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A study of alcohol and temperature effects on aggregation of β-lactoglobulin by viscosity and small-angle X-ray scattering measurements

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ABSTRACT

Small-angle X-ray scattering (SAXS) of aqueous solutions of methanol and 2,2,2-trifluoroethanol (TFE) of β-lactoglobulin (β-Lg) in concentrations of 30 and 63 mg/mL was measured as a function of alcohol concentration in a temperature range of 298–363 K to investigate the effects of alcohol and temperature on aggregation of the protein. The viscosity of aqueous solutions of methanol, ethanol and TFE of β-Lg in a concentration of 2 mg/mL was also measured as a function of alcohol concentration at an ambient temperature. The relative viscosity, η / η_0, where η and η_0 are the viscosities of the protein solutions and the solvents, respectively, showed a maximum at around 50%, 40% and 20% of the methanol, ethanol and TFE solutions, respectively. This finding suggests that the aggregation of β-Lg of a low concentration occurs at the specific alcohol concentrations. The SAXS data showed that the aggregation of β-Lg in terms of the aggregation number and the radius of gyration initiates at an alcohol concentration of ~40% for the methanol solutions and ~10% for the TFE solutions and increases with alcohol concentration at ambient temperature. Furthermore, the temperature of thermal denaturation of β-Lg decreased by ~30 K for the TFE solution and by ~20 K for the methanol solution, compared with the case for water (348 K). Thus, the addition of TFE promoted the thermal denaturation of the protein more effectively than methanol. The aggregation structure of the thermally denatured protein was independent of type and concentration of alcohol in the concentrated protein solutions. Furthermore, the fractal dimension of the aggregates was ~2.6, indicating a three dimensional network structure. However, in the diluted protein solutions, the aggregate structure after the thermal denaturation was likely to be a low cross-linked network gel in the methanol solution, which was similar to that in water. On the other hand, more complex aggregates tended to form in the TFE solution.

1. Introduction

Aggregation of proteins has recently been revisited intensively in connection with amyloid fibrils that have often been discussed as an origin of amyloidosis, such as Alzheimer disease [1,2], e.g. the amyloid fibrils are formed by stacking the β-sheet structure of proteins led by a misfolding process induced by various factors. Proteins are known to be denatured, followed by aggregation and gelation by heating [3–28], pressurizing [29–33] and addition of co-solvent [17,18,21,24,26,34–38]. The properties of aggregates also depend on protein concentration, pH of the solution, ionic strength, and several other parameters.

Alcohols with a small hydrophobic alkyl group are miscible with water at any concentration, and thus the hydrophilic/hydrophobic environment around proteins can be controlled by changing alcohol concentration in alcohol–water mixture. Therefore, a study of co-solvent effect of alcohol on protein aggregation would reveal how the change of the hydrophilic/hydrophobic environment around a protein affects denaturation and aggregation of the protein. Among various proteins so far investigated, the properties of amyloid fibrils of β-Lg have drawn much attention [24,25,28]. Hence, β-Lg would be a good target to study the mechanism of protein aggregation.

To this aim, previously we performed small-angle neutron scattering (SANS), neutron spin echo (NSE), and dynamic light scattering measurements of β-Lg in aqueous solutions of various alcohols: methanol, ethanol, 1-proponaol, 2-propanol, *tert*-butanol, trifluoroethanol (TFE) and hexafluoro-isopropanol (HFIP) as a function of alcohol concentration [37]. It has been found that β-Lg in the alcohol–water solutions undergoes gelation at specific alcohol concentrations where the alcohol-induced α-helical structure of β-Lg is stabilized. Furthermore, the structure and dynamic properties of β-Lg were investigated as a function of alcohol concentration in ethanol– and TFE–water solutions by SANS and quasi-elastic neutron scattering (QENS) measurements [38]. At low concentration of TFE (10%), the mean square amplitudes of vibration $\langle u^2 \rangle$ increase with a retention of a native-like structure. Addition of 20% of TFE induces nucleation, going along with

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a further increase of $<u^2>$. A further increase in TFE concentration to 30% changes the nanoscale structure of the oligomeric nucleate, but induces no further significant changes in $<u^2>$. To our knowledge, there are only a few studies about an alcohol effect on thermal denaturation of β-Lg [15,20,27].

Small-angle X-ray scattering (SAXS) is a powerful tool to investigate the aggregate structure at a nano-meter scale. The development of a laboratory X-ray source and a two-dimensional detector has enabled us to produce high quality SAXS data even in a laboratory, compared to that obtained in synchrotron radiation sources. One of the advantages of laboratory SAXS is any beam time availability so that we could measure SAXS of a protein systematically at various types and concentrations of alcohols.

The macroscopic properties of protein aggregates are reflected on viscosity of protein solutions. The advantage in viscosity is its sensitivity to the formation of aggregates in dilute solutions (about several mg/mL), whereas SAXS requires a solution of high concentration (over several tens of mg/mL) to obtain an enough contrast of protein aggregates with the solvent.

In the present study, SAXS of β-Lg was measured in aqueous solutions of methanol and TFE at 298–363 K as a function of alcohol concentration. An alcohol-induced protein aggregation and an alcohol effect on thermal denaturation of the protein were investigated. The viscosities of aqueous solutions of methanol, ethanol and TFE of β-Lg were also measured as a function of alcohol concentration. The alcohols were chosen so that the strength in hydrophobicity changes in an order of methanol, ethanol and TFE [39,40]. What we would like to clarify is as follows: (1) whether the structure of aggregates changes with type and concentration of alcohols and the protein concentration (2) whether the structure of aggregates induced by alcohols is the same as that by heat, and (3) how alcohols affect the thermal denaturation of protein.

2. Experimental

2.1. Samples

β-Lg from bovine milk, 90% PAGE (L0130, lot 095K7006), was purchased from SIGMA-ALDRICH as lyophilized powder and used without further purification. Methanol, ethanol and TFE were purchased from Kanto Kagaku. The β-Lg powder was dissolved in a KCl solution. To adjust pH of the solution to 7, a small amount of a KOH solution was added adequately. Then, alcohol and water were added to the β-Lg solution. In the final solutions, the concentration of KCl was 50 mM and the concentrations of the protein were 2 mg/mL for viscosity measurements and 30 and 63 mg/mL for SAXS measurements, irrespective of the ratio of water and alcohol. The mixed solutions were stirred with a vortex mixer for 30 s. All samples measured are summarized in Table 1.

2.2. Viscosity measurements

The densities, ρ, were measured on a vibrating densitometer DMA-48 (Anton Paar) which had been calibrated with dried air and distilled water at 298 ± 0.1 K. The kinematic viscosities, $\nu = \eta / \rho$, where η is the viscosity, were measured with a Cannon-Fenske type viscometer in a water bath whose temperature was controlled to within 298 ± 0.1 K.

Table 1
Experimental conditions of viscosity and SAXS measurements of the β-Lg solutions.

	Concentration of β-Lg (mg/mL)	Alcohols (v/v%)	Temperature (K)
Viscosity	2	Methanol (0–80), ethanol (0–80) and TFE (0–60)	298
SAXS	63	Methanol (0–20), TFE (10)	298–363
SAXS	30	Methanol (0–40), TFE (0–5, 10, 40)	298–363
SAXS	30	Methanol (0–70), TFE (0–70)	298

The relation between the flow time, *t*, and the kinematic viscosity, *ν*, is given by the following equation:

$$\nu = Ct - K/t \quad (1)$$

where *C* and *K* are constants. Since the contribution of the kinetic energy correction term, K / t, on the relative viscosity of a solution was at most 0.05%, the K / t term was neglected. The constant, *C*, was determined by the measurements of water at 298 K. The flow time of the sample solutions was measured within ±0.1 s and averaged over at least 5 runs.

2.3. SAXS measurements

SAXS measurements were made on a small-angle X-ray diffractometer, Nano-Viewer (Rigaku), using Cu*Kα* radiation (wavelength λ = 1.5418 Å) under the operating condition of 40 kV and 30 mA. The X-ray beam was focused with a confocal max flux mirror. Flight paths before and after a sample were kept in vacuum to avoid air scattering.

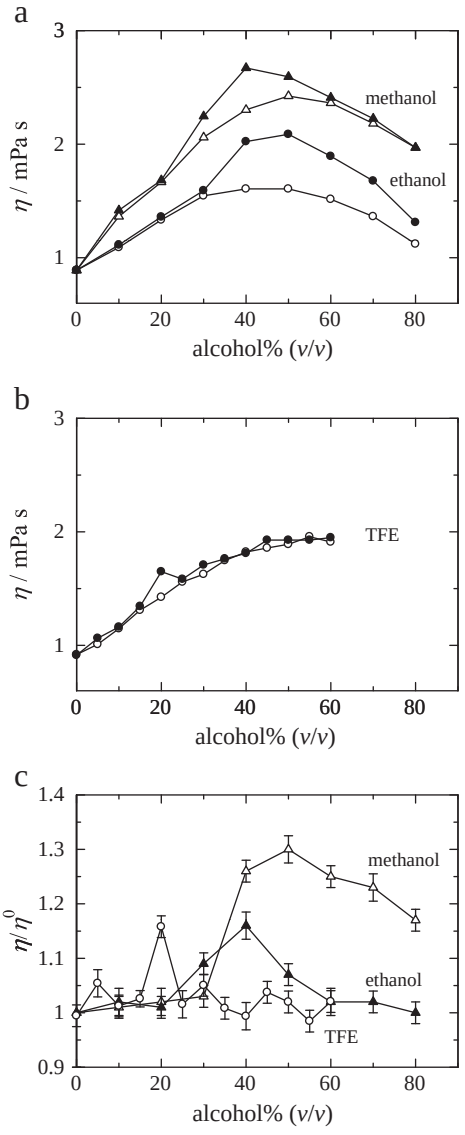

Fig. 1. Absolute viscosity, η, of the methanol- (▲) and ethanol- (●) solutions of β-Lg (2 mg/mL) and those, η_0, of the solvent (△, ○) as a function of alcohol concentration, (b) the corresponding values of the TFE solutions (●) and the solvent (○), and (c) the relative viscosities, η / η_0, obtained. All data were taken at 298 K.