

## Bovine Serum Albumin 수용액의 초음파 측정

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## Ultrasonic Measurement in Bovine Serum Albumin Solution

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**요 약.** 단백질 수용액 중의 초음파흡수 mechanism을 규명하기 위하여 pH 7의 소혈청 albumin(BSA) 수용액의 초음파흡수 측정을 주파수 100 kHz에서 1600 MHz에 걸쳐 행하였다. 측정방법은 plano-concave 공명법(0.1~10 MHz), Bragg 반사법(12~100 MHz)과 고분해능 Bragg 반사법(120~1600 MHz)을 사용하였다. pH 7에서의 초음파흡수스펙트럼은 Davidson-Cole 분포식의 거울상인 분포함수를 사용한 완화곡선과 일치하였다. 이 완화현상을 BSA 분자의 수화평행에 의한 것으로 해석하였다.

**ABSTRACT.** Ultrasonic absorption was measured in bovine serum albumin (BSA) aqueous solution (50 g/l) in the frequency range from 100 kHz to 1600 MHz at neutral pH. Three experimental techniques were used to cover the wide frequency range: plano-concave resonator, conventional Bragg reflection, and high-resolution Bragg reflection methods. The absorption spectrum at neutral pH fitted the relaxation curve well using the distribution function of a mirror image of Davidson-Cole function. The relaxation behavior was interpreted in terms of various degree of hydration of BSA molecules.

### INTRODUCTION

Bovine serum albumin (BSA) is one of the typical and popular proteins, and a number of ultrasonic works have been made on BSA to understand the mechanism of ultrasonic absorption in protein solutions<sup>1-3</sup>. Kessler and Dunn<sup>1</sup> first measured the absorption spectra up to 163 MHz over the pH range 2.3~11.8. The excess absorption below pH 4.3 and above pH 10 was attributed to conformational changes. Lang *et al.*<sup>2</sup>, however, showed that proton transfer reactions at the acidic and alkaline side chains were responsible for the excess absorption peaks. Recently Barnes *et al.*<sup>4-6</sup> measured the absorption in the frequency range 60 kHz to 1 MHz using a spherical ultrasonic resonator. They showed that a maximum absorption

per wavelength existed at 400 kHz in the acidic region and at 3 MHz in the alkaline region, and ascribed those to the proton transfer reactions at carboxyl and amino groups, respectively.

Another problem unsettled so far, however, is the relaxation mechanism operating at neutral pH. Kessler and Dunn<sup>1</sup> proposed a solute-solvent equilibrium as the relaxation mechanism on the basis of the distribution of relaxation times. Broad distributions of relaxation time were also observed in hemoglobin solutions by Carstensen and Schwan<sup>7</sup>. O'Brien and Dunn<sup>8</sup>, and Schneider *et al.*<sup>9</sup>, and in myoglobin and some peptides by Strom-Jensen and Dunn<sup>10</sup>. Most of these authors indicated the solute-solvent equilibrium as the relaxation mechanism. Cho *et al.*<sup>11</sup>, on the other hand, sugge-

sted volume fluctuations of protein molecules are responsible for the pH-independent absorption. The greatest difficulty to be encountered in measuring ultrasonic properties of protein solutions lies in the fact that the relaxation region extends to a very wide frequency range. The frequency range of previous experiments has been confined to two orders of magnitude at the widest, for example 1 to 100 MHz, because of experimental difficulties. In the situation, more extensive and precise ultrasonic study is needed as functions of frequency to gain clearer insight into the relaxation mechanisms.

This paper describes ultrasonic absorption measurements in BSA aqueous solutions over the frequency range 0.1~1600 MHz in the neutral pH. The absorption spectrum observed at neutral pH was successfully analyzed with the distribution of relaxation time assuming a mirror-image curve of the Davidson-Cole function. This distribution function suggests that hydration of BSA molecules is responsible for the absorption.

## EXPERIMENT

The crystallized and lyophilized sample of bovine serum albumin (Sigma Chemical Co., A7638) was dissolved in distilled water of chromatography grade to make solutions with the concentration

of 50 g/l. The pH after the dissolution was 7. The measurement was carried out at the temperature of 20°C. The temperature was controlled to within 0.1°C.

We used three experimental techniques for measuring ultrasonic absorption to cover the wide frequency range 0.1~1600 MHz: a plano-concave resonator in the range 0.1~10 MHz<sup>12</sup>, Bragg reflection method in the range 12~100 MHz<sup>13</sup>, and high-resolution Bragg reflection method (HRB) in the range 120~1600 MHz<sup>14</sup>. The key apparatus of the present work is the plano-concave resonator that covers the lowest frequency range where the data have been scarce. The method is briefly described here.

A block diagram of the new resonator method is shown in Fig. 1. A quartz transducer driven by a frequency synthesiser excites continuous waves and standing waves are set up in the plano-concave cavity. We can observe the intensity of the standing waves by monitoring the Raman-Nath diffracted light. The amplitude of the first-order diffracted light is proportional to that of the sound. A resonance spectrum is obtained with an optical heterodyne system. The bandwidth of one resonance curve gives absorption coefficient of the sample liquid. The high-quality factor attained with this resonator allowed the reliable absorption measurements in the frequency range below 1 MHz, where the conventional plano-concave resonator has poor accuracy. Fig. 2 show a cross sec-

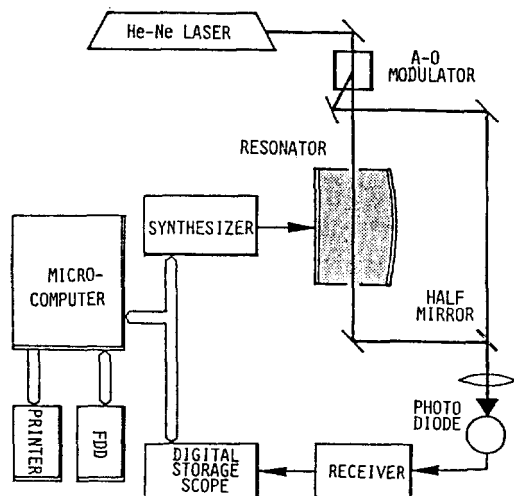


Fig. 1. Block diagram of the plano-concave resonator.

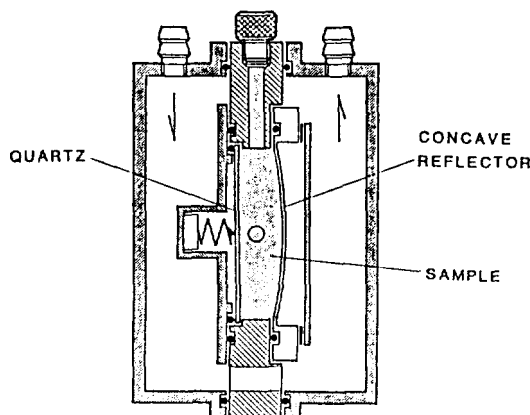


Fig. 2. Cross section of the plano-concave resonator.

tion of the resonator. The cylindrical cell is made of stainless steel and has two glass windows to allow the laser beam to pass through. An X-cut quartz transducer with a fundamental frequency of 2 MHz and a 56 mm effective diameter, and a concave reflector of stainless steel, are mounted at each end of the cell wall. The distance between them is about 15 mm, and this cell contains approximately 50 ml of sample liquid. The cell wall was made taking special care that the cell ends be parallel; therefore, no extra mechanism to adjust the parallelism between the quartz and reflector was necessary.

## RESULTS AND DISCUSSION

**Analysis of the absorption spectrum.** Results of absorption measurements at pH 7 are shown in Fig. 3 as a function of frequency. The data obtained by the three different techniques are smoothly connected with each other without any normalization procedure. The value of absorption divided by the square of frequency  $\alpha/f^2$  decreases linearly with frequency in the lower frequency range, and approaches the high-frequency limiting

value  $(\alpha/f^2)_\infty = 27 \times 10^{-15} \text{ s}^2/\text{m}$ . This value is almost equal to the absorption in water  $\alpha/f^2 = 25.3 \times 10^{-15} \text{ s}^2/\text{m}^{15}$ , suggesting the relaxation process does not contribute at frequencies above 1 GHz. The variation in  $\alpha/f^2$  in the wide frequency range indicates a distribution of relaxation times. To discuss only the relaxation process, we subtracted the limiting value  $(\alpha/f^2)_\infty$  from the experimental data, and then plotted the results in the form of absorption per wavelength  $\alpha\lambda$ , as shown in Fig. 4. The features to be described are the peak at about 100 MHz and the long low-frequency tail. At the first stage, we have tried to fit the relaxation curve assuming the Davidson-Cole distribution, as was used in poly-L-glutamic acid solutions by Burke *et al.*<sup>16</sup>. However, we did not succeed in fitting it to the data because this type of function has a low-frequency cutoff and high-frequency tail that are just contrary to the features observed. We have therefore introduced a new distribution function of relaxation times  $g(\tau)$  having a high-frequency cutoff and low-frequency tail by making a mirror image of the Davidson-Cole function around the cutoff frequency. Thus we obtain the expression for the complex bulk modulus  $K^*$ <sup>17</sup>.

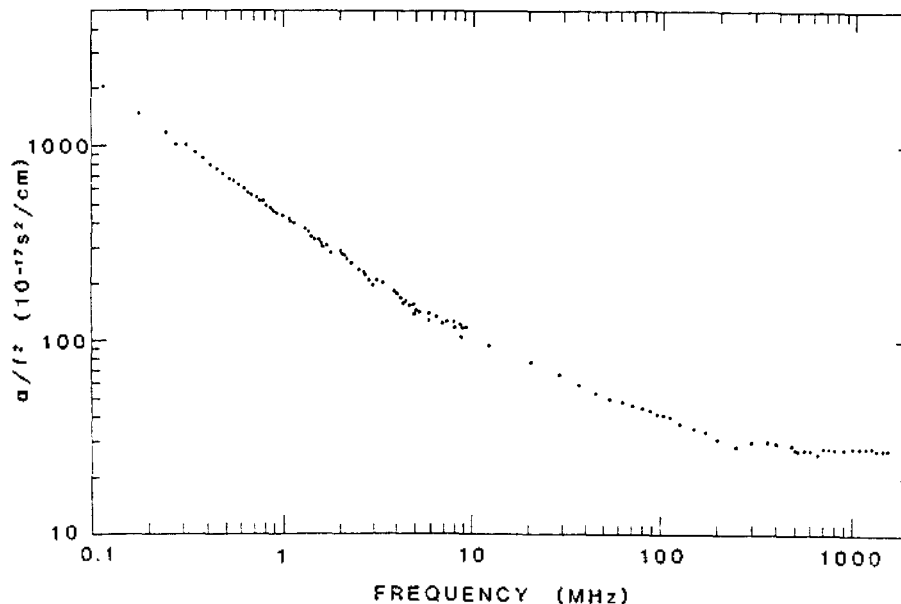


Fig. 3. Ultrasonic absorption  $\alpha/f^2$  versus frequency in the bovine serum solution with the concentration of 50 g/l at pH 7 and 20°C.

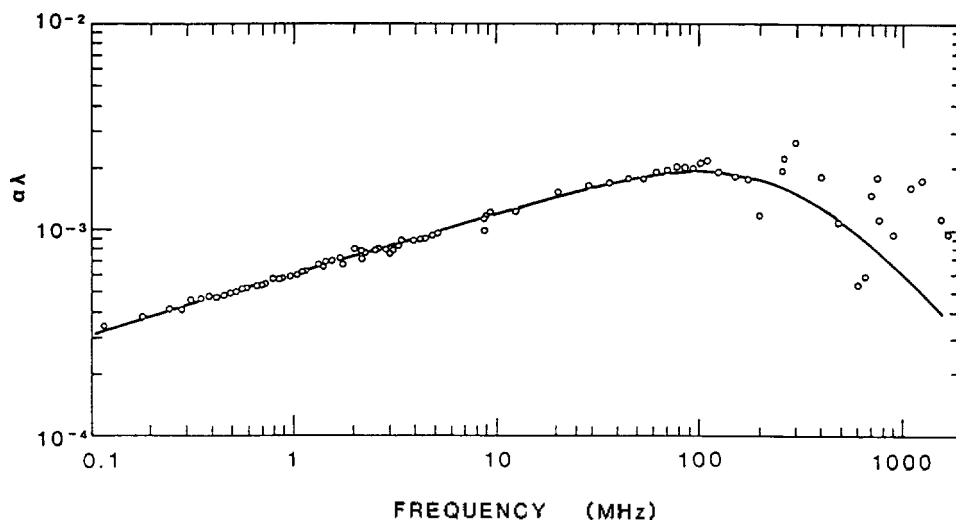


Fig. 4. Relaxation absorption per wavelength versus frequency in the bovine serum albumin solution at pH 7. The absorption at the high-frequency limit was subtracted from the raw data. The solid line represents the fitted curve of Eq. (3) with  $\tau_0 = 5.5 \times 10^{-10}$  s,  $\beta = 0.29$  and  $K_\infty - K_0 = 5.15 \times 10^6$  N/m<sup>2</sup>.

$$K^*(i\omega\tau_0) = K_0 + (K_\infty - K_0) \int_0^\infty \frac{g(\tau/\tau_0) i\omega\tau}{1 + i\omega\tau} d\left(\frac{\tau}{\tau_0}\right), \quad (1)$$

where

$$\begin{aligned} & g\left(\frac{\tau}{\tau_0}\right) d\left(\frac{\tau}{\tau_0}\right) \\ &= \frac{\sin \beta\pi}{\pi} \left(\frac{1}{\tau/\tau_0} - 1\right)^\beta d \ln\left(\frac{\tau}{\tau_0}\right), \text{ for } 1 < \tau/\tau_0 < \infty \\ &= 0, \text{ for } 0 < \tau/\tau_0 < 1. \end{aligned} \quad (2)$$

Here,  $K_0$  and  $K_\infty$  are the low- and high-frequency limits of bulk modulus, respectively,  $\tau_0$  is the cutoff relaxation time, and  $\beta$  is the parameter characterizing the width of the distribution. Substituting Eq. (2) into Eq. (1) leads to

$$\alpha\lambda = \frac{\pi}{K_0} (K_\infty - K_0) (1 + \omega^2\tau_0^2)^{\beta/2} (\omega\tau_0)^\beta \sin\beta\theta, \quad (3)$$

$$K' = K_0 + (K_\infty - K_0) (1 + \omega^2\tau_0^2)^{-\beta/2} (\omega\tau_0)^\beta \cos\beta\theta, \quad (4)$$

$$\theta = \arctan(1/\omega\tau_0)$$

where  $K'$  is the real part of the bulk modulus. Fitting of Eq. (3) can be readily done if the data are plotted in double logarithmic scale. The position and height of the  $\alpha\lambda$  peak gives  $\tau_0$  and  $K_\infty - K_0$ , respectively, and the gradient of the low-frequency tail determines  $\beta$ . The best fit was achieved

when  $\tau_0 = 5.5 \times 10^{-10}$  s,  $\beta = 0.29$ , and  $K_\infty - K_0 = 5.15 \times 10^6$  N/m<sup>2</sup>, and is represented by the solid line in Fig. 4.

#### Discussion on the mechanism of the relaxation.

In hemoglobine solutions Schneider *et al.*<sup>9</sup> obtained a broadband absorption spectrum that bears a great resemblance to that in Fig. 4. They suggested that a four-step relaxation process was sufficient to explain the spectrum, but did not identify the relaxation mechanism. Possible mechanisms predicted at neutral pH are the proton transfer reaction of the imidazole group at histidine residues (expected  $pK = 6.5 \sim 7.0$ ), structural fluctuation of protein molecules, and hydration (solute-solvent interaction). Contribution of the proton transfer reaction of the imidazole group was suggested in hemoglobin solutions<sup>8</sup> from the result that a weak absorption peak was observed around pH 7. In BSA solutions, however, the fact that no such peak has been observed<sup>18</sup> and that the histidine residue content is one fifth of that in hemoglobin indicate that the proton transfer reaction is a minor cause of the absorption<sup>19</sup>. Structural fluctuation, such as internal motions of side chains, may not show a broad absorption spectrum. The relaxations associated with internal ro-

tation of side chains in polymer solutions are expected to yield single or double relaxations<sup>20</sup>. Thus the broad distribution of relaxation times suggests that the dominant relaxation mechanism is the perturbation of hydration equilibrium by sound waves.

It is well known that there exists hydration of 0.2~0.7 g of water per 1 g of protein<sup>21</sup>. Studies of sorption isotherm<sup>22</sup> showed that there are multiple degrees of hydration water having a different enthalpy of hydration from that of bulk water. Recent NMR studies also show that there are at least four regions of distinct hydration behavior of lysozyme<sup>23</sup>. The first layer is tightly bound to available polar group on the protein, and the residence time of the water molecules is the order of  $10^{-6}$  s<sup>21</sup>. Successive water layers are weakly restricted to protein, and the residence time would be  $10^{-6}$ ~ $10^{-9}$  s<sup>24-26</sup> depending upon the degree of the hydration. Bulk-free water surrounds this hydration shell. Water molecules exchange their positions among different layers and between the outer layer and bulk water. It may be this hydration equilibrium that is responsible for the relaxation. The distribution of relaxation times likely corresponds to various structures of hydrated water. The present experimental results are consistent with this model in two respects. First, the range of the distribution of relaxation times  $1.5 \times 10^{-6}$ ~ $5 \times 10^{-10}$  s, agrees with that of the residence times of hydrated water molecules. Second, the observation that the relaxation strength is larger at higher frequencies can be explained by considering that the high-frequency parts of the relaxation are associated with the outer layers of hydration (smaller residence times) in which the quantity of water molecules is much larger than that in the inner layers<sup>23</sup>.

### CONCLUSION

The absorption spectrum at neutral pH in BSA aqueous solution was well fitted to the relaxation curve assuming a distribution of relaxation frequency with a high-frequency cutoff and long low-frequency tail. The relaxation behavior was inter-

preted in terms of various degrees of hydrations of BSA molecules. However, these measurements are not enough to clarify the whole relaxation mechanisms. Especially the accurate technique for measuring absorption lower than 100 kHz with small sample volume should be explored

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