Journal of Chromatography, 147 (1978) 205–212

CHROM. 10,296

ADSORPTION PHENOMENA ON SEPHACRYL® S-200 SUPERFINE

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(Received June 7th, 1977)

SUMMARY

The adsorption properties of Sephacryl S-200 are dependent upon the pH of the eluent buffer employed and the net surface charge of the solutes under investigation. At pH 3.5 the gel adsorbs acidic, neutral and basic proteins. By raising the pH of the eluent to 5.5 all adsorbed material can be desorbed from the gel.

At pH 5.5 the gel showed no tendency to adsorb proteins of any surface charge. At this pH the gel acts as a passive molecular sieve.

At pH 8 the gel begins to behave as a cation exchanger and strongly adsorbs any protein that is positively charged at this pH. Acidic or neutral proteins are eluted unretarded. These cation-exchange properties can be eliminated by including at least 0.2 *M* NaCl in the eluent buffer.

At low ionic strength of the eluent buffer, DNA, tRNA and rRNA are adsorbed on to the gel at pH 3.5 or 5.5 but are completely desorbed by stepwise elution with 0.5 *M* NaCl in equilibrating buffer.

The HETP values calculated for several solutes indicate that Sephacryl S-200 gives higher resolution and less zone-spreading than does Sephadex G-200.

INTRODUCTION

Sephacryl® S-200 Superfine is a new chromatographic medium recently introduced by Pharmacia Fine Chemicals, Uppsala, Sweden for molecular-sieve chromatography of proteins and other biopolymers. Its separation range is nominally equivalent to that of Sephadex G-200 Superfine. The gel is a hybrid prepared by covalently cross-linking allyl dextran with N,N'-methylenebisacrylamide, resulting in a rigid matrix that can withstand high flow-rates. Its general properties are outlined in a pamphlet published by Pharmacia[1].

Experiments done at the Institute of Biochemistry, Uppsala, Sweden, showed that the gel gives high resolution with low zone-spreading compared with Sephadex G-75 or G-200. However, the separation observed was not strictly size-dependent,

indicating that the gel does not act solely as a passive molecular sieve. These observations prompted a systematic study of the chromatographic properties of Sephacryl S-200 using a set of homologous proteins which were of essentially the same size, but which differed in isoelectric point. The efficiencies of columns (HETP values) packed with Sephacryl S-200 and Sephadex G-200 were also determined and compared using well-defined dextran fractions.

MATERIALS AND METHODS

The four homologous proteins used in this study were fractionated from the venom of the Australian snake Taipan (*Oxyuranus s. scutellatus*) by gel filtration on Sephadex G-75 (ref. 2) followed by ion-exchange chromatography on SP-Sephadex C-25. The venom was purchased from Mr. C. Tanner, Cooktown, Australia. They are designated as fractions I–IV in order of their elution from the column. Each protein is composed of about 120–130 amino-acid residues in a single polypeptide chain cross-linked by seven disulphide bridges. Their isoelectric points range from pH 4 to 12. DNA from calf thymus and rRNA and tRNA from *E. coli* B were provided by Dr. U. Hellman of the Institute of Biochemistry, Uppsala, Sweden.

Sephacryl S-200 Superfine (hydrated diameter 40–105 μm), Sephadex G-200 (particle size 40–120 μm corresponding to a hydrated diameter of 130–400 μm), dextran fractions with a narrow range of molecular weights and Pharmacia K-16 (1.6 × 40 cm) chromatographic columns were obtained from Pharmacia. All chemicals used were of analytical grade.

Column packing

Sephadex G-200 was packed in the conventional manner[3], whereas Sephacryl S-200 was packed according to a modified procedure in order to avoid shrinkage at high flow-rates. The packing operation was carried out at a flow-rate greater than the desired elution rate and resulted in a tightly and uniformly packed column. The following procedure was used: (i) insert a plunger into the bottom of the vertically mounted column; (ii) close the outlet and fill the column with a thin slurry of the deaerated gel; (iii) remove the membrane or nylon net from another plunger and insert it into the upper end of the column; (iv) connect this end to a peristaltic pump, open the lower outlet and continuously feed the column with the thin slurry at a flow-rate of *ca.* 50 $ml \cdot cm^{-2} \cdot h^{-1}$. The slurry should be gently stirred throughout the operation. Packing is complete within 90 min for a 30 cm column. (v) Remove the upper plunger, fit it with its membrane or nylon net and reinsert it into the column, which is then ready for use after equilibration with the appropriate eluent.

Chromatographic procedure

Three identical columns were packed with Sephacryl S-200 and equilibrated, respectively, with 0.05 *M* of each of the following buffers: glycine–HCl, pH 3.5; sodium acetate, pH 5.5; Tris–HCl, pH 8.0. A fourth identical column was packed with Sephadex G-200 and equilibrated with the Tris–HCl buffer, pH 8.0.

About 2 mg of a test protein was dissolved in 0.5 ml of each of the equilibrating buffers and applied to the appropriate column. The flow-rate was maintained at about 2.7 $ml \cdot cm^{-2} \cdot h^{-1}$ and fractions of 1 ml were collected. The distribution of protein

in the column effluents was determined by absorbance measurements at 276 nm, whereas the elution profile of the dextran samples was monitored by means of a refractometer.

RESULTS AND DISCUSSION

Comparison of column performance

The bed height equivalent to a theoretical plate (HETP) was calculated from the elution curves of the various dextran fractions chromatographed on each type of column under identical experimental conditions using a standard formula[4]:

$$\text{HETP} = \frac{L}{5.545}\left(\frac{w_{1/2}}{l}\right)^2$$

where L = length of the column (in cm)
l = the elution volume of the zone, and
$w_{1/2}$ = width of the elution curve (in ml) at a point corresponding to one-half of the maximum peak height.

The values are recorded in Table I.

TABLE I

COMPARISON OF THE COLUMN PERFORMANCE (HETP) FOR SEPHACRYL S-200 AND SEPHADEX G-200 USING VARIOUS DEXTRAN FRACTIONS

Experimental conditions: column, 1.6 × 32 cm; sample volume, 0.5 ml (3 mg/ml of solute); flow-rate, 2.5 ml·cm^{-2}·h^{-1}; V_0 (ml), 23 (Sephadex G-200) and 24 (Sephacryl S-200); V_t (ml), 64 (Sephadex G-200) and 57 (Sephacryl S-200); eluent, 0.05 *M* Tris–HCl, pH 8.

Solute	*Mol. wt.*	*HETP (mm)*	
		G-200	*S-200*
Dextran 20	20,000	3.2	1.3
Dextran 40	40,000	5.7	1.9
Dextran 70	70,000	4.3	1.6
Dextran 150	150,000	1.5	0.8
Chymotrypsinogen A	25,000	1.1	1.2

The HETP values vary with the type of solute and are lowest for both Sephadex G-200 and Sephacryl S-200 when higher-molecular-weight solutes are used. The values for Sephadex G-200 are consistently higher by a factor of 2 to 3 compared with Sephacryl S-200. The results show that Sephacryl S-200 gives relatively narrower elution curves and thereby better resolution than is attainable with Sephadex G-200. According to Walton[5] the theoretical lower limit for HETP that may be achieved under ideal experimental conditions (perfect packing, ideal flow conditions, *etc.*) approaches the average diameter of the particles making up the chromatographic bed. In both cases, however, the ratio of the particle diameter to HETP is greater than 10. The lower HETP observed with Sephacryl S-200 indicates that this gel has better packing properties than G-200. In any case, the results obtained confirm the impression that Sephacryl S-200 gives better resolution with less zone-spreading than Sephadex G-200.

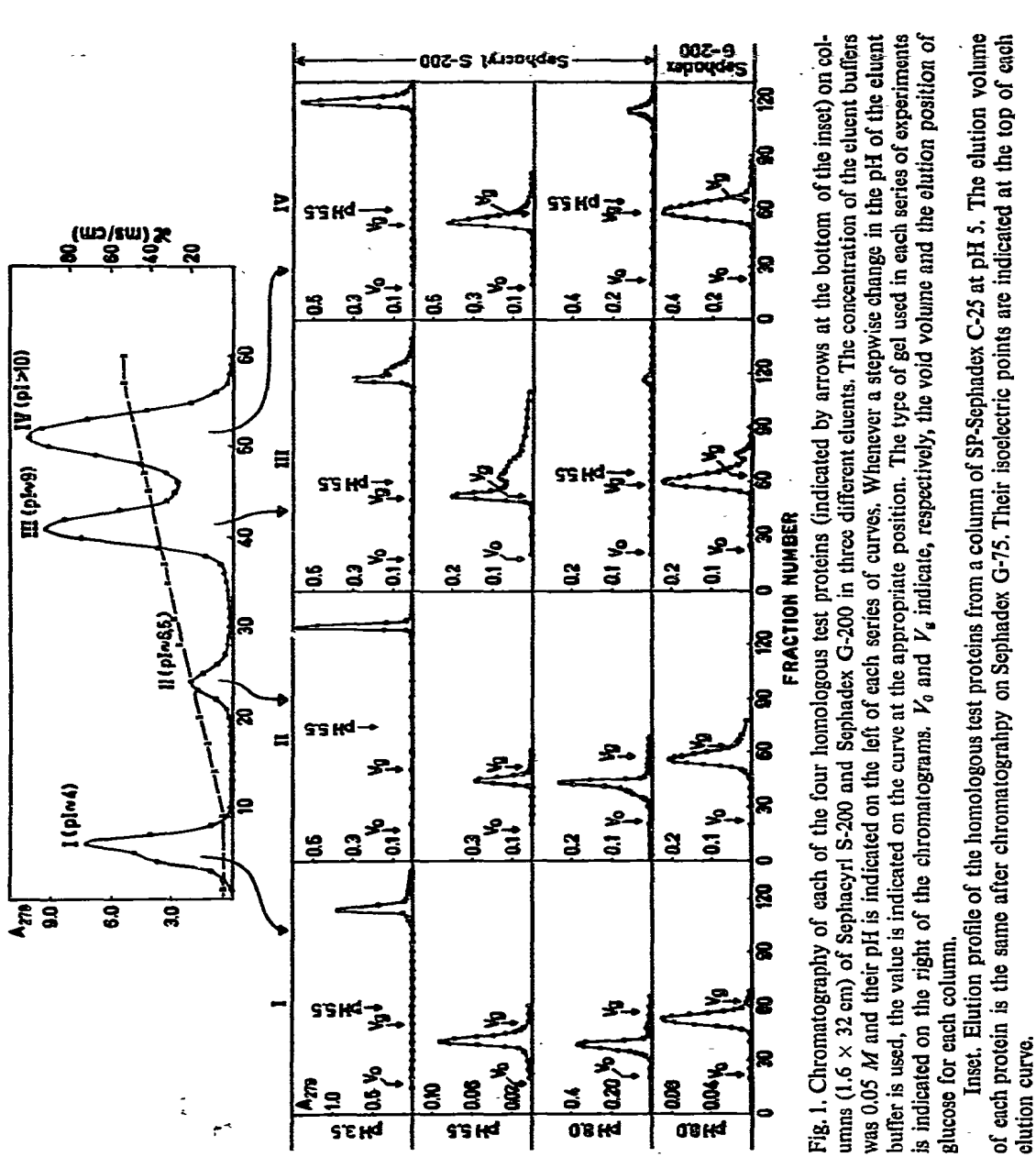

Fig. 1. Chromatography of each of the four homologous test proteins (indicated by arrows at the bottom of the inset) on columns (1.6 × 32 cm) of Sephacryl S-200 and Sephadex G-200 in three different eluents. The concentration of the eluent buffers was 0.05 *M* and their pH is indicated on the left of each series of curves. Whenever a stepwise change in the pH of the eluent buffer is used, the value is indicated on the curve at the appropriate position. The type of gel used in each series of experiments is indicated on the right of the chromatograms. V_0 and V_t indicate, respectively, the void volume and the elution position of glucose for each column.

Inset. Elution profile of the homologous test proteins from a column of SP-Sephadex C-25 at pH 5. The elution volume of each protein is the same after chromatography on Sephadex G-75. Their isoelectric points are indicated at the top of each elution curve.

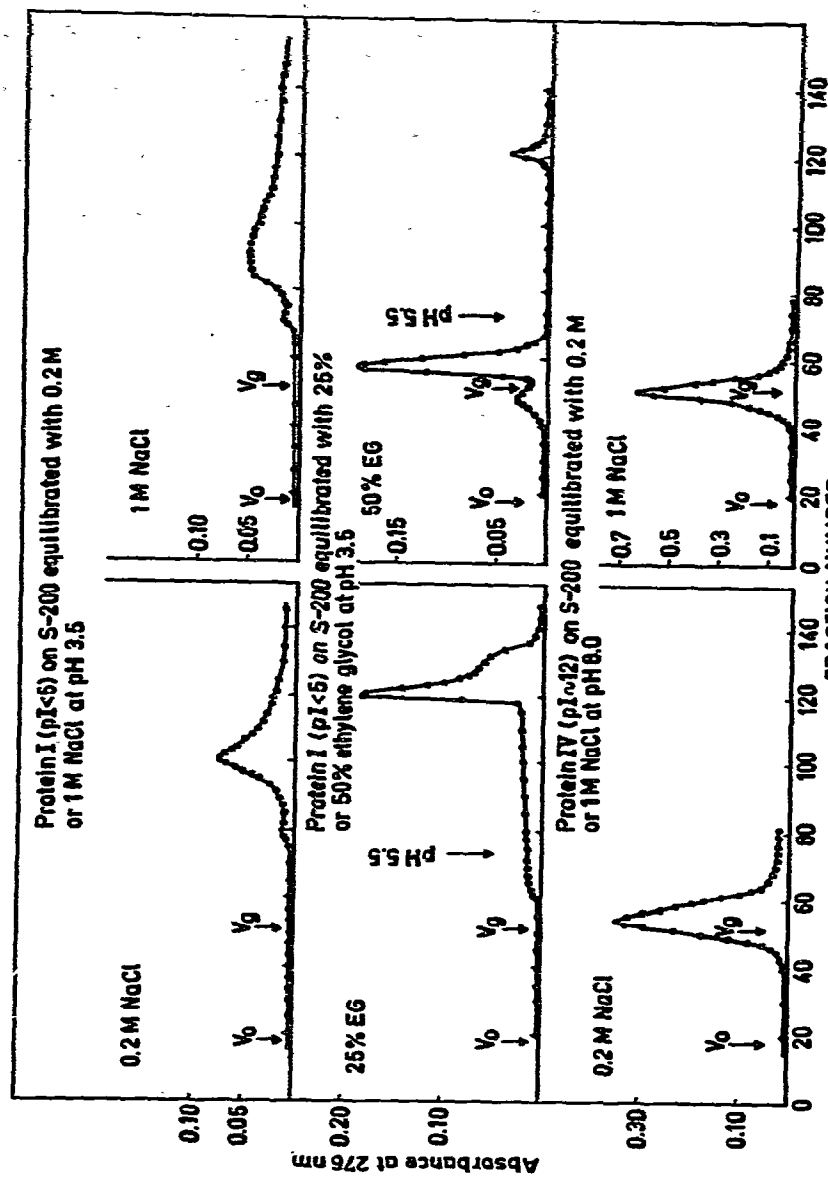

Fig. 2. Chromatography of an acid and a basic protein from the venom of the Australian snake Taipan (*Oxyuranus s. scutellatus*) on columns of Sephacryl S-200 equilibrated with various eluents. The isoelectric point (p*I*) of each protein and the type of eluents employed are indicated above each set of figures. Column dimensions, 1.6 × 32 cm; sample volume, 0.5 ml containing 2 mg protein; flow-rate, 5.5 ml/h; fraction volumes, 1.1 ml; V_o = void volume; V_e = elution volume of glucose; EG = ethylene glycol.

Chromatographic properties of Sephacryl S-200 at various pH values

The results obtained with the four test proteins on identical columns of Sephacryl S-200 equilibrated with buffers of pH 3.5, 5.5 and 8.0 are shown in Fig. 1. For comparison, the corresponding elution patterns from a similar column of Sephadex G-200 equilibrated at pH 8 are also included. The isoelectric point of the individual proteins and their elution positions from a column of SP-Sephadex C-25 equilibrated at pH 5 are inserted at the top of the figure for reference.

Chromatography at pH 3.5. Regardless of the net charge of the individual test proteins, Sephacryl S-200 equilibrated and eluted at pH 3.5 behaved as a non-specific adsorbent. None of the proteins tested could be displaced from the column by continuous elution with several column volumes of the equilibrating buffer. However, upon elution with buffer of pH 5.5, each protein was completely desorbed after passage of one column volume of the eluent. The pH of the eluent at which the different individual proteins were eluted was between 4 and 4.5. The results thus indicate that the non-selective adsorption at pH 3.5 is probably due to hydrogen-bonding effects and other non-ionic solute–gel interactions. Although it is difficult to ascertain the nature of the forces that are responsible for the observed adsorption effects on Sephacryl S-200 at pH 3.5, these results clearly show that they can be eliminated simply by increasing the pH of the eluent buffer toward neutrality.

The nature of the adsorption phenomenon at pH 3.5 was investigated further by employing different kinds of eluents to displace the adsorbed proteins (Fig. 2). Incorporation of 0.2 or 1 *M* sodium chloride in the eluent buffer weakens the gel–protein interactions but the elution patterns show considerable zone-spreading. The results show that increasing the salt concentration increases the zone-spreading. On the other hand, the inclusion of a high concentration of ethylene glycol in the eluent decreases the adsorption of solutes on to the gel. The desorption capacity increases and becomes effective at higher concentrations (*i.e.*, 50%) of the ethylene glycol. The moderate but significant retention in the presence of ethylene glycol is remarkable, an effect which could be exploited to advantage in certain types of separation work. The effects of salt and ethylene glycol indicate that at pH 3.5 the forces responsible for adsorption of proteins to Sephacryl S-200 are hydrophobic interactions and possibly also hydrogen-bonding between the solutes and the gel matrix.

Chromatography at pH 5.5. The conclusions reached above are supported by the results obtained following chromatography of each of the same test proteins on a column of Sephacryl S-200 equilibrated and eluted at pH 5.5. None of the proteins is adsorbed under these conditions and elution positions are therefore comparable to those obtained on Sephadex G-200. At pH 5.5 solute–gel interactions appear to be minimal regardless of the net surface charge of the solute under investigation. The results thus strongly suggest that Sephacryl S-200 behaves as a "passive molecular sieve" (like Sephadex G-200) in the pH range 4.5–6. If non-steric solute–gel interactions are to be avoided the gel should thus be used within this pH interval whatever the nature of the proteins to be chromatographed.

The pH dependence of the adsorption phenomena on Sephacryl S-200 can be exploited to solve various separation problems. Since the results obtained here can serve as guidelines for predicting the behaviour of the gel at acidic and near neutral pH values, its use as a separation medium can be further extended by manipulating (*e.g.*, using a pH gradient between pH 3.5 and 5.5) or eliminating (*e.g.*, by stepwise

change of eluent pH from 3.5 to 5.5) the non steric effects which are responsible for the observed adsorption. Such applications are analogous to the well-established aromatic effects that are operative on Sephadex gels[6].

Chromatography at pH 8. The elution behaviour of the test proteins on Sephacryl S-200 at pH 8 depended on the net surface charge of the proteins at that pH. Proteins I (p*I ca.* 4) and II (p*I ca.* 6.5) were not adsorbed, whereas proteins III (p*I ca.* 9) and IV (p*I ca.* 12) were adsorbed (see Fig. 1). The latter two proteins could not be displaced from the column by elution with buffer of pH 5.5, which did elute them when they had been adsorbed at pH 3.5. Even Tris was strongly adsorbed on to the gel at pH 8 and was not easily displaced by subsequent elution with sodium acetate at pH 5.5.

The adsorption of basic proteins on to Sephacryl S-200 at pH 8 strongly suggests that the gel acts as a cation exchanger, a conclusion supported by the results shown in Fig. 2 (bottom curves). When 0.2 or 1 *M* sodium chloride was included in the eluent the basic protein IV eluted from the column at the same position as was observed with an eluent of pH 5.5. Moreover, zone-spreading decreased with increasing concentration of salt in the eluent buffer. Such ionic adsorption effects on Sephacryl S-200 are evidently disadvantageous, especially when size estimates are to be made from gel filtration data in simple aqueous media or when the gel is used for separating basic substances that are positively charged at or above pH 8. If the gel is to be used as a passive molecular sieve at alkaline pH, sodium chloride must be included in the eluent buffer at a concentration of at least 0.2 *M*. On the other hand, such adsorption effects can be exploited for the group separation of solutes in a mixture based on differences in their surface charges employing simple adsorption chromatographic techniques.

Chromatography of nucleic acids. Sephacryl S-200 equilibrated with 0.05 *M* buffer at pH 3.5 or 5.5 was found to adsorb DNA, rRNA and tRNA. The adsorbed solutes could not be displaced by elution with 50% ethylene glycol in equilibrating buffer as was the case for proteins adsorbed at pH 3.5 (see Fig. 2). However, upon stepwise elution with 0.5 *M* NaCl in the equilibrating buffers the adsorbed solutes were completely displaced. Since polysaccharides and all the model proteins used in this study were not adsorbed onto the gel at pH 5.5, these results indicate that proteins and polysaccharides can be separated from nucleic acids by simple elution chromatography on Sephacryl S-200 at low ionic strength.

CONCLUSIONS

Although this investigation is not exhaustive, the results serve as guidelines for predicting the behaviour of Sephacryl S-200 at various pH values which are commonly used in separation work with biological macromolecules. The use of homologous proteins of the same size but different isoelectric points facilitated the study of non-steric factors which could influence chromatographic behaviour on Sephacryl S-200. The results show that solute–gel interactions are minimal at or near neutral pH values. At acidic pH the gel acts as a non-specific adsorbent for all of the test proteins. At pH 8 the gel behaves as a cation exchanger and adsorbs only basic proteins.

The exhibition of such adsorption properties by a gel designed to serve as a

molecular-sieving medium for macromolecules might at first seem disadvantageous. However, these results show how the gel can be used with advantage either as a passive molecular sieve or as a cation exchanger for the group separation of basic from acidic or neutral proteins simply by varying the pH of the eluent buffer. However, the indiscriminate adsorption of all proteins at pH 3.5, irrespective of differences in surface charge, makes the gel unsuitable for separation work at this pH (except possibly for group separation) unless the adsorbed proteins can be selectively fractionated by use of pH gradients. The high resolution and reduced zone-spreading obtainable on Sephacryl S-200 might be due to a combination of its molecular-sieve and ionic adsorption properties. An understanding of the adsorption phenomena on Sephacryl S-200 as outlined here will thus make it possible to manipulate, or eliminate at will, the adsorption effects on the gel according to the requirements of the separation problem at hand.

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