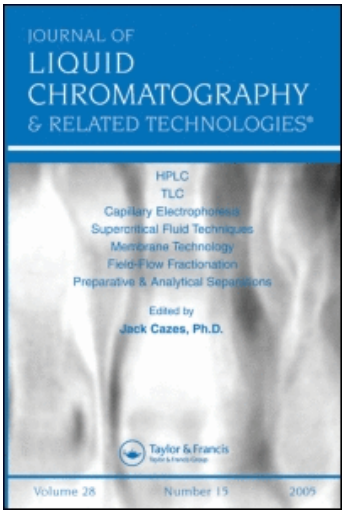

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Estimation of Hydrodynamic Volume of Proteins Using High-Performance Size-Exclusion Chromatography and Intrinsic Viscosity Measurement: An Attempt At Universal Calibration

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ESTIMATION OF HYDRODYNAMIC VOLUME OF PROTEINS USING HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY AND INTRINSIC VISCOSITY MEASUREMENT: AN ATTEMPT AT UNIVERSAL CALIBRATION

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ABSTRACT

Hydrodynamic volume was estimated by using high-performance size-exclusion chromatography (SEC) with a silica-based column and intrinsic viscosity ([η]) measurement, for native proteins and those reduced in sodium dodecyl sulphate (SDS) or guanidine hydrochloride (Gu-HCl). In solutions of 6M Gu-HCl, proteins in their reduced state behaved hydrodynamically as randomly coiled linear homopolymers and exhibited good linearity between an

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equivalent hydrodynamic radius (R_e) obtained from [η] data against $erf^{-1}(1-k_d)$ obtained from SEC data. However, [η] values of SDS-reduced protein complex in the SEC solvent were considerably lower than those reported by Reynolds and Tanford, and their SEC behavior demonstrated several undesirable phenomena. The proteins reduced in 6M Gu-HCl solution are recommended as polymer standards for the universal calibration of proteins and polypeptides.

INTRODUCTION

In recent years, high-performance size-exclusion chromatography (SEC), also known as gel permeation chromatography (GPC), has become popular for estimating the molecular weight of proteins and polypeptides in the presence of denaturants such as 6M guanidine hydrochloride (Gu-HCl) and sodium dodecyl sulphonate (SDS). Although SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a highly efficient and convenient method to determine the molecular weight of biological macromolecules, this method is not well suited to the precise determination of mobilities or recovery of samples. SEC is free from such shortcomings, and its sensitivity and resolution have improved greatly following the development of columns of silica-based aqueous gel for high-performance liquid chromatography (HPLC) (1). The evaluation of columns such as TSK-SW columns has been reported by many investigators (2-7). Several attempts have been made to evaluate the hydrodynamic volume of proteins in examinations of correction of a distribution coefficient, K_d, with an equivalent hydrodynamic radius, R_e (8-12).

Before the appearance of HPLC, Reynolds, Tanford and co-workers (13-17) investigated in detail the relationships between the molecular weight and hydrodynamic volume of proteins, using experiments employing gel filtration, intrinsic viscosties ([η]), sedimentation coefficient and so on. It is important to verify their work in light of new experimental information obtained from HPLC.

A method of calibration for hydrodynamic volume, termed the universal calibration procedure, proposed by Grubisic and co-workers (18) has been highly rated for various organic solvents examined by high-performance SEC (19-22). There are several articles (23-25, 40-43) on the validity of the universal calibration procedure for water-soluble polymers, however, there are few articles (37-39) that for proteins because of the lack of polymer standards having a narrow molecular weight distribution with a silica-basd column.

This paper describes the estimation of hydrodynamic volume of native proteins and those reduced in SDS or GU-HCl solutions using high-performance SEC with a silica-based TSK-SW column and intrinsic viscosity ([η]) measurement. The universal calibration procedure for proteins and polypeptides is also discussed.

MATERIALS

The protein samples, thyroglobulin, γ-globulin, bovine serum albumin (BSA), α-chymotrypsinogen, cytochrome c,

insulin and bacitracin were obtained from Sigma; ferritin, catalase and aldose from Boehinger Mannheim; lysozyme chloride from Tokyo Kasei (Japan) and ovalbumin from Seikagaku Kogyo (Japan). The proteins are denoted by their respective symbols of abbreviation in the figures. SDS, Gu-HCl and dithiothreitol (DTT) were obtained from Nakarai Chemicals (Japan) in especially pure grades. All other chemicals and reagents were of analytical grade.

METHODS

SEC Measurement

SEC was carried out at 25°C with a Model HLC-803 high-performance liquid chromatograph (Tosoh, Japan) equipped with a UV detector set at 280 nm and multi-wavelength UVILOG 7 TYPE UV detector (Ohyoubunkou, Japan) set at 260 nm. TSK G3000SW and G4000SW columns (both 600 × 7.5 mm I.D.), packed with silica-based aqueous gels of different pore sizes, were obtained from Tosoh. The sample solvents and eluents for the sample proteins are shown in Table 1. The buffer conditions and concentrations of the denaturants for the sample solvent and HPLC eluent were determined by preliminary evaluations of SEC behavior. As no reoxidation of reduced proteins occurred in this SEC medium, the reduced protein samples were applied to the column directly, without being alkylated. Every sample solvent and HPLC eluent was filtrated by a 0.2 μm membrane filter (Nucleopore) and

TABLE 1

Solvents for Proteins Used in This Study

Protein	Native state	SDS-denatured reduced state	Gu-HCl-denatured reduced state
sample solvent*	1/15M sodium phosphate 0.2M NaCl (pH 6.8)	0.1M sodium phosphate 2.0% SDS 0.02M DTT (pH 7.0)	10 mM Tris-HCl 1 mM EDTA 6M Gu-HCl 0.02M DTT (pH 7.5)
HPLC eluent	1/15M sodium phosphate 0.2M NaCl (pH 6.8)	0.1M sodium phosphate 0.1% SDS (pH 7.0)	10 mM Tris-HCl 1 mM EDTA 6M Gu-HCl (pH 7.5)

* Sample solvents were used to disolve the protein samples for measuring SEC and viscosity.

degassed by ultrasonic treatment for 30 min and replaced by N_2 gas purge for 10 min before use. The concentrations of protein samples and reagents were 0.1-0.2% (wt/v), and measurement was done at a flow-rate of 1.0 ml/min.

Viscosity Measurement

Viscosity was measured with an Ostwald-type viscometer with a flow time for water of 270 sec. Intrinsic viscosities were determined in the viscometer immersed in a thermostated water bath at 25± 0.015°C. The protein sample

was dissolved in the sample solvent, the same as that used in SEC measurement shown in Table 1, at several concentrations in a range between 0.5 mg/ml and 5.0 mg/ml. Sample solution (3 ml) was incubated in the viscometer for at least 15 min before the reduced viscosity, η_{sp}/C, was measured. Intrinsic viosity, $[\eta]$, was obtained by extraporation of η_{sp}/C, to $C = 0$. Protein concentrations were determined by a dry weight method in which samples were dried at 105°C for 24 hours using a vacuum pump. Judging from the SEC patterns, all the proteins measuring viscosity were free of aggregates in the sample solvents shown in Table 1.

Treatment of Data

The distribution coefficient, K_d, was calculated from the SEC data by the equation, $K_d = (V_e - V_0)/ (V_t - V_0)$, where V_e is the elution volume of the protein, V_0 is the void volume and V_t is the total available volume. Each elution volume of the sample was measured as the distance between the point of sample application and location of each peak top on the recorder chart. The value of V_0 was determined from the elution volume of blue dextran 2000 (Pharmacia). Phenylalanine (Nippon Rikagaku Yakuhin, Japan) was selected from among β-alanine (Tokyo Kasei, Japan), benzoic acid (Wako, Japan), L-tryptophan (Nippon Riakgaku Yakuhin) and phenylalanine as a substance to determine the value of V_t becausc it showed the finest SEC resolution and the same elution volume when three different HPLC eluents (Table 1) were used.

In order to relate K_d to equivalent hydrodynamic radius, R_e, we used the following equations 1 (26, 27) and 2 (28)

$$R_e = A + B\ erf^{-1}(1 - K_d) \quad (1)$$

$$K_d^{1/3} = a - b\ R_e \quad (2)$$

where $erf^{-1}(1 - K_d)$ is the inverse error function of $(1 - K_d)$, and A, B, a and b are the empirical constants for a given chromatographic system. For the proteins reduced in 6M Gu-HCl and SDS solutions, we used the following equations 3 (14, 27) and 4 (16, 27)

$$R_G = (\text{constant})\ M^{0.555} \quad (\text{in 6M Gu-HCl}) \quad (3)$$

$$R_e = (\text{constant})\ M^{0.73} \quad (\text{SDS complex}) \quad (4)$$

where R_G represents the radius of gyration. As R_e is directly proportional to R_G, the relationships between $M^{0.555}$ and $erf^{-1}(1 - K_d)$ can be represented from equations 1 and 3 as follows

$$M^{0.555} = A' + B' erf^{-1}(1 - K_d) \quad (5)$$

The relationships between $M^{0.73}$ and $erf^{-1}(1 - K_d)$ can be represented from equations 1 and 4 as follows

$$M^{0.73} = A'' + B'' erf^{-1}(1 - K_d) \quad (6)$$

where A', B', A" and B" are empirical constants for a given chromatographic system.

According to the Mark-Houwink equation, the relationships between intrinsic viscosity, $[\eta]$, and the molecular weight, M, can be represented as

$$[\eta] = K M^a \quad (7)$$

where K and a are constants. In order to relate the intrinsic viscosity, $[\eta]$, to R_e, we used the following equation

$$[\eta] = 2.5 \frac{N}{M} \frac{4\pi}{3} R_e^3 \qquad (8)$$

where N and M are Avogadro's number and the molecular weight of protein, respectively.

RESULTS

SEC Measurements

Figure 1 shows the log M versus V_e plots for proteins, in both native state and reduced in SDS or Gu-HCl solution using (A) 3000SW and (B) 4000SW. Three calibration graphs of proteins present unique characteristics that were different from the others. The linearity of calibration graphs for proteins in both denatured states was far better than that of the native state. A remarkable difference appeared between calibration graphs for proteins in SDS and those in Gu-HCl, at the molecular weight of polypeptides below 10,000; viz., the plots of the SDS system deviated from linearity and became steeper, whereas the plots of the Gu-HCl system maintained their linearity at low molecular weights.

Figure 2 shows the relationships beween $M^{0.555}$ and $erf^{-1}(1 - K_d)$ of reduced and unreduced proteins in 6M Gu-HCl solution with G3000SW. According to Fish et al. (27), a linear relationship between $M^{0.555}$ and $erf^{-1}(1 - K_d)$ holds, as shown

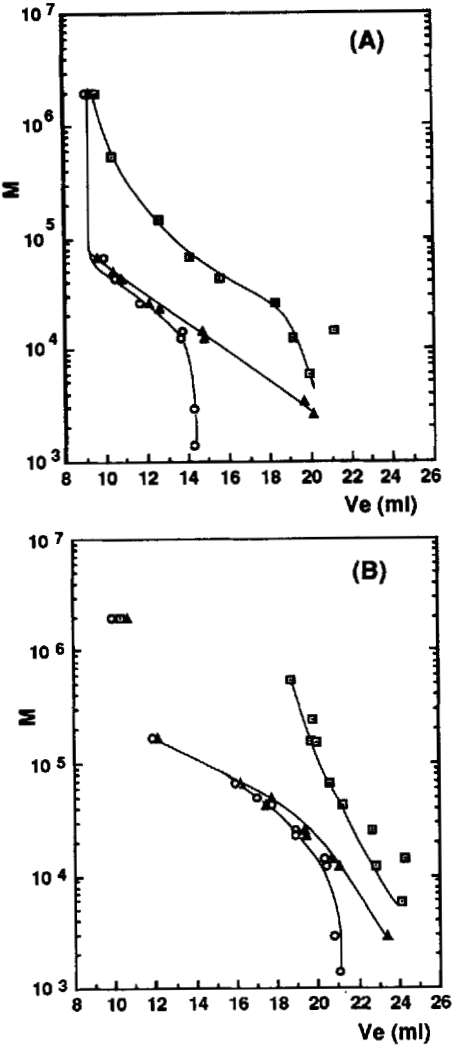

FIGURE 1. The log M vs. elution volume (V_e) calibration graphs for (□) native protein, (○) SDS-reduced protein complex, (▲) protein reduced in Gu-HCl solution with (A) TSK G 3000SW and (B) TSK G 4000SW; both columns are 600 × 7.5 mm I.D. HPLC instrument, Model HLC-803; flowrate, 1.0 ml/min; detector, UV (280 nm); column temp., 25°C. Sample solvent and HPLC eluent at each stage are listed in Table 1.

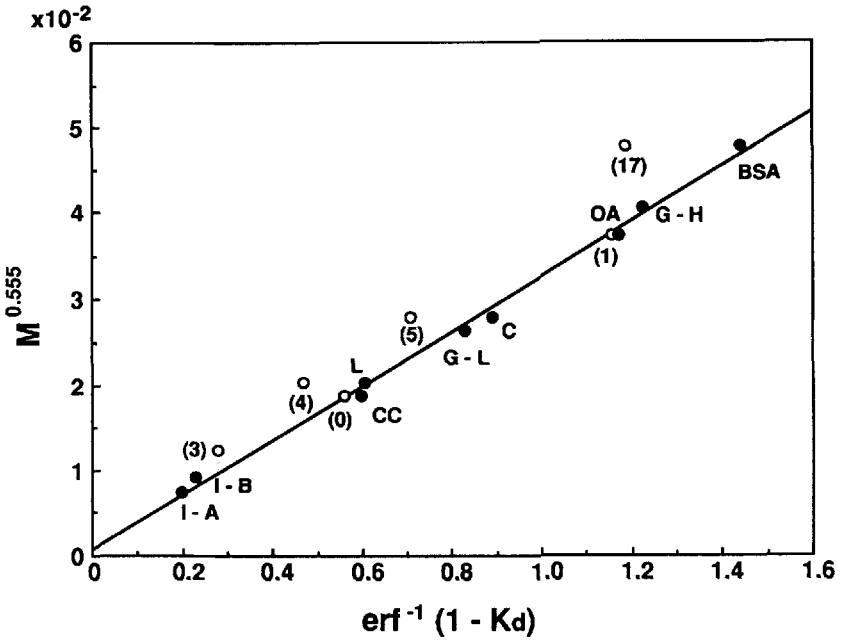

FIGURE 2. The relationships between $M^{0.555}$ and $erf^{-1}(1 - K_d)$ for (●) proteins reduced in 6M Gu-HCl solution and (○) unreduced proteins in 6M Gu-HCl solution with TSK G 3000SW. The SEC measurements for unreduced proteins were carried out in the absence of 0.02M DTT at the sample solvent shown in Table 1. The numbers of S-S bonds in the proteins are shown in paraentheses. The plots of the proteins reduced in 6M Gu-HCl solution were determined by the method of least squares. The correlation coefficient was 0.995. The analytical conditions of HPLC were the same as those given in Figure 1. Abbreviations used are BSA : bovine serum albumin; G-H : γ-globulin heavy chain; OA : ovalbumin; C: α-chymotrypsinogen; G-L : γ-globulin light chain; L : lysozyme chroride; CC : cytochrome c; I-B : insulin B chain; I-A : insulin A chain.

in equation 5, for gel filtration of agarose gel in 6M Gu-HCl. Consistent results were obtained with HPLC, that is, good linearity was exhibited among the plots of reduced proteins with disulfide (S-S) bond, whereas most plots of unreduced proteins with S-S bond did not fall on the line of reduced proteins. A similar result was reported by Ui (10), namely, that satisfactory linearity was obtained between $M^{0.555}$ and $K_d^{1/3}$ for the proteins reduced in 6M Gu-HCl solution with 3000SW and 4000SW from the equations 2 and 3, but linearity was not better when $erf^{-1}K_d$ was used instead of $K_d^{1/3}$. In this study, we confirmed that a linear relationship was obtained between both $M^{0.555}$ against $K_d^{1/3}$, and $M^{0.555}$ against $erf^{-1}(1 - K_d)$ with both 3000SW and 4000SW.

Figure 3 shows the relationship between $M^{0.73}$ and $erf^{-1}(1 - K_d)$ of the SDS-reduced protein complexes with the columns of (A) 3000SW and (B) 4000SW. The linearities were poor with both columns in comparison with those in which Gu-HCl solution was used, as shown in Fig. 2.

Viscosity Measurements

The intrinsic viscosities ($[\eta]$) of bovine serum albumin (BSA) were measured in the different solvents shown in Table 1, to determine whether $[\eta]$ data obtained by Reynolds & Tanford (13-17, 27) are applicable to our study. Our data were in reasonable agreement with those obtained by Reynolds & Tanford (15), except for those obtained in SDS

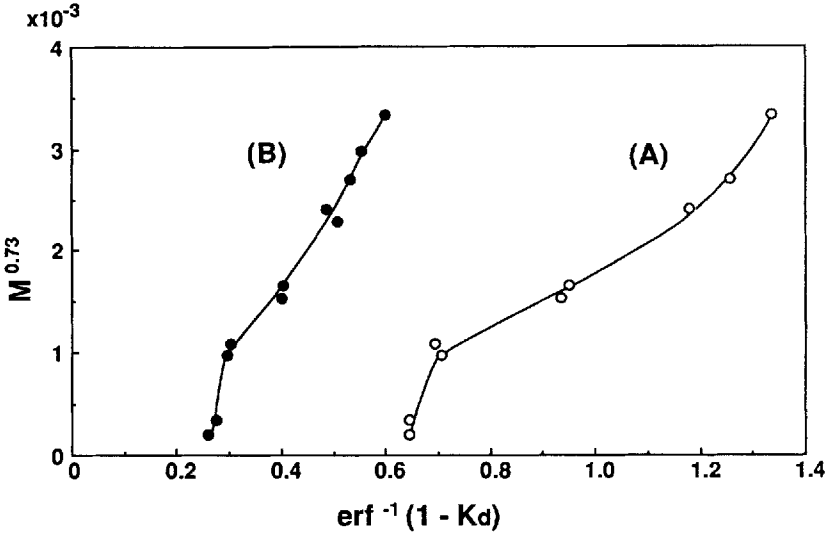

FIGURE 3. The relationships between $M^{0.73}$ and $erf^{-1}(1 - K_d)$ for the SDS-reduced protein complex with (A) TSK G 3000SW and (B) TSK G 4000SW. The sample solvent and HPLC eluent are shown in Table 1. The analytical conditions of HPLC were the same as those given in Figure 1.

solution. Our [η] values of BSA in native, unreduced and reduced states in Gu-HCl solution were 5.2, 24.8 and 50.1 ml/g, while those determined by Reynolds & Tanford were 3.7, 22.9 and 52.2 ml/g, respectively. Our figure of 32.2 ml/g, for the [η] value of SDS-reduced BSA complex, was considerably lower than that of Reynolds & Tanford, 54.2 ml/g.

The viscosity of BSA, ovalbumin, α-chymotrypsinogen, lysozyme chloride, cytochrome c and insulin in their reduced states in SDS solution were measured. According to the

equation 7, the result of $[\eta]$ values can be represented as

$$[\eta] = 1.74 \cdot 10^{-4} M^{1.09} \quad (9)$$

where M is the molecular weight of the proteins, excluding insulin, whose plot does not fall on the line of equation 9.

Table 2 summarizes the $[\eta]$ values of six proteins in native state and reduced in SDS or Gu-HCl solution as determined by our experiments and derived from the data of Reynolds & Tanford (14-17, 27).

Hydrodynamic volume of protein

Figure 4 shows the relationships between equivalent hydrodynamic redius, R_e, and $erf^{-1}(1 - K_d)$ for several proteins with (A) 3000SW and (B) 4000SW in different solvents. According to equation 8, the R_e values were calculated from the $[\eta]$ values shown in Table 2. The values of $erf^{-1}(1 - K_d)$ were obtained from the SEC data. Good linearity was obtained from the plots of the proteins reduced in 6M Gu-HCl solution, giving the following equations:

$$R_e = 1.96 + 53.1\ erf^{-1}(1 - K_d) \quad (10)$$

$$R_e = 1.62 + 130.0\ erf^{-1}(1 - K_d) \quad (11)$$

with 3000SW and 4000SW, respectively.

Figure 5 shows the universal calibration graphs of several proteins, the plots of log $[\eta]M$ against V_e, with (A) 3000SW and (B) 4000SW in different solvents. In this case, the linearity of the plots of proteins reduced in 6M Gu-HCl solution was satisfactory with 3000SW, but poor with

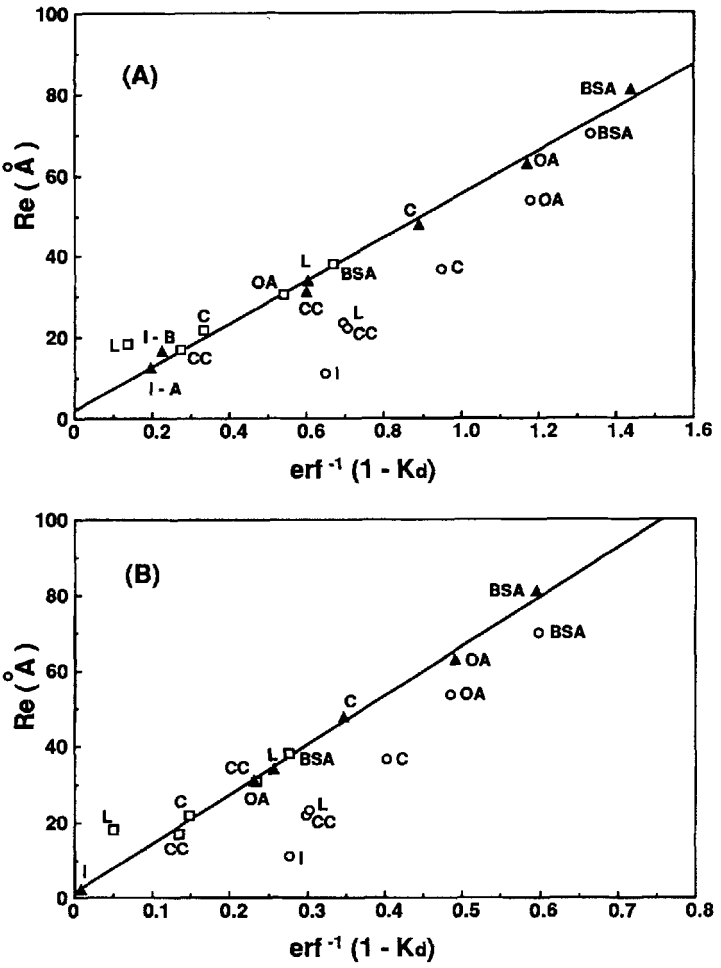

FIGURE 4. The relationships between R_e and $erf^{-1}(1 - K_d)$ for (□) native protein, (○) SDS-reduced protein complex, and (▲) protein reduced in Gu-HCl solution with (A) TSK G 3000SW and (B) TSK G 4000SW. The plots of the proteins reduced in Gu-HCl solution were obtained by the method of least squares. The correlation coefficients with (A) TSK 3000SW and (B) TSK 4000SW were 0.993 and 0.994, respectively. The analytical conditions of HPLC were the same as those given in Figure 1. Abbreviations used are I : insulin. Other abbreviations are shown in Figure 2.

TABLE 2

Intrinsic Viscosity (ml/g) of Proteins in Different Solvents

Protein	Native*	SDS-denatured** (S-S reduced)	Gu-HCl-denatured* (S-S reduced)
BSA	5.2	32.2	50.1
Ovalbumin	4.2	22.6	36.3
Chymotrypsinogen	2.5	12.2	26.8
Lysozyme chloride	2.7	5.6	17.1
Cytochrome c	2.5	5.4	15.1
Insulin	-	2.9	6.1

* Intrinsic viscosity values except for BSA are data reported by Reynolds and Tanford (13-17).

** Intrinsic viscosity values of BSA (M = 67000), ovalbumin (M = 43000), α-chymotrypsinogen (M = 25700), lysozyme chloride (M =14400) and cytochrome c (M = 12400) at reduced states in SDS solutions, as reported by Reynolds and Tanford (15), are 54.2, 33.5, 15.8, 9.0 and 9.7 ml/g, respectively.

4000SW. As the elution volume is more a function of effective volume than of molecular weight in SEC, the universal calibration shown in Fig. 5 is a much more suitable profile for estimating the hydrodynamic volume of proteins than the conventional calibration shown in Fig. 1.

DISCUSSION

The universal calibration procedure is based on the assumption that molecules separate on the basis of their

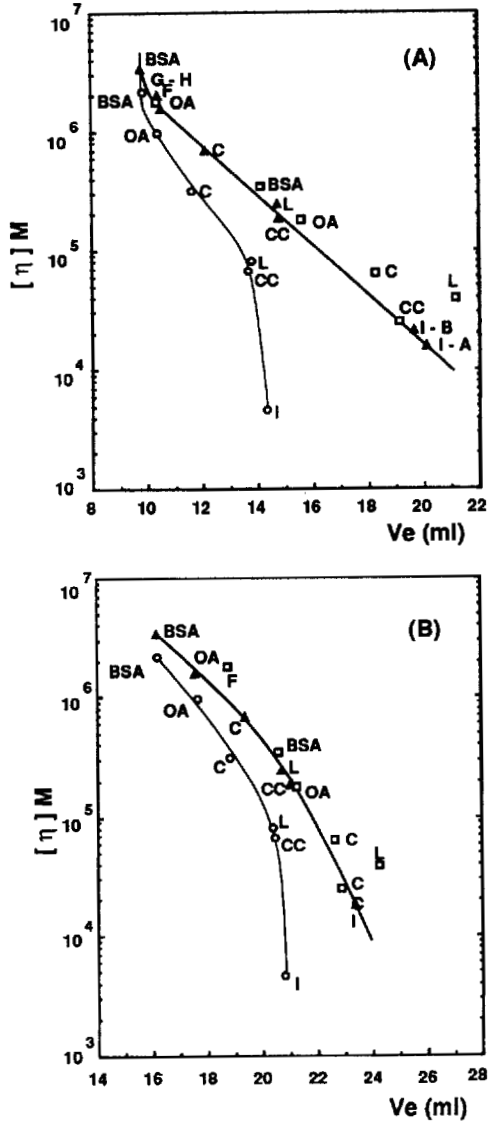

FIGURE 5. Universal calibration plots for (□) native protein, (○) SDS-reduced protein complex and (▲) protein reduced in Gu-HCl solution with (A) TSK G 3000SW and (B) TSK G 4000SW. The analytical conditions of HPLC were the same as those given in Figure 1. Abbreviations used are F : ferritin; I : insulin. Other abbreviations are shown in Figure 2.

hydrodynamic volumes, $[\eta]M$ (18). The hydrodynamic volume of any solute at a given elution volume can then be determined if a log $[\eta]M$ versus elution volume (V_e) plot (i.e., universal calibration plot) is established (19-22). In this case, adsorption, elecrostatic interactions and SEC concentration effects must be absent, and also $[\eta]$ values used to calculate hydrodynamic volumes must be accurate. The universal calibration procedure for proteins and polypeptides presents some serious problems that need to be solved.

The most serious problem is the lack of reliable water-soluble polymer standards for the silica-based column with a narrow molecular weight distribution in the range from 10^3 to more than 10^6, such as polystyrene standards (29) in the case of organic polymers. Although the available water-soluble polymer standards, such as PET, pullulan, dextran and poly (ethylene oxide) are capable to use as standards with a polymer-based column (23, 41), these polymer standards are generally unsuitable for use as standards for proteins and polypeptides with silica-based column (38, 39).

The second problem is specific interactions between the protein and gel. The elution volume of a solute is governed not only by the steric elution mechanism of separation but also by a second mechanism resulting from the preferential affinity among the main components in the SEC system. From our experience, remarkable adsorption can be observed with basic proteins at native state using polymer-based column.

The best way to reduce these drawbacks is to use denatured proteins and polypeptides in their reduced states as a standard with silica-based column. In this study, the hydrodynamic volumes of native and denatured proteins were investigated with SEC and [η] values using the same analytical solvents, as shown in Table 1. The buffer conditions (3, 8, 12) and the concentrations of the denaturants (7, 10) for the sample solvent and the HPLC eluent were determined by preliminary evaluations of SEC behavior (data not shown).

On the basis of the SEC results shown in Fig. 2 and the relationships between [η] data and SEC data shown in Figs. 4 and 5, the proteins reduced in 6M Gu-HCl solution are considered to behave hydrodynamically as randomly coiled homopolymers.

In contrast, the behavior of SDS-reduced protein complexes is considerably complicated and differs from that of proteins reduced in Gu-HCl solution. Several models have been proposed for these complexes, such as the 'rod-like model' of Reynolds and Tanford (15), 'flexible nature of the complex' proposed subsequently and 'neckless model' proposed by Takagi et al. (30, 31).

In this paper, we would like to point out two serious problems of SDS-reduced protein complexes. The first is the strange phenomenon observed in SEC measurements of the proteins with molecular weights below 10,000, as shown in Fig. 1. Imamura et al. (7) reported that this effect depended

upon the concentration of SDS, and 0.1% SDS and 0.05M sodium phophate buffer at pH 7.0 was suitable for maintaining linearity of the plot, even in the low molecular weight range.

In the case of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (32, 33), Swank and Munkres (34) recommended the addition of 8M urea to the SDS buffer, because the presence of both 8M urea and SDS improves the resolution of the system for proteins with molecular weights less than 10,000 and facilitates the dissociation of protein aggregates. Unfortunately, this suggestion is not applicable to the SEC system. Although 8M urea has been extensively used as a strong protein denaturant like 6M Gu-HCl, urea is fundamentally unsuitable for SEC measurements using silica-based columns. Moreover, Takagi et al. (31) reported that all conventional gels for molecular sieving deteriorated in aqueous solutions containing both 3.5 mM SDS and 8M urea (solution for SDS-PAGE), making gel filtration chromatography impracticable.

The second problem is that [η] values of our data for SDS-reduced protein complexes were considerably lower than those previously reported (15, 35). The main reason may be that the previous measurements were made in media with salt conditions significantly lower than that of the buffer solution used in the present study. For example, the ionic strength of the buffer used to measure the [η] values for SDS-

reduced protein complexes by Reynolds & Tanford was 0.026, much lower than that of our solvent shown in Table 1. Takagi et al. (9) reported that [η] values for SDS-reduced protein complexes, as well as the elution behavior of SEC, were highly sensitive to salt concentration, and the SEC resolution of protein polypeptides was satisfactory only at the buffer concentration between 0.05M and 0.15M. Our experimental results support their work, and it is suggested that the [η] values for SDS-reduced protein complexes obtained by Reynolds & Tanford (15) are unsuitable for adoption as the buffer condition for SEC measurements in general (36).

Recently, a new method for the universal calibration in SEC with on-line differential viscometry has been developed (40-44). We expect that these problems for SDS-reduced protein complexes and the propriety of proteins reduced in 6M Gu-HCl solution as polymer standards for the universal calibration procedure for proteins will be investigated in detail using this new method.

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