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Preferential hydration and solubility of proteins in aqueous solutions of polyethylene glycol

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Abstract

This paper is focused on the local composition around a protein molecule in aqueous mixtures containing polyethylene glycol (PEG) and the solubility of proteins in water+PEG mixed solvents. Experimental data from literature regarding the preferential binding parameter were used to calculate the excesses (or deficits) of water and PEG in the vicinity of β-lactoglobulin, bovine serum albumin, lysozyme, chymotrypsinogen and ribonuclease A. It was concluded that the protein molecule is preferentially hydrated in all cases (for all proteins and PEGs investigated). The excesses of water and deficits of PEG in the vicinity of a protein molecule could be explained by a steric exclusion mechanism, i.e. the large difference in the sizes of water and PEG molecules.

The solubility of different proteins in water+PEG mixed solvent was expressed in terms of the preferential binding parameter. The slope of the logarithm of protein (lysozyme, β-lactoglobulin and bovine serum albumin) solubility versus the PEG concentration could be predicted on the basis of experimental data regarding the preferential binding parameter. For all the cases considered (various proteins, various PEGs molecular weights and various pHs), our theory predicted that PEG acts as a salting-out agent, conclusion in full agreement with experimental observations. The predicted slopes were compared with experimental values and while in some cases good agreement was found, in other cases the agreement was less satisfactory. Because the established equation is a rigorous thermodynamic one, the disagreement might occur because the experimental results used for the solubility and/or the preferential binding parameter do not correspond to thermodynamic equilibrium.

Keywords: Protein; Aqueous polyethylene glycol; Preferential binding parameter; Solubility; Fluctuation theory

1. Introduction

Polyethy1ene glycol (PEG), one of the most useful protein salting-out agents, is considered to be the most successful precipitant for protein crystallization [1]. Unlike ethanol and other organic precipitating agents, PEG has little tendency to denature or to specifically interact with the proteins even when present in high concentrations and at elevated temperatures [2]. One can also vary its molecular weight in order to select the best choice for the precipitation of a particular protein. McPherson considers that PEG may be the best reagent for crystallizing proteins and that the optimum PEG molecular weight for this purpose is between 2000 and 6000 [2]. The optimum PEG molecular weight was selected on the basis of the viscosity of the solution, the denaturation action of aqueous PEG and protein solubility. It should also be mentioned that PEG is neither corrosive nor toxic, is not inflammable and has a very low vapor pressure [1]. In addition, polyethylene glycol is available commercially in good quality at reasonable prices.

However, the mechanism of PEG induced precipitation is almost unknown. The interactions between the protein (component 2), PEG (3) and water (1) as well as the local properties of PEG+water mixed solvent near the protein surface are of particular interest from this point of view.

One of the most informative experimental quantity for the understanding of the above issues is the preferential binding parameter [3–9]. The preferential binding parameter can be expressed at different concentration scales:

1) in molal concentrations

$$\Gamma_{23}^{(m)} \equiv \lim_{m_2 \to 0} \left(\partial m_3 / \partial m_2\right)_{T,P,\mu_3} \tag{1}$$

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where m_i is the molality of component i, P is the pressure, T the temperature (throughout this paper only isothermal–isobaric conditions are considered), and μ_i is the chemical potential of component i.

2) in molar concentrations

$$\Gamma_{23}^{(c)} \equiv \lim_{c_2 \to 0} (\partial c_3 / \partial c_2)_{T,P,\mu_3} \quad (2)$$

where c_i is the molar concentration of component i. It should be noted that $\Gamma_{23}^{(m)}$ and $\Gamma_{23}^{(c)}$ are defined at the infinite dilution of the protein.

Several authors reported measurements of the preferential binding parameter in the system water (1)/protein (2)/PEG (3) [10–14]. It was found that for various proteins, various PEGs molecular weights, and various PEG concentrations, the protein is preferentially hydrated and the PEG is excluded from the vicinity of the protein molecule. The prevalent viewpoint which explains such a behavior is based on the steric exclusion mechanism suggested by Kauzmann and cited in Ref. [15]. According to this mechanism [12,14], the deficit of PEG and the excess of water (in comparison with the bulk concentrations) are located in the shell (volume of exclusion) between the protein surface and a sphere of radius R (see Fig. 1) [12,14]. However, Lee and Lee [10,11] suggested that the preferential exclusion of the PEG from the protein surface also involves the protein hydrophobicity and charge.

In our recent publication [16], it was shown how the experimental data regarding the preferential binding parameter can be used to calculate the excess (or deficit) of water and cosolvent in the vicinity of a protein molecule. The methodology, based on the Kirkwood–Buff theory of solutions [17], allowed one to compare the concentrations of water and cosolvent molecules in the vicinity of the protein molecule with the bulk solution concentrations (water+cosolvent mixture) in absence of the protein molecule and ultimately to draw a conclusion about the preferential hydration or preferential solvation. Furthermore, such data allowed one to analyze the ability of a cosolvent to stabilize a protein, because the preferential hydration of a protein in an aqueous solution containing an organic compound is related to the ability of the latter to stabilize the structure of the protein [18–20].

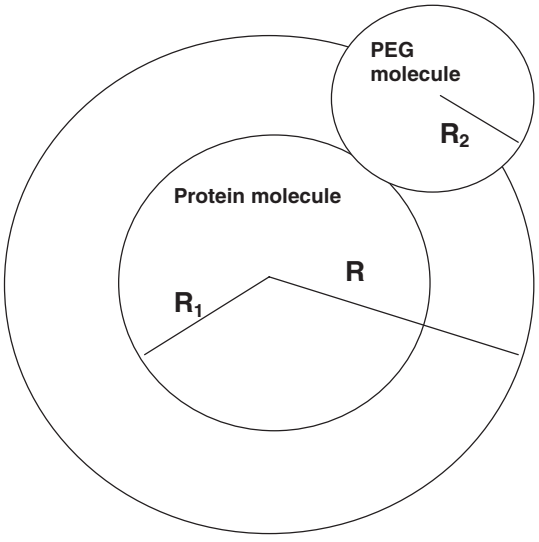

Fig. 1. The excess of water (in comparison with the bulk concentration) is located in the shell (volume of exclusion) between the protein surface and a sphere with an effective radius $R=R_1+R_2$, where R_1 is the radius of the protein molecule and R_2 is the radius of the PEG molecule (it is supposed that both the protein and the PEG molecules have spherical shapes). This figure is adapted from Refs. [12,14].

Another important use of the preferential binding parameter is its connection to the protein solubility. The authors of the present paper [21] showed that the preferential binding parameter is closely related to the solubility of a protein in a mixed solvent and that the experimental data regarding the preferential binding parameter can be used to predict how a cosolvent changes the solubility (salting-in or salting-out) or even to predict the solubility in a wide range of cosolvent concentrations.

Consequently, three important characteristics of the protein behavior in aqueous solutions (stability, preferential hydration (or solvation) and solubility) can be related to the preferential binding parameter. For instance, the addition of glycerol leads to an excess of water in the vicinity of the protein [16], i.e. the protein is preferentially hydrated; in addition, glycerol can be used to stabilize the native structure of the protein [18]. Glycerol also decreases the solubility of the protein, i.e. glycerol is a salting-out agent [21]. In contrast, the addition of urea leads to an excess of urea in the vicinity of the protein [22], i.e. the protein is preferentially solvated [18]. Urea increases the solubility of the protein, i.e. urea induces a salting-in effect [21]. In addition, it is well-known that urea can cause protein denaturation [4–6,22]. A similar analysis can be carried out for such an important cosolvent as the polyethy1ene glycol.

In the present paper, the Kirkwood–Buff theory of solutions will be used to examine dilute mixtures of various proteins in aqueous solutions containing PEGs of different molecular weights in terms of the preferential binding parameter. As already mentioned, extensive experimental data regarding the preferential binding parameter in the systems water/protein/PEG are available in the literature [10–14].

The purpose of the present analysis is two-fold: (i) to examine the local composition of a mixed solvent around different proteins in dilute solutions of proteins in water/PEG mixed solvents as a function of the PEG concentration and molecular weight and to use the obtained results to identify the mechanism of protein hydration in the presence of PEG, and (ii) to predict the solubility of proteins in water/PEG mixed solvents. Finally, the obtained results are compared with experiments available in the literature.

From a theoretical viewpoint, the present analysis can allow one to better understand the interactions between a protein and the constituents of a mixed solvent in the system water/protein/PEG and how these interactions differ from those between a protein and the constituents of a mixed solvent containing "regular" and not polymeric cosolvents. From a practical perspective, the results of this paper could allow one to better understand and improve the design of protein precipitation techniques with polyethylene glycol.

2. Theoretical part

2.1. Expressions for the preferential binding parameter via the Kirkwood–Buff integrals

It was demonstrated that the preferential binding parameters $\Gamma_{23}^{(m)}$ and $\Gamma_{23}^{(c)}$ can be expressed via the Kirkwood–Buff integrals as follows [23]:

$$\Gamma_{23}^{(m)} = \frac{c_3}{c_1} + c_3(G_{23} - G_{12} + G_{11} - G_{13}) \tag{3}$$

and [16]

$$\Gamma_{23}^{(c)} = c_3(G_{23} - G_{13}) \tag{4}$$

where $G_{\alpha\beta}$ are the Kirkwood–Buff integrals defined as [17]

$$G_{\alpha\beta} = \int_0^\infty (g_{\alpha\beta} - 1) 4\pi r^2 \mathrm{d}r \tag{5}$$

$g_{\alpha\beta}$ is the radial distribution function between species α and β, and r is the distance between the centers of molecules α and β.

The Kirkwood–Buff integrals G_{11} and G_{13} can be calculated from the characteristics of the protein-free mixed solvents, whereas G_{12} and G_{23} depend on the properties of the infinitely dilute protein solutions [16].

One can also write the following expression for the partial molar volume of a protein at infinite dilution in a mixed solvent (V_2^∞) in terms of the Kirkwood–Buff theory of solution [24]

$$V_2^\infty = -c_1 V_1 G_{12} - c_3 V_3 G_{23} + kTk_T \cong -c_1 V_1 G_{12} - c_3 V_3 G_{23} \tag{6}$$

where V_i is the partial molar volume of component i, k is the Boltzmann constant, and k_T is the isothermal compressibility of the mixed solvent. The partial molar volumes V_1 and V_3 are those for the protein- free mixed solvent, and the contribution of kTk_T is usually negligible in comparison with $c_1 V_1 G_{12}$ and $c_3 V_3 G_{23}$.

Experimental data regarding $\Gamma_{23}^{(m)}$ and V_2^∞ are available in the literature for many water/protein/cosolvent systems [4–14,18–20,22]. The Kirkwood–Buff integrals G_{11} and G_{13} are for the binary mixture water/cosolvent and can be calculated as described in the literature [24–28]. The Kirkwood–Buff integrals G_{12} and G_{23} can be calculated from Eqs. (3) and (6) using experimental data for $\Gamma_{23}^{(m)}$, V_2^∞, V_1 and V_3.

2.2. Analytical expressions for the Kirkwood–Buff integrals G_{12}, G_{23}, G_{11} and G_{13} at infinite dilution of a protein

The analytical expressions for the Kirkwood–Buff integrals G_{12}, G_{23}, G_{11} and G_{13} at infinite dilution of a protein are helpful in analyzing the different factors that affect the preferential binding parameter and ultimately the preferential hydration or solvation. The following analytical expressions for the Kirkwood–Buff integrals G_{12} and G_{23} can be written [16]:

$$G_{12} = kTk_T - \frac{J_{21} V_3 c_3 + J_{11} V_2^\infty c_1}{(c_1 + c_1 J_{11} + c_3)} - \frac{V_3 c_3 (c_1 + c_3)(V_1 - V_3) + V_2^\infty (c_1 + c_3)}{(c_1 + c_1 J_{11} + c_3)} \tag{7}$$

and

$$G_{23} = kTk_T + \frac{J_{21} V_1 c_1 - J_{11} c_1 V_2^\infty}{(c_1 + c_1 J_{11} + c_3)} + \frac{c_1 V_1 (c_1 + c_3)(V_1 - V_3) - V_2^\infty (c_1 + c_3)}{(c_1 + c_1 J_{11} + c_3)} \tag{8}$$

where $J_{11} = \lim_{x_2 \to 0} \left(\frac{\partial \ln \gamma_1}{\partial x_1} \right)_{x_2}$, $J_{21} = \lim_{x_2 \to 0} \left(\frac{\partial \ln \gamma_2}{\partial x_1} \right)_{x_2}$, x_i is the mole fraction of component i, and γ_i is the activity coefficient of component i in a mole fraction scale. The expressions for the Kirkwood–Buff integrals G_{11} and G_{13} are also well-known from the literature (see for example [26,27])

$$G_{11} = kTk_T - \frac{(c_1 + c_3)^2 V_1 V_3}{(c_1 + c_1 J_{11} + c_3)} + \frac{(c_1 + c_3)(V_3 - V_1) - J_{11}}{(c_1 + c_1 J_{11} + c_3)} \tag{9}$$

and

$$G_{13} = kTk_T - \frac{(c_1 + c_3)^2 V_1 V_3}{(c_1 + c_1 J_{11} + c_3)}. \tag{10}$$

Expressions for G_{12}, G_{23}, G_{11} and G_{13} for an ideal ternary mixture at infinite dilution of a protein can be obtained from Eqs. (7)–(10) by taking into account that according to the definition of an ideal mixture [29], the activities of the components are equal to their mole fractions (x_i) and their partial molar volumes are equal to those of the pure components ($V_i = V_i^0$). These expressions are [16]

$$G_{12}^{(\mathrm{id})} = kTk_T^{(\mathrm{id})} - V_3^0 c_3 (V_1^0 - V_3^0) - V_2^0 \tag{11}$$

$$G_{23}^{(\mathrm{id})} = kTk_T^{(\mathrm{id})} + V_1^0 c_1 (V_1^0 - V_3^0) - V_2^0 \tag{12}$$

$$G_{11}^{(\mathrm{id})} = kTk_T^{(\mathrm{id})} - (c_1 + c_3) V_1^0 V_3^0 + V_3^0 - V_1^0 \tag{13}$$

and

$$G_{13}^{(\mathrm{id})} = kTk_T^{(\mathrm{id})} - (c_1 + c_3) V_1^0 V_3^0. \tag{14}$$

All the above equations will be later used in the calculations of the excesses and deficits of the constituents of a mixed solvent in the vicinity of a protein surface.

2.3. Excess and deficit numbers of molecules of water and cosolvent around a protein molecule

The conventional way to calculate the excess number of molecules i around a molecule j ($\Delta n_{ij}'$) is provided by the relation [25]:

$$\Delta n_{ij}' = c_i G_{ij}. \tag{15}$$

However, as noted by Matteoli and Lepori [30] and Matteoli [31], the above expression leads for an ideal binary mixture to non-zero values, even though they are expected to vanish. For the above reasons, Eq. (15) was replaced by [27,30,31]:

$$\Delta n_{ij} = c_i(G_{ij} - G_{ij}^R) \tag{16}$$

where G_{ij}^R is the Kirkwood–Buff integral of a reference state. Matteoli and Lepori [30] and Matteoli [31] suggested the ideal solution ($^{(id)}$) as the reference state because then Δn_{ij} becomes zero for an ideal solution, as intuition suggests that it should be. Shulgin and Ruckenstein [27] suggested a reference state in which all the activity coefficients are equal to unity but there are no constraints on the partial molar volumes of the components (*superscript* $^{(SR)}$). This reference state provides zero excesses for ideal mixtures for both binary and ternary mixtures [27,32]. It also satisfies the volume conservation condition, which for a binary mixture can be expressed as "the volume occupied by the excess i molecules around a j molecule must be equal to the volume left free by the j molecules around the same j molecule" [30,31]. Far from critical conditions, the above two reference states provide almost the same results [27,33]. In this paper, we consider $G_{ij}^R = G_{ij}^{(SR)}$.

For the ($^{(SR)}$) reference state, Eqs. (7) and (8) for G_{12} and G_{23} for an infinitely dilute protein in a mixed solvent can be recast as follows [16]:

$$G_{12}^{(SR)} = kTk_T^{(SR)} - V_3c_3(V_1 - V_3) - V_2^{\infty} \tag{17}$$

and

$$G_{23}^{(SR)} = kTk_T^{(SR)} - V_1c_1(V_1 - V_3) - V_2^{\infty}. \tag{18}$$

G_{12} and G_{23} will be first calculated by combining Eqs. (3) and (6) with experimental data regarding the preferential binding parameter $\Gamma_{23}^{(m)}$ and the partial molar volumes V_2^{∞}, V_1 and V_3. Furthermore the excess (or deficit) number of molecules of water and PEG around a protein molecule will be calculated using Eq. (16).

2.4. Relationship between preferential interaction of a protein in an aqueous PEG solution and protein solubility

The solubility of a protein in a water+cosolvent mixture depends on many factors such as temperature, cosolvent concentration, pH, type of buffer used, etc. The solubilities of proteins in aqueous PEGs solutions have been investigated both experimentally [34–42] and theoretically [38,41,43–48].

The experimental results showed that: (i) the addition of PEG decreases the protein solubility; (ii) the low molecular weight PEG is a less effective precipitant than the high molecular weight PEG, and (iii) the log of protein solubility versus PEG concentration is linear. The authors of the present paper [21] recently derived the following relation for the solubility of a protein in a mixed solvent as a function of the cosolvent mole fraction

$$\left(\frac{\partial \ln y_2}{\partial x_3}\right) = -\frac{c_3(c_1 + c_3)V_1 - \Gamma_{23}^{(m)}(1 - c_3V_3)(c_1 + c_1J_{11} + c_3)}{c_1c_3V_1} \tag{19}$$

where y_2 is the protein solubility in mole fraction.

Eq. (19) is a rigorous thermodynamic equation at infinite protein dilution. It allows one to derive a simple criteria for salting-in or salting-out for low cosolvent concentrations. At low cosolvent concentrations ($c_3 \to 0$)

$$\left(\frac{\partial \ln y_2}{\partial x_3}\right) = -\left(\frac{\partial \ln y_2}{\partial x_1}\right) = \frac{\alpha}{V_1^0} - 1 \tag{20}$$

where $\alpha = \lim_{c_3 \to 0} \frac{\Gamma_{23}^{(m)}}{c_3}$ and V_1^0 is the molar volume of pure water. Consequently, one can conclude that for low cosolvent concentrations salting-in occurs when

$$\left(\frac{\partial \ln y_2}{\partial x_3}\right) > 0, \qquad \text{hence when } \alpha > V_1^0 \tag{21}$$

and salting-out occurs when

$$\left(\frac{\partial \ln y_2}{\partial x_3}\right) < 0, \qquad \text{hence when } \alpha < V_1^0. \tag{22}$$

It is well-known [8,49,50] that the preferential binding parameter $\Gamma_{23}^{(m)}$ is, at least at low cosolvent concentrations, proportional to the cosolvent concentration. Consequently salting-in or salting-out depends on the slope of the curve $\Gamma_{23}^{(m)}$ versus concentration for small values of c_3.

The above criteria for salting-in or salting-out (Eqs. (21), (22)) are valid [21]: (i) for $c_3 \to 0$, hence when a small amount of cosolvent is added to pure water; (ii) for ternary mixtures (water (1)–protein (2)–cosolvent (3)) (it should be emphasized that the experimental results regarding the preferential binding parameter $\Gamma_{23}^{(m)}$ and the solubilities are usually for mixtures which involve in addition a buffer, and the effect of the buffer is taken into account only indirectly via the preferential binding parameter $\Gamma_{23}^{(m)}$); (iii) for infinite dilution (this means that the protein solubility is supposed to be small enough to satisfy the infinite dilution approximation ($\gamma_2 \cong \gamma_2^{\infty}$, where γ_2^{∞} is the activity coefficient of a protein at infinite dilution)); (iiii) for experimental preferential binding parameters $\Gamma_{23}^{(m)}$ and solubilities determined at low cosolvent concentrations.

Physically speaking, Eq. (20) provides the slope of a curve representing the dependence of log mole fraction of the solubility versus the cosolvent mole fraction.

The following expression for protein solubility in a dilute cosolvent solution can be derived from Eq. (19), when $\Gamma_{23}^{(m)}$ is proportional to c_3 [21]:

$$\ln\frac{y_2}{y_2^w} = -\frac{(\alpha - V_3\Gamma_{23}^{(m)})\ln a_w}{V_1} + \ln x_1 \approx -\frac{(\alpha - V_3\Gamma_{23}^{(m)})\ln a_w}{V_1} = -\frac{(1 - V_3c_3)\alpha \ln a_w}{V_1} = -c_1\alpha \ln a_w \tag{23}$$

where y_2^w is the protein solubility in the cosolvent-free water plus buffer and a_w is the water activity in the protein-free mixed solvent. Eq. (23) allows one to calculate the protein solubility if the composition dependence of $\Gamma_{23}^{(m)}$ is available (a_w and the partial molar volumes V_1 and V_3 are characteristics of the protein-free mixed solvent).

3. Numerical estimations for various water/protein/PEG systems

3.1. Kirkwood–Buff integrals and the excess (or deficit) number of molecules of water and PEG around a protein molecule

The Kirkwood–Buff integrals and the excess (or deficit) number of molecules of water and PEG around a protein molecule were calculated for β-lactoglobulin (β-LG), bovine serum albumin (BSA), lysozyme, chymotrypsinogen and ribonuclease A (RNase A). Experimental data regarding $\Gamma_{23}^{(m)}$ and V_2^{∞} for these systems are available in the literature [11,12,14]. The partial molar volumes V_1 and V_3 in the aqueous solutions of PEGs were calculated using the experimental data and the correlations suggested in Ref. [51].

The Kirkwood–Buff integrals G_{12} and G_{23} were calculated from Eqs. (3) and (6) using experimental data for $\Gamma_{23}^{(m)}$, V_2^{∞}, V_1 and V_3. The difference between G_{11} and G_{13} in the right hand side of Eq. (3) was calculated using the expression (see Eqs. (9) and (10)):

$$G_{11}-G_{13}=\frac{(c_1+c_3)(V_3-V_1)-J_{11}}{(c_1+c_1J_{11}+c_3)}\approx V_3-V_1. \tag{24}$$

The above approximation simplifies the calculations without affecting much the accuracy (see Appendix).

The calculated Kirkwood–Buff integrals G_{12} and G_{23} can be found in the Supplementary Material.

The excess (or deficit) numbers of molecules of water and PEG (Δn_{12} and Δn_{23}) around a protein molecule were calculated using Eq. (16) and are listed in Table 1. The results demonstrate that in all cases there is preferential exclusion of PEG from the surface of the protein, conclusion in agreement with previous observations [10–14]. There is only one exception (water/lysozyme/PEG 200, at a concentration of 10 g PEG/100 ml solution). However, this is probably caused by the inaccuracy in the experimental value of $\Gamma_{23}^{(m)}$ (according to the authors of Ref. [12], for this composition, $\Gamma_{23}^{(m)}=0.66\pm1.32$ [mol/mol]).

In order to better understand why PEG is preferentially excluded from the vicinity of a protein molecule, we calculated the excess (or deficit) number of molecules of water and PEG around a protein molecule as a function of the PEG molecular weight at a constant PEG weight concentration. The results of the calculations are presented in Fig. 2 (only the Δn_{12}s are plotted; Δn_{23} can be calculated using the balance relation $V_3\Delta n_{23}=-V_1\Delta n_{12}$). One can see from Fig. 2 that the excess number of molecules of water around a protein increases monotonically with increasing PEG molecular weight (and, respectively, molar volume) in agreement with the above mentioned steric exclusion mechanism.

Table 1
The excess (or deficit) number of molecules of water and PEG around a protein molecule as a function of cosolvent concentration

System	g of PEG/100 ml of solution	Δn_{12} [mol/mol]	Δn_{23} [mol/mol]	ΔV [l/mol protein][A]
Water/lysozyme/PEG 400 (pH=3.0) [11][B]	2.8	109.9	−5.9	2.0
	5.6	160.6	−8.6	2.9
	11.2	186.1	−10.0	3.4
	22.4	211.6	−11.4	3.8
	33.6	240.8	−12.9	4.4
	44.8	211.1	−11.1	3.8
Water/lysozyme/PEG 1000 (pH=3.0) [11]	2.5	45.6	−1.0	0.8
	5	44.6	−1.0	0.8
	10	85.1	−1.8	1.5
	20	115.8	−2.5	2.1
	30	138.8	−3.9	2.5
Water/lysozyme/PEG 4000 (pH=3.0) [11]	0.5	45.5	−0.2	0.8
	1.25	135.6	−0.7	2.5
	2.5	166.1	−0.9	3.0
	3.75	170.5	−0.9	3.1
Water/β-LG/PEG 200 (pH=2.0) [12]	10	−5.0	0.5	−0.1
	20	18.1	−2.0	0.3
	30	18.8	−2.0	0.3
	40	14.2	−1.5	0.3
Water/1.2 β-LG/PEG 400 (pH=2.0) [12]	30	66.0	−3.5	1.2
	40	57.2	−3.0	1.0
Water/β-LG/PEG 600 (pH=2.0) [12]	10	53.7	−1.9	1.0
	20	79.4	−2.9	1.4
	30	59.6	−2.1	1.1
Water/β-LG/PEG 1000 (pH=2.0) [12]	10	104.3	−2.2	1.9
	20	109.6	−2.4	2.0
	30	105.5	−2.3	1.9

[A] ΔV is the volume occupied by the excess of water (or by the deficit of PEG) molecules around a protein molecule, [B] the source of experimental data regarding $\Gamma_{23}^{(m)}$ and V_2^{∞} used in calculations.

In order to provide additional insight on the PEG exclusion, the dependence of the excess number of molecules of water in the vicinity of a protein molecule is plotted against the volume of exclusion (V_S), volume inaccessible to the PEG molecules in the vicinity of a protein molecule (see Fig. 1). The volume of exclusion (cm^3/mol protein) was calculated using the expression [12,14]:

$$V_S=(4\pi N_A/3)[(R_1+R_2)^3-R_1^3]\cdot10^{-24} \tag{25}$$

where N_A is Avogadro's number.

The radii of various protein molecules were taken from Ref. [14] where they were estimated from the partial specific volumes of the proteins and protein molecular weights. The radii of the PEG molecules were taken equal to their radii of gyration [14]. The dependence of the excess number of molecules of water in the vicinity of a protein molecule against the volume of exclusion is presented in Fig. 3. It shows that in all cases the excess number of molecules of water in the vicinity of a protein molecule is almost proportional to the exclusion volume. This proportionality constitutes an argument in the favor of the steric exclusion mechanism. However, one should note that the excess water molecules are assumed to be located in the volume of exclusion and it is supposed that both the protein and the PEG molecules have spherical shapes.

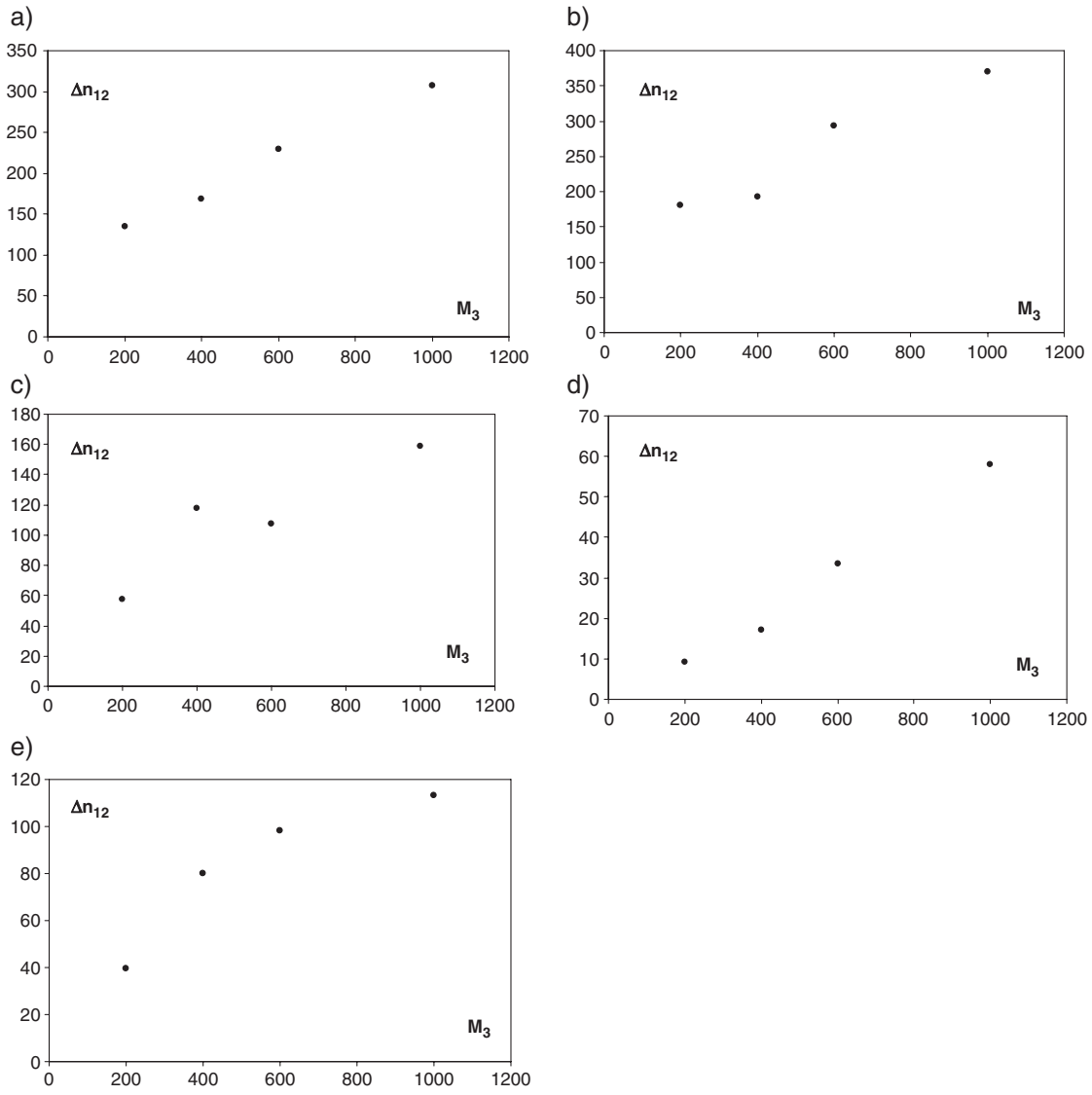

Fig. 2. Dependence of Δn_{12} [mol/mol] on molecular weight of PEG (M_3) for various proteins: a) BSA (pH=3.0), b) BSA (pH=7.0), c) chymotrypsinogen (pH=3.0), d) lysozyme (pH=7.0), e) RNase A (pH=2.0). Experimental data regarding $\Gamma_{23}^{(m)}$ and V_2^{∞} for these systems were taken from Ref. [14].

A steric exclusion mechanism implies that a geometric factor and not an energetic one, such as the differences in the intermolecular interactions between the constituents of the water+protein+PEG mixtures is responsible for the local composition around a protein molecule. This constitutes the main difference between the preferential binding in water+protein+PEG mixtures and water+protein+low molecular weight cosolvents (such as urea, glycerol, alcohol, etc.) mixtures.

3.2. Solubility of different proteins in water+PEG mixed solvents

Generally the experimental data regarding the solubility of a protein in a water+PEG mixture are presented as a linear dependence of the logarithm of the solubility versus the PEG concentration. Eq. (23) can be used for solubility calculations when the values of y_2^{w} are available; unfortunately, such data could not be found in the literature. Eq. (20) can be, however, used to predict the slope of the solubility curve and this prediction can be compared with experiment. The calculated and measured results are listed in Table 2. Table 2 reveals that for various proteins and various PEGs molecular weights, Eq. (20) predicts a negative slope $\left(\frac{\partial \ln y_2}{\partial x_3}\right)$ at $c_3=0$, and hence a salting-out effect of PEG on protein solubility. Such a conclusion is in agreement with most experimental data regarding the protein solubility in aqueous PEG mixtures [34–42]. However, there are a few investigations [52,53] in which a salting-in effect of PEG on protein solubility was found. One can also see from Table 2 that there is no complete agreement between the experimental solubilities of various proteins in aqueous PEG mixtures obtained in different laboratories. For example, the value of $\left(\frac{\partial \ln y_2}{\partial x_3}\right) = -0.08$ at $c_3=0$ for the solubility of BSA in water+PEG 3350 at pH=4.6 [41] is very different from $\left(\frac{\partial \ln y_2}{\partial x_3}\right) = -0.23$ at $c_3=0$ for the solubility of HSA in water+PEG 4000 at pH=4.5 [38]. These two cases are, however, very similar and one should expect the slopes to be comparable.

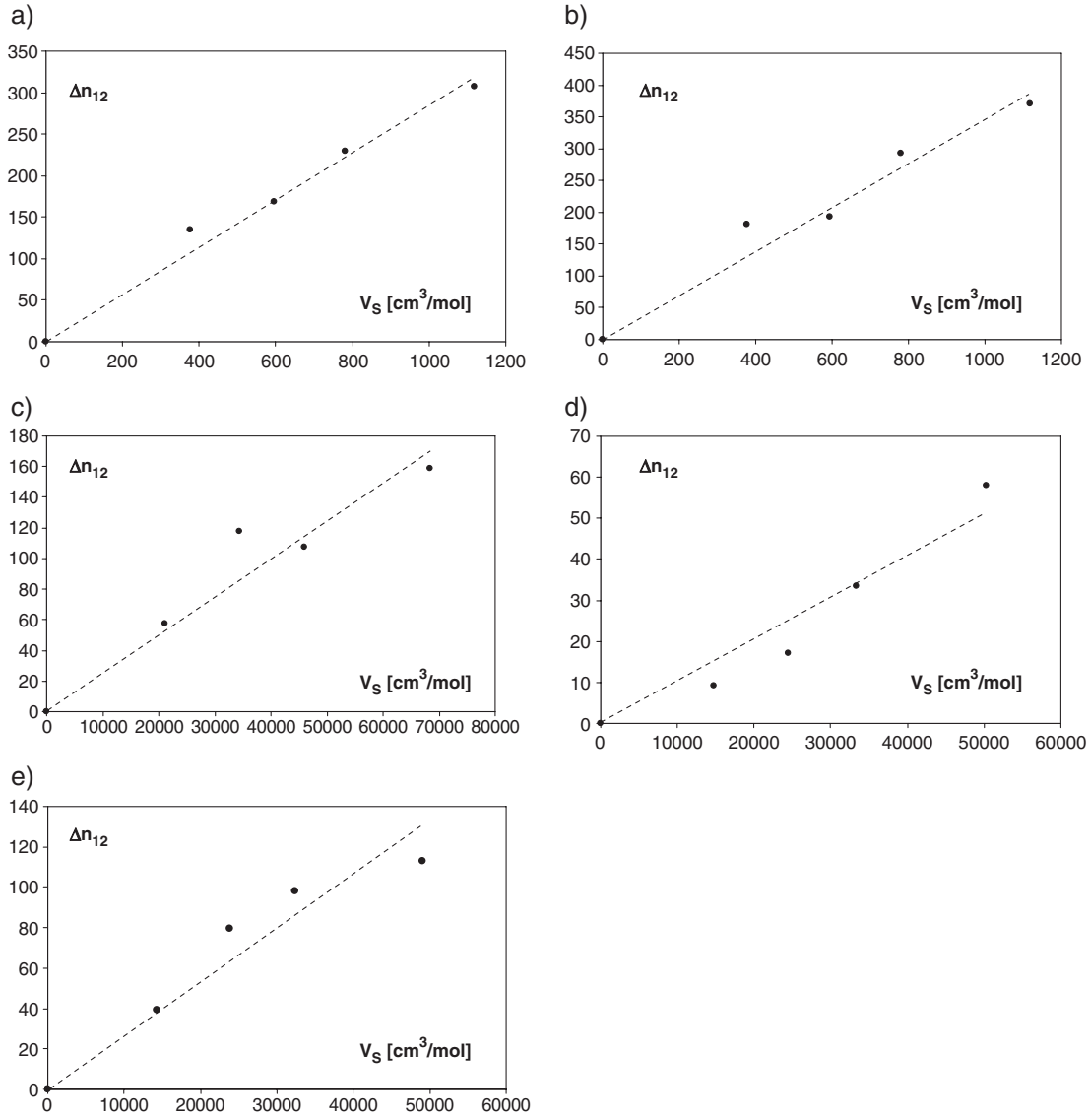

Fig. 3. Dependence of Δn_{12} [mol/mol] on the volume of exclusion (V_S) for various proteins: a) BSA (pH=3.0), b) BSA (pH=7.0), c) chymotrypsinogen (pH=3.0), d) lysozyme (pH=7.0), e) RNase A (pH=2.0). The dashed lines are shown for comparison. Experimental data regarding $\Gamma_{23}^{(m)}$ and V_2^{∞} for these systems were taken from Ref. [14].

It should also be mentioned that there are large discrepancies between the experimental values for the preferential binding parameter $\Gamma_{23}^{(m)}$ obtained by different authors. For example, $\Gamma_{23}^{(m)}=-2.0$ [mol/mol] for BSA in water/PEG 1000 at 10% PEG (w/v) and pH=3 [11], and for the same concentration $\Gamma_{23}^{(m)}=-7.21$ [mol/mol] (at pH=2) [14]. Another example, $\Gamma_{23}^{(m)}=-2.45$ [mol/mol] for β-LG in water/PEG 1000 at 10% PEG (w/v) and pH=2 [12], and for the same concentration $\Gamma_{23}^{(m)}=-0.18$ [mol/mol] (at pH=3) [11]. Such large differences led to the scattering in predicted solubilities.

Our Eqs. (19) and (23) are rigorous thermodynamic relations which provide a relation between the protein solubility and the preferential binding parameter $\Gamma_{23}^{(m)}$. These thermodynamic equations provide a consistency test between the protein solubility $\left(\frac{\partial \ln y_2}{\partial x_3}\right)$ and the preferential binding parameter $\Gamma_{23}^{(m)}$. If either the protein solubility and/or the preferential binding parameter do not correspond to thermodynamic equilibrium, then Eqs. (19) and (23) cannot be satisfied. We employed all available experimental data regarding the preferential binding parameter for the systems water (1)+protein (2)+PEG (3) to calculate the slope of the protein solubility $\left(\frac{\partial \ln y_2}{\partial x_3}\right)$ at $c_3=0$ and compared the calculated values with the experimental ones. Table 2 shows that there are cases in which there is a reasonable agreement between the experimental and the predicted values. This occurs, for instance, for the solubility of BSA (HSA) in water+high (larger or equal to 4000) molecular weight PEG. There are, however, also cases in which there are differences as large as a factor of six. As already emphasized, in the latter cases either the solubility and/or the preferential binding parameter determined experimentally do not correspond to thermodynamic equilibrium.

Table 2
Comparison between the experimental slopes of the solubility versus PEG mole fraction curve $\left(\frac{\partial \ln y_2}{\partial x_3}\right)$ with the results predicted by Eq. (20)

Experiment			Prediction		
Protein+PEG	pH, reference[A]	Slope	Protein+PEG	pH, reference[B]	Slope
Lysozyme+PEG 4000	pH=7.0, [38]	−0.02	Lysozyme+PEG 200	pH=7.0, [14]	−0.04
			Lysozyme+PEG 400	pH=7.0, [14]	−0.04
			Lysozyme+PEG 400	pH=3.0, [11]	−0.16
			Lysozyme+PEG 600	pH=7.0, [14]	−0.04
			Lysozyme+PEG 1000	pH=7.0, [14]	−0.05
			Lysozyme+PEG 1000	pH=3.0, [11]	−0.01
			Lysozyme+PEG 2000	pH=7.0, [14]	−0.07
			Lysozyme+PEG 3000	pH=7.0, [14]	−0.05
			Lysozyme+PEG 4000	pH=7.0, [14]	−0.11
			Lysozyme+PEG 4000	pH=3.0, [11]	−0.13
			Lysozyme+PEG 6000	pH=7.0, [14]	−0.09
β-LG+PEG 20,000	pH=5.0, [36]	−0.04 and −0.05[C]	β-LG+PEG 200	pH=2.0, [12]	−0.02
			β-LG+PEG 400	pH=2.0, [12]	−0.04
			β-LG+PEG 600	pH=2.0, [12]	−0.04
			β-LG+PEG 1000	pH=2.0, [12]	−0.04
			β-LG+PEG 1000	pH=3.0, [11]	−0.01
			β-LG+PEG 2000	pH=2.0, [14]	−0.21
			β-LG+PEG 3000	pH=2.0, [14]	−0.18
			β-LG+PEG 4000	pH=2.0, [14]	−0.17
			β-LG+PEG 6000	pH=2.0, [14]	−0.13
HSA[D]+PEG 400	pH=4.5, [38]	−0.09	BSA+PEG 200	pH=7.0, [14]	−0.84
HSA+PEG 600	pH=4.5, [38]	−0.11			
HSA+PEG 1000	pH=4.5, [38]	−0.14			
BSA+PEG 1450	pH=4.6, [41]	−0.09 and −0.10	BSA+PEG 400	pH=7.0, [14]	−0.45
BSA+PEG 1450	pH=7.0, [41]	−0.09 and −0.10			
BSA+PEG 3350	pH=4.6, [41]	−0.08 and −0.08			
BSA+PEG 3350	pH=7.0, [41]	−0.11 and −0.10	BSA+PEG 600	pH=7.0, [14]	−0.46
BSA+PEG 3350	pH=8.0, [41]	−0.02 and −0.03			
HSA+PEG 4000	pH=3.8, [38]	−0.16	BSA+PEG 1000	pH=7.0, [14]	−0.29
HSA+PEG 4000	pH=4.5, [38]	−0.23			
HSA+PEG 4000	pH=5.2, [38]	−0.21			
HSA+PEG 4000	pH=4.5, [38]	−0.15	BSA+PEG 1000	pH=3.0, [11]	−0.08
HSA+PEG 6000	pH=4.5, [38]	−0.27			
BSA+PEG 6000	pH=4.0, [34]	−0.29			
BSA+PEG 6000	pH=5.1, [34]	−0.27	BSA+PEG 2000	pH=7.0, [14]	−0.33
BSA+PEG 6000	pH=5.8, [34]	−0.26			
BSA+PEG 8000	pH=4.6, [41]	−0.13 and −0.12	BSA+PEG 3000	pH=7.0, [14]	−0.20
BSA+PEG 8000	pH=7.0, [41]	−0.20 and −0.12			
BSA+PEG 8000	pH=8.0, [41]	−0.07 and −0.07			
BSA+PEG 10,000	pH=4.6, [41]	−0.13 and −0.12	BSA+PEG 4000	pH=7.0, [14]	−0.21
BSA+PEG 10,000	pH=7.0, [41]	−0.08 and −0.20			
BSA+PEG 10,000	pH=8.0, [41]	−0.04 and −0.06			
HSA+PEG 20,000	pH=4.5, [38]	−0.27	BSA+PEG 6000	pH=7.0, [14]	−0.19
BSA+PEG 20,000	pH=5.0, [34]	−0.22 and −0.24			

[A]References regarding the experimental values of solubility data, [B]references regarding the experimental values of the preferential binding parameter, [C]two experimental data sets are available and [D]HSA designates human serum albumin.

According to experimental observations [34–41], the logarithm of the protein solubility versus PEG concentration exhibits linearity over a wide range of PEG concentrations. Eq. (23) allows one to examine this issue. For the sake of simplicity only the dilute region ($m_3 < 0.5$) was considered. It is worth noting that most of the experimental measurements of the preferential binding parameter $\Gamma_{23}^{(m)}$ and protein solubility for the system water+protein+PEG were carried out in this composition range [11–14,34–42]. In this composition range the preferential binding parameter $\Gamma_{23}^{(m)}$ is proportional to the concentration of the cosolvent [8,49,50] and Eq. (23) becomes a rigorous one. Eq. (23) reveals that the linearity (or nonlinearity) of $\ln y_2$ versus the PEG concentration depends entirely on the characteristics of the protein-free mixed solvent water/PEG. The dependence of the product $c_1 \ln a_w$ on PEG concentration is plotted in Fig. 4 for various PEG molecular weights. It shows an almost linear behavior of the logarithm of the protein solubility versus PEG concentration for PEG 1000 and PEG 4000. However, the logarithm of protein solubility versus the PEG concentration for PEG 6000 is nonlinear and one can expect the same nonlinear behavior to occur for higher molecular weight PEGs.

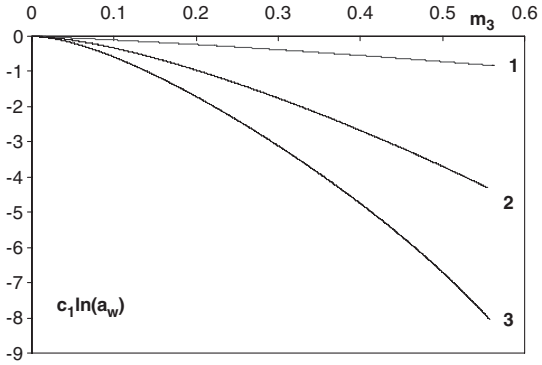

Fig. 4. Dependence of the product $c_1 \ln a_w$ in protein-free mixed solvent water/PEG on the PEG molality: 1) PEG 1000, 2) PEG 4000, 3) PEG 6000. Water activities in water/PEG mixtures were calculated as indicated in Ref. [54].

Another important issue is the salting-out strength of various molecular weight PEGs. It is well-known from literature that the low molecular weight PEGs are less effective precipitants than the high molecular weight PEG [11–14,34–42]. Eq. (20) provides the same result, because the salting-out effectiveness is proportional to the slope $\left(\frac{\partial \ln \gamma_2}{\partial x_3}\right)$ at $c_3=0$ and hence to $\alpha = \lim_{c_3 \to 0} \frac{\Gamma_{23}^{(m)}}{c_3}$ which, according to the $\Gamma_{23}^{(m)}$ experimental data [11–14], increases with increasing molecular weight of PEG [11–14].

4. Conclusion

In this paper, the Kirkwood–Buff theory of solutions is used to examine the effect of PEG on aqueous protein solutions, the focus being on the local composition of the mixed solvent in the vicinity of the protein molecule and on the protein solubility. The theoretical considerations led to equations that connect the experimental preferential binding parameter with the excess (or deficit) numbers of water and cosolvent molecules around a protein molecule. Calculations were carried out for various proteins in various PEG solutions. The results showed that in all cases the proteins were preferentially hydrated. Evidence was also brought that the hydration is a result of steric exclusion.

In addition, the solubility of a protein in water+PEG mixed solvent was examined. For this purpose, a previously [21] derived relationship between the preferential binding parameter and the solubility of a protein in a binary aqueous solution was used to predict the slope of the logarithm of the protein solubility versus the PEG concentration in terms of the experimental preferential binding parameter. Slopes were predicted for the solubilities of lysozyme, β-lactoglobulin and bovine serum albumin in water+PEG mixtures for various pHs and various PEG molecular weights and compared with experiment. For all considered cases (various proteins, various PEG molecular weights and various pHs), the theory predicts that the PEG acts as a salting-out agent, conclusion in agreement with experimental observations. Numerical comparison between the predicted and the experimental slopes showed good agreement in some cases (the solubility of BSA (HSA) in water+high (larger or equal to 4000) molecular weight PEG). In other cases, such as the case of the solubility of BSA in water+low molecular weight PEG the agreement was not satisfactory probably because the experimental data regarding the preferential binding parameter and/or the solubility do not correspond to the thermodynamic equilibrium. The equations were also used to shed some light on the linearity of the logarithm of protein solubility versus PEG concentration and on the salting-out effectiveness of PEG of various molecular weights.

It is noteworthy to point out that the preferential binding parameter provides an interconnection between the local and bulk properties in water+protein+cosolvent mixtures. Indeed, when the preferential binding parameter $\Gamma_{23}^{(m)}$ is negative, a protein is preferentially hydrated (water is in excess), the protein is additionally stabilized and its solubility is decreased by the cosolvent. It seems that there is no exception to this rule.

Appendix A

The purpose of this Appendix is to compare the Kirkwood–Buff integrals G_{12} and G_{23} calculated by combining Eqs. (3) and (6) with two expressions for $(G_{11}-G_{13})$:

1) a rigorous expression

$$G_{11}-G_{13} = \frac{(c_1+c_3)(V_3-V_1)-J_{11}}{(c_1+c_1J_{11}+c_3)} \qquad (A-1)$$

2) a simplified expression in which $J_{11}=0$

$$G_{11}-G_{13} = V_3-V_1 \qquad (A-2)$$

The results of the calculations of G_{12} and G_{23} for the system water (1)/β-lactoglobulin (2)/PEG 1000 (3) are listed in Table 3.

The partial molar volumes V_1 and V_3 for water/PEG 1000 were calculated using the experimental data and correlations provided in Ref. [51] and J_{11} was calculated using the concentration dependence of the water activity in water/PEG 1000 mixture [54].

Table 3 shows that the simplified expression for $(G_{11}-G_{13})$ can be used without essential change in accuracy.

Table 3
G_{12} and G_{23} for the system water (1)/β-lactoglobulin (2)/PEG 1000 (3)

g of PEG/100 ml of solution	G_{12} [cm^3/mol]		G_{23} [cm^3/mol]	
	Calculated using expression (A-1)	Calculated using expression (A-2)	Calculated using expression (A-1)	Calculated using expression (A-2)
10	−11,750	−11,770	−37,060	−36,840
20	−11,360	−11,430	−26,390	−26,050
30	−11,120	−11,250	−22,080	−21,690

Appendix B. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bpc.2005.11.010.

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