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Title

Cross-linking controls the mechanical properties of protein crystals

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Abstract

Mechanical properties such as plasticity of materials are fundamental and important properties in the processing and applications. Protein crystals are one of the macroscopic molecular crystals composed of the protein molecules with nanometer size. It has been explored the application of protein crystals not only for the structure analysis of proteins, but also for the application as solids. Generally, it is known that native protein crystals

are quite brittle. On the other hand, it is empirically known that the mechanical property of protein crystals is enhanced by a cross-linking technique. One of qualitative reasons is due to that the chemical cross-linking enhances the mechanical strength but the detailed mechanical properties are not clear. Herein, we investigated the macroscopic elastic and plastic behaviours of cross-linked tetragonal hen-egg-white lysozyme crystals under compression test. The cross-linked crystals exhibited plastic deformation whereas the native crystals showed brittle fracture. The plastic behaviour of the cross-linked crystals clearly showed yield phenomena with upper and lower yield points. Stress-induced dislocations were also noted. The Burgers vectors of the moving dislocations of the crystals were characterised by synchrotron X-ray topography, and the results indicated that the slip systems were controlled by cross-linking. Thus, cross-linking leads to the macroscopic plastic deformation of protein crystals. The ductile properties of the cross-linked protein crystals indicate their great potential for material applications such as biocatalysis and bio-sensing.

I. Introduction

Plasticity and ductility are fundamental and important properties in the processing and applications of various materials. The plastic deformation of crystalline materials is caused by dislocation multiplication and motion which have been widely investigated for many metals and semiconductor crystals [1]. Studying the dislocation behaviours of crystalline materials is important to understand their mechanical properties.

Protein crystals are solid materials composed of biomacromolecules. Obtaining single protein crystals is crucial for understanding the structure of protein molecules. The

1 molecular size of protein crystals is in the order of nanometres; thus, a large
2 intermolecular distance and pore size of over 10 nm distinguish these materials from
3 common inorganic and low-molecular organic crystals. In addition, protein crystals
4 contain large amounts of intracrystalline water (~70 vol.%) [2,3]. Thus, the presence of
5 porosity within protein crystals enables the introduction of additives to bring out new
6 material properties. The application of such porous structures holds promise for the
7 development of new biomaterials. Therefore, the application of protein crystals has been
8 explored not only for the structure analysis of proteins, but also for the application as the
9 biomaterials [4].

11 The mechanical properties of protein crystals, such as their elasticity and plasticity, have
12 been evaluated so far. Native protein crystals are generally fragile [5] and their hardness
13 is sensitive to environmental conditions such as humidity [6] because these crystals are
14 crystallised by weak interactions, such as van der Waals forces, electrostatic forces, and
15 the hydrogen bonds associated with intracrystalline water. Recently, it has been reported
16 that crystallisation with additives such as gel media is useful to improve the fragility of
17 these crystals [7]. The fracture stress of a crystal grown with 1.0 wt.% gel (2.12 MPa) is
18 approximately 10 times larger than that of native crystals (0.29 MPa) [7]. Additives can
19 also drastically improve the fracture stress of these crystals.

21 Cross-linking method has been much attracted for enhancing the strength of protein
22 crystals. Cross-linked protein crystals are empirically strong and can be handled directly
23 by mechanical contact without breakage [8]. Moreover, they are usually stable and

1 insoluble in water and organic solutions [9]. In the case of crystals comprised of atoms,
2 such as metals and inorganic materials, the precise modulation of inter-site bonding forces
3 within the established crystal lattice structure presents a significant challenge. In contrast,
4 protein crystals exhibit a unique capacity to exert control over these bonding forces
5 through the strategic introduction of covalent bonds facilitated by cross-linking between
6 lattice sites. This feature can be regarded as a significant characteristic inherent to protein
7 crystals with inherent porosity. Cross-linking forms covalent bonds between the amino
8 acid residues of protein molecules [10]. Glutaraldehyde is a commonly used cross-linker
9 because of its ease of handling and high efficacy.

10
11 In pioneering research, the mechanical properties, such as Young's modulus, of cross-
12 linked triclinic hen-egg-white lysozyme (HEWL) crystals was estimated to be 210 MPa
13 by using transverse resonance vibrations in the frequency range of 1–100 kHz [11].
14 Dynamic Young's moduli of 3.21 and 0.58–3.20 GPa in ultra-high frequency (MHz and
15 GHz) regions have been measured in tetragonal HEWL (T-HEWL) crystals by using
16 ultrasonic pulse echo and the Brillouin scattering method, respectively [12,13,14]. The
17 microscopic mechanical properties of cross-linked T-HEWL crystals have been
18 investigated using the nanoindentation method [9,15]. The macroscopic mechanical
19 properties of cross-linked protein crystals must be characterised to obtain a
20 comprehensive understanding of these crystals and expand their applications. However,
21 to the best of our knowledge, no reports on such characterisations have yet been published.

22
23 Herein, we investigated the macroscopic elastic and plastic behaviours of cross-linked T-
24 HEWL crystals using an Instron autograph instrument. The results revealed that the cross-

linked protein crystals had noticeably improved mechanical properties, which transitioned from brittle to ductile characteristics owing to cross-linking among the protein molecules. To the best of our knowledge, this phenomenon has not been observed in any other material, although the brittle–ductile transition owing to temperature changes has been observed. In addition, the plastic behaviour of the crystals exhibited yield phenomena with upper and lower yield points. The appearance of a slip line suggests that plastic deformation is controlled by a dislocation mechanism. The slip plane and Burgers vector of the slip dislocation were determined using synchrotron X-ray topography. Macroscopic plastic deformation occurred even in brittle protein crystals because of cross-linking.

II. Experimental methods

A. Preparation of the specimens

Once-crystallised HEWL was purchased from Nacalai Tesque, Inc. and used without further purification. T-HEWL crystals ($P4_32_12$, $a = b = 79.1 \text{ \AA}$, $c = 37.9 \text{ \AA}$, $Z = 8$) were grown via a salt-concentration gradient method at 296 K in test tubes held vertically using NiCl_2 as a precipitant [16]. Almost all crystals exhibited similar growth planes, such as the $\{110\}$ and $\{101\}$ planes. The crystal size was approximately 2–4 mm.

Chemical cross-linking was performed by soaking. Glutaraldehyde was purchased from Wako Pure Chemical Industries, Ltd. and used without further purification. The chemical cross-linking solution was prepared by adding 2.5 wt.% glutaraldehyde to a solution containing the same precipitant (11.6 wt.% NiCl_2) as the original solution used for

crystallisation. The cross-linked crystals were obtained by controlling the cross-linking time. Native crystals were immersed in this solution at 296 K for 1–7 day. The lattice parameters of cross-linked HEWL crystals are slightly shorter than those of native ones [17]. For comparison, the lattice parameters of both types of crystals are summarised in Table 1. The crystal size was measured using an optical microscope. The contact areas and heights of the specimens were measured using optical micrographs and ImageJ software [18].

B. Compression test

Compression tests were performed on an Instron autograph machine (SHIMADZU, AGS-X) at 296 ± 2 K. The crystals were covered with the precipitant solution to prevent water evaporation and placed on the stage of the instrument. The crystals were compressed using two parallel stainless-steel plates, one with a load cell attached and the other with a sample stage, at a compression rate of 50 $\mu\text{m}/\text{min}$. The strain rate was $4 \pm 2 \times 10^{-4} \text{ s}^{-1}$ because the compression tests were conducted on grown crystals with heights of 1.5–3.5 mm. During the measurements, the compressive plane and axis corresponded to the (110) and $[\bar{1}\bar{1}0]$ planes, respectively.

C. Characterisation of dislocations by X-ray topography

Synchrotron X-ray topography with a monochromatic beam was performed at 296 K on the BL20B beamline at the Photon Factory of the High-Energy Accelerator Research Organization (KEK). A monochromatic beam of 1.2 $\text{\AA}$ was selected by adjusting the double-crystal monochromator. The crystals were gently transferred into a transparent polypropylene straw. The evaporation of the water contained in the crystal was avoided

by covering it with the precipitant solution, and both sides of the straw were sealed with Parafilm. The sealed straw was mounted on a precision goniometer and rotated in high-resolution angular steps around the Bragg diffraction angle. A series of diffraction images were collected using a high-spatial-resolution 2D digital X-ray CCD camera (Photonics Science X-RAY FDI 1.00:1, effective pixel size = $6.45 \times 6.45 \mu\text{m}^2$) with a total exposure time of several minutes. The CCD camera outputs sequential X-ray topographs as 16-bit greyscale TIFFs. These images are easily analysed using free software such as *ImageJ* [18]. *ImageJ* software can draw three stack-type images as Z-projections. The dislocation images were reconstructed using the median value of the diffraction intensity, as reported previously [19]. X-ray topographs with a spatial resolution of $2 \mu\text{m}$ were obtained using X-ray films (STRUCTURIX D2, Agfa) with an exposure time of 180 s. X-ray topography is a powerful tool for identifying crystal defects, such as dislocations.

III. Results and discussion

A. *Stress–strain curves of native and cross-linked protein crystals*

Fig. 1(a) and (b) show the stress–strain curves of native and one-week-cross-linked T-HEWL crystals obtained under compression. Here, stress and strain refer to the nominal stress and strain, respectively. Stress and strain showed a linear relationship in the curves of both the native and one-week cross-linked crystals. This linear region corresponds to elastic deformation. Red lines on Fig. 1 (a) and (b) were extrapolated from the slope of the elastic region.

We assessed the elastic and plastic regions of the stress–strain curves. The static Young's

modulus of the crystals was estimated from the slope of the linear region of their stress–strain curves. The Young’s moduli of the native and one-week-cross-linked crystals were 67.5 ± 22.6 and 379 ± 80.6 MPa, respectively. The static Young’s modulus of the native crystals was comparable with that of previously reported native and gel-incorporated crystals (63 MPa) [7]. By contrast, the static Young’s modulus of the one-week-cross-linked crystals increased drastically compared with that of the native crystals owing to the strong intermolecular forces induced by cross-linking with glutaraldehyde. The Young’s modulus of the one-week-cross-linked crystals was comparable with that of a previously reported cross-linked crystal (210 MPa) [11] but lower than that of cross-linked crystals measured using the nanoindentation method (1080 MPa) [15]. This discrepancy may be related to the test scale between macroscopic and microscopic deformations.

When the maximum stress was achieved in the elastic region of the stress–strain curve of the native crystals, the stress abruptly decreased with increasing strain and fracture occurred simultaneously. Optical micrographs of the native crystals before and after compression are shown in Fig. 1(c) and (d), respectively. Numerous cracks and fragments could be observed in the compressed crystals. This behaviour corresponds to typical brittle fractures. The maximum stress in the stress–strain curve of the native crystals corresponds to the fracture stress. The fracture stress was 0.57 ± 0.26 MPa, which is similar to a previous report [7].

In previous studies, the plastic deformation due to the slip deformation has been observed in the native protein crystals by the microindentation method [6,20,21,22]. However, in

1 this study of the macroscopic deformation, rather than plastic deformation, brittle
2 fractures occurred in the native crystals. This difference of the deformation behaviour
3 could be considered by the relationship between the specimen and the crack size, as
4 reported as the brittle materials such as ceramics [23].

5
6 The stress–strain behaviour of the one-week-cross-linked crystals showed interesting
7 phenomena. Rather than brittle fractures, plastic deformation occurred in these crystals.
8 This plastic deformation exhibited yield-point phenomena with upper and lower yield
9 points. In other words, when the maximum stress was achieved, the stress decreased with
10 increasing strain and reached a minimum value, as shown in Fig. 1(b). Beyond the lower
11 yield point, the stress gradually increased with increasing strain. The deformation
12 observed may be classified as stage I deformation, which manifests as an easy-glide
13 region of dislocations in typical face-centred cubic crystals such as Cu crystals [24]. Stage
14 I deformation was observed in most of the cross-linked protein crystals at strains of up to
15 approximately 60% during compression.

16
17 Macroscopic observation of the crystal surfaces revealed many slip lines. A bundle of
18 inhomogeneous slip lines appeared as slip bands, as shown in Fig. 1(f). Moreover, slip
19 traces along the $[112]$ and $[11\bar{2}]$ directions were observed on the crystal surface and the
20 width of the bundle was approximately 100 μm , shown as line A in Fig. 1(f). The angle
21 between the trace of line A and the $[001]$ direction was approximately 57° . The angle
22 between the $[112]$ and $[001]$ directions was 55° , which agreed with the measured angle.
23 This means that the double slips with $[112]$ and $[11\bar{2}]$ occur. The appearance of the slip

lines indicates that plastic deformation is controlled by a dislocation mechanism. However, work hardening was not observed in the stress–strain curves. The two types of dislocations in the $[112]$ and $[11\bar{2}]$ directions do not appear to interact with each other; therefore, sessile dislocations could not nucleate owing to dislocation reactions.

According to the dislocation model, the yield-point phenomena of crystalline materials can be explained by the nucleation, multiplication, and motion of dislocations. Two types of yield-point phenomena have been reported. One is due to the strong dislocation-locking effect of impurity atoms or the so-called Cottrell effect, which leads to the pronounced stress drop required for deformation after yielding in some alloys [25] and is typically seen in low-carbon Fe crystals. The other is due to the dynamic behaviours of dislocation multiplication and motion in accordance with the Johnston–Gilman yielding theory [26]. Three conditions are important in this theory. First, the initial mobile-dislocation density must be low. Second, the dislocation velocity corresponding to the applied stress must be low. Finally, the dislocations should multiply rapidly. These behaviours have been observed in single crystals with free and/or low dislocation density, such as in semiconducting crystals composed of Ge and Si [27–30]. In a previous report, the grown-in dislocation density of native T-HEWL crystals was estimated to be $\sim 10^2 \text{ cm}^{-2}$ [31] which means high perfection. As the grown-in dislocation density of cross-linked protein crystals is expected to be low, they are also expected to have higher perfection. In the case of the cross-linked T-HEWL crystals, the stress drops in the stress–strain curve could be mainly explained by the Johnson–Gilman theory. The fracture stress of the cross-linked crystals is considered to be higher than their yield stress because of cross-linking, although fracture primarily occurs in the native crystals because of their relatively lower

fracture stress. The characteristics of the plastic deformation associated with induced dislocations are discussed later. The results thus far indicate that brittle materials are transformed into ductile materials via cross-linking.

We investigated the relationship between the cross-linking time and mechanical properties, such as Young's modulus and lower yield stress, of the crystals. Fig. 2 shows the dependence of the Young's modulus and lower yield stress of the crystals on the cross-linking time. The one-day-cross-linked protein crystals showed brittle fractures and did not exhibit a plastic deformation. As seen in Fig. 2(a), the Young's modulus greatly increased and reached the maximum value after 1 day of cross-linking. The Young's modulus of the 1–7-day-cross-linked crystals was 389 ± 36 MPa, which is approximately 5.8 times higher than that of native crystals. On the other hand, the lower yield points appeared for samples with cross-linking time for 2 days. It gradually increased with increasing cross-linking time for samples cross-linked over 3 days, as shown in Fig. 2(b). The average lower yield stress of the 3–7-day-cross-linked crystals was 12.5 ± 0.8 MPa. Both the Young's modulus and lower yield stress depended on the cross-linking time, although the times required to reach stable values differed. The cross-linking reaction is predominantly controlled by the interactions of Lys13 and Arg45. Buch *et al.* revealed through X-ray structural analysis that the cross-linking reaction at Arg45 occurs faster than that at Lys13 [32]. The inter-(111)-plane-bonding, which is slip plane, are only Lys13-Lys13. Therefore, the yield stress required to cut the bond and induce slip deformation is a delayed increase. Details regarding the slip planes will be discussed later. In addition, the discrepancy in the time dependence of these parameters may be attributed to the fundamental mechanisms of both phenomena. Also, it is considered that this time

dependence is due to the difference of the amount of the cross-linking. In the short reaction period such as 1 day, the inside of the crystal is brittle similar to the native crystal, and the crack nucleation and propagation easily occurs. Young's modulus is insensitive to the number of cross-linked molecules because of the deformation of the entire sample, so it increases quickly after one day of cross-linking. After the inside of the crystal is cross-linked fully by the longer time, the lower yield stress reaches the maximum values. The dislocation motion could be more sensitive for number of cross-linked molecules because the core structure of dislocations depends on the intermolecular potential [33].

B. Identification of slip systems activated by cross-linking

We consider the slip systems of the one-week-cross-linked crystals. Fig. 3(a) shows a schematic diagram of the as-grown form of a T-HEWL crystal. Fig. 3(b), (c), and (d) show X-ray topographic images of the 110, $\bar{1}10$, and 200 diffractions, respectively. The corresponding schematics of the crystals are shown in each figure. Fig. 3(c) corresponds to Fig. 3(b) rotated by 90° around the [001] direction, and Fig. 3(d) corresponds to Fig. 3(b) rotated by -45° around the [001] direction. Here, we focus on the two contrasts in these topographs. A clear contrast in crystal shape can be observed for each diffraction, indicating that the compressed crystal maintains its single crystallinity. Another contrast in the lines denoted A and B is observed in the 110 reflection, as shown in Fig. 3(b). The contrast in these lines is in good agreement with the slip traces observed in the optical images shown in Fig. 1(f). However, the line contrast seen in Fig. 3(b) disappeared, as shown in Fig. 3(c). Moreover, planar shapes with a white contrast, denoted planes A and B, appeared in the 200 reflection, as shown in Fig. 3(d).

Next, we consider the slip systems introduced during the compression tests. The Burgers vectors of the induced dislocations were identified using synchrotron X-ray topography. The Burgers vectors of dislocations can be determined based on the invisibility criterion for dislocation images ($\mathbf{g} \cdot \mathbf{b} = 0$, where $\mathbf{g}$ and $\mathbf{b}$ are the diffraction and Burgers vectors, respectively). The contrasts of lines A and B in Fig. 3(b) are invisible in the $\bar{1}10$ reflection, as shown in Fig. 3(c). According to the invisibility criterion, the possible Burgers vectors for the slip traces denoted by line A lie in the $[11\bar{1}]$, $[11\bar{2}]$, and $[22\bar{1}]$. Thus, the Burgers vector was assigned to the $[11\bar{2}]$ because the $[11\bar{1}]$ and $[22\bar{1}]$ are not parallel to the slip traces observed in the optical images, as shown in Fig. 1(f) and (g) (the angles between the $[001]$ and $[11\bar{2}]$ and the slip line observed in the crystal are 54° and 57° , respectively). Subsequently, the slip planes were determined. Based on Weiss' law of zones, the (111) , (201) , and (110) planes are possible slip planes along the Burgers vector $[11\bar{2}]$. We considered the Schmidt factor to identify the possible slip planes. For reference, the d-spacing and Burgers vectors of the native and cross-linked crystals are listed in Tables 2 and 3, respectively. The Schmidt factor is proportional to the critical resolved shear stress [34]. The Schmidt factors for the (111) , (201) , and (110) planes are estimated as 0.46, 0.40, and 0.00, respectively, as summarised in Table 4. Thus, one of the slip systems for the slip traces denoted by line A in Fig. 1(f) was assigned as $(111)[11\bar{2}]$. Similarly, the slip system for the slip traces denoted by line B in Fig. 1(f) was characterised as $(11\bar{1})[112]$ (the Schmidt factor for this line is 0.46, similar to that of line A). In previous investigation, the slip deformation of native crystals with $(110)[1\bar{1}0]$ was detected through microindentation test [6]. However, in this study, the slip system remains inactive due to a Schmidt factor of 0.00. Additionally, the slip system of $(111)[11\bar{2}]$ was not observed in

native crystals. This suggests that cross-linking enables the activation of the slip system.

Fig. 4(a) shows a schematic of the $(111)[11\bar{2}]$ slip system in a unit cell of the cross-linked crystals when compressed in the $[\bar{1}\bar{1}0]$ direction. Two slip systems move at an angle in response to compression and intersect. In general, slip planes have the highest density of atoms or molecules, that is, they are the most widely spaced, and the slip direction is always parallel to the Burgers vector of the dislocation responsible for slip deformation [34]. As shown in Tables 2 and 3, the assigned $(111)[11\bar{2}]$ slip system in the cross-linked crystals has a large Burgers vector (11.31 nm) on planes with a narrow lattice spacing (3.07 nm). In continuous elastic bodies, the self-energy of a dislocation E is proportional to the shear modulus μ and the square of the Burgers vector b^2 , that is, $E \sim \mu b^2$ [34]. Therefore, as a general criterion, the dislocation in a crystal should have the minimum Burgers vector, that is, the minimum lattice vector. However, previous studies in which the slip systems of tetragonal, orthorhombic, and triclinic HEWL crystals were characterised using Vickers hardness experiments demonstrated that the slip systems in these crystals are unique because various slip systems are activated by the applied stress [19,20,21]. Almost all slip systems without the minimum dislocation energy estimated by dislocation theory have been observed in other protein crystals using X-ray topography [35], which means stress-induced dislocations without the shortest Burgers vector and with the largest d -spacing are activated in the protein crystals. The characteristics of this slip system may be associated with the strong bonds formed between HEWL molecules owing to cross-linking.

Cross-linking reactions between the glutaraldehyde and HEWL molecules are not random

1 events. Cross-linking between amino acid residues, such as lysine and arginine, and
2 glutaraldehyde forms $-C=N-$ bonds [10]. Cross-linking with glutaraldehyde mainly
3 involves lysine (Lys13) and arginine (Arg45) residues in HEWL molecules [32]. The
4 reaction is initiated at the preferred cross-linking sites depending on the arrangement of
5 the HEWL molecules. The free aldehyde groups of glutaraldehyde are highly reactive and
6 exhibit different tendencies for self-polymerisation depending on environmental factors
7 such as pH and linker concentration in aqueous media [36].

8
9 Fig. 4(b) shows schematics of the molecular arrangement and slip system of the cross-
10 linked crystals based on their $[111]$ (top view of the (111) direction) and $[1\bar{1}0]$ (side view
11 of the (111) direction) views, which were drawn based on their structural information
12 (PDB ID: 2hu3 [10]). The HEWL molecules are schematically drawn as ellipses and the
13 waviness of the crystallographic planes is ignored because protein molecules have
14 complex shapes. As shown in Fig. 4(b), the amino acid residues Lys13 and Arg45 are
15 diagonally arranged on the HEWL molecule. Cross-linking between HEWL molecules
16 occurs at Lys13–Lys13 and Arg45–Arg45. In the side view of (111) , interplanar bonds
17 corresponding to the bonds ☆ and ★ in the top view are indicated by lines. The only
18 interplanar bonds are Lys13–Lys13 cross-linking bonds. Six intra-cross-linking bonds and
19 two inter-cross-linking bonds are observed on the crystallographic (111) plane of the unit
20 cell (the area is 37.3 nm^2). Thus, the molecular interactions in the (111) plane of the cross-
21 linked HEWL crystals are stronger than those of the native crystals. We considered that
22 the cross-linked (111) plane becomes more stable against stress so that the (111) plane
23 can act as the slip plane.

1 The appearance of plastic deformation in the crystals depends on the nucleation and
2 motion of dislocations under an applied stress. In native HEWL crystals with weak
3 intermolecular interactions, the fracture stress is much lower than the yield stress required
4 for the nucleation and motion of dislocations. Consequently, fracture is favoured over
5 plastic deformation. However, in the case of cross-linked protein crystals, the fracture
6 stress is higher than the yield stress because of the strong intermolecular interactions
7 induced by cross-linking, resulting in plastic deformation. The ductile properties of cross-
8 linked protein crystals indicate their great potential for various material applications with
9 enzymatic activity for bio-catalysis and bio-sensing [37,38].

11 **IV. Conclusion**

12 In summary, cross-linking is widely known to be useful for strengthening protein crystals.
13 In this study, we quantitatively evaluated the mechanical properties of cross-linked
14 protein crystals by compression test. It was found that cross-linked protein crystals were
15 plastically deformed and showed typical stress-strain curves with the upper- and lower-
16 yield points. Thus, brittle native protein crystals were transformed into ductile protein
17 crystals by cross-linking. After compression testing, slip traces were clearly observed in
18 the cross-linked protein crystals, with the Burgers vectors of the slip systems determined
19 by using X-ray topography. This shows that plastic deformation during compression
20 primarily occurs due to dislocation multiplication and motion, causing slip deformation
21 within the crystal. The ductility of protein crystals exhibited by cross-linking indicate
22 their immense potential for material applications with enzymatic activity for bio-catalysis
23 and bio-sensing. It is anticipated to open up new possibilities for biomaterials
24 development.

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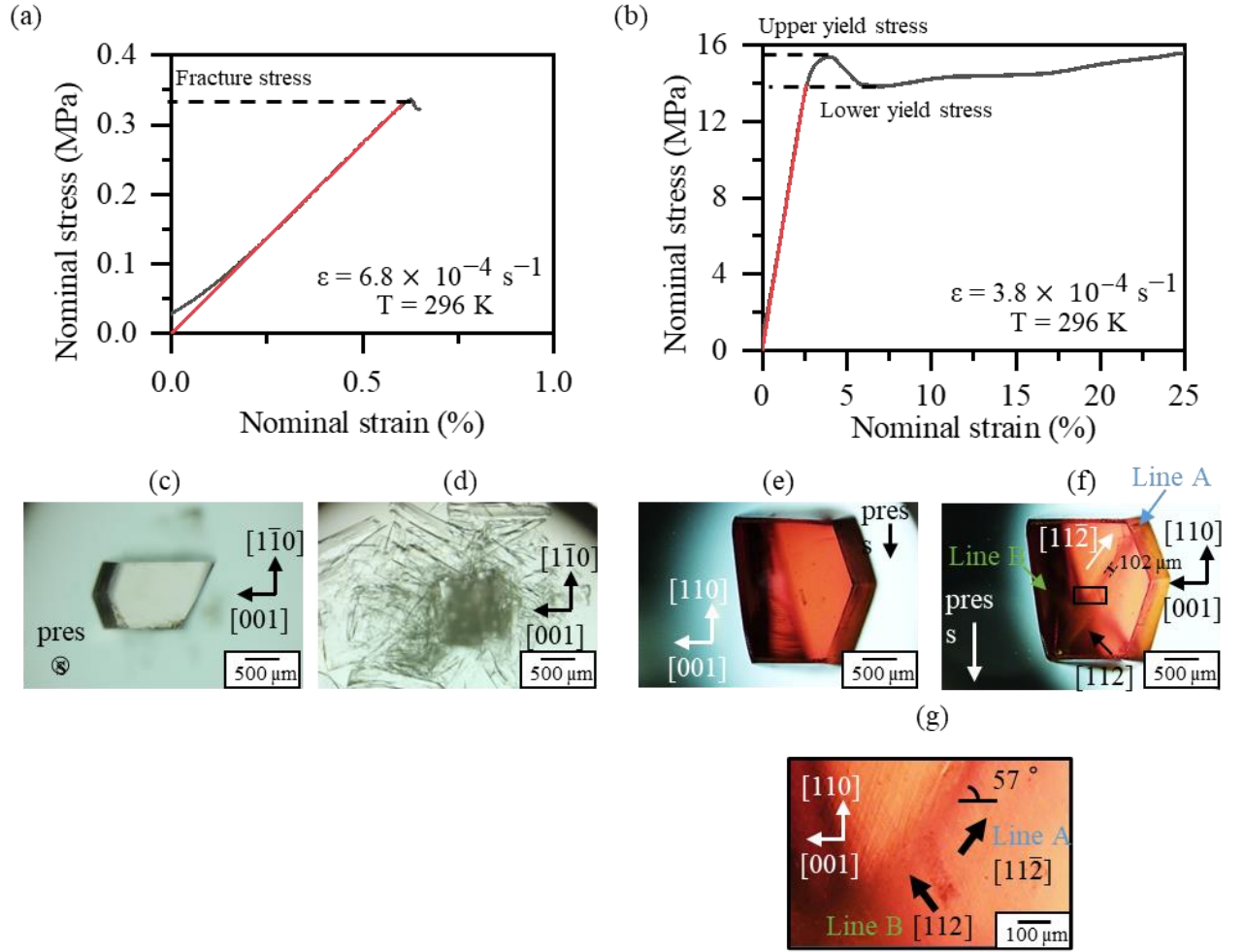
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1 **Figures**



2

3 **Figure 1.** Typical stress–strain curves of (a) native and (b) one-week-cross-linked protein
4 crystals at 296 K. The strain rate is $\sim 10^{-4} \text{ s}^{-1}$. Red lines are extrapolated as the slope of the
5 elastic region. ϵ and T in the figure indicate the strain rate and temperature during the
6 compression test, respectively. (c, d) and (e, f) Optical micrographs of the native and 1-
7 week cross-linked crystals (c, e) before and (d, f) after the compression test. (g)
8 Magnification of the area marked by the rectangle in (f). Slip traces in the $[112]$ and $[1\bar{1}\bar{2}]$
9 directions are denoted by arrows.

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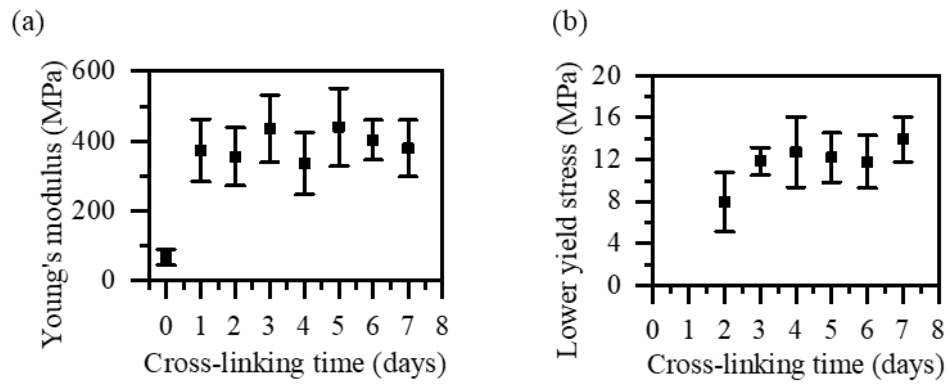


Figure 2. Relationship between the cross-linking time and (a) Young's modulus and (b) lower yield stress of the cross-linked crystals. The error bars in (a) and (b) indicate the standard deviation (plots: average value).

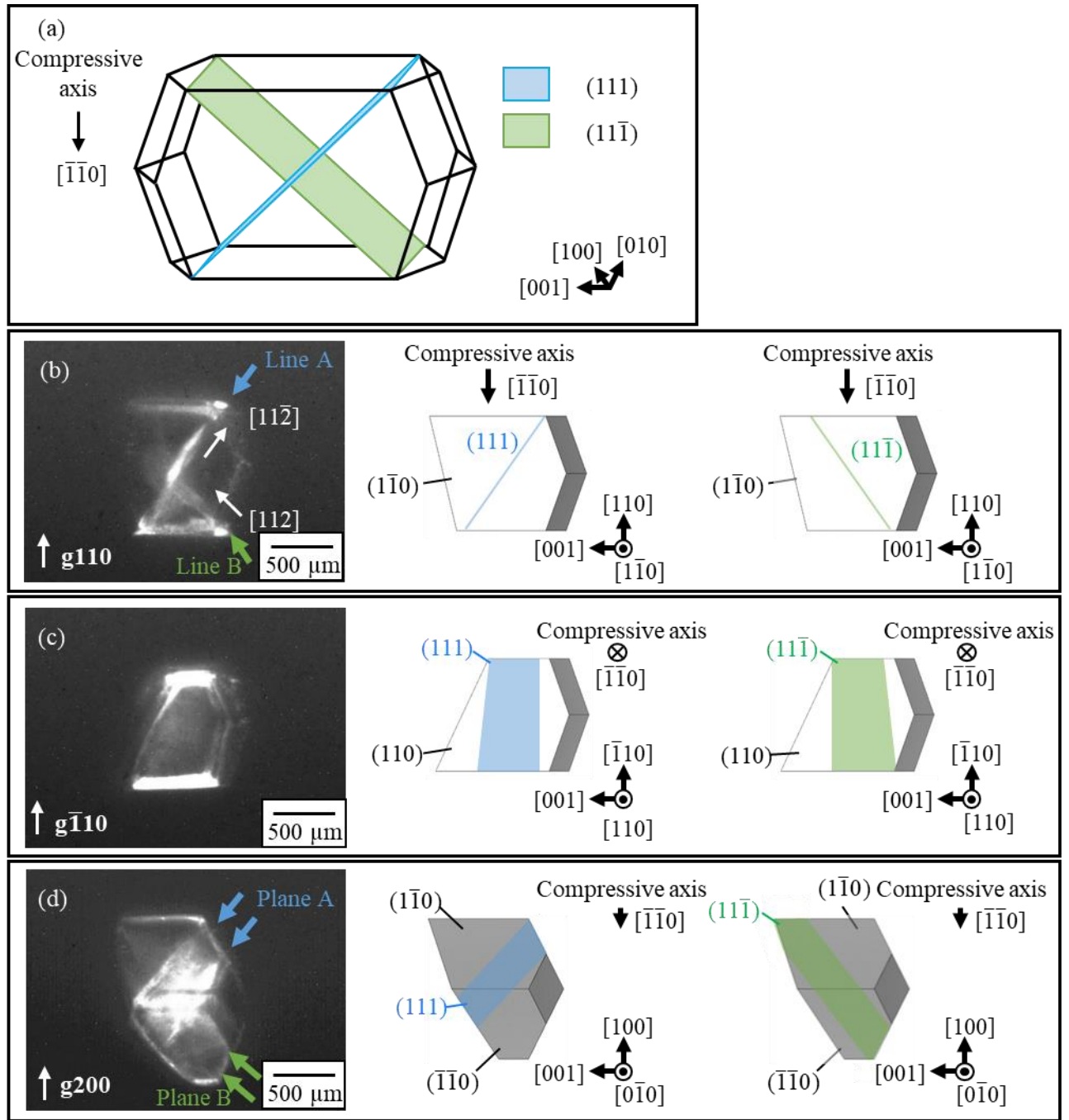


Figure 3. (a) Schematic diagram of a T-HEWL crystal with the ideal form. (b–d) Typical X-ray topographs (median filtered images) and corresponding schematics of the crystal orientation taken from the (b) 110 , (c) $\bar{1}10$, and (d) 200 reflections after the compression test. Based on the compression test, this sample had approximately 3.5% plastic strain. The schematic figures of crystal morphology were prepared using VESTA software [39].

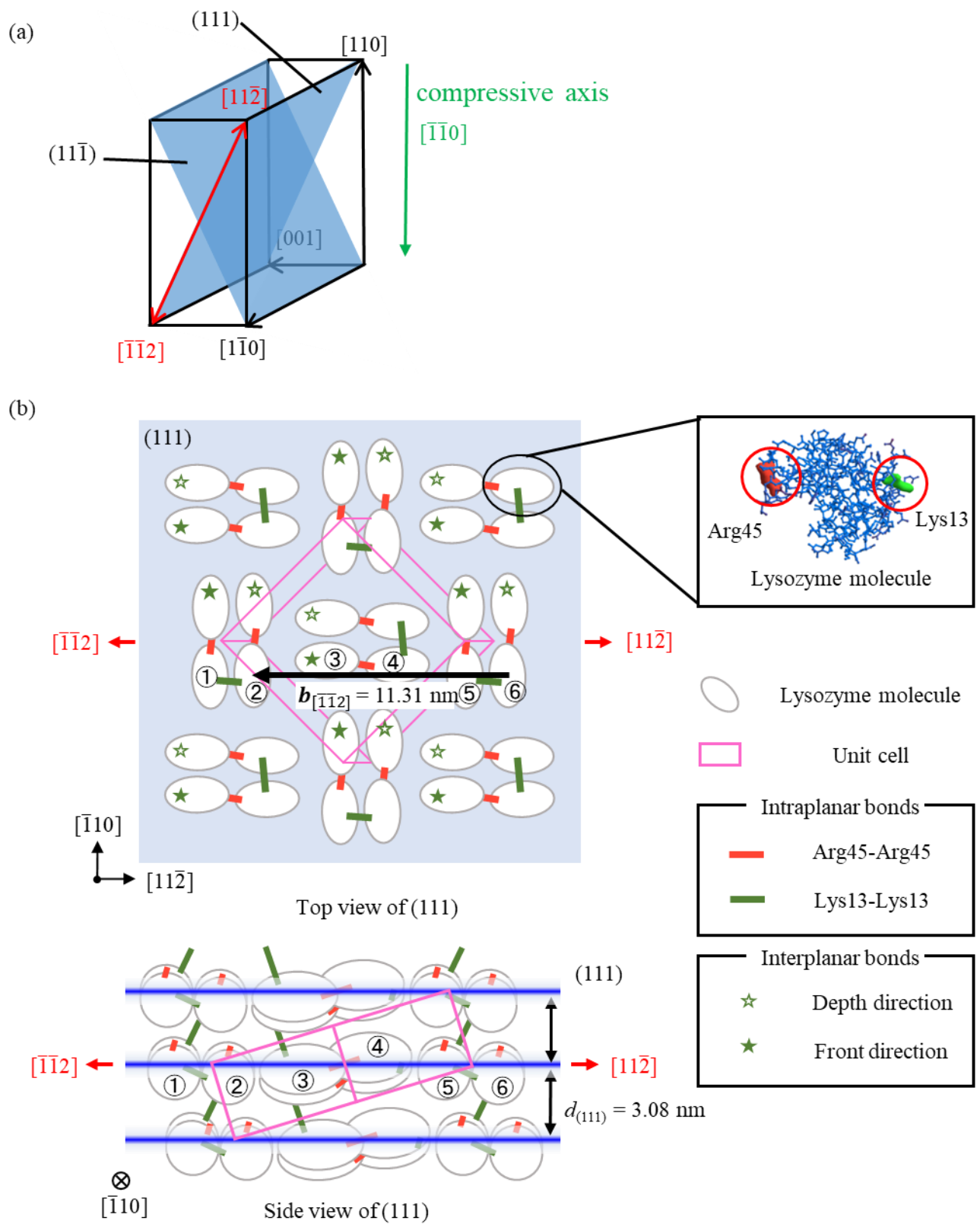


Figure 4. Schematic illustrations of the slip systems and molecular arrangements of the cross-linked crystals. (a) Slip system $(111)[11\bar{2}]$ when compressed in the $[\bar{1}\bar{1}0]$ direction. The two blue planes represent $\{111\}$, and the red line shows the $\langle 112 \rangle$ direction on $\{111\}$ planes. (b) Molecular arrangement based on the $[111]$ (top view of the (111) direction) and $[1\bar{1}0]$ (side view of the (111) direction) planes. White ellipses indicate lysozyme molecules, and red, green, and pink parallelepipeds represent Arg–Arg bonds, Lys–Lys bonds, and a unit cell, respectively. The cross-links of Lys13–Lys13 (denoted as green lines) and Arg45–Arg45 (denoted as red lines) appear in the (111) slip plane. The bonds in the (111) slip plane are drawn as lines, while the bonds in the depth and front directions are represented by ☆ and ★, respectively. Here, ☆ is directed toward the lower region of the (111) plane, and ★ is directed toward the upper region of the (111) plane. In the side view of the (111) plane, the blue lines on the molecular packing represent the (111) slip plane with a d-spacing of 3.08 nm. In (b), the numbers 1-6 assigned to the HEWL molecules in top view correspond to those of side view.

1 **Tables**

2 **Table I.** Lattice parameters of native and cross-linked T-HEWL crystals.

Lattice parameters [nm]	Native	Cross-linked
	[PDB: 1lyz [17]]	[PDB: 2hu3 [10]]
<i>a</i>	7.91	7.89
<i>b</i>	7.91	7.89
<i>c</i>	3.79	3.70

3 Note that the cross-linking time was 24 h [10].

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1 **Table II.** Magnitude of the d -spacing of the lattice plane in native and cross-linked HEWL
2 crystals.

Lattice plane	d -spacing [nm]	
	Native	Cross-linked
(100)	7.91	7.89
(001)	3.79	3.70
(101)	3.42	3.35
(110)	5.59	5.58
(111)	3.14	3.08
(201)	2.74	2.70

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1 **Table III.** Magnitude of the Burgers vector in native and cross-linked HEWL crystals.

Burgers vector	<i>b</i> [nm]	
	Native	Cross-linked
[100]	7.91	7.89
[001]	3.79	3.68
[101]	8.77	8.71
[110]	11.19	11.16
[111]	11.79	11.75
[112]	11.34	11.31

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1 **Table IV.** Estimated Schmidt factor of cross-linked HEWL crystals.

Slip system	Schmidt factor
(111)[11 $\bar{2}$]	0.46
(11 $\bar{1}$)[112]	0.46
(201)[11 $\bar{2}$]	0.40
(1 $\bar{1}$ 0)[11 $\bar{2}$]	0

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