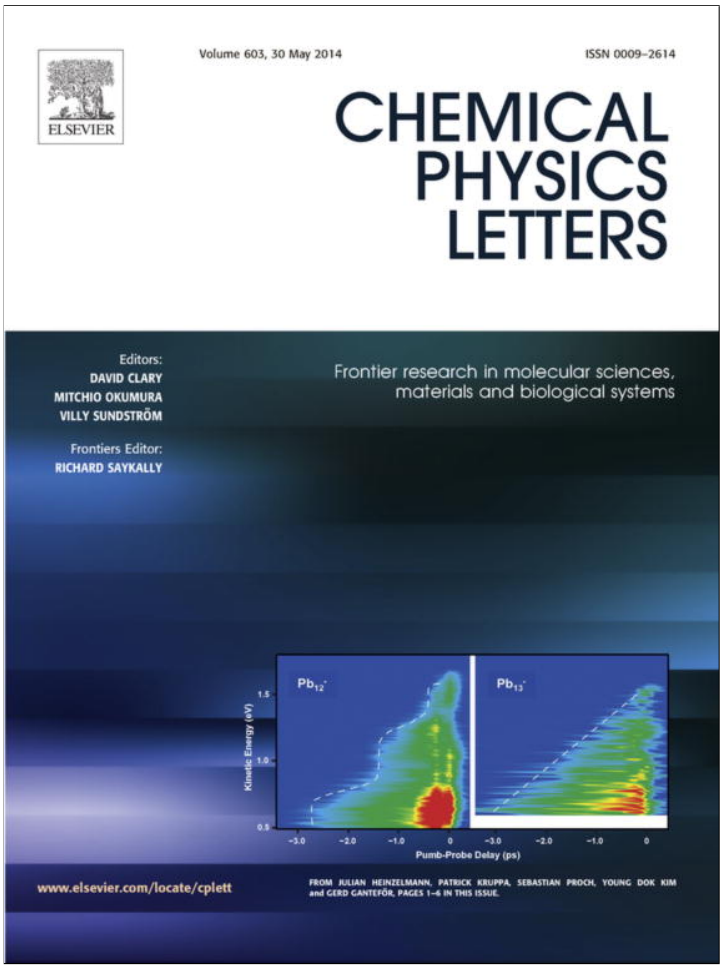

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A study of the hydration of ribonuclease A using densitometry: Effect of the protein hydrophobicity and polarity

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ABSTRACT

The excess volumes of the binary system of ribonuclease A (RNase A) with water were obtained as a function of composition at 25 °C. The excess quantities for RNase A were compared with the published data for several unrelated proteins (lysozyme, serum albumin, lactoglobulin, and chymotrypsinogen A). The hydrophobicity of these proteins is gradually changed over a wide range. It was found that the more hydrophilic a protein is, the more significant the hydrophilic hydration contribution is. RNase A is the most hydrophilic protein in the present study, and it has the most significant hydrophilic hydration contribution.

1. Introduction

Water binding (hydration or biological water) plays a key role in determining the structure, stability, dynamics, and functions of proteins [1–7]. On the other hand, there are essential differences between hydration and the bulk water surrounding a protein [1–9]. Thus, a characterisation of the hydration of proteins requires elucidating the effects of the protein on water and *vice versa*. Therefore, a quantitative estimation of the protein and water contributions to the thermodynamic functions of binary protein–water systems is of considerable importance and practical interest.

Thermodynamic studies have traditionally been very important in ascertaining a better understanding of protein–water interactions. Volume is an important thermodynamic quantity directly associated to the compactness or globularity of a protein, and is generally dependent on a combination of factors [6,9–13]. The hydration of charged and polar groups reduces volume. However, the volume changes associated with the exposure of hydrophobic groups depend on the model compounds selected, ranging from small negative to positive values. Moreover, it is not clear whether the volume change associated with hydrophobic hydration plays an important role in the total volume change.

Several studies have investigated the water density (volume) in the hydration shell of the proteins. A combined neutron and X-ray scattering study [14] showed that the average density of the first hydration shell of proteins is significantly higher than that of bulk water. This finding is consistent with the results obtained from the molecular simulation, crystallographic, and solution studies [10,15–17]. The higher density of preferentially bound water and a comparison of hydration values in solution are also given in Ref. [6].

The partial specific volumes for the majority of globular proteins in aqueous solutions fall within a narrow range between 0.70 and 0.75 $cm^3\ g^{-1}$ [3,6,10–13,18,19]. The concentration dependences of the apparent volumes of serum albumin, ovalbumin, and oxyhemoglobin were measured at high protein concentrations (protein mass fraction, w_2, ~0.3–0.4) [18]. No effect of protein on the solvent was observed at these concentrations, suggesting a constant partial specific volume of the solvent in the studied concentration range. The dilatometric measurements of serum albumin and oxyhemoglobin are consistent with this conclusion [19].

Direct volumetric studies of binary water–protein systems at low hydration levels are relatively rare. Volumes of protein–water systems with a w_1 (water mass fraction) between 0 and 0.56 were measured in Refs. [20–23]. It was shown that at a $w_1 > 0.2$–0.3, the volumes of the binary protein–water systems depend linearly on the water content. However, at the lowest w_1 values, there are deviations from this linearity. Thus, these studies [20–23] suggest that the partial specific volumes of proteins are lower at high hydration levels than in the dried state. Bull and Breese [20] estimated that at a $w_1 < 0.2$, the partial specific volume of water is lower than that of bulk water.

The measured volumes [10–13,18–23] contain total information on the binary water–protein systems, including the corresponding conformational changes in the protein structure and the glass transition. However, the simultaneous estimation of the protein and water contributions to the volume of binary protein–water systems in the entire range of water contents has not been attempted.

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In our previous studies [9,24], we proposed a novel methodology to investigate the protein–water interactions. This methodology is based on the analysis of the excess thermodynamic functions of mixing. It facilitates the individual evaluation of the protein and water partial quantities in the entire range of water content. The present study is aimed at understanding which molecular parameters control the excess volumes of binary protein–water systems.

To test the predictive ability of the proposed method, a system containing, as a model protein, bovine pancreatic ribonuclease A (RNase A) was studied in this work. The excess partial quantities for RNase A were compared with the published data for several unrelated globular proteins (lysozyme, chymotrypsinogen A, serum albumin, and lactoglobulin) [9]. These biomacromolecules represent a series of proteins in which the hydrophobicity of proteins is gradually changed over a wide range. RNase A is one of the most hydrophilic proteins applied in biomolecular investigations [25–27].

Ribonuclease A is a small monomeric enzyme of 124 amino acids, containing no non-protein components. Ribonuclease is an example of an + protein. RNase A hydrolyses single-stranded RNA through cleavage of the phosphodiester linkage at the 3′ side of pyrimidine nucleotides [25–27].

2. Methodology

2.1. Excess partial volumes

The thermodynamic properties (volume V, enthalpy H, entropy S, and Gibbs free energy G) of a real binary water-protein system can be expressed in terms of the excess functions, Z^E. They are the difference between the thermodynamic function of mixing in a real system and the value corresponding to an ideal system at the same temperature, pressure and composition. For an ideal system, all excess functions are zero. Deviations of the excess functions from zero indicate the extent to which the studied binary system is non-ideal due to strong specific interactions between components (i.e., hydrogen bonding and charge–charge interactions). More comprehensive reviews of excess functions are given in Refs. [28–30].

The Z^E values can be calculated using Eq. (1):

$$Z^E = Z^m - Z^m_{id} \tag{1}$$

where Z^m is the thermodynamic function of mixing for a real system; Z^m_{id} is the thermodynamic function for an ideal mixture.

For binary mixtures, the Z^m_{id} values can be calculated using Eq. (2):

$$Z^m_{id} = w_1 Z^0_1 + w_2 Z^0_2 \tag{2}$$

where Z^0_1 and Z^0_2 are the thermodynamic function values for pure water and pure protein, and w_1 and w_2 are the mass fractions of water and protein, respectively ($w_2 = 1 - w_1$).

For binary mixtures, the Z^E values are composed of two components:

$$Z^E = w_1 \overline{Z}^E_1 + w_2 \overline{Z}^E_2 \tag{3}$$

where $\overline{Z}^E_1$ is the excess function for component 1 (water), and $\overline{Z}^E_2$ is the excess function for component 2 (protein).

The excess partial quantities of components 1 and 2 are calculated using Eqs. (4) and (5):

$$\overline{Z}^E_1 = Z^E - w_2 \left(\frac{dZ^E}{dw_2} \right)_{T,P} \tag{4}$$

$$\overline{Z}^E_2 = Z^E - w_1 \left(\frac{dZ^E}{dw_1} \right)_{T,P} \tag{5}$$

where $\overline{Z}^E_1$ and $\overline{Z}^E_2$ are the excess partial quantities of components 1 and 2.

The excess partial volumes of RNase A and water are calculated using Eqs. (6) and (7):

$$\overline{V}^E_1 = V^E - w_2 \left(\frac{dV^E}{dw_2} \right)_{T,P} \tag{6}$$

$$\overline{V}^E_2 = V^E - w_1 \left(\frac{dV^E}{dw_1} \right)_{T,P} \tag{7}$$

where $\overline{V}^E_1$ and $\overline{V}^E_2$ are the excess partial volumes ($cm^3\ g^{-1}$) of the water and protein.

3. Experimental

3.1. Materials

Bovine pancreatic ribonuclease A (R-5500) was purchased from Sigma Chemical Company (St. Louis, MO, USA) and was used without further purification. The purity of protein samples was verified through electrophoresis and dynamic light scattering measurements (90Plus Particle Size Analyzer, Brookhaven Instruments Corporation, USA) to be more than 95%. The molecular mass of ribonuclease A was taken as 13 700 Da. The water was doubly distilled.

3.2. Water sorption measurements

The dried protein (zero hydration level) was obtained through vacuum drying using a microthermoanalyser 'Setaram' MGDTD-17S (±0.00001 g) at 25 °C and 0.1 Pa, until a constant sample mass was reached. The water content of the dried protein was estimated as 0.003 ± 0.002 g water g^{-1} protein using the Karl Fischer titration method, according to the recommendations [31,32].

At the lowest and intermediate water mass fractions, the protein preparations were prepared as follows. The initially dehydrated protein samples were exposed to pure water vapour. The water vapour was consecutively flowed through a thermostated glass tube with drying agent (P_2O_5), a thermostated saturator filled with saturated salt solution, and a cell containing the protein sample. The schematic representation of the experimental setup is given in Ref. [24]. An external ethylene glycol thermostat (RC 6 from Lauda, Germany) was used to control the temperature with a precision of 0.1 °C. The sorption equilibrium was reached after 180 min. The water activity (a_w) in the vapour phase was adjusted by changing the saturated salt solution in the saturator. The water activities of the saturated salt solutions were taken from [33,34]. The following salts were used: LiBr (a_w = 0.064), KOH (a_w = 0.078), LiCl (a_w = 0.11), $CaBr_2$ (a_w = 0.17), CH_3COOK (a_w = 0.22), $MgCl_2$ (a_w = 0.33), K_2CO_3 (a_w = 0.44), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75), KCl (a_w = 0.84), KNO_3 (a_w = 0.94), and K_2SO_4 (a_w = 0.97). The salts used for sample conditioning were of analytical pure grade. The water content of the samples after equilibration was measured by drying under vacuum using a 'Setaram' MGDTD-17S microthermoanalyser at 25 °C and 0.1 Pa until a constant sample mass was reached.

3.3. Densitometry

The volumes of the enzyme–water mixtures, V^m, were calculated using Eq. (8):

$$V^m = \frac{1}{d_m} \tag{8}$$

where d_m is the density of the enzyme–water mixtures, g cm^{-3}.

At the highest water mass fractions (w_1~0.6–1.0), the densities of the enzyme–water mixtures were measured using a high precision densitometer (DMA-5000 M, Anton Paar, Austria, precision ± 1 × 10^{-6} g cm^{-3}) at 25 °C. The instrument constant was determined by calibrating the densitometer using double-distilled water of a known density.

At the lowest and intermediate water mass fractions (w_1 = 0–0.5), the volumes of the protein–water mixtures were determined using a helium pycnometer (AccuPyc 1330, Micromeritics, 1.0 ml cell) at 25 °C. Helium was used as the displacement gas at an equilibration rate of 0.1 kPa min^{-1}. Ten sample runs were sufficient for values of the volumes significant to the third decimal place. At the lowest and intermediate water mass fractions (w_1 = 0–0.5), the protein preparations were prepared as described in Section 3.2.

4. Results and discussion

4.1. Partial volumes of water and RNase A

Figure 1 presents the dependence of the volumes of binary RNase A-water mixtures, V^m, on the water mass fraction, w_1. At high w_1 values, the volumes of the RNase A-water mixtures depend linearly on the water content. However, at low w_1 values, there is a deviation from linearity.

The volumes (densities) of the RNase A-water systems were measured in Refs. [10,13,35,36]. As shown in Figure 1 and Table 1, the V^m and $\overline{V}_2$ values are consistent with the previously published results for RNase A [10,13,35,36], indicating that the apparatus and the experimental procedure are adequate to calculate the volumes.

Figure 2 shows the dependence of the excess volumes of the studied binary systems, V^E, on the hydration level of RNase A. The V^E values were calculated using Eq. (1). In ideal binary mixtures (mixtures of two components, W [water] and P [protein]) the average W–P interactions are the same as the average W–W and P–P interactions. The excess volumes are zero for ideal mixtures. Non-ideal mixtures are composed of particles for which the W–W, P–P, and W–P interactions are all different. As shown in Figure 2, the excess volumes of binary protein–water mixtures are negative over the whole composition range. A well-defined minima in the excess volume values exists at w_1~0.1, which indicates the densest structure packing of the studied binary systems. This means that the RNase A-water mixtures are non-ideal.

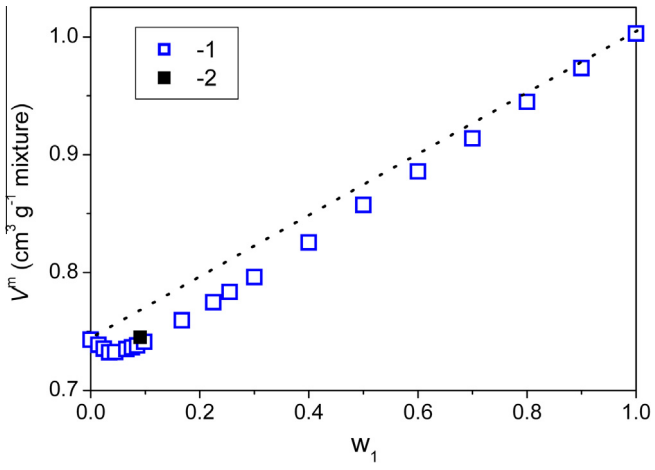

Figure 1. The V^m values for the water-RNase A mixtures as a function of the mass fraction of water, w_1, at 25 °C: (1) Ribonuclease A (this study). The standard errors of estimation of the V^m values were 0.002–0.004 cm^3 g^{-1}. Each experiment was performed 3–4 times. The dashed line corresponds to the ideal binary mixture. (2) Ribonuclease A. (Adapted data from Ref. [36].)

Table 1
Partial specific volume of ribonuclease A, $\overline{V}_2$,[a] at w_1 = 1.0, w_1 = 0 and 25 °C.

Protein	$\overline{V}_2$ (w_1 = 1.0), cm^3 g^{-1}	$\overline{V}_2$ (w_1 = 0), cm^3 g^{-1}
Ribonuclease	0.704 [10]; 0.704 [13]; 0.703 [this study]	0.743 [this study]

[a] The $\overline{V}_2$ values were estimated using the following equation: $V^m = w_1\overline{V}_1 + w_2\overline{V}_2$.

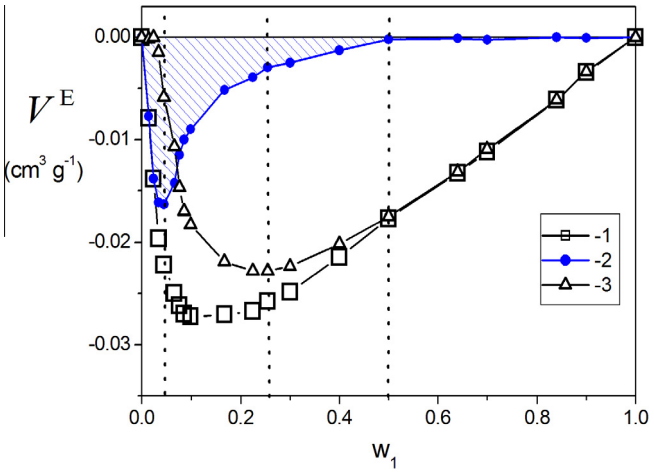

Figure 2. Excess thermodynamic functions plotted versus the mass fraction of water, w_1, at 25 °C: (1) V^E; (2) $w_1\overline{V}_1^E$; (3) $w_2\overline{V}_2^E$.

Figure 2 presents the dependencies of the $w_i\overline{V}_i^E$ ($w_1\overline{V}_1^E$ and $w_2\overline{V}_2^E$) functions plotted versus the water mass fraction. These functions were calculated using Eq. (3). They show the individual protein and water contributions to the excess thermodynamic functions in the entire range of water content. Figure 2 demonstrates that the protein contribution ($w_2\overline{V}_2^E$) reaches maximal values at w_1~0.25. However, the $w_1\overline{V}_1^E$ function reaches maximal values at low water content (w_1~0.05).

Figure 3 shows the dependence of the $\overline{V}_1^E$ values on the hydration level of RNase A. The $\overline{V}_1^E$ values were calculated using Eq. (6). Figure 4 presents the $\overline{V}_2^E$ values as a function of the mass fraction of water. The $\overline{V}_2^E$ values were calculated using Eq. (7). The excess partial volume curves presented in Figures 2–4 can be divided into four parts. At the lowest water content (w_1 = 0–0.05), the $\overline{V}_2^E$ values are close to zero, reflecting the fact that all of the protein molecules contact the same protein molecules dur-

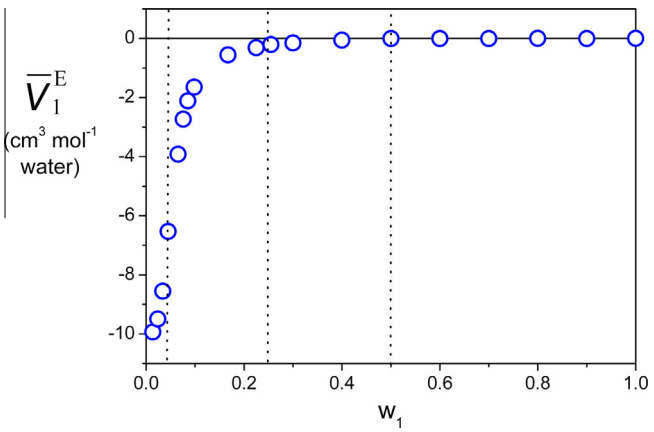

Figure 3. Excess partial volume of water, $\overline{V}_1^E$, as a function of the mass fraction of water, w_1, at 25 °C.

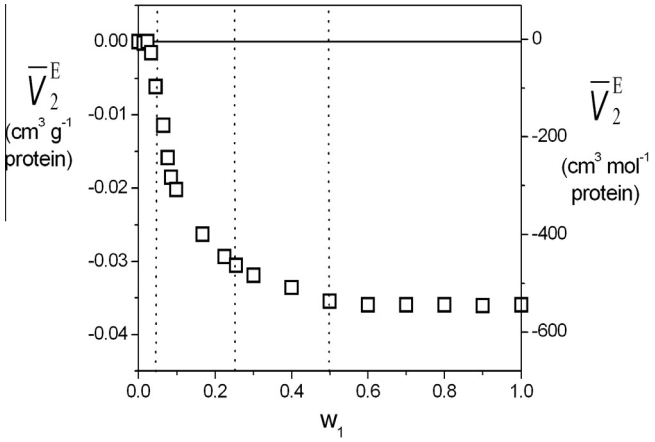

Figure 4. Excess partial volume of RNase, $\overline{V}^E_2$, as a function of the mass fraction of water, w_1, at 25 °C.

ing this range of water content (Figure 4). The proteins are in a glassy (rigid) state [1]. In the glassy state, the dehydration-induced conformational changes and restrictions on conformational transitions cause the protein to become frozen into a broad distribution of conformational states. No biological activity was observed at $w_1 < 0.05$ (Figure 4 from Ref. [8]).

At $w_1 < 0.05$, the $w_2\overline{V}^E_2$ values are close to zero (Figure 2). At $w_1 \sim 0.05$, the $w_1\overline{V}^E_1$ values reach maximal values. These facts imply that, at the lowest water content, changes in the excess volume, V^E, solely reflect water addition.

Hutchens et al. [37] examined the heat capacities of insulin at $w_1 = 0$ and 0.038 and chymotrypsinogen A at $w_1 = 0$ and 0.096, from −263 to 37 °C. No phase transition corresponding to the ice–liquid water transition was observed at low hydration.

This region corresponds to the hydration of charged groups [1,2,38]. At low water content, the $\overline{V}^E_1$ values differ significantly from zero (Figure 3). The $\overline{V}^E_1$ values are highly negative. As concluded in Ref. [9], the $\overline{V}^E_1$ ($w_1 = 0$) values are close to those observed for diethylamine, suggesting that, at the lowest w_1 values, the interaction with the protein elements containing the amino groups might be a dominant factor controlling the state of water molecules.

Part 2 ($w_1 = 0.05$–0.25) corresponds to the hydration of polar groups [1,2,38]. In this water content range, the $\overline{V}^E_1$ values change sharply from highly negative to moderate values (Figure 3). This sharp transition reflects the formation of a spanning hydrogen-bonded network of water at the protein surface [2,4]. The formation of this network occurs via a quasi-two-dimensional percolation transition of the hydration water at the enzyme surface [4].

The results obtained for biopolymers using several experimental methods have been summarised in Ref. [1]. The results suggest that proteins undergo a glasslike dynamic transition at a water content of approximately 10 wt% at 25 °C. This water content falls within part 2 in the present study. The transition from the glassy (rigid) to the flexible (elastic) state is accompanied by significant changes in the properties of the proteins [1]. For example, the apparent heat capacity of lysozyme, ϕC_{p2}, determined from isothermal experiments using a drop calorimeter, increases from extremely low values to high values in this water content interval [39]. During the isothermal sorption of water, a glasslike transition results in a step on the excess partial volume of RNase (Figure 4). The $\overline{V}^E_2$ values change sharply from extremely low values to highly negative values.

Part 3 ($w_1 = 0.25$–0.5) indicates the appearance of the 'structured' water [38]. 'Structured' water is composed of molecules that interact with hydrophobic surface patches on the protein-bridging between bound water molecules. Rupley and Careri [39] asserted that this region contains condensed water molecules over weakly interacting surface elements, which are likely nonpolar atoms, not polar groups or adjacent-charged.

The onset of biological activity was observed in this region. The enzyme-catalysed hydrolysis of the hexasaccharide of N-acetylglucosamine [$(GlcNAc)_6$] ([2], Figure 4 from Ref. [8]) was examined as a function of the water content and showed that the reaction grows sharply at $w_1 > 0.15$. The biological activity was suppressed within parts 1 and 2 (Figure 4 from Ref. [8]).

At the highest water content ($w_1 > 0.5$), the proteins are in a flexible (elastic) state [1]. Excess partial quantities attain their fully hydrated values. The $\overline{V}^E_2$ values (Figure 4) reach minimal values. At $w_1 < 0.5$, the $w_1\overline{V}^E_1$ values are near zero (Figure 2). Bulk water was observed in this region through proton NMR measurements [38]. The molecular motion of these water molecules is solely determined through the interaction characteristics of water molecules. Water addition has no significant effect on the excess thermodynamic functions. At $w_1 > 0.5$, the changes in the excess thermodynamic functions primarily reflect changes in the state of RNase A.

4.2. Effect of the protein hydrophobicity and polarity

To show the predictive ability of the proposed methodology, we related the obtained results to the general properties of the proteins. Hydrophobicity and polarity were selected as possible common parameters. The hydrophobicity of the studied proteins has been estimated as the average hydrophobicity of the side chains of the constitutive amino acids, H_ϕ (J per residue). The calculated H_ϕ values for many proteins were collected in [40].

We calculated the areas under the $w_1\overline{V}^E_1 = f(w_1)$ and $w_2\overline{V}^E_2 = f(w_1)$ curves for RNase A (this study), lysozyme, chymotrypsinogen A, α-chymotrypsin, β-lactoglobulin, and serum albumin [9]. These areas were defined as $A(w_1\overline{V}^E_1)$ and $A(w_2\overline{V}^E_2)$, respectively. The $A(w_1\overline{V}^E_1)$ area for RNase A is shown shaded in Figure 2.

The $A(w_2\overline{V}^E_2)$ values are plotted as a function of H_ϕ in Figure 5A. There is a clear correlation between the $A(w_2\overline{V}^E_2)$ values and H_ϕ. This correlation shows that the more hydrophobic a protein is, the more significant the $A(w_2\overline{V}^E_2)$ value is.

A polarity parameter, ***P***, was proposed by Bigelow [41]. It was defined as a volume ratio of polar amino acids to non-polar amino acids. There is a good correlation between the $A(w_1\overline{V}^E_1)$ values and Bigelow's polarity parameter (Figure 5B). This correlation means that the more hydrophilic a protein is, the more significant the $A(w_1\overline{V}^E_1)$ value is.

As a rule, most hydrophobic groups of globular proteins are buried in the interior of the protein molecule. On the other hand, charged and polar residues are located preferentially on the surface. H_ϕ may be regarded as a parameter characteristic mostly of the hydrophobic interior of a protein. ***P*** reflects the degree of the polarity of the protein surface.

RNase A is the most hydrophilic protein in this study. As shown in Figure 5B, this protein has the most significant hydrophilic hydration contribution, $A(w_1\overline{V}^E_1)$. Lactoglobulin is the most hydrophobic protein. As shown in Figure 5A, this protein has the most significant hydrophobic hydration contribution, $A(w_2\overline{V}^E_2)$. These correlations show that the contributions corresponding to the hydration of the protein interior and the protein surface are coupled differently to the excess volumes. The changes in the partial quantities corresponding to the protein interior are largely described by the $A(w_2\overline{V}^E_2)$ values. The changes in the partial quantities corresponding to the protein surface are described by the $A(w_1\overline{V}^E_1)$ values.

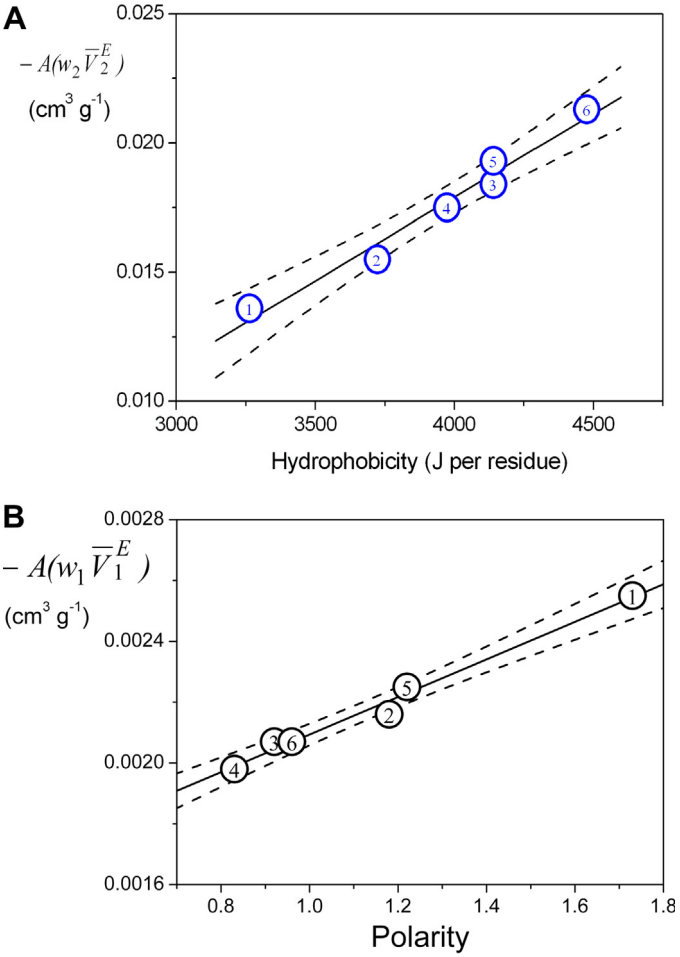

Figure 5. (1) RNase A; (2) Lysozyme; (3) Ovalbumin; (4) Chymotrypsinogen A; (5) Serum albumin; (6) β-Lactoglobulin. (A) Relationship between the $A(w_2\overline{V}_2^E)$ value and the hydrophobicity, H_ϕ, of proteins. $-A(w_2\overline{V}_2^E) = 0.008(0.002) + 6.5\cdot10^{-6}(0.6\cdot10^{-6})^*H_\phi$, where the number of the experimental points is $N = 6$, the standard error of estimation is $S_o = 4.6 * 10^{-4}$, and the correlation coefficient is $R = 0.992$. The dashed lines show the 95% confidence interval. (B) Relationship between the $A(w_1\overline{V}_1^E)$ value and Bigelow's polarity parameter, P, of proteins. $-A(w_1\overline{V}_1^E) = 0.015(0.001) + 6.2\cdot10^{-4}(0.4\cdot10^{-4})^*P$, where the number of the experimental points is $N = 6$, the standard error of estimation is $S_o = 2.8{*}10^{-5}$, and the correlation coefficient is R = 0.992. The dashed lines show the 95% confidence interval.

5. Conclusions

High-precision densitometry and water sorption measurements were applied to study the hydration of ribonuclease A. The hydration process was characterised by analysing the excess functions of mixing. This method facilitates the individual evaluation of the protein and water partial quantities in the entire range of water content. The excess partial volumes are extremely sensitive to changes in the state of water and the protein. It was shown that the protein and water contributions to the excess functions markedly depend on the hydration level. At the lowest water content, changes of the excess functions solely reflect water addition. At the highest water content, water addition has no significant effect on the excess functions. At $w_1 > 0.5$, the changes of the excess functions primarily reflect changes in the state of the protein.

The excess partial quantities for RNase A are compared with the published data for several unrelated globular proteins (lysozyme, chymotrypsinogen A, serum albumin, and lactoglobulin). These biomacromolecules represent a series of proteins in which the hydrophobicity is gradually changed over a wide range. It was found that the excess volumes for the studied proteins are determined by the hydration of hydrophilic and hydrophobic protein groups. The more hydrophilic a protein is, the more significant the hydrophilic hydration contribution is and *vice versa*. RNase A is the most hydrophilic protein in the present study, and it has the most significant hydrophilic hydration contribution. Lactoglobulin is the most hydrophobic protein in this study, and it has the most significant hydrophobic hydration contribution.

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