PHYSICAL REVIEW E **79**, 061917 (2009)

Elastic constants in tetragonal hen egg-white lysozyme crystals containing large amount of water

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(Received 6 October 2008; revised manuscript received 7 May 2009; published 12 June 2009)

Transverse sound velocity of cross-linked tetragonal hen egg-white (HEW) lysozyme crystals containing large amount of water in the crystal was measured using ultrasonic pulse-echo method. All elastic constants of cross-linked crystals were observed to be $C_{11}=C_{22}=5.50$ GPa, $C_{12}=4.33$ GPa, $C_{13}=C_{23}=3.94$ GPa, $C_{33}=5.22$ GPa, $C_{44}=C_{55}=0.68$ GPa, and $C_{66}=0.84$ GPa, respectively. We found that the elastic constants of the cross-linked crystals are identical to those of the intrinsic ones without cross-linking. Moreover, we found that tetragonal HEW lysozyme crystals that enclose large amount of water show decreased elastic constants (softening). In particular, the shear elastic constants $C_{44}=C_{55}$ and C_{66} showed more softening effect comparing with other elastic components.

DOI: 10.1103/PhysRevE.79.061917 PACS number(s): 87.15.−v, 62.20.−x, 62.30.+d, 62.65.+k

I. INTRODUCTION

In general, protein crystals enclose large amount of water within it. It is known that there is as much as water of 30–80 vol % in protein crystals. In addition, the water is qualitatively classified into two types in terms of their behaviors: (i) mobile water among the protein molecules and (ii) immobile water strongly bound to the protein molecules. Such peculiarity of protein crystals are specific compared with those of inorganic and organic crystals. Accordingly, it is important to investigate the physical properties of the protein-water system in order to understand the intrinsic physical properties of protein crystals.

Researches into the mechanical and physical properties of protein crystals have been carried out by various groups [1–11]. In a previous work [4,5] we have observed the combination of elastic constants, $(C_{11}+C_{12}+2C_{66})/2$, of tetragonal hen egg-white (HEW) lysozyme crystals using ultrasonic pulse-echo method, which corresponds to the longitudinal sound velocity along the [110] direction in the MHz frequency regime. Furthermore, Speziale *et al.* determined the elastic constants, C_{11} and C_{33}, of tetragonal HEW lysozyme crystals by measuring the longitudinal wave in GHz frequency regime along [100] and [001] directions using Brillouin scattering method [6,7].

More recently, all components of elastic constants in tetragonal HEW lysozyme crystals dehydrated at 42% relative humidity (hereafter denoting 42% RH) have been determined using ultrasonic pulse-echo method in MHz frequency regime [10]. However, the shear components of elastic constants at 98% RH have not been observed yet because of brittleness of the crystals. In fact to generate a transverse ultrasonic wave in the crystal using ultrasonic pulse-echo method, a transducer must be in close contact with the crystal. However, it is difficult to glue a transducer to a protein crystal since they are quite brittle. Thus, it is important to improve the strength of protein crystals in order to succeed with the transverse ultrasonic wave measure. In general, the chemical cross-linking is used to improve the brittleness of protein [12–14]. This chemical cross-linking is preformed by adding glutaraldehyde to protein crystals, which forms some bonds between lysine residues on neighboring protein molecules. In particular, Wine *et al.* [15] revealed that the chemical cross-linking is produced between the two ϵ-amines of Lys13 residues of two neighboring lysozyme molecules in recent paper. In this way, we improved the strength and we applied transverse ultrasonic wave without breaking the protein. As results, all of elastic constants in tetragonal HEW lysozyme crystals with large amount of intracrystalline water were determined.

II. EXPERIMENT

Lysozyme crystals were grown through a salt-concentration gradient method at 23 °C vertically in test tubes using $NiCl_2$ as a precipitant [16,17]. Large crystals up to 4 mm in size were grown over a period of two weeks. The as-grown crystals (we call those crystals “non-cross-linked crystals” hereafter) had a tetragonal structure with the space group $P4_32_12$, lattice constants $a=b=79.1$ Å and $c=37.9$ Å, and eight molecules per unit cell at room temperature. Almost all the crystals had growth habits such as {110} and {101} crystallographic faces. The high quality of non-cross-linked crystals was confirmed by x-ray topography [18–21].

The chemical cross-linking was performed using a method similar to Iimura *et al.* [22]. The solution used for the chemical cross-linking was prepared by adding glutaraldehyde to a solution containing the same precipitant as the original solution used for crystallization. The final concentrations were 2.5 wt % glutaraldehyde and 11.6 wt % $NiCl_2$. Non-cross-linked crystals were immersed in such a solution at room temperature for a week. After the chemical cross-linking, the lattice constants were investigated by x-ray diffraction method.

Sound velocity measurements were carried out using the pulse-echo mode of operation of an ultrasonic pulser/receiver

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TABLE I. The propagation direction and polarization direction and the corresponding ρv^2 in term of the elastic constants, where ρ is density and v is velocity.

				Sound velocity (m/s)	
Propagation direction	Polarization direction	Measured velocity	ρv^2	Cross-linked crystal at 98% RH	Non-cross-linked crystal at 100% RH[a],[b]
[110]	Longitudinal [110]	v_1	$(C_{11}+C_{12}+2C_{66})/2$	2182 ± 54	2070 ± 34
	Transverse [001]	v_2	C_{44}	752 ± 48	
	Transverse $[1\bar{1}0]$	v_3	$(C_{11}-C_{12})/2$	695 ± 44	
In (010) plane, at an angle of θ=22.8° to [001] axis.	Quasilongitudinal	v_4	C [c]	2086 ± 32	2001 ± 33
	Quasitransverse [010]	v_5	$(C_{44}\cos^2\theta+C_{66}\sin^2\theta)/2$	768 ± 66	
	Quasitransverse $[10\bar{1}]$	v_6	C [d]	760 ± 78	

[a] The values measured in solution.

[b] Reference [10].

[c] $C=1/2\{C_{11}\sin^2\theta+C_{33}\cos^2\theta+C_{44}+\sqrt{(C_{11}\sin^2\theta-C_{33}\cos^2\theta+C_{44}\cos 2\theta)^2+(C_{13}+C_{44})^2\sin^2 2\theta}\}$.

[d] $C=1/2\{C_{11}\sin^2\theta+C_{33}\cos^2\theta+C_{44}-\sqrt{(C_{11}\sin^2\theta-C_{33}\cos^2\theta+C_{44}\cos 2\theta)^2+(C_{13}+C_{44})^2\sin^2 2\theta}\}$.

(PR35, JSR Ultrasonics) at room temperature. The transverse and longitudinal ultrasonic measurements were preformed using the transducer working at 5 and 18 MHz, respectively. A perfect contact between the transducer and crystal is essential for the transverse ultrasonic measurements. Thus, a shear gel (SONOTECH Co., Ltd.) was used as adhesive to glue the cross-linked tetragonal HEW lysozyme crystals to the transducer. The large (110) and (101) crystallographic faces were used for the sound velocity measurements. Transverse and longitudinal ultrasonic waves were propagated perpendicular to each face, respectively. For (110) crystallographic face, thus, all the wave propagation directions were along the [110] direction. So, one longitudinal sound velocity, v_1, and two transverse sound velocities, v_2 with polarization direction in [001] and v_3 with $[1\bar{1}0]$, were measured. On the other hand, for (101) crystallographic face the wave propagation directions were not along the [101] direction. Therefore the quasilongitudinal sound velocity, v_4, the quasitransverse sound velocity, v_5, with a polarization direction in [010], and the quasitransverse sound velocity, v_6, with a polarization direction in $[10\bar{1}]$ propagated perpendicular to (101) crystallographic face were measured. That is, the measured sound velocities v_1, v_2, and v_3 were pure modes, while the measured ones v_4, v_5, and v_6 were quasimodes. The propagation directions and polarization directions of these sound velocities from v_1 to v_6 are summarized in Table I.

In order to observe the improvement on the strength of lysozyme crystals cross-linked by glutaraldehyde, the Vickers hardness test was carried out. The crystal was set on the specimen stage of the apparatus to keep the (110) crystal face perpendicular to the indenter. The indenter, with a load of 4.9 MPa (0.5 g weight), was pulled down to the crystal surface at a velocity of 0.01 mm/s. The contact period of the indenter with the surface was 5 s. The Vickers hardness was estimated by an equation: $1.854(F/d^2)$, where F (N) and d (mm) are the load and length of the diagonal of the indentation, respectively, and then the unit kgf was changed by MPa.

III. RESULT AND DISCUSSION

X-ray diffraction revealed that the cross-linked HEW lysozyme crystals had tetragonal structure with the space group $P4_32_12$, lattice constants $a=b=79.3$ Å, $c=37.8$ Å, and eight molecules per unit cell. From this result, it was found that there was little change in the crystal structure for the cross-linked tetragonal HEW lysozyme crystals at 98% RH compared with those for the non-cross-linked ones. This result is in agreement with that of previous reports [15,22].

The traveling time for the transverse ultrasonic wave was measured in a number of crystals with various thicknesses of ~0.5–~1.0 mm. The typical relationship between crystal thickness and traveling time was shown in Fig. 1. From the inclination of the straight line fitting to the curve in Fig. 1, for instance, the average transverse sound velocity, v_2, of the cross-linked crystals was determined to be 752 ± 48 m/s. Similarly, v_1, v_3, v_4, v_5, and v_6 for the cross-linked crystals were measured to be 2182 ± 54, 695 ± 44, 2086 ± 32, 768 ± 66, and 760 ± 78 m/s, respectively. The velocities measured using the cross-linked tetragonal HEW lysozyme

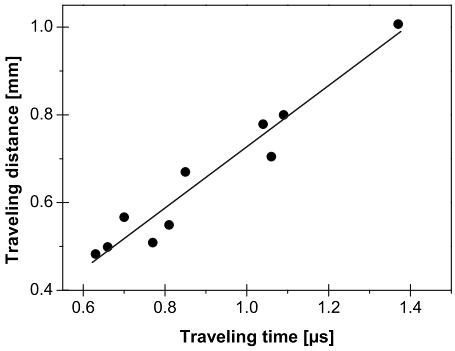

FIG. 1. The relationship between traveling distance and traveling time for the reflected transverse ultrasonic wave along the [110] direction of a cross-linked tetragonal HEW lysozyme crystals. The wave is polarized along the [001] direction.

TABLE II. Comparison of longitudinal sound velocity measured in this work and by Edwards *et al.* [23].

	Material	Longitudinal sound velocity (m/s)
Our result	v_1 (lysozyme crystal)	2070 ± 34
	v_4 (lysozyme crystal)	2001 ± 33
Edwards *et al.*[a]	Lysozyme compacted disk	2004 ± 23
	Ribonuclease crystal	1784 ± 72
	Hemoglobin crystal	1828

[a] Reference [23].

crystals are summarized in Table I. The sound velocities for the non-cross-linked tetragonal HEW lysozyme crystals in solution (at 100% RH) reported previously [4,10] are also listed in this table. It is clear that the longitudinal sound velocities, v_1 and v_4, of the cross-linked crystals at 98% RH were almost comparable with those of the non-cross-linked ones in solution (100% RH). This suggests that the chemical cross-linking had little contribution to ultra sound velocity in MHz regime. Indeed, considering the result of Wine *et al.* [15], the chemical cross-linking is produced only between two particular molecules. Thus, most of intermolecular interactions in the cross-linked crystals would be the same as those in the non-cross-linked ones. However the sound velocities of the cross-linked crystals, v_1 and v_4, are a little bit smaller than those of non-cross-linked ones. It seems that the cross-linking has a small effect on the intermolecular potentials.

Edwards *et al.* measured the longitudinal sound velocities of lysozyme compacted disk, ribonuclease crystals, and hemoglobin crystals using piezo and laser methods. As results it seems that these longitudinal velocities on protein crystals are almost the same [23], which are summarized in Table II. Therefore, it is revealed that there is little dispersion in the frequency range from 5 MHz to 2 GHz. Recently, in addition, Svanidze *et al.* [11] observed the sound velocities along the [110] direction in the non-cross-linked crystals at 98% RH using Brillouin scattering method. The longitudinal velocity corresponding to the velocity, v_1, has been observed to be ~ 2190 m/s. Thus, it should be noticed that the cross-linking by glutaraldehyde has a small effect on the ultrasonic sound velocity in MHz and GHz regions.

The cross-linked crystals with a tetragonal phase (422 class) have six independent elastic constants: $C_{11}=C_{22}$, C_{12}, $C_{13}=C_{23}$, C_{33}, $C_{44}=C_{55}$, and C_{66}. For sound waves observed in this crystal, the solution of the Christoffel equation is described in details in our previous paper [10]. Here, the density of the crystal, ρ, and the angular direction of the ultrasonic wave vector with respect to the [001] direction, θ, were set to be 1.21 $\mathrm{Mg/m^3}$ and 25.5°, respectively. Thus, the six elastic constants for the cross-linked tetragonal HEW lysozyme crystals were thus determined to be $C_{11}=C_{22}=5.50$ GPa, $C_{12}=4.33$ GPa, $C_{13}=C_{23}=3.94$ GPa, $C_{33}=5.22$ GPa, $C_{44}=C_{55}=0.68$ GPa, and $C_{66}=0.84$ GPa, respectively, as shown in Table III. Indeed, the elastic constants, $C_{11}=C_{22}$ and C_{33}, observed in this work were in good agreement with those of the non-cross-linked crystals at 98% RH, which have been observed to be 5.49 and 5.48 GPa, respectively, using Brillouin scattering method [6,7] as shown in Table IV. Although Speziale *et al.* [7] could not observed all of elastic constants of non-cross-linked crystals at 98% RH, they observed that the combination of elastic constants, $C_{11}+C_{12}+2C_{66}$ was 12.81 GPa. These values were almost coincident with our results as shown in Table IV. Thus, the observed elastic constants of the cross-linked crystals at 98% RH were coincident with those of the non-cross-linked ones at 98% RH. This means that we were able to estimate the elastic constants in intrinsic HEW lysozyme crystals using the lysozyme crystals cross-linked by glutaraldehyde.

Thus, it seems that the cross-linking by glutaraldehyde does not affect the microscopic small displacements such as

TABLE III. All the six elastic constants, the Young's modulus E, shear modulus μ, bulk modulus K, compressibility β, and Poisson's ratio σ of cross-linked at 98% RH and non-cross-linked at 42% RH crystals. In addition, the values extrapolated from non-cross-linked at 42% RH crystals are listed.

		Cross-linked crystal	Non-cross-linked crystal[a]	
		98% RH	42% RH	98% RH (extrapolated values)
Elastic constant (GPa)	$C_{11}=C_{22}$	5.50	12.44	5.52
	C_{12}	4.33	7.03	3.12
	$C_{13}=C_{23}$	3.94	8.38	3.71
	C_{33}	5.22	12.79	5.68
	$C_{44}=C_{55}$	0.68	2.97	1.32
	C_{66}	0.84	2.63	1.16
Young's modulus (GPa)	E	2.01	7.25	3.21
Shear modulus (GPa)	μ	0.70	2.64	1.17
Bulk modulus (GPa)	K	4.51	9.46	4.20
Compressibility (1/Pa)	β	2.21×10^{-10}	1.05×10^{-10}	2.38×10^{-10}
Poisson's ratio	σ	0.42	0.37	0.37

[a] Reference [10].

TABLE IV. Comparison of elastic constants at 98% RH using ultrasonic pulse-echo method and Brillouin scattering method.

	Cross-linked crystals at 98% RH (present results)	Non-cross-linked crystals at 98% RH (Brillouin scattering)[a]
C_{11} (GPa)	5.50	5.49
C_{33} (GPa)	5.22	5.48
$C_{11}+C_{12}+2C_{66}$ (GPa)	12.4	12.8

[a] Reference [7].

the lattice vibration and the elastic deformation. On the other hand, the brittleness clearly was improved by the cross-linking as shown in our experiment. In order to clarify the influence of the cross-linking on macroscopic behaviors such as the brittleness, the hardness of the cross-linked crystals at 98% RH was measured by Vickers hardness test. It reached around 60 MPa at room temperature. The non-cross-linked one was about 20 MPa at the same environment [24]. This clearly demonstrates that the cross-linking by glutaraldehyde affected the macroscopic deformation. As a result, the cross-linking has little influence on the mechanical properties due to microscopic displacements such as elastic constants. Contrary to this, it affects macroscopic mechanical properties.

Moreover, the Voigt averages can be employed to estimate other physical properties. The averages for dynamic average shear modulus μ and the Lame constant λ in a tetragonal crystal give the following equations [10,25]:

$$\mu = 1/15(2C_{11} + C_{33} + 6C_{44} + 3C_{66} + C_{12} + 2C_{13}),$$

$$\lambda = 1/15(2C_{11} + C_{33} + 4C_{44} + 2C_{66} + 4C_{12} + 8C_{13}). \quad (1)$$

These averages, μ and λ, were calculated to be 0.70 and 4.04 GPa, respectively, by substituting the elastic constants C_{ij} of the cross-linked tetragonal HEW lysozyme crystals at 98% RH into Eq. (1). Using these averages, an average dynamic Young's modulus E can also be estimated [10,25]. The Young's modulus of the cross-linked crystals at 98% RH was evaluated to be 2.01 GPa. In addition, the bulk modulus $K = \lambda + 2/3\mu$ was also evaluated to be 4.51 GPa, while the compressibility $\beta = 1/K$ of the cross-linked crystals at 98% RH was evaluated to be 2.21×10^{-10}/Pa. The Poisson's ratio $\sigma = \lambda/2(\lambda + \mu)$ was 0.42. These evaluated mechanical properties of the cross-linked crystals at 98% RH are summarized in Table III.

To understand the effect on intracrystalline water in a crystal, it is worthwhile to investigate a change in elastic constants due to dehydration. Here, it should be noticed that only mobile water in the crystal was evaporated due to dehydration. That is, this mobile water in the crystal could play a key role in intermolecular interactions. Hereafter the intracrystalline water means mobile water.

Previously, we have reported all the elastic constants in tetragonal HEW lysozyme crystals dehydrated at 42% RH [10], which are listed in Table III. The elastic constants of the cross-linked crystals at 98% RH except $C_{11}=C_{22}$ and C_{33} were about one half as small as those of the non-cross-linked ones at 42% RH, while the shear components, $C_{44}=C_{55}$ and C_{66}, of the cross-linked crystals at 98% RH were one third and one fourth as small as those of the non-cross-linked ones at 42% RH. To clarify this result, the ratio of elastic constants of 98% RH to those of 42% RH vs a relative humidity is shown in Fig. 2. This plot clearly shows that the ratio was influenced by the intracrystalline water. The ratio on shear components of elastic constants, $C_{44}=C_{55}$ and C_{66}, particularly depended on the contents of intracrystalline water comparing with other elastic components. This demonstrated that the shear components $C_{44}=C_{55}$ and C_{66} were much softer than those of normal components, $C_{11}=C_{22}$ and C_{33}, due to the intracrystalline water. Thus, the intracrystalline water induced the softening of the shear elastic constants.

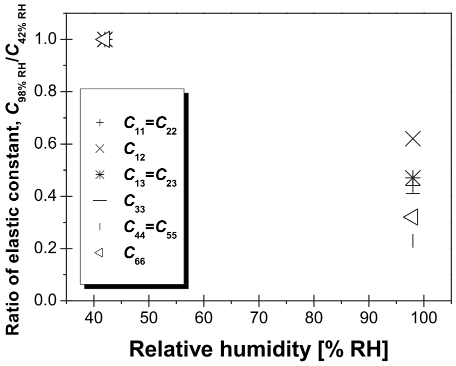

FIG. 2. The ratio of elastic constants of 42% RH to elastic constants of 98% RH versus a relative humidity.

Generally speaking, the intermolecular potentials between two protein molecules are not found yet as a function of interatomic distances in the protein crystals because the molecules contain a lot of atoms so that the interactions among molecules is very complex. Thus we could not estimate even the cohesive energy in a protein crystal. Therefore, unfortunately, this softening effect of shear elastic constants due to intracrystalline water cannot be interpreted by the intermolecular potentials.

Moreover, the previous shear elastic constants extrapolated from the longitudinal sound velocity in the dehydrated crystals [10] ($C_{44}=C_{55}$ and C_{66}), which are listed in Table III, are larger than the values measured in this work. This discrepancy comes from the softening of shear elastic constants.

In order to compare elastic constants with chemical bonds, typical elastic constants, C_{11} and C_{44}, of crystals with various kinds of chemical bonds were listed in Table V. The observed elastic constants C_{11} and $C_{44}=C_{55}$ of the cross-linked crystals at 98% RH were smaller than those of organic crystal composed of small molecules, for instance benzophenone crystals as shown in Table V. On the other hand, those values obtained for the non-cross-linked crystals at 42% RH are almost in agreement with those for organic crystals with small molecules, which mean that the intermolecular interactions in the dehydrated protein crystals are mainly due to van der Waals bonds [10].

On the contrary, it is also possible that the intermolecular interactions in tetragonal HEW lysozyme crystals containing large amount of water are due to other interactions different

TABLE V. Comparison of the elastic constants of tetragonal HEW lysozyme crystals with those of other crystals. *H*: hydrogen bonds, *V*: van der Waals bonds, *I*: ionic bonds, *M*: metallic bonds, and *C*: covalent bonds.

Crystals	Bonding type	C_{11} (GPa)	C_{44} (GPa)	Ref.
Cross-linked lysozyme at 98% RH	H,V,I	5.50	0.68	This result
Non-cross-linked lysozyme at 42% RH	H,V,I	12.44	2.97	[10]
Benzophenone	V	10.8	2.10	[27]
H_2O	H	15.3	4.46	[28]
NaCl	I	48.7	12.6	[25]
Cu	M	168	75.4	[25]
Si	C	165	79.6	[25]

from van der Waals forces. That is, the intracrystalline water would prevent van der Waals bonds between the molecules. Therefore, hydrogen bonds could be dominant for the intermolecular interactions in protein crystals with large amount of water. Concerning intermolecular interactions using the macrobond approach [26], we estimate that the influence of hydrogen bonds is larger than that of van der Waals bonds. In other words, it appears that the intermolecular interactions in protein crystals are mainly due to water-mediated hydrogen bonds, i.e., amino acid-water and water-water interactions. Therefore, it is concluded that the softening on elastic constants due to the intracrystalline water is related to the change in the intermolecular interaction from van der Waals bonds to water-meditated hydrogen bonds.

IV. CONCLUSION

We have observed the shear components of elastic constants, $C_{44}=C_{55}$ and C_{66}, for tetragonal HEW lysozyme crystals containing large amount of water in the crystal by improving the brittleness of protein using the chemical cross-linking method. The six elastic constants of the cross-linked tetragonal HEW lysozyme crystals were determined to be $C_{11}=C_{22}=5.50$ GPa, $C_{12}=4.33$ GPa, $C_{13}=C_{23}=3.94$ GPa, $C_{33}=5.22$ GPa, $C_{44}=C_{55}=0.68$ GPa, and $C_{66}=0.84$ GPa, respectively. Those elastic constants of the cross-linked crystals observed in this work are in almost agreement with those of the intrinsic ones without cross-linking. Compared with the elastic constants of non-cross-linked crystals at 42% RH, it was found that intracrystalline water in the crystal have a strongly influence on the softening of elastic constants, in particular, the shear components, $C_{44}=C_{55}$ and C_{66}. This suggests that the intracrystalline water in the crystal play an important role in intermolecular interactions in protein crystals. In addition, it seems that the intermolecular interactions in protein crystals containing large amount of water are mainly due to water-mediated hydrogen bonds.

ACKNOWLEDGMENTS

The authors thank Professor M. Satoh and Dr. H. Hashimoto at Yokohama City University for x-ray analysis of lysozyme crystals and Professor R. Micheletto at Yokohama City University for critically reading and discussing this paper. This research was partially supported by KAKENHI Grant-in Aid for Scientific Research C (Grant No. 18560648).

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