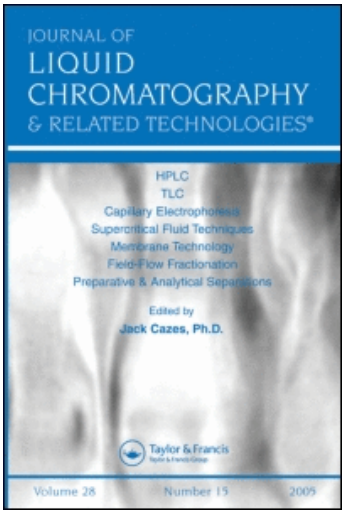

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PROTEIN ADSORPTION OF SEPHACRYL S-TYPE GELS USING HIGH CONCENTRATIONS OF AMMONIUM SULFATE

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ABSTRACT

A variety of proteins adsorbed to Sephacryl gels, S-200, S-500 and S-1000 and Fractogel TSK-HW65(F) in the presence of high concentrations of ammonium sulfate. The adsorption of these proteins to individual gel matrices showed some variations. The strength of adsorption of cytochrome c, myoglobin and chymotrypsinogen A to Sephacryl S-200 increased with temperature and pH, suggesting that hydrophobic interaction is involved in the adsorption. The chromatographic behaviour of these three proteins on Sephacryl S-type gels and Fractogel varied with the ammonium sulfate concentration of the eluant buffer. At 30% saturation, cytochrome c and myoglobin eluted together and followed by chymotrypsinogen A. At 45% saturation, however cytochrome c eluted before myoglobin and chymotrypsinogen A remained tightly bound to the column. Temperature, pH, flow-rate and glycerol were some of the factors affecting the separation of these three proteins on Sephacryl S-200.

Introduction

Sephacryl S-200, a gel matrix prepared by covalently cross-linking N-N'-methylenebisacrylamide and allyl dextran, is commonly used for molecular-sieve chromatography (1). An earlier study showed that it adsorbs proteins at high concentrations of ammonium sulfate (2). Recently, we have

used it as an adsorbent for separating crude extracts of cyanobacteria (3-5). This was achieved by binding the cyanobacterial proteins to the gel matrix at a high concentration of ammonium sulfate and then eluting them with a lower concentration of the same salt. The elution order observed was not related to the molecular size of the protein. Using ammonium sulfate gradients to separate proteins on solid supports has previously been attempted on other gel filtration matrices. In some cases, protein adsorption to the gel matrix involved hydrophobic interaction (6-9). The existence of such interactions in Sephacryl S-200 gels has not been investigated. Moreover, very little is known about the chromatographic behaviour of proteins on Sephacryl S-200 when highly concentrated ammonium sulfate is present in the running buffer.

Here, we report a systematic study on protein adsorption and chromatographic behaviour using Sephacryl type gels and high concentrations of ammonium sulfate. In addition, another gel filtration matrix, Fractogel TSK-HW 65(F), has been studied and compared with the Sephacryl S-type gels.

MATERIALS AND METHODS

Sephacryl gels, S-200, S-500 and S-1000, bovine serum albumin, chymotrypsinogen A, ovalbumin were purchased from Pharmacia. Conalbumin, glucose oxidase, horse-heart cytochrome c and myoglobin were obtained from Sigma Company and Fractogel TSK HW-65(F) from Merck Chemical Company. All other chemicals were of analytical grade.

Measurement of degree of protein adsorption on gel matrix

Bovine serum albumin, chymotrypsinogen A, myoglobin, cytochrome c, conalbumin, glucose oxidase and ovalbumin were used. The adsorption of these individual proteins on Sephacryl gels, S-200, S-500 and S-1000, and Fractogel TSK HW-65(F) was studied at room temperature, using ammonium sulfate at 25%, 40%, 50%, 60% 70%, 80% and 90% saturation.

Firstly, 0.2 ml of a gel matrix was mixed with 2.8 ml Tris/HCl buffer (pH 7.5, 50 mM) containing 200 mM NaCl and an appropriate amount of ammonium sulfate. Then the gel was mixed with 20 ul of a protein solution (20 ug/ul) and

centrifuged for 10 min. at 10,000 rpm. To estimate the protein adsorbed to the gel, the absorbance of the supernatant was measured at 280 nm. A plot of the percentage of protein adsorbed versus ammonium sulfate concentration was constructed for each gel type. From the plot, the ammonium sulfate concentration corresponding to 50% of protein adsorption was estimated.

For chymotrypsinogen A, myoglobin and cytochrome c, the adsorption studies were repeated at different temperatures (27°C, 20°C and 5°C) and pHs (6.5 and 8), using Sephacryl S-200.

Chromatographic Procedure

Four identical columns (20 x 1.5 cm), each packed with a different gel matrix, were used throughout the study.

Sephacryl gels, S-200, S-500 and S-1000, and Fractogel were packed according to Belew et al. (2). These gel matrices, each packed to a height of 18 cm, were pre-equilibrated with a running buffer containing 50mM Tris/HCl (pH 8), 200 mM NaCl and 0%, 20%, 35% or 40% saturation of ammonium sulfate. In these studies, only chymotrypsinogen A, myoglobin and cytochrome c were used. Firstly, a stock solution (5mg/ml) of each individual protein was prepared, using the running buffer. Then, a protein sample, prepared by mixing 15 ul of myglobin, 7.5 ul of cytochrome c and 30 ul of chymotrypsinogen A, was loaded onto each column and the columns eluted at room temperature. The flow-rate was adjusted to 0.24 ml/min. The above procedure was repeated using the individual proteins. The elution profiles of individual proteins were compared with that of the protein mixture. The results show that the elution of individual proteins was not affected by the presence of the other two proteins.

The effects of using different temperatures (5°C, 10°C and 20°C), pHs (6.5 and 7.5), flow rates (0.48, 0.96 and 1.6 ml/min.) and additives (Tween 20, Tween 80, Triton X-100 and glycerol) on protein adsorption to Sephacryl S-200 were also investigated.

RESULTS AND DISCUSSION

Adsorption of proteins to Sephacryl gels, S-200, S-500 and S-1000 and Fractogel TSK-HW 65(F)

Cytochrome c, myoglobin, chymotrypsinogen A, bovine serum albumin, ovalbumin, conalbumin and glucose oxidase were used for the adsorption study. Table 1 shows the ammonium sulfate concentrations corresponding to 50% adsorption of the individual proteins to various gel matrices. When Sephacryl gels were used for protein adsorption, the ammonium sulfate concentrations estimated for individual proteins were found to follow the pattern: cytochrome c > myoglobin > chymotrypsinogen A, bovine serum albumin, ovalbumin, conalbumin > glucose oxidase. Among the Sephacryl gels, no marked difference in ammonium sulfate concentration needed to elute the individual proteins was observed. It is worth mentioning that the small decrease in ammonium sulfate concentration observed for cytochrome c, myoglobin or chymotrypsinogen A, when Sephacryl S-1000 was used as the adsorbent, was probably not due to experimental errors. Such decrease, found in repeated experiments, suggests that Sephacryl S-1000 may be better adsorbent than Sephacryl S-200 for small proteins. This may simply be due to its larger pore size. As for the adsorption of proteins on Fractogel TSK-HW65(F), the ammonium sulfate concentrations required for elution of proteins were somewhat different from those of Sephacryl gels. For chymotrypsinogen A and glucose oxidase, the required concentrations were 6-13% lower in Fractogel as compared to Sephacryl gels, whereas for ovalbumin and conalbumin, the concentrations were 6-9% higher. The ammonium sulfate concentrations were also found to follow a slightly different pattern: cytochrome c > myoglobin > bovine serum albumin, ovalbumin, conalbumin > chymotrypsinogen A > glucose oxidase. All these results, found in repeated experiments, probably reflect the differences in strength of binding of individual proteins to different adsorbents.

Fractogel TSK-HW65(F) has the same material as Toyopearl HW-65(F) (10), and its matrix, designed for gel filtration chromatography, is characterized as a crosslinked hydroxylated

Table 1. The ammonium sulfate concentration (% saturation) providing a half-maximal adsorption for individual proteins on Sephacryl gels and Fractogel.

Protein	Sephacryl S-200	Sephacryl S-500	Sephacryl S-1000	Fractogel HW-65 (F)
Cytochrome c	79	72	71	73
Myoglobin	62	61	56	60.5
Chymotrypsinogen A	49	42	42	36.5
Bovine Serum Albumin	48.5	47	47	49.5
Ovalbumin	47.5	44	45	53.5
Conalbumin	47	47	45	51
Glucose oxidase	40	40	39	30.5

Table 2. The ammonium sulfate concentration (%) saturation providing a half-maximal adsorption for individual proteins on Sephacryl S-200 at different temperature.

Proteins	Temperature (°C) 27	20	5
Cytochrome c	79	88	>95
Myoglobin	62	76	92
Chymotrypsinogen A	49	52	58

polyether (11). Previous studies (12,13) showed that Toyopearl HW-type gels adsorb proteins in the presence of high concentrations of ammonium sulfate, and attributed such adsorption phenomena to hydrophobic interaction. The involvement of such interaction in the Sephacryl gels is supported by the results of this investigation, especially the effects of temperature and pH on adsorption of cytochrome c, myoglobin and chymotrypsinogen A. Table 2 shows that protein adsorption to Sephacryl S-200 decreases at low temperature. This result agrees well with the finding that the strength of hydrophobic interaction decreases with a decrease in temperature (14). All three proteins were found to adsorb strongly to Sephacryl S-200 at elevated pH values (Table 3). Since pH can affect the net charge of a protein, hydrophobic adsorption is expected to be greatest when the mobile phase pH is close to the pI value of that protein. This may explain why cytochrome c and chymotrypsinogen A adsorbed more strongly to the Sephacryl S-200 at increasing pH. Both of these proteins have pI values of greater than 9 (15). It is hard to explain the results obtained with myoglobin on the basis of hydrophobic interaction alone, since this protein has a pI value of about 7.1 (16). Myoglobin is assumed to be more negatively charged at pH 8, and hence would be thought to bind less strongly to the gel, yet the result shows that a lower

ammonium sulfate concentration was required, suggesting that the protein binds more strongly. Part of the interaction between myoglobin and Sephacryl S-200 might be attributed to residual positive charge on the gel matrix, but this is unlikely since an earlier study (17), conducted in an absence of ammonium sulfate, shows that Sephacryl S-200 does not adsorb acidic protein at pH 8. However, some changes in the protein structure of myoglobin may not be excluded at pH 8, thus allowing some of the previously buried hydrophobic amino acids to participate in the adsorption.

Chromatographic properties of Sephacryl S-200 at different ammonium sulfate concentrations

These studies were conducted at pH 8.0 as indicated in the materials and methods. In the absence of ammonium sulfate, cytochrome c, myoglobin and chymotrypsinogen A were all eluted together, whereas at 20% saturation, they were retained to an equal extent and were eluted later. Figure 1 shows the elution profiles at 30% and 45% saturation of ammonium sulfate. At 30% saturation, chymotrypsinogen A was more strongly adsorbed to the gel than the other proteins and was eluted separately, whereas at 45% saturation, it was not eluted. It was eluted only when the ammonium sulfate concentration was lowered to 30% saturation. The difference in the retention of myoglobin and cytochrome c on Sephacryl S-200 was only manifested at 45% saturation when myoglobin was eluted after cytochrome c.

pH effects. The above study was repeated at pH 7.5 and 6.5. The results obtained with 30% and 45% saturation of ammonium sulfate are also given in Figure 1. At 30% saturation and pH 7.5 or 6.5, the unresolved peak of cytochrome c and myoglobin was found to elute off the column slightly earlier than at pH 8.0. In addition, the heights of the chymotrypsinogen A peaks were found to vary at different pHs. At 45% saturation , peaks of cytochrome c and myoglobin showed increasing resolution as pH was raised from 6.5 through 7.5 to 8.0. There was also a sharp rise in the elution volume of myoglobin as the pH was increased above pH 6.5. This agrees with the earlier finding that Sephacryl S-200 adsorbs myoglobin more strongly at higher pH (Table 3).

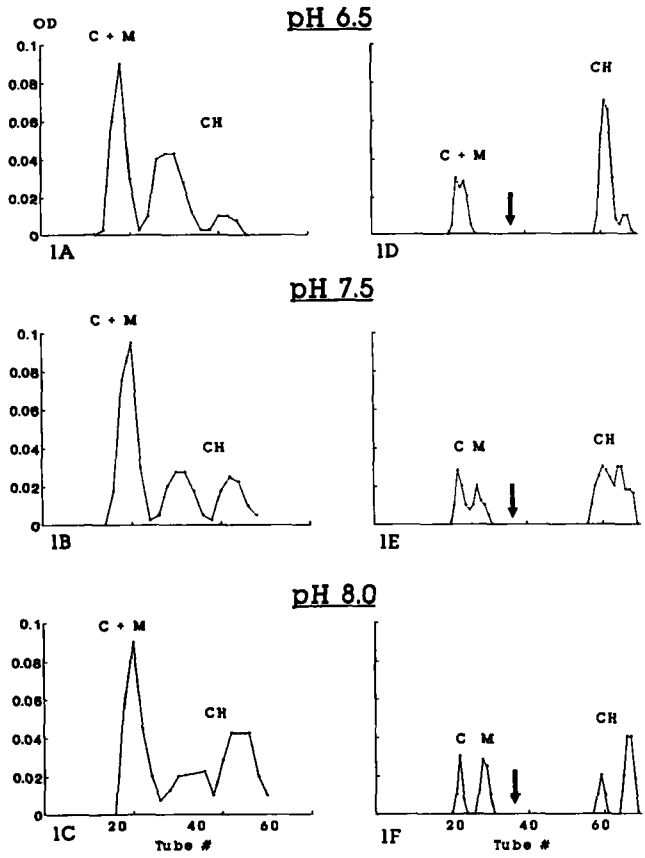

Figure 1. Elution profiles of cytochrome c (C), myoglobin (M) and chymotrypsinogen A (CH) off a Sephacryl S-200 column using 30% (A-C) and 45% (D-F) saturated ammonium sulphate at different pHs. The arrows indicate a change in ammonium sulphate concentration to 30% saturation.

Chymotrypsinogen A was not eluted at 45% saturation at any pH. During all these studies, it was noticed that the initial condition of the chymotrypsinogen A stock solution could affect the elution profile of the protein. If the stock solution had been kept overnight at 4°C or had been subjected to freezing and thawing prior to usage, the protein was found to elute as two distinct peaks as indicated in Figure 1. In

Table 3. The ammonium sulfate concentration (% saturation) providing a half-maximal adsorption for individual proteins on Sephacryl S-200 at different pH.

Proteins	pH 8.0	7.5	6.5
Cytochrome c	86	90	>95
Myoglobin	62	76	80
Chymotrypsinogen A	48	52	54

all the subsequent experiments, freshly prepared protein was used and it was found to elute from the gel matrix as a single peak.

Temperature effects. Figure 2 shows the elution profiles obtained with 30% and 45% saturations of ammonium sulfate at three different temperatures, 5°C, 10°C and 20°C. In general, there was an obvious drop in protein resolution as the temperature was lowered. This was accompanied by a drop in elution volume for individual proteins. These findings, indicating a drop in the strength of interaction between the proteins and the gel matrix at lower temperatures, coincide with those of the adsorption studies which show that protein adsorption to Sephacryl S-200 decreases at low temperature (Table 2).

Flow-rate. Figure 3 shows the elution profiles at 45% saturation of ammonium sulfate and different flow-rates. Better resolution was achieved at 0.48 ml/min. or lower for cytochrome c and myoglobin. At 1.6 ml/min., the two proteins were not resolved at all. In addition, the protein bands eluted earlier than at lower flow-rates.

Additives. Different detergents including Tween 20 and 80 (0.25%, 0.5% and 1% v/v) and Triton X-100 (0.025%, 0.05% and 0.1% v/v) were incorporated separately into the washing buffer along with 45% saturation of ammonium sulfate. These

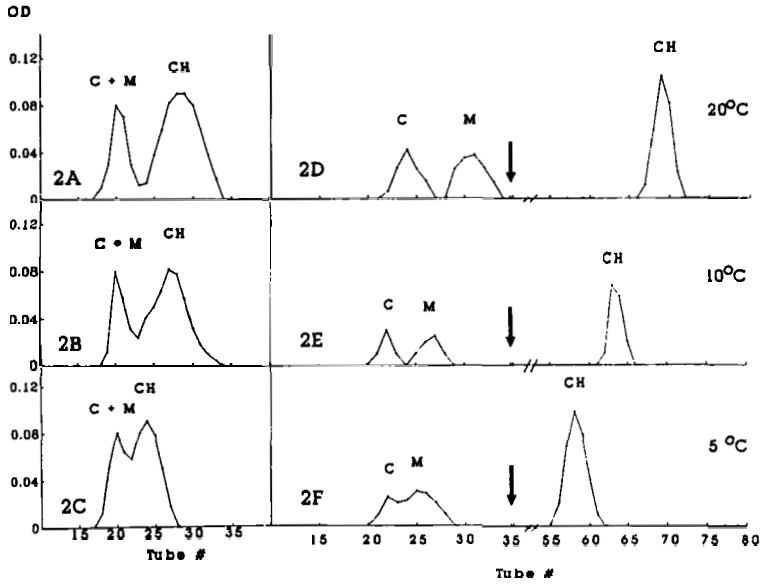

Figure 2. Elution profiles of cytochrome c (C), myoglobin (M) and chymotrypsinogen A (CH) off a Sephacryl S-200 column using 30% (A-C) and 45% (D-F) saturated ammonium sulphate at different temperatures. The arrows indicate a change in ammonium sulphate concentration to 30% saturation.

detergents had no effect on protein adsorption to the gel matrix. Figure 4 shows that all three proteins were not retained at all at 45% saturation and eluted as a single peak when glycerol (50% v/v) was incorporated into the running buffer. This finding supports the involvement of hydrophobic interaction in protein adsorption.

Chromatographic properties of other Sephacryl gels and Fractogel TSK-HW(65) at different ammonium sulfate concentrations

The studies were conducted at pH 8.0 as described in materials and methods. As with Sephacryl S-200, these other gel matrices had no molecular sieving effect on the test proteins in the absence of ammonium sulfate. At 20%

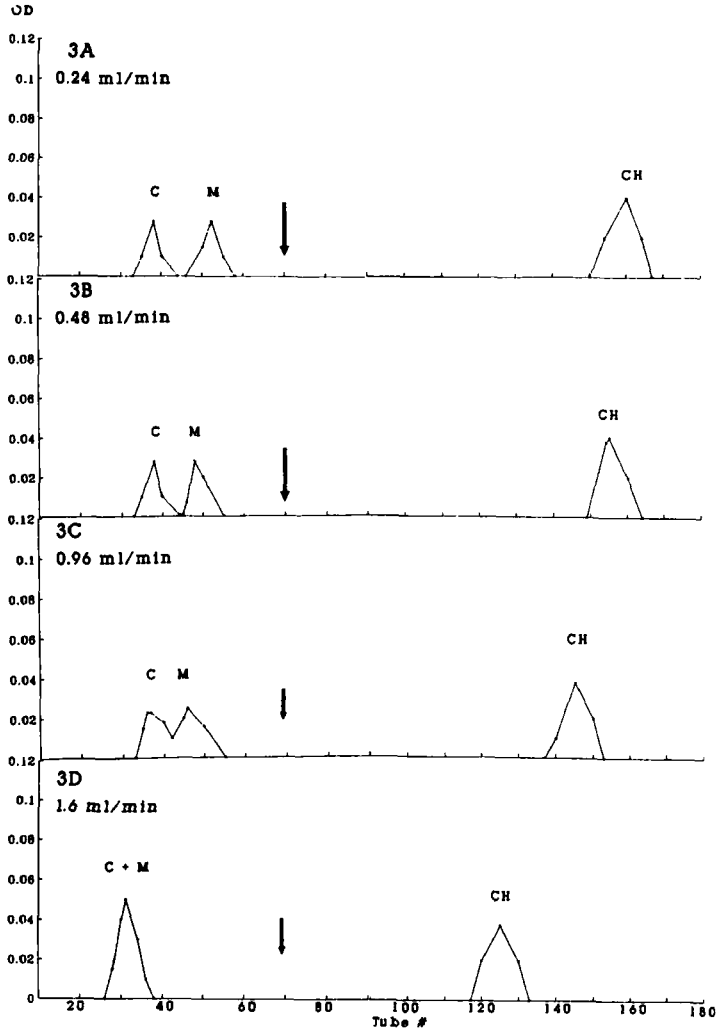

Figure 3. Elution profiles of cytochrome c (C), myoglobin (M) and chymotrypsinogen A (CH) off a Sephacryl S-200 column using 45% saturated ammonium sulphate at different flow-rates. The arrows indicate a change in ammonium sulphate concentration to 30% saturation.

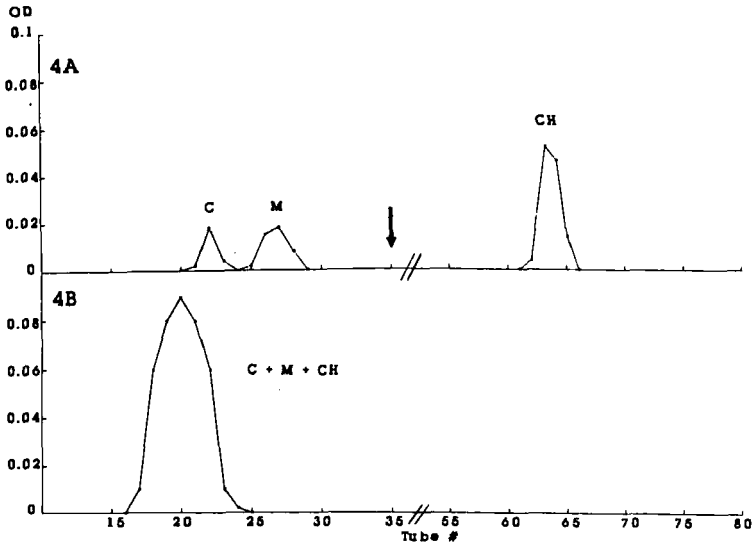

Figure 4. Elution profiles of cytochrome c (C), myoglobin (M) and chymotrypsinogen A (CH) off a Sephacryl S-200 column using 45% saturated ammonium sulphate with and without and without added glycerol (4B and 4A respectively). The arrows indicate a change in ammonium sulphate concentration to 30% saturation.

saturation of ammonium sulfate, the proteins were found to elute from other gel matrices later than when Sephacryl S-200 was used. Chymotrypsinogen A could be partially separated from the other two proteins at this saturation which was not the case with Sephacryl S-200. The elution profiles obtained at 30% and 45% saturation are shown in Figure 5. As with Sephacryl S-200, all the other gel matrices were able to resolve cytochrome c and myoglobin at 45% saturation. In addition, the extent of resolution and the elution volume of these two proteins were found to follow the same pattern: S-1000 > S-500 > S-200 > Fractogel. At 30% saturation, chymotrypsinogen A was much better resolved on other gels matrices than on Sephacryl S-200; whereas at 45% saturation, it was not eluted from any of the gels unless the ammonium sulfate concentration was reduced to 30% saturation. At the

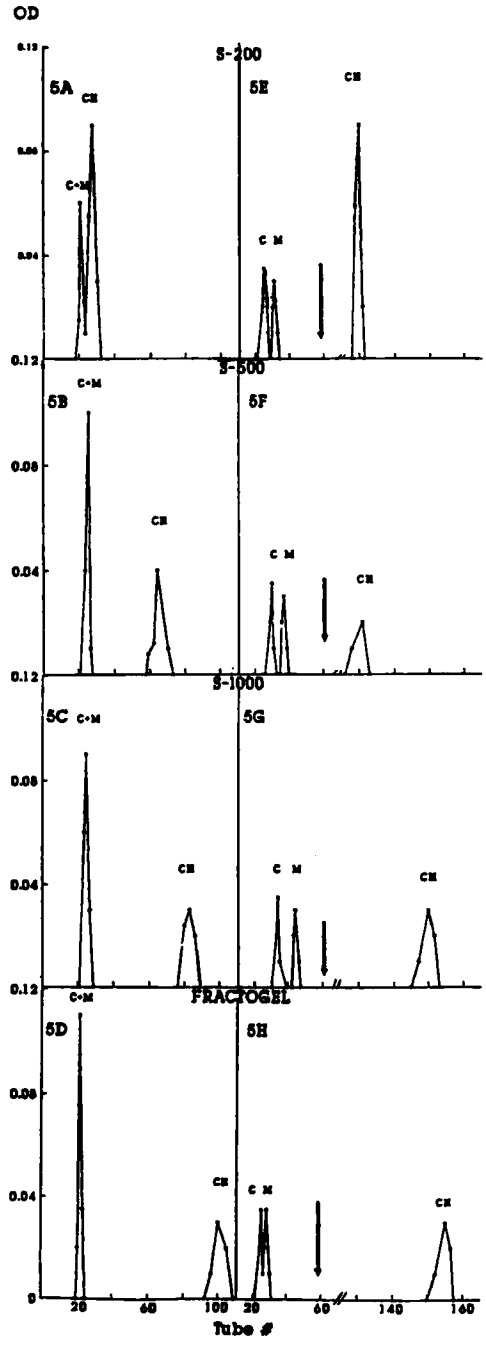

Figure 5. Elution profiles of cytochrome c (C), myoglobin (M) and chymotrypsinogen A (CH) off columns containing various Sephacryl gels and Fractogel using 30% (A-D) and 45% (E-H) ammonium sulphate. The arrows indicate a change in ammonium sulphate concentration to 30% saturation.

latter concentration, the elution volume of chymotrypsinogen A was seen to follow a series: Fractogel > S-1000 > S-500 > S-200. All these findings agree with the previous results indicating that Sephacryl S-1000 adsorbs cytochrome c and myoglobin more strongly than all the other gel matrices and that Fractogel adsorbs chymotrypsinogen A better than the Sephacryl gels (Table 1).

CONCLUSION

Sephacryl S-type gels show adsorption of proteins in the presence of high concentrations of ammonium sulfate. Such adsorption is likely to involve hydrophobic interaction. The chromatographic results presented here show that protein separation is based on the affinity of the protein to the matrix. The results also show that a variety of factors including pH, temperature, flow-rate and glycerol affect protein adsorption.

The exhibition of protein adsorption by commercial matrices used for gel-filtration chromatography has been well documented (6-9). Surprisingly, very few of them have been used as an adsorbent for protein separation. This is partly due to the lack of information on their adsorption properties. Thus, it is interesting to note that Sephacryl S-type gels have very similar adsorption properties to Fractogel, which has been useful to hydrophobic interaction chromatography (7, 9, 10, 12, 13).

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