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THERMOCHEMICAL, VOLUMETRIC AND SPECTROSCOPIC PROPERTIES OF LYSOZYME–POLY(ETHYLENE) GLYCOL SYSTEM

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Investigations of lysozyme–polyethylene glycol system were made by differential scanning calorimetry, fluorescence and density techniques. The values of unfolding enthalpies, ΔH_N^U, unfolding temperatures, T_m, excess molar heat capacities, ΔC_p, and apparent molar volumes, V_Φ, were determined as functions of PEG concentration. The three PEGs of average molecular mass (*MW*) 6000, 10000, 20000 were used as macromolecular crowding agents. The concentration of polymers was changed in the range 0–30% mass per volume (*w*/*v*). The values of ΔH_N^U remained constant with no dependence on PEG concentration, while PEG addition to buffered lysozyme solutions caused linear decrease of T_m. The values of ΔC_p and V_Φ of lysozyme dramatically changed in the range of 8–10% of PEG concentration. The fluorescence spectroscopy was used in order to investigate the polymer influence on possible solvent–lysozyme interactions. The electrical properties of polymer–water and polymer–buffer systems, the dielectric constants of solutions were determined with use of impedance spectroscopy.

Keywords: *density, DSC, fluorescence intensity, lysozyme unfolding, macromolecular crowding, PEG, preferential hydration*

Introduction

The investigations of protein–polymer systems are one of the most needed objects for pharmaceutical and biomedical usage. Some of the polymers i.e. polyethylene glycol (PEG), dextran are often used as modificators of the biological macromolecules reaction environment in order to improve the biochemical activity of proteins [1–3] or bioavailability of drugs [4]. One of the applications of PEG is as a macromolecular crowding agent [5–8]. The primary target of the study presented here was obtaining of some new information on lysozyme unfolding under macromolecular crowding conditions by the way of thermodynamic investigations. Differential scanning calorimetry (DSC) investigations allow direct determination of the values of enthalpy of unfolding (ΔH_N^U), the unfolding temperature (T_m) and molar heat capacities (ΔC_p) for buffered solutions of lysozyme with PEG of various mass and concentration.

Thermal stability of lysozyme in the presence of PEG has already been investigated by spectrophotometry [9–11]. It was observed that the addition of PEG to the buffered lysozyme solution changes the thermal stability of lysozyme. It is reported in these papers that unfolding temperature of lysozyme decreases with the increase of PEG concentration. According to Lee and Lee [9] PEG decreases the thermal stability of lysozyme because of the hydrophobic properties of polymer and lysozyme. The unfolding process of lysozyme causes an exposition of hydrophobic groups of peptide chain of protein to the solvent. These groups are then surrounded with water molecules which form clusters around hydrophobic residues of protein. The presence of hydrophobic PEG chains leads to the formation of some new hydrophobic interactions between PEG and protein molecules. This observation does not agree with some experimental data reported earlier [12] where completely opposite effect of PEG on protein stability is reported. The most popular methods applied in investigations like these, are spectroscopic. On the basis of spectroscopic data it is possible to estimate some of the thermodynamic parameters like biological activity; it is also possible to determine the polymer excluded volume effect on thermal stability of proteins in systems where polymers are used as macromolecular crowding agents [13, 14]. On the other hand – there is a great lack of the data which could have its origin from direct thermodynamic methods.

Differential scanning calorimetry investigations enable to determine free Gibbs energy, ΔG. Thus, the correlation between experimental results and the result calculated according to Minton *et al*. [13] would be possible, as well as the comparison between spectroscopic data of T_m with calorimetrically received values and literature data.

Experimental

Hen egg-white lysozyme received from Fluka (lyophilized, dialyzed) was used without further purification in acetate buffered solutions (pH=4.25). Polyethylene glycols (PEG) 6000, 10000 (Fluka) and

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PEG 20000 (Polysciences Inc.) were checked using mass spectroscopy method in order to determine the average molecular mass. The lysozyme concentration in buffered solutions was determined with the use of UV/Vis Spectrophotometer Varian 400 BIO CARY (Varian Inc., Palo Alto, CA, US) at 280 nm.

Calorimetric and densimetric measurements

All measurements were made differentially with buffer-PEG solutions as references in order to observe only protein behaviour as a result of PEG influence. Solutions were prepared freshly each day, with protein concentration ranging 0.38–0.39 mM; PEG concentration was changed in the range 0–30% *w*/*v* where *w*/*v* corresponds to the ratio of PEG mass in grams per volume unit expressed in percents. The calorimetric measurements in the case of lysozyme – PEG 6000, 10000, 20000 in buffered solutions of pH=4.25 were made using a differential adiabatic microcalorimeter DASM-4 (NPO, 'Biopribor', Pushchino, Russia) [15, 16] for which the volume of each calorimetric vessel was 0.47 mL. On the basis of recorded DSC scans, the ΔC_p dependence on temperature of systems under studies, the values of molar enthalpies of unfolding (ΔH_N^U), the corresponding values of van't Hoff enthalpies and denaturation temperatures (T_m) were determined. The values of excess molar heat capacity (ΔC_p) in 25°C were also calculated. The measurements were performed in the range from 20 to 100°C at a fixed rate of 1 K min^{-1} using the procedure described by Privalov and Khechinashvili [17]. Received value of ΔH_N^U and T_m=77°C (350 K), for buffered lysozyme solution (pH=4.25) was in agreement with the data published by Pfeil and Privalov [18]. The determined value of ΔC_p in 25°C (21.3 kJ mol^{-1} K^{-1}) agreed with literature data [19] which equals 20.0 kJ mol^{-1} K^{-1}.

The density measurements were done using Anton Paar DMA 60/602 digital densimeter (Graz, Austria) equipped with a Hetoterm thermostat, Model C8-7, to ensure temperature stability ±0.001°C. The values of apparent molar volume, V_Φ of lysozyme in buffered PEG solutions at 25°C were calculated on the basis of density measurements. All measurements were repeated two-three times and the results were obtained with a measurement error ranging 0.3–0.5%.

The DSC measurements were enlarged for pH=3.0 and 7.5 in order to check the pH influence on ΔH_N^U and T_m values in the presence of PEG. These measurements were made using differential scanning calorimeter VP-DSC Microcal (MicroCal LLC, Northampton, MA, US) [20] according to the procedure described by Michnik *et al*. [21]. The analysis of results are in accord with the recommendation for DSC experiments reported by Hinz *et al*. [22].

Fluorescence measurements

Water solutions of 0.5 mg mL^{-1} lysozyme were purified by gel filtration method using Bio-Gel P6 (Bio-Rad, USA). The final concentration of protein (ranging 5–6·10^{-6} M) was obtained by adding acetate buffer of pH=4.25. The spectra of buffer–PEG solutions were carried out and subtracted to the spectra of protein solution. The fluorescence emission spectra were collected using Luminescence Spectrometer Perkin Elmer LS 50 B (Perkin-Elmer Ltd., Beaconsfield, UK). Temperature was controlled with the use of the programmable external water bath (Lauda RC 6 CP thermostat) and monitored inside the cell with a microthermistor coupled with Hanna Microcomputer Thermometer (Hanna Instruments, Woonsocket, RI, USA). The lysozyme spectra in presence or in absence of PEG were collected in 25°C as a function of polymer increasing concentration as well as temperature dependent spectra from 25 to 100°C. The scan rate was 1 K min^{-1}, as in DSC technique. It is generally assumed that excitation at 295 nm results in fluorescence emission primarily from Trp. The fluorescence measurements of lysozyme–PEG systems at pH=4.25 were carried out either following directly the emission intensity change at 350 nm or following the emission spectra (from 297 to 450 nm) at different temperature. In the latter case intensity of the spectra at 350 nm of emission wavelength at different temperature was considered. Excitation and emission slit widths were set at 4.5 nm. Additionally, the fluorescence measurements of lysozyme–PEG in citrate/phosphate buffer solution of pH=3.00 and 7.50 were also collected in order to monitor pH influence on the spectra courses.

Dielectric constant measurements

Impedance spectroscopy was used in order to determine dielectric constants of water solutions of three PEG (6000, 10000, 20000) and for buffered solutions for PEG 20000. The measurements were carried out in a two-electrode system at open circuit potential. The laboratory-made teflon vessel consisting of two platinum electrodes of the surface of 1 cm^2 was used; the distance between electrodes was constant and equalled 1.00 mm. Impedance spectra were collected in the frequency range from 10^7 to 10^3 Hz using Impedance/Gain-Phase Analyzer (Frequency Response Analyser – FRA) SI1260 Solartron (Solartron Analytical, Hampshire, UK). The voltage amplitude was limited to 0.6 V. Six solvents of well-known dielectric constant values (distillated water, acetone, DMSO, MeOH, EtOH, methylacetate ester) were used to calibrate the measuring system. The dielectric constants were determined as a function of PEG concentration in all solutions investigated.

pH measurements

Mettler Toledo MP 220 pH meter (Switzerland) with pH InLab Electrode with integrated NTC temperature sensor was used to determine the pH of all solutions investigated. The measurements were made at 25°C. An increase in pH values of solutions (ranging from 0.3–0.5 pH) was observed in the presence of PEG, dependently on molecular mass of polymer used. The largest increase of pH (0.5 of pH) was observed for PEG 20000 solutions as a linear function of PEG concentration. The pH changes are connected with PEG tendency to decrease the values of dielectric constants of solutions [23].

Results

Calorimetric determinations

The results of calorimetric experiments, ΔH_N^U, T_m values and corresponding van't Hoff enthalpy changes, ΔH_{vH}, are presented in Table 1. No dependence of determined enthalpies of lysozyme unfolding on PEG concentration was observed in the range of the experimental error (±11 kJ mol^{-1}).

The vant't Hoff enthalpies (presented in Table 1) were calculated with an assumption of two-state model of the process investigated according to the relation:

$$\Delta H_{vH} = ART_m^2 \frac{\Delta C_p^{\Phi}}{Q_t} \quad (1)$$

where ΔC_p^{Φ} is the apparent heat capacity at transition temperature T_m, $T_m=t_{1/2}+273.15$; $t_{1/2}$ is the temperature in °C at which the process has been half-completed, Q_t is the total heat of reaction and the factor A has a value of 4.00 [24].

The temperature of lysozyme unfolding, T_m decreases as the concentration of PEG in protein solution increases. T_m values appeared to be dependant on PEG molecular mass and concentration in solution. The largest decrease of lysozyme T_m values was observed for the lowest molecular mass of PEG – 6000; the smallest T_m decrease was observed for lysozyme solutions with PEG 20000. The T_m values for lysozyme in presence of PEG 10000 were found to be characterised by average decrease between PEG 6000 and 20000. These observations suggested the T_m of lysozyme dependence on molecular mass of PEG and the direction of tendency: the larger decrease of T_m values of lysozyme is accompanied by increasing PEG concentration of lower molecular mass. The T_m could be expressed using the following equations:

$$T_{m(\text{lys vs. PEG 6000})} = -0.1835C_{\%\text{PEG 6000}} + 349.64 \quad (R^2=0.990) \quad (2)$$

$$T_{m(\text{lys vs. PEG 10000})} = -0.1603C_{\%\text{PEG 10000}} + 349.83 \quad (R^2=0.996) \quad (3)$$

$$T_{m(\text{lys vs. PEG 20000})} = -0.1151C_{\%\text{PEG 20000}} + 350.00 \quad (R^2=0.991) \quad (4)$$

where $C_{\%}$ is PEG concentration.

Table 1 The received results of: calorimetrically determinated values of enthalpies of denaturation of lysozyme in the presence of PEG (ΔH_N^U), calculated van't Hoff enthalpies (ΔH_{vH}) and the temperatures of denaturation (T_m). The scanning calorimetry measurements were repeated 3 times. The average measurement error of ΔH_N^U is ±11 kJ mol^{-1}. The average error of lysozyme unfolding temperature determination is ±0.2 K

PEG/*w*/*v*%	ΔH_N^U/kJ mol^{-1}			ΔH_{vH}/kJ mol^{-1}			T_m/K		
	PEG								
	6000	10000	20000	6000	10000	20000	6000	10000	20000
0	512	512	512	541	541	541	350.0	350.0	350.0
1	523	489	–	544	540	–	349.5	349.6	–
3	–	519	531	–	544	531	–	349.3	349.6
5	510	540	–	548	556	–	348.6	349.0	–
8	519	–	519	565	–	535	348.0	–	349.1
9	–	540	–	–	548	–	–	348.3	–
10	498	–	506	556	–	527	347.6	–	348.8
15	544	510	514	552	573	527	349.9	347.6	348.3
19	531	523	523	569	577	535	346.1	346.7	347.7
22	–	494	535	–	581	540	–	346.3	347.4
25	523	–	–	557	–	–	345.2	–	–
26	–	506	548	–	577	531	–	345.7	347.2
28	531	–	–	561	–	–	344.7	–	–

lysozyme concentration=0.38 mM; acetate buffer solutions, pH=4.25

The dependence of Gibbs free energy changes *vs*. polymer concentration was calculated according to the procedure described by Sasahara *et al*. [13]. Using calorimetrically determined values of ΔH_N^U and T_m and the Gibbs–Helmholtz equation:

$$\Delta G_u(T) = \Delta H_u(T_m)\left(1 - \frac{T}{T_m}\right) - \\ -\Delta C_p\left[(T_m - T) + T\ln\frac{T}{T_m}\right] \quad (5)$$

where T_m is transition temperature at which the $\Delta G(T_m)$ is equal to 0, $\Delta H_u(T_m)$ is the value of calorimetrically determined enthalpy change of lysozyme unfolding process at T_m, and ΔC_p reflects the heat capacity change on unfolding. The ΔC_p value of lysozyme unfolding was assumed 6.5 kJ mol^{-1} [25]. The results are presented in Fig. 1.

It was assumed that the increase of Gibbs free energy from the value of ΔG_u (T_m^0) that reflects the value of ΔG for buffered protein solution without polymer, to the values of $\Delta\Delta G_u(T_m)$ that represent the ΔG in different PEG concentrations equals mw_{PEG} in which m reflects the linear slope of $\Delta\Delta G_u(T_m)$ *vs*. the concentration of PEG (w_{PEG}).

The theoretical calculations of linear slope (m coefficient) for the dependence of $\Delta\Delta G_u(T_m)$= f(% PEG) were based on the relation [13]:

$$m_{U-F} = \left[\left(1 + \frac{r_U}{r_{PEG}}\right)^2\left(1 + \frac{r_F}{r_{PEG}}\right)^2\right]v_{PEG}RT \quad (6)$$

where r_U and r_F are the effective radius of unfolded and folded form of lysozyme, respectively; r_{PEG} is an effective radius of PEG, and v_{PEG} is specific excluded volume of PEG used. The r_U, r_F and v_{PEG} parameters used in theoretical calculations are presented in Table 2. The results of calculated m coefficient values are presented in Table 3. In this table, the calculated values of m coefficient according to macromolecular crowding theory and with use of structural parameters obtained on the basis of SPT model are also presented.

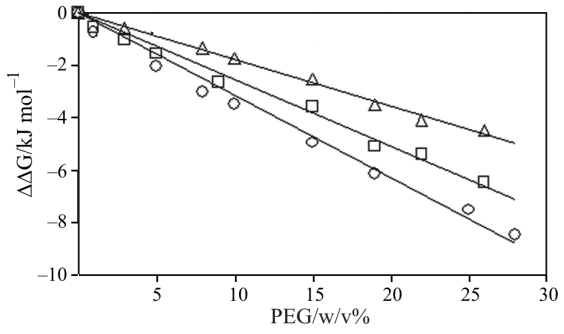

Fig. 1 The $\Delta\Delta G_u(T_m)$ dependence on PEG concentration: △ – PEG 20000; □ – PEG 10000; ○ – PEG 6000

Table 2 The values of structural parameters: r_U, r_F, r_{PEG} or v_{PEG} used for m coefficient calculations

r_U/nm	r_F/nm	r_{PEG}/nm	v_{PEG}/nm^3	
			PEG 6000	PEG 20000
1.9~2.1 [33]	1.5~1.6 [33]	0.22 [34]	1.3 [35]	1.8 [35]

Table 3 The experimental and calculated values of m coefficients

PEG	m_{U-F} (experimental)	m_{U-F} (calculated)
6000	–0.31 (R^2=0.9831)	–0.32~–0.42 (R^2=0.9294~0.9411*)
20000	–0.18 (R^2=0.9953)	–0.13~–0.17 (R^2=0.8996~0.9302*)

*depended on r_U and r_F used for calculations

Volumetric determinations

The densimetric measurements were conducted in order to monitor the PEG influence on possible changes of lysozyme apparent molar volume, $V_{\Phi,lys}$. The obtained results of $V_{\Phi,lys}$ were used to calculate the values of excess molar heat capacities, ΔC_p of lysozyme as a dependence on PEG concentration in 25°C. Both $V_{\Phi,lys}$ and ΔC_p values are presented in Table 4 as a function of increasing PEG concentration. Figure 2a presents the dependence of relative apparent molar volume difference, $\Delta V_{\Phi,lys}$ of lysozyme in buffered PEG solutions on the concentration of three PEGs used. The $\Delta V_{\Phi,lys}$ is expressed as:

$$\Delta V_{\Phi,lys} = \frac{V - V_0}{V} \quad (7)$$

where V_0 and V denotes the apparent molar volume of buffered lysozyme solution and apparent molar volumes of lysozyme–PEG–buffer system, respectively.

As it is shown in the Fig. 2a the curve courses of lysozyme volume changes strongly depend on molecular mass and concentration of PEG used in investigated solutions. The most significant change in $V_{\Phi,lys}$ is associated with PEG 20000. Initially constant values of $V_{\Phi,lys}$ begin to increase when polymer concentration reaches approx. 8% *w*/*v*. The characteristic maximal values of $V_{\Phi,lys}$ are then observed at about 17% of PEG 20000 concentration. At higher PEG 20000 concentrations – about 30% – the values of $V_{\Phi,lys}$ appeared to be lower than the maximal value observed for this PEG. Essentially no changes in $V_{\Phi,lys}$ were observed for PEG 6000. The calculated values of $V_{\Phi,lys}$ were constant in the range of the experimental error and did not depend on PEG 6000 concentration.

In the case of PEG 10000 rapid increase in values of $V_{\Phi,lys}$ was observed when the polymer concentration of 10% *w*/*v* had been reached. No

Table 4 The values of apparent molar volumes of lysozyme $V_{\Phi,lys}$ determined on the basis of densimetric measurements in 298 K in 0.1 M acetate buffer solution at apparent pH=4.25 and calculated values of excess molar heat capcities of lysozyme ΔC_p in 298 K – as a function of each PEG used concentration

PEG/*w*/*v*%	$V_{\Phi,lys}$ (298 K)/cm^3 mol^{-1}			$\Delta C_{p,lys}$ (298 K)/kJ mol^{-1} K^{-1}		
	PEG 6000	PEG 10000	PEG 20000	PEG 6000	PEG 10000	PEG 20000
0	9833±142	9833±142	9833±142	21.3±0.4	21.3±0.4	21.3±0.4
1	9836±105	9845±15	9967±91	20.2±0.7	20.9±0.5	–
3	–	–	9690±117	–	–	20.2±0.8
5	9820±124	9575±164	9765±77	20.4±0.3	22.6±0.9	19.8±0.5
8	9826±48	9502±48	9898±102	20.2±0.5	22.0±0.8	19.1±0.5
9	–	–	10206±140	–	–	20.7±0.7
10	9763±93	9502±78	11120±210	20.2±0.4	23.2±0.6	23.2±0.4
12	–	–	11569±58	–	–	29.0±0.6
15	9714±137	–	12399±121	19.6±0.8	–	35.4±0.9
17	9725±76	10941±106	13228±197	–	29.9±0.7	42.4±1.2
19	9690±36	–	–	20.2±0.3	–	–
22	9651±113	12337±67	12038±44	19.1±0.6	34.4±0.8	46.9±0.8
26	–	–	12084±137	–	–	42.8±0.5
28	9595±122	–	–	19.3±0.7	–	–
29	–	13707±123	–	–	–	–

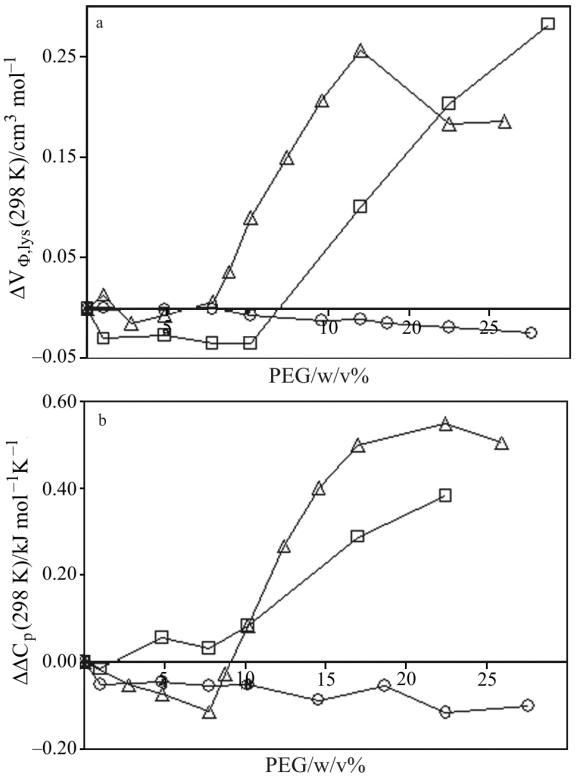

Fig. 2 The a – $\Delta V_{\Phi,lys}$ and b – $\Delta\Delta C_p$ dependence on PEG concentration: △ – PEG 20000; □ – PEG 10000; ○ – PEG 6000; acetate buffer solutions, pH=4.25; c_{lys}=0.38 mM

significant changes in $V_{\Phi,lys}$ values in lower PEG 10000 were observed.

The $\Delta\Delta C_{p,lys}$ values for three PEGs at 25°C are presented in Fig. 2b. The inflexion point of about 8% for PEG 20000 is characteristic for both volume and heat capacity dependencies on PEG concentration. The $\Delta C_{p,lys}$ values, similar to those of apparent molar volumes, are initially constant. The large increase in lysozyme $\Delta C_{p,lys}$ values for investigated polyethylene glycol 20000, in consequence of increasing PEG concentration after inflexion point of 8% of polymer in solutions, was also observed. The characteristic maximal value of $\Delta C_{p,lys}$ for about 22% of PEG 20000 concentration was noticed. In case of PEG 6000 there were no significant changes in $\Delta C_{p,lys}$ values in the investigated PEG concentration range. The calculated values remain constant in this case. The ΔC_p values of lysozyme solutions in presence of PEG 10000 remain constant till 8% of PEG concentration. Then the increase of $\Delta C_{p,lys}$ upon PEG concentration is observed. The slope of increasing part of curve for PEG 10000 is smaller than the slope of increasing curve part for PEG 20000. This observation suggests that increasing molar mass of polymer used causes more evident changes in excess molar heat capacities and the maximum value of $\Delta C_{p,lys}$ could be reached considerably quickly in the case of PEGs of higher molar mass.

It is interesting to notice that for both, $V_{\Phi,lys}$ and $\Delta C_{p,lys}$ the dependencies on different PEG's concentrations are similar. Firstly, considering the ranges of polymers concentration where the changes are observed as well as the characteristic inflection points. Secondly, in both cases no dependence on PEG 6000 concentration was noticed.

Table 5 The estimated values of enthalpies and temperatures of lysozyme unfolding as a function of increasing PEG concentration received on the way of temperature-scanning fluorescence measurements

	pH	PEG 20000/*w*/*v*%					
		0	3	6	12	26	30
ΔH_{fluo}/kJ mol^{-1}	3.0	493	472	–	474	–	462
	4.25	538	520	530	530	536	525
	7.5	522	513	–	–	–	505
$T_{m,fluo}$/K	3.0	345.0	344.6	–	344.0	–	343.0
	4.25	250.0	349.6	349.1	348.7	347.4	346.9
	7.5	346.0	345.3	–	–	–	343.0

Fluorescence measurements

The lysozyme–PEG 20000 system was investigated under three different pH conditions: pH=3.00; pH=4.25 (as in the case of calorimetric measurements) and pH=7.50 in order to obtain the pH dependence of possible PEG–lysozyme–solvent interactions.

HEWL thermally induced transition could be monitored by changes in tryptophan exposition in fluorescence. The thermal unfolding of lysozyme is reflected by a shift of the spectrum towards shorter wavenumber and by a deflection of the temperature dependence of the fluorescence intensity from the usual thermal dependence curve. Figure 3 shows the changes of wavenumber for lysozyme at pH=3.00, 4.25, 7.50 (Figs 3a–c, respectively) at different concentrations of PEG in function of the temperature are shown. In all cases relatively to the native and unfolding state, adding PEG determines a blue shift of the wavenumber related to both states, respectively.

Figure 4a reports the temperature dependence of fluorescence intensity at 350 nm for lysozyme in the absence or in the presence of PEG at pH=3.00. In Fig. 4b the progress degrees of the unfolding reaction and the related fitting curves for the thermodynamic analysis are represented.

Taking into account that the intensity of fluorescence signal at values not corresponding to the maximum are linearly related to the fraction of conversion from native to unfolded state and considering the two-state model, the correspondent equilibrium constant, k, is given by:

$$k=U/N\ ,\ \alpha=k/(1+k)\rightarrow k=\alpha/(\alpha+1) \qquad (8)$$

where α is the degree of advancement of the unfolding process. This assumption makes it possible to calculate the thermodynamic parameters of $\Delta H^{U}_{N,fluo}$ and $T_{m,fluo}$ by the way of spectroscopic investigations of lysozyme unfolding process. The results of the estimated values of the enthalpies and temperatures of lysozyme unfolding are presented in Table 5.

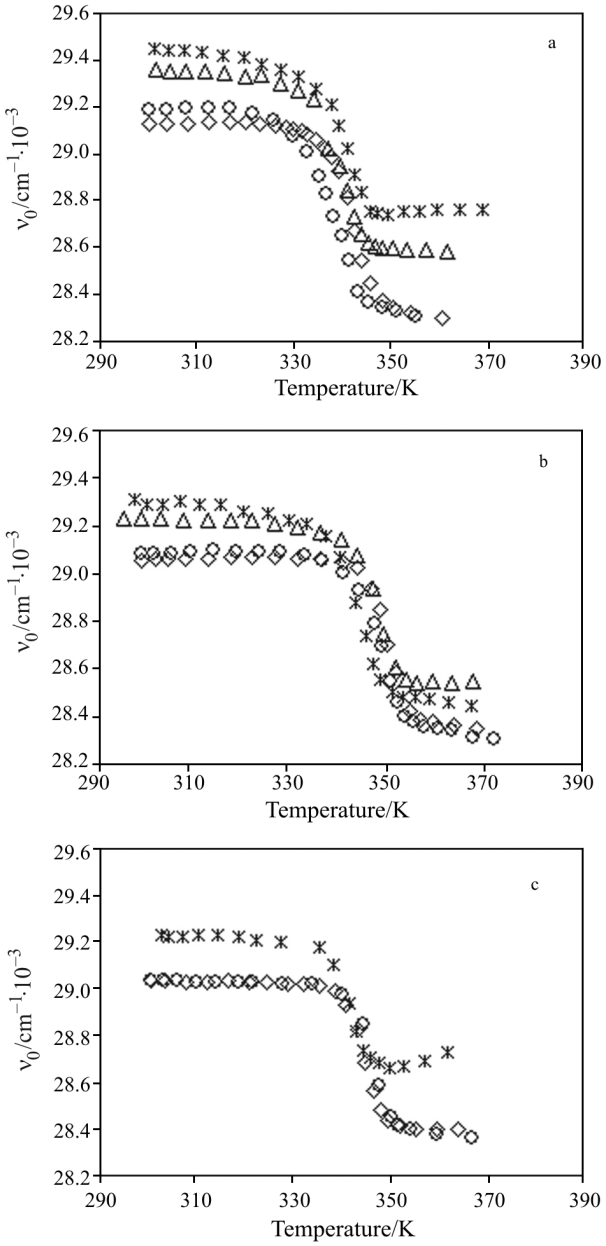

Fig. 3 The dependence of the wavenumber maximum of the fluorescence spectra of lysozyme in presence of different amount of PEG in function of the temperature: a – pH=3.00, b – pH=4.26, c – pH=7.50; ◇ – lysozyme, ○ – 3% PEG, △ – 12% PEG, ∗ – 30% PEG. Lysozyme concentration $5\cdot10^{-6}$ M, 0.05 M phosphate citrate buffer solutions

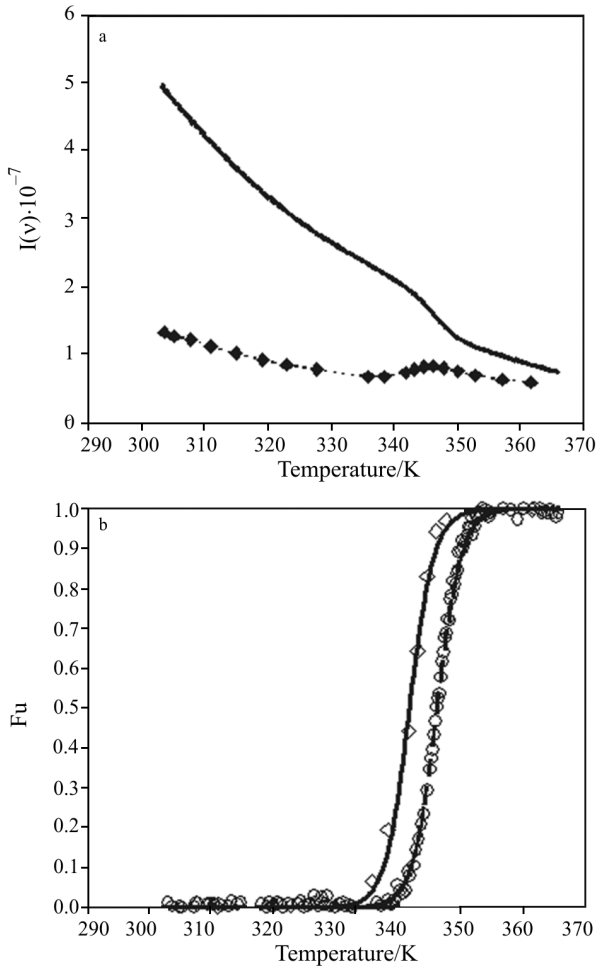

Fig. 4 The temperature dependence of fluorescence intensity at 350 nm, λ_{ex}=295 nm, a – for lysozyme, solid line, and lysozyme in the presence of PEG 10%, ◆---◆ – at pH=4.25; b – the ○ – temperature dependence of unfolding fraction for lysozyme, and ◇ – lysozyme in the presence of PEG 10%, and the related fitting curves, respectively dashed and solid line, for the thermodynamic analysis using a two-state model. Lysozyme concentration $5\cdot10^{-6}$ M, 0.05 M phosphate citrate buffer solutions

Discussion and conclusions

According to the classification given by von Hippel and Schleich [26] hydrophobic polymers like PEG belong to one of the modificator groups, which have a significant effect on biomolecule environment. Proteins are preferentially hydrated in the presence of these modificators, regarded as a protein structure stabilisers, precipitators and self-assembly inductors. The special emphasis should be put on the particular role of water in all biological systems investigations. The water environment, where bioreactions take place, can be easily modified by adding the agent i.e. PEG, and the consequences in physico-chemical properties of environment will depend on the different affinities of the protein to water and particular agent to water [27]. Some substances like PEG interact with water what has an indirect effect on the interaction between protein and water. The water structure is generally perturbed at an interface as defined by molecule-hydration shell and hydration shell-bulk water. Non-protein-associated (bulk) water plays a very important role in determining the strength of the hydrophobic effects on protein folding/unfolding. Addition of PEG well-known for its hydrophobic properties to water-buffered protein solution significantly changes these structures of water and the electrical properties of solution. Zimmerman and Minton in their review publication [5] describe that PEG could force protein molecules to associate. The decrease of apparent molar volumes V_Φ of dimmer or trimmer forming would be expected in such a process. The effect is caused by exclusion of accessible area among associated protein molecules for solvent molecules [28]. The obtained results of apparent molar volume of lysozyme and its changes in the presence of PEG investigations, shown in Fig. 2a, are significantly connected with PEG's tendency to reorganise the structure of the protein-solvent interaction system. Assuming a hydrodynamic radius R=1.7 nm for lysozyme [29] the increase of one or two water molecule layers, i.e. 0.2–0.4 nm [30], determines an increase of about 30–40% in the V_Φ. This fact is in agreement with the increase of the hydration shell in presence of a modificator [27]. In Fig. 2a it can be observed that the protein at 298 K at pH 4.25 and the same ionic strength of the DSC experiments does not show any volume change if the PEG added has a low MW. Increase of PEG molecular mass appears a critical concentration (8–10%) where ΔV_Φ is observed. Therefore, as it is suggested by Creighton [31], the bound water has a 15% greater heat capacity than the bulk water. The increase in ΔC_p reported in Fig. 2b reflects this fact.

As it was reported in this paper, PEG addition to lysozyme buffered solutions does not affect the ΔH_N^U values with large differences.

Calorimetric-DSC studies of lysozyme unfolding process for tertiary system: PEG–lysozyme–buffer (pH=4.25) showed that the average value of the enthalpy of conformational change, ΔH_N^U is 520.4±29.7 kJ mol^{-1} and does not depend on the molecular mass and concentration of PEG's used in investigated range of concentrations of these polymers. The calorimetrically determined value of the unfolding enthalpy for lysozyme in acetate buffer solution without PEG equals 509.6±8.4 kJ mol^{-1}. This observation suggests that PEG addition does not influence the values of lysozyme conformational change.

The ΔH_N^U values determined on the basis of the calorimetric measurements were compared with the calculated van't Hoff enthalpies, ΔH_{vH}. The average values of ΔH_{vH} are: 549.3±34.3 and 542.6±13.8 kJ mol^{-1} for lysozyme–PEG–buffer and lysozyme–buffer system, re-

spectively. The comparison of both values of ΔH_N^U and ΔH_{vH} allowed for an assumption that the investigated process is a two-state process, although the van't Hoff enthalpies are insignificantly higher than the enthalpies determined by DSC.

The calorimetrically determined values of ΔH_N^U are in good agreement with the data received by the way of temperature-scanning fluorescence spectroscopy measurements.

Although the enthalpies ΔH_N^U can be regarded as practically constant, the values of T_m were found to decrease with the increasing PEG concentration, as it was observed from calorimetric as well as from fluorescence measurements. There are two hypotheses concerning this fact. According to the first one the increase in PEG concentration produces a decrease in dielectric constant values as reported in [23]. In spite of the fact that buffered solutions – containing ions – were used in all experiments reported here, the same effect of decreasing dielectric constant values was observed with the increasing PEG concentration (as reported in the Experimental section). Therefore, in the presence of PEG an increase in pH values of solutions was observed. This observation could have been considered as one of the possible explanations for T_m values of lysozyme unfolding lowering in the presence of increasing of PEG concentration. But the direction of T_m changes with PEG concentration is not in agreement with the direction of T_m changes with increasing pH. The latest data [32] showed that the T_m of lysozyme unfolding does not change significantly in the pH range (4.25–4.70) used in our investigations. Even if pH variation would be taken into considerations because of PEG ability to change the dialectical constant of solutions under studies and in consequence – to change the pH of solutions, there should be observed an increase of T_m values for lysozyme unfolding. This observation has completely opposite direction than the one observed during our investigations.

The temperatures of lysozyme unfolding change linearly in function of the increasing PEG concentration. This observation suggests that the increasing PEG concentration causes a decrease of lysozyme thermal stability. Thus, the values of T_m determined by the way of calorimetric measurements are in good agreement with the values determined in spectroscopic experiments [9].

According to Hermans *et al*. the observation of lowering T_m values with the increasing PEG concentration could be reflected by the fact that lysozyme unfolded structure is favourable in the presence of PEG with respect to non-PEG solvent. In spite of PEG ability to lower the polarity of a solvent, so as to stabilize the unfolded state, it is also expected that PEG would favour the compact, native state of protein due to larger excluded volume of its unfolded state [11]. On the other hand, it is possible that because of high values of excluded volumes which characterize the unfolded form of lysozyme, PEG would stabilize the native, folded form of the protein under studies.

On the basis of calorimetric measurements the changes of Gibbs free energy $\Delta\Delta G$ for lysozyme unfolding as a function of increasing PEG concentration were calculated. The same dependences were calculated according to model of calculations presented by Minton [13]. The good agreement between the received results of linear slope coefficient, m, for the dependence $\Delta\Delta G_u(T_m)=f(\%\ \text{PEG})$ was obtained. So the model proposed by Minton and the way of excluded volume effects on the lysozyme unfolding with PEG as a crowding agent, could be used for interpretation of calorimetrically – directly determined – thermodynamical parameters of the process under studies. The obtained agreement between both experimental and theoretical of the m coefficient for lysozyme unfolding process in the presence of PEG, could also suggest that PEG chains are able to exclude proteins from the polymer–water interfaces what results lowering of unfolding temperature.

On the basis of volumetric and fluorescence measurements the changes in solvent–lysozyme molecules were observed. It is quite obvious that the water concentration decreases with the increasing volume occupancy by background species like PEG. In spite of this fact, the increase of apparent molar volume of lysozyme suggests an increase of hydration shell of this protein.

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