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The SAXS and Rheological Studies of HEWL Amyloid Formation

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We performed small angle X-ray scattering and rheological experiments in order to analyze the aggregation and denaturation processes of hen egg white lysozyme initiated by the presence of ethanol molecule. At low ethanol concentrations (below 60% (v/v)) we did not observe any change of the radius of gyration of lysozyme and no drastic changes in viscosity of the protein solution. With the increase in ethanol concentration up to the final concentration of 85% (v/v) the viscosity of protein solution dramatically increased. For high ethanol concentration a pseudoplastic behavior of lysozyme solution was observed, indicating a process of aggregation and reorientation of the protein molecules. Similar effects were observed in small angle X-ray scattering experiments. We assume that the analysis of the aggregation processes of the hen egg white lysozyme could contribute to our understanding of the mechanism of lysozyme amyloid formation.

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1. Introduction

Amyloid fibrils forming deposits of insoluble aggregation are associated with human diseases such as Alzheimer's and Parkinson's diseases [1]. Although, in general any protein can form an amyloid fibril, the final structure of the latter shows very similar morphological and histochemical characteristics [2]. The mature amyloid fibers appear essentially the same in the electron micrograph. These are stiff, unbranched fibres with a diameter of about 6÷12 nm, of undetermined length,

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built of protofilaments, which are twisted along the axis of the fibril [3]. It has been found that several non-pathological proteins and short peptides can form amyloid fibrils under conditions determined by pH [4, 5], temperature [6, 7], pressure [8, 9] and ethanol solution of different concentrations [10]. The need of understanding the process of amyloid formation has drawn intense *in vitro* studies. Analysis of the hitherto obtained results has indicated that the tendency towards amyloid aggregations is a generic property of all polypeptides [11], but its mechanism has not been recognized yet. It is then very important to determine all structural changes of a protein molecule leading to amyloid structure formation.

The amyloid structure formation has been studied by several experimental techniques — radiation scattering measurements [12–16], circular dichroism [17] and X-ray diffraction [18]. In this paper we present studies of the aggregation and denaturation processes of hen egg white lysozyme (HEWL) initiated by the presence of ethanol molecules using the methods of small angle X-ray scattering (SAXS) and rheological experiments. These experiments enable us to determine the hydrodynamic properties of the protein solution, providing information about the overall structure and dynamic behavior of protein molecule. In particular, viscosity of the solution can sensitively reflect changes in the protein conformation, denaturation and aggregation processes.

Ideal fluids exhibit a Newtonian behavior which means that the viscosity is independent of shear strain rate and a relation betwen shear strain rate and a shear stress is a linear function. However, some condensed-phased materials are neither simple liquids nor simple crystalline solids. In many cases, the relationship between stress and deformation for a complex fluid is not linear and depends on the fluid structure. Therefore, the study of the rheological response, which is then called a non-Newtonian behavior, can bring information on the nature and strength of the interparticle interactions governing the fluid structure. For example, suspensions of

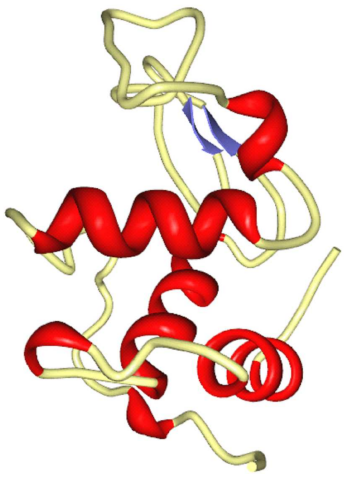

Fig. 1. Structure of HEWL (pdb code: 2VB1 [20]).

long biological molecules are shear-thinning materials — their viscosity decreases with the increase in the shear strain rate. This is often a consequence of high molecular weight molecules being untangled by the flow. Generally, this behavior became more intensive with the increase in concentration.

HEWL (Fig. 1), which was analyzed in our experiments, is a small globular protein consisting of two domains, one containing four α-helices and the other including two β-strands. The native structure of the protein is stabilized by four disulfide bridges [19, 20]. HEWL is closely related to the human lysozyme, so extensive study of HEWL in different conditions is expected to be of interest for medical sciences as it should contribute to our understanding the mechanism of amyloid formation.

2. Material and methods

HEWL was purchased from SIGMA. The protein was dissolved in a small amount of water and then dialyzed against 40% (v/v) ethanol solution. Finally, appropriate amount of the lysozyme solution and 96% (v/v) ethanol were mixed 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 taking into account the excitation coefficient is $\varepsilon^{280\mathrm{nm}} = 2.64$ ml mg^{-1} cm^{-1} [21].

SAXS studies were carried out on the EMBL SAXS-WAXS beamline X33 at the DORIS storage ring of the DESY (Hamburg, Germany) using a MAR-345 image plate detector. The measurements were performed within the scattering vector s ranging from 0.15 to 5 nm^{-1} (where $s = 4\pi \sin\theta/\lambda$ and $\lambda = 0.15$ nm). The data were collected at 290 K using 1 mm cells with mica windows (20 μm) and the sample volume was 100 μL. The background scattering of the buffer was quantified before and after measurements of the protein solution and the signals obtained were subtracted from the protein SAXS patterns. All experimental data were normalized to the intensity of the incident beam and corrected for nonhomogeneous detector response. The scattering of the buffer was subtracted and the final scattering curve was obtained using the program PRIMUS [22]. Glucose iso-

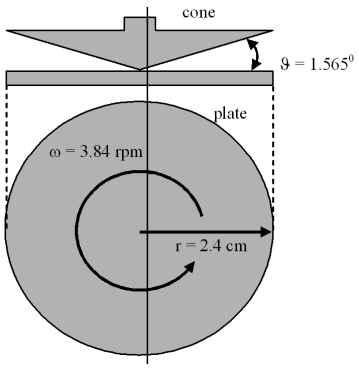

Fig. 2. Cross-section of the cone used in our rheological measurements.

merase from *Streptomyces rubiginosus*, with a known molecular weight of 173 kDa, was used as a molecular weight standard [23]. The pair distance distribution function $p(r)$ as well as the radii of gyration R_G were calculated using the program GNOM [24].

Viscosity of the lysozyme in water–ethanol solution was measured using a Brookfield rheometer with the cone-plate geometry (Fig. 2). The measurements were performed at temperature 298 K, which was stabilized with the accuracy of 0.1 K.

3. Results and discussion

We have determined the viscosity of the lysozyme in the water ethanol solution. A low increase in the viscosity of the protein solution, from 1.32 cP (c_{EtOH} = 10%) up to 2.58 cP (c_{EtOH} = 60%), was observed until the ethanol concentration in the solution reaches a value of 60% (v/v) (Fig. 3). According to our rheological data the lysozyme solution is a Newtonian fluid at low ethanol concentration — 10% (v/v) (Fig. 4). The shear stress of the solvent increases linearly with increasing shear rate of the solution and the protein viscosity in certain ethanol concentrations is constant and independent of the values of shear rate.

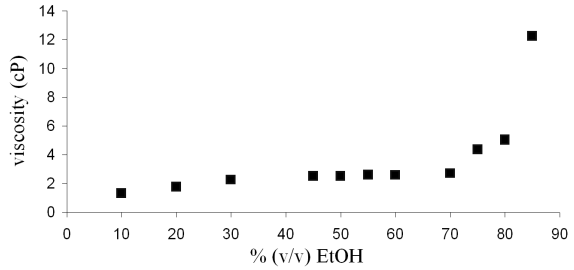

Fig. 3. Changes of the protein solution viscosity as a function of the ethanol concentration.

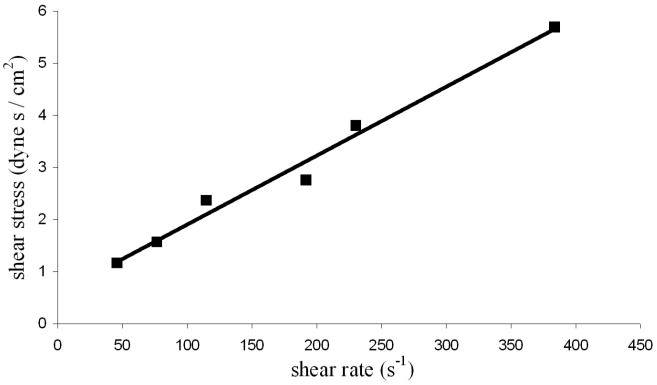

Fig. 4. Newtonian behavior of HEWL solution in 10% (v/v) ethanol.

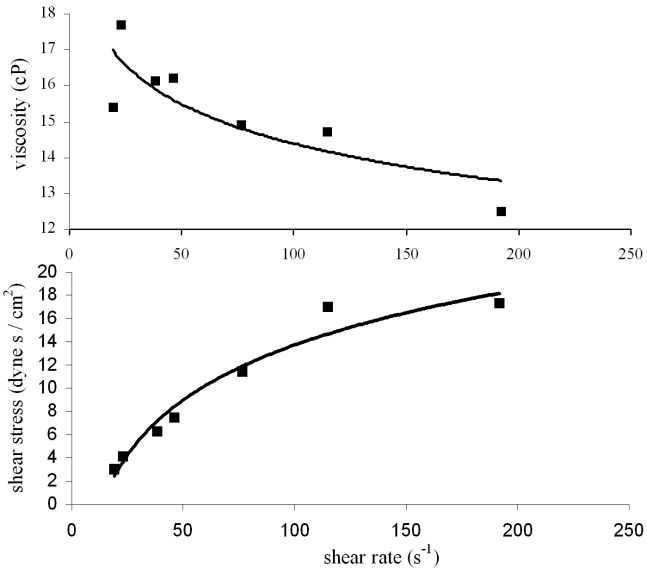

Fig. 5. Pseudoplasticity shown by HEWL solution in 85% (v/v) ethanol: a) viscosity — shear rate characteristics, b) shear stress — shear rate characteristics.

Over the value of 60% of the ethanol concentration the solution viscosity dramatically increased and the experimental value of the viscosity was equal to 12.25 cP (c_{EtOH} = 85%). When the ethanol concentration is increased above 80% (v/v) a typical non-Newtonian pseudoplastic behavior of the lysozyme is observed, as indicated by the non-linear variation of the shear stress with the shear rate and by a decrease in the viscosity with increasing shear rate (Fig. 5). This observation suggests that the process can be explained by the reorientation of macromolecules under the flow conditions or by unfolding of the macromolecular chains and their rearrangement under the acting force.

The rheological studies were accompanied with the synchrotron SAXS measurements. The exemplary SAXS data are presented in Fig. 6. The significant changes of the curves shapes and intensity with increasing ethanol concentration were observed. On the basis of the SAXS data, the radii of gyration characterizing the lysozyme molecule in particular ethanol concentrations were calculated. The results are given in Fig. 7. At low ethanol concentrations (below 60% (v/v)) no drastic changes in radii of gyration have been observed (R_{G} was about 1.48 nm). In the solutions containing higher amount of ethanol (c_{EtOH} from 60% to 65% (v/v)) the radius of gyration increased to about 1.6 nm. The process of lysozyme aggregation started for $c_{\mathrm{EtOH}} > 65\%$. The radius of gyration jumped to R_{G} = 1.96 nm (c_{EtOH} = 70%) and 3.1 nm for c_{EtOH} = 75%. For the last two samples (c_{EtOH} = 80% and c_{EtOH} = 85% (v/v)) the values of the gyration radius increased above 4 nm, but the system also exhibited high polydispersity. The observed changes are in agreement with the earlier described tendency [15].

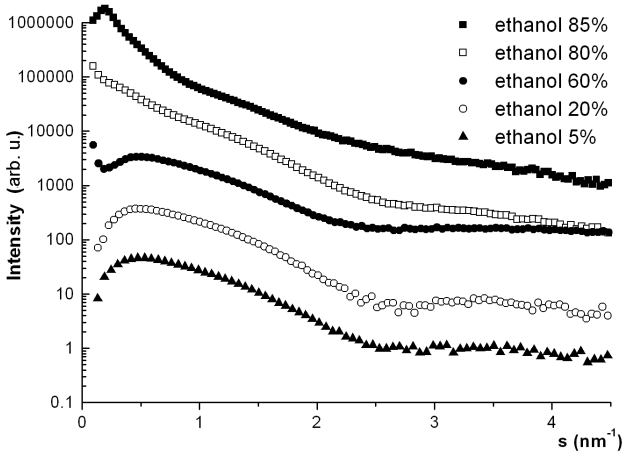

Fig. 6. SAXS curves recorded for mixtures of HEWL with ethanol.

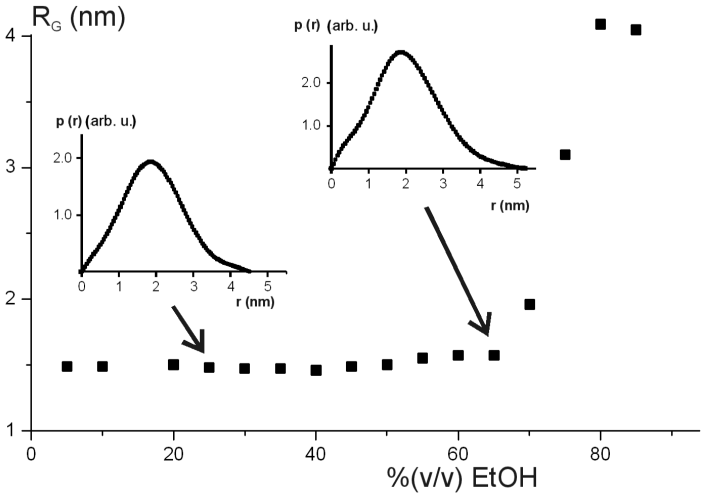

Fig. 7. Radii of gyration characterizing HEWL versus ethanol concentration and the pair distance distribution function $p(r)$.

In general, the increase in the viscosity of the protein solution can be explained by the process of a protein denaturation and simultaneous aggregation [25]. The effect of alcohol on structure and stability of the HEWL molecule has been extensively studied [7, 10, 15, 16]. Yonezawa et al. [15] reported that at low ethanol concentration the lysozyme molecule is a monomer. The process of dimer formation and subsequent association into the structural form of protofilaments was induced in ethanol solution up to $c_{\mathrm{EtOH}} = 70\%$ (v/v). Finally, fibrils were reported to exist in a $c_{\mathrm{EtOH}} = 90\%$ (v/v). As follows from earlier studies alcohol can induce

protein helical structure formation and destabilize the tertiary structure [10, 26]. Similar effects were observed in trifluoroethanol [27] and methanol [28]. Furthermore, it has been suggested that alcohol acts as protein binding ligand causing the structural transformation of the hydration shell by reducing the polarity on the protein surface [16].

A high increase in the protein viscosity was also induced by other organic solvents like tetramethylurea (TMU) [29] and dimethylsulfoxide (DMSO) [29, 30]. The pseudoplastic behavior of the protein in TMU indicated that the lysozyme existed in the elongated conformation and was able to reorient under shear stress.

4. Conclusions

On the basis of the experimental results presented here we can conclude that the aggregation processes are induced by the presence of ethanol in the solution with the ethanol concentration higher than 65% (v/v). Under these experimental conditions we observe a strong increase in the solution viscosity and a substantial increase in the gyration radius of the macromolecules. We shall continue our studies with analysis of samples containing a higher concentration of lysozyme. We expect that this would enable us to determine a phase diagram for all structural forms of the protein.

Acknowledgments

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