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CONTROLLED PORE GLASS CHROMATOGRAPHY OF PROTEIN–SODIUM DODECYL SULPHATE COMPLEXES

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SUMMARY

The inclusion of urea has been found to eliminate adsorption of protein–sodium dodecyl sulphate (SDS) complexes to controlled pore glass. Using buffer containing 6 *M* urea, 0.5 % SDS and glass with pore diameter 12.3 nm, it is possible to determine protein molecular weights in the range 3500–12,000. Results with glass of larger pore diameter (25.5 nm) are similar to those reported in the absence of urea in the molecular-weight range 12,000–140,000. Controlled pore glass chromatography also permits the study of the relative importance of conformation free of charge effects for those proteins which deviate from the normal calibration curve for SDS–polyacrylamide gels.

INTRODUCTION

Although gel filtration of proteins in denaturing solvents over controlled pore glass (CPG) is a potentially attractive method for molecular-weight determination, there have been few reports of its use in the literature. Truman *et al.*[1] used a 70-nm pore size glass in 7 *M* urea for proteins in the molecular-weight range 12,000–25,000, while Collins and Haller[2] found that a pore size of 50 nm was suited to the range 17,000–385,000 for protein–sodium dodecyl sulphate (SDS) complexes. It would appear from these studies that adsorption of proteins to the CPG support is not the problem in denaturing solvents that it is in non-denaturing solvents[3]. Contrary to these reports, we have found that adsorption can occur in solvents containing either urea or SDS.

Molecular-weight determination of protein subunit polypeptides by gel filtration in 6 *M* guanidine hydrochloride (GuHCl) over agarose is becoming increasingly popular[4]. Because of the instability of the bed volume under pressure, which necessitates the use of low flow-rates, this is a very slow technique. It would be a great advantage if CPG could be used instead of agarose but we have been unable to prevent adsorption of proteins to CPG in 6 *M* GuHCl either by raising the pH or using Carbowax 20M[3]. This contrasts with the successful use of 4 *M* GuHCl for the molecular-weight determination of amylose and dextran[5].

The aim of the present investigation was to extend the molecular-weight range

that can be studied on CPG below 12,000 in order to apply the technique to the high-tyrosine protein group of wool (mol. wt. <10,000). These proteins are retarded in denaturing solvents on Sephadex and agarose columns[6] and also give anomalous low-molecular-weight values in SDS–polyacrylamide gels. In general, the studies reported here show that by extending the range down to mol. wt. 3500, CPG is a comparable support medium to Sephadex and agarose but has the advantage of high flow-rate and bed stability.

EXPERIMENTAL

Materials

Controlled pore glass CPG-10, 25.5 and 12.3 nm mean pore diameter, 120–200 mesh and pore volumes of 0.96 and 0.90 ml/g, respectively, was supplied by Electro-Nucleonics (Fairfield, N.J., U.S.A.). The SDS was BDH (Poole, Great Britain) specially pure grade while all other chemicals were of analytical or equivalent grade. The values assumed for the molecular weights of the dissociated proteins are given in Table I.

TABLE I

MOLECULAR WEIGHTS OF POLYPEPTIDE CHAINS

No.	*Protein*	*Reference*	*Subunit mol. wt.*
1	Insulin B-chain*	7	3400
2	Glucagon	7	3500
3	High-tyrosine component 0.62	8	7000
4	Cytochrome *c*	9	11,700
5	Apomyoglobin	9	17,200
6	α-Chymotrypsinogen A	9	25,700
7	Rabbit tropomyosin**	10	33,500
8	Aldolase**	9	40,000
9	Ovalbumin	9	43,000
10	Bovine serum albumin***	9	68,000

* Prepared as described in ref. 11.
** These proteins exist as oligomers under native conditions
*** Small amount of dimer present.

Methods

The protein–SDS complexes were prepared by incubating approximately 10 mg of protein with 20 mg of SDS in 1 ml of 6 *M* urea, 0.5% SDS, and 0.05 *M* phosphate buffer (sodium salts) pH 7 at 90° for 10 min. For those proteins which were not in the S-carboxymethyl form, 2% β-mercaptoethanol was included in the incubation mixture. Subsequently, 20% (w/v) sucrose was added to facilitate loading and the samples were passed through a 1.2-μm Millipore filter.

Preparation and operation of column

The required amount of CPG (the gravity packed volume is approximately 25% more than the final bed volume) was immersed in a large volume of degassed distilled water until evolution of bubbles had ceased. Considerable difficulty was

encountered in obtaining uniformly packed CPG columns, particularly when the diameter was of the order of a centimetre or less. A satisfactory procedure involved fitting the glass column (175 × 0.9 cm) with a rigid 15-cm extension arm connected by a short length of flexible silicone tubing to a clamped filter funnel. The column was half filled with water and the bottom securely attached to a laboratory vortex mixer. Applying strong vibration, the CPG slurry was added through the funnel and agitation continued until a stable bed height had been reached (approx. 90 min). In order to remove excess glass added during packing or adventitious dirt particles, it was found advisable to stir the liquid above the glass and remove the suspended material. The CPG was equilibrated by pumping at least 500 ml of filtered eluent (6 *M* urea, 0.5% SDS, 0.05 *M* phosphate buffer pH 7) through the column. A peristaltic pump was used to maintain a flow-rate of 0.66 ml/min·cm². The sample (0.2 ml), was layered carefully under the solvent onto the glass. In order to obtain sufficient precision, we found it necessary to use weight instead of volume as the measure of elution position. Following Fish *et al.*[4], we shall continue to use the symbol V_e, and the term elution volume, on the understanding that the elution positions are based on weight determinations of a constant density eluent. The expression used to normalize data from different runs was a distribution coefficient, K_d, defined as

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

where V_e is the weight of solvent corresponding to the peak concentration of eluting solute, V_0 is the weight of solvent in the column external to the CPG matrix and V_t is the weight of solvent contained within and without the CPG matrix. The void volume, V_0, and the total intrusion volume, V_t, were measured with Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) and tryptophan, respectively. The void volume was 43–45% of the bed volume. In a typical experiment with the 25.5 nm CPG column and a bed volume of 105 ml (packed height 165 cm), the void volume and total intrusion volume were 49.3 and 93.9 g, respectively.

RESULTS AND DISCUSSION

Initially, attempts were made to extend the work of Truman *et al.*[1] to include low-molecular-weight proteins in 8 *M* urea. In our hands, however, most proteins were either partially or completely adsorbed to the CPG support. This problem could not be alleviated by raising the pH or ionic strength, adding alcohols or Carbowax 6000 or 20M (ref. 3). Closer examination revealed several unsatisfactory aspects of the results reported by Truman *et al.*[1]. Firstly, their void volume seems much too high[2] for the bed volume of the column used. Secondly, the authors do not comment about retardation of their standard proteins and yet they report that cytochrome *c* elutes near 40 ml from a column whose total intrusion volume is approximately 30 ml. Finally, it is surprising that they found that only proteins below molecular weight 40,000 are suited to CPG of 70-nm pore diameter, bearing in mind that CPG with a pore diameter of 37 nm is suitable for amylose and dextran with molecular weights in the range 20,000–200,000[5].

The use of 0.1% SDS in phosphate buffer pH 7, as described by Collins and

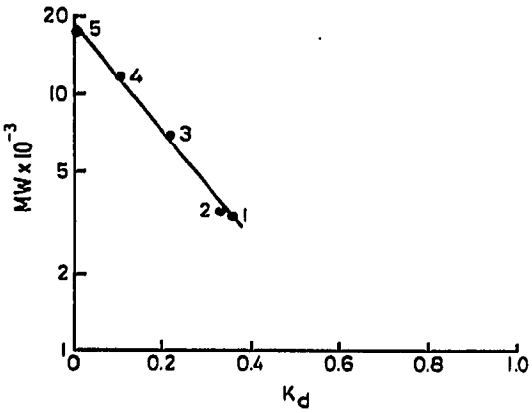

Fig. 1. Semilogarithmic plot of molecular weight *versus* K_d for a 12.3-nm pore diameter column using urea–SDS–phosphate as eluent. Numbers correspond to those given to the proteins listed in Table I.

Haller[2], was investigated with a column of 12.3-nm CPG. The peak corresponding to ovalbumin showed a long trailing edge while a high-tyrosine protein from wool failed to elute during the passage of five column volumes of buffer. However, increasing the concentration of SDS to 0.5% and including 6 *M* urea in the phosphate buffer prevented the adsorption onto CPG of all the proteins we have examined including the high-tyrosine wool protein which bound so strongly in the absence of urea. In this buffer, sharp symmetrical peaks were observed without the trailing seen for other denaturing solvents. Fig. 1 shows the plot of log molecular weight *versus* distribution coefficient, K_d, for proteins in the molecular-weight range 3500–12,000 chromatographed over CPG of pore diameter 12.3 nm. This is in contrast to a previous report[2], which claimed that it is not possible to discriminate between protein–SDS complexes on CPG when the protein molecular weight is less than 17,000. Besides the point shown on the plot in Fig. 1, this column gave molecular-weight values consistent with those found in the analytical ultracentrifuge for other high-tyrosine proteins.

The plot of log molecular weight *versus* distribution coefficient for proteins in the molecular-weight range 12,000–140,000 chromatographed over CPG of pore

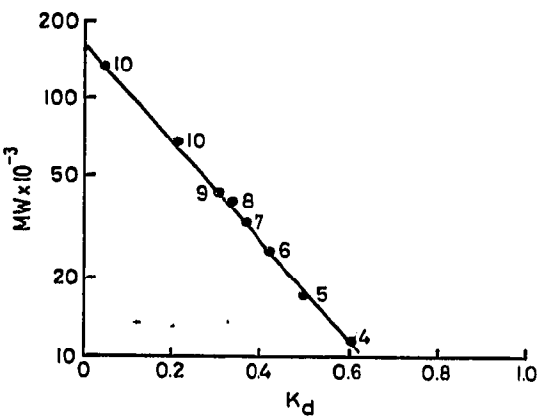

Fig. 2. Semilogarithmic plot of molecular weight *versus* K_d for a 25.5-nm pore diameter column using urea–SDS–phosphate as eluent. Numbers correspond to those given to the proteins listed in Table I.

diameter 25.5 nm is shown in Fig. 2. This result is similar to that reported by Collins and Haller[2] for a 28-nm column except that the linear portion extends down to molecular weight 12,000 rather than 17,000. The extremely good linearity of the calibration curves for these columns allows molecular-weight determination with an experimental error of ± 10% or better assuming that the unknown polypeptide chain adopts the same conformation as the proteins used for calibration. However, the relatively low volume within the glass pores in relation to the packed column volume means that CPG does not resolve a mixture of polypeptides as well as an equivalent column volume of Sephadex or agarose.

CONCLUSIONS

Chromatography over CPG appears to be a convenient method for the determination of the molecular weight of proteins under denaturing conditions. Although the conformation of proteins in the SDS–urea mixture recommended has not been studied, it has been reported that urea may weaken the binding of SDS to proteins[12]. However, the similarity of the calibration curve for the 25.5-nm CPG column shown in Fig. 2 with that for a 28-nm CPG column reported by Collins and Haller[2] suggests that the presence of urea in the eluent, while reducing the likelihood of adsorption, does not greatly affect the hydrodynamic properties of protein–SDS complexes.

In common with all empirical column procedures for molecular-weight determination, chromatography in the presence of SDS assumes that each polypeptide chain adopts the same conformation such that its dimensions are a unique function of molecular weight. It has been reported[13] that at low molecular weight, the rod-like shape of a protein–SDS complex begins to approximate a sphere and thus the relationship between molecular weight and hydrodynamic shape becomes unpredictable. Fish *et al.*[13] have set a lower limit of 15,000 to molecular weights that may be reliably measured by gel filtration in SDS solutions while Collins and Haller[2] were unable to show any size discrimination in the molecular-weight region 12,000–17,000. Neither of these groups appear to have investigated proteins with molecular weights below 10,000. From Fig. 1, it can be seen that a reliable calibration curve can be established for polypeptides in the molecular-weight range 3500–12,000. It thus appears that at these low molecular weights the protein–SDS complexes, although they may not have a rod-like shape and although they are in the presence of urea, do exhibit a common gross conformation. It is interesting to note that for SDS–polyacrylamide gel electrophoresis of oligopeptides[7], both in the presence and in the absence of 8 *M* urea, a linear calibration curve can be drawn for the molecular-weight range 2000–8000 (which has a different slope from that obtained for proteins in the molecular-weight range 12,000–45,000).

The use of CPG for the determination of molecular weight in the presence of SDS offers a means of studying those proteins which display anomalous behaviour on SDS–polyacrylamide gels. The high-tyrosine proteins isolated from wool have an apparent molecular weight of the order of 4000 when determined by electrophoresis in SDS containing urea. One of these proteins (component 0.62) has been extensively studied and a molecular weight of 6950 calculated from its amino acid sequence[8]. The fact that the molecular weight of this protein determined on CPG is approximately 7000 suggests that its anomalous migration in gels is due to an unusually high

charge per unit mass (*i.e.*, higher degree of SDS binding) rather than to an unexpectedly small hydrodynamic size. By way of contrast, a purified low-sulphur protein component from wool of molecular weight near 60,000 in the ultracentrifuge, gave a value of 71,000 using the 25.5-nm CPG column and a similar anomalous high value in SDS–polyacrylamide gel. For this protein, the unexpected retardation in polyacrylamide gel seems to be due to the conformation of the protein–SDS complex although charge could still play a minor role.

Although agarose gel filtration with 6 *M* guanidine hydrochloride as the denaturing solvent provides the best means at present for the chromatographic determination of the molecular weight of protein subunit polypeptides, the speed of measurement and the bed stability of CPG in urea–SDS makes this a very attractive technique. Once two or three columns covering the range of sizes normally encountered in protein chemistry have been calibrated, they can be stored for subsequent use. In this respect, although the 12.3-nm pore glass reported here gave adequate discrimination for proteins below molecular weight 12,000, it is possible that a slightly lower pore size would allow the characterization of even smaller polypeptides.

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