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Note

Molecular-weight estimation of proteins using Sepharose CL-6B in guanidine hydrochloride

AFTAB A. ANSARI and ROSE G. MAGE

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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It has been almost two decades since Lathe and Ruthven[1] and Porath and Flodin[2] first reported that gels can fractionate molecules on the basis of molecular size. Since then, several kinds of gel filtration media (dextran, polyacrylamide, agarose) have been introduced. Andrews[3–5] proposed that the elution characteristics of proteins are uniquely determined by molecular weight. Gel filtration techniques therefore became widely used tools not only for fractionation of macromolecules on the basis of size but also for molecular-weight determinations. However, the contention that the relative elution rates of proteins are uniquely determined by molecular weight, holds true (even approximately) only when all proteins being compared belong to the same conformational type. To avoid this discrepancy Fish *et al.*[6,7] introduced gel chromatography in presence of strong denaturants such as 6 *M* guanidine hydrochloride (Gdn·HCl) and sodium dodecyl sulfate. Gel filtration in denaturing solvents is a useful technique for separation and molecular weight estimation of polypeptides. However, most of the soft gels in denaturants like 6 *M* Gdn·HCl present the problems of high flow resistance causing low flow-rates and long operation periods. Further, they are not stable in 6 *M* Gdn·HCl for long periods of time. The gel beads sometimes start breaking in this medium within a week. During the course of our search for a more stable molecular-sieving medium[8] we came across a cross-linked Sepharose gel called Sepharose CL (Pharmacia, Uppsala, Sweden), which is prepared from Sepharose by reaction with 2,3-dibromopropanol under strongly alkaline conditions. This product is claimed by the manufacturers to be more stable in Gdn·HCl (and other strong solvents) than any other gel filtration medium. However, no report exists on the separation range of these gels for proteins in 6 *M* Gdn·HCl.

We wish to report some results showing that Sepharose CL-6B is highly stable in 6 *M* Gdn·HCl and can be successfully used over a period of at least ten months for separation and molecular-weight determination of polypeptides. Moreover, proteins which were very poorly resolved when Bio-Glas was used to estimate their molecular weights in 6 *M* Gdn·HCl[8] were well resolved in the present study.

EXPERIMENTAL

The proteins used and their sources are shown in Table I. The heavy chain of rabbit IgG was prepared as described[15].

TABLE I

PROTEINS USED IN GEL FILTRATION EXPERIMENTS

Protein	*Source*	*Molecular weight*	*Reference*
B Chain of insulin	Sigma, I-2379	2900	9
Insulin	Sigma, I-5500	5750	10
Cytochrome *c*	Sigma, C-2506	12,400	11
Hemoglobin	Sigma, H-2500	15,500	9
α-Chymotrypsinogen	Sigma, C-4879	25,700	9
Rabbit IgG, H chain	Prepared in this lab.	49,000	12
Bovine serum albumin	Sigma, A-4378	69,000	9
Transferrin	Sigma, T-2252	76,600	13
Rabbit IgG	Prepared in this lab.	148,000	14

Sepharose CL-6B and Blue Dextran 2000 were from Pharmacia. 2,4-Dinitrophenyl-alanine (DNP-Ala) was a Sigma product. Gdn·HCl was an ultrapure grade from Schwarz/Mann (Orangeburg, N.Y., U.S.A.) and had an absorbance of less than 0.03 at 280 nm as a 6 *M* solution.

Column packing and operation

The gel was washed on a funnel with four volumes of water and suspended in the column solvent (6 *M* Gdn·HCl plus 0.1 *M* sodium phosphate buffer, pH 7.0). The solvent was changed several times over a 6-h period. The gel was then washed on the funnel with four volumes of the column solvent. After deaeration under vacuum, the slurry was poured into a Pharmacia column (90 × 1.5 cm), and was permitted to pack under gravity. Final equilibration was achieved by running 2–3 volumes of the column solvent through the column. The column was run at a flow-rate of 12 ml/h (6.8 ml per cm^2 column cross-section per h). A pressure drop of approximately 130 cm was required to maintain this flow-rate.

Proteins were reduced with 0.1 *M* dithiothreitol and alkylated with 0.22 *M* iodoacetamide as described previously[15]. In cases where reduction of disulfide bonds was not intended, the protein was simply dissolved in the column solvent containing 0.1 *M* iodoacetamide.

Proteins (0.5–1.0 mg of each) were run through the column individually as well as in a mixture. The samples were brought to a volume of 200 μl and applied on to the top of the gel bed through a small tube. Fractions of 2 ml were collected and monitored by measuring absorbance at 280 nm. Blue Dextran and DNP-Ala were routinely run through the column to check the void volume (V_0) and the "inner" volume (V_i).

All chromatographic experiments were carried out at room temperature.

RESULTS AND DISCUSSION

All the results presented in this paper were obtained from a single Sepharose CL-6B column, run over a period of 10 months. Each protein was passed through the column three to six times over this period, and no significant change in the elution pattern or elution position was noticed for any of the proteins. Two typical elution profiles, one for reduced and alkylated proteins, and the other for unreduced proteins,

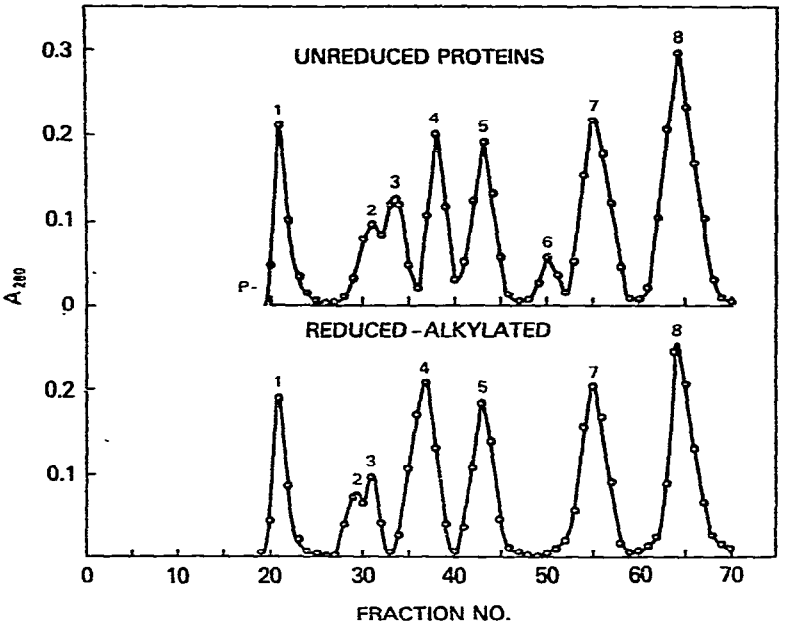

Fig. 1. Elution profiles for reduced-alkylated proteins and unreduced proteins from a Sepharose CL-6B column (90 × 1.5 cm) in 6 *M* Gdn·HCl, 0.1 *M* sodium phosphate, pH 7.0. 0.5–1 mg of each protein was mixed to give a total volume of *ca.* 200 μl which was applied on to the column and eluted at a flow-rate of 12 ml/h; 2-ml fractions were collected. Peaks: 1 = Blue Dextran; 2 = bovine serum albumin; 3 = rabbit IgG, H chain; 4 = α-chymotrypsinogen; 5 = cytochrome *c*; 6 = insulin; 7 = B chain of insulin; 8 = DNP-Ala.

are shown in Fig. 1. Most evident are the sharp, symmetrical peaks and the resolving power of this gel.

Another point worth mentioning is the stability of the reduced-alkylated protein solutions. The reduced-alkylated protein samples described in our previous paper[8] were stored in 6 *M* Gdn·HCl refrigerated for over a year. Their elution behavior from the Sepharose CL column was found to be the same as that for freshly prepared solutions of corresponding proteins. A similar check for unreduced protein solutions was not made.

Fig. 2 shows the logarithmic plots between molecular weight and distribution coefficient, K_d, for the reduced-alkylated and unreduced proteins. Both the plots are linear up to 80,000 daltons. Thus the use of Sepharose CL-6B in 6 *M* Gdn·HCl permits useful molecular-weight estimates between the extreme limits of 3000 and 80,000.

In Fig. 2, the plot for reduced-alkylated proteins is deviated from the plot for the unreduced porteins, and the pattern of this deviation is in the direction that one would predict from the expected size difference between denatured proteins with disulfide bonds intact and those with disulfide bonds reduced. Even if the proteins are fully denatured in 6 *M* Gdn·HCl, the intact disulfide bonds exert a certain amount of physical constraint on the polypeptide chain. The effect of this physical constraint is to reduce the hydrodynamic volume of the polypeptide chain in comparison to the same polypeptide chain with its disulfide bonds broken. This effect should obviously

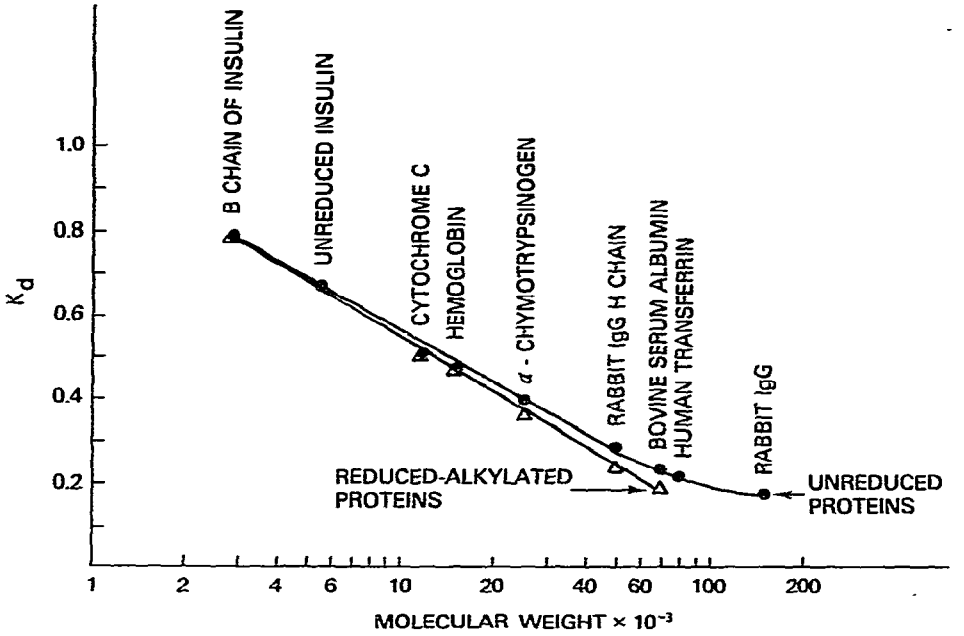

Fig. 2. Semilogarithmic plots of the molecular weights *versus* K_d values obtained by running the proteins through a Sepharose CL-6B column (90 × 1.5 cm) in 6 *M* Gdn·HCl, 0.1 *M* sodium phosphate, pH 7.0. The distribution coefficient, K_d, was calculated according to equation: $K_d = (V_e - V_0)/V_t$, where V_e is the elution volume of solvent at the peak concentration of eluting solute, V_0 is the void volume of the column determined by running Blue Dextran through the column, and V_t is the volume of solvent contained within the gel bed determined by subtracting the value of V_0 from the elution volume of DNP-Ala.

get increasingly noticeable with increasing size of the polypeptide chain and increasing number of the disulfide bonds. This effect should also be dependent upon the size of the "loop" formed by the disulfide bond. Thus the B chain of insulin does not have any disulfide bond (ref. 16, p. 378) and cytochrome *c* has only one disulfide bond forming a very small loop between residues 14 and 17 (ref. 16, p. 282). Therefore their K_d values do not differ significantly under the two conditions. At the other end, bovine serum albumin, for instance, has 17 disulfide bonds, many of them forming large loops (ref. 16, p. 497). This causes a reduction in the hydrodynamic volume of the unreduced chain as compared to the reduced protein resulting in a high K_d value.

A recent report by Nozaki *et al.*[17] has shown that anomalous retardation in gel chromatography is a general property of all large asymmetric particles, most probably because of end-on insertion into the gel pores. The fact that linear calibration curves are obtained up to a molecular weight of approximately 75,000 in Gdn·HCl, shows that the technique is suitable for molecular-weight estimations up to this molecular weight.

In conclusion, Sepharose CL-6B is highly stable in 6 *M* Gdn·HCl, 0.1 *M* sodium phosphate, pH 7.0, and is suitable for molecular-weight determinations in the range 3000–80,000 daltons, without presenting the problems of flow-rates and low chemical and physical stability commonly encountered with other soft gels in 6 *M* Gdn·HCl.

This gel may also prove to be more suitable than the conventional Sepharose for making immunoabsorbents, especially when Gdn·HCl is to be used as the eluting agent.

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