects of solubility change under high pressure. Waghmare *et al.* [9] and Pan *et al.* [10] assumed that the final number of subtilisin crystals was proportional to $J$, and they did not observe the transient number of the crystals. Thus, this study successfully clarified, for the first time, that the 3D nucleation of GI crystals was kinetically accelerated under high pressure. The acceleration is probably due to the decrease in the surface free energy $\alpha$ of a nucleus with pressure, since we have clarified the decrease in the molecular surface energy of GI crystals with pressure [4]. The change in the activation energy $\varepsilon$ for a solute molecule to be incorporated into a critical nucleus with pressure also affects the acceleration. From the precise analysis of the dependence of $J$ on $\sigma$, $\alpha$ and $\varepsilon$ are estimated. However,
at this stage, the error of $\sigma$ is still large, and the number of the data is not large enough for the precise analysis. Further data accumulation of $J$ and more precise solubility measurements [11] should be indispensable for more detailed analysis of the acceleration of the 3D nucleation.

![Figure 3](image1.png)

**Figure 3.** Time course of the number of observed microcrystals at $T = 20 \, ^{\circ}\text{C}$, $C = 27.07 \, \text{mgmL}^{-1}$, and $P = 100 \, \text{MPa}$. Solid curve indicate Gompertz function

![Figure 4](image2.png)

**Figure 4.** $J$ and $\tau^{-1}$ with supersaturation $\sigma$. Open and closed symbols indicate $J$ and $\tau^{-1}$, respectively. Circles and squares indicate the data measured under 0.1 and 100 MPa, respectively.

4. Conclusions

In this study, first, the solubility $C_e$ of GI crystals under high pressure was determined from the dependence of the growth and dissolution rates of the crystals on concentration of GI at 0.1 and 100 MPa. Second, using the solubility data, we determined the supersaturation $\sigma$ at 0.1 and 100 MPa. Third, the number of the crystals per unit volume $N$ was observed *in situ* with time, and the nucleation rate $J$ and induction time $\tau$ were calculated. Our key findings are as follows.

1. $C_e$ decreased with increasing pressure as described previously.
2. Both $J$ and $\tau^{-1}$ increased with increasing pressure at the same $\sigma$. This indicates that the 3D nucleation of GI crystals was kinetically accelerated under high pressure.

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