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Denaturation and aggregation of lysozyme in water–ethanol solution

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We have applied rheological methods for the analysis of ethanol-lysozyme interaction during the process of denaturation and aggregation of the protein. At low concentration of ethanol a destruction of the hydration shell of lysozyme is observed. With the increase in the ethanol concentration a structural transformation takes place. It leads to the formation of a protein aggregate with an elongated structure. The rheological characteristics of lysozyme-water–ethanol solution changes from Newtonian to pseudoplastic.

Key words: lysozyme, viscosity, monomer–to–filament structural transition, volumetric studies

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INTRODUCTION

Considerable attention is presently focused on a group of human diseases known as amyloidoses. On the basis of intensive studies (Stefani & Dobson, 2003; Uversky an & Fink, 2004) it is now well known that one of the most important problems is the misfolding and pathological aggregation of certain proteins. About twenty proteins are known to be involved in human diseases of that type. However, it was shown that many other proteins could form amyloid fibrils (Stefani & Dobson, 2003). These observations have opened a new scientific area of studies on folding and aggregation of protein molecules.

It is known from the literature that hen egg-white lysozyme (HEWL) can form ordered aggregates in water–ethanol solution (Goda *et al.,* 2000; Krebs *et al.,* 2000; Fujiwara *et al.,* 2003; Cao *et al.,* 2004; Trexler and Nilsson 2007; Holley *et al.,* 2008). On the basis of small-angle *X*-ray and neutron scattering studies a phase diagram for structural transformation was determined and the pathway of the amyloid fibril formation of HEWL was proposed (Yonezawa *et al.,* 2002). There was, however, no clear description of an intermediate state, which is a part of the folding/unfolding pathway (Goda *et al.,* 2000; Matagne *et al.,* 1997; 2000; Frare *et al.,* 2004). It is not clear, among others, what the real structure of this state is. A part of the primary structure (53–101), which includes helix C and a part of the β domain, has been assigned to a highly amyloidogenic region of the lysozyme molecule (Frare *et al.,* 2004). Some other studies lead to the conclusion that in water–dimethyl sulfoxide (DMSO) solution an ordered core in the intermediate state should be assigned to the α domain (Bhattachacjya and Balaram 1997).

In this paper we present a new analysis of HEWL structural transformations. Our experimental data have enabled us to draw conclusions about the process of formation of an intermediate state and subsequent aggregation of the protein in the water–ethanol system. We have applied the methods of macrorheology since the viscosity measurements could be very useful in the detection of conformational changes, in particular in the analysis of ordered or disordered aggregation.

Theoretical (Zhou, 1995) and experimental (Ahmad & Salahuddin, 1974; Lefebvre 1982; Areas *et al.,* 1996; Monkos, 1997; Kamiyama *et al.,* 2004; Pan *et al.,* 2009) studies on the viscosity of various lysozyme solutions have been reported previously. According to a theoretical analysis (Zhou, 1995) the intrinsic viscosity is strongly influenced by the hydration level of the protein. The best reproduction of experimental data was obtained under the assumption of a 0.9 Å thickness of the hydration shell of the lysozyme molecule, which corresponds to the hydration level of 0.3÷0.4 $g_{water}/g_{protein}$. The calculated value for intrinsic viscosity equaled 2.99 $cm^3 \cdot g^{-1}$. For native globular proteins the intrinsic viscosity, determined experimentally, is low — 3÷4 $cm^3 \cdot g^{-1}$ (Trexler & Nilsson, 2007) — and in the case of HEWL this value (at T = 298 K) changes from 2.66 $cm^3 \cdot g^{-1}$ (Monkos 1997) to (2.84±0.43) $cm^3 \cdot g^{-1}$ (Kamiyama *et al.,* 2004). However, under denaturating conditions the intrinsic viscosity of lysozyme increases significantly up to 18.4 $cm^3 \cdot g^{-1}$ (in 6M guanidine hydrochloride at 298 K) (Lefebvre, 1982).

The other parameter, which changes with the structural transformations of a protein is its volume. The volume change is directly related to the compactness or globularity of the protein molecule (Sasahara *et al.,* 1999; Chalikian & Filfil, 2003; Schweiker *et al.,* 2009; Sirotkin & Winter, 2010). There is a combination of factors which determine the volume change. The most important are three of them: hydration, internal cavities and molecular vibrations in the protein. However, in the case of chemical denaturation (when the process takes place under the conditions of stable temperature) the effects of molecular vibrations do not significantly contribute to the volumetric changes. Therefore a partial specific volume gives information about the protein–solvent interaction and about the changes in protein conformation. Studies of the partial specific volume of HEWL in water–DMSO solution (Kamiyama *et al.,* 2003), water–ethanol solution (Sirotkin & Winter, 2010) and water–guanidinium chloride solution (Sasahara *et al.,* 1999; Sirotkin & Winter, 2010) were reported before.

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Abbreviations: HEWL, hen egg-white lysozyme; DMSO, dimethyl sulfoxide; TMU, tetramethylurea

MATERIALS AND METHODS

Hen egg-white lysozyme was purchased from SIGMA. The protein was dissolved in a small amount of water and then an appropriate amount of the lysozyme solution and 96% (v/v) ethanol were mixed by magnetic stirring in order to achieve a desired protein and ethanol concentration (c_{EtOH} varied from 10% to 85% (v/v)). Final protein concentration was determined by UV/VIS spectrophotometer. We have measured the extinction coefficient (at λ=280 nm) of HEWL in water-ethanol solution [ε_{280nm}=(2.54±0.08) ml·mg^{-1}·cm^{-1}] and we have not observed any changes of this parameter as a function of ethanol concentration. Information about the protein concentration is presented in Fig. 1 (see legend). The protein concentration was determined with an accuracy of about 10^{-5} g·cm^{-3}. The viscosity and partial specific volume measurements were performed 24 hours after sample preparation.

Analysis of the time course of protein aggregation was performed in a separate experiment. The aggregation was determined on the basis of fluorescence intensity (λ=482 nm) of thioflavin T (ThT). Measurements of the time dependence of fluorescence intensity were performed at room temperature using a HNA 188S – spectrophotometer. The sample contained the protein (4.5 mg/ml), ethanol (80%, v/v) and ThT (2.5 mM). Measurements started 24 hours after sample preparation. Under the experimental conditions the rate constant k=0.8 day and time lag t_{lag}=3.9 day. An increase in protein concentration caused a decrease in these parameters.

A Brookfield Cone/Plate Low Volume DV-II+ viscometer with a CPE-42 cone coupled to a water bath to control the temperature of the sample (Brookfield TC 500) was used for the measurements of viscosity. The data were collected at the temperature of 298 K, which was stabilized with the accuracy of 0.1 K. The cone angle equaled 1.565°, its diameter — 2.4 cm. For samples containing a high concentration of the protein a CP-52 cone was used with the angle of 3° and the diameter of 1.2 cm. In both experimental setups the sample volume was 1ml. The accuracy of viscosity measurements was better than 0.01 mPa·s.

We have measured the viscosity of lysozyme–water–ethanol solutions as a function of the ethanol as well as the lysozyme concentration. On the basis of the experimental data the intrinsic viscosity has been calculated according to the definition:

$$[\eta]=\lim_{c\to 0}\frac{1}{c}\left(\frac{\eta}{\eta_0}-1\right), \quad (1)$$

where η is the viscosity of the colloid and η_0 denotes the viscosity of the dispersion medium — water–ethanol system and c is the protein concentration.

Density of the protein solutions was measured using Anton-Paar microprocessor gauge of the DMA 38 type with an oscillating glass U-tube. This instrument permitted density measurements with an accuracy of 10^{-4} g·cm^{-3}. All measurements were performed under the conditions of constant temperature 298 K. On the basis of the experimental data a partial specific volume of lysozyme was calculated according to the equation (Gekko and Hasegawa1986):

$$\bar{v}=\lim_{c\to 0}\frac{1}{c}\left(1-\frac{d-c}{d_0}\right), \quad (2)$$

where d is the density of lysozyme solution, d_0 — the density of the dispersion medium and c — the concentration of protein solution expressed in g·cm^{-3}.

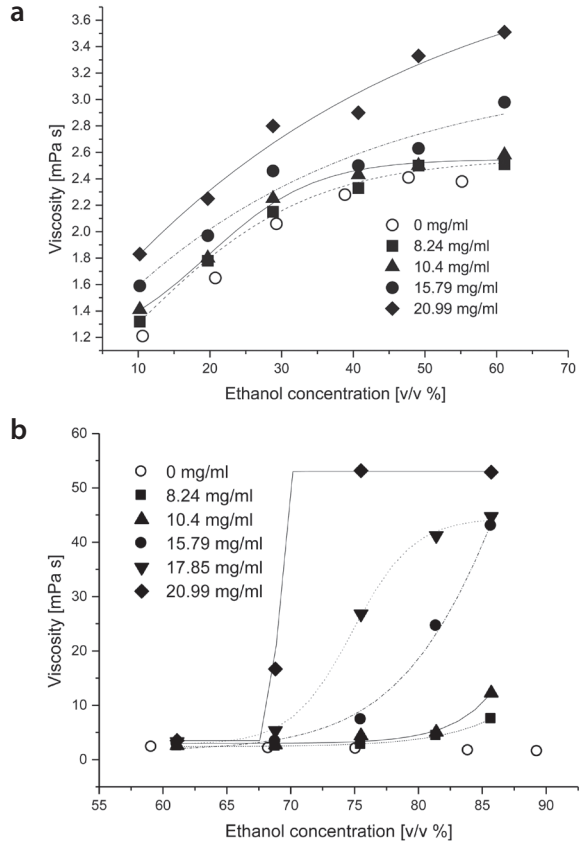

Figure 1. Viscosity of the lysozyme–water–ethanol solutions as a function of ethanol concentration
(a) at low ethanol content; **(b)** at high ethanol content for different concentration of protein (in the figure).

The setup for light scattering experiments was equipped with a Kr laser (λ=647 nm) from Carl Zeiss, Jena, Germany. The intensity of the scattered light was measured at an angle θ=90° using an ALV5000 correlator from ALV, Langen, Germany. The measurements were performed at room temperature.

RESULTS AND DISCUSSION

Results of our studies on the viscosity of water–ethanol solutions of lysozyme are presented in Fig. 1. Lysozyme concentration was changed within the range of 8÷20 mg·ml^{-1}. For all solutions the viscosity increased slowly with the addition of ethanol until the ethanol concentration reached the value of about 60% (v/v) (Fig. 1a). For higher ethanol concentrations the viscosity increased much more dramatically (Fig. 1b). For example, in the sample with the protein concentration of 20 mg·ml^{-1}, the viscosity increased very sharply from η=3.51 mPa·s (ethanol concentration c_{EtOH}=60%) to the plateau value of about η=53 mPa·s (c_{EtOH}=75%). The viscosity of water–ethanol solution is lower than the viscosity of the colloid and changes from 1.05 mPa·s (c_{EtOH}=0%) to 2.38 mPa·s (c_{EtOH}=55.16%) and 1.53 mPa·s (c_{EtOH}=89.3%).

At low ethanol concentration the solution under consideration is a Newtonian fluid (Fig. 2a). The shear stress increases linearly with increasing shear rate and the viscosity is constant. At higher ethanol concentration (Fig. 2b) the rheological characteristics of lysozyme solution changes from Newtonian to pseudoplastic as

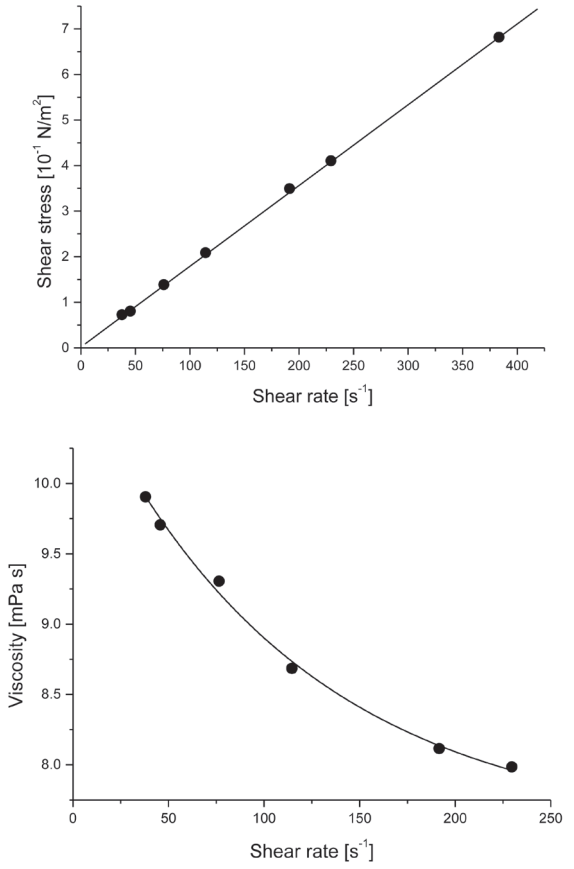

Figure 2. (a) Shear–flow curve of the lysozyme–water–ethanol solution (protein concentration — 8.24 mg/ml) — Newtonian flow at c_{EtOH}=10% (v/v); (b) Viscosity curve of the lysozyme–water–ethanol solution (protein concentration — 8.24 mg/ml) – pseudoplastic flow at c_{EtOH}= 85% (v/v).

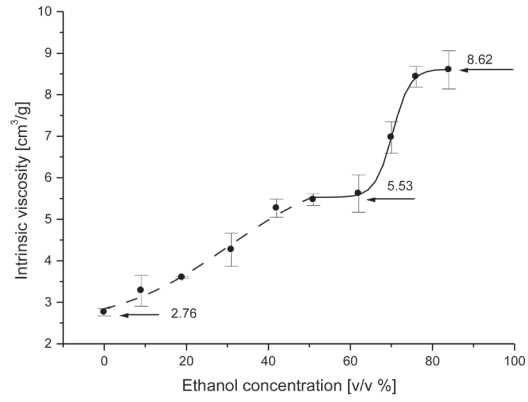

Figure 3. Intrinsic viscosity of the lysozyme–water–ethanol solution as a function of ethanol concentration.

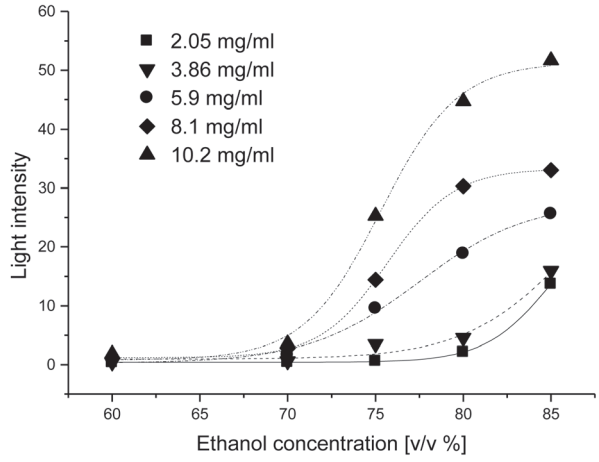

Figure 4. Intensity of the scattered light defined as $(I–I_{buff})/I_{toluen}$ as a function of ethanol concentration.

indicated by a drastic decrease in the viscosity with increasing shear rate. Similar effects have been observed for lysozyme in the tetramethylurea (TMU) and DMSO solutions (Areas *et al.,* 1996). One can draw the conclusion that organic cosolvents induce changes in protein conformation leading to the formation of elongated structures, which are able to reorient themselves under shear stress. These are not elongated chains of the protein, since under the completely denaturating conditions the flow of the lysozyme solution was Newtonian (Lefebvre 1982).

Figure 3 shows the intrinsic viscosity of the lysozyme–water–ethanol solution as a function of ethanol concentration. According to our data the intrinsic viscosity of the water solution of lysozyme equals 2.69 $cm^3 \cdot g^{-1}$, which is in agreement with other experimental results (2.66 $cm^3 \cdot g^{-1}$ (Monkos 1997), 2.7 $cm^3 \cdot g^{-1}$ (Lefebvre 1982), 2.84 $cm^3 \cdot g^{-1}$ (Kamiyama *et al.,* 2004)). Upon the addition of ethanol we have observed a systematic increase in the intrinsic viscosity. We have approximated our final results with two sigmoidal functions with plateau values of 2.69 $cm^3 \cdot g^{-1}$, 5.56 $cm^3 \cdot g^{-1}$ and 8.62 $cm^3 \cdot g^{-1}$. This choice was dictated by the fact that the sigmoidal function well approximates changes of intrinsic viscosity in the case of protein denaturation, as it was observed for thermal denaturation of ribonuclease (Harding, 1997). According to our experimental data we can draw the conclusion that there are two stages of lysozyme structural transformations. The first stage, which ends up with the intrinsic viscosity of about 5.56 $cm^3 \cdot g^{-1}$, corresponds to the transformations correlated with reconstruction of the hydration shell, while the second stage leads to the formation of an elongated structure. In this structural form the intrinsic viscosity achieves the value of 8.63 $cm^3 \cdot g^{-1}$. One should notice that the second plateau corresponds to the region of a strong increase in viscosity and the change of the flow from Newtonian to pseudoplastic. None of the determined stages of structural transformations ends up with full denaturation of the protein. According to the simplified relation given by Tanford (Tanford *et al.,* 1967) the intrinsic viscosity of a protein in the random coil state can be expressed as a power function of the number of amino acid residues, n

$$\eta = 0.716 \cdot n^{0.67}. \quad (3)$$

Since the molecule of lysozyme from hen egg-white consists of 129 amino acid residues, the intrinsic viscosity of the protein under the conditions of complete denaturation should equal 18.85 $cm^3 \cdot g^{-1}$. This is in agreement with the experimental value of 18.4 $cm^3 \cdot g^{-1}$ obtained for lysozyme in 6 M guanidine hydrochloride (Lefebvre, 1982). In our experiment the maximum value of the intrinsic viscosity is much lower and equals 8.63 $cm^3 \cdot g^{-1}$.

The aggregation of lysozyme molecules has been observed also in the studies using light scattering. The corresponding data are presented in Fig. 4. It is well known that the intensity of the scattered light increas-

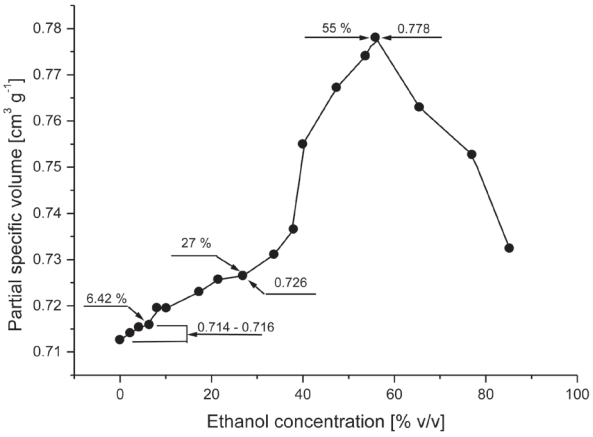

Figure 5. Change of the partial specific volume of lysozyme as a function of ethanol concentration in the lysozyme–water–ethanol solution.

es with the size of the macromolecule. The tendency of the aggregation is very similar to that observed in the analysis of rheological data. The method of light scattering is much more sensitive and the process is observed already at low lysozyme concentration.

In order to analyze the structural transformations of the protein more precisely we have also performed the measurements of the partial specific volume of lysozyme as a function of ethanol concentration (Fig. 5). At very low ethanol concentration (in the range 0÷6%, v/v) the specific volume slightly increases and its average value of 0.715 $cm^3 \cdot g^{-1}$ is in very good agreement with the literature (Kamiyama *et al.,* 2003). With the increase in the ethanol concentration, up to about 30% (v/v), the specific volume increases up to the value of 0.726 $cm^3 \cdot g^{-1}$. These data are in a good correlation with our observations of the changes of lysozyme hydrodynamic radius, R_H (Szymańska & Ślósarek, 2012). According to the data from dynamic light scattering R_H remains constant and equals (1.856±0.023) nm when the ethanol concentration increases up to about 4.3% (v/v) and then starts to increase with a growing amount of alcohol. This means that at very low ethanol concentration (within the range 0÷6%, v/v) the protein–ethanol interaction is very weak. According to the crystal structure analysis (Lehmann *et al.,* 1985) there are only about ten sites on the surface of the protein that could be occupied by ethanol molecules. They are placed in the region of the b domain and the C-terminus of the protein. These ethanol molecules do not affect the structure and thermal motion of the atoms of the protein. However, according to the data presented by Deshpande and co-workers (Deshpande *et al.,* 2005), the denaturation process starts when the mutual interaction between ethanol and lysozyme causes dehydration of the protein.

A very strong increase in the specific volume of the protein is observed when the ethanol concentration changes within the range of 30÷55% (v/v). The partial specific volume reaches the maximum value of 0.778 $cm^3 \cdot g^{-1}$. The change of the partial specific volume equals 0.052 $cm^3 \cdot g^{-1}$, which is close to the result (Dn = 0.040 $cm^3 \cdot g^{-1}$) obtained for the disulfide-deficient variant of hen egg-white lysozyme (Akasaka *et al.*, 2007). During subsequent studies the formation of protofibrils and the change in the partial specific volume were observed for the protein dissolved in 20 mM sodium acetate buffer containing 30 mM NaCl (pH 4.0) (protein concentration — 5.07 mg/ml). The change in the partial specific volume was assigned to the monomer — to — protofibril transition at temperature 298 K. Evaluation of the partial specific volume of lysozyme was also observed under conditions of chemical denaturation, when the protein was dissolved in the water–DMSO solutions (Kamiyama *et al.,* 2003). In this case the maximum value of the patrial specific volume was only 0.734 $cm^3 \cdot g^{-1}$ (Δv=0.023 $cm^3 \cdot g^{-1}$). In all these cases the increase in the volume is related to the formation of a new protein structure ready for the formation of the filaments. The volume change is mainly related to the increase in the internal cavities.

However, when the ethanol concentration was greater than 55% (v/v) we have observed a decrease in the partial specific volume (Fig. 5). The same process was observed in water–DMSO solution (Kamiyama *et al.,* 2003). The authors described it as a disaggregation of the filaments. According to our results when the ethanol concentration is greater than 55% (v/v) there is a strong increase in viscosity (Fig. 1) and a change from the Newtonian to the pseudoplastic flow (Fig. 2). In our opinion the strong decrease in the partial specific volume indicates the process of structural transformations of the filaments associated with the formation of a more compact structure and the subsequent rearrangement of the hydration shell.

CONCLUSIONS

In the present paper we have described experimental data obtained from the measurements of viscosity and density of lysozyme-water-ethanol solution as well as from the static light scattering experiment performed on similar sample. Using all these methods it was possible to follow any changes of the size and dispersion of the lysozyme molecule. The rheological characteristics of the solution enabled us to follow the process of gradual unfolding of the peptide chain. These data were correlated with the change of the protein specific volume, which could be determined by densitometry. Finally, the light scattering experiment enabled control of the possible aggregation of the protein. For these reasons applied methods could be considered as complementary.

The analysis based on our rheological data of the structural transformation of the lysozyme molecule indicates the formation of linear aggregates in a two step evolution of the protein molecule from the native state to the aggregates. According to the analysis of the volumetric studies we can assign the first stage of the process to the formation of the intermediate state with ethanol molecules located on the surface of the protein. The volume of the protein is slightly increased. The viscosity of the solution increases from about 0.5 mPa·s to 3 mPa·s and the flow of the solution remains Newtonian. The second stage can be attributed to the formation of the new state ready for the aggregation into the filaments. We observe a significant increase in the partial specific volume up to the value of 0.774 $cm^3 \cdot g^{-1}$. For higher ethanol concentrations the viscosity of the lysozyme–water–ethanol solution strongly increases and the flow changes form Newtonian to pseudoplastic. The intrinsic viscosity reaches the maximum value of 8.7 $cm^3 \cdot g^{-1}$.

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