Supporting Information

Precipitant-free lysozyme crystals grown by centrifugal concentration reveal structural changes

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1. Materials and Methods

Samples

Hen egg-white lysozyme (HEWL) (Seikagaku Co., Tokyo; 6 times recrystallized) was used as a model protein without further purification. This grade of HEWL contains 1.5% impurity materials, and the major component is a covalently bound HEWL dimer. Distilled water was further deionized and filtered with an ultra-pure water production apparatus (Simpli-Lab, Merck Millipore, Darmstadt, Germany; resistivity > 18.2 MΩcm). HEWL shows the best activity at 0.0 M NaCl (*6*), and its activity monotonically decreases with the NaCl concentration; in the precipitant-free condition, the 3D structure of a HEWL molecule should be that of a native protein.

Glucose isomerase (GI) (Spezyme® Gipf, Nagase Chemical, Osaka, Japan) was used for the applicability confirmation. This sample contains sorbitol for the inhibition of enzymatic activities of GI molecules.

Apparatus

The desalination and concentration of HEWL molecules were performed using a centrifugal concentrator (Ultracel 3K, Amicon Ultra-15 centrifugal filter units, Merck Millipore, Cork, Ireland). First, 0.6 g of HEWL was dissolved in approx. 15 mL of ultra-pure water in the filter unit. A pair of dissolved samples was set in a high-speed and cooled centrifuge (6200, Kubota, Tokyo). Centrifugal concentration was conducted at 7500 g for 40 min, after which the volume of residual solution became approx. 1 mL. After the addition of approx. 14 mL of ultra-pure water, the above deionization and concentration procedures were repeated. This centrifugal concentration process from 15 mL to 1 mL was repeated five times in total.

In the case of GI molecules, the centrifugal concentration and dilution with 30% aqueous glycerol solution was repeated five times for the removal of the sorbitol and anti-freezing solvent was substituted for the water for the purpose of flash cooling followed by X-ray diffraction data collection.

Xray diffraction data collection

The obtained HEWL crystals were washed with 30% aqueous glycerol solution, picked up with a nylon loop, and flash-cooled. Roughly 0.2 mm-sized crystals were set on a goniometer on the BL-5A beamline at the Photon Factory of the High Energy Accelerator Research Organization (KEK-PF, Tsukuba, Japan). Data were measured using a CCD detector (ADSC Quantum 315r, Area Detector Systems, Poway, CA) and were processed using the software program HKL-2000.

GI crystals grown in 30% aqueous glycerol solution were picked up directly and flash-cooled. Roughly 0.5 mm-sized crystals were used for data collection. Data were collected using an in-house X-ray generator (rotating anode Rigaku FR-E+ with an R-AXIS VII detector) at a maximum power of 2025 W at the Institute of Advanced Medical Sciences, Tokushima University (Tokushima, Japan). The camera length *d* was equal to 220 mm.

Structural determination and refinement

The structure of HEWL was determined by molecular replacement using the program Molrep from Collaborative Computational Project Number 4 software for macromolecular X-ray crystallography graphical user interface (CCP4i)[1] with the structure of lysozyme (Protein Data Bank (PDB) entry 2ZQ3) as a model. In this model, a sodium ion was detected as a part of the

unit cell. The refinement was carried out using Refmac5 from the CCP4i and manually fitted with winCoot0.7. [2] Using PDB_REDO 7.09, we finally refined the model until R_{value} = 0.173 and R_{free} = 0.213. The structure was released in the PDB (PDB ID 5YIN).

2. Fig. S1

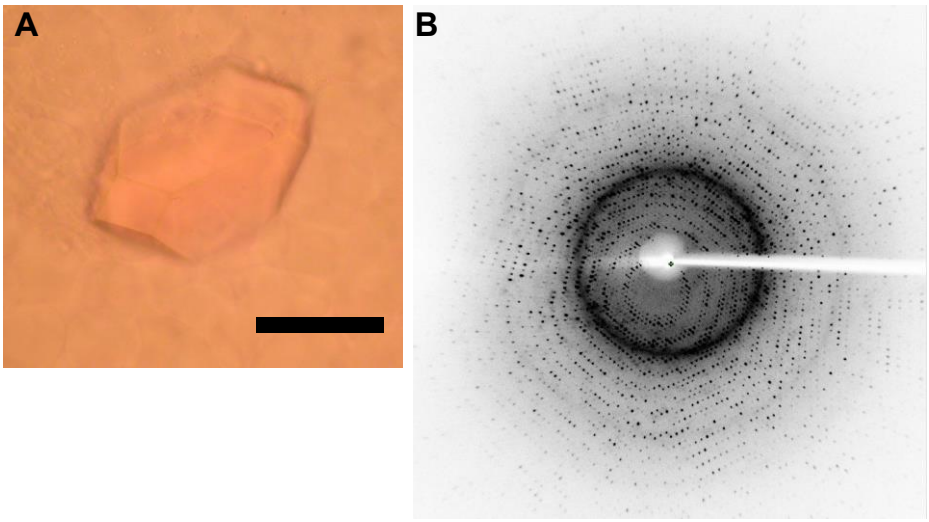

Fig. S1. A precipitant-free glucose isomerase crystal obtained by centrifugal concentration and typical diffraction spots of the crystal.

(A) A crystal nucleated in the dense liquid phase which was separated during the centrifugal concentration of an aqueous glucose isomerase solution. The crystal is surrounded by planar surfaces. Scale bar: 100 μm. (B) An oscillation photograph of a crystal. The oscillation angle is 1°, and the exposure time was 1 min.

3. Table S1

Table S1 Data collection statistics.

Data collection statistics of the precipitant-free HEWL crystal.

Beamline	KEK-PF-BL5A
Detector	ADSC Quantum 315r
Wave length	1.0000 Å
Space group	$P2_12_12_1$ (a = 30.49 Å, b = 58.04 Å, c = 67.33 Å)
Resolution range	50.00 Å – 1.65 Å
No of reflections (Unique)	103142 (14851)
Completeness	98.2%
Total linear R_{merge}	0.041
Mean I/σ	54.2
Mosaicity	0.45 – 0.55

4. Captions for Movies S1 and S2

Movie S1. Structure of HEWL of a salted-out crystal (PDB ID = 2ZQ3) and precipitant-free crystal (this study).

Whole backbone atoms of a HEWL molecule of the salted-out crystal and precipitant-free crystal are shown as stick models alternately. The model of the salted-out crystal (2ZQ3) is shown as light green sticks with its activation site residues as red sticks, and the model of the precipitant-free crystal (this study) is shown as light blue sticks. The sodium ion located in the salted-out crystal disappears in the precipitant-free crystal. Asp^{101} flips significantly, whereas the positions of other important residues (Glu^{35}, Asp^{52}, Trp^{62}, Trp^{63}, and Trp^{108}) in the active site remain almost unchanged.

Movie S2. Tandem flips between side chains of Asp^{101} and Asn^{103}.

Backbone atoms around Asp^{101} and Asn^{103} of a HEWL molecule of the salted-out crystal and precipitant-free crystal are shown as stick models. The model of the salted-out crystal (2ZQ3) is shown as light green sticks, and the model of the precipitant-free crystal (this study) is shown as light blue sticks. Asp^{101} and Asn^{103} are shown as red and blue sticks. Side chains of Asp^{101} and Asn^{103} are separated maximally in the salted-out crystal, whereas they approach each other in the precipitant-free crystal.

5. Rough Estimates of Crystallization Conditions

For the readers' convenience, rough estimates were done. For instance, when concentration in a dilute phase was 96 $mgmL^{-1}$ and that in a dense phase was 296 $mgmL^{-1}$, that in a gel-like part was roughly calculated to be around 550 ~ 650 $mgmL^{-1}$. Although crystallization was started in the gel-like part, the dense phase could be used as a supersaturated solution, also. Additional separate experiments were done to confirm whether nucleation occurs in the dense phase or not. At 25 °C, 352 and 250 $mgmL^{-1}$ dense HEWL aqueous solutions started nucleation in a day and two weeks, respectively. Between these concentrations, we could find an appropriate condition for obtaining high quality crystals reproducibly.

6. References

1. Collaborative Computational Project, Number 4. The CCP4 suite: programs for protein crystallography. *Acta Cryst. D***50**, 760-763, (1994).
2. P. Emsley, B. Lohkamp, W. G. Scott, and K. Cowtan, *Acta Cryst. D***66**, 486-501, (2010).