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Analysis of protein denaturation by high-performance continuous differential viscometry

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ABSTRACT

The intrinsic viscosity of protein solutions changes when denaturation occurs. Therefore, viscosity measurement using traditional glass capillary viscometers has been a key method to study the process of protein denaturation. Such measurements are laborious, time consuming and need at least 10 ml of sample. The Viscotek differential viscometer can be used as an on-line detector for high-performance liquid chromatography to monitor the viscosity of column effluent. In this study, samples were injected using an autosampler onto a "delay" column containing glass beads in place of the high-performance liquid chromatography columns. Results indicate guanidine hydrochloride, heat and pH act as denaturing agents and changes the intrinsic viscosities of serum albumin, turkey egg albumin, and ovalbumin solutions. The differential viscometer is sensitive and provides accurate measurements of minor changes in viscosities of very dilute protein solutions undergoing denaturation. The advantage of using the differential viscometer instead of conventional glass capillary viscometer is the increased sensitivity, precision, speed and operational ease that permits measurements of solution viscosity of low sample concentrations up to 1.2 μg of pure proteins.

INTRODUCTION

Most proteins, globular and fibrous in their native states, are folded into well-defined, essentially rigid, three-dimensional structures [1,2]. The macromolecular nature of these proteins is best understood when they are in a denatured or unfolded state [3]. Denaturation is a non-proteolytic modification of a native protein that leads to definite changes in chemical, physical and biological properties [4]. There are many physical and chemical properties that change during denaturation [5]. These physicochemical changes that occur can be monitored by viscometry, optical rotatory dispersion, circular dichroism, nuclear magnetic resonance and electron spin resonance. The gross conformational changes generally result in the modification of the rheological behavior of proteins in solution. Therefore, viscosity measurement has been a key method in many denaturation studies [3–7]. Viscometry is a useful tool because of its extreme sensitivity and technical simplicity. Since the intrinsic viscosity provides information on the overall size and shape of the molecule, it is a sensitive and simple indicator of denaturation. The intrinsic viscosity of proteins in solution increases when denaturation occurs. Such an increase may correspond to an aggregation of the denatured molecules, a change in the shape of hydrated protein

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molecules, unfolding of the polypeptide chains or anisotropic swelling of the particles. The increase in viscosity is often due to several of these processes which may occur almost simultaneously in a protein solution undergoing denaturation. Thus, it has been found that the globular proteins have very low values of intrinsic viscosity, little more than predicted for a nonsolvated sphere and they increase markedly upon denaturation [7].

Despite its great utility, the solution viscosity method of studying protein denaturation has declined in popularity in recent years. Until very recently, viscometric methods have not kept pace with the rapid evolution of most analytical methods towards sophisticated instrumentation capable of greater sensitivity, speed and ease of operation. Traditional viscometers such as the glass capillary tubes of Ostwald or Ubbelohde [8] are still the most widely used viscometers for solution viscosity measurement today. However, these viscometers require high solute concentrations and lack the speed and sensitivity to meet the needs of viscosity measurements of dilute protein solutions.

In this paper, we have used the Viscotek differential viscometer (DV) [9–12] detector on-line with an isocratic high-performance liquid chromatography (HPLC) setup for measuring viscosity of dilute protein solutions undergoing denaturation. The solutions are dilute enough that a single-point determination of intrinsic viscosity can be made, without the need to exrapolate the viscosities of several sample concentrations. We show that the DV yields a solution viscosity measurement of unmatched sensitivity, precision, speed and operational ease using microlitre injection volumes of very low sample concentration.

EXPERIMENTAL

Chemicals and reagents

Proteins such as bovine serum albumin, chicken egg albumin (ovalbumin), turkey egg albumin, β-lactoglobulin, lysozyme and hemoglobin were obtained from Sigma (St. Louis, MO, U.S.A.). All proteins were used as received, without further purification. Water was HPLC Omnisolv grade from EM Science (Cherry Hill, NJ, U.S.A.) and monobasic sodium phosphate was from Mallinckrodt (Paris, KY, U.S.A.). All other chemicals obtained from commercial sources were used without purification.

The buffer used in the mobile phase was adjusted to the appropriate pH by addition of potassium hydroxide. The mobile phase was filtered through a 0.45-μm membrane (Micro Filtration Systems, Dublin, CA, U.S.A.) and vacuum-degassed.

Sample preparation

Protein solutions were prepared in 5 m*M* NaH_2PO_4 at pH 7.0. Concentrations ranged from 1.5–2.8 mg/ml. The proteins were first dissolved in 5 m*M* NaH_2PO_4 (pH 7.0) and allowed to stand for 30 min. The proteins at this stage show their native state. The protein solutions were heated in a 60°C water-bath in the presence and absence of 0.1 *M* 2-mercaptoethanol for 30 min for the thermal denaturation experiment. In the experiment showing denaturation by guanidine hydrochloride (GuHCl), the proteins were dissolved directly in 4 *M* GuHCl and 0.1 *M* 2-mercaptoethanol to obtain randomly coiled polypeptide chains. For denaturation by changing pH, proteins were dissolved directly in 5 m*M* NaH_2PO_4 having different pH values.

Equipment and condition

The HPLC–DV system was modular in design and consisted of a Micromeritics (Norcross, GA, U.S.A.) Model 728 autosampler, Valco (Houston, TX, U.S.A.) Model EQ60 remote switching valve fitted with a 50-μl loop, Scientific System (State College, PA, U.S.A.) Model 222B HPLC pump and Viscotek (Porter, TX, U.S.A.) Model 110-02 (range adjusted at 4) viscometer detector. Experiments were performed on a "delay" column that was packed with DMCS-treated glass beads (100–200 mesh) in 450 mm × 3 mm I.D. stainless-steel tube (Alltech, Deerfield, IL, U.SA.) in place of usual HPLC packing. The purpose of the delay column is to provide the dispersion effect needed to generate an easily detectable elution profile at finite time. No retentive separation of protein samples was intended. The elution buffer consisted of 5 m*M* NaH_2PO_4 (pH 7.0) with flow-rate maintained at 0.5 ml/min. The protein solutions were analyzed by duplicate injections on the DV while measuring both the inlet pressure and the differential pressure.

A schematic of the DV detector [11,12] is shown in Fig. 1. The pneumatic pulse dampener shown is a PTFE tube (304 mm × 2.5 mm I.D.). Briefly, the protein solution from the delay column flows continuously through the balanced bridge network, which consists of four capillaries (R1–R4). Res 1 is located out of the flowstream and act as a compensate volume reservoir so that any flow-rate fluctuations cause equal pressure changes on each side of the differential pressure transducer. The other reservoir (Res 2) holds up the protein solution and prevents it from entering capillary R4. For any time slice in the elution profile, protein solution will be in capillaries R1, R2 and R3 but the elution buffer will be in capillary R4. Res 1 and Res 2 are large (30

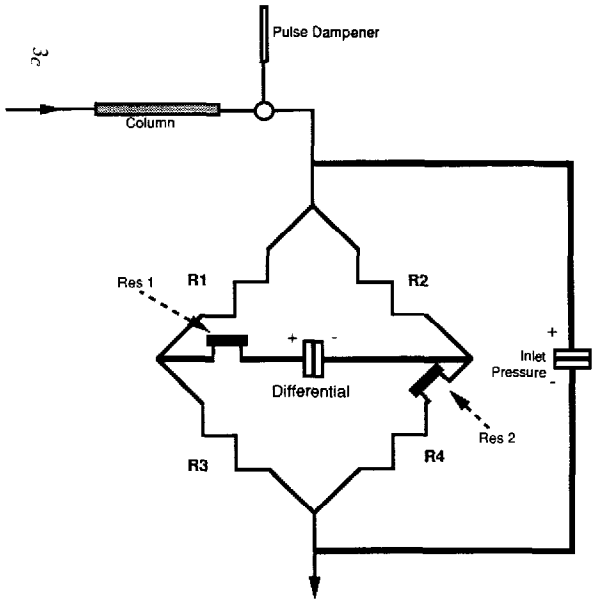

Fig. 1. Simplified schematic diagram of the differential viscometer. See text.

ml) reservoirs so that the 5 m*M* NaH_2PO_4 (pH 7.0) elution buffer is not entirely displaced until after the chromatographic peak is completely eluted. Two pressure measurements are normally made by use of transducers. The differential pressure, ΔP, is due to the difference in the viscosity of the protein solution in capillary R3 and viscosity of the elution buffer in capillary R4. Protein solution viscosities in R1 and R2 cancel each other. Another transducer measures P_i, the inlet pressure into the bridge. The DV detector nulls the solvent pressure as desired yielding a differential (ΔP) signal proportional to the specific viscosity of the protein solution. The DV being highly sensitive, can measure the specific viscosity near zero concentration, thereby obtaining the intrinsic viscosity from a single measurement. The following expression relates the specific viscosity (η_{sp}) to these two pressure measurements [10].

$$\eta_{sp} = 4\Delta P/(P_i - 2\Delta P)$$

The intrinsic viscosity $[\eta]$, is defined as,

$$[\eta] = (\eta_{sp}/c)_{c \to 0}$$

where c is the concentration of the protein solution in g/dl. In the present experiments, the concentrations and specific viscosities are low enough that the extrapolation implied in the above equation can be neglected.

Calculation

Intrinsic viscosity was calculated [10–12] from data collected using the above equations on an IBM compatible personal computer system with Viscotek's UNICAL software.

RESULTS AND DISCUSSION

In the first experiment, the intrinsic viscosity determinations were made on native proteins. The high sensitivity of the balanced bridge design is illustrated in Fig. 2. The integrated area under the elution peak was used to calculate intrinsic viscosity. In its native state, β-lactoglobulin had higher intrinsic viscosity than other native proteins (Table I). The total time required for analysis of a sample was about 3 minutes, from injection to final computer report. The negative dips (Fig. 2) in the baseline before the peaks seems anomalous and is difficult to explain. This behaviour is not normally observed in size-exclusion chromatography peaks with the viscometer detector. In the present case, with no chromatographic columns to disperse the injected sample, the negative dip may be a result of a very sharp concentration gradient in the viscometer bridge. It is likely that for the very sharp peaks observed in these experiments, a bridge with shorter capillaries would be required. More experiments are presently being conducted to confirm this. However, the intrinsic viscosity values (Table I) reported of the various native proteins are very close to values reported elsewhere [3–5]. The small difference between the observed values reflects the difference in the type of salt concentration and temperature at which the intrinsic viscosity was measured. The precision of the DV technique for determining intrinsic

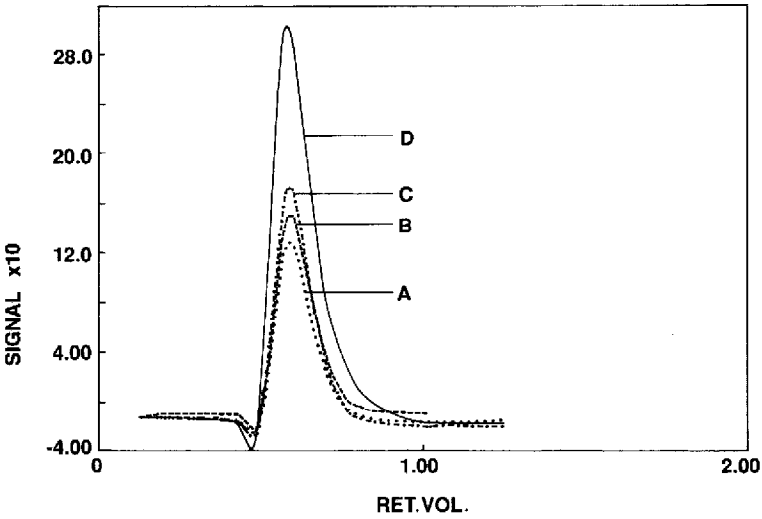

Fig. 2. Elution profile of native proteins dissolved in 5 m*M* NaH_2PO_4 (pH 7). A = Hemoglobin; B = ovalbumin; C = serum albumin; D = β-lactoglobulin. RET. VOL. = Retention volume in ml; signal in mV.

viscosity was excellent. It had a standard deviation of less than 0.002 for duplicate injections. In a separate experiment, we made dilutions of the native protein solutions. It was seen that the differential viscometer was sensitive and precise enough to measure intrinsic viscosity of 1.2 μg of protein samples. There was no direct correlation [13] between molecular weight and intrinsic viscosity (Table I).

Next, we explored the use of the DV as a tool for investigation of the denaturation process. For any protein, the value of intrinsic viscosity after denaturation largely depends on the denaturing agent. Therefore, we investigated the effect of some well known denaturing agents. As illustrated in Figs. 3 and 4, there is an appreciable increase in intrinsic viscosity of both ovalbumin and serum albumin solutions at both acidic and basic pH conditions. The results clearly demonstrate that

TABLE I

INTRINSIC VISCOSITIES OF PROTEINS IN NATIVE STATE AND 4 *M* GUANIDINE HYDROCHLORIDE

Protein[a]	Mol. wt.	Native state, $[\eta]$ (dl/g)[b]	Denatured state, $[\eta]$ (dl/g)[b]
Hemoglobin (A)[a]	64 500	0.017	0.069
Ovalbumin (B)[a]	45 000	0.020	0.163
Serum albumin (C)[a]	66 000	0.022	0.361
β-Lactoglobulin (D)[a]	36 800	0.037	0.113
Turkey egg albumin	45 000	0.020	0.389

[a] This designation corresponds to elution profiles shown in Fig. 2.
[b] Mean of three samples, each injected twice.

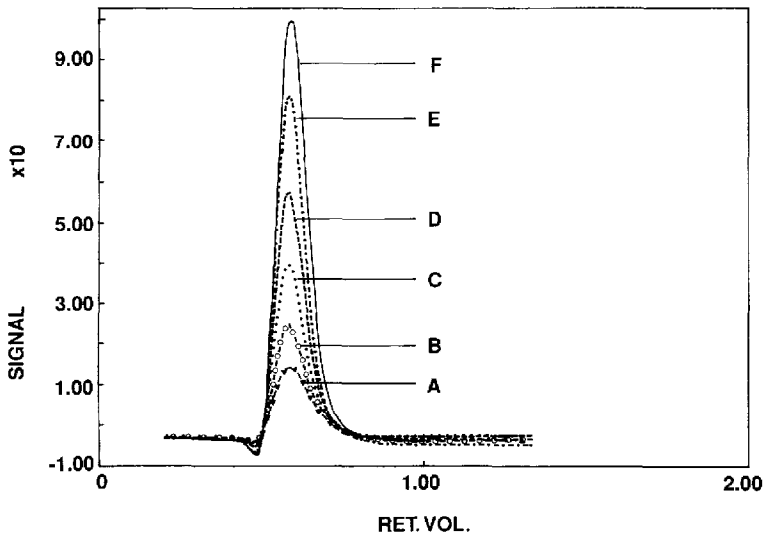

Fig. 3. Elution profile of ovalbumin when exposed to varying pH conditions. A = pH 7; B = pH 2.5; C = pH 4.5; D = pH 6.0; E = pH 8.0; F = pH 12.0. RET. VOL. = Retention volume in ml; signal in mV.

both ovalbumin and serum albumin were subject to structural changes as the pH of these solutions were altered. Increasing acidity possibly caused expansion of the molecules [14] that resulted in the increase of intrinsic viscosity. In the alkaline state, there is a huge increase in intrinsic viscosity because intermolecular disulfide bonds are readily formed, which result in aggregation, gelation or precipitation. Several other reactions leading to irreversible products are also possible [4]. Thus, it is evident that viscosity of protein solutions is complicated by the electrostatically interacting anionic and cationic protein side chains and by their action on water molecules. The viscosity

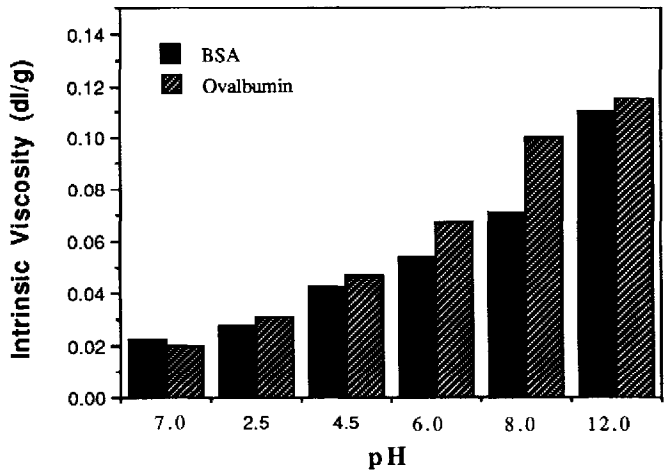

Fig. 4. Effect of varying pH conditions on bovine serum albumin (BSA) and ovalbumin.

of a protein solution therefore depends on the pH of the solution. The rate of denaturation is low at the isoelectric point of the protein and increases in acid or alkaline solutions [4].

The acid denaturation of the serum albumin is interesting. The intrinsic viscosity, which is 0.020 dl/g for the native serum albumin (Fig. 4) rises to only 0.031 dl/g when pH is lowered to 2.5. It proves that only limited expansion of the molecule occurs, which leaves the major globular regions of the molecule intact [15,16].

The effect of temperature on serum albumin, ovalbumin and turkey egg albumin was also investigated (Fig. 5, Table II). There was an increase of intrinsic viscosity upon heating the ovalbumin solution (Fig. 5A). This may be due to aggregation. However, it also immediately suggests a positive enthalpy effect associated with the previously postulated expansion equilibrium [17]. This thermal effect is reversible. When solutions were cooled after brief exposure to 60°C, they regained their original room temperature viscosity values. However, when turkey egg albumin (Fig. 5B) and serum albumin were heated, the intrinsic viscosity value decreased. This is probably due in part to the hydrolysis of the protein [17]. However, when heating was done in the presence of 0.1 m*M* 2-mercaptoethanol, which reduces disulfide bonds, the intrinsic viscosity increased in oval albumin, serum albumin and turkey egg albumin (Fig. 5).

Organic solutes such as GuHCl are powerful denaturating agents for proteins [18]. The action of 4 *M* GuHCl in the presence of 0.1 m*M* 2-mercaptoethanol to reduce disulfide bonds on some native globular proteins was monitored. In all cases (Fig. 5, Table I) the intrinsic viscosity increased [19]. Results obtained here and from optical rotatory measurement [4] clearly demonstrate that proteins in concentrated GuHCl solutions are randomly coiled without their disulfide bonds. The effect of GuHCl on protein conformation can be explained on the basis of free energy effects localized at hydrophobic interactions and peptide groups [18].

The differential viscometry technology can be also very useful in the determination of size [14,18], shape and solvation of proteins together with detection of chemical modification [20] by combination with other techniques. Work on these application areas is presently being performed in our laboratory. Research is also being conducted in our laboratory to develop a HPLC/size-exclusion chromato-

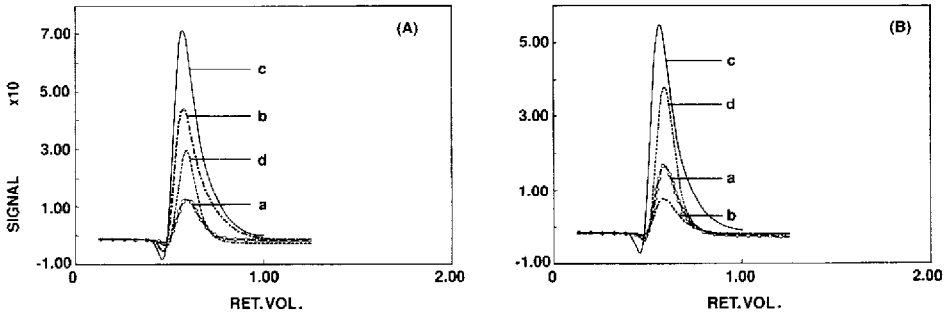

Fig. 5. Elution profile of (A) ovalbumin and (B) turkey egg albumin when exposed to varying denaturating conditions. a = Native protein; b = protein solution heated at 60°C for 30 min; c = protein solution heated at 60°C for 30 min in presence of 2-mercaptoethanol; d = native proteins dissolved in 4 *M* guanidine hydrochloride. RET. VOL. = Retention volume in ml; signal in mV.

TABLE II

EFFECT OF TEMPERATURE ON INTRINSIC VISCOSITIES OF NATIVE PROTEINS

Protein	Intrinsic viscosity (dl/g)[a]		
	Native state	Denatured state	
		Heat[b]	Heat with 2-mercaptoethanol[c]
Ovalbumin	0.020	0.056	0.449
Turkey egg albumin	0.020	0.012	0.360
Serum albumin	0.022	0.010	0.296

[a] Mean of three samples, each injected twice.
[b] Heated in water bath at 60°C for 30 min without any 2-mercaptoethanol.
[c] Heated in water bath at 60°C for 30 min in the presence of 0.1 m*M* 2-mercaptoethanol.

graphy–DV approach that would provide specific information about proteins undergoing the process of denaturation.

The following general statements are made regarding intrinsic viscosity measurement by high-performance continuous differential viscometry technology. (1) The balanced bridge design of the differential viscometer allows measurement of solution viscosity in a routine manner. The precison of the 2 min per sample analysis run time is much superior to that of the Ubbelohde tube for solution viscosity measurement. (2) Intrinsic viscosity measurements on proteins are particularly useful in showing changes in molecular configuration due to denaturation. (3) When globular proteins "denature" by the action of either GuHCl, heat or changing pH, the intrinsic viscosity normally increases. (4) It is possible to use only 1.2 μg of protein for measuring intrinsic viscosities.

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