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HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS AND PEPTIDES ON SPHERON P-300

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SUMMARY

Hydrophobic interaction chromatography of proteins and peptides on Spheron P-300 has been studied. Proteins such as human serum albumin, chymotrypsinogen and lysozyme are retained by this support; the higher the concentration of the salt added, the more protein is retained. The sorption and desorption were examined quantitatively with lysozyme. The theoretical plate height and its dependence on temperature during hydrophobic interaction chromatography of the lysozyme were determined. The hydrophobic interaction of the support was demonstrated by hydrophobic interaction chromatography of the protein components of human serum proteins, hog pancreatic amylase extract, and a peptide mixture obtained from tryptic digests of lysozyme. The effect of pH on the elution of human serum albumin, chymotrypsinogen and lysozyme was observed.

The fractionation of crude hog pancreatic amylase by gradient elution was used to demonstrate the effect of alcohols as polarity-reducing agents. The properties required of materials used for hydrophobic interaction chromatography of biopolymers are discussed.

INTRODUCTION

The first examples of the biochemical application of reversed-phase liquid column chromatography, *i.e.* a separation technique that, for solute sorption, makes use of a stationary phase with a polarity lower than that of the eluant, were reported around 1950[1,2]. This separation technique, one modification of which was named "salting-out chromatography" by Tiselius[2], has sometimes been called "hydro-

phobic interaction"[33] or "hydrophobic salting-out"[4] chromatography, when relatively polar, water-soluble low molecular weight compounds were separated; it has also been called "solvophobic" chromatography[5]. Hydrophobic interaction chromatography has not been as widely applied in the separation of proteins as have, for example, ion-exchange and gel permeation chromatography. Protein interactions with non-polar groups of the stationary phase during column chromatography have received increased attention lately in studies on the effect of hydrophobic spacing arms in affinity chromatography[6–13]. A number of papers have appeared devoted to hydrophobic interaction chromatography on long-chain alkyl derivatives of agarose prepared after activation of the support with cyanogen bromide[6–25]. When such supports bear both hydrophobic and polar charged groups, hydrophobic interaction and ion exchange can operate against each other. This led to the preparation of uncharged agarose derivatives and to studies of the effect on the sorption and elution of proteins of the size and density of the non-polar substituents. The possible denaturation of proteins, their yields, and the effect of type of salt and its concentration on the hydrophobic interaction have also been investigated[3,4,15,25–28]. Far less attention has been paid to synthetic polymers. The effect of hydrophobic interactions of alcohol dehydrogenase with copolymers of acrylamide and methylacrylate of different composition and hydrophobicity on the action of the enzyme after its binding to the polymer has been studied[29].

An earlier paper described hydrophobic interaction chromatography of uracil alkyl derivatives on a Spheron support[30], *i.e.* a macroporous, highly cross-linked copolymer of 2-hydroxyethyl methacrylate and ethylene glycol dimethacrylate. Because of the presence of the hydroxyl groups supplied by the former co-monomer, this support is sufficiently hydrophilic to swell in aqueous solutions. On the other hand, the aliphatic polymer backbone provides numerous non-polar binding sites for hydrophobic interactions. The retention of uracil alkyl derivatives by a Spheron P-300 column, expressed in ln K_D, was found to be proportional to the area of the non-polar surface of the individual derivatives[30].

The possibility of separating proteins by hydrophobic interaction chromatography on Spheron P-300 is examined in this paper.

EXPERIMENTAL

Chemicals

All the chemicals used were of analytical purity grade (Lachema, Brno, Czechoslovakia). *tert.*-Butyl alcohol (for chromatography), methanol, ethanol and ethylene glycol (spectral grade) were also supplied by Lachema.

Human serum albumin (HSA; min. cont. 98% according to electrophoresis) was obtained from the Institute of Sera and Vaccines, Prague. The HSA contained 2.1 mole of fatty acids according to gas–liquid chromatography. After the charcoal treatment[31] the fatty acid content dropped to 0.1. Chymotrypsinogen A (from bovine pancreas) (CHTG), recrystallized five times, and lysozyme (LZ), twice recrystallized, were obtained from Worthington N.J., U.S.A.*, and human blood plasma from

* Certain other CHTG preparations, though identical in *N*-terminal end-groups, amino-acid composition and activity, were not homogeneous when subjected to hydrophobic interaction chromatography.

the Institute of Sera and Vaccines. Crude hog pancreatic amylase was prepared according to Caldwell[32]. α-Amylase was prepared from hog pancreas by the method of Fischer[33], recrystallized five times and lyophilized. The tryptic digest of lysozyme was prepared according to a reported method[34], except that incubation was prolonged five times.

Chromatography material

Spheron P-300™, particle size 20–40 microns, was obtained from Lachema. The sorbent was extracted and traces of carboxylic groups removed by reaction with diazomethane, as described elsewhere[35]. The exclusion limit of the material was 500,000 daltons for polydextran. Additional characteristics of the material, such as distribution of particle size, pore size, internal surface area, solvent regain, column permeability, etc., have been previously reported[35]. Spheron P-1000™ (exclusion limit for polydextran 1,000,000 daltons) was of the same origin.

Equipment

The chromatographic apparatus consisted of a type 68005 proportional minipump connected to a 2-ml forecolumn, a 75-μl sample injection loop, a 420 × 6.28 mm I.D. precision bore glass column with adjustable column ends, and a thermostated water jacket. The absorbance was recorded in an absorbance monitor (type DVD, 254 nm) or in a spectral analyser (type UVM-4), both manufactured by the Instrument Development Workshops of the Czechoslovak Academy of Sciences. An RI detector (Knauer 2050) or conductivity monitor (30-μl LKB-53215 flow cell combined with an OK-102/1 conductometer) (Radelkisz, Hungary) were used.

Methods

The column was packed by the slurry technique in 20% $(NH_4)_2SO_4$ at a flow-rate of 930 $ml \cdot h^{-1} \cdot cm^{-2}$. Interparticle porosity[36] was determined in water using 1% Dextran T-2000 solution (Pharmacia, Uppsala, Sweden). Void volumes[36] for proteins were calculated from the elution volumes of HSA and LZ determined in a mixture of 20% (v/v) of methanol in 0.01 *M* sodium phosphate, pH 6.20, containing 0.2 or 0.4 *M* Na_2SO_4. Disc electrophoreses were carried out in the apparatus (GE-4) manufactured by Pharmacia, and the gels were stained with Coomassie Brillant Blue G 250 (Serva, Heilberg, G.F.R.).

Amylase activity was determined using starch (Merck, Darmstadt, G.F.R.) according to the method of Zulkowski and using 3,5-dinitrosalicylic acid (analytical grade, Lachema) according to the method described by Bernfeld[37].

Protein concentrations in effluents were measured spectrophotometrically at 280 nm in an Opton PMQ-II spectrophotometer.

pH Values were measured by a combined glass–calomel electrode (type GK-2302-C; Radiometer, Copenhagen, Denmark). Corrections for high salt content or for organic solvents were not made[38], and therefore some of the pH values obtained are only approximate.

RESULTS

Differences in the content and hydrophobicity of amino acids on the surface of molecules of globular proteins are mostly responsible for differences in their interactions with the non-polar groups of the stationary phase and for differences in their retention times during hydrophobic interaction chromatography.

The values of distribution coefficients, K_D, characterizing hydrophobic interaction chromatography of some proteins on Spheron P-300 at various salt concentrations of elution buffer at constant pH and temperature are given in Table I. It is obvious from these values that the retention of the proteins examined increases with increasing KCl concentration (increasing K_D), and that the accessible non-polar surface area of these proteins increases in the order HSA < CHTG < LZ < α-amylase. There are considerable differences in the K_D values of the individual proteins even though the values of hydrophobicity indexes calculated from total amino-acid composition differ only a little[39]. HSA is known to adsorb a number of substances, some of them probably by hydrophobic forces, but it is relatively weakly adsorbed on the column. The retention is the same for HSA both before and after treatment to remove fatty acids. It was suggested that HSA consists of three domains, each containing two trough-like subdomain structures formed by three helical regions[40]. Models show that contacts between helices and the inside trough are hydrophobic,

TABLE I

DISTRIBUTION COEFFICIENTS K_D FOR HYDROPHOBIC CHROMATOGRAPHY OF LYSOZYME (LZ), CHYMOTRYPSINOGEN (CHTG), HUMAN SERUM ALBUMIN (HSA) AND α-AMYLASE ON SPHERON P-300 AT DIFFERENT SALT CONCENTRATIONS

Enzyme	*Salt concentration*	K_D
LZ	without salt	0.3
	0.2 *M* KCl	1.34
	0.4 *M* KCl	1.95
	0.6 *M* KCl	2.58
	0.85 *M* KCl	3.45
	1.0 *M* KCl	4.29
CHTG	0.6 *M* KCl	0.41
	0.75 *M* KCl	0.85
	0.85 *M* KCl	1.24
	1.5 *M* KCl	3.16
	2.0 *M* KCl	3.69
HSA	1.0 *M* KCl	0
	2.0 *M* KCl	0.03
	3.0 *M* KCl	0.06
	4.0 *M* KCl	0.26*
	0.4 *M* Na_2SO_4	0
	0.6 *M* Na_2SO_4	0.18
	0.7 *M* Na_2SO_4	0.37
	0.8 *M* Na_2SO_4	1.27*
α-Amylase	without salt	>20

* For the fast-moving peak. All experiments were done in 0.01 phosphate buffer. pH reading always 6.13, column 240 × 6.28 mm, Spheron P-300. Flow-rate 40 ml/h; temperature 20°.

whereas the outside trough is hydrophilic[40]. Such a structure would enable HSA to interact strongly with small molecules but only slightly with a rigid internal surface of the gel. We have observed a stronger interaction on exposing HSA to thermal or pH destabilization. LZ and α-amylase especially are characterized by a very high content of tryptophan residues, several times greater than the average content of tryptophan in proteins[41,42]. The strong interaction of α-amylase with the gel could be caused by some of these residues being located on the surface of the protein molecule.

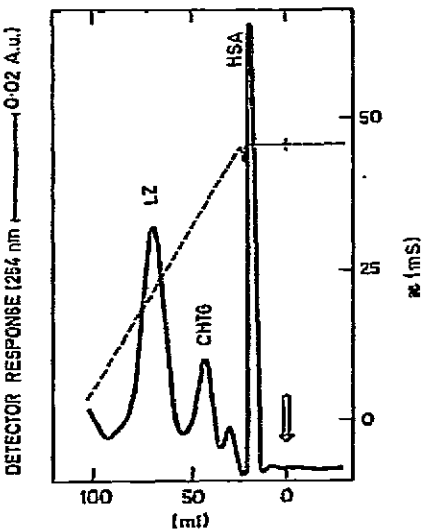

Fig. 1. Separation of human serum albumin (HSA), chymotrypsinogen (CHTG), and lysozyme (LZ) by hydrophobic interaction chromatography on Spheron P-300. Column 250 × 6.28 mm, flow-rate 68.5 ml/h; temperature 25°. Linear gradient of 50 ml of buffer A plus 50 ml of buffer B. Buffers: A, 0.75 mole/l of KCl in 0.02 *M* sodium acetate, pH 5.65; B, 0.02 *M* sodium acetate, pH 5.65. Solid line, absorbance at 254 nm; broken line, conductivity of effluent.

The differences in the K_D values for HSA, CHTG, and LZ were used for separation of these proteins by hydrophobic interaction chromatography (Fig. 1). The sorption capacity of Spheron P-300 for lysozyme is shown graphically in Fig. 2. Lysozyme was applied to the column in a buffer containing 2.5 mole/l of KCl, the protein concentration being 2.5 mg/ml. The column was subsequently washed by the same buffer (18 times the column volume). The peak in Fig. 2 represents the elution of sorbed lysozyme after application of the buffer without KCl. The capacity of Spheron P-300 for lysozyme (*ca.* 50 mg/g support) was determined in the effluent. It was shown in a similar experiment where the amount of lysozyme applied was much less than the column capacity, that the desorption of LZ was quantitative. The recovery, repeatedly determined spectrophotometrically, was 100 ± 4%.

Fig. 3 shows the separation of a mixture of human plasma components on Spheron P-300. The elution profile is similar to that obtained with Phenyl-Sepharose® CI-4B (ref. 43). In this case the separation is inferior to that obtained using ion-exchange chromatography[44,45].

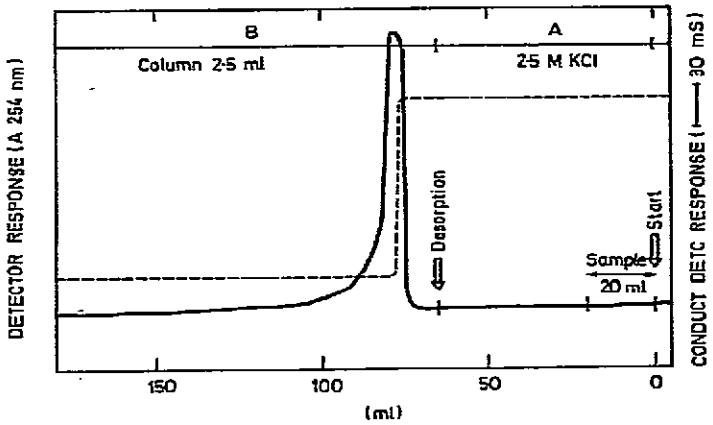

Fig. 2. Sorption of lysozyme on Spheron P-300. Column 65 × 7.0 mm (2.5 ml); 50 mg of lysozyme in 20 ml 0.02 *M* phosphate buffer containing 2.5 mole/l of KCl, pH 6.20 (A) was applied. The column was subsequently washed with 45 ml of 0.02 *M* phosphate buffer containing 2.5 mole/l of KCl, pH 6.20 (A). Desorption of lysozyme was effected by 0.02 *M* phosphate buffer without KCl, pH 5.60 (B). Flow-rate 40 ml/h; temperature 20°. Solid line, absorbancy at 254 nm; broken line, conductivity of effluent.

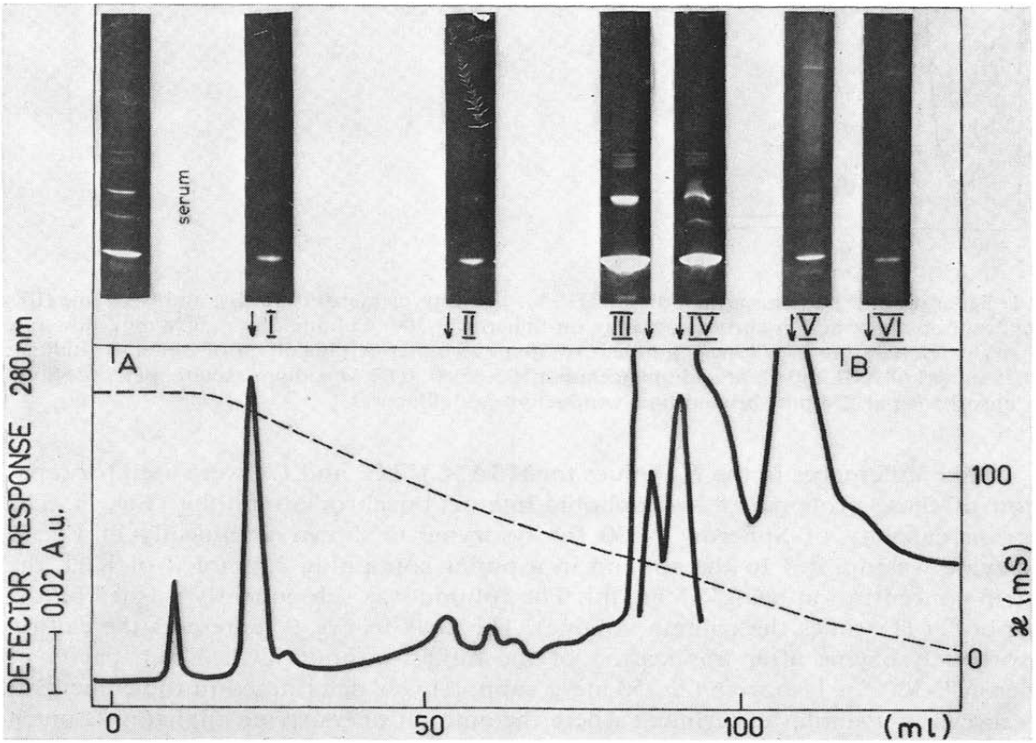

Fig. 3. Separation of components of human serum proteins by hydrophobic interaction chromatography on Spheron P-300. Column 250 × 6.28 mm, flow-rate 37.5 ml/h, temperature 20°. Sample loading, 100 µl of plasma. Linear gradient of 60 ml of buffer A plus 60 ml of buffer B. Buffers: A, 36% (w/w) of $(NH_4)_2SO_4$ in 0.05 *M* phosphate buffer, pH 6.62; B, 50% (v/v) of ethylene glycol in 0.05 *M* phosphate buffer, pH 6.62. After the elution by the gradient the column was eluted by 25 ml of buffer B. Continuous line, absorbancy at 280 nm; dashed line, conductivity of effluent. Gradient gel electrophoresis of fractions was performed on Pharmacia Gradient Gel PPA/30, after concentration of the fractions on an Amicon UM-20 membrane and desalting on a Sephadex G-15 column. Electrophoretic results are numbered with respect to the fractions analysed.

Fig. 4 shows the separation of crude amylase prepared from hog pancreas according to the method of Caldwell[32] by hydrophobic interaction chromatography on Spheron P-300. Three linear gradients, containing 20% (w/w) of $(NH_4)_2SO_4$ in starting buffer to 60% (v/v) of *tert.*-butyl alcohol in terminal buffer, were used. Hog pancreatic α-amylase is an enzyme with strong hydrophobic interactions and is therefore eluted at a very high alcohol concentration of the eluent. This is probably caused by high tryptophan content[41,42], localized at least partly on the surface of the molecule, because the average hydrophobicity index of α-amylase does not exceed a mean value[39]. We tested the activity of hog pancreatic α-amylase in a medium with a high content of an alcohol of low polarity. We did not observe any decrease of activity after incubation for 1 h with up to 60% (v/v) of *tert.*-butyl alcohol in 0.2 *M* phosphate (pH 6.62).

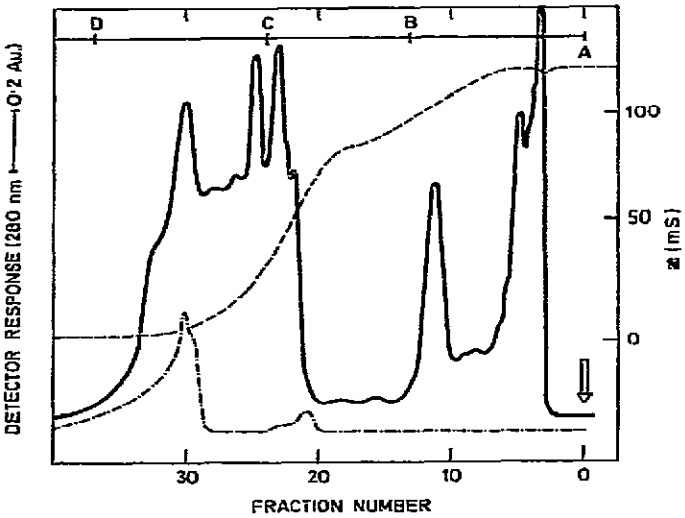

Fig. 4. Hydrophobic interaction chromatography on Spheron P-300 of components of crude hog pancreatic amylase, prepared according to Caldwell[32]. Column 242 × 6.28 mm, flow-rate 16.5 ml/h, temperature 20°. Sample loading, 5 mg of protein mixture in 200 μl of starting buffer. Three linear gradients were used: first gradient, 10 ml of buffer A plus 10 ml of buffer B; second gradient, 10 ml of buffer B plus 10 ml of buffer C; third gradient, 10 ml of buffer C plus 10 ml of buffer D. The elution of the column was completed by 25 ml of buffer D. Buffers: A, 20% (w/w) of $(NH_4)_2SO_4$ in 0.1 M phosphate buffer, pH 6.59; B, 5% (w/w) of $(NH_4)_2SO_4$ in 0.1 M phosphate buffer, pH 6.59; C, 40% (v/v) of *tert.*-butyl alcohol in the same buffer; D, 60% (v/v) of *tert.*-butyl alcohol in the same buffer. ———, conductivity of effluent; —·—·—, amylase activity.

In the separation of amylase from a crude hog pancreatic extract (Fig. 4) enzyme activity was detected only in effluent with a relatively high content of the polarity-reducing agent (50% (v/v) of *tert.*-butyl alcohol). This fractionation was performed at a pH close to the isoelectric point of α-amylase, where the solubility of the enzyme is low. Another fractionation of the crude amylase at lower pH, where the enzyme is much more soluble, is shown in Fig. 5. In this case amylase was also eluted at a relatively high alcohol concentration of eluent. Chromatography of pure, crystallized α-amylase from hog pancreas (Fig. 5) afforded three main components

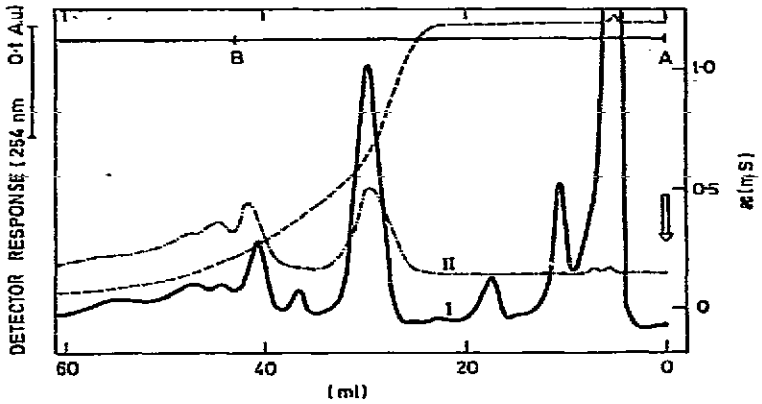

Fig. 5. Separation of crude hog pancreatic amylase, prepared according to Caldwell[32], by hydrophobic interaction chromatography on Spheron P-300. Column 235 × 6.28 mm, flow-rate 40 ml/h, temperature 20°. Sample loading, 5 mg of proteins in 200 µl of starting buffer after centrifugation. Linear gradient of 22 ml of buffer A plus 22 ml of buffer B, subsequently elution by 50 ml of buffer B. Buffers: A, 0.5 *M* acetate buffer, pH 3.00; B, 50% (v/v) of *tert.*-butyl alcohol in buffer A. — — —, conductivity of effluent; — · — · —, elution profile of crystallized and lyophilized α-amylase.

differing in hydrophobicity. Hydrophobic interaction chromatography is relatively effective for the separation of crude amylase components of considerably different hydrophobicity. Crude amylase (characterized by the disc electrophoresis pattern shown in Fig. 6) has been used before for testing the isoelectric free-flow fractionation apparatus[46]. The separation achieved by hydrophobic chromatography (Figs. 4 and 5) is better, even when the amount of sample applied is relatively large. The fractionation of crude amylase shown in Fig. 4 was carried out with loadings of from 1 to 30 mg per 7.5 ml of column without a noticeable loss of resolving power.

Strong interactions of amylase with non-polar surface areas of the Spheron support can be exploited for the immobilization and one-step isolation of the enzyme (Fig. 6). Crude amylase in 0.02 *M* phosphate (pH 6.62) was applied to a small, 2-ml Spheron P-300 column until the column was saturated with sorbed proteins (determined by absorbance and activity assays of effluent). Less hydrophobic proteins were eluted by the same buffer, and hydrophobic proteins, *i.e.* mainly α-amylase, after the addition of 45% (v/v) of *tert.*-butyl alcohol to the buffer. A 15–30 fold enrichment of the enzyme was thus achieved, judging by the specific activity of the α-amylase. The purification is indicated by the disc electrophoresis shown in Fig. 6.

Whereas the literature on hydrophobic interaction chromatography of proteins is relatively rich, the method has been little exploited for the separation of peptides even though it is equally promising in this field. The possibilities of hydrophobic interaction chromatography of peptides on Spheron P-300 are indicated in Fig. 7, which shows the separation of a tryptic digest of lysozyme.

The tryptic digestion of lysozyme leads to the formation of small peptides. If isocratic elution of these peptides is carried out (using a medium of low polarity, *i.e.* water or alcohol–water mixtures), all the peptides are eluted in small elution volumes (5.0–5.8 ml) with the gel acting as a molecular sieve. When a buffer with a high concentration of a salt is used for elution, the peptides are mostly retained in the column.

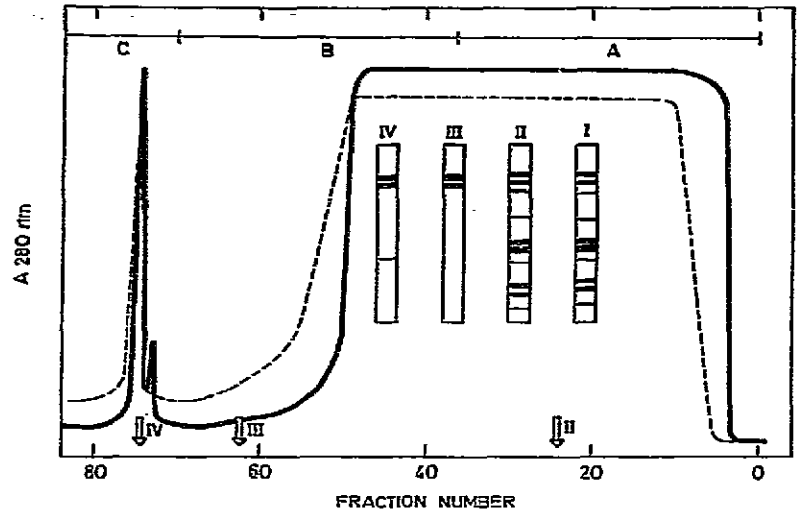

Fig. 6. Stepwise isolation of α-amylase from hog pancreatic α-amylase, prepared according to Caldwell[32]. A 50 mg sample of crude amylase in 30 ml of 0.02 *M* phosphate buffer, pH 6.62, was applied to a 35 × 7.0 mm column of Spheron P-300. The pH of the sample solution was adjusted to 9.5, and after 5 min, to 6.50. Flow-rate 40 ml/h, temperature 20° (A). As soon as the specific activity became constant, the column was eluted by the buffer until the absorbance at 280 nm dropped below 0.02 (B). Elution was then performed with the buffer, which contained 45% (v/v) of *tert.*-butyl alcohol (C). Full line, absorbance at 280 nm; dashed line, relative amylase activity. The purification of amylase is documented by disc electrophoreses, numbered with respect to the site from which samples were taken. I, disc electrophoresis of crude hog pancreatic amylase.

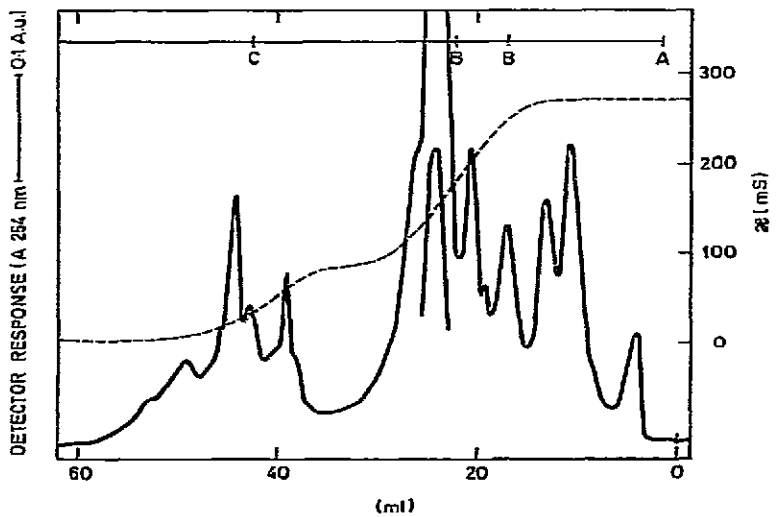

Fig. 7. Elution profile of peptides absorbing at 254 nm, obtained by separation of a tryptic digest of lysozyme by hydrophobic interaction chromatography on Spheron P-300. Column 215 × 6.28 mm, flow-rate 40.0 ml/h, temperature 20°. Sample loading, 3.5 mg of peptides in 200 μl of starting buffer. Three linear gradients were used: first gradient, 10 ml of buffer A plus 10 ml of buffer B; second gradient 10 ml of buffer B plus 10 ml of buffer C. Elution was continued by 15 ml of buffer C plus 15 ml of buffer D. Buffers: A, 17.5% (w/w) of $(NH_4)_2SO_4$ in 0.05 *M* phosphate buffer, pH 6.67; B, 5% (w/w) of $(NH_4)_2SO_4$ in the same buffer; C, 50% (v/v) of *tert.*-butyl alcohol in 0.05 *M* phosphate buffer, pH 6.67; D, 65% (v/v) of *tert.*-butyl alcohol in the same phosphate buffer. Broken line, conductivity of effluent.

The peptides are not eluted even in volumes many times exceeding the elution volumes of small inert solutes like glucose or KCl. The peptides are eluted when the polarity of the eluent falls. Because of the very high initial ionic strength and a very low content of ionic groups in Spheron gels, the contribution of ion-exchange interactions to the retention of peptides is negligible. Ionic interactions could only participate when the salt concentration is low. However, we have not observed such an effect, even for basic amino acids such as arginine or proteins such as lysozyme. Other types of interaction (such as Van der Waals or charge dipole–induced dipole) could be hardly strong enough to retain various peptides to the observed extent.

Generally, the hydrophobic interaction effect results rather from various polar interactions of the surrounding medium than from the interactions of the hydrophobic species. Hydrophobic interactions are enhanced in polar media, and weakened in less polar solvents. This behaviour was observed for interactions of peptides with the Spheron support. We can thus surmise that the peptides separated as shown in Fig. 7 are mostly retained on Spheron by hydrophobic interactions. Up to now little attention has been paid to the effect of the fundamental conditions, such as pH, temperature, type of eluent, etc., on the chromatographic process.

Effect of pH

We examined the effect of pH on the elution of HSA, CHTG and LZ at constant salt concentration (Fig. 8). We observed increased retention with decreasing pH for all three proteins studied. There is little variation in the middle pH region, but the effect is more significant at pH values below 5.5.

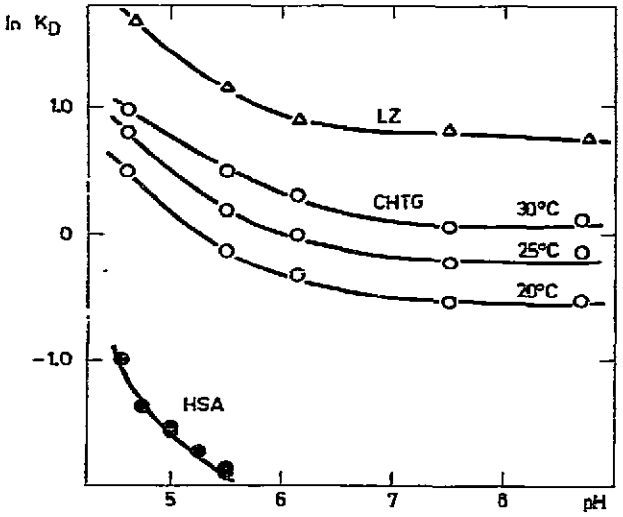

Fig. 8. Dependence of distribution coefficients K_D for hydrophobic chromatography of chymotrypsinogen (CHTG), human serum albumin (HSA) and lysozyme (LZ) on the pH of the elution buffer. Column 240 × 6.28 mm, Spheron P-300; flow-rate 40 ml/h. CHTG (circles), 0.75 mole/l of KCl in 0.01 *M* phosphate buffer; HSA (full points), 0.7 mole/l $(NH_4)_2SO_4$ in 0.01 *M* phosphate buffer; LZ (triangles), 0.6 mole/l of KCl in 0.01 *M* phosphate buffer. Data for LZ and HSA were recorded at 20°.

A similar observation with Octyl-Sepharose in the pH range 7–9 has been reported[25].

Effect of temperature

The temperature dependence of K_D for CHTG is shown in Fig. 8. The retention of CHTG and also of HSA is higher at higher temperatures in pH region 5–9 and at temperatures between 20 and 40°. An opposite effect was observed for lysozyme. Lysozyme is slightly less adsorbed on Spheron P-300 when the temperature is raised from 20 to 40°.

Effect of eluent

To decrease the polarity of the eluent used for elution of more hydrophobic proteins during hydrophobic interaction chromatography, a number of water-soluble organic compounds were used, such as aliphatic alcohols and amines, acetone and ionic and non-ionic detergents[20,25]. We also examined the effect of the organic, polarity-reducing phase during gradient hydrophobic interaction chromatography of proteins on Spheron P-300. The fractionation of crude hog pancreatic amylase was effected by *tert.*-butyl alcohol (Figs. 4, 5 and 6), and the elution profiles obtained with methanol, ethanol, and ethylene glycol (Fig. 9) were compared and found to be very similar. The best separation was observed in ethylene glycol (Fig. 9), because

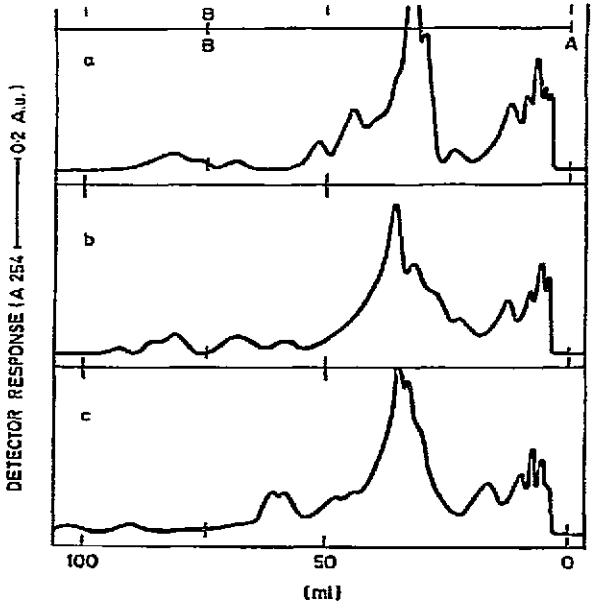

Fig. 9. Elution profiles of components of crude hog pancreatic amylase, prepared according to Caldwell[32], resulting from separation of the enzyme by hydrophobic interaction chromatography on Spheron P-300. Various alcohols were used as polarity-reducing agents: a, ethanol; b, ethylene glycol; c, methanol. Column 240 × 6.28 mm, flow-rate 40.0 ml/h, temperature 20°. Sample loading, 5 mg of amylase in 100 µl of starting buffer. The elution was always effected by a linear gradient of 40 ml of buffer A plus 40 ml of buffer B. Buffers: A, 35% (w/w) of $(NH_4)_2SO_4$ in 0.05 *M* phosphate buffer, pH 6.67; B, 50% (v/v) of alcohol in 0.05 *M* phosphate buffer, pH 6.67.

the polarity gradient shows the smallest slope; this was favourable for the separation of the given mixture.

Efficiency of chromatographic support

In the preceding paper[30] we described the dependence of the reduced theoretical plate height (h_r) on reduced linear flow velocity (v_r) and temperature for solvophobic chromatography of uracil alkyl derivatives on Spheron P-300. These functions are plotted for a high molecular weight solute, lysozyme, in Figs. 10 and 11. The efficiency of separation of low molecular weight compounds (uracil derivatives, curve I) and of lysozyme (curve II) as a function of v_r (from 0.37 mm/sec to 3.5 mm/sec) is shown for the same support in Fig. 10. The curves are relatively flat, so separation is rapid without much loss of efficiency. Curve II is shifted toward higher h_r values than curve I because the measurements with uracil derivatives were made at 60° whereas those with lysozyme were carried out at 20°. From the curves characterizing the efficiency of separation of uracil alkyl derivatives[30] and of lysozyme (Fig. 11) as function of temperature, it can be concluded that the efficiency of the Spheron P-300 column (exclusion limit 500,000), calculated from reduced magnitudes, is similar for low molecular weight solutes (uracil alkyl derivatives, mol. wt. 116–168) and for high molecular weight solutes (lysozyme, mol. wt. 14,000, monomer, or 28,000 for dimer, at pH 4–9)[48]. When hydrophobic interaction chromatography of lysozyme on Spheron P-300 (Fig. 11) was examined from the viewpoint of dependence of theoretical plate height on temperature, we observed that the separation efficiency increases (the theoretical plate height decreases) with increasing temperature. This is most likely caused by an increase of the diffusion rate in the stationary phase. This effect cannot

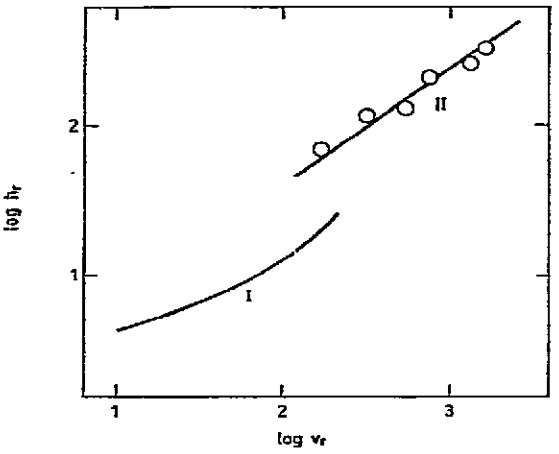

Fig. 10. Reduced theoretical plate height (h_r) for hydrophobic interaction chromatography of lysozyme on Spheron P-300 as a function of linear reduced flow velocity (v_r). Column 240 × 6.28 mm, temperature 20°. Sample loading, 0.5 mg of lysozyme in 75 μl of elution buffer. The elution was effected by 0.6 mol/l of KCl in 0.02 *M* phosphate buffer, pH 6.62. Curve I shows the dependence determined with alkyl derivatives of uracil[30], curve II the dependence determined with lysozyme. The following values were plotted: maximal diameter of Spheron particles 40 μm; interparticle porosity 0.314; diffusion constant $7.25 \cdot 10^{-6}$ cm²/sec for uracil derivatives and $8.6 \cdot 10^{-7}$ cm²/sec for lysozyme[47].

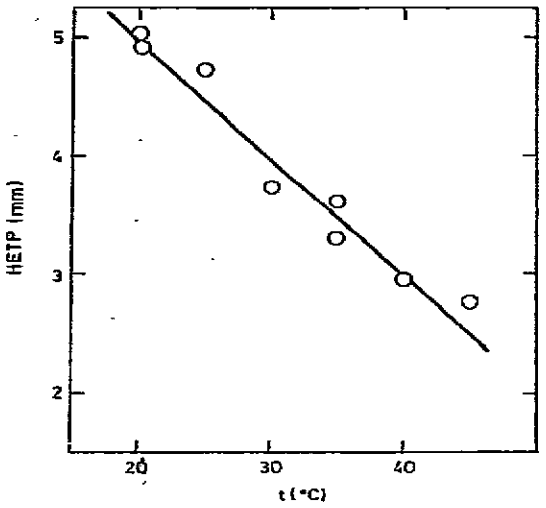

Fig. 11. Height equivalent of theoretical plate (HETP) as a function of temperature for hydrophobic chromatography of lysozyme on Spheron P-300. Flow-rate 40.0 ml/h. See legend to Fig. 10 for the other conditions.

be made use of for the separation of native proteins because of their thermal lability, but it could, however, be exploited for the separation of peptides or denatured proteins.

DISCUSSION

Two opposing properties must be balanced in the chromatographic material used for hydrophobic chromatography of biological substances (mostly proteins and peptides). The material must be hydrophilic enough to become deterged in aqueous media where biopolymers and their fragments are dissolved and where hydrophobic interactions can take place. On the other hand, the material must have a sufficient number of hydrophobic binding sites interacting during chromatography. These binding centres should be as homogeneous as possible and should yield the same, appropriate, binding power (corresponding to mean K_D values) in order to keep denaturation to a minimum. Besides these more or less specific requirements, the material should possess all the other features of good chromatographic material, *i.e.* it should have good flow and mechanical properties, chemical stability, etc. It has been shown with the alkyl derivatives of Sepharose that a small number of lipophilic groups means a low binding capacity whereas a high number decreases the deterge-ability of the gel, and that a big non-polar moiety increases the risk of denaturation[25,27].

We may conclude from the results presented here that Spheron P-300 has most of the features required for hydrophobic interaction chromatography of biopolymers. The great number of hydroxyl groups supplied by the 2-hydroxyethyl methacrylate co-monomer (6.0 mequiv./g in bulk, 2.5 mequiv./g determined on the surface by titration) lends a sufficiently hydrophilic character. The aliphatic side-chains of the

gel backbone form hydrophobic regions of the proper size to perform binding interactions with non-polar groups of proteins and peptides at higher ionic strengths. It has been demonstrated by use of the spectral polarity label that the polarity on the surface of Spheron gels in water is similar to that of ethanol, whereas values close to those of water were obtained with polydextran derivatives[49]. Only enzymes with a great number of hydrophobic groups, such as hog pancreatic α-amylase, are bound more strongly even at low ionic strengths (buffer molarity below 0.3). Amylase is retarded even on more hydrophilic materials, such as polydextran gels. This retardation was originally ascribed to a specific interaction between amylase and polydextran (a polysaccharide similar to starch, the substrate of amylase[50]); a hydrophobic interaction with the cross-linking agent, however, is more probable. The interaction of Spheron P-300 with the other proteins examined, *i.e.* human serum albumin, chymotrypsinogen, chymotrypsin, trypsin, trypsinogen, and lysozyme, is relatively weak (see Table I) in the middle pH range and in dilute buffers (about 0.2 *M*). This renders Spheron-type materials suitable for affinity chromatography[51] because the biospecific interactions are mostly stronger than the hydrophobic interactions under these conditions.

In principle, any chromatographic material developed so far for the separation of biopolymers possesses hydrophobic interactions with protein molecules under appropriate conditions. The retardation of amylase on dextran gels has been mentioned above. The interaction of hydrophobic amino acids with Sephadex G-10 and G-25 has been exploited for the separation of hydrophobic amino acids[52]. The reported retardation of phenol on polydextran gels with a higher degree of cross-linking can probably be ascribed to hydrophobic bonding[53]. We have tested the hydrophobic interactions of proteins and low molecular weight compounds with a coated porous glass ion-exchange support (CM-glycophase™/CPG-250; Pierce, Rockford, Ill., U.S.A.). We observed that human serum albumin and the alkyl derivatives of uracil (5-methyluracil, 5-ethyluracil, 5-propyluracil, and 5-cyclobutyluracil) are all slightly retained in 0.74 *M* $(NH_4)_2SO_4$ by hydrophobic forces. The low molecular weight solute with the largest hydrophobic interacting surface, *i.e.* 5-cyclobutyluracil, is retained only a little more than serum albumin. On the contrary, the retention of 5-cyclobutyluracil by the Spheron support is much greater, because the number of binding sites for low molecular weight compounds, which can partly diffuse even into the highly cross-linked polymer support, is considerably higher than the number for high molecular weight compounds (proteins), which can diffuse into macropores only. Consequently, a higher retention of low molecular weight compounds than of high molecular weight proteins is observed, even though the latter have a larger total hydrophobic surface. When the surfaces of the macropores of the Spheron support were coated with hydrophilic glucose units, the hydrophobic interactions of proteins were reduced, while low molecular weight compounds were retained by hydrophobic interactions to the same degree.

Column performance

The column of Spheron P-300 (dimensions 250 × 6.28 mm) was used for one year, without repacking, for more than 350 chromatography runs with proteins; the average loading was 1–4 mg. The column retained its efficiency throughout. At the end of the period a weak sorption of strongly basis proteins (*e.g.* of lysozyme) in

buffers of low ionic strength (<0.05) and in water was observed, probably because a small number of carboxyl groups had been formed by hydrolysis of the gel matrix. This undesirable phenomenon was completely suppressed by pumping a solution of diazomethane in dichloroethane over the column and washing the column with methanol and water. The pressure in the column was 5–10 atm at a flow-rate of 40 ml/h. This pressure increased to 20–50 atm in mixed solvents containing alcohols, as a result of small changes in bed swelling. As high pressures can be applied to Spheron supports[35] this increase does not cause any difficulties. Spheron P-1000 showed the same chromatographic characteristics as Spheron P-300 when used for hydrophobic interaction chromatography; in some cases increased tailing was observed.

So far all materials used for hydrophobic interaction chromatography have been prepared from an inert or hydrophilic matrix by introduction of hydrophobic groups of appropriate size and concentration. It has been shown in this study that a material possessing good chromatographic properties can be obtained directly from monomers having both hydrophilic and hydrophobic character.

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