Observation of all the components of elastic constants using tetragonal hen egg-white lysozyme crystals dehydrated at 42% relative humidity

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Success in measuring transverse sound velocity allowed us to determine, for the first time, all six elastic constants of a protein crystal. An ultrasonic pulse-echo method was used to perform sound velocity measurements on tetragonal hen egg-white (HEW) lysozyme crystals that were partially dehydrated at 42% relative humidity. The measurements were performed using the (110), (101), and (001) crystallographic faces. Thus, all six elastic constants of the dehydrated tetragonal HEW lysozyme crystals were determined: $C_{11}=C_{22}=12.44$ GPa, $C_{12}=7.03$ GPa, $C_{13}=C_{23}=8.36$ GPa, $C_{33}=12.79$ GPa, $C_{44}=C_{55}=2.97$ GPa, and $C_{66}=2.63$ GPa. In addition, for the hydrated crystals, the longitudinal sound velocities along the [110] direction and the direction normal to the (101) face were measured. From these results, all the components of elastic constants in the hydrated crystals were extrapolated.

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I. INTRODUCTION

In order to advance research on protein molecules and protein crystals, it is necessary to grow highly perfect crystals. This is because the defect structures of the crystals hinder the determination of three-dimensional protein structures using x-ray diffraction and neutron diffraction methods. Moreover, even if highly perfect crystals could be grown, the crystals can be damaged and/or deformed by poor post-growth treatments. To obtain highly perfect protein crystals, it is important to know the defect structure, deformation, and fracture. The perfection strongly depends on crystal defects such as point defects (including impurities), dislocations, segregations, voids, and microcracks. Energy calculations for the defects involving the theory of elasticity require that all elastic constants, C_{ij}, be known. In particular, for protein crystals with low symmetry, the anisotropic elastic constants are essential for estimating the stress fields and their energies. Thus, it is important to determine all the components of elastic constants in a protein crystal.

Measurements of Young's modulus in protein crystals were previously carried out using the static or vibration method for the frequency range below KHz in cross-linked hen egg-white (HEW) lysozyme crystals with a monoclinic crystal structure [1–3]. Meanwhile, we measured the combination of some elastic constants of hydrated and dehydrated tetragonal HEW lysozyme crystals without cross-linking using a longitudinal ultrasonic wave (10 MHz) by the pulse-echo method [4,5]. Moreover, Speziale *et al.* have determined some components of elastic constants in tetragonal HEW lysozyme crystals as a function of humidity for the frequency range of 1 GHz using Brillouin scattering [6,7]. They determined two elastic constants, C_{11} and C_{33}. Recently, Fourme *et al.* measured the compressibility in hydrated tetragonal HEW lysozyme crystals under high pressure using x-ray analysis [8]. Chernov has measured the static Young's modulus of monoclinic HEW lysozyme crystals [9]. Thus, there has not yet been a measurement of all the components of elastic constants, C_{ij}, in HEW lysozyme crystals.

To determine all the components of elastic constants in protein crystals, both the longitudinal and transverse sound velocities need to be measured. In the pulse-echo method, in order to generate a transverse ultrasonic wave in the crystal, a transducer must be in close contact with the crystal. However, since hydrated protein crystals are fragile, it was difficult to glue a transducer to a protein crystal. Thus, measurements of transverse sound velocity have not been previously carried out on protein crystals. Recently, we found that the sound velocity increases with exposure to air and approaches a constant value [5]. This suggested that the protein crystals hardened due to dehydration. Thus, we succeeded in establishing contact between a crystal and transducer using dehydrated crystals. As a result, we were able to generate a transverse ultrasonic wave through the dehydrated HEW lysozyme crystals at 42% relative humidity (RH) and determine all the components of elastic constants in dehydrated crystals. In this paper, we report all the six elastic constants of tetragonal HEW lysozyme crystals dehydrated at 42% RH obtained using an ultrasonic pulse-echo method.

II. EXPERIMENTAL

Lysozyme crystals were grown through a salt-concentration gradient method at 23 °C vertically in test tubes, using $NiCl_2$ as a precipitant [10,11]. Large crystals up to 4 mm in size were grown over a period of two weeks. The crystals had a tetragonal structure with the space group $P4_32_12$, lattice constants $a=b=79.1$ Å, $c=37.9$ 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 morphology of HEW lysozyme crystals is schematized in Fig. 1. The high quality of as-grown crystals was characterized by x-ray topography [12,13]. These highly perfect hydrated crystals were slowly dehydrated at 42% RH for three months. The crystal structure and lattice constants of the dehydrated crystals were investigated using x-ray diffraction.

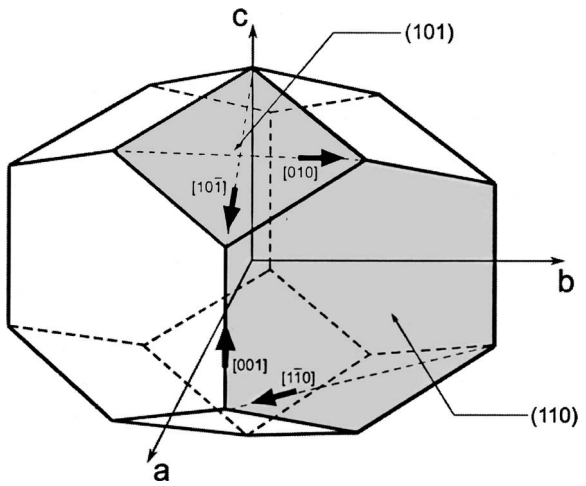

FIG. 1. A schematic of the morphology of HEW lysozyme crystals. The arrows show the polarization direction.

Sound velocity measurements were carried out using the pulse-echo mode of operation of an ultrasonic pulser/receiver (PR35, JSR Ultrasonics) at room temperature and ambient humidity. Longitudinal and transverse ultrasonic transducers with frequencies of 18 MHz and 5 MHz, respectively, were used. A close contact between the transducer and crystal is essential for the measurement of the transverse ultrasonic wave. Thus, a shear gel (Sonotech Co., Ltd.) was used as an adhesive to glue the dehydrated crystal to the transducer. Measurements of the transverse ultrasonic wave were carried out using the larger (110) and (101) habit faces of the dehydrated tetragonal HEW lysozyme crystals. The transverse ultrasonic waves were propagated vertically along each face. Measurements of the longitudinal ultrasonic wave were also carried out using the larger (110) and (101) habit face. In addition, to introduce the longitudinal ultrasonic wave perpendicular to the (001) face of the dehydrated crystal, the crystal was cut parallel to the (001) face. The traveling time of the ultrasonic wave, spent passing through the crystal, was measured as the time interval between reflected echoes from the bottom and top faces of the crystal. The traveling distance is twice the thickness of the crystal. The crystal thickness was measured with a micrometer just after the ultrasonic measurements.

To extrapolate the elastic constants of the hydrated HEW lysozyme crystals, the measurement of a longitudinal ultrasonic wave through crystals in solution was carried out using a transducer with a frequency of 18 MHz. The larger (110) and (101) habit faces of the hydrated tetragonal HEW lysozyme crystals were used in the measurements of the longitudinal sound velocity.

III. RESULTS AND DISCUSSION

X-ray diffraction revealed that the dehydrated HEW lysozyme crystals had a tetragonal structure with the space group $P4_32_12$, lattice constants $a=b=73.9$ Å, $c=31.2$ Å, and eight molecules per unit cell. The lattice constants for the dehydrated and hydrated crystals are compared in Table I. As shown in Table I, the a and b axes shrank by 6.5%, while the c axis shrank by 17.6% upon dehydration. Dobrianov *et al.* have measured the changes in the lattice constants of tetragonal HEW lysozyme crystals during dehydration to be ~4% and ~15% in the case of the a and b axes and the c axis, respectively [14], which are comparable to our results.

The volume percent of water molecule in the crystal can be estimated from the lattice constants of the dehydrated crystals. The volume percent of water molecules in the crystal can be estimated using the following equation [15]:

$$V_{solv.} = 1 - \frac{1.66\bar{v}}{V_M} \approx 1 - \frac{1.23}{V_M}, \tag{1}$$

where $\bar{v}$ is the partial specific volume of the protein in the crystal, and V_M is the unit cell in Å^3 per protein molecular weight in the unit cell. For most proteins, $\bar{v}$ is approximately 0.74 cm^3/g. From Eq. (1), the volume percent of water in the crystal, $V_{solv.}$, in the dehydrated tetragonal HEW lysozyme crystal was estimated to be 16 vol %, assuming that the molecular weight of lysozyme is 14 600 and the number of molecules per unit cell is eight. Moreover, the density of the crystal ρ was estimated to be 1.29 Mg/m^3 for the dehydrated tetragonal HEW lysozyme crystals. The water content (vol %) and density of the dehydrated and hydrated crystals are compared in Table I. Here, taking the water content of the crystal to be 16 vol %, the amount of residual water per unit cell was estimated to be 2.7×10^{-20} g/unit cell.

The water in the protein crystals was qualitatively classified into two types in terms of its behavior: (i) mobile water between the protein molecules and (ii) immobile water strongly bound to the protein molecules [15,16]. Both types will be present in the crystal after exposure to air. Mobile water easily evaporates from the crystal, while immobile water will typically remain in the crystal. According to a neutron crystallographic study using tetragonal HEW lysozyme crystals grown by a salt concentration gradient method similar to that used in the present study, 157 bound water mol-

TABLE I. The lattice constants, space groups, solvent contents, and densities of dehydrated and hydrated crystals.

	Lattice constant				
	a, b axis	c axis	Space group	Solvent content	crystal density ρ(Mg/m^3)
Hydrated crystal	79.1 Å	37.9 Å	$P4_32_12$	39 vol %	1.21
Dehydrated crystal	73.9 Å	31.2 Å	$P4_32_12$	16 vol %	1.29

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

Propagation direction	Polarization direction	Measured velocity	ρv^2	Sound velocity (m/s)	
				Dehydrated crystal	Hydrated crystal
[110]	Longitudina	v_1	$(C_{11}+C_{12}+2C_{66})/2$	3097	2070
	Transverse [001]	v_2	C_{44}	1518	—
	Transverse $[1\bar{1}0]$	v_3	$(C_{11}-C_{12})/2$	1448	—
[001]	Longitudinal	v_4	C_{33}	3149	—
In (010) plane, at an angle of θ=22.8° to [001] axis.	Transverse [010]	v_5	$C_{44}\cos^2\theta+C_{66}\sin^2\theta$	1505	—
	Quasilongitudinal	v_6	$C^{\dagger}$	3197	2001
	Quasitransverse $[10\bar{1}]$	v_7	$C^{\ddagger}$	1481	—

$C^{\dagger}=\frac{1}{2}\{C_{11}\sin^2\theta+C_{33}\cos^2\theta+C_{44}+\sqrt{(C_{11}\sin^2\theta-C_{33}\cos^2\theta+C_{44}\cos 2\theta)^2+(C_{13}+C_{44})^2\sin^2 2\theta}\}$
$C^{\ddagger}=\frac{1}{2}\{C_{11}\sin^2\theta+C_{33}\cos^2\theta+C_{44}-\sqrt{(C_{11}\sin^2\theta-C_{33}\cos^2\theta+C_{44}\cos 2\theta)^2+(C_{13}+C_{44})^2\sin^2 2\theta}\}$

ecules were observed on a lysozyme molecule [17]. From this study, the corresponding weight of the bound water can be estimated to be 3.7×10^{-20} g/unit cell. This is in moderate agreement with the estimate of 2.7×10^{-20} g/unit cell for residual water obtained in the present study. This suggests that almost all the mobile water in the protein molecules evaporated. Thus, it can be concluded that the residual water molecules in the dehydrated tetragonal HEW lysozyme crystals represent immobile water strongly bound to the protein molecules.

The sound velocity measurements were carried out using the (110), (101), and (001) planes, as shown in Fig. 1. The propagation and polarization directions, together with the corresponding velocities in terms of the elastic constants, are presented in Table II. For the (110) face, all the wave propagation directions were along the [110] direction. One longitudinal sound velocity, v_1, and two transverse sound velocities, v_2, with a polarization direction in the [001] direction and, v_3, with a polarization direction in the $[1\bar{1}0]$ direction, were measured. For the (001) face, a longitudinal sound velocity, v_4, was measured as well, and this wave was propagated along the [001] direction. The measured sound velocities $v_1$and v_4 were pure longitudinal mode velocities, while v_2 and v_3 were pure transverse mode velocities. On the other hand, for the (101) face, the wave propagation directions were not along the [101] direction. Thus, only the transverse sound velocity, v_5, with a polarization in the [010] direction was a pure mode. The quasilongitudinal wave, v_6, propagated perpendicular to the (101) plane. The quasitransverse sound velocity, v_7, with a polarization in the $[10\bar{1}]$ direction propagated perpendicular to the (101) plane. Figure 2 shows the typical wave form of the reflected transverse ultrasonic wave along the [110] direction, polarized in the [001] direction, for the dehydrated tetragonal HEW lysozyme crystals. As seen in the figure, it is found that a transverse ultrasonic wave is generated in the dehydrated tetragonal HEW lysozyme crystals, although the transverse ultrasonic wave signal was weak relative to the longitudinal ultrasonic wave signal. The first reflected wave shows the reflected echo from the bottom of the crystal and the second reflected wave comes from the top of the crystal surface. The traveling time for the transverse ultrasonic wave was measured in a number of crystals with different thicknesses of ~1.5 mm to ~3 mm. The relationship between crystal thickness and traveling time is shown in Fig. 3. From the slope of the straight line fit to the data in Fig. 3, the transverse sound velocity, v_2, of the dehydrated crystals was determined to be 1518 m/s. Similarly, v_1, v_3, v_4, v_5, v_6, and v_7 for the dehydrated crystals

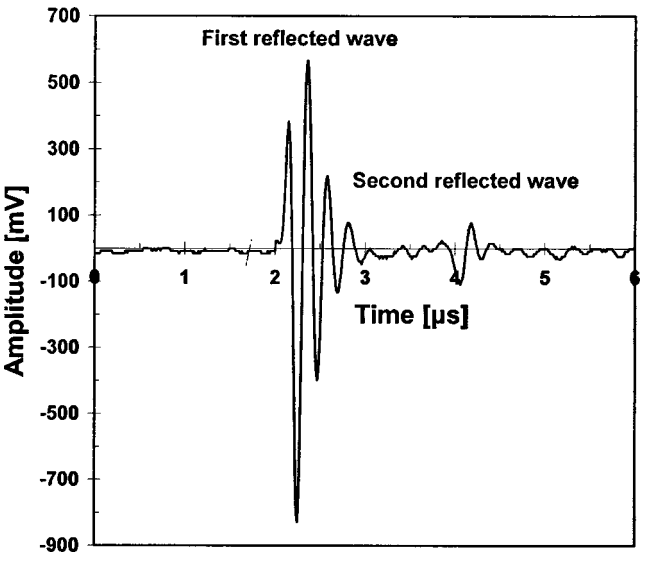

FIG. 2. The typical wave form of the reflected transverse ultrasonic wave along the [110] direction of a tetragonal HEW lysozyme crystal dehydrated at 42% RH, obtained using a transducer at a frequency of 5 MHz. The wave was polarized along the [001] direction.

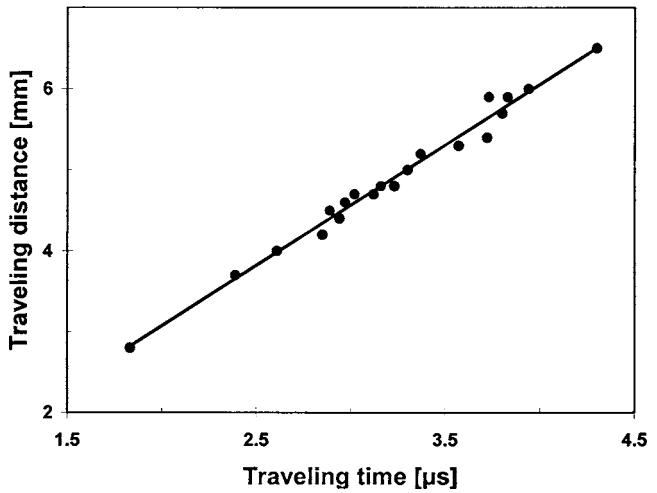

FIG. 3. The relationship between traveling distance and traveling time for the reflected transverse ultrasonic wave along the [110] direction of a tetragonal HEW lysozyme crystal dehydrated at 42% RH. The wave was polarized along the [001] direction.

were measured to be 3097, 1448, 3149, 1505, 3197, and 1481 m/s, respectively. The values of the sound velocity measured for the dehydrated tetragonal HEW lysozyme crystals are tabulated in Table II.

Elastic constants for the crystals were estimated from the sound velocity values. The dehydrated 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 a sound wave along the [110] direction in this crystal, the solution for the Christoffel equation is given by following equations:

$$\rho v_1^2 = \frac{1}{2}(C_{11} + C_{12} + 2C_{66}), \quad \rho v_2^2 = C_{44},$$

$$\text{and} \quad \rho v_3^2 = \frac{1}{2}(C_{11} - C_{12}), \tag{2}$$

where ρ is the density of the crystal. In addition, for a sound wave along the [001] direction in this crystal, the solution for the Christoffel equation is given by following equation:

$$\rho v_4^2 = C_{33}. \tag{3}$$

Similarly, for the (101) face in this crystal, the solution of the Christoffel equation is given by [18],

$$\rho v_5^2 = C_{44}\cos^2\theta + C_{66}\sin^2\theta, \tag{4}$$

$$\rho v_6^2 = \frac{1}{2}\{C_{11}\sin^2\theta + C_{33}\cos^2\theta + C_{44} + \sqrt{(C_{11}\sin^2\theta - C_{33}\cos^2\theta + C_{44}\cos 2\theta)^2 + (C_{13}+C_{44})^2\sin^2 2\theta}\}, \tag{5}$$

$$\rho v_7^2 = \frac{1}{2}\{C_{11}\sin^2\theta + C_{33}\cos^2\theta + C_{44} - \sqrt{(C_{11}\sin^2\theta - C_{33}\cos^2\theta + C_{44}\cos 2\theta)^2 + (C_{13}+C_{44})^2\sin^2 2\theta}\}, \tag{6}$$

where θ is the angular direction of the ultrasonic wave vector with respect to the [001] direction. From the x-ray diffraction of the dehydrated crystals, $a=b=73.9$ Å, $c=31.2$ Å, and consequently $\theta=22.8°$. By substituting the measured sound velocities obtained in this work into the above expressions, C_{ij} was calculated. Here, the sound velocities v_1, v_2, v_3, v_4, and v_5 are pure mode, whereas the sound velocities v_6 and v_7 are quasilongitudinal and quasitransverse mode, respectively. From Eqs. (2) and (4), all elastic constants except C_{13} were determined. C_{13} was then calculated by substituting the determined elastic constants into Eq. (5). The six elastic constants for the dehydrated tetragonal HEW lysozyme crystals were thus determined to be $C_{11}=C_{22}=12.44$ GPa, $C_{12}=7.03$ GPa, $C_{13}=C_{23}=8.36$ GPa, $C_{33}=12.79$ GPa, $C_{44}=C_{55}=2.97$ GPa, and $C_{66}=2.63$ GPa, as presented in Table III. According to Born's stability criterion [19], the elastic strain energy density of crystals should be positive for all combinations of strain components. In a tetragonal crystal, Born's stability criterion is given by

$$C_{11} - |C_{12}| > 0,$$

$$(C_{11} + C_{12})C_{33} - 2C_{13}^2 > 0,$$

$$C_{66} > 0. \tag{7}$$

The elastic constants estimated in this work are found to satisfy Born's stability criterion.

To determine the origin of the elastic constants in protein crystals, Table IV compares the elastic constants of the dehydrated crystals with those of other crystals. As can be seen in Table IV, the values of the elastic constants of the dehydrated crystals are mostly in agreement with those of benzophenone, which is a molecular crystal, and ice, which is a crystal formed by hydrogen bonding. It seems that the intermolecular interactions in the dehydrated tetragonal HEW lysozyme crystals are mainly due to van der Waals interactions and hydrogen bonds. In general, elastic constants will increase with increasing total bonding energy. Thus, the elastic constants of the lysozyme crystals would be expected to be larger than those of the benzophenone crystals. However, the magnitudes of the two sets of elastic constants were almost the same. Thus, the elastic constants of protein crystals

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 the dehydrated and hydrated crystals.

		Dehydrated crystal	Hydrated crystal (Extrapolated values)
Elastic constant (GPa)	$C_{11}=C_{22}$	12.44	5.52
	C_{12}	7.03	3.12
	$C_{13}=C_{23}$	8.36	3.71
	C_{33}	12.79	5.68
	$C_{44}=C_{55}$	2.97	1.32
	C_{66}	2.63	1.16
Young's modulus (GPa)	E	7.25	3.21
Shear modulus (GPa)	μ	2.64	1.17
Bulk modulus (GPa)	K	9.46	4.20
Compressibility (1/Pa)	β	1.05×10^{-10}	2.38×10^{-10}
Poisson's ratio	σ	0.37	0.37

presumably originate from the intermolecular interactions in the crystals. These interactions can be understood through the macrobond approach in which a macrobond contact is defined if at least one atom pair whose distance is less than 4 Å exists between neighboring molecules with bound water molecules [20,21]. That is, only atoms near the surface of each lysozyme molecule are important in the overall intermolecular interactions [22].

As shown in Table III, C_{66} is equal to C_{44}. Thus, the anisotropic factor A for a cubic crystal was employed for the tetragonal crystal. Using elastic theory [23], the anisotropic factor $A=2C_{44}/(C_{11}-C_{12})$ for the dehydrated crystals was estimated to be 1.09. This suggests that the dehydrated tetragonal HEW lysozyme crystals can be assumed to be isotropic bodies. Thus, the Voigt averages were used to estimate an average dynamic Young's modulus [23]. The Voigt averages for μ and λ in the tetragonal crystal give the following equations:

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

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

Crystal	Bonding type	C_{11} (GPa)	C_{44} (GPa)
Dehydrated lysozyme crystal	H, V, I	12.44	2.97
Hydrated lysozyme crystal[a]	H, V, I	5.52	1.32
Benzophenone (Ref. [25])	V	10.8	2.10
H_2O (Ref. [26])	H	15.3	4.46
NaCl (Ref. [23])	I	48.7	12.6
Cu (Ref. [23])	M	168	75.4
Si (Ref. [23])	C	165	79.6

[a]Extrapolated values.

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

We obtained $\mu=2.64$ GPa and $\lambda=7.70$ GPa by substituting the elastic constants C_{ij} of the dehydrated crystals into Eq. (8). Using these Voigt averages, an average dynamic Young's modulus E can be estimated from the following equation:

$$E = \frac{\mu(3\lambda + 2\mu)}{\lambda + \mu}. \quad (9)$$

The Young's modulus of the dehydrated crystals was evaluated to be 7.25 GPa. Moreover, we also obtained the bulk modulus $K=\lambda+2/3\mu=9.46$ GPa, while the compressibility $\beta=1/K$ of the dehydrated crystals was evaluated to be 1.05×10^{-10}/Pa. In addition, the Poisson's ratio was $\sigma=\lambda/2(\lambda+\mu)=0.37$. Recently, the compressibility of tetragonal HEW lysozyme crystals under high pressure ($\sim$1 GPa) was estimated to be 0.98×10^{-10}/Pa from x-ray analysis [8]. This value roughly agrees with that obtained in our study. This means that the dehydrated crystals are compressed to a higher pressure ($\sim$1 GPa) relative to the hydrated crystal. In addition, the compressibility of the lysozyme molecule itself is 0.77×10^{-10}/Pa, which is comparable to the values obtained in our study [24].

It is worth extrapolating the elastic constants of the hydrated crystals on the basis of C_{ij} in the case of dehydrated crystals. However, the transverse ultrasonic wave could not be measured in the hydrated crystals. Thus, the elastic constants of the hydrated crystals were extrapolated from the ratio of the longitudinal sound velocity between the dehydrated and hydrated crystals. The longitudinal sound velocities along the [110] direction, v_1, and along the direction normal to the (101) face, v_6, of the hydrated crystals were measured to be 2070 m/s and 2001 m/s, respectively. The values of the sound velocity along the [110] direction were higher than previously measured values [4]. One possible explanation for this is that the perfection of tetragonal HEW lysozyme crystals was improved. The sound velocities along the [110] and [101] directions for the hydrated and dehy-

TABLE V. Comparison of elastic constants measured at different relative humidities using the ultrasonic pulse-echo method and Brillouin scattering.

	Ultrasonic pulse-echo method (our work)			Brillouin scattering (Refs. [6,7])		
	Experimental		Extrapolated			
	Hydrated crystal	Dehydrated crystal at 42% RH	Hydrated crystal	98% RH	79% RH	67% RH
ρ(Mg/m^3)	1.21	1.29	1.21	1.21	1.25	1.27
$C_{11}+C_{12}+2C_{66}$ (GPa)	10.36	24.74	—	12.81	18.2	22.2
C_{33} (GPa)	—	12.79	5.68	5.48	9.57	11.7
C_{11} (GPa)	—	12.44	5.52	5.49	—	—

drated crystals are compared in Table II. The longitudinal sound velocity of the dehydrated crystals is about 1.5 times as fast as that of the hydrated crystals. Consequently, the elastic constant $C_{11}+C_{12}+2C_{66}$ of the dehydrated crystals corresponding to v_l was about 2.25 times as high as that of the hydrated crystal. Thus, it was assumed that all the elastic constants of the hydrated crystals are 1/2.25 times that of the dehydrated crystals. The extrapolated elastic constants of the hydrated crystals were evaluated to be $C_{11}=C_{22}=5.52$ GPa, $C_{12}=3.12$ GPa, $C_{13}=C_{23}=3.71$ GPa, $C_{33}=5.68$ GPa, $C_{44}=C_{55}=1.32$ GPa, and $C_{66}=1.16$ GPa, as presented in Table III. These elastic constants satisfy Born's stability criterion, as those of the dehydrated crystals. In addition, the Young's modulus E, Shear modulus μ, Bulk modulus K, compressibility β, and Poisson's ratio σ of the hydrated crystals using the Voigt average elastic constants were estimated to be 3.21 GPa, 1.17 GPa, 4.20 GPa, 2.38×10^{-10}/Pa and 0.37, respectively.

Recently, the longitudinal sound velocity and some elastic constants of the hydrated tetragonal HEW lysozyme crystals have been determined using Brillouin scattering by Speziale *et al.* [6,7]. The measurement of a transverse ultrasonic wave could not be achieved, but measurements of the longitudinal ultrasonic wave along the [110], [001], and [100] directions were performed as a function of relative humidity. The values of $C_{11}+C_{12}+2C_{66}$, C_{33}, and C_{11} obtained using our ultrasonic pulse echo and the Brillouin scattering methods are tabulated in Table V. As shown in Table V, the values of $C_{11}+C_{12}+2C_{66}$ and C_{33} obtained using our ultrasonic pulse-echo method and the Brillouin scattering method increased with decreasing relative humidity. Here, let us note the values of $C_{11}+C_{12}+2C_{66}$. The value of $C_{11}+C_{12}+2C_{66}$ at 98% RH obtained using the Brillouin scattering method agree with that obtained using our ultrasonic pulse-echo method despite the fact that the frequency of the excited wave differs by three orders of magnitude. This suggests that there is little dispersion in the frequency range from ~5 MHz to ~1 GHz. Next, let us examine the extrapolated values for the hydrated crystals. These values are in good agreement with those at 98% RH obtained using the Brillouin scattering method. Thus, it seems that the extrapolated elastic constants of the hydrated HEW lysozyme crystals are reasonable.

More recently, Chernov has determined the static Young's modulus for hydrated monoclinic HEW lysozyme crystals to be 0.1–0.5 GPa through bending experiments [9]. This value is much smaller than that obtained by dynamic methods such as the ultrasonic pulse-echo method. This suggests that there is a frequency dependence of the Young's modulus in the quasistatic regime. That is, while there is no dispersion in the dynamic regime (from 5 MHz to 1 GHz), there may be at lower frequencies.

IV. CONCLUSIONS

We have demonstrated that transverse sound velocity measurements in tetragonal HEW lysozyme crystals dehydrated at 42% RH can be performed using the ultrasonic pulse-echo method. Thus, the elastic constants of dehydrated tetragonal HEW lysozyme crystals were determined to be $C_{11}=C_{22}=12.44$ GPa, $C_{12}=7.03$ GPa, $C_{13}=C_{23}=8.36$ GPa, $C_{33}=12.79$ GPa, $C_{44}=C_{55}=2.97$ GPa, and $C_{66}=2.63$ GPa.

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[1] V. N. Morozov and T. Y. Morozova, Biopolymers **20**, 451 (1981).

[2] V. N. Morozov, T. N. Morozova, G. S. Kachalova, and E. T. Myachin, Int. J. Biol. Macromol. **10**, 329 (1988).

[3] V. N. Morozov and T. Y. Morozova, J. Biomol. Struct. Dyn. **11**, 459 (1993).

[4] M. Tachibana, K. Kojima, R. Ikuyama, Y. Kobayashi, and M. Ataka, Chem. Phys. Lett. **332**, 259 (2000). **354**, 360 (2002).

[5] M. Tachibana, H. Koizumi, and K. Kojima, Phys. Rev. E **69**, 051921 (2004).
[6] C. L. Caylor, S. Speziale, S. Kriminski, T. Duffy, C.-S. Zha, and R. E. Thorne, J. Cryst. Growth **232**, 498 (2001).
[7] S. Speziale, F. Jiang, S. Kriminski, C.-S. Zha, R. E. Thorne, and T. S. Duffy, Biophys. J. **85**, 3202 (2003).
[8] R. Fourme, R. Kahn, M. Mezouar, E. Girard, C. Hoerentrup, T. Prangé, and I. Ascone, J. Synchrotron Radiat. **8**, 1149 (2001).
[9] A. A. Chernov, J. Struct. Biol. **142**, 3 (2004).
[10] M. Ataka and T. Katsura, JAERI-M 61, 92 (1992)..
[11] M. Tachibana and K. Kojima, Curr. Top. Cryst. Growth Res. **6**, 35 (2002).
[12] K. Izumi, K. Taguchi, Y. Kobayashi, M. Tachibana, K. Kojima, and M. Ataka, J. Cryst. Growth **206**, 155 (1999).
[13] M. Tachibana, H. Koizumi, K. Izumi, K. Kajiwara, and K. Kojima, J. Synchrotron Radiat. **10**, 416 (2003).
[14] I. Dobrianov, S. Kriminski, C. L. Caylor, S. G. Lemay, C. Kimmer, A. Kisselev, K. D. Finkelstein, and R. E. Thorne, Acta Crystallogr. **D57**, 61 (2001).
[15] B. W. Matthews, J. Mol. Biol. **33**, 491 (1968).
[16] G. Otting, E. Liepinsh, and K. Wuthrich, Science **254**, 974 (1996).
[17] N. Niimura, Y. Minezaki, T. Nonaka, J.-C. Castagna, F. Cipriani, P. Høghøj, M. S. Lehmann, and C. Wilkinson, Nat. Struct. Biol. **4**, 909 (1997).
[18] C. G. Winterheimer and A. K. McCurdy, Phys. Rev. B **18**, 6576 (1978).
[19] M. Born and K. Huang, *Dynamical Theory of Crystal Lattices* (Oxford, New York, 1969).
[20] H. Oki, Y. Matsuura, H. Komatsu, and A. A. Chernov, Acta Crystallogr. **D55**, 114 (1999).
[21] Y. Matsuura and A. A. Chernov, Acta Crystallogr. **D59**, 1347 (2003).
[22] H. Koizumi, M. Tachibana, H. Kawamoto, and K. Kojima, Philos. Mag. **84**, 2961 (2004).
[23] J. P. Hirth and J. Lothe, *Theory of Dislocations*, 2nd ed. (Wiley, New York, 1982).
[24] K. Gekko and Y. Hasegawa, Biochemistry **25**, 6563 (1986).
[25] R. A. Pethrick, J. N. Sherwood, and C. S. Yoon, Philos. Mag. A **65**, 1033 (1992).
[26] C. A. Tulk, R. E. Gagnon, H. Kiefte, and J. Clouter, J. Chem. Phys. **101**, 2350 (1994).