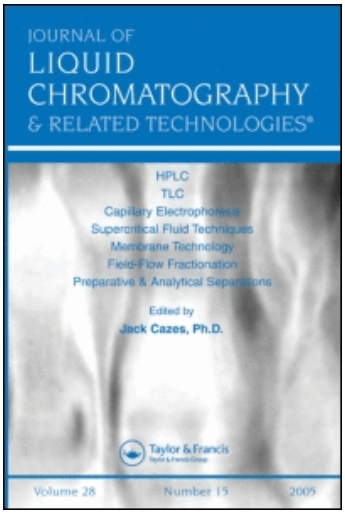

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SEPARATION OF PROTEINS ON POLAR BONDED PHASES BY HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

Hydrophobic interaction chromatography (HIC) with a polar bonded phase ("Acetamide") developped for size exclusion chromatography (SEC) is described. Retention of proteins depends on the surface area of the stationary phase, the pH and ionic strength of the eluent. For efficient separation the pore diameter should be 25 nm or more. The surface area should be large to achieve retention even at low ionic strength. Separation is only possible with a gradient from high to low ionic strength. Gradient volumes of 10 empty column volumes with column lengths above 15 cm are recommended. Selectivity can be optimized via pH adjustment. The advantage of this column packing is its applicability for two different separation modes: SEC and HIC.

INTRODUCTION

The application of modern liquid chromatographic techniques (high performance liquid chromatography, HPLC) has led to an improvement of separation, characterization, and purification of proteins. The separations are based on native properties of the proteins like size (or molecular weight), charge at a given pH and hydrophobicity (originating from amino acid composition and tertiary structure). These differences have been used in classi-

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cal protein separation procedures as well as with chromatographic techniques. The differences in charge are used in electrophoresis and in ion exchange chromatography. Size differences are utilized in ultra centrifuge separation and in size exclusion chromatography (SEC). The differences in hydrophobicity of the individual proteins have been widely applied in fractionated precipitation by the addition of neutral salts. The chromatographic separation with hydrophobic stationary (reversed) phases has proven to be a highly efficient and versatile tool for protein separation and characterization (1-4). However, the hydrophobic interaction and hence retention with these stationary phases with alkyl groups ranging from butyl to octadecyl and phenyl are usually too strong. To achieve elution, a high organic modulator concentration is required. To suppress ionic interactions, a low pH value has to be employed additionally. Therefore, the highly efficient separation of proteins with reversed phases has primarily its range of application for the separation and characterization of protein fragments after degradation. Because of the harsh elution conditions required, only few proteins retain their biological activity.

On the other hand, SEC with chemically modified silicas differing in pore size distribution and pore volume has proven to be a chromatographic system where proteins can be separated according to size under almost physiological conditions with retention of their biological activity (5-9). Separations based on exclusion effects are always finished with the elution of the smallest molecules, i.e. eluent molecules, the point where separation by adsorption starts. The separation capacity of exclusion chromatography is, therefore, very limited. It has been shown, that it is possible to increase separation capacity by working at optimal (low) linear velocities with spherical stationary phases with a large pore volume (10); but still SEC cannot compete in separation capacity with sorptive chromatographic techniques.

It has been demonstrated (9) that some proteins even in the SEC mode may show ionic and hydrophobic interactions with the stationary phase. The ionic interaction can be eliminated by increasing the ionic strength of the aqueous buffer eluent to 0.3 to 0.5 mol/L. By doing so with hydrophobic peptides and proteins an increase in retention may be observed. In this case the so-

lutes were salted out into the polar stationary phase. The hydrophobic interaction of the solutes with the stationary phase increased, caused by an apparent increase of the "hydrophilicity" of the aqueous eluent.

Consequently, a chromatographic separation can be achieved by injecting the solutes on to a column at high eluent salt concentration; the solutes are retarded. Elution and separation can then be accomplished by decreasing the salt concentration in a gradient mode. This separation system, based on protein solubility and their fractionate precipitation has been used with soft gels (11-16), and under HPLC conditions with silica based stationary phases (9,17-19).

The solubility of proteins depends on temperature, pH value, and ionic strength of the aqueous system. The influence of temperature is limited. By choosing the pH of the buffer at a given ionic strength more or less close to the isoelectric point (pI value) of the protein its solubility can be reduced. The danger of denaturation increases the further off the pH is from the pI value of the protein. Hence, it is recommendable to adjust the pH close to that of the protein's in vivo surroundings.

In this paper the application of chemically bonded phases with polar functional groups - originally developed for SEC of proteins - for HIC of proteins will be described. Pathways for optimization of stationary phase properties (surface area, pore diameter, etc.), of eluent composition and gradient conditions will be discussed in this paper.

EXPERIMENTAL CONDITIONS

The chromatographic measurements were done with a Waters (Milford, Mass., USA) gradient system (two M 6000 A pumps and a 660 programmer). In the HIC mode the pump plunger had to be rinsed periodically with water to prevent damage by salt residues. A cyclon type mixer (25) was used in connection with a 10 cm column packed with 10 µm glass particles to smooth gradient fluctuations and pulsations.

The columns (stainless steel) were packed with isopropanol - carbon tetrachloride as slurry liquid. The stationary phases

("Acetamide") were prepared as described (5,6,9) by reacting acetylaminopropyl-triethoxy silane with silica. The only difference was the coating of the silica surface with a monolayer of water before adding the silane to the silica (26).

Silica types of Grace (Worms, FRG) were used for the preparation of bonded phases (type HPLC 250 A with 7 µm or 5 µm particle diameter, and type XWP 500 A). Nucleosil 100 (Macherey und Nagel, Düren, FRG) was used as standard silica type.

The amide phase was stable in the aqueous buffers used, as proven by constant nitrogen content and identical C/N relationship, before and after use.

Standard protein samples were obtained from Sigma (München) and Serva (Heidelberg). Buffer substances were purchased from Fluka (Neu Ulm) or Merck (Darmstadt).

RESULTS AND DISCUSSIONS

Properties of the Stationary Phase

Polar bonded phases with diol or amide functionalities have been used for size exclusion chromatography of proteins. Here, the aim has been to reduce the interaction of the proteins with the surface to a minimum so that a single mechanism - separation according to molecular sizes - is responsible for separation. Because on bonding the surface never can be covered completely silanophilic interaction always takes place. It has been shown (9) that these ionic interactions (Donnan exclusion and/or ionic sorption) can be minimized by changing the pH of the eluent (altering dissociation and charge of the proteins), or by increasing the ionic strength of the eluent (reduction of Donnan exclusion and ion exchange). Additionally, hydrophobic sorption on the bonded groups resulted in larger retention volumes than expected from molecular size. In Figure 1 the separation of a standard protein mixture in the SEC mode with a diol and an amide phase is compared. The most striking difference here is the strong retention of lysozyme with the diol phase. Two effects may be responsible, hydrophobic interaction with the carbon chain and ionic

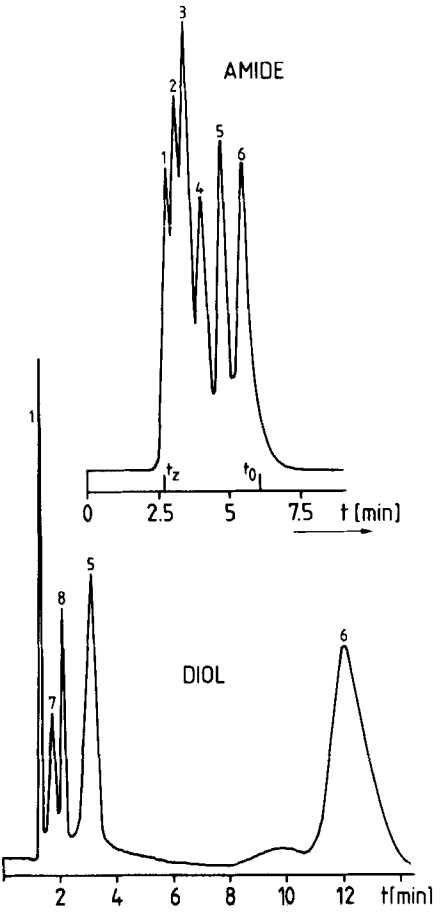

FIGURE 1. Comparison of DIOL and Amide phase.
Upper chromatogram: Acetamide on Grace silica 100 A, adjusted to pH 9, dp = 5 µm. Column = 250 x 4.1 mm; Eluent = 0.1 M Tris-buffer; pH = 7.5; 0.4 M NaCl; u = 0.7 mm/sec.
Lower chromatogram: LiChrospher Si 100 DIOL. Column = 250 x 4.6 mm; Eluent = Soerensen-buffer pH 7.4. I = 0.4.
Solute 1 = ferritin; 2 = aldolase; 3 = BSA; 4 = hemoglobin; 5 = cytochrom C; 6 = lysozyme; 7 = ovalbumin; 8 = Myoglobin.

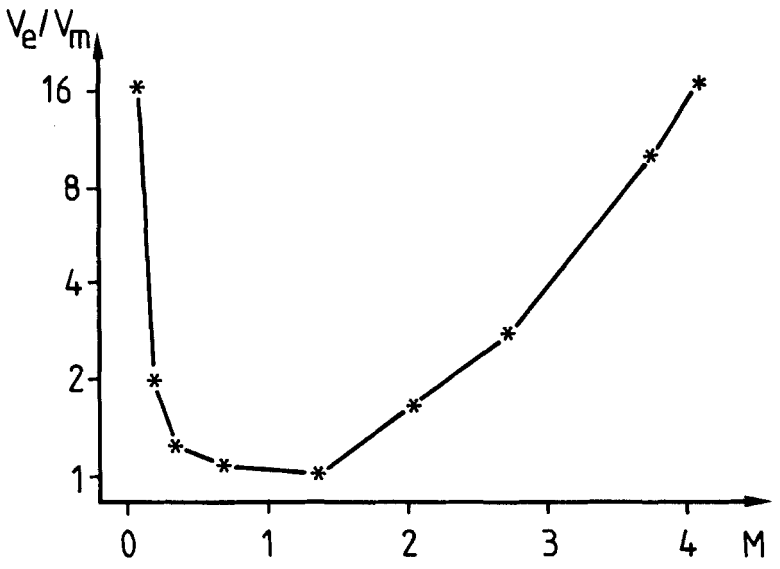

FIGURE 2. Influence of ionic strength on elution volume of lysozyme.
Column: Acetamide (as in Figure 1); Eluent: Acetate buffer pH 5; ionic strength adjusted by NaCl.

interaction with surface silanols. With the "Acetamide" phase the sorptive interactions are less pronounced.

By additionally adjusting the surface pH of the silica to 9 (20) before bonding of the "Acetamide", lysozyme with a pI value around 11 could be brought into the SEC separation range, where it is eluted before V_m. However, even with this system lysozyme is always eluted later than the other proteins of comparable size and basicity like cytochrome C, ribonuclease A and histone. Consequently, hydrophobic interactions of this "soluble oil drop" are also playing a role even on this short chain amide phase. The hydrophobic interaction can be increased by increasing the "hydrophilicity" of the eluent by adding neutral salts. Figure 2 demonstrates the different mechanisms of retention of lysozyme on this chemically modified silica. At low ionic strength the strongly basic lysozyme is retarded by an ion exchange mechanism. By increasing the ionic strength this retention decreases due to

the law of mass action. In the range of ionic strength between 0.5 and 1.5 no interaction with the stationary phase takes place and elution depends on the molecular size, whereas at an ionic strength above 1.5 mol/l the retention of lysozyme increases with increasing ionic strength. In this region retention or elution can be achieved by modifying the ionic strength of the buffer. A similar behaviour can be observed with other proteins as well.

In Figure 3 the dependance of k' values on eluent ionic strength with two different stationary phases is shown for several proteins. In both cases, amide groups have been bonded on silica differing in specific surface area (250 m^2/g and 80 m^2/g) and average pore diameter (25 nm and 50 nm respectively). As eluent a 0.1 m phosphate buffer of pH 7 was used. The ionic strength was adjusted by ammonium sulfate. It is surprising that the slopes of these curves are very similar and seem to be independent of protein nature and type of stationary phase. With the 50 nm silica the curves are only shifted to higher ionic strengths. In order to achieve identical retention, the ionic strength has to be higher with the low surface area material. This may cause technical problems. Similar influence of surface area on solute retention has been discussed for low molecular chromatography. In analogy to reversed phase chromatography of proteins (4) the slopes of these k' vs ionic strength curves are steep. The (molar) slopes S are around two and are slightly larger than reported for sepharose systems (14). Because in RP chromatography the slopes are usually related to the volume percentage of organic modulator, it seems not permissible to directly compare the slopes for proteins in both types of hydrophobic interaction chromatography.

In isocratic analysis peak broadening is very large and comparable to that in RP chromatography of proteins. The H values can approach the mm range despite optimal and expected column performance for small molecules. This peak broadening can be attributed to slow sorption/desorption kinetics. When the same column is used in the SEC mode, where the proteins are eluted unretained, the H values are about two orders of magnitude smaller.

Consequently, efficient separation of proteins is only possible in the gradient elution mode, starting with a highly con-

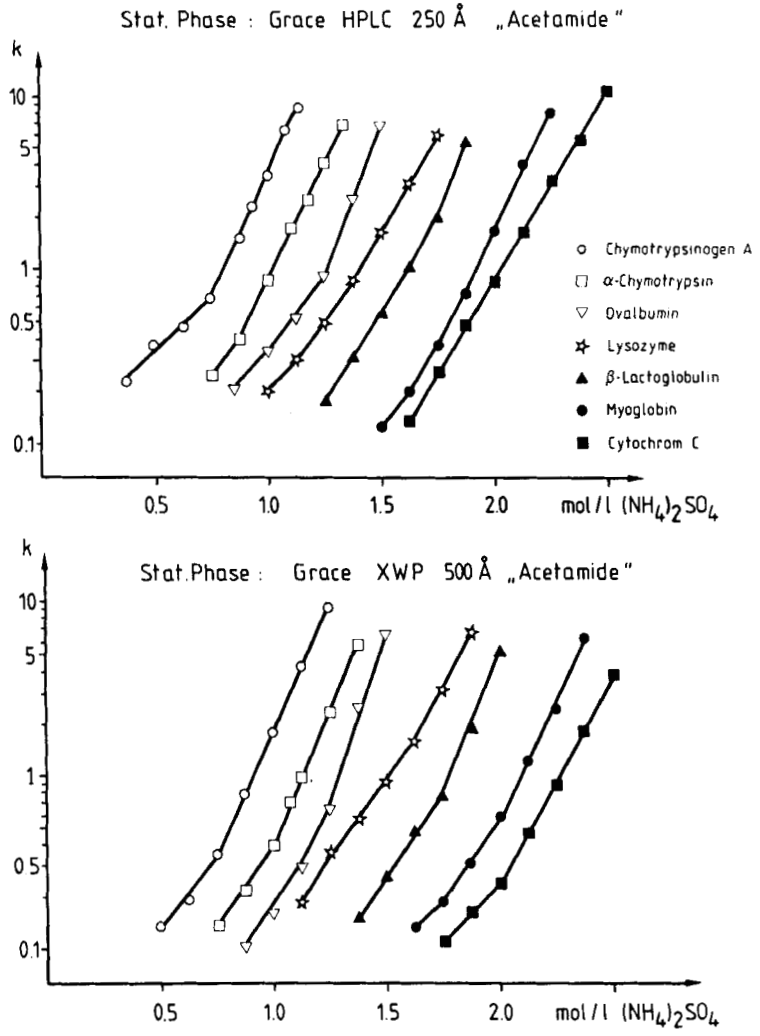

FIGURE 3. Influence of ionic strength and silica surface area on protein retention.
Eluent: 0.1 M phosphate buffer pH 7; ionic strength adjusted by ammonium sulfate.

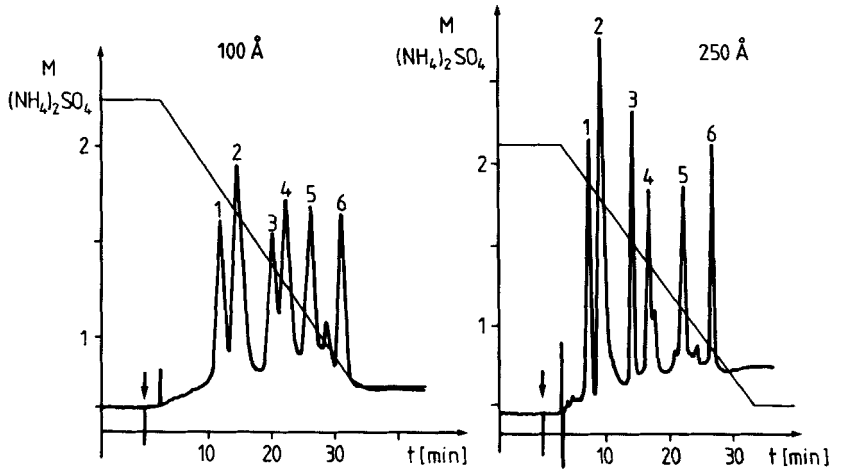

FIGURE 4. Influence of silica pore diameter on protein separation.
Left chromatogram: Acetamide on Nucleosil Si100
Right chromatogram: Acetamide on Grace XWP 250 A
Gradient A: 2.5 mol/L ammonium sulfate in B
B: 0.1 M phosphate buffer pH 7
Gradient: 10% A to 80% B; Gradient volume 30 ml;
Solutes: 1 = myoglobin; 2 = β-lactoglobulin; 3 = lysozyme; 4 = ovalbumin; 5 = α-chymotrypsin; 6 = chymotrypsinogen A.

centrated solution of ammonium sulfate in the appropriate buffer. The solutes are injected into this eluent. Elution is then achieved by continuously reducing the ionic strength of the buffer. Pure dilute buffer solution is preferentially used as the "stronger" eluent B. Peak broadening in gradient elution is reduced, and efficient separations are possible as can be seen in Figure 4. A mixture of protein standards is separated on a column packed with 25 nm pore diameter "Acetamide" phase (surface area 250 m^2/g) and compared to that on a column packed with 10 nm silica (surface area 300 m^2/g). The average particle diameter of both stationary phases was identical. Other conditions were also kept constant. As can be seen, peak broadening is worse with the narrow pore silica. This additional preak broadening is caused by hindered diffusion of the proteins to the surface within the pores (4). From this discussion it can be deduced, that an opti-

mum stationary phase for HIC of proteins should have a pore diameter large enough for unhindered diffusion of the proteins (25 nm and larger), and on the other hand a still large enough surface area to achieve retention at relatively low ionic strength. High salt concentrations negatively affect both solute diffusion and equipment performance.

Optimization of Gradient Conditions

In gradient elution of small molecules resolution is a complex function of column length, particle diameter, program time, and eluent flow rate. Peak capacity increases always with program time and decreasing flow rate. For practical reasons minimum possible flow rate with conventional equipment is around 1 ml/min. This flow rate was used throughout the following measurements. For optimization the program time, i.e. the gradient volume (21), was varied. In Figure 5 the protein standards are separated with a small gradient volume of 10 ml and with a larger one (40 ml). As can easily be seen, resolution is much better with the larger gradient volume, in part due to reduced peak broadening because of the slower effective protein movement through the column. Gradient volumina of 30 ml or more were always used in the following chromatograms.

For low molecular weight solutes, it has been found (22) that with gradient volumina around 10 empty column volumes the peaks are eluted with an eluent composition in which their isocratic k' value is around 1. With standard columns of 250 x 4.1 mm this corresponds to gradient volumes of 30 to 35 ml or to program times of 30 to 35 minutes at flow rates of 1 ml/min. Increasing gradient volume results in higher isocratic k' values. For gradient elution of macromolecules it has been found (23), that at column end they move with the velocity of an unretained solute. Solute acceleration occurs within a very short distance of the column packing. In other words, their velocity is either zero or equal to that of the eluent. Consequently, one might extrapolate that the "ideal" column length for polymer gradient elution approaches zero. This has been found valid for synthetic polymers as well as for proteins in reversed phase chromatography (23).

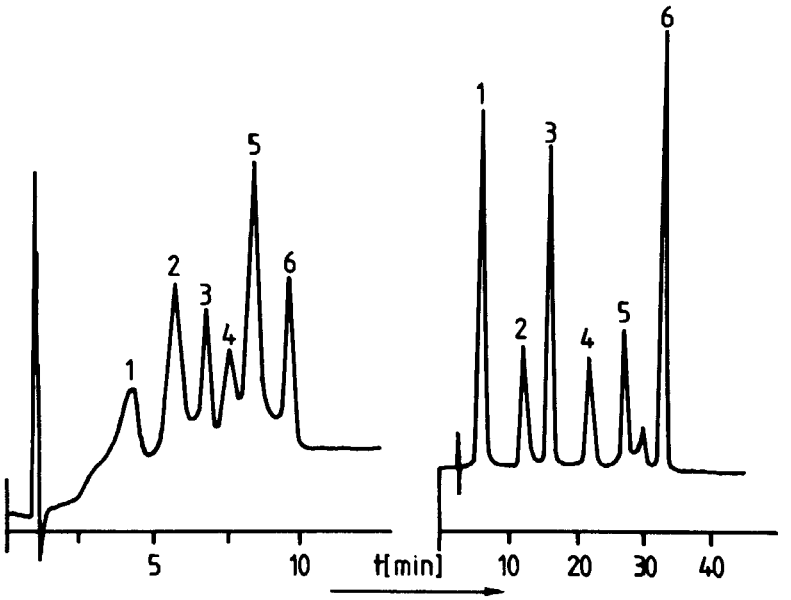

FIGURE 5. Influence of program time on resolution.
Column: Acetamide on Grace XWP 250 A
Gradient conditions as in Figure 4
Left chromatogram: program time 10 min (gradient volume 10 ml)
Right chromatogram: program time 40 min (gradient volume 40 ml)

With HIC on polar stationary phases this is not the case as can be easily seen in Figure 6, where the column length was varied between 5 and 20 cm. The gradient volume was kept constant at 30 ml, thus varying from 50 empty column volumes with the 5 cm column to 11 with the 20 cm column. Resolution is worse with the shorter column, especially for the early eluting peaks, despite the higher peak capacity due to the bigger gradient volume. This demonstrates, that the solutes already have a definite velocity early in the gradient. As can also be seen, the proteins are eluted at a constant eluent composition, as expected from low molecular gradient elution. Consequently, for HIC of proteins column length should be 15 cm or longer for optimum resolution.

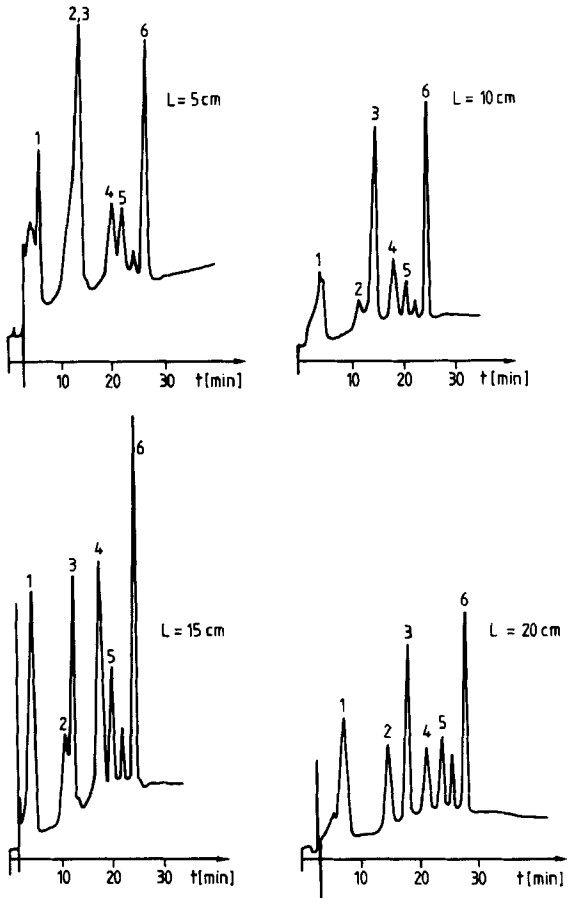

FIGURE 6. Influence of column length on resolution. Conditions as in Figure 5, except pH = 5.5 and gradient volume 30 ml.

Influence of pH on Protein Retention

The solubility of proteins depends among others on the net charge of the macromolecule. The charge and hence the solubility can be altered by changing the ionic strength and the pH value. Generally, protein solubility is lowest at a pH close to its pI value, where the net charge equals zero. Two problems may arise

in discussing pH influence on protein retention. The pI values are defined only for a certain milieu (usually low ionic strength) and are mainly determined via electrophoresis. The buffer capacity of 0.1 molar phosphate buffer is insufficient to keep the pH value constant over the whole range of ammonium sulfate concentrations applied in HIC. Due to dilution effects and changes of activity coefficients the pH adjusted to 7 for eluent A and B decreases to 6.7 during the gradient run. The decreasing salt concentration is superimposed first with a decreasing and then with an increasing pH gradient. Consequently, it is not surprising when the chromatographic behaviour of proteins by changing the pH cannot always be correlated to their pI values. Figure 7 shows the correlation of elution behaviour (under standard gradient conditions) with eluent pH for several proteins. Lipase with a pI value of 5.5 is not eluted from the column at this pH. On the other hand, the retention of the basic proteins like lysozyme, cytochrome C, myoglobin is smallest at pH = 7 and increases with decreasing pH.

The different influence of pH on retention of the individual proteins permits to change the elution order by varying the pH. Figure 8 compares the separation of standard proteins at pH 4.4 and 7. This opens an additional way for optimization of a separation. It should be mentioned, that with RP chromatography of proteins the pH has to be reduced to values below 3 to achieve efficient separations (4). HIC has, therefore, the advantage to work at pH values close to physiological requirements.

Protein Stability

The biological activity can be affected due to sorption processes or incompatibility with elution conditions. In SEC mode with "Acetamide" phases it has been shown that the biological activity is retained (9). In RP chromatography proteins are affected by the low pH and/or by the high organic modulator concentrations required for elution.

It has also been shown, that the contact time of the proteins with the hydrophobic surface can cause denaturation (24). A

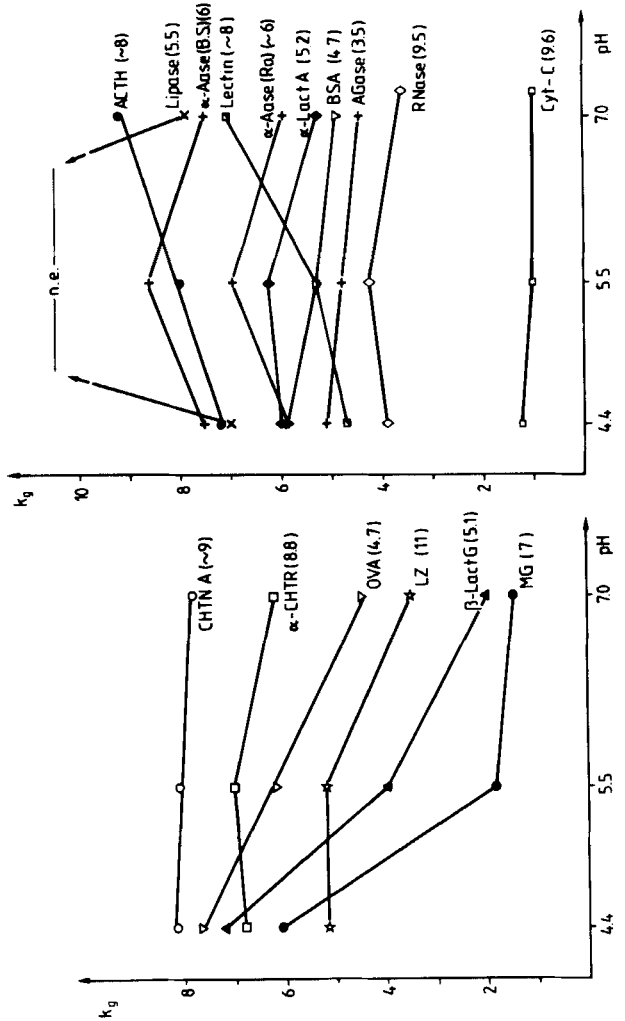

FIGURE 7. Influence of eluent pH on protein retention. Conditions as in Figure 6.

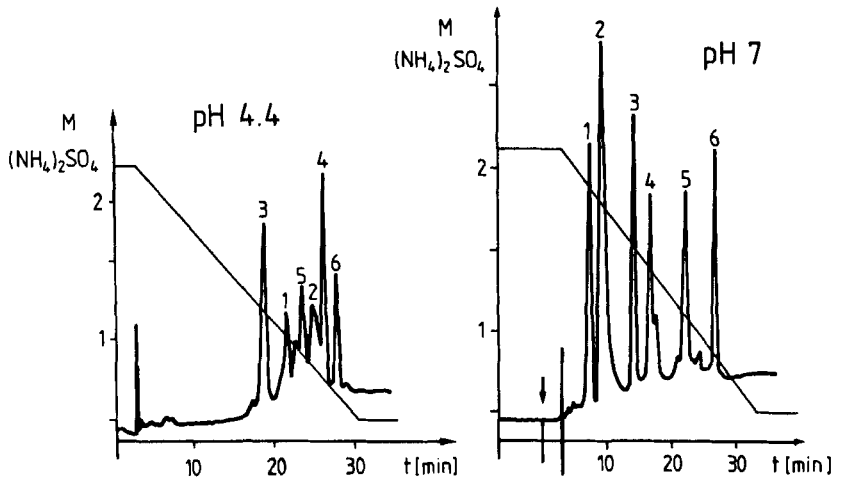

FIGURE 8. Influence of eluent pH on selectivity.
Conditions as in Figure 6; eluent pH as noted.

method has been proposed there to determine chromatographically the stability of proteins into the chromatographic system. Four different enzymes were injected in the chromatographic system and eluted by identical gradient either immediately after injection or after 30 min residence time on the stationary phase (flow stopped). As can be seen in Figure 9, peak height, peak shape, and elution volume are identical in every case. This indicates that neither the contact with the "Acetamide" phase nor the elution conditions affect enzyme stability. Of course, with proteolytic enzymes like trypsin which is self-digesting at pH 7 a complex chromatogram is obtained as shown in Figure 10. This decomposition, of course, is not related to the chromatographic process. Reducing the pH value reduces its proteolytic activity. Consequently, trypsin stability is improved and the chromatogram obtained is less complex as shown also in this Figure for a freshly prepared sample.

The stability of proteins in this chromatographic system can easily be correlated to protein stability in the classical protein purification process of ammonium sulfate precipitation. When

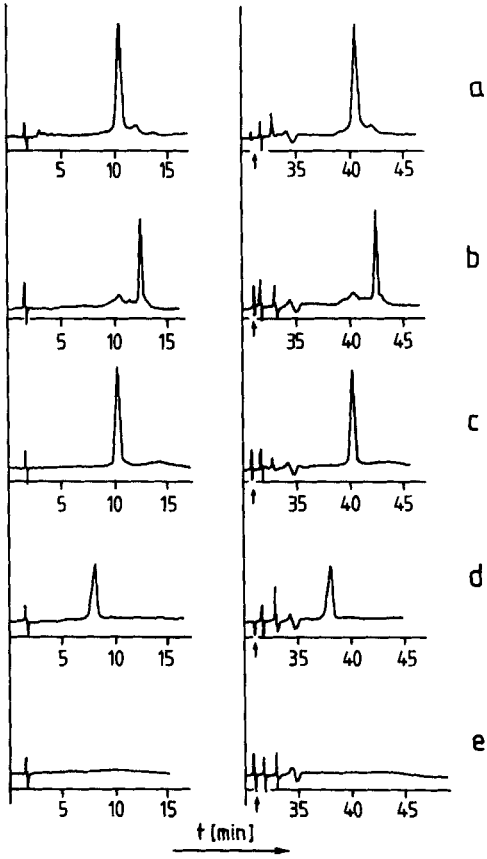

FIGURE 9. Chromatographic test for protein stability.
Conditions as in Figure 5, except column 150 x 4.1 mm, gradient volume 10 ml, and for solute b: acetamide on Grace silica XWP 500 A.
The left chromatograms were obtained directly after injection, the right ones with a 30 min stopped flow after injection.
Solutes: a = α-chrymotrypsinogen; b = ß-glucoxidase; c = α-aldolase (Ro); d = lysozyme; e = without any sample.

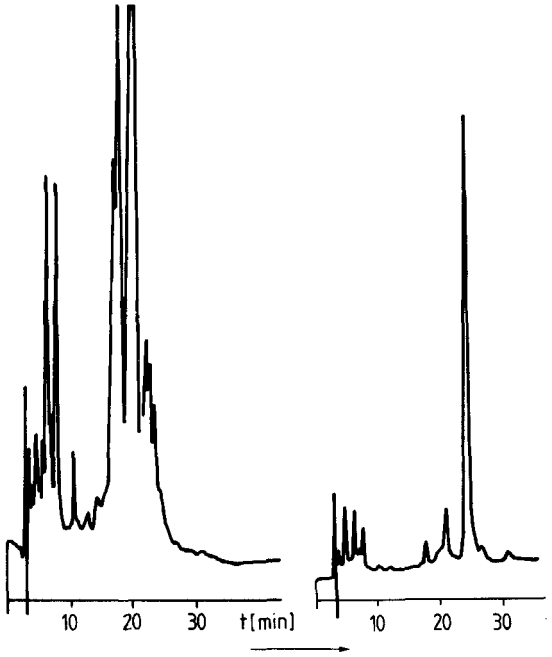

FIGURE 10. Elution of trypsin.
Conditions as in Figure 6
left: 20 h at pH 7, room temperature
right: freshly prepared solution pH 4.4, T = 278 K

the chromatographic conditions follow those described there to prevent denaturation, biological activity will also be retained in the chromatographic separation.

SEC and HIC
A Single Phase Bidimensional Separation System.

The polar bonded phases with "Acetamide" groups have been used for both SEC (9) and sorption chromatography as described here. With a single column the separation mechanism can easily be changed from a separation according to molecular size to a mechanism based on hydrophobic interaction of the proteins with the

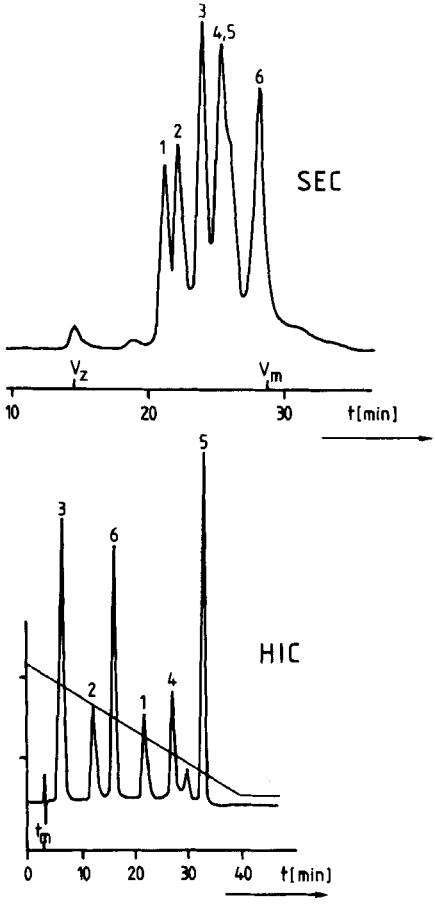

FIGURE 11. One column - two separation mechanisms.
Lower chromatogram: Conditions as in Figure 6
Upper chromatogram: eluent pH 7.5; 0.1 M TRIS/HCl buffer, 0.4 M NaCl.

surface of the stationary phase, just by a modification of the eluent composition. With a buffer of pH 7.5 and an ionic strength of 0.5 the separation of standard proteins on the "Acetamide" column is according to size, as can be seen in the upper chromatogram of Figure 11. Chymotrypsin and chymotrypsinogen which are similar in size (molecular weight) cannot be separated in this system. However, with the same column this separation causes no

problems in the HIC mode. Here, the solutes are injected at high ionic strength and the ionic strength decreases in a gradient (lower chromatogram in Figure 11).

This demonstrates clearly the potential of bidimensional protein separation with two principally different separation mechanisms: SEC and HIC. Because of technical problems (low ionic strength) the SEC mode is recommended as a first step, the HIC separation as the second one, and if desalting or a buffer change is required, a third step in SEC mode can be added. For all these three separation steps one type of column packing is sufficient.

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