Use of Multiple Cryoprotectants to Improve Diffraction Quality from Protein Crystals

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ABSTRACT: Current X-ray sources—such as high-flux synchrotron radiation and XFEL—enable us to determine the tertiary structures of proteins at medium or low resolution even from crystals with poor quality. However, high-resolution crystal structures are still required to obtain detailed information about protein structures in the pharmaceutical and biochemical sciences. To improve the crystal quality, several types of post-crystallization treatments, including soaking experiments, have been utilized. However, such treatments do not always improve the crystal quality, and a new method of crystal-quality enhancement is eagerly awaited. Here, we propose a novel strategy for the post-crystallization treatment, the multistep soaking method, in which a crystal is sequentially soaked in 2−3 different cryoprotectant solutions. The sequential use of different cryoprotectants significantly expands the number of options for subsequent post-crystallization treatment. Using the new method, we successfully improved the crystal quality of CagA, a type IV secretion effector derived from Helicobacter pylori, from 7.5 to 3.1 Å resolution, in addition to improving the crystal quality of phosphatidylinositol 5-phosphate 4-kinase β.

INTRODUCTION

Crystallization has been a bottleneck in protein crystallography. While the development of crystallization techniques using crystallization robots, screening kits, and other approaches has allowed the examination of a huge number of crystallization conditions at the initial screening,1,2 the obtained crystals do not always diffract to sufficient resolution for crystal structure determination of proteins. To improve the crystal quality, several types of post-crystallization treatments have been developed.3−4 Cryoprotection, dehydration, crystal annealing, and flash annealing techniques can occasionally improve the crystal quality, leading to successful crystal-structure determination in the case of especially challenging crystals.5−10 Post-crystallization treatments, however, do not always improve the crystal quality. Furthermore, while the structural information on multidomain proteins and large molecular complexes has become increasingly important in the field of structural biology, crystals of these difficult targets tend to be of poor quality. Therefore, there is a critical need to develop a new method for improving crystal quality.

Over the last two decades, most diffraction data have been collected at cryogenic temperature to avoid radiation damage caused by strong synchrotron X-ray beams.11,12 In order to avoid the formation of crystalline ice during the protein crystal freezing, several kinds of cryoprotectants have been utilized.5,13,14 The screening of a proper cryoprotectant has thus been an important step for the crystal structure determination of biological macromolecules with synchrotron radiation. Several groups have reported that the crystal quality was improved by optimizing the conditions of crystal soaking in the cryoprotectant solution.15,16 We also succeeded in improving the crystal quality of human TAF-Iβ, which is a histone...
chaperone with histone H3–H4 preference, by combining the optimization of cryo-conditions and mutations. On the basis of this experience, we have developed a novel approach to the post-crystallization treatment, the multistep soaking method, which utilizes 2–3 different cryoprotectant solutions for soaking. Using this method, we succeeded in improving the crystal quality of CagA from 7.5 to 3.1 Å resolution, and thereby determined its crystal structure. CagA, an effector protein of Helicobacter pylori, plays a key role in the development of gastric cancer, the second-leading cause of cancer-related deaths worldwide. In the crystal structure determination of CagA, a huge number of data sets were obtained from the CagA crystals that were treated under a variety of soaking conditions, including multistep soaking. The conditions and results of the soaking experiments—the lot number of the purified protein sample, mutant name, crystallization conditions, soaking conditions, crystal size, resolution limit, and so forth—were recorded for all crystals. We analyzed these data and concluded that the multistep soaking was significantly effective in improving the CagA crystal. In addition, the crystal quality of phosphatidylinositol-5-phosphate 4-kinase β (PI5P4Kβ) was improved by this method. Here, we propose the multistep soaking method as an effective method for post-crystallization treatment.

**Experimental Section**

**Purification and Crystallization of CagA.** In this study, CagA (1186 amino acid residues, Mw = 132 kDa) derived from H. pylori strain 26695 was utilized for crystallization. Since the C-terminal region of CagA, residues 877–1186, was expected to be an intrinsically disordered region, these residues were deleted from full-length CagA for the crystallization study. CagA(1–876), which is composed of residues 1–876 of CagA, was overexpressed in Esherichia coli and purified as described previously. The purified CagA(1–876) was concentrated to 50–60 mg/mL using an Amicon Ultra-4 centrifugal filter (50 000 MWCO; Millipore) in 500 mM sodium chloride and 20 mM Tris-HCl, pH 8.0, and then crystallized by the sitting-drop vapor-diffusion method at 293 K. The sitting drop was prepared by mixing 1.0 μL of each protein and reservoir solutions. While many mutant CagA proteins were utilized for the structure determination of CagA, only the wild type CagA crystals were statistically analyzed in this study to avoid crystal-quality deviations in mutant CagA proteins.

**Soaking and Cryocooling of CagA Crystals.** Artificial mother liquor should maintain the crystal for at least 2–3 days without damage. The conditions of the artificial mother liquor were determined based on those of the crystallization solutions (7–10% (v/v) ethanol, 50 mM Tris-HCl, pH 7.0–8.8). First, the pH of the artificial mother liquor was adjusted to that of the droplet solution. Second, the concentration of the precipitant, ethanol in this case, was optimized; the concentration needs to be increased to avoid dissolution of crystals in the mother liquor solution. In the case of the CagA crystal, 10%, 20%, and 40% (v/v) ethanol concentrations were tested and 20% (v/v) ethanol in 50 mM Tris-HCl (pH 7.0–9.1) showed the least damage on the crystal. The conditions of the artificial mother liquor were therefore determined to be 20% (v/v) ethanol, 50 mM Tris-HCl, pH 7.0–9.1. The CagA(1–876) crystals could be maintained in the artificial mother liquor for 3 days without damage. Screening of cryoprotectant reagents was performed using a CryoPro kit (Hampton Research), which includes 36 cryoprotectant reagents. In this study, 20 cryoprotectant reagents, glycerol, ethylene glycol, PEG200, PEG400, PEG600, PEG6000, polyvinylpyrrolidone K 15 (PVP), (±)-2-methyl-4-pentanediol (MPD), 1,6-hexanediol, 1,2-propanediol, dimethyl sulfoxide (DMSO), 2-propanol, ethanol, methanol, D-(+)-sucrose, meso-erythritol, xylitol, D-(+)-raffinose, D-(+)-trehalose dehydrate (THL), and D-(+)-glucose, were used for the initial screening. The artificial mother liquor solution supplemented with a cryoprotectant (hereafter “the cryoprotectant solution”) was utilized for the soaking experiments. In the typical case, the cryoprotectant solution contained 20% (v/v) ethanol, 50 mM Tris/HCl, pH 7.0–8.5, and 17.5–30% (w/v or v/v) of a cryoprotectant reagent. Crystals soaked in the cryoprotectant solution were mounted on a cryoloop (Hampton Research), and flash cooled in liquid nitrogen.

**Purification and Crystallization of Phosphatidylinositol-5-Phosphate 4-kinase β (PI5P4Kβ).** His-tagged PI5P4Kβ with a PreScission-protease cleavage site was overexpressed in E. coli BL21 (DE3) using the pET28 vector. The overexpressed PI5P4Kβ was purified by Ni-affinity chromatography. The cell lysate was applied to Ni-NTA agarose (QIAGEN) equilibrated with buffer A [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM β-mercaptoethanol (β-Me), 10 mM imidazole]. The column was then washed with buffer A and His-tagged PI5P4Kβ was eluted from the column with buffer B [50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 10 mM β-Me, 300 mM imidazole]. The fraction containing His-tagged PI5P4Kβ was concentrated using an Amicon Ultra centrifugal filter (10 000 MWCO; Millipore) and the His-tag was cleaved by using PreScission protease (GE Healthcare). The solution was dialyzed against Buffer C [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM DTT] at 277 K and applied to a Resource Q column (GE Healthcare) equilibrated with Buffer C. PI5P4Kβ was eluted with a linear gradient of NaCl up to 400 mM. The fractions corresponding to PI5P4Kβ were collected and concentrated to 20 mg/mL.

PI5P4Kβ was crystallized with the sitting-drop vapor diffusion method by mixing 1.5 μL of PI5P4Kβ solution (20 mg/mL) and 1.5 μL of reservoir solution [100 mM sodium-citrate (pH 5.5), 100 mM magnesium-acetate, 100 mM lithium-acetate, 10% (v/v) PEG-4000] at 277 K; these conditions were optimized based on the earlier crystallizing conditions. Plate-shaped crystals with approximate dimensions of 0.4 × 0.4 × 0.1 mm3 appeared in 3–4 days.

The contents of the artificial mother liquor for PI5P4Kβ [100 mM sodium-citrate (pH 5.5), 100 mM magnesium-acetate, 100 mM lithium-acetate, 7% (v/v) PEG-4000] were determined based on those of the reservoir solutions. The artificial mother liquor was utilized for soaking experiments.

**Snapshot Images and Their Analysis.** To check the resolution of crystals, snapshot diffraction images of the crystals were taken at 95 K using ADSC Quantum CCD detectors on beamlines PF-AR NE3A, PF-AR NW12A, and BL-5A, and Pilatus 2 M (Dectris) on BL-1A of the Photon Factory, KEK (Tsukuba, Japan). The resolution of each snapshot image (hereafter “snapshot resolution”) was determined using method 2 of the program Labelit in the Phenix suite. It is of note that the resolution determined by Labelit is usually lower than that determined by full data collection. For example, a CagA(1–876) crystal showing a snapshot resolution of 4.5 Å typically gives a maximum resolution of ~3.5 Å when using fully collected diffraction data (see below). Because crystals showing poor snapshot images were not utilized for full data collection, the resolution and quality of crystals used in the data collection were somewhat biased (e.g., only good crystals were utilized for data collection). In the statistical analysis, therefore, we corrected for this bias by using snapshot images collected from all the mounted crystals.

**Diffraction Data Collection.** Diffraction data were collected at 95 K using ADSC Quantum CCD detectors on beamlines PF-AR NE3A, PF-AR NW12A, and BL-5A, and Pilatus 2 M (Dectris) on BL-1A of the Photon Factory, KEK (Tsukuba, Japan). Crystals for data collection were selected on the basis of snapshot images. The collected diffraction data were processed and scaled using the programs XDS and XS SCALE, respectively. The maximum resolution of the processed data set was determined using the R-merge and I/σ(I) values at the outmost resolution shell, which should be less than 0.50 and more than 3.0, respectively.

**Results and Discussion**

**Quality of the Initially Obtained CagA(1–876) Crystals.** Crystals of CagA(1–876) were obtained at 293 K
in approximately 5–10 days using the sitting-drop vapor-diffusion method. The dimensions of typical crystals were 0.3 × 0.3 × 0.1 mm³ and the crystals belong to the space group P4₁2₁2. However, the obtained crystals diffracted to no more than 7.5 Å at room temperature, suggesting that they were intrinsically of poor quality. Crystals that were cryoprotected by a glycerol solution [30% (v/v) glycerol, 40% (v/v) ethanol, 50 mM Tris-HCl, pH 9.0] diffracted to only approximately 6.6 Å at 95 K (Figure 1). Therefore, improvement of the CagA(1–876) crystal quality was required to solve the crystal structure.

**Figure 1.** Diffraction image of a CagA(1–876) crystal cryoprotected by a glycerol solution [30% (v/v) glycerol, 40% (v/v) ethanol, 50 mM Tris-HCl, pH 9.0].

**Initial Screening of Cryoprotectants for the CagA(1–876) Crystals.** Screening of suitable cryoprotectants was performed using 20 reagents from the CryoPro kit (Hampton Research) to improve the crystal quality of CagA(1–876) (Table 1). The effect of each cryoprotectant was examined using snapshot diffraction images from several crystals soaked in the cryoprotectant solution under the same conditions, and the snapshot resolution of each image was determined using the program Labelit. The screening of 20 cryoprotectants revealed that CagA(1–876) crystals soaked in the meso-erythritol (hereafter “erythritol”) or THL solution showed better snapshot resolutions than those soaked in the other cryoprotectant solutions (Table 1). Thus, the soaking time and temperature for the THL and erythritol solutions were optimized.

The results using the optimized soaking conditions showed that the soaking time for the THL solution did not affect the crystal quality significantly. While CagA(1–876) crystals soaked in the THL solution [30% (w/v) THL, 20% (v/v) ethanol, 50 mM Tris-HCl pH 9.0] sometimes showed a snapshot resolution better than 4.5 Å, ice-ring patterns could not be removed from the diffraction image, suggesting that the cryo-protection was not sufficient with 30% (w/v) THL.

The optimization experiments revealed that soaking in the erythritol solution [17.5% (w/v) erythritol, 40% (v/v) ethanol, 50 mM Tris-HCl pH 9.0] frequently damaged CagA(1–876) crystals. The CagA(1–876) crystals soaked in the erythritol solution for more than 30 s at 293 K generally showed surface cracks and diffracted to no more than 6 Å resolution. Soaking CagA(1–876) crystals in the erythritol solution at 277 K, however, reduced the crystal damage and the soaked crystal sometimes showed a snapshot resolution better than 4 Å. However, because the quality of the crystal changed rapidly (within about 30 s) in the erythritol solution, it was extremely difficult to consistently obtain high quality crystals using this solution. The best diffraction data showed a maximum resolution of 3.2 Å for the crystals soaked in erythritol, but only 5 of 93 crystals had diffraction data better of than 3.5 Å resolution (Table 2).

**Concept of the Multistep Soaking Method.** The effects of THL and erythritol were marginal and the success rate for obtaining a crystal with a maximum resolution better than 3.5 Å was less than 20%, even when data collections were performed using crystals selected by the snapshot screening (Table 2, Figure 2). To further improve the crystal quality, we developed

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**Table 1. Screening of Cryoprotectants for CagA(1–876)**

<table>
<thead>
<tr>
<th>soaking no.</th>
<th>cryoprotectant</th>
<th>snapshot resolution (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None (293 K)</td>
<td>7.5</td>
</tr>
<tr>
<td>1</td>
<td>Glycerol</td>
<td>6.3</td>
</tr>
<tr>
<td>2</td>
<td>Ethylene glycol</td>
<td>No diffraction</td>
</tr>
<tr>
<td>3</td>
<td>PEG200</td>
<td>No diffraction</td>
</tr>
<tr>
<td>4</td>
<td>PEG400</td>
<td>7.9</td>
</tr>
<tr>
<td>5</td>
<td>PEG600</td>
<td>No diffraction</td>
</tr>
<tr>
<td>6</td>
<td>PEG4000</td>
<td>No diffraction</td>
</tr>
<tr>
<td>7</td>
<td>Polyvinylpyrrolidone K15</td>
<td>No diffraction</td>
</tr>
<tr>
<td>8</td>
<td>MPD</td>
<td>No diffraction</td>
</tr>
<tr>
<td>9</td>
<td>1,6-Hexanediol</td>
<td>No diffraction</td>
</tr>
<tr>
<td>10</td>
<td>1,2-Propanediol</td>
<td>No diffraction</td>
</tr>
<tr>
<td>11</td>
<td>DMSO</td>
<td>No diffraction</td>
</tr>
<tr>
<td>12</td>
<td>2-Propanol</td>
<td>No diffraction</td>
</tr>
<tr>
<td>13</td>
<td>Ethanol</td>
<td>No diffraction</td>
</tr>
<tr>
<td>14</td>
<td>Methanol</td>
<td>No diffraction</td>
</tr>
<tr>
<td>15</td>
<td>D-(+)-Sucrose</td>
<td>6.4</td>
</tr>
<tr>
<td>16</td>
<td>meso-Erythritol</td>
<td>3.9</td>
</tr>
<tr>
<td>17</td>
<td>Xylitol</td>
<td>5.0</td>
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<tr>
<td>18</td>
<td>D-(+)-Raffinose</td>
<td>7.1</td>
</tr>
<tr>
<td>19</td>
<td>D-(+)-Trehalose (THL)</td>
<td>3.9</td>
</tr>
<tr>
<td>20</td>
<td>D-(+)-Glucose</td>
<td>7.8</td>
</tr>
</tbody>
</table>

*The best value of screened crystals is given, when more than one crystal was utilized for the cryoprotectant screening.

**Table 2. Summary of Diffraction Data of CagA Processed by XDS under Various Soaking Conditions**

<table>
<thead>
<tr>
<th>soaking conditions</th>
<th>all crystals</th>
<th>selected crystals for data collection</th>
<th>maximum resolution (Å)</th>
<th>best resolution (in maximum resolution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THL</td>
<td>6.4 ± 1.6 (49)</td>
<td>5.2 ± 1.7 (11)</td>
<td>4.0 ± 0.28 (11)</td>
<td>3.6</td>
</tr>
<tr>
<td>Erythritol</td>
<td>5.6 ± 1.2 (93)</td>
<td>4.5 ± 0.42 (19)</td>
<td>3.7 ± 0.23 (19)</td>
<td>3.2</td>
</tr>
<tr>
<td>THL + Erythritol</td>
<td>5.1 ± 0.97 (40)</td>
<td>4.1 ± 0.21 (7)</td>
<td>3.7 ± 0.17 (7)</td>
<td>3.5</td>
</tr>
<tr>
<td>THL + PEG1000</td>
<td>4.2 ± 0.24 (8)</td>
<td>4.2 ± 0.24 (6)</td>
<td>3.2 ± 0.09 (6)</td>
<td>3.1</td>
</tr>
<tr>
<td>THL + PEG1000</td>
<td>5.3 ± 1.1 (82)</td>
<td>4.6 ± 0.70 (25)</td>
<td>3.6 ± 0.26 (25)</td>
<td>3.3</td>
</tr>
<tr>
<td>THL + PEG4000</td>
<td>4.5 ± 1.0 (60)</td>
<td>4.0 ± 0.19 (11)</td>
<td>3.3 ± 0.11 (11)</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*Standard deviations are used for errors. Values in parentheses are the number of crystals. The wild type and also mutant CagA were included.
a novel soaking method, the multistep soaking method, based on our earlier experiences in the crystal structure analysis of TAF-Iβ.18 The crystal structure of TAF-Iβ showed that THL molecules used as a cryoprotectant specifically bound to TAF-Iβ in the crystal. THL molecules were located at the interface of domains and seemed to stabilize the protein structure of TAF-Iβ. Since protein stabilization in the crystal would contribute to improvement of the crystal quality,24−28 we attempted to utilize cryoprotectants not only as cryoprotectants per se, but also as protein-stabilizing reagents in the post-crystalization treatment. It is noteworthy that protein stabilization could occur in both a direct and indirect manner, and thus that direct and specific interactions between a target protein and a cryoprotectant would not always be required. Further, we considered that the utilization of more than one cryoprotectant would be effective. If the effects of two distinct cryoprotectants on a crystal differ from each other, the effects of various cryoprotectants could be additive. Based on this idea, we attempted to improve the CagA(1–876) crystals by using more than one cryoprotectant.

Crystal Quality Improvement of CagA(1–876) by the Multistep Soaking Method. As a first step in analyzing the multistep soaking method, we examined the combined effect of THL and erythritol on the crystal quality of CagA(1–876). Snapshot resolution, which can be calculated by Labelit,22 was utilized for the analysis. Since the CagA(1–876) crystal was stable only in the THL solution, CagA(1–876) crystals were initially soaked in 4 μL of THL solution [30% (w/v) THL, 20% (v/v) ethanol, 50 mM Tris-HCl, pH 9.0]. Then, 4 μL of the erythritol solution [17.5% (w/v) erythritol, 40% (v/v) ethanol, 50 mM Tris-HCl pH 9.0] was added to the THL solution (Supporting Information Figure S1). When the THL-soaked crystals were transferred to the erythritol solution [17.5% (w/v) erythritol, 40% (v/v) ethanol, 50 mM Tris-HCl pH 9.0], some of them cracked, suggesting that the coexistence of THL and erythritol is critical in the second step. The soaking time in each cryoprotectant solution was optimized, resulting in best soaking times of 14 h for the THL solution and 20 s for the erythritol−THL solution. Interestingly, after soaking in the THL solution more than 30 min, no cracks appeared on the CagA(1–876) crystal during 3 min of soaking in the erythritol−THL solution. A diffraction study of the CagA(1–876) crystals treated with the multistep soaking method showed significantly improved snapshot resolution (Figure 3). While only approximately 10% of the crystals soaked in the THL solution showed snapshot resolution better than 4.5 Å, approximately 35% of the crystals treated by the multistep soaking with THL and erythritol showed snapshot resolution better that 4.5 Å.

Screening of Cryoprotectants for the Second Soaking. We then examined another combination of cryoprotectants in multistep soaking for the CagA(1–876) crystal. Since THL is the only cryoprotectant that can maintain the CagA(1–876) crystal without damage for more than 1 day as described above, the THL solution should be the first cryoprotectant solution. To choose a second cryoprotectant, we screened 13 reagents [{(+)}-sucrose, xylitol, {(+)}-raffinose, {(+)}-glucose, glycerol, ethylene glycol, PEG200, PEG400, PEG1000, PEG2000MME, PEG4000, PEG6000, and PEG8000], and found that PEG400, PEG1000, and PEG4000 were effective as the second cryoprotectant (Table 2, Figure 3). While PEG400, PEG1000, and PEG4000 damaged the CagA(1–876) crystals when used as the first cryoprotectant (Table 1), the CagA(1–876) crystals became tolerant to these cryoprotectants after soaking in the THL solution. These facts suggested that some of the interaction sites for PEG400, PEG4000, and PEG1000

Figure 2. Distribution of maximum resolutions of CagA(1–876) crystals under several soaking conditions. Crystals for diffraction data collection were selected on the basis of snapshot images; only relatively good crystals were used for data collection (Table 2, Figure 3). Average maximum resolutions with standard deviations and the number of crystals used for the experiments are given.

Figure 3. (a) Distribution of snapshot resolutions of CagA(1–876) crystals under several soaking conditions. (b) Statistical analysis of the snapshot-resolution distribution. **p < 0.05 and ****p < 0.0001 by Student’s t-test.
were blocked by THL, which may have been why the crystals became tolerant to these cryoprotectants.

Next, various combinations of THL and these cryoprotectants were tested, and the best combination for the CagA(1–876) crystal was THL and PEG1000. This combination improved not only the snapshot resolution, but also the reproducibility of the diffraction experiments (Figure 3, Table 2). CagA(1–876) crystals that were treated with THL and PEG1000 showed snapshot resolutions better than 4.5 Å resolution with more than 85% probability. We were able to collect the full diffraction data of CagA(1–876) at 3.1 Å resolution using two-step soaking with THL and PEG1000 (Table 2). The combination of THL and PEG1000 showed much better resolution than the use of either THL or PEG1000 individually, suggesting that two cryoprotectants acted synergistically.

**Multistep Soaking Improved the Crystal Quality of PISP4Kβ.** To confirm the effectiveness of the multistep soaking method, we applied this technique to another crystal, and found that the crystal quality of PISP4Kβ was also improved by the multistep soaking method. PISP4Kβ is an emerging target for cancer therapy and controls the levels of the lipid second messenger, PI(5)P.29 Initially, we tested 14 cryoprotectants for PISP4Kβ (glycerol, ethylene glycol, PEG600, PEG4000, 1,6-hexanediol, 1,2-propandiol, DMSO, sucrose, erythritol, xylitol, inositol, trehalose, glucose, and PVP). Two of these, ethylene glycol and PVP, were relatively good for cryoprotection, but the fraction of crystals diffracting to better than 3 Å (snapshot resolution) was less than 15%. Moreover, the crystal soaked in PVP solution showed ice rings, indicating that further cryoprotection was required. The ethylene glycol solution damaged PISP4Kβ, forming several cracks on the surface of the crystals. We therefore utilized both PVP and ethylene glycol and optimized the conditions for multistep soaking. The best result was obtained by two-step soaking using PVP and ethylene glycol. First, a PISP4Kβ crystal was soaked in the PVP solution [25% PVP, 7% (w/v) PEG4000, 100 mM magnesium acetate, 100 mM sodium citrate pH 5.5] for 24 h and then the ethylene glycol solution [30% ethylene glycol, 7% (w/v) PEG4000, 100 mM magnesium acetate, 100 mM sodium citrate pH 5.5] was added to the PVP solution. After 20 s of soaking, the crystal was mounted on a cryo-loop and flash-cooled by liquid nitrogen. As shown in Figure 4, the multistep soaking significantly improved the resolution of PISP4Kβ. Notably, soaking with a single cryoprotectant solution, i.e., PVP or ethylene glycol, did not realize a clear improvement in crystal quality (Figure 4). The combination of the two cryoprotectants was required to improve the crystal quality of PISP4Kβ, suggesting that the two cryoprotectants acted synergistically.

**CONCLUSION**

We demonstrated that the multistep soaking method is effective for improving crystal quality. Importantly, the effect of multistep soaking was much greater than the additive effects of soaking in the constituent cryoprotectants. Even when either of two cryoprotectants showed only a marginal effect on a crystal, the combination of the two (or sometimes more) cryoprotectants achieved a significant improvement in crystal quality. This study provided two examples of crystals whose quality was improved by the multistep soaking method, CagA(1–876) and PISP4Kβ, along with the relevant statistical analyses. We also observed other cases of substantial crystal quality improvement by the multistep soaking method, CagA(261–829), BphA4, and so forth. The multistep soaking should thus be an effective protocol for improving the crystal quality.

Here, we propose the following as a general protocol for the multistep soaking method. First, the screening of cryoprotectants should be performed as usual. A cryoprotectant solution(s) that can stably maintain the crystal of the target protein should be selected in the first screening, even when the effect of the cryoprotectant is marginal. If the crystal quality is not significantly improved at this stage, a second cryoprotectant should be screened. For the second step, a protectant solution can be added to the first cryoprotectant solution containing the crystals (Supporting Information Figure S1). All available cryoprotectants should be examined for potential use in the second soaking, even if they showed no positive effects at the initial screening. Since the order of the post-crystallization treatment is generally critical,18 the order of the cryoprotectant solutions should be examined in the multistep soaking method. The soaking time and temperature should also be optimized for each cryoprotectant.
**Crystal Growth & Design**

**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.cgd.5b01692.

- Diffraction image of CagA(1−876) cryoprotected with glycerol and distribution of maximum resolutions of CagA(1−876) crystals (PDF)

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Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This study was supported in part by JST, CREST, and by JSPS KAKENHI Grant Number 24657079.

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