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Note

Monitoring of the elution from a high-performance gel chromatography column by a spectrophotometer, a low-angle laser light scattering photometer and a precision differential refractometer as a versatile way to determine protein molecular weight

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It has become common practice to estimate protein molecular weight by methods which depend on the molecular sieving effect, such as gel electrophoresis and gel chromatography. These methods enable estimation of molecular weights of several or more proteins by a single run using very small amounts of samples. Classical physicochemical methods cannot satisfy the present demand for a method that is both time- and sample-saving.

The high-resolution separation of proteins became possible because of the development of prepacked high-performance gel chromatography columns such as the TSK-GEL SW series[1]. Molecular weights of proteins eluted from such a column can be determined by a tandem array of a low-angle laser light scattering photometer and a precision differential refractometer, as has been reported by the present authors[2,3]. The technique has since been successfully applied to membrane proteins solubilized by surfactants[4,5] and to the proteins of calf eye lens[6]. The technique is not only as accurate as the classical physicochemical methods but also as convenient as the methods depending on the molecular sieving effect.

We have tried further to improve the technique to establish it as a reliable and versatile technique for the determination of protein molecular weight. The measuring system was made more sophisticated by the addition of a spectrophotometer to the system. In this paper we present the standard instrumentation and procedure of the improved technique. Techniques depending on the molecular sieving effect fail to estimate molecular weights of proteins which behave "anomalously", such as glycoproteins. We thought, therefore, that the high performance of our measuring system would be most persuasively demonstrated if it could determine the molecular weights of typical examples of such proteins. Human α_1-acid glycoprotein and chicken ovomucoid, both with high carbohydrate content, were selected as the examples. The molecular weights of the glycoproteins thus obtained showed excellent agreement with those determined by the amino acid sequential and the carbohydrate compositional analyses.

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EXPERIMENTAL

Molecular weights of proteins were measured with a system consisting of a spectrophotometer, a low-angle laser light scattering photometer and a precision differential refractometer each equipped with a flow-through cell. An outline of the instrumentation is shown in Fig. 1. Measurements were carried out at room temperature (25 ± 2°C).

Both human α_1-acid glycoprotein (Lot 81F-9630) and chicken ovomucoid (Lot 60F-8110) were obtained from Sigma. Bovine serum albumin [66,267[7], 0.670 ml mg^{-1} cm^{-1} (ref. 8)], hen's ovalbumin (42,700[9], 0.735[8]), bovine carbonic anhydrase (29,000[10], 1.90[8]) and bovine pancreatic ribonuclease A (13,700[11], 0.706[8]) were the best commercially available products, and assumed to have molecular weights and extinction coefficients at 280 nm as given in the parentheses.

RESULTS

Fig. 2a and b shows the elution curves of human α_1-acid glycoprotein and chicken ovomucoid, respectively, recorded by the three detectors shown in Fig. 1. Each of the glycoproteins gave a single major peak.

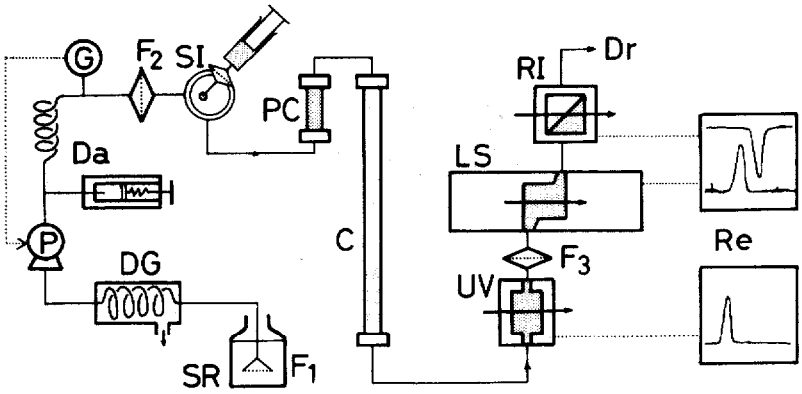

Fig. 1. Outline of instrumentation. SR, solvent reservoir (3.5 l); F_1, sintered stainless-steel filter (Umetani Seiki, Model SYF); DG, degasser (Elma Optical, Model ERC-3310); P, high-pressure pump (Milton-Roy, Model NSI-33R); Da, bellows-type damper (Umetani Seiki, Model S-100 and helically coiled stainless-steel tube (2 m × 0.2 mm I.D.); G, pressure gauge with a provision for switching off the pump when the pressure exceeds the value permissible for the column; F_2, sintered stainless-steel filter (Umetani Seiki, Model SLF); SI, syringe-loading sample injector (Rheodyne, Model 7125) with a loop of volume 100 μl; PC, Pre-column (Toyo Soda, TSK-GEL GSWP, 10 × 0.75 cm); C, column (Toyo Soda, TSK-GEL G3000SW, 60 × 0.75 cm); F_3, ultrafilter with a pore size of 0.5 μm (Millipore, type FHLP 01300); UV, ultraviolet spectrophotometer (Toyo Soda, TSK UV-8, Model II); LS, low-angle laser-light-scattering photometer (Toyo Soda, TSK Model LS-8, He–Ne laser); RI, precision differential refractometer (Toyo Soda, TSK model RI-8); Re, double-pen recorder; Dr, drain.

As has been reported previously[2], the molecular weight, M, of a protein can be determined according to the equation

$$M = k' \cdot (dn/dc)^{-1} \cdot (\text{Output})_{LS} \cdot (\text{Output})_{RI}^{-1} \quad (1)$$

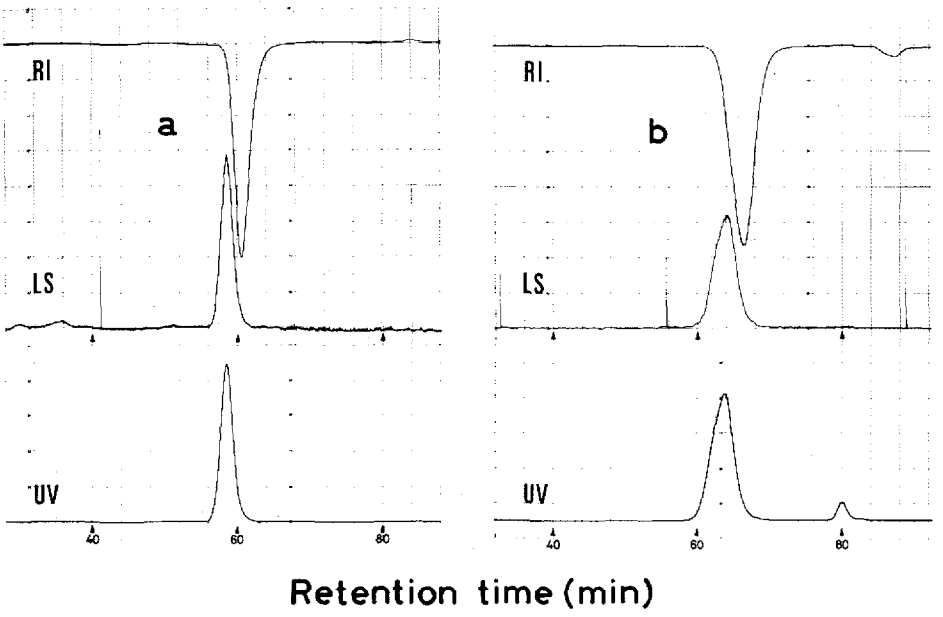

Fig. 2. Elution patterns of (a) α_1-acid glycoprotein and (b) ovomucoid recorded by the spectrophotometer at 280 nm (lower), the low-angle laser light scattering photometer (middle) and the precision differential refractometer (upper). The sample contained (a) 0.5 mg of α_1-acid glycoprotein and (b) 1.0 mg of ovomucoid in 100 μl of the solvent. Solvent: 0.025 *M* NaH_2PO_4, 0.075 *M* Na_2HPO_4, 0.2 *M* sodium chloride and 0.003 *M* sodium azide, pH 7.1. Gain setting: spectrophotometer, 1.28 a.u.f.s.; light scattering photometer, 32; refractometer, 128. Flow-rate: (a) 0.33 ml/min; (b) 0.31 ml/min.

where k' is a constant determined by the instrumental and experimental conditions, dn/dc is the specific refractive index increment and $(\text{Output})_{LS}$ and $(\text{Output})_{RI}$ are the output of the scattering photometer and of the refractometer respectively. The specific refractive index increment can be described as

$$(dn/dc) = k'' \cdot E \cdot (\text{Output})_{RI} \cdot (\text{Output})_{UV}^{-1} \quad (2)$$

where k'' is a constant, E is the extinction coefficient expressed in terms of weight concentration and $(\text{Output})_{UV}$ is the output of the spectrophotometer. Thus, the molecular weight can be expressed as

$$M = k \cdot E^{-1} \cdot (\text{Output})_{UV} \cdot (\text{Output})_{LS} \cdot (\text{Output})_{RI}^{-2} \quad (3)$$

The constant, k, can be estimated by monitoring the elution of a standard protein with known molecular weight and extinction coefficient. To ensure accuracy, a mixture of four standard proteins was applied to the measuring system. Fig. 3 shows the elution curves thus obtained.

The elution curves shown in Figs. 2 and 3 explicitly show the high resolution, sensitivity and stability of the measuring system used in the present study. When the elution curves are compared with those in our first publication on the application of

this technique[2], it is clear that the performance of our measuring system has since been significantly improved.

A plot of the values of the right side of eqn. 3, except the constant k, for standard proteins with known molecular weights and extinction coefficients gives a universal calibration line with a slope equal to k. The molecular weight of a protein thus can be determined uniquely when its accurate extinction coefficient is available. Line A in Fig. 4 was obtained using the data shown in Fig. 3. The peak heights were adopted as the measures of the corresponding outputs. The instrumental constant k in eqn. 3 was fairly invariant. It was sufficient to determine the k value once a day in a series of experiments during which the solvent was steadily flowing through the system. Lines B and C in Fig. 4 were obtained in different series of experiments carried out to determine the molecular weights of α_1-acid glycoprotein and ovomucoid, respectively.

The extinction coefficients of α_1-acid glycoprotein and ovomucoid were assumed to be 0.881 and 0.406 ml mg^{-1} cm^{-1} at 280 nm, respectively, values calculated using the reported values at 278 nm[12,13] and their absorption spectra. The average value of $E^{-1} \cdot (\text{Output})_{UV} \cdot (\text{Output})_{LS} \cdot (\text{Output})_{RI}^{-2}$ in eqn. 3 was evaluated from experimental data such as that shown in Fig. 2. The height of a peak was taken as the output for each of the detectors. The molecular weight of α_1-acid glycoprotein was thus determined to be 38,300 ± 400 ($n=4$) by using line B of Fig. 4 as the

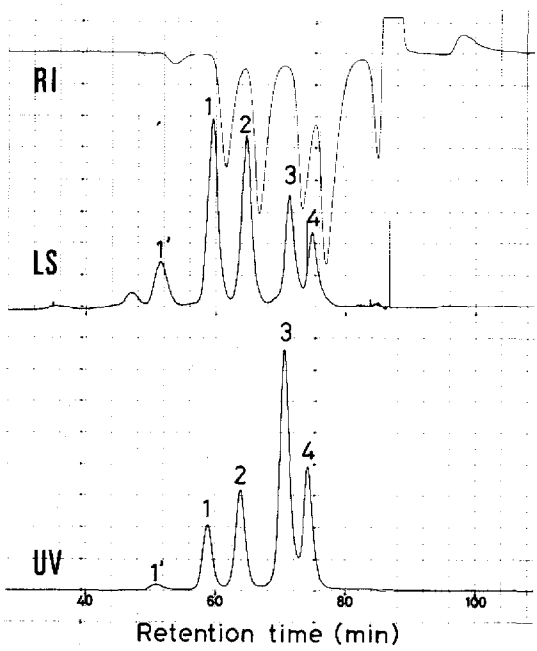

Fig. 3. Elution patterns of the mixture of four standard proteins obtained in an experiment carried out under the same conditions as those in Fig. 2 except for the flow-rate. The sample contained bovine serum albumin (330 μg), ovalbumin (400 μg), carbonic anhydrase (500 μg) and ribonuclease A (610 μg) in 100 μl of the eluant. Flow-rate: 0.31 ml/min. Peaks: 1′ = dimer of bovine serum albumin; 1 = bovine serum albumin; 2 = ovalbumin; 3 = carbonic anhydrase; 4 = ribonuclease A. Gain settings are identical to those in Fig. 2.

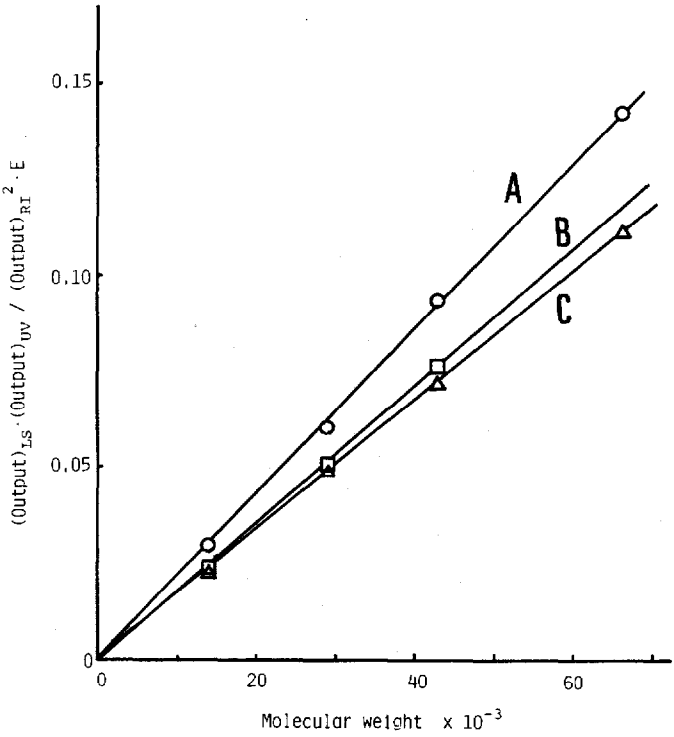

Fig. 4. Calibration lines for molecular weight determination of proteins. For details, see text.

calibration line. The molecular weight of ovomucoid was determined to be 25,000 ± 500 ($n=7$) by using line C.

The peaks of ovomucoid in Fig. 2b shared a common feature of each having an obscure shoulder in their fronts. Since the light scattering photometer emphasizes the presence of a species with higher molecular weight, the above feature precludes this possibility. In fact, no heterogeneity in molecular weight was detected in the region around the top of the peaks. One possible explanation might be that the ovomucoid, which has an acidic protein nature according to its heterogeneity in charge[14], is incompletely resolved by the TSK-GEL column which is expected to show an ionic-exclusion effect to such a protein[15].

DISCUSSION

The molecular weight of the protein moiety of human α_1-acid glycoprotein has been determined to be 21,270 by a sequence study[16]. The carbohydrate content of the glycoprotein was calculated to be 44.1% (w/w)[17], based on the result of a study of the primary structure of the carbohydrate moieties[18]. Thus the molecular weight of the glycoprotein can be assumed to be 38,000. The value obtained in the present study, 38,300, is in excellent agreement with this value.

Various techniques have been applied to the determination of the molecular weight of human α_1-acid glycoprotein. Gel chromatography gave much higher esti-

mate, 70,000, in the absence of denaturant[19] and a lower estimate, 31,500, in the presence of 6 *M* guanidine hydrochloride[20]. In the present experiments, the glycoprotein showed an elution behaviour comparable to that of bovine serum albumin in phosphate buffer in the absence of denaturant. This is in agreement with the observation of Kawasaki *et al.*[19]. Gel electrophoresis in the presence of sodium dodecyl sulphate gave values ranging from 53,000 to 40,000 with increasing concentration of polyacrylamide gel[21]. Conventional physicochemical techniques using equipment such as an osmometer, an ultracentrifuge or a light scattering photometer have frequently been used for the determination of the molecular weight of the glycoprotein. The values obtained (compiled in ref. 22) were fairly consistent, ranging between 39,000 and 41,000 with a general tendency to overestimate.

The molecular weight of chicken ovomucoid was determined to be 25,000. The molecular weight of the protein moiety of the glycoprotein has been previously determined to be 20,080 by a sequence study[23]. The carbohydrate content has been estimated to be between 20 and 25% (w/w)[14]. Thus the total molecular weight of the glycoprotein is estimated to be between 25,100 and 26,800 from its composition. This glycoprotein has been demonstrated to behave anomalously in both SDS–polyacrylamide gel electrophoresis and gel chromatography[21,24]. The glycoprotein also behaved anomalously in the present experiments being eluted at a retention time comparable to that of ovalbumin.

The results described above clearly indicate that the technique described in this paper can be used to determine the molecular weight of proteins to which no technique depending on the molecular sieving effect can be applied. The reported value for the molecular weight of α_1-acid glycoprotein next to ours in accuracy is that (39,000) obtained by Kawahara *et al.*[25] under extreme care based on their long experience. In the case of ovomucoid, the sedimentation equilibrium technique gave a molecular weight of *ca.* 28,000 which is a significantly higher estimate.

The present technique also requires experience in the operation and in becoming familiar with the performance of the measuring system shown in Fig. 1. Once one has become acquainted with the system, a series of measurements can be finished within several hours. For a protein whose extinction coefficient is unknown, the determination of the coefficient will require more time than that required for the main experiments. To make the procedure both time- and sample-saving, the quantitative amino acid analysis technique[26] might be the best choice.

The present technique can be applied to any protein which can be applied to presently available high-performance gel chromatography columns. Exceptions are proteins which absorb in the visible region. As has been discussed in a previous paper[27], the situation will be improved by changing the light source of the refractometer. Proteins which absorb at or near the wavelength of the He–Ne laser will still be outside the range of the present technique.

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