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

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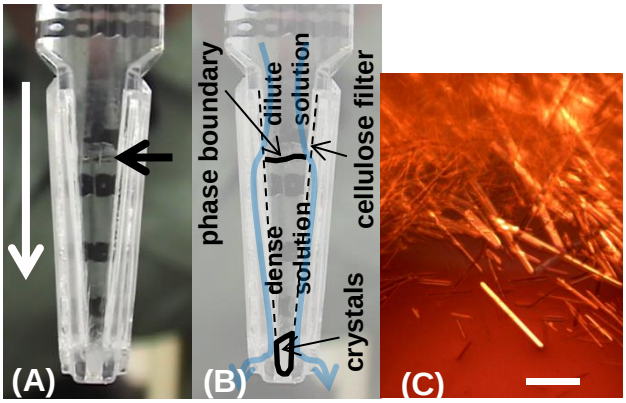
The three-dimensional (3D) structure of a protein molecule in its crystal need not correspond to that found *in vivo* in many cases, since we usually crystallize protein molecules using precipitants (salts, organic solvents, polymeric electrolytes, etc.), and the precipitants are often incorporated into crystals along with the protein molecules. Although precipitant-free crystallization methods would solve these problems, such methods had not yet been established. We have achieved a novel precipitant-free crystallization method by liquid-liquid phase separation during the centrifugal concentration of lysozyme in ultra-pure water. In the 3D structure of the precipitant-free crystal, lysozyme loses a sodium cation and changes the position of Ser 72. Deionization of the solution also appears to induce a change in the position of Asp 101 and an increase in the activity of lysozyme.



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

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precipitant-free, lysozyme, centrifugal concentration, synchrotron x-ray crystallography

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incorporated into crystals along with the protein molecules. Although precipitant-free crystallization methods would solve these problems, such methods had not yet been established. We have achieved a novel precipitant-free crystallization method by liquid-liquid phase separation during the centrifugal concentration of lysozyme in ultra-pure water. In the 3D structure of the precipitant-free crystal, lysozyme loses a sodium cation and changes the position of Ser 72. Deionization of the solution also appears to induce a change in the position of Asp 101 and an increase in the activity of lysozyme.

Structure-based drug design (SBDD) is defined as a method to optimize the potency of a drug using the precise structure information of a target protein,[1] and this is one of the most important methods used to design and synthesize newly-developed drugs such as HIV protease inhibitors, influenza neuraminidase inhibitors, and more.[2,3] Although SBDD often requires high-quality crystals that can be analyzed at high resolution (< 1.5 Å) in order to minimize the difficulty of determining the structure of target proteins,[4] the Protein Data Bank (PDB) reported that less than 10 percent (10798 structures of 123784 as of February 28, 2018) of structures revealed by X-ray analysis showed their X-ray resolution < 1.5 Å.[5] Furthermore, strictly speaking, almost all structural entries in the PDB do not represent their *in vivo* structures, since these crystals were grown with a large amount of precipitants (salts, polymeric electrolytes, organic solvents and so on). The precipitants almost certainly will affect the structure of proteins significantly, since, for example, lysozyme shows its best activity in a precipitant-free condition, and its activity is strongly inhibited at high NaCl concenrations.[6] Such a significant change in structures would result in changes in the positions of molecules larger than 1.5 Å.

If we can crystallize proteins without using any precipitants, such potential problems will be eliminated, because we can freely set precipitant concentrations from 0 to an arbitrary value in crystallization processes. As the first achievement of a precipitant-free method, Pitts crystallized aspartic proteinase by centrifugation without using precipitants in 1992.[7] Although he showed the picture of a crystal obtained in this way and a diffraction pattern at 2.3 Å resolution, the electron density map of the three-dimensional (3D) structure of aspartic proteinase was not shown and the crystal growth processes were not detailed. Retailleau et al. showed very high solubility data at 0.0 M NaCl.[8] Although this possibly means that they could crystallize lysozyme without using precipitants, they did not reveal the precise crystal growth processes at 0.0 M NaCl and did not provide information on crystal forms.

Here we present a novel desalination and condensation technique that uses a centrifugal filtration apparatus with which we successfully separated a stable dense liquid phase of hen egg-white lysozyme (HEWL). We then discuss the nucleation and growth of orthorhombic crystals (precipitant-free crystals) in the dense liquid phase. Finally, the results of X-ray crystallography of the crystals are discussed in comparison with the reported structure of orthorhombic HEWL crystals which were prepared by a conventional salting-out method (salted-out crystals).

During the centrifugal concentration processes, stable liquid-liquid (L-L) phase separation into dense and dilute phases occurred (Figures 1A, B). Although the L-L phase separation itself has been studied by many researchers,[9-11] and Taratuta et al. also succeeded in separating a bulky dense phase,[10] all of the solutions used in the previous studies contained salts. In the bottom-most part of the dense phase, a gel-like and transparent phase existed. The gel-like phase could not be pipetted easily, and the average concentration of residual solution in the dense phase was 223 ± 4 mgmL^{-1}; it appears that the gel-like phase contains higher-density HEWL molecules. At

T = 298 K, the bottom part of the dense liquid phase became opaque after several hours, and many needle-like crystals were observed with the use of a transmitted light microscope (Figure 1C). The density and viscosity in the bottom part of the dense phase were extraordinarily high, and the crystals nucleated in that part. Therefore, the growth process in the dense phase should be no longer normal solution growth-like (from dilute environmental phases) but rather is melt growth-like or solid phase growth-like (from dense environmental phases). The crystals show a faceted outer shape, which grew in a lateral (layer by layer) manner.

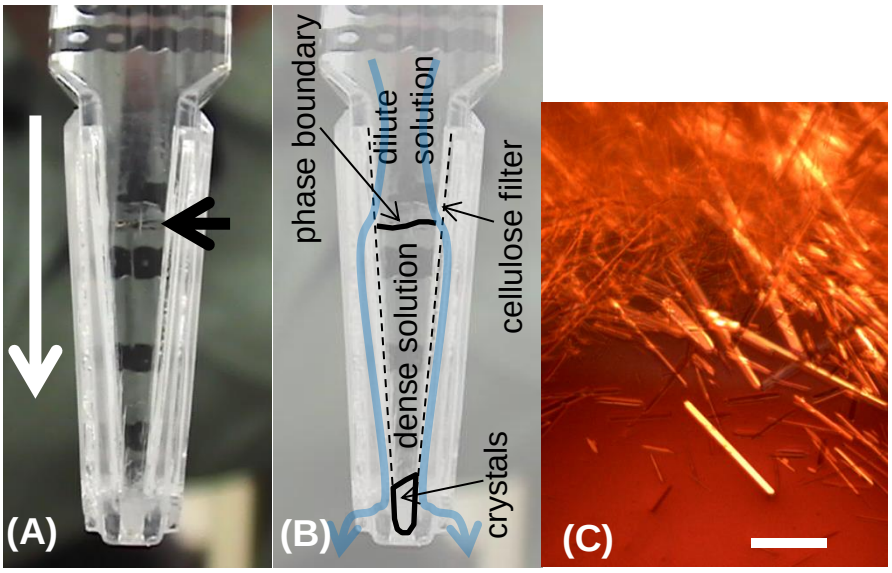

Figure 1. Liquid-liquid phase separation in an aqueous lysozyme solution and crystallization in the dense phase. (A) A filtering unit of the centrifugal concentration apparatus and separated liquid phases. The white arrow represents the direction of centrifugal acceleration. The white opaque region at the bottom of the unit contains crystals. The phase boundary between the dense and dilute solutions is denoted by a black arrow. (B) Details of panel A are shown. Broken lines show the positions of cellulose filters. Curved blue arrows schematically show the paths of water through the filters. (C) Crystals which were nucleated at the bottom of the filtering unit after a

liquid-liquid phase separation by centrifugal concentration. In fact, at the very bottom-most of the dense liquid phase, a gel-like transparent amorphous substance was present in which the crystals nucleated. Each crystal is surrounded by planar surfaces. Scale bar: 100 μm.

Sufficiently clear (up to the resolution limit of 1.65 Å) diffraction spots in an oscillation photo were obtained using a crystal obtained in this way (Fig. 2). The data collection statistics are summarized in Table S1. The comparison of a specific site of a HEWL molecule in the

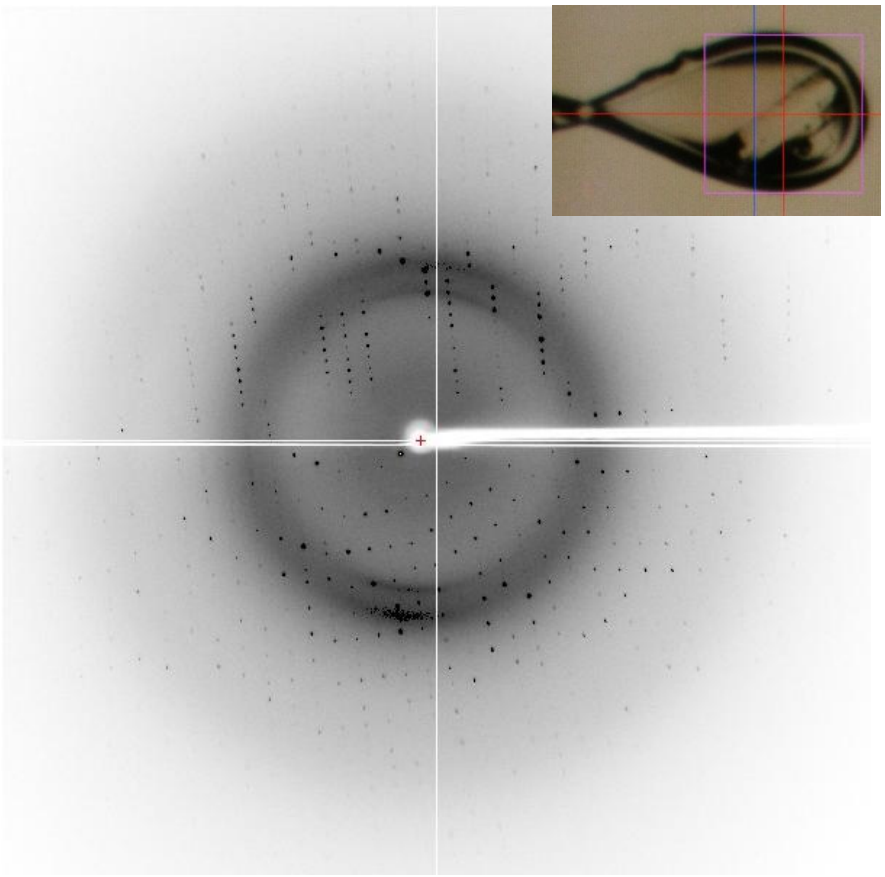

Figure 2. Typical diffraction spots of a crystal used in this study. An oscillation photograph of a crystal is presented. The oscillation angle is 1°, and the exposure time was 1s. Inset: A crystal in a 30% aqueous glycerol solution trapped in a nylon loop that had been flash-cooled for data collection. The width of the pink square window shown in this inset represents 0.2 mm.

precipitant-free crystal (PDB ID = 5YIN) with that of the salted-out crystal (PDB ID = 2ZQ3) is summarized in Figure 3. The root mean square deviation (defined as the square root of the mean of the square of the distances between matched atoms) between the 3D backbone structure in the precipitant-free and that in the salted-out lysozyme crystal is calculated to be 0.31 Å using PyMol. The left and right columns in Figure 3 show the same position from different viewpoints.

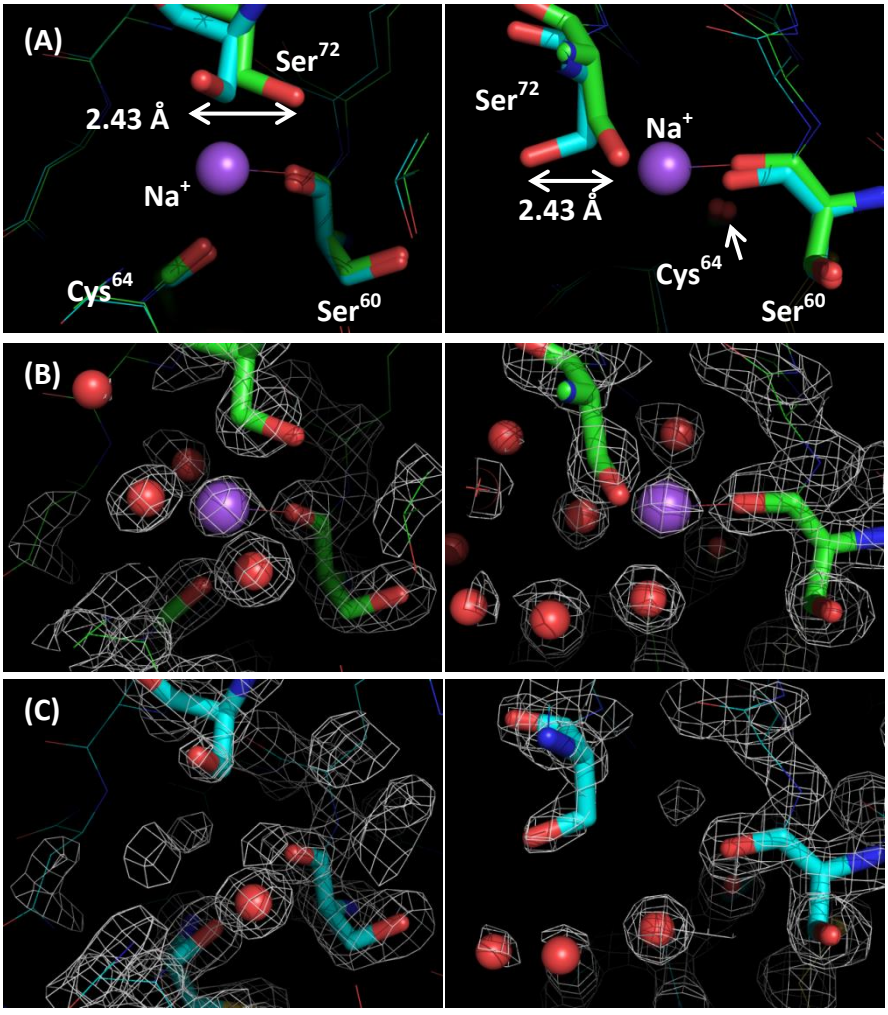

Figure 3. Positions of residues of HEWL molecules and water molecules around a specific site at which a sodium ion is detected in the salted-out crystal. The upper and lower raws show the same pictures at the same position from different viewpoints. (A) A superimposition of HEWL

molecules in the salted-out crystal (light green sticks) and the precipitant-free crystal (light blue sticks) around a sodium ion (violet spheres) site. (B) Pictures at the same site in the salted-out model are shown with electron density maps ($2mF_o$ - DF_c maps contoured at 2.0 σ) and the oxygen atoms (red spheres) of water molecules. Three oxygen atoms of water molecules, two oxygen atoms of the hydroxyl group of Ser72 side chains and one oxygen atom of the carbonyl group of the Cys64 main chain form an octahedral molecular geometry around the sodium ion. (C) Pictures at the same site in the precipitant-free crystal used in this study are shown with electron density maps ($2mF_o$ - DF_c maps contoured at 2.0 σ) and oxygen atoms of water molecules. The sodium ion has clearly disappeared. The direction of the hydroxyl group of Ser72 is completely different from that shown in panel B, since the electron density meshes clearly align the molecular backbones of both the model and the results of this study.

Figure 3A is composed of two superimposed images of the main residues (Ser60, Cys64, and Ser72, which are shown as light green sticks) with a sodium ion in the salted-out model and those (light blue sticks) without the sodium ion in the precipitant-free crystal used in this study. Among these three residues, in particular, the direction of the hydroxyl group of Ser72 in the precipitant-free crystal was clearly different from that in the salted-out crystal. This is probably due to the disappearance of an ion-dipole interaction between the hydroxyl group of Ser72 and the sodium ion. To confirm whether or not this change is significant, we superimposed electron density maps on the backbone structures of the salted-out crystal and the precipitant-free crystal (Fig. 3B). We can clearly confirm that a sodium ion in the salted-out crystal is surrounded by three oxygen atoms of water molecules, two oxygen atoms of the hydroxyl group of Ser side chains and one oxygen atom of the carbonyl group of the Cys64 in the main chain. These oxygen atoms form an octahedral molecular geometry around the sodium ion. The bond lengths suggest

that there are dipole-ion interactions between six oxygen atoms and a sodium cation due to their bond lengths. On the other hand, as shown in Figure 3C, the sodium ion has disappeared, since the centrifugal desalination of the HEWL solution with ultra-pure water was repeated more than five times before crystallization. Two water molecules are also excluded from this site. The oxygen atom of the hydroxyl group of Ser72 clearly moves 2.43 Å, as shown in Figure 3A, since the length of the C-O bond of the hydroxyl group is 1.40 Å, and the C-O bond is rotated around its carbon atom. This change is presumably due to the disappearance of the above ion-dipole attractive interactions. Although Ser72 is not the part of the active site of lysozyme, the change in the position of the oxygen atom is clearly > 1.5 Å and cannot meet the criterion of SBDD.

Other parts of the HEWL molecule are also modified in the precipitant-free crystal (Movie S1). Although no drastic changes in the overall 3D molecular structure were observed, the structure in the precipitant-free crystal seemed to shrink a bit as a whole, and several residues at water accessible surfaces seem to move significantly. In particular, the Asp101 residue clearly flipped in tandem with the flip of Asn103 (Figure 4 and Movie S2). Asp101 is known to be an important residue that interacts with substrate molecules at the active site of lysozyme, whereas the other much more important catalytic center residues (Glu35 and Asp52) and the other important residues (Trp62, Trp63, and Trp108) in the active site do not change their positions significantly[12] while at the same time, the activity of lysozyme is inhibited at high NaCl concentrations.[6] Thus the inhibition is probably due to the change in the position of Asp101. Why does only Asp101 flip significantly? As shown in Movie S2, Asp101 flips in tandem with the flip of Asn103. Both Asp101 and Asn103 are located on the surface of a lysozyme molecule and are easily accessed by water molecules, cations, and anions in a solution. Therefore, they are

probably influenced by a change in the solution properties, since Asp101 and Asn103 are acidic and polar residues, respectively. An electron-density map between the side chains of Asp101 and

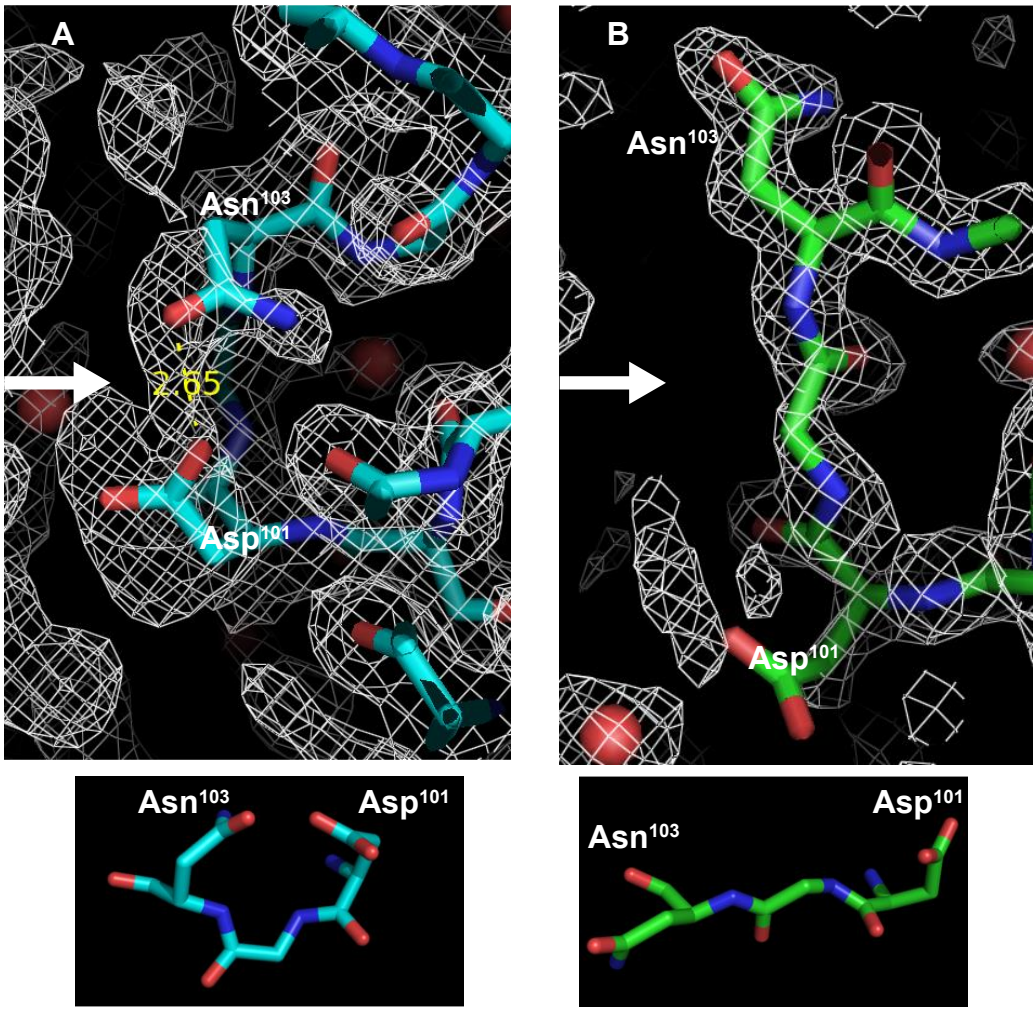

Figure 4. Electron-density maps between side chains of Asp101 and Asn103. Backbone atoms as sticks and an electron density map as meshes around Asp101 and Asn103 of HEWL molecules of the precipitant-free (A) and salted-out (B) crystals are shown. Smaller figures indicate selected backbone atoms (from Asp101 to Asn103) in the upper maps. White arrows indicate directions of side views of the backbone atoms. 2mF_o - DF_c maps shown in A are contoured at 0.5 σ, and those shown in B are contoured at 1.0 σ. An oxygen atom (red stick) of the carboxyl group of Asp101 and oxygen atom (red stick) of the amide group of Asn103

approach together in the precipitant-free crystal. The distance between these atoms is 2.65 Å. This is classified as moderate hydrogen bond length (2.5 ~ 3.2 Å);[13] there would be a hydrogen bond between the side chains. In the case of salted-out crystals, these atoms are completely separated from each other (B).

Asn103 of a HEWL molecule of the precipitant-free crystal is shown in Figure 4A. The distance between an oxygen atom of the carboxyl group of Asp101 and the oxygen atom of the amide group of Asn103 is 2.65 Å. This is classified as a moderate hydrogen bond length (2.5 ~ 3.2 Å);[13] there would be a hydrogen bond between these side chains (O-H···O). On the other hand, in the case of the salted-out crystals, these atoms are completely separated from each other (Figure 4B). Large amounts of sodium cations probably shield negatively charged oxygen atoms of the carboxyl group of Asp101; the hydrogen bond between the above two oxygen atoms of the precipitant-free crystals would be broken by electrostatic shielding of charged residues of the salted-out crystals containing large amounts of cations. This indicates that changes in solution properties owing to the removal of precipitants would affect the structure of the active site significantly; the change in the distance between Asp101 and Asn103 is clearly > 1.5 Å and cannot meet the criterion of SBDD.

The applicability of our precipitant-free crystallization method to other proteins should be confirmed for a generalization of the method. In fact, we have succeeded in crystallizing glucose isomerase (GI) by centrifugal concentration (Figure S1A). As observed in the HEWL system, liquid-liquid phase separation also occurred. The nucleation and growth of GI crystals occurred

in the dense liquid phase. An oscillation photograph clearly shows that the quality of the obtained crystal is sufficiently high for structure analysis at the atomic level (Figure S1B).

In conclusion, we have designed a novel desalination and condensation technique that uses a centrifugal filtration apparatus with which we successfully separated a stable dense liquid phase of HEWL and obtained precipitant-free HEWL crystals there. Using this technique, we can freely set precipitant concentrations from 0 to an arbitrary value, including in vivo conditions, in protein crystallization processes. Synchrotron X-ray crystallography of the precipitant-free crystals revealed significant structural changes. The oxygen atom of the hydroxyl group of Ser72 moves 2.43 Å, since a sodium ion which interacted with Ser72 in salted-out crystals disappeared. Asp101 clearly flipped in tandem with the flip of Asn103. The flip would result in the increase in the activity of HEWL at low NaCl concentrations,[6] since Asp101 directly interact with substrate molecules at the active site.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Materials and methods, an image of a precipitant-free GI crystal and its oscillation photograph (Fig. S1), X-ray data collection statistics (Table S1), captions for movies S1 and S2, and Rough estimate of crystallization conditions (PDF)

Whole backbone atoms of the salted-out crystal and precipitant-free crystal (Movie S1), tandem flips between side chains (Movie S2) (AVI)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SBDD, structure-based drug design; HEWL, hen egg-white lysozyme; GI, glucose isomerase.

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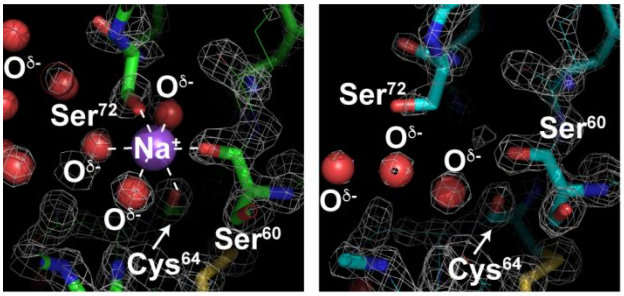

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