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COMPARISON OF ELUTION PATTERNS OF PROTEINS CHROMATOGRAPHED ON CONTROLLED-PORE GLASS AND CARBOXYMETHYL-CELLULOSE

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SUMMARY

As controlled-pore glass (CPG) has anionic silanol groups, we compared the elution patterns of proteins chromatographed on CPG with those on carboxymethyl (CM)-cellulose using standard proteins and two protein mixtures, rabbit serum and bovine parotid extracts. The results showed that the order of proteins eluted from CPG was similar to that from CM-cellulose, although some differences were found. The adsorption of proteins on CPG was slightly stronger than that on CM-cellulose and the separation of proteins on CPG was slightly better. Some conditions, such as amounts of proteins loaded on the column, pH, temperature and kind of buffer solution for elution, were investigated. The pH of buffer solutions was important in the adsorption chromatography of proteins on CPG and the useful pH range was 7.0–8.5.

INTRODUCTION

The use of adsorption chromatography for the separation of proteins on controlled-pore glass (CPG) has been reported independently by us[1] and Bock *et al.*[2]. We determined the amount of biological materials adsorbed on the glass in order to clarify the mechanisms of such adsorption. CPG (1 g, 97 m^2) adsorbed approximately 5 μmole of cationic biological materials, such as lysine, histidine, arginine, hexosamines and cytidine, in distilled water medium.[3] The amount of other amino acids and neutral carbohydrates was 0–0.8 μmole per gram of CPG, and we therefore concluded that one major force for the adsorption of proteins on glass surfaces was ionic amine–silanol bonding and CPG behaved like a cation exchanger.[3] We also concluded that another major property of the adsorption of proteins on glass was cooperative aggregation between proteins and glass, based on the experimental results that proteins were adsorbed the greatest at their isoelectric points[4]. Glycine and leucine were also adsorbed at their isoelectric points and this adsorption of glycine might prevent the adsorption of proteins on CPG[5]. Adsorption studies using detergent solutions showed that bovine serum albumin in urea, guanidine chloride, Triton

X-100, Brij 35 and cationic soap solutions at pH 5.4 was well adsorbed on glass surfaces but albumin in anionic soap, such as sodium dodecyl sulphate (SDS) and hard soap solutions, was not adsorbed[6].

In this work, we compared the elution patterns of proteins chromatographed on CPG by a stepwise elution with that on CM-cellulose, which is a cation exchanger used for the separation of proteins, and we investigated some conditions, such as pH of buffers, temperature and amount of proteins loaded on the column.

MATERIALS AND METHODS

The CPG used was CPG-10 240 Å (Electro-Nucleonics, Fairfield, N.J., U.S.A.), which was composed of 96% silica and had a surface area of 97 m^2/g and a particle size of 100 μm. After being washed with water, 0.1% SDS, water, chromic acid mixture and water, the glass was packed in a column (20 × 1.2 cm, 22.6 ml, 10 g weight). After the column had been equilibrated with buffer, the solution containing proteins (20 mg/ml) was applied on the column and subsequently the column was eluted stepwise with buffers at 4° and a flow-rate of 0.5 $ml/cm^2 \cdot min$. The fraction volumes collected were 5 ml.

Column operation at a constant temperature of 37° was carried out on a 35 × 0.9 cm column. One protein used as a reference for protein mixtures was rabbit serum, which was dialysed against distilled water and lyophilized. Another was an aqueous extract of the acetone-dried powder, which was prepared from a precipitate at pH 5.4 of the aqueous extract of bovine parotid gland[7].

Standard proteins used were bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill., U.S.A.), lysozyme (Sigma, St. Louis, Mo., U.S.A.), chymotrypsinogen A (Boehringer, Mannheim, G.F.R.), catalase (Boehringer) and haemoglobin (Sigma). Buffers used for the elution of proteins were 0.2 *M* sodium chloride in 0.01 *M* phosphate, Tris–hydrochloric acid, glycine, 0.1% SDS in phosphate, sodium acetate and phosphate (pH 7.4). The CM-cellulose used (Tohoku-Pulp Co., Tokyo, Japan) had an exchange capacity of 0.63 mequiv./g (11 ml/g dry weight); DEAE-cellulose (DE-11, Whatman, Clifton, N.J., U.S.A.) had an exchange capacity of 0.97 mequiv./g (6.4 ml/g dry weight). CM-cellulose (dry weight 1.8 g) was washed and packed in a 15 × 1.3 cm column. After the protein solution had been applied on the column, the column was eluted stepwise with buffers at 4° and a flow-rate of 0.5 $ml/cm^2 \cdot min$. DEAE-cellulose (0.4 g) was packed in an 8 × 0.63 cm column. Protein concentrations in the solution were determined by measurement of the absorbance at 280 nm. Analytical polyacrylamide gel disc electrophoresis was carried out by the Davis method[8].

RESULTS

Chromatography of rabbit serum on CPG and CM-cellulose

Fig. 1A shows the elution pattern of rabbit serum (100 mg) at pH 8.0 on a CPG column (20 × 1.2 cm) using the following buffers: sodium chloride–phosphate, Tris–hydrochloric acid, glycine and SDS–phosphate. Disc electrophoretic patterns of the fractions in Fig. 1A are shown on the left-hand side of the figure. The maximal amounts of bovine serum albumin and chymotrypsin adsorbed on 1 g of CPG in

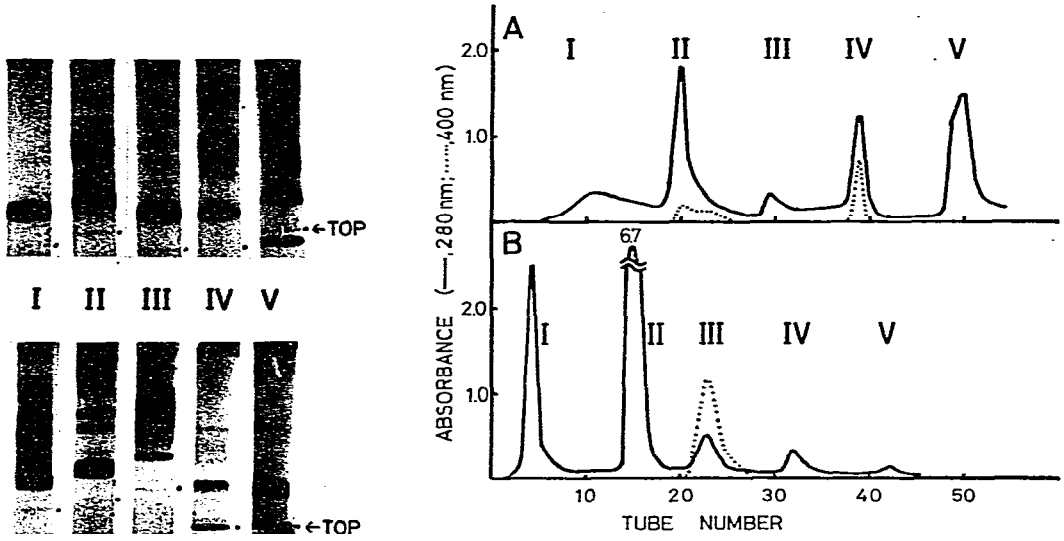

Fig. 1. Elution patterns of rabbit serum (100 mg) on (A) a CPG column (20 × 1.2 cm) and (B) a CM-cellulose column (15 × 1.3 cm) at 4° and (left) disc gel patterns of separated fractions. The fraction volumes collected were 5 ml. Eluents: (A) I, 0.2 *M* NaCl–0.01 *M* phosphate (pH 8.0); II, 0.01 *M* Tris–HCl (pH 8.0); III, 0.2 *M* Tris–HCl (pH 8.0); IV, 0.2 *M* glycine (pH 8.0); V, 0.1% SDS in 0.05 *M* phosphate (pH 7.4); (B) I, 0.01 *M* sodium acetate (pH 5.6); II, 0.2 *M* sodium acetate (pH 5.6); III, 0.5 *M* sodium acetate (pH 5.6); IV, 0.2 *M* phosphate (pH 7.4); V, 0.2 *M* HCl.

distilled water were 136 and 233 mg, respectively[4]. A 10-g amount of glass in the column should be able to adsorb 1–2 g of proteins, and therefore the amount (100 mg) of protein applied on the column was one tenth to one twentieth of the adsorption capacity of the glass.

Yields of the fractions in Fig. 1A are shown in Table I. The elution patterns using 20 mg of rabbit serum (one fiftieth to one hundredth of the adsorption capacity of CPG) on an identical column and with an identical elution system at pH 8.0 with that in Fig. 1A was the same as shown in Fig. 1A. Yields of the fractions are shown in Table I and the ratios of the yields of the fractions with the use of 20 mg of proteins are very similar to those in Fig. 1A, as shown in Table I.

From these results, we concluded that the reproducibility of the elution pattern of proteins is good for a range of proteins one tenth to one hundredth of the adsorption capacity of CPG (2–10 mg of protein per gram of CPG). The elution pattern at a constant temperature of 37° on CPG using 100 mg of rabbit serum was similar to that at 4° and the yields are also similar, as shown in Table I. It was concluded that the influence of temperature on chromatography on CPG was not strong. After being eluted with SDS solution, the column had not adsorbed any proteins, because the column was not darkened on elution of chromic acid mixture. When a CPG column is binding proteins, the column is darkened on elution of chromic acid mixture.

Fig. 1B shows the elution pattern of rabbit serum on a CM-cellulose column (15 × 1.2 cm) and coloured protein measured at 400 nm was eluted at fraction III. Disc electrophoretic patterns of proteins in the fractions in Fig. 1B are shown on the left-hand side of the figure. From a comparison of Fig. 1A and B, the protein gel

TABLE I

PERCENTAGE YIELDS OF FRACTIONS OBTAINED ON CHROMATOGRAPHY UNDER VARIOUS CONDITIONS

Fraction	*Conditions*					
	CPG, serum, 100 mg, 4° (Fig. 1A)	*CPG, serum, 20 mg, 4°*	*CPG, serum, 100 mg, 37°*	*CM-cellulose, serum, 100 mg, 4° (Fig. 1B)*	*CPG, parotid, 100 mg, 4° (Fig. 3A)*	*CM-cellulose, parotid, 100 mg, 4° (Fig. 3B)*
I	15.6	16.5	22.4	19.7	48.3	48.2
II	21.6	25.0	21.6	44.2	11.2	13.3
III	6.8	7.5	0	7.3	1.7	2.1
IV	9.3	14.1	8.8	3.0	2.1	10.7
V	19.0	21.2	22.0	1.8	26.0	1.7
Other minor fractions	3.4	2.5	4.0	0.8	5.2	8.0
Total	75.7	86.8	78.8	76.8	94.5	84.0

pattern in fraction I from the CPG column was not found in any fractions eluted from the CM-cellulose column, but the gel patterns of other fractions from the CPG and CM-cellulose columns are similar, as follows. The disc gel pattern of proteins in fraction I from CM-cellulose is similar to that in fraction II from CPG and the pattern of fraction II from CM-cellulose was coincident with that of fraction IV from CPG, as both fractions contained proteins that have low mobility and broad gel patterns. Protein with a relative mobility of 0.4 (α-globulin) in fraction III on CM-cellulose was eluted at fractions IV and V from CPG. The similarity of the fractions eluted from both CM-cellulose and CPG columns was established by analyses by SDS gel electrophoresis.

The order of elution of proteins from CM-cellulose and CPG is similar and it was concluded that CPG behaved like a cation exchanger such as CM-cellulose in the chromatography of proteins. The separation of proteins on CPG by this elution system using a similar column size was better than that on CM-cellulose and the overall yields of proteins from both columns are identical, as shown in Table I.

The elution patterns at pH 6.0, 7.0, 7.5 and 8.5 are shown in Fig. 2. Proteins were well adsorbed on CPG at pH 6.0 and eluted with the SDS solution. At pH 7.0, some components of the rabbit serum protein mixture were eluted with 0.01 *M* Tris–hydrochloric acid but the major proportion was adsorbed and eluted with the SDS solution. The proteins were fractionated at pH 7.5 but the separation and yield were not better than those at pH 8.0. The separation at pH 8.5 was also not better than that at pH 8.0. The values shown in Fig. 2 were those of the buffer solutions used and were different from those of the effluent, the pH values of which were changed by ± 0.5 of the pH of the buffer by the ion-exchange character of the glass.

Chromatography of parotid protein mixture

Fig. 3 shows the elution patterns of bovine parotid protein mixture (100 mg) on a CPG column (20 × 1.2 cm) at pH 8.0 and on a CM-cellulose column (15 × 1.3 cm) and stained disc gel patterns of the fractions. Milky turbid proteins measured at 650 nm are eluted at an early point in fraction I eluted from a CPG column by the exclu-

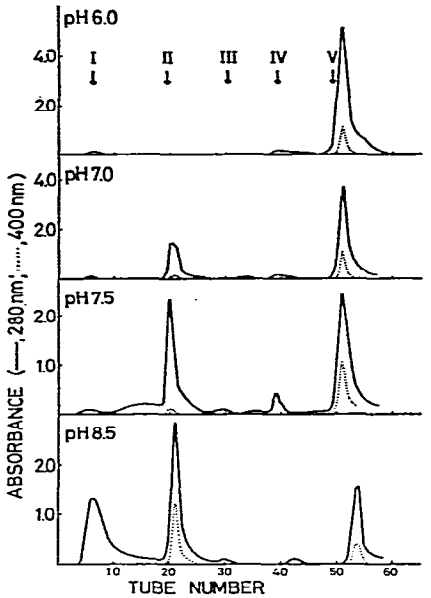

Fig. 2. Elution patterns of rabbit serum (100 mg) on a CPG column (20 × 1.2 cm) at 4° and various pH values. The fractions volumes collected were 5 ml. Eluents: I, 0.2 *M* NaCl–0.01 *M* phosphate; II, 0.01 *M* Tris–HCl; III, 0.2 *M* Tris–HCl; IV, 0.2 *M* glycine; V, 0.1% SDS in 0.05 *M* phosphate. Tris–HCl of pH 6.0 contains phosphate of one-twentieth the concentration of Tris–HCl.

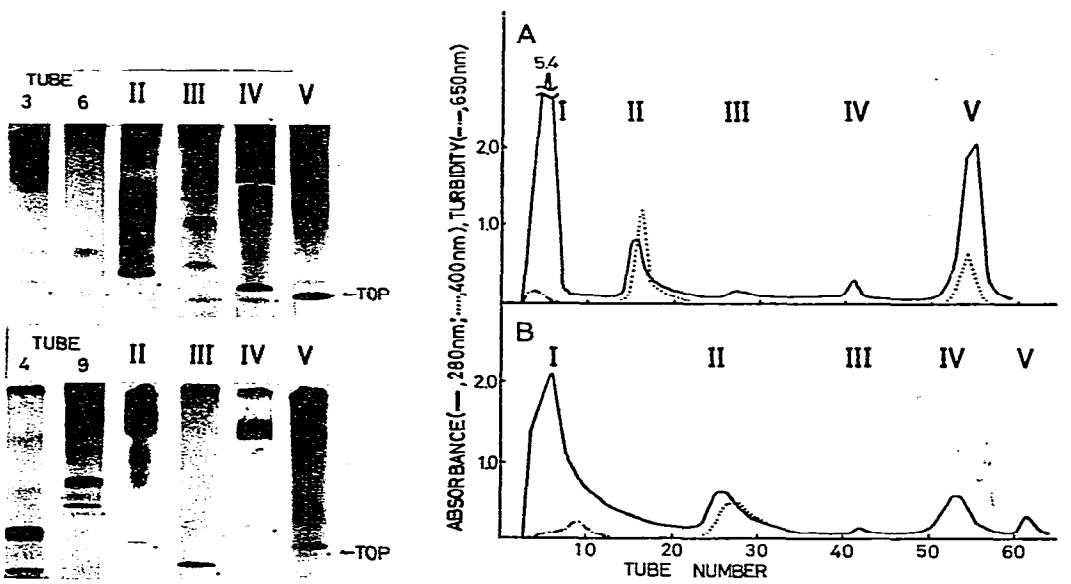

Fig. 3. Comparison of elution patterns of bovine parotid protein mixture (100 mg) on (A) CPG and (B) CM-cellulose columns and (left) disc gel patterns of separated fractions. Other conditions as in Fig. 1.

sion chromatographic effect and a late point in fraction I eluted from a CM-cellulose column. Similar results are obtained from the gel patterns, because proteins with a relative mobility of 0.3 were eluted at an early point in fraction I from CPG and at a late point in fraction I from CM-cellulose, and other proteins with a mobility of 0.8 were eluted at a late point in fraction I from CPG and at an early point in fraction I from CM-cellulose.

The parotid protein mixture was obtained from a precipitate at pH 5.4, and hence many proteins are negatively charged at pH 8.0 and yields of fraction I from CPG and CM-cellulose are high, as shown in Table I, for repulsion to negatively charged adsorbents.

Coloured proteins measured at 400 nm were eluted at similar position in Fig. 3A and B. Fraction II from CPG and CM-cellulose contain an identical protein of relative mobility 0.5 and fraction II from CM-cellulose contains the protein of relative mobility 0.2 in fraction IV from CPG. From the results of the chromatography of bovine parotid proteins, it was also concluded that the orders of elution of proteins from CPG and CM-cellulose columns were similar. CP 3 is preferable to other ion exchangers on account of its ease of sterilization.

Chromatography of standard proteins

Fig. 4 shows the elution patterns on CPG and CM-cellulose columns using standard proteins, such as bovine serum albumin, haemoglobin, catalase, lysozyme and chymotrypsinogen A. As shown in the disc gel patterns on the left-hand side of Fig. 4, albumin, haemoglobin and part of the catalase were eluted in fractions I, II and IV in Fig. 4A, respectively, and the proteins were eluted in the same order in Fig. 4B using CM-cellulose, as follows: albumin at an early point (tube 24) in fraction II, haemoglobin at a late point (tubes 25 and 26) in fraction II and catalase in fraction IV.

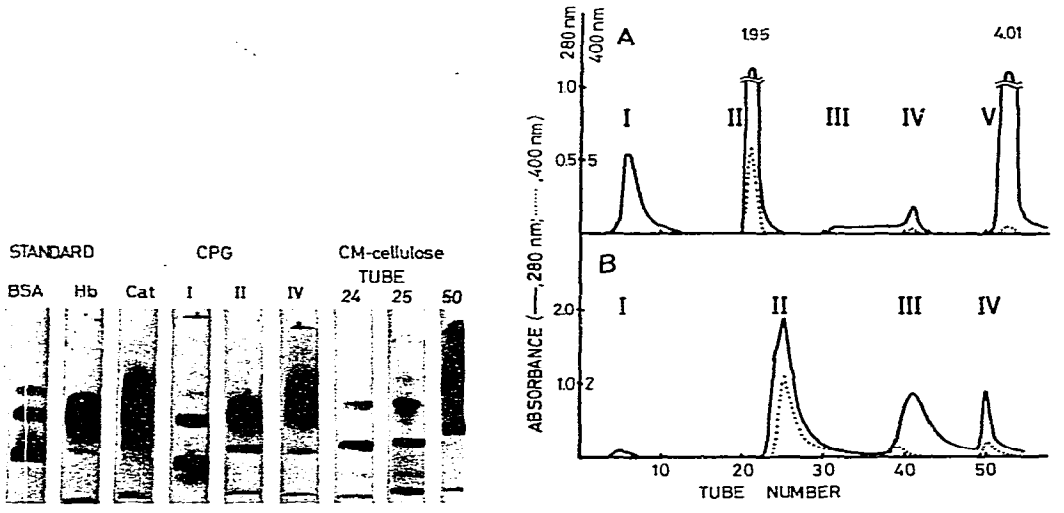

Fig. 4. Elution patterns of standard proteins (10 mg) on (A) CPG and (B) CM-cellulose columns. Other conditions as in Fig. 1, except the solution IV in (B) was 0.1 *N* NaOH. BSA = bovine serum albumin; Hb = haemoglobin; Cat = catalase.

The band of haemoglobin on the gel was clearly ascertained by a brown colour before staining and also by the absorbance at 400 nm.

Chymotrypsinogen A and lysozyme were eluted in fraction V in Fig. 4A and the existence of both proteins in the fraction was examined by SDS gel electrophoresis, whose patterns are not shown because neither protein moved on analytical disc gel at pH 8.6. Chymotrypsinogen A (isoelectric point pH 8.6) and lysozyme (isoelectric point 11.5) have a cationic charge at pH 8.0, and therefore the both proteins were strongly adsorbed on the negatively charged silanol of CPG. The order of elution of proteins on CPG might be depend on repulsion between the negatively charged proteins and silanol, and the cohesive force of proteins to glass surfaces. In Fig. 4B, chymotrypsinogen A was eluted in fraction II and lysozyme in fraction III, which is different to the results on CPG. The elution points of chymotrypsinogen A and lysozyme in Fig. 4A and B were also ascertained by using one kind of each protein as well as SDS gel electrophoresis. The adsorption of proteins on CPG was stronger than that on CM-cellulose.

The results using standard proteins support our contention that the order of elution of proteins on CPG are similar to those on CM-cellulose, even if some differences were found with of chymotrypsinogen A and lysozyme. However, the differences are useful for the separation of proteins that are not separated on CM-cellulose.

DISCUSSION

The pH range used in chromatography on CM-cellulose column is acidic (4–6), but the range with CPG is 7.0–8.5, as shown in Fig. 2, and this pH range might be valuable for maintaining the native conformation of some proteins. The use of a pH higher than 9.0 is not suitable for chromatography on CPG, because glass is labile in alkaline solutions[9]. By comparison of the adsorption capacity of bovine serum albumin, the maximal amount of the protein adsorbed on 1 ml of DEAE-cellulose was 35 mg in 0.01 *M* phosphate (pH 7.4) and 60 mg in 0.01 *M* sodium acetate (pH 5.6), whereas the amount on 1 ml of CPG in distilled water was 54 mg, similar to that on DEAE-cellulose. We separated proteins on similarly sized CPG and CM-cellulose columns using the same amount of proteins (100 mg), and concluded that the separation and adsorption capacity of CPG was similar to or better than that of the cellulose ion exchangers.

We examined the influence of several solution at pH 8.0 for adsorption of proteins on CPG. The order of eluting force of the buffers, as shown in Fig. 1A, was phosphate < Tris–hydrochloric acid < glycine. SDS ions were the strongest for elution but SDS ions bound on protein molecules were repulsed by silanol residues. The elution strengths of sodium chloride, sodium sulphate and sodium acetate at pH 8.0 were weaker than that of phosphate at pH 8.0. This order of elution strengths is due mainly to interference to binding of $-NH_3^+$ ions on protein molecules to silanol residues on glass surfaces and by the differences in the strengths of adsorption of inorganic cations in buffers to cation exchangers. However, we assume that the separation of proteins on CPG was partially caused by another major adsorption force due to the cooperative aggregative action between the glass and proteins and the difference in the elution patterns on CPG and CM-cellulose must depend on this force.

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