Preparation of Large-Volume Crystal of Cellulase Under Microgravity to Investigate the Mechanism of Thermal Stabilization

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Abstract: Enzymatic saccharification of cellulose by cellulases is expected to enable energy-efficient production of soluble sugars from biomass, and thus the catalytic mechanisms of cellulases have been intensively studied. We have shown that mutation of non-catalytic cysteine residues of cellobiohydrolase Cel6A from the basidiomycete Phanerochaete chrysosporium improves the thermal stability of the enzyme. To understand why, we considered visualizing the hydrogen bond network within the mutant enzyme by neutron diffraction analysis. In this study, we first examined the optimum concentration ranges of NaCl and polyethylene glycol to produce the required large-volume crystal, and based on the results, we chose nine sets of conditions for crystallization under microgravity at the International Space Station. A large-volume crystal of the mutant enzyme was obtained successfully.

Keywords: Protein crystallization, Glycoside hydrolase, Hydrogen bond, Large-volume crystal, Kirara.

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1. Background

Enzymatic saccharification by cellulases is expected to be an efficient approach to produce soluble sugars for fuels and chemicals from reproducible biomass with a lower energy requirement than chemical or physical treatments. Cellulose is the most abundant biomass in nature, and cellobiohydrolases (CBHs) are indispensable for the complete saccharification of cellulosic biomass, because they can degrade crystalline cellulose, which is resistant to enzymatic hydrolysis. Nevertheless, the degradation of highly crystalline cellulose remains a key bottleneck for achieving efficient enzymatic saccharification.

Among CBHs, Cel6 belongs to glycoside hydrolase (GH) family 6 (EC.3.2.1.91) in the Carbohydrate-Active enZyme database (CAZy) (http://www.cazy.org/). It shows excellent hydrolytic activity, comparable to that of Cel7 belonging to GH family 7 (EC.3.2.1.176), and moreover, synergistic hydrolysis occurs when crystalline cellulose is incubated with both Cel6 and Cel7 together. However, the thermal stability of Cel6 CBHs is generally lower than that of Cel7 CBHs. This is problematic, because the synergy between Cel6 CBHs and Cel7 CBHs is reduced due to the lower thermal stability of Cel6 CBHs during industrial-scale enzymatic saccharification, which is conducted at an elevated temperature to increase the hydrolytic rate and
prevent microbial contamination. Therefore, increasing the thermal stability of Cel6 CBHs should immediately lead to an increase in the efficiency of commercial enzymatic saccharification.

Previous studies aimed at improving the thermal stability of Cel6 CBHs suggested that certain mutant enzymes, in which free cysteine residues that do not form a disulfide bond are substituted, exhibit increased thermal stability\(^6,7\). To investigate the reason for this, we introduced mutations into the gene encoding Cel6 CBH from the basidiomycete *Phanerochaete chrysosporium* (*PcCel6A*), and obtained a thermostable mutant (*PcCel6AΔfreeCys*)\(^8\). X-Ray crystal structure analysis focusing on the catalytic domain of the mutant indicated that the hydrogen bond network of the mutant might be more stable than that of wild-type enzyme.

However, to confirm this idea by visualizing the hydrogen bond network of the mutant would require a neutron diffraction analysis, because only neutron diffraction can identify hydrogen atoms\(^9\). This is not straightforward, as is apparent from the rather small number of structures so far determined by neutron diffraction analysis, because neutron crystallography requires a very large-volume crystal compared with X-ray crystallography\(^10\). We considered that this obstacle might be overcome by conducting crystallization under microgravity in space, because previous studies have shown that the volume and quality of crystals grown in space are superior to those of lab-grown crystals on earth\(^11\)–\(^21\).

In preparation for a precious experiment on the International Space Station (ISS), whose trial and sample number are limited, the applicability of many kinds of crystallization methods and conditions has been examined so far. The major crystallization techniques include vapor diffusion (VD), batch, and counter diffusion (CD) methods\(^22,23\). In the VD method, a protein sample and a reservoir solution are mixed to form a drop and then moisture in the drop evaporates and migrates to the reservoir solution via the air, concentrating both the protein and the crystallization reagents in the drop. This method is suitable for evaluating a large number of crystallization conditions\(^24\). In the batch method, a mixture of the protein sample and the reservoir solution is loaded into a capillary. In the CD method, the protein sample diffuses out of a large capillary while crystallization reagents diffuse into the capillary. This bidirectional diffusion makes it possible to scan a wide range of concentrations of protein sample and reservoir solution components\(^25–27\). However, even with the use of these methodologies and the accumulated scientific knowledge of crystallization over a long history of study\(^28–31\), it still often takes a long time and requires a large amount of protein sample to identify optimum crystallization conditions for obtaining large-volume crystals.

In addition to the crystallization method, the choice of crystallization reagents can be a key factor in producing large-volume crystals. Recently, the combination of a principal crystallization reagent such as polyethylene glycol (PEG), which is frequently used for protein crystallization, and a salt such as NaCl, which is usually contained in the protein sample, has been developed to control crystal generation\(^32\). Thus, optimization by changing the concentration of PEG was conducted for wild-type *PcCel6A*\(^33\). On the other hand, 2-methyl-2,4-pentanediol (MPD) has often been added as one of the crystallization reagents\(^34\), despite concern that it might have a negative effect on the protein, because it tends to be easily oxidized during storage.

In the present study, therefore, we aimed to systematically establish suitable methodology to obtain large-volume crystals of *PcCel6AΔfreeCys* under microgravity in space using only NaCl and PEG as crystallization reagents. Initially, we began on a small scale, using vapor diffusion and batch methods to test many combinations of conditions for crystallization. Then, we utilized the information obtained to move to a larger-scale reaction using the counter diffusion method, and identified suitable sets of conditions for the experiment on the ISS. The crystals obtained under microgravity conditions were subjected to neutron/X-ray diffraction measurements. Based on our findings, we discuss the strategy for obtaining large-volume crystals under microgravity, as well as the requirements for successful neutron diffraction measurements.

### 2. Methods

#### 2.1. Materials

*Pichia pastoris* strain KM71H (Thermo Fisher Scientific Inc., MA, USA) was used for heterologous production of the mutant enzyme. Sodium acetate trihydrate, acetic acid, ammonium sulfate, tris(hydroxymethyl)aminomethane (Tris), 6 mol/L hydrochloric acid, and sodium chloride for the purification buffer were purchased from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. The crystallization solution consisted of 50% (w/v) polyethylene glycol (PEG) 4000 (Qiagen Sciences, MD, USA), sodium chloride, sodium acetate trihydrate, and acetic acid.

Int. J. Microgravity Sci. Appl. 38 (1) 380103 (2021) 2 of 12
2.2. Production of mutant protein

pPIC\text{Z}\alpha vector containing the gene of the catalytic domain of \textit{PcCel6A}\text{ΔfreeCys} was prepared by deleting the N-terminal CBM-linker region (residues 1-81) of the whole-length \textit{PcCel6A}\text{ΔfreeCys} gene in the pPIC\text{Z}\alpha vector synthesized previously\textsuperscript{8).} The mutant protein was expressed in \textit{P. pastoris} using a 5 L jar fermenter according to the reported method\textsuperscript{35).} After 4 days of methanol-fed batch culture, the culture solution was centrifuged at 4°C with $3,000 \times g$ for 10 min and centrifuged again at $10,000 \times g$ for 10 min. The supernatant was ultrafiltered and the filtrate was concentrated using a Pellicon\textsuperscript{®} XL50 with a Biomax\textsuperscript{®} Membrane 300 kDa and 5 kDa (Merck KGaA, Darmstadt, Germany).

2.3. Purification of protein for terrestrial crystallization

The protein sample for terrestrial crystallization was prepared as follows. Ammonium sulfate was added to the enzyme solution to give a final concentration of 0.5 or 0.6 M and the solution was applied to a 175 mL TOYOPEARL Phenyl-650M column (Tosoh Corporation, Tokyo, Japan) equilibrated with the same molarity of ammonium sulfate in 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with an 875 mL linear gradient from the same molarity to 0 mM ammonium sulfate in the same buffer. The enzyme fractions were concentrated and equilibrated with 20 mM Tris-HCl (pH 8.0) using ultrafiltration discs (5 kDa NMW; Merck KGaA). The solution was applied to a 150 mL TOYOPEARL DEAE-650 column (Tosoh Corporation) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The enzyme was eluted with a 1,500 mL linear gradient from 0 to 20 mM NaCl in the same buffer. The molecular weight of the protein was estimated by SDS-PAGE using 12% polyacrylamide gel. Since doublet bands were seen at approximately 37 kDa, presumably reflecting a difference in glycosylation, the fractions with lower molecular weight were collected.

2.4. Purification of protein for crystallization in space

The protein sample for crystallization in space was prepared as follows. The enzyme precipitated by 3.3 M ammonium sulfate (70% saturation) after ultrafiltration and concentration as described above was diluted with 20 mM sodium acetate buffer (pH 5.0) to make a final concentration of ammonium sulfate of 1 M. The solution was applied to a 175 mL TOYOPEARL Phenyl-650M column (Tosoh Corporation) equilibrated with the same molarity of ammonium sulfate in 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with an 875 mL linear gradient from the same molarity to 0 mM ammonium sulfate in the same buffer. The fractions with lower molecular weight were concentrated and equilibrated with 20 mM Tris-HCl (pH 7.0) using ultrafiltration discs (5 kDa NMW; Merck KGaA). The solution was applied to a 26 mL TOYOPEARL SuperQ-650 column (Tosoh Corporation) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The enzyme was eluted with a 78 or 130 mL linear gradient from 0 to 0.15 or 0.25 M NaCl in the same buffer. The molecular weight of the protein was estimated by SDS-PAGE on 12% polyacrylamide gel. The fractions with lower molecular weight were collected and NaCl contained in the fractions was diluted with 20 mM Tris-HCl (pH 7.0).

2.5. Screening by the vapor diffusion method

Crystallization by the sitting drop vapor diffusion method was conducted using 96-Well CrystalQuick\textsuperscript{™} (Greiner Bio-One International GmbH, Kremsmünster, Austria), as illustrated in Fig. 1A. The concentrations of reagents chosen to form crystals of \textit{PcCel6A}\text{ΔfreeCys} were based on the crystallization conditions of wild-type \textit{PcCel6A} (\textit{PcCel6A}\text{WT})\textsuperscript{34), because the pl and charge density at pH 4.5 in the crystallization buffer were expected to be similar to those of the wild-type enzyme, based on the physicochemical properties of the protein calculated from the amino acid sequence by using the C-Profile program (Confocal Science Inc., Tokyo, Japan).

Each droplet was formed in a round well by adding 0.75 µL of the reservoir solution composed of 25 to 35% (w/v) PEG 4000, 50 mM sodium acetate buffer (pH 4.5), and 75 to 125 mM NaCl to the same volume of 34 mg/mL protein sample containing about 8 mM NaCl. Therefore, the initial concentration of droplets before diffusion was about 41.5 to 66.5 mM NaCl, 12.5 to 17.5% (w/v) PEG 4000, and 25 mM sodium acetate buffer (pH 4.5). 150 µL of the reservoir solution was also placed in a flat-bottomed well. The mixtures were incubated at 20°C.
2.6. Screening by the batch method

Crystallization by the batch method was conducted using Minicaps® capillaries (5 µL; Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany) as illustrated in Fig. 1B. The protein sample and the reservoir solution, 1 µL each, were mixed on Parafilm® (Amcor, Zürich, Switzerland) to give final concentrations of 18 mg/mL protein, 28.95 to 100.00 mM NaCl, 10 to 25% (w/v) PEG 4000, and 25 mM sodium acetate buffer (pH 4.5). The mixture was introduced into the capillaries by utilizing the capillarity phenomenon, and then both ends of the capillaries were sealed with KIMBLE® Cha-seal™ Tube Sealing Compound (DWK Life Sciences, Mainz, Germany). Each capillary was put in a 1.5 mL plastic tube and incubated at 20°C.

2.7. Screening by the counter diffusion method

Crystallization by the counter diffusion (CD) method was conducted in 0.3 mm capillaries (Confocal Science Inc.) consisting of a straight glass capillary in which the protein sample is loaded and a gel-tube made of agarose gel in silicone tubing, as illustrated in Fig. 1C. The gel-tubes were soaked for several days in 1 mL of the gel immersion solution composed of 30 to 55 mM NaCl, 10% (w/v) PEG 4000, and 25 mM sodium acetate buffer (pH 4.5). Aliquots of 5 µl of the protein sample (35 mg/mL in 20 mM Tris-HCl (pH 8.0) containing about 8 mM NaCl) were put in 200 µL plastic tubes and introduced into the capillaries up to 30 mm from the bottom via capillarity. The top of each capillary was sealed with KIMBLE® Cha-seal™ Tube Sealing Compound and a piece of gel tube with 2 µL of the gel immersion solution on the top was attached to the capillary bottom. The bottom of the gel-tube was cut diagonally and the set was placed in a 5 mL plastic tube containing 1 mL of the reservoir solution composed of 30 to 55 mM NaCl, 25% (w/v) PEG 4000, and 25 mM sodium acetate buffer (pH 4.5). Tubes were held in a vertical direction and incubated at 20°C.

Finally, crystal growth by the CD method was checked using a Crystallization Cell for Larger Crystal (2.0 mm bore; Confocal Science Inc.), consisting of a glass capillary and a gel-tube, as a rehearsal for the microgravity experiment. The gel-tube was soaked for 4 days in 1 mL of the gel immersion solution composed of 40 mM NaCl, 30% (w/v) PEG 4000, and 25 mM sodium acetate buffer (pH 4.5). Then 36 µL of the protein solution composed of 35 mg/mL protein, 4.9 mM NaCl, and 12% (w/v) PEG 4000 were put in a 200 µL tube and sucked up into the capillary by pulling air up from the top of the capillary using a syringe equipped with a needle. Then the capillary was sealed and a piece of gel tube was attached and cut diagonally as described above. The set was placed in a 5 mL plastic tube containing 1 mL of reservoir solution composed of 40 mM NaCl, 30% (w/v) PEG 4000, and 25 mM sodium acetate buffer (pH 4.5) and incubated at 20°C while held in a vertical direction.

![Figure 1](image)

**Figure 1.** Schematic illustration of the sitting drop vapor diffusion method (A), the batch method (B), and the counter diffusion method (C). The reaction volumes in the vapor diffusion and batch methods were 1.5 and 2 µL, respectively. This volume was scaled up by an order of magnitude in the counter diffusion method.


2.8. Crystallization under microgravity

Crystallization under microgravity was conducted by the CD method in the first trial of the High Quality Protein Crystal Service (Kirara#1) provided by Japan Manned Space Systems Corporation (JAMSS, Tokyo, Japan). The gel-tube was soaked for about a week in gel immersion solution composed of 35 to 45 mM NaCl, 15 to 30% (w/v) PEG 4000, and 25 mM sodium acetate buffer (pH 4.5). The protein solution, composed of 35 mg/mL protein, 0 to 45 mM NaCl, and 8 to 25% (w/v) PEG 4000, was loaded in a Crystallization Cell for Larger Crystal. The cell was sealed with sealing compound and C-Cap (Confocal Science Inc.), and a piece of gel tube was attached. The set was put in C-SGT (Confocal Science Inc.) containing the reservoir solution with the same components as the gel immersion solution, and the gel-tube was nipped with a clip to inhibit diffusion between the solutions.

The clip was removed at the launch site (John F. Kennedy Space Center) and the set was placed in a small incubator developed by JAMSS. The incubator was transported to the ISS by the Dragon spacecraft (Space Exploration Technologies Corp., CA, USA) and placed in the International Commercial Experiment Cubes Facility (Space Application Services NV/SA, Sint-Stevens-Woluwe, Belgium) in the Columbus experiment module of ISS. After crystallization at 20 ± 2°C for about a month, the small incubator was splashed down to the Pacific Ocean by Dragon spacecraft and transported to Japan.

2.9. X-Ray and neutron diffraction measurements

X-Ray diffraction data of the crystals grown by the vapor diffusion and counter diffusion methods were obtained using synchrotron radiation at the BL-5A X-ray beamline of the High Energy Accelerator Research Organization (KEK), Tsukuba, Japan and processed using XDS. X-Ray diffraction of crystals grown by the batch method was measured using synchrotron radiation at Diamond Light Source (DLS) i04 X-ray beamline, Oxfordshire, UK. Data for a crystal (leftmost in Table 2) were processed using the DIALS pipeline in xia2, and those for others were processed using the autoPROC pipeline. Most of the crystals other than space grown ones for X-ray diffraction measurement at low temperature were immersed in cryoprotectant solution containing 40% (w/v) PEG 4000, prepared by mixing the reservoir solution and 50% (w/v) PEG 4000, and the measurement was conducted at around 95 K.

A large microgravity-grown crystal for neutron diffraction measurement was transferred from the capillary to a solution whose composition was the same as that in which the crystal had been grown. Subsequently, the crystal was encapsulated in a Mark tube made of quartz glass (outside Φ = 3.5 mm and wall thickness 0.01 mm; Hilgenberg GmbH, Malsfeld, Germany) together with the solution. Measurement was performed at around 24°C with an iBIX diffractometer at the BL03 neutron beamline of the Japan Photon Accelerator Research Complex (J-PARC). X-Ray diffraction measurement of a space-grown crystal in the quartz capillary was also conducted at room temperature using BL-5A at KEK.

3. Results and discussion

3.1. Screening by the vapor diffusion method

In the sitting drop vapor diffusion method, when the initial concentration of NaCl was 41.5 mM, tabular and columnar crystals of PtCel6AΔfreeCys appeared after 3 days with 12.5 to 14.5% (w/v) PEG 4000, while when the concentration of NaCl was 54 mM, small acicular crystals appeared after 5 days with only 12.5% (w/v) PEG 4000, as shown in Fig. 2. No crystals were observed when the concentration of NaCl was 66.5 mM. Thus, we presumed that crystals could be obtained in the concentration ranges of approximately 41.5 to 54.0 mM NaCl and 12.5 to 14.5% (w/v) PEG 4000.

The space group of the crystal generated in 41.5 mM NaCl and 13.5% (w/v) PEG 4000 was P2₁2₁2₁, which is the same as the crystal of PtCel6A_WT (PDB ID 5XCY). In addition, the resolution obtained was 1.23 Å, which was also similar to that in the case of the crystal of PtCel6A_WT obtained by the vapor diffusion method. Therefore, we concluded that high-quality crystals can be obtained without using MPD by optimizing the concentrations of NaCl and PEG.
Figure 2. Crystallization by the sitting drop vapor diffusion method. The shape of the crystals was tabular (■), columnar (●), or acicular (▲), depending on the NaCl and PEG concentrations. NA means the condition in which crystals were not observed. The numbers indicate the initial concentration of each droplet.

3.2. Screening by the batch method

Since the droplet could be condensed in the vapor diffusion method, the batch method was conducted to determine the exact concentration of a protein sample and a reservoir solution when the crystals are grown by checking the concentration presumed in the vapor diffusion method. Crystals appeared sporadically in the concentration ranges of 28.95 to 53.95 mM NaCl and 11.75 to 14.00% (w/v) PEG 4000 from the 3rd day, as shown in Table 1A. To examine the individual effects of NaCl and PEG on the crystallization, the concentration range of each reagent was broadened while keeping the concentration of the other fixed, as shown in Table 1B and 1C. When the concentration of NaCl was raised to 75 and 100 mM at a fixed PEG 4000 concentration of 12.5% (w/v), crystals appeared after 8 and 3 days, respectively (Table 1B). On the other hand, when concentrations of 10, 15, 20, and 25% (w/v) PEG 4000 were tested at a fixed NaCl concentration of 28.95 mM, crystals only appeared with 15% (w/v) PEG 4000 after 8 days (Table 1C). Throughout these three tests, it was often the case that clusters of crystals gradually covered the bottom of the capillary.

Even though most crystals obtained by the batch method was acicular, tabular and columnar crystals were also formed under some conditions (Table 1A). To see if the crystal shape is critical for X-ray diffraction measurement, we compared the diffraction data of thick tabular or columnar crystals and thin acicular crystals. As shown in Table 2, the highest resolution of thick crystals was 1.09 or 1.15 Å while that of thin crystals was 1.36 or 1.58 Å. One of the diffraction data of a thin acicular crystal could not be processed correctly. Further, the space group of thick crystals was the same as before, whereas every thin crystal measured showed a different space group. Other parameters such as higher values of Mean<\(I/\sigma(I)\)> and lower values of \(R_{merge}\) also indicated that the quality of thick crystals was superior to that of thin crystals and contributed to the successful X-ray diffraction measurement. Accordingly, we concluded that thick crystals are more desirable than thin crystals for acquiring high-resolution data in the correct space group.

Since the shape of all the crystals obtained by both additional experiments was acicular (Table 1B and 1C), we assumed that suitable crystals would be formed at 28.95 to 53.95 mM NaCl and 12.25 to 13.75% (w/v) PEG 4000 (Table 1A).
Table 1. Crystallization by the batch method. Based on the result of the first trial (A), the concentration ranges of NaCl (B) and PEG (C) were extended. NA means the condition in which crystals were not observed.

<table>
<thead>
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<th>PEG 4000 [%]</th>
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NaCl [mM]

Table 2. Effect of crystal shape on X-ray diffraction. Thick or plate-like crystals were obtained only from 12.25 to 13.75% (w/v) PEG 4000, while thin needle-like crystals appeared under a wide range of crystallization conditions. Values in parentheses are for the highest resolution shell.

<table>
<thead>
<tr>
<th>PEG 4000 [%]</th>
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<td>75</td>
<td>100</td>
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</table>

NaCl [mM]

3.3. Screening by the counter diffusion method

Since the crystallization experiment under microgravity is performed by the counter diffusion method, the demonstration of this method is essential. The PEG 4000 concentration of 25% (w/v) was chosen as the initial concentration of the reservoir solution because it was expected that the concentration of PEG would be raised gradually, and that the crystal growth would be started when the concentration of PEG passed around
ten-odd percent as inferred by the batch method. Although the possibility of crystal growth at a higher PEG concentration was less in the batch method, the concentration of PEG was not kept at a dozen percent and raised to 25% (w/v) because a higher PEG concentration generally brings about higher resolution on the crystal42). Besides, the initial PEG 4000 concentration of the gel immersion solution was set at 10% (w/v) to save the time required for the diffusion by raising from the highest PEG concentration in which the crystals were not likely to appear. When we examined crystallization conditions of 30 to 55 mM NaCl as the initial concentration of the gel immersion solution and the reservoir solution at a fixed PEG 4000 of 10% (w/v) in the gel immersion solution and 25% (w/v) in the reservoir solution, crystals appeared within 2 weeks only when the NaCl concentration in the gel immersion solution and the reservoir solution was 40 mM, as shown in Fig. 3A. Since the duration of the microgravity environment was planned to be about 4 weeks, 40 mM NaCl seemed to be the most appropriate concentration for the reservoir solution.

When the crystallization was performed in the large bore capillary with a diameter of 2.0 mm which is the same cell as planned for the microgravity experiment, the initial concentration of PEG 4000 in the capillary was set at 12% (w/v). That was because thick crystals were expected to appear by keeping the low concentration of PEG for a longer period according to the results in the batch method. As shown in Fig. 3B, large numbers of small crystals appeared and gradually accumulated at the bottom of the capillary. The reason for this might be that crystal nucleation is accelerated at the starting concentration of 12% (w/v) PEG 4000 in the capillary.

![Figure 3](image-url)

**Figure 3.** Crystallization by the counter diffusion method. A capillary with a diameter of 0.3 mm was used for the initial examination, where numbers indicate the initial concentration of the gel immersion solution and the reservoir solution (A). A rehearsal of the microgravity experiment was performed using a cell with a diameter of 2.0 mm (B). NA means the condition in which crystals were not observed.
3.4. Crystallization under microgravity

Nine different crystallization conditions were tested under microgravity at the ISS, based on the promising NaCl and PEG concentration ranges identified on the ground (Fig. 4). In addition to the strategy of increasing both NaCl and PEG concentrations in the capillary (P6-P8) in a similar manner to the rehearsal on the ground (Fig. 3B), two other contrasting strategies of bidirectional diffusion were examined in the microgravity experiment; increasing only the PEG concentration in the capillary (P-P2), or increasing only the NaCl concentration in the capillary (P3-P5). In each strategy, the two different NaCl concentration around the optimum concentration presumed by the counter diffusion method or the two different PEG concentration higher than the optimum concentration presumed by the batch method was chosen to see if the thick crystals also appear at a higher/lower NaCl and PEG concentration under microgravity. The initial concentration of PEG in the capillary of P6-P8 was lowered to 10% (w/v) because crystal nucleation was excessive when the concentration was set at 12% (w/v) in the rehearsal of the microgravity experiment on the ground. While NaCl was mostly diffused into the capillary during the microgravity condition, PEG concentration in the capillary of P-P2 and P6-P8 was calculated to reach a little less than 20% during the microgravity condition. Based on the result that a crystal did not appear when the PEG concentration was fixed at 25% (w/v) (P5), it is assumed that crystals were grown under microgravity with 35 to 45 mM NaCl and 8 to a little less than 20% PEG 4000. We found that large crystals were obtained when the NaCl concentration in the capillary was raised from 0 to 40 mM at the fixed PEG 4000 concentration of 15% (w/v) (Fig. 4; P3). The size of the largest crystal was 1.46 mm^2 × 0.3 mm. When the initial PEG concentration of the reservoir solution was raised to 20% (P4) or 25% (P5), many fine crystals or no crystals appeared, respectively. In contrast to the strategy of raising only the NaCl concentration in the capillary (P3-P5), slender crystals appeared when only the PEG concentration in the capillary was raised (P-P2). Moreover, the crystals became thinner when the strategy of raising both NaCl and PEG concentration in the capillary was applied (P6-P8). Narrower and longer crystals were formed as the initial NaCl concentration of the reservoir solution was raised from 35, 40 to 45 mM. All of these findings suggest that a low concentration of PEG at the first phase of crystallization and a relatively higher concentration of NaCl lead to more crystal nucleation of PcCel6AAΔfreeCys, which could inhibit the growth of large individual crystals. Conversely, the largest crystal might appear on condition that the concentration of PEG was retained at the optimum value without diffusion (P3).

Neutron diffraction spots up to 5.2 Å were observed by the measurement of the large-volume crystal P3 in Fig. 4 for 30 minutes, which implied that the space-grown crystal was feasible for neutron crystallography. However, longer measurement to increase the resolution could not be performed because cracks appeared in the crystal and it became cloudy during storage in the thermostated chamber at 20°C for half a day. This deterioration during storage might have been caused by the stress imposed on the microgravity-grown crystal due to physical contact and changes in environmental temperature during transfer from the crystallization cell to the quartz capillary for the diffraction measurement. It also appeared that the thickness (0.3 mm) of the crystals might influence the stability.

X-Ray diffraction measurement was conducted using crystals grown by raising the PEG concentration in the capillary from 8 to 30% (w/v) at a fixed NaCl concentration of 45 mM (Fig. 4; P2). The crystal was plate-like shape and the space group was P2_12_1. These traits resembled the crystals produced by the batch method that showed a higher resolution than the needle-like crystals (Table 2). In fact, the resolution of the crystals was 1.71 Å at room temperature and 1.08 Å at around 95 K. This high resolution indicates that the crystallization strategy of adjusting the NaCl and PEG concentrations does have the potential to produce high-quality crystals under microgravity in space.
4. Conclusions

In this work, we aimed to establish methodology for obtaining large-volume crystals of a thermostable mutant of \textit{PcCel6A} under microgravity in space by changing the concentration of two kinds of crystallization reagents (NaCl and PEG) two-dimensionally. Extensive optimization starting from small-scale testing of a wide range of conditions and progressing to large-scale testing of promising combinations identified NaCl and PEG concentration ranges that appeared suitable to make large-volume crystals under microgravity. Although we successfully obtained a large-volume crystal from the ISS, it was not sufficiently stable for us to complete neutron diffraction measurements. We are planning to perform crystallization using a capillary made of quartz and currently working to improve the stability and handling of microgravity-grown crystals sufficiently to enable neutron diffraction measurements to be completed.

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Conflicts of Interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References


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