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Pressure dependence of the apparent specific volume of bovine serum albumin: insight into the difference between isothermal and adiabatic compressibilities

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Abbreviation: BSA, bovine serum albumin.

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ABSTRACT

There are some theoretical arguments related to interpreting the adiabatic compressibility (β_s) of a protein determined from the sound velocity and the difference between β_s and isothermal compressibility (β_T). To address these problems experimentally, we constructed a high-pressure oscillating densitometer and used it to measure the apparent specific volume of bovine serum albumin as a function of pressure (0.1–78 MPa) and temperature (5–35^{o}C). The β_T determined from plots of the apparent specific volume vs. pressure was slightly larger than β_s at all temperatures examined, with the difference between the two compressibilities increasing as the temperature was decreased. Only at room temperature did the observed β_T agree with those estimated from β_s using the heat capacity and the thermal expansibility of the protein, suggesting that there are significant as-yet-unknown mechanisms that affect protein compressibility.

Keywords: isothermal compressibility, adiabatic compressibility, bovine serum albumin, specific volume

1. Introduction

Compressibility is an important thermodynamic quantity for understanding the pressure response and volume fluctuation of a protein molecule in solution [1]. Two types of partial compressibility (isothermal and adiabatic) are defined as the pressure derivative of the partial volume at constant temperature and constant entropy. The partial isothermal compressibility (β_T) can be determined by directly measuring the partial volume under variable pressure, but its measurement is technically difficult at least to the precision of determining the adiabatic compressibility (β_s) due to the small pressure dependence of the partial volume and to the denaturation or conformational change of a protein under high pressure. On the other hand, the β_s value can be more easily and accurately determined by measuring the sound velocity (U) and density (d) using the Newton-Laplace equation: $\beta_s = 1/dU^2$. Therefore, the β_s values of many proteins have been measured by this method and widely used for investigating the hydration state, compactness or flexibility (volume fluctuation), conformational change, and structure–function relationship of proteins [2–12].

However, there are some theoretical arguments related to interpreting the β_s value of a protein determined from the sound velocity. Nölting suggested that the interior of a protein molecule is close to the isothermal condition during sound velocity measurements so that the experimentally obtained β_s values fall between the adiabatic and isothermal compressibilities (pseudoadiabatic theory) [13]. On the other hand, Pinfield and Povey suggested that the propagation of sound remains adiabatic under the usual experimental conditions but that the thermal scattering effect of the sound must be taken into account when calculating the adiabatic compressibility (thermal scattering theory) [14]. A rigorous thermodynamic equation for the difference between β_T and β_s was derived [11,15,16], but this has not been confirmed experimentally with proteins. Experimental evidence for the difference between β_T and β_s would be indispensable for answering these theoretical problems and for deepening the understanding of the compressibility of protein molecules in terms of protein dynamics.

A novel technique for directly measuring β_T is oscillating densitometry, which can reliably record the solution density under high pressure [17,18]. This method was used by Seemann *et al*. [18] to investigate the temperature- and pressure-induced unfolding of staphylococcal nuclease, but no subsequent applications have been reported, probably due to technical difficulties.

In the present study, we constructed an oscillating densitometer and used it to measure the apparent specific volume of a protein, bovine serum albumin (BSA), at varying pressure (0.1–78 MPa) and temperature (5–35^{o}C) in the native state. This

protein was used as a model protein because it has a large adiabatic compressibility [5] that allows comparatively accurate measurements of the pressure dependence of the partial volume. The apparent β_T values obtained therefrom were compared with the β_s values determined from sound velocity measurements to gain insight into the mechanism underlying the difference between the two compressibilities.

2. Materials and Methods

2.1. Materials

BSA (99% pure on gel electrophoresis) was purchased from Sigma Chem. Co. and used without further purification. The protein sample was dissolved in double-distilled water at a concentration of about 40 mg/mL and was deionized by exhaustive dialysis against water at 4°C. The protein solution was purified by passing it through a 0.2-μm membrane filter and degassed under vacuum for 5 min before the density measurement. The concentration of the protein was determined by absorption measurement using an extinction coefficient of 6.58 dL/(g·cm) at 278 nm [19]. Fluorescence spectrometry (Jasco FP-750 with a Teramecs PCI-400 high-pressure cell unit) confirmed that the protein was not denatured under the conditions of the density measurements (5–35°C below 80 MPa).

2.2. Densitometry

A density measurement system was constructed using a high-precision oscillating-tube cell (DMA 512P, Anton Paar, Austria), which was designed to measure the density under pressures up to 70 MPa and temperatures from –10°C to 150°C. A block diagram depicting the main components of the apparatus is shown in Fig. 1. The oscillating-tube cell, which was constructed from alloy (Hastelloy C-256) and had an inner volume of about 1 mL, was connected to a stainless high-pressure vessel (total volume of about 25 mL) and a hand pump both made by Akico, Tokyo. The period of harmonic oscillation of the tube was fed to a personal computer at 10-s intervals using a universal counter (Iwatsu SC-7205, Japan) and an RS-232C interface. The resolution of the period corresponds to a density of approximately 1×10^{-6} g/mL. The pressure was monitored using a digital pressure gauge (Naganokeiki TY-KH 15, Japan) to an accuracy of 0.1 MPa. Temperature was controlled to within 0.01°C using a thermostat (Neslab RTE-7). The high-pressure densitometer constructed in the present study is similar to that used by Seemann *et al.* [18].

(Fig. 1)

The instrument constant was determined by calibrating the densitometer with double-distilled water of known density for each set of temperature and pressure values. We used the following equation of state for water because it provides a good fit to the experimental data of density (or volume) over the range of 0–150°C and up to 100 MPa [20]:

$$1/d_o^* = (1/d_o)(1 + a_T P + b_T P^2)/(1 + c_T P) \quad (1)$$

where d_o and d_o^* are the densities of water at atmospheric pressure and pressure P, respectively, and coefficients a_T, b_T, and c_T are functions of temperature T that vary according to the fourth or fifth power of T. The instrument constant (A_p) at pressure P was determined from the densities and the periods of oscillation of water, f_o and f_o^*, at atmospheric pressure and pressure P, respectively, according to the relation

$$A_p = (1/f_o^{*2} - 1/f_o^2)/(d_o^* - d_o) \quad (2)$$

The A_p values at any given pressures in sample measurements were calculated by assuming a quadratic function of pressure for A_p determined using Eq. (2).

Density was measured under high pressure by introducing the protein solution into the pressure vessel and the oscillating-tube cell. After thermal equilibrium was established, the period of oscillation was recorded at atmospheric pressure, and the pressure was then increased to about 80 MPa before being set to the next lower pressure, with this process being repeated many times down to atmospheric pressure. The period of oscillation (f_s^*) recorded at each pressure was adopted only when the values of f_s at atmospheric pressure before and after the compression were identical within the experimental error. The density of the solution (d_s^*) and the apparent specific volume of the protein (v^*) at pressure P were calculated based on the periods of oscillation for the solution (f_s^* and f_s):

$$d_s^* - d_s = (1/f_s^{*2} - 1/f_s^2)/ A_p \quad (3)$$

$$v^* = (1/m^*) [1 - (d_s^* - m^*)/d_o^*] \quad (4)$$

The protein concentration (m^*) was calibrated to the pressure using the relation $m^* = m (d_s^*/d_s)$, where m and d_s are the protein concentration and the solution density,

respectively, at atmospheric pressure. The apparent isothermal compressibility (β_T) at atmospheric pressure was calculated from the equation $\beta_T = -(1/v)(\partial v^*/\partial P)_T$ using the apparent specific volume (v) at atmospheric pressure.

The effects of temperature and protein concentration on v were examined by separately measuring the solution density with an accuracy of 1×10^{-6} g/mL using an oscillating densitometer (DMA5000, Anton Paar) at atmospheric pressure.

3. Results and discussion

3.1. Temperature dependence of the experimental β_T and β_s values

The constructed high-pressure densitometer requires a high protein concentration (~ 40 mg/mL) because of the low repeatability of density data obtained under high pressure. However, since no significant concentration dependence of v was observed for BSA below 40 mg/mL at atmospheric pressure, the v^* value was measured at a protein concentration of 32.0–37.3 mg/mL by neglecting the protein concentration dependence of v^* under high pressure. Fig. 1 plots v^* as a function of pressure below 78 MPa at four temperatures, and indicates that v^* decreases linearly with increasing pressure at all temperatures within an experimental error of $\pm3\times10^{-3}$ mL/g. The β_T values calculated from the slopes by least-squares regression analysis are listed in Table 1 with the v values at atmospheric pressure. These v values differ slightly from the reported partial specific volumes at infinite dilution [6,7,21] because the protein concentration dependence of v was neglected in the present study. Evidently, the observed β_T values involve large experimental errors ($0.78–1.62\times10^{-11}$ Pa^{-1}) compared with those ($0.1–0.5\times10^{-11}$ Pa^{-1}) of β_s values [5]. Table 1 includes the β_s values previously determined from the sound velocity and density measurements at atmospheric pressure [7,21].

The experimentally determined β_T and β_s values are plotted against temperature in Fig. 3. We note that both β_T and β_s increase with increasing temperature in the range of 5–35°C. Furthermore, we note that β_T is distinctly larger than β_s in the low temperature range (5–20°C), while the difference becomes smaller in the high temperature range (25–35°C), β_T almost coinciding with β_s within experimental error. To our knowledge, this result represents the first experimental evidence that the β_T and β_s values of a protein are different.

(Fig. 2) (Fig. 3) (Table 1)

3.2. Comparison of the experimental β_T and β_s values with existing theories

The isothermal compressibility β_T of a pure substance is related to adiabatic compressibility β_s by the equation

$$\beta_T = \beta_s + T\alpha^2/dC_p \tag{5}$$

where d is the density, α is the thermal expansibility, T is the temperature, and C_p is the specific heat capacity at constant pressure. This equation has been used to estimate roughly the β_T value of a protein from the β_s value by replacing the parameters (α, d, and C_p) in Eq. (5) with the partial (or apparent) quantities of the protein [5,7]. However, this procedure is clearly an oversimplification since the thermal expansion of bulk solvent is not taken into consideration. A more rigorous equation has been proposed to estimate the isothermal compressibility of a solute in solution from the experimentally determined β_s [11,15,16]:

$$\beta_T = \beta_s + T(\alpha_o{}^2/d_o c_{p,o})(2\alpha/\alpha_o - c_p/vd_o c_{p,o}) \tag{6}$$

where d_o is the density of the solvent, α_o is the thermal expansibility of the solvent, $c_{p,o}$ is the specific heat capacity of the solvent at constant pressure, $\alpha = (1/v)(\partial v/\partial T)$ is the partial thermal expansibility of the solute, and c_p is the partial specific heat capacity of the solute at constant pressure. This equation will apply at any given pressure, but there are no data on the pressure dependence of each partial quantity including β_s, and hence the difference between β_T and β_s of the protein could be evaluated only at atmospheric pressure. The significant difference between β_T and β_s was found with small organic salts [16]. Recently, Sminovas *et al*. applied this equation to evaluate β_T from β_s of insulin aggregate and discussed the origin of the difference between the two compressibilities [22]. However, there has been no attempt to compare the β_T value estimated from Eq. (6) with the experimentally observed β_T of protein at varying temperature.

Here we estimated the β_T value from with β_s for BSA using Eq. (6). For BSA in water, α was determined to be 5.04×10^{-4} K^{-1} from the temperature dependence of v over 5–40°C (Table 1), which is very close to the value (4.97×10^{-4} K^{-1}) reported previously [5,7]. The c_p value has been determined to be 1.34 J/(g·K) by calorimetry [23]. The α and c_p values were used to estimate the values of β_T from Eq. (6). The estimated β_T values are listed in the fifth column of Table 1 and plotted against temperature in Fig. 3. The difference between the β_T from Eq.(6) and the experimentally observed β_s decreases with decreasing temperature, from 1.90×10^{-11} Pa^{-1} at 40°C to 0.55×10^{-11} Pa^{-1}

at 10°C, and disappears at 4°C where α_o is zero mainly due to the large decrease in ($\alpha_o^2/d_o c_{p,o}$) or α_o. This diminished difference between the β_T and β_s values (Fig. 3) at lower temperature as predicted from Eq. (6) is just the opposite to what is observed in the experiment. The true origin must be sought in factors that have been neglected in Eq. (6), in which the protein system is treated as rigid as a small compound.

Further, contrary to prediction from Eq. (6), the difference between the observed β_T and β_s values decreases with increasing temperature, and becomes negligible above 35°C. The β_T values estimated using Eq. (6) are close to the experimentally observed β_T values at room temperature, but are overestimated at higher temperatures and underestimated at lower temperatures, with a maximum difference of 2×10^{-11} Pa^{-1}. If the observed β_T values were regarded as identical to the β_T values estimated from Eq. (6), the β_s values under pure adiabatic conditions should be 1.5–2×10^{-11} Pa^{-1} smaller than the observed β_s values (Fig. 3). The quantitative meaning of these differences in compressibilities may be reduced by experimental errors, but a distinctly different feature in temperature dependence of β_T and β_s is worthy of discussion on the basis of compressibility theories.

According to the theory of Nölting [13], the β_s value determined from the sound velocity should fall between the pure adiabatic and isothermal compressibilities at high temperature, but the sound velocity measurements should approach pure isothermal conditions at low temperature where the temperature ratio (R) of the protein molecule ($T_{protein}$) to bulk water (T_{bulk}) during a pressure perturbation increases due to the decreased expansibility of water:

$$R = T_{protein}/T_{bulk} = [d_o c_{p,o}\alpha/(d\, c_p \alpha_o)\,] - 1 \qquad (7)$$

As shown in Fig. 3, the experimentally observed β_s values range between the β_s estimated for pure adiabatic conditions and the experimentally observed β_T at high temperature as suggested by Nölting [13], but they deviate largely from the β_T at low temperature or under isothermal conditions.

On the other hand, according to Pinfield and Povey [14], the larger β_s for pure adiabatic conditions than the observed one could be ascribed to the thermal scattering effect ($\theta > 0$) because this effect contributes to reduce sound velocity more significantly at lower temperature, as suggested by θ being proportional to the square of R in Eq. (7):

$$(U_o/U)^2 = 1 + [\beta/\beta_o + d/d_o + \theta\,]\,\phi \qquad (8)$$

where U and U_o are the sound velocities of the protein solution and the solvent,

respectively, β and β_0 are the adiabatic compressibilities of the protein solution and the solvent, respectively, ϕ is the volume fraction of the protein, and the higher-order term of ϕ is neglected [14]. Our calculation of sound velocity is essentially based on the Urick equation without the thermal scattering effect of the sound wave. Our results are then consistent with the prediction of Pinfield and Povey at lower temperatures, but they contradict it at higher temperatures because the observed β_S values are larger than those under a pure adiabatic condition. Thus, the experimental data on β_T and β_S are only partly consistent with the theoretical predictions over a limited temperature range (around 20^{o}C for BSA).

3.3. Possible origins for the temperature-dependent differences between the β_T and β_S values

Fig. 3 represents the first experimental evidence that the β_T and β_S values of BSA are uniquely different with varying temperature. Although the generality of this phenomenon must await further studies on other proteins, the distinct feature of the difference of the two compressibilities shown in Fig. 3 may be worthy of further discussion on the origin of this phenomenon.

The adiabatic or isothermal compressibility of a protein is determined by two major contributions, the cavity and the hydration, because the constitutive atoms can be assumed to be incompressible [3,5]:

$$\beta_T \text{ or } \beta_S = -(1/v)\,[(\partial V_{cav}/\partial P) + (\partial \Delta V_{sol}/\partial P)] \quad (9)$$

where V_{cav} is the cavity volume in a protein molecule generated by imperfect atomic packing and ΔV_{sol} is the volume change due to hydration. Generally, cavity contributes positively and hydration contributes negatively to β_T and β_S, and hence the observed compressibility can be positive or negative depending on the relative values of the two terms. Since most cavities are essentially in a vacuum, thermal exchange during sound scattering would occur adiabatically. The apparent adiabatic compressibility of cavity is significantly larger than that of bulk water [3,5], making it the most compressible part of a protein molecule. Therefore, even a small perturbation of a cavity would cause large changes in β_T and β_S.

Hydration accounts for about 30% of the weight of a protein, which corresponds to one or two layers of water molecules around the protein surface. Because the water of hydration is different from bulk water in various thermodynamic quantities like density and heat capacity, the thermal conductivities of hydrated and bulk water should

also differ significantly, which would induce a temperature difference between the protein and the bulk water, thereby perturbing the sound velocity measurements from a pure adiabatic condition. Further, the contribution of the thermal volume of a protein to its compressibility has not been evaluated quantitatively, although the average thickness of about 1 Å on the protein surface has been assumed for the thermal volume [21]. However, since this thickness is twice the estimate of 0.5 Å for small compounds, the thermal volume may induce additional modes in the mutual thermal motion of proteins and solvating waters.

A recent high-pressure NMR study has revealed that the structure of protein is highly dynamic and its fluctuation is heterogeneous over the folded architecture not only in space, but also in time range of fluctuation, as disclosed by pressure-induced chemical shifts [24, 25]. The linear or non-linear chemical shifts are observed even at low pressure below 100 MPa. The fluctuations that lead to linear pressure-induced shifts are believed to occur generally at sufficiently high frequency ($>10^6$ sec^{-1}) so that their contribution to the compressibility would be reflected rather equally both in β_S as well as in β_T. On the other hand, the slower fluctuations (10^3~10^6 sec^{-1}) represented by non-linear pressure shifts are often accompanied by significant changes in partial molar volume of the system, meaning significant changes in cavity and hydration. Although the presence of slower motions has not been clarified yet in BSA within the pressure range (< 80 MPa) employed in the present experiment, it is likely that the increasing discrepancy between the experimental β_T and β_S values (Fig. 3) is due to the increase of slower internal motions in BSA at lower temperatures.

As shown above, there are many possible origins for the difference between β_T and β_S of a protein different from a small compound. Because of the technical difficulties in its measurements, β_T seems irreplaceable for β_S at present, which is of practical value in investigations of the hydration, conformation, and dynamics of protein molecules under usual experimental conditions. However, the experimental comparison of β_T and β_S values will not only have its theoretical interest, but will also have importance as a generally applicable method for characterizing slow fluctuations in proteins.

4. Conclusions

The present study has demonstrated experimentally that β_T is larger than β_S for BSA in the temperature range between 5°C and 40°C, but that the difference tends to increase with decreasing temperature. These experimental data are only partly consistent with the theoretical predictions, suggesting that there are as-yet unknown effects excluded in both the experiments and the current theories of protein compressibility. Accumulation

of experimental data of β_T along with β_s on other proteins is definitely needed for the generalization of the above proposal. The experimental comparison of β_T and β_s would be a new source of information in future for probing functionally important internal dynamics of a protein.

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Figure Captions

Fig. 1. A schematic diagram of the high-pressure densitometer used for sound velocity measurements under high pressure.

Fig. 2. Pressure dependence of the apparent specific volume (v^*) of BSA in water at 5°C (■), 15°C (▲), 25°C (●), and 35°C (▼). For clarity the v^* data are offset relative to one other. The protein concentrations were 32.0–37.3 mg/mL.

Fig. 3. Temperature dependence of the isothermal (β_T) and adiabatic (β_s) compressibilities of BSA in water. ○: β_s experimentally determined (refs. 7 and 21); ●: β_T experimentally determined (this study); ▲: β_T estimated using Eq. (6); □: β_s under pure adiabatic conditions predicted by regarding the experimentally observed β_T values as identical to those estimated using Eq. (6).

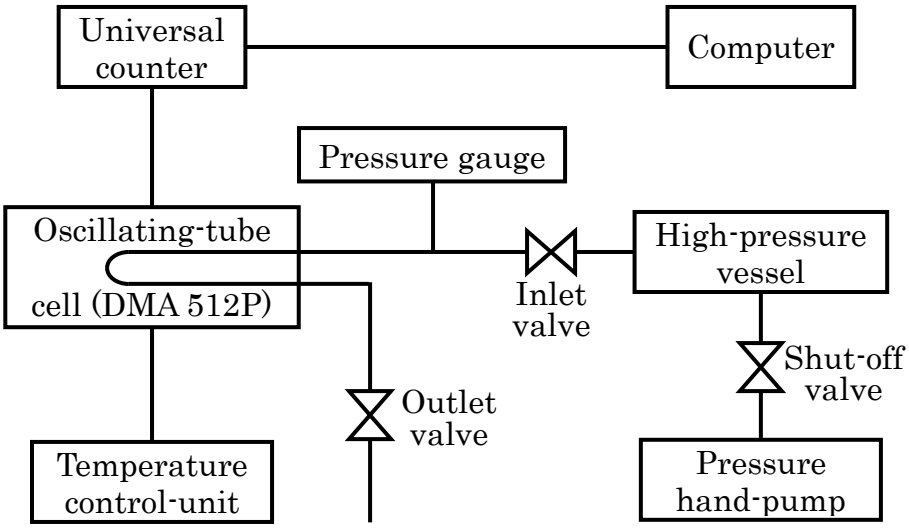

Universal counter
Computer
Pressure gauge
Oscillating-tube cell (DMA 512P)
Inlet valve
High-pressure vessel
Shut-off valve
Outlet valve
Temperature control-unit
Pressure hand-pump

Fig. 1

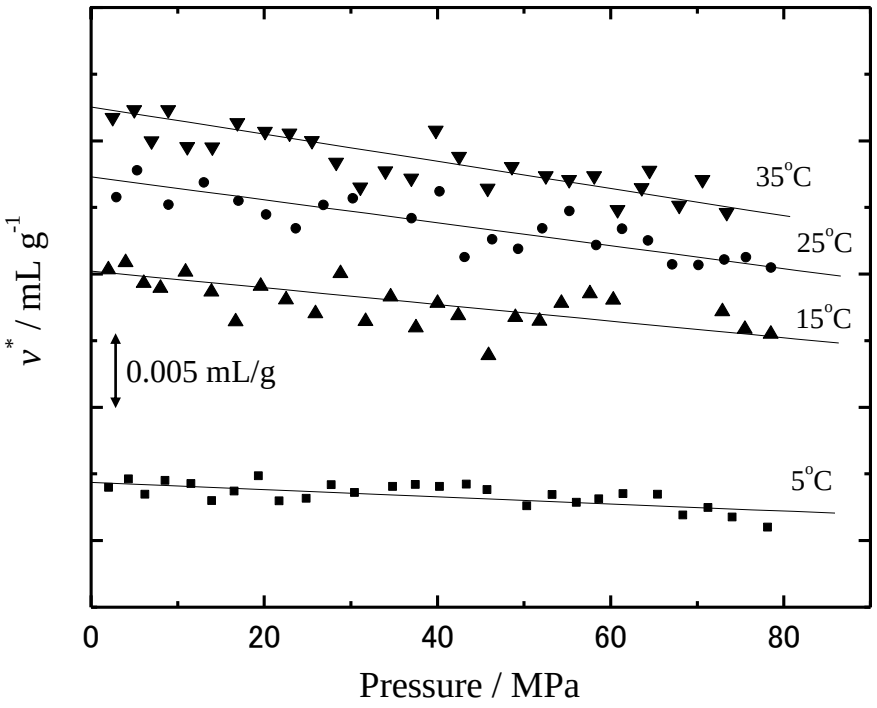
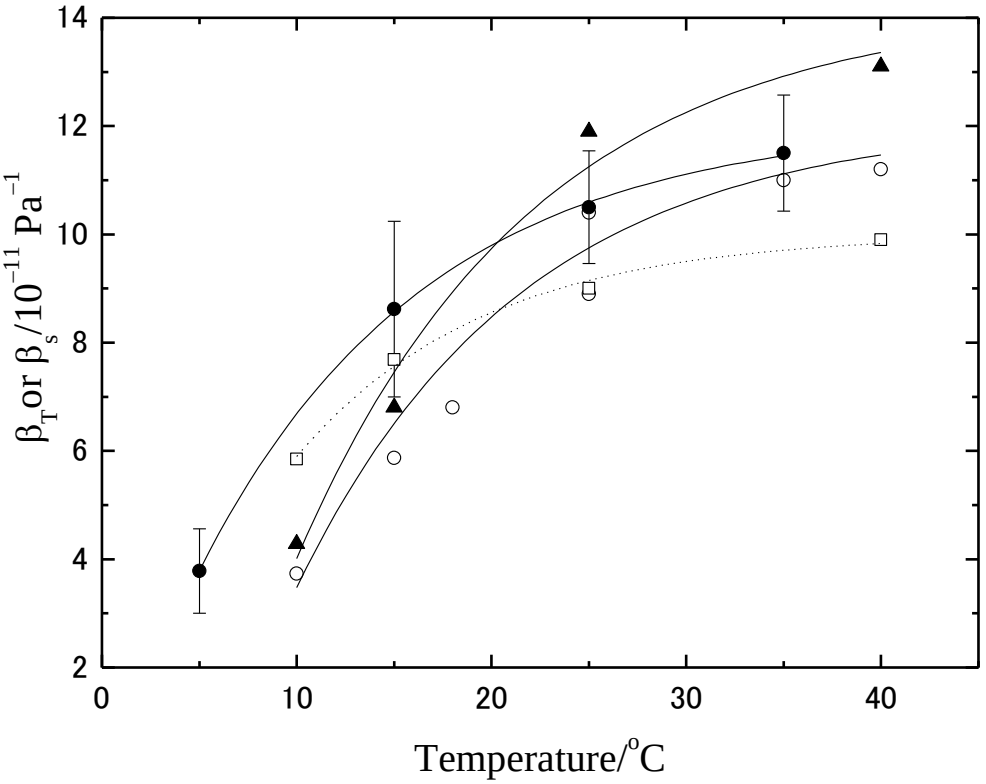


Fig. 2


14
12
10
8
6
4
2
0
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20
30
40
β_T or $\beta_s/10^{-11}$ Pa^{-1}
Temperature/^{o}C

Fig. 3

Table 1

Apparent isothermal (β_T) and adiabatic (β_s) compressibilities of BSA in water

Temperature (°C)	v (this study) (mL·g^{-1})	β_T (this study) (10^{-11}Pa^{-1})	β_s (measured) (10^{-11}Pa^{-1})	$\beta_{T\ estimated}$ (Eq. 6) (10^{-11}Pa^{-1})
5	0.719	3.78±0.78	-	-
10	0.721	-	3.73	4.28
15	0.724	8.62±1.62	5.87	6.80
18	-	-	(6.8)	-
25	0.729	10.5±1.04	10.4 (8.9)	11.9
35	0.734	11.5±1.07	(11)	-
40	0.737	-	11.2	13.1

Parenthesized and unparenthesized β_s values were taken from refs. 21 and 7, respectively.