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COMPARISON OF HYDROPHOBIC-INTERACTION AND REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS*

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SUMMARY

The variable hydropohobic nature of proteins allows their separation through differential hydrophobic surface interactions. From these observations two modes of protein chromatography have been developed, hydrophobic-interaction chromatography (HIC) and reversed-phase chromatography (RPC). Selectivity of the HIC column can be easily manipulated by changing mobile phase variables. Protein retention was increased by decreasing the pH from neutrality or by using a salt with a greater "salting-out" ability. In addition, selectivity can be altered through chemical modification of the matrix surface. Protein retention and resolution decreased concomitantly with matrix ligand density. There were several major differences in HIC and RPC selectivity. Hydrophilic proteins such as cytochrome *c* and myoglobin were weakly retained on the HIC coluumn but strongly retained on the RPC column. In contrast, a hydrophobic protein such as β-glucosidase was strongly retained on the HIC column and only weakly retained on the RPC column. Other proteins were retained equally by RPC and HIC columns. Load capacity on the HIC column was determined by plotting resolution as a function of protein load. Resolution decreased significantly after 7.5 mg of total protein had been loaded onto the column per cm^3 of column material. Samples of lactic dehydrogenase and α-chymotrypsin ranging in size from 10–200 μg were recovered from an HIC column with greater than 86% enzymatic activity in all cases. The recovery of enzymatic activity of α-chymotrypsin ranged from 55–91%, while none of the activity of β-glucosidase was recovered from the RPC column.

INTRODUCTION

The chromatographic separation of proteins based on their hydrophobic properties was first developed in the early 1970's. Support materials for this type of hydropohobic chromatography were first synthesized by linking alkylamines to agarose gels[1,2]. Elution of proteins from hydrophobic matrices was generally achieved through the use of deforming buffers such as imidazole-citrate, which cause conformational changes in the protein. Arfmann and Shaltiel[3] found that protein adsorp-

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tion could be optimized by using high salt concentrations, such as 1 *M* ammonium sulfate. They also found that the affinity of the proteins for the hydrophobic matrices was proportional to the chain length of the derivative. The name hydropohobic-interaction chromatography (HIC) was introduced by Hjertén[4] to describe salt-mediated separations of proteins on weakly hydrophobic carbohydrate gel matrices. Although the basic retention process is probably the same as that of reversed-phase chromatography (RPC), native protein structure is more likely to be maintained in HIC. In this paper, all separations achieved by the elution of hydrophobic columns with organic solvents will be classified as RPC while those achieved with aqueous mobile phases will be referred to as HIC.

During an HIC fractionation, proteins are induced to bind to weakly hydrophobic materials through the use of high-ionic-strength mobile phases; the driving force being an increase in entropy. In a polar environment, hydrophobic moieties cause an ordering of the water structure in the immediate vicinity. Entropy is increased when the hydropohobic groups are excluded from the polar environment as in the interaction of the protein with the matrix surface. Adsorbed proteins are then selectively desorbed during a linear descending salt gradient. As the ionic strength of the mobile phase decreases, entropy is no longer a driving force in the protein–matrix interaction; weakening the hydropohobic interaction and allowing the protein to be eluted from the column.

HIC mobile phases are generally compatible with most proteins. Fujita *et al.*[5] recovered as much as 75% of the initial enzymatic activity of several yeast enzymes after chromatography in 3 *M* ammonium sulfate on several derivatized cellulose columns. Fausnaugh *et al.*[6] found recoveries of α-amylase and β-glucosidase exceeding 92% after chromatography in 1 *M* sodium sulfate on a silica-based HIC column with butyrate ligands.

In RPC, proteins are bound to matrices that are generally more hydrophobic than the HIC matrix. This increased hydrophobicity is achieved through a higher density of alkyl ligands on the matrix surface[7]. As in HIC, entropy drives the interaction of the protein with the hydropohobic surface, but the interaction is so strong on RPC columns that proteins may not be eluted with buffers. Desorption is accomplished through the introduction of an organic additive in the mobile phase. The organic solvent may interact with both the hydropohobic matrix and the protein, thereby displacing the protein; or it may disorder the water structure so that the protein may be eluted from the column. Numerous reports[8–10] have shown that RPC has very high resolving power for proteins and peptides.

The use of organic solvents in RPC can be very detrimental to the native protein structure. Protein denaturation becomes thermodynamically favorable as organic solvents are added to the mobile phase. This denaturation is generally irreversible, resulting in a loss of enzymatic activity. Luiken *et al.*[11] found that, although the activity of ribonuclease was unaffected, only 1–5% of the enzymatic activity of horse radish peroxidase was recovered after RPC.

The prospect of increasing resolution and performance by decreasing particle size led to the development of rigid microparticulate matrices for HIC. For example, Chang *et al.*[12] bonded low-molecular-weight polyethylene glycols to the surface of 37–74 μm particle diameter controlled-porosity glass. Although these materials were stable to hundreds of atmospheres of pressure and had the elution properties of

gel-type HIC columns, they were not truly microparticulate. Engelhardt and Mathes[13] later showed that matrices which were too hydropohobic for size exclusion-chromatography (SEC) could be used effectively in the fractionation of proteins in the hydrophobic interaction mode. In fact, many high-performance SEC matrices have some hydrophobic character with high-ionic-strength mobile phases[14]. Kato *et al.*[15] have synthesized HIC matrices by coupling butyl and phenyl glycidyl ethers to TSK G3000SW SEC packings which gave good resolution of several proteins. More recently, Fausnaugh *et al.*[6] have shown that silica-based matrices with a weakly hydropohobic polymeric layer of butyrate groups may also be used for HIC.

This paper examines protein separation by HIC and RPC. The two types of column materials were compared with respect to selectivity, resolution, and enzymatic activity of a series of proteins. Further studies were conducted on HIC columns, examining mobile phase variables, matrix ligand density, and protein load capacity.

EXPERIMENTAL

Materials

Vydac 101TPB5 was a gift from The Separations Group (Hisperia, CA, U.S.A.). 2-(Carbomethoxy)ethyltrichlorosilane (CMETS) was purchased from Petrarch Systems (Levittown, PA, U.S.A.). Tetraethylenepentamine (TEPA), butyric anhydride, and benzoic anhydride were obtained from Aldrich (Milwaukee, WI, U.S.A.). Trifluoroacetic anhydride was obtained from Sigma (St. Louis, MO, U.S.A.). Acetic anhydride was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Synchropak RP-8 was purchased from Synchrom (Linden, IN, U.S.A.).

All proteins were obtained from Sigma, except for β-glucosidase (β-GLU) which was purchased from Worthington (Freehold, NJ, U.S.A.). All buffers and solvents were analytical-reagent grade.

Instrumentation

HIC columns were tested on a Micromeritics 7000B liquid chromatograph with a variable-wavelength Chromonitor 785 UV detector, a Linear 260 chart recorder, and a Rheodyne 7120 injector valve. RPC was performed on a liquid chromatographic system consisting of an LDC solvent programmer, two Altex pumps, an Altex mixer, a Varian UV-50 variable-wavelength detector, a Rheodyne 7120 injector valve, and a Fisher Recordall Series 500 recorder.

Synthesis of packing material

HIC packings were synthesized in a three-step coating process as previously described[6]. In the initial reaction, 5 μm macroporous silica was silylated with CMETS in carbon tetrachloride. TEPA in N,N-dimethylformamide was subsequently coupled to the matrix at multiple sites by nucleophilic displacement of the methoxy group from the organosilane, as previously described[16,17]. In the final step of the synthesis, the TEPA-coated packing material was derivatized with an excess of anhydride in a reaction mixture of pyridine and dioxane. Trifluoroacetate and butyrate column materials were synthesized in this manner. In the ligand density study, density was controlled in the final step by derivatizing the polymer-coated packing material with a series of mixtures of benzoic and acetic anhydride in varying ratios. In this exper-

iment, a control column was used in which only the first two steps of the synthesis were completed, giving an underivatized TEPA-coated packing material. The total amount of ligand/g of support was determined via a picric acid assay[18]. By adding a trace amount of [^{3}H]acetic anhydride to the reaction mixture in the final step of the synthesis, the number of acetyl ligands/g of matrix could be quantitated. From the difference in the total and the acetyl ligand values, the number of phenyl ligands/g of support was calculated. In this manner a series of seven phenyl/acetyl columns were synthesized having a phenyl ligand density ranging from 0–73.5 μmole/g of matrix.

Gradient hydrophobic-interaction chromatography

A mixture of cytochrome *c* (CYT c) (2.5 mg/ml), conalbumin (CON) (10 mg/ml), and β-GLU (20 mg/ml) was chromatographed on the butyrate column. The initial buffer contained 1.0 *M* sodium citrate, sodium sulfate, ammonium sulfate, sodium chloride, or ammonium thiocyanate in 10 m*M* potassium phosphate buffer, pH 7.0. The proteins were chromatographed in a 20-min linear descending salt gradient to 10 m*M* potassium phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min.

The same mixture was loaded onto the column in 1.0 *M* sodium sulfate in 10 m*M* buffer at a pH ranging from 3 to 8. The proteins were chromatographed in a 20-min linear descending salt gradient to 10 m*M* buffer at the same pH and a flow-rate of 1 ml/min. A second mixture of ovalbumin (OVA) (15 mg/ml) and lysozyme (LYZ) (5 mg/ml) was chromatographed under the same conditions.

Gradient chromatography of phenyl–acetyl and TSK-phenyl columns

A series of twelve proteins of various molecular weights and isoelectric points were chromatographed individually on each of the seven phenyl–acetyl columns and on the commercial HIC column from Toya Soda, TSKgel Phenyl-5PW. A 20-min linear descending salt gradient was run from 1.0 *M* sodium sulfate in 10 m*M* potassium phosphate buffer, pH 7.0 to 10 m*M* potassium phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min. The proteins were CYT c (2.5 mg/ml) myoglobin (MYO) (2 mg/ml), OVA (15 mg/ml), ribonuclease A (RNAse) (10 mg/ml), LYZ (5 mg/ml), β-GLU (20 mg/ml), α-chymotrypsin (α-CHY) (10 mg/ml), α-chymotrypsinogen-A (α-CHYGEN) (5 mg/ml), bovine serum albumin (BSA) (10 mg/ml), CON (15 mg/ml), ferritin (2 mg/ml), and lactoperoxidase (2 mg/ml). A mixture of CYT c, MYO, RNAse, OVA, LYZ, and β-GLU was chromatographed under the same conditions.

Reversed-phase chromatography

The same twelve proteins were chromatographed on the Synchropak RP-8 column. A 20-min linear gradient was run from 0.1% trifluoroacetic acid (TFA) in water to 0.1% TFA in 2-propanol–water (60:40) at a flow-rate of 1 ml/min.

Recovery of enzymatic activity

Protein samples were eluted at 1 ml/min during a 20-min linear gradient from 1.0 *M* sodium sulfate in 10 m*M* potassium phosphate buffer (pH 7.0) to 10 m*M* potassium phosphate buffer (pH 7.0) for HIC. The reversed-phase samples were eluted at 1 ml/min during a 20-min linear gradient from 0.1% TFA in water to 0.1% TFA in 2-propanol–water (60:40). Control samples were injected into empty capillary

tubing. HIC α-CHY and RPC β-GLU samples were dialyzed against 10 m*M* potassium phosphate buffer (pH 7.0) before they were assayed for activity. α-CHY was assayed by measuring the increase in absorbance at 256 nm as benzoyl-L-tryosine ethyl ester (BTEE) was hydrolyzed by the enzyme[19]. Lactic dehydrogenase was assayed in a modification of the procedure used by Morgenstern *et al.*[20]. The increase in absorbance at 340 nm was measured as L-lactate was converted to pyruvate in the presence of NAD at pH 8.8. β-GLU was assayed with *p*-nitrophenyl-β-D-glucopyranoside[21].

Load capacity

Load capacity for the butyrate and the TSK-phenyl column was determined by measuring the protein resolution as a function of the amount of protein loaded onto the column. A mixture of CON (15 mg/ml) and β-GLU (20 mg/ml) was loaded onto each of the columns in 1.0 *M* sodium sulfate in 10 m*M* potassium phosphate buffer (pH 7.0). The proteins were chromatographed during a 20-min linear descending salt gradient to 10 m*M* potassium phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min.

RESULTS AND DISCUSSION

Protein retention, resolution, and selectivity

HIC column selectivity can be easily manipulated by changing such mobile phase variables as salt concentration, salt type, and pH. Retention increases concomitantly with salt concentration, as previously noted[6]. Melander and Horváth[22] have developed a simple theory explaining the effect of neutral salts on the solubility and chromatography of proteins. Protein solubility, being dependent upon salt concentration, initially increases with concentration, but then decreases essentially linearly once a certain concentration is reached. The decrease in solubility, or salting-out region, can be characterized by a molal salting-out constant which relates the change in solubility with the change in salt concentration[23]. Melander and Horváth have calculated molal salting-out constants for hemoglobin with a series of neutral salts and have shown that higher values indicate a greater salting-out ability of the salt.

Protein retention and resolution on the butyrate column was examined as a function of the chemical nature of the salt. Four salts with differing hemoglobin molal salting-out constants were tried along with ammonium thiocyanate, which is a chaotropic agent and would be expected to interfere with hydrophobic interactions and decrease retention. Salts with higher hemoglobin salting-out constants, such as sodium citrate, gave increased retention of the proteins tested (Fig. 1). When a salt with a lower hemoglobin molal salting-out constant, such as sodium chloride, or ammonium thiocyanate was used, the proteins were not retained. The more hydrophilic proteins were best resolved by salts with higher hemoglobin molal salting-out contants, while salts with lower hemoglobin molal salting-out constants gave better resolution of more hydrophobic proteins (Fig. 2).

From the results of the studies on salts, a general approach to HIC of proteins can be constructed. In choosing mobile phase conditions to separate a protein from a mixture, initial experiments should be performed with a salt of intermediate salting-out ability, *e.g.* sodium sulfate, at a concentration of at least one molar. Subsequent

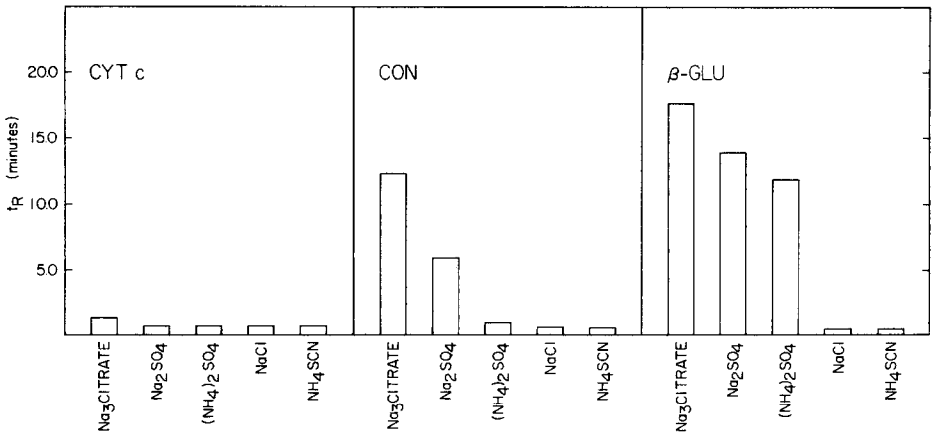

Fig. 1. The effect of salt type on protein retention on the butyrate column was determined with a 20 μl injection of a mixture of cytochrome *c* (2.5 mg/ml), conalbumin (10 mg/ml), and β-glucosidase (20 mg/ml). The proteins were separated in a 20-min linear gradient from 1.0 *M* salt in 10 m*M* potassium phosphate buffer (pH 7.0) to 10 m*M* potassium phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. t_R = Retention time.

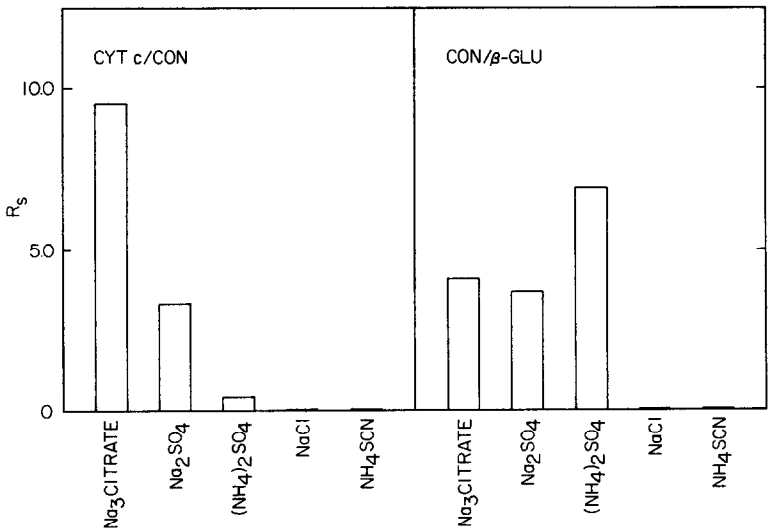

Fig. 2. The effect of salt type on resolution was determined for the mixture of three proteins chromatographed as in Fig. 1.

conditions can be altered so that selectivity is tailored to a given protein. For example, retention can be increased by increasing the initial salt concentration or by using a salt with a greater salting-out ability.

Protein retention and resolution was also investigated as a function of pH with five proteins of differing molecular weight and isoelectric points (Table I). All five proteins were retained the most tenaciously between pH 4 and 5 (Fig. 3). At lower pH, retention of all five proteins decreased. Between pH 5 and 6, the retention of all

TABLE I

PHYSICAL PARAMETERS OF PROTEINS

Protein (origin)	*Molecular weight*	*pI**
Cytochrome c (horse heart)	12,200	9.0, 9.4
Ribonuclease A (bovine pancreas)	13,500	8.7, 8.8 (guinea pig pancreas)
Lysozyme (egg white)	13,900 (human)	11 (human serum)
Myoglobin (horse heart)	17,500	6.9, 7.3
Ferritin	24 subunits, 18,500	4.27, 4.35, 4.40 (horse spleen)
α-Chrymotrypsin (bovine pancreas)	21,600	8.38, 8.76 (ox pancreas)
α-Chymotrypsinogen-A (bovine pancreas)	25,000	8.8, 9.2, 9.6
Ovalbumin (egg white)	43,500	4.7
β-Glucosidase (almonds)	2 subunits, 65,150	7.3
Bovine serum albumin	69,000	4.98, 5.07, 5.18
Conalbumin (chicken egg white)	77,000	6.0, 6.3, 6.6
Lactoperoxidase (bovine milk)	85,000	9.16, 9.32, 9.49, 9.68, 9.7, 9.80

* From refs. 24–26.

five proteins decreased. At higher pH, the retention LYZ, CON, and CYT c increased while the retention of β-GLU and OVA decreased. This has not been found to be a general rule for all HIC columns[27]. Part of these observed results might be attributed to the residual positive charge on the packing material, but this is unlikely since

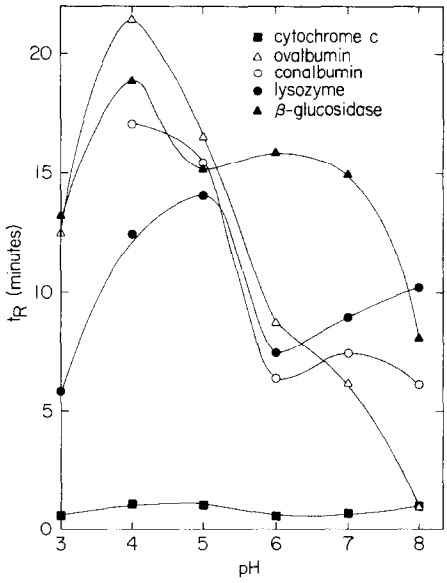

Fig. 3. The effect of pH on protein retention on the butyrate column was determined by chromatographing 20-μl injections of two protein mixtures at a pH ranging from 3 to 8. The first mixture contained cytochrome *c* (2.5 mg/ml), conalbumin (10 mg/ml), and β-glucosidase (20 mg/ml). The second mixture contained ovalbumin (15 mg/ml) and lysozyme (5 mg/ml). Each mixture was loaded onto the column in 1.0 *M* sodium sulfate in 10 m*M* buffer at a pH from 3 to 8 and chromatographed in a 20-min linear descending salt gradient to 10 m*M* buffer at the same pH at a flow-rate of 1 ml/min. Conalbumin was insoluble below pH 4.0.

acidic proteins were not retained when chromatographed on a phenyl–acetyl column in the anion-exchange mode with 10 m*M* potassium phosphate buffer, pH 6.0. The increased protein retention at low pH could be due to partial denaturation of these proteins under acidic conditions. There was no obvious relationship between retention and isoelectric point of individual proteins. The best resolution between these five proteins would be obtained at a pH between 7 and 8.

In RPC, protein retention can be altered through the selection of the organic solvent and the ion-pairing agent (IPA). Organic solvents can be ranked according to decreasing elutrophic strength as compared to water.

propanol > acetonitrile > methanol > water

Accordingly, it would take less propanol to elute a protein from a column than acetonitrile. IPAs can have substantial effects on protein selectivity and resolution. Commonly used polar IPAs include phosphoric acid, perfluorinated carboxylic acids, and trialkylammonium phosphate[28]. Of the perfluorinated carboyxlic acids, TFA and heptafluorobutyric acid (HFBA) are often used as IPAs. TFA is a weak hydrophobic IPA, separation being based mainly on overall hydrophobicity, while HFBA is a strong hydrophobic IPA and the basic character of the protein forms the basis for separation[9]. TFA generally causes a decrease in retention time. Using TFA, O'Hare *et al.*[10] found greater recovery of several proteins from an RPC column than when sodium chloride, phosphate, or hydrochloric acid was used.

Besides varying the mobile phase, it is also possible to alter protein retention in HIC by altering the ligand density on the surface of the matrix. TEPA-derivatized silica was further derivatized with a series of benzoic and acetic anhydride mixtures. This resulted in a series of column materials with phenyl ligand densities up to 73.5 μmoles/g of matrix. The average area of the matrix surface per ligand can be calculated from the ligand density/m^2 and the surface area of the silica in m^2/g. For example, a phenyl/acetyl column material having 73.5 μmoles of phenyl/g of matrix and a surface area of 56.4 m^2/g has a ligand density of 1.3 μmoles of phenyl/m^2. This is equivalent to 127 $Å^2$/ligand. In contrast, a phenyl/acetyl column material with 9.28 μmoles of phenyl/g of matrix and the same surface area would have a ligand density of 0.165 μmoles of phenyl/m^2. This is equivalent to 1010 $Å^2$/ligand. An average protein with a spherical diameter of 40–50 Å would be in contact with possibly twelve phenyl ligands on the 1.30 μmoles of phenyl/m^2 column material and only two phenyl ligands on the 0.165 μmole of phenyl/m^2 column material. When there is a limited number of hydrophobic residues at the surface of a protein, the availability of ligands on the matrix surface could become a determinant in the adsorption process.

Twelve proteins of varying molecular weights and isoelectric points as presented in Table I, were chromatographed on each of the phenyl–acetyl columns and on the Toya Soda TSKgel Phenyl-5PW HIC column. On the TSK-phenyl column the elution order of the proteins was identical to that of a phenyl–acetyl column of comparable hydrophobicity. However, the peaks on the TSK-phenyl column were sharper for proteins that were strongly retained by the column. From the results on the phenyl/acetyl columns, the proteins were divided into three groups. LYZ, RNAse, MYO, and CYT c showed a concomitant decrease in retention with phenyl ligand density until the ligand density had been reduced by a factor of two (Fig. 4A). After

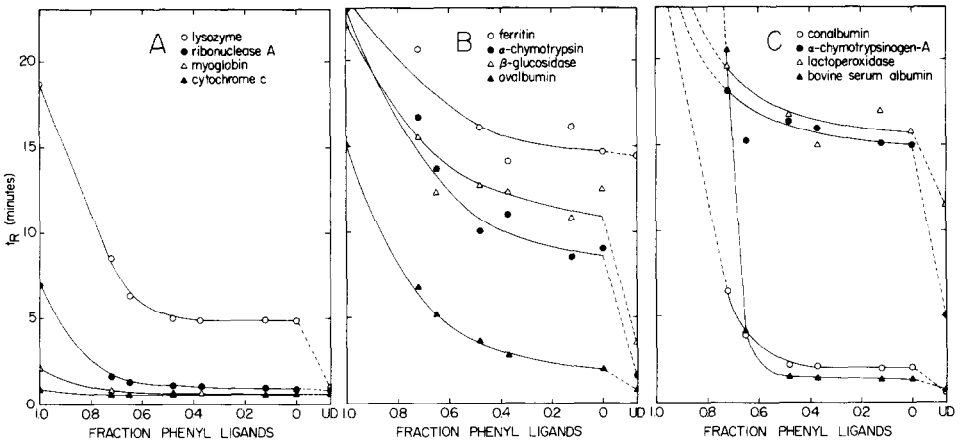

Fig. 4. The effect of phenyl ligand density on protein retention was determined by chromatographing 20-μl injections of twelve individual proteins on a series of columns with decreasing phenyl ligand densities. Each protein was chromatographed in a 20-min linear gradient from 1.0 *M* sodium sulfate in 10 m*M* potassium phosphate buffer (pH 7.0) to 10 m*M* potassium phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. "UD" refers to the control column of underivatized TEPA-coated column material.

that point, no further decrease in retention was observed. The four proteins in this group were the smallest of the twelve standard proteins used as test substances. If one assumes that the proteins are spherical, then the smaller proteins would interact with the support over a smaller surface area, thus contacting fewer phenyl groups. The leveling-off of the retention was not due to interaction with the underlying polymer, since a control column of underivatized TEPA-coated packing material gave a further decrease in protein retention. Ferritin, α-CHY, β-GLU, and OVA showed a large initial decrease in retention as the phenyl ligand density was decreased to approximately two-thirds of the initial value (Fig. 4B). The retention continued to decrease as the phenyl ligand density was decreased further, but at a lesser rate. These four proteins were the next largest in size. Retention also decreased when this group was chromatographed on the control column of underivatized TEPA-coated packing material. At higher phenyl ligand densities BSA, CON, lactoperoxidase, and α-CHYGEN were not eluted from the column during the gradient (Fig. 4C). In fact, once adsorbed, these proteins required an organic solvent for elution. A further decrease in retention was seen when these proteins were chromatographed on the underivatized TEPA-coated packing material. With one exception, α-CHYGEN, these four proteins were the largest of the twelve test proteins.

Although the chromatographic behavior allows these proteins to be grouped by molecular weight, the proteins in each group did not have the same retention characteristics in that some proteins were retained longer than others. Although size may be a contributing factor in HIC, surface hydrophobicity determines protein retention.

α-CHY and α-CHYGEN exhibited quite different chromatographic behavior even though there is not much structural difference between the enzyme and its zymogen. In the activation of α-CHYGEN four amino acids are excised: Ser-13, Arg-14, Thr-147, and Asn-148. The main chain rotates 180°, and this buries the formerly

exposed residues Ile-16 and Val-17 and exposes Met-192, Gly-193 and Arg-145 (ref. 29). This would make the surface of the enzyme more hydrophilic than the zymogen. Thus, the enzyme might be expected to be eluted before the zymogen on an HIC column, and this was the case.

The same twelve proteins were individually chromatographed on a Synchropak RP-8 RPC column. The elution order in comparison with an HIC phenyl–acetyl column is shown in Table II. MYO, β-GLU, and α-CHYGEN each showed multiple peaks upon elution. The largest of the peaks in terms of area was taken to be the major peak in each case.

When compared to the retention times of these same twelve proteins on the HIC phenyl–acetyl columns, those on the RP-8 column exhibited several major differences. CYT c and MYO, which are very hydrophilic on an HIC column, are both fairly strongly retained by the RPC column. In contrast, OVA is strongly retained by the RPC column, but it is generally eluted less than halfway through an HIC gradient. β-GLU, which is strongly retained by all the HIC columns, is only weakly retained by the RPC column.

TABLE II

HIC AND RPC SELECTIVITY

HIC		*RPC*	
Protein	t_R *(min)*	*Protein*	t_R *(min)*
CYT c	0.6	β-GLU	5.3
MYO	0.8	RNAse	10.7
RNAse	1.6	CYT c	12.6
CON	6.3	α-CHY	13.6
OVA	6.5	LYZ	14.3
LYZ	8.5	MYO	14.6
β-GLU	15.6	Ferritin	16.6
α-CHY	16.6	α-CHYGEN	16.8
α-CHYGEN	18.1	BSA	17.1
Lactoperoxidase	19.5	CON	17.3
BSA	20.5	OVA	18.5
Ferritin	20.8	Lactoperoxidase	20.3

These differences in retention times can be explained by examining the structure of the proteins during each of these chromatographic processes. In HIC, the proteins retain their native structure, and most of the hydrophobic residues are buried in the interior of the molecule. In RPC, the acids and organic solvents partially denature the proteins, thereby exposing the more hydrophobic interiors. Therefore, the RPC of proteins with more hydrophilic exteriors, such as CYT c and MYO, exhibit a larger degree of hydrophobicity and interact strongly with the hydrophobic surface of the reversed-phase column.

A mixture of six proteins was chromatographed on each of the phenyl–acetyl columns and on the TSK-phenyl column (Table III). In general, there was a decrease in protein retention and resolution (R_s) with decreasing phenyl ligand density. The major exception is the resolution between LYZ and β-GLU, where resolution in-

creased by a factor of three as the phenyl ligand density decreased to zero. This is due to the fact that the retention time of β-GLU did not decrease at the same rate as that of LYZ when the phenyl ligand density was decreased. Although the proteins were widely separated on the high phenyl ligand density columns, the peaks were fairly broad. As the phenyl ligand density decreased, the peaks became somewhat sharper, but resolution was lost due to decreased retention and/or loss of selectivity. The TSK-phenyl column gave very good resolution of the six proteins. The selectivity was not as good as the high ligand density phenyl–acetyl column, but the peaks were sharper, and this accounted for the better resolving power of the TSK-phenyl column.

The Synchropak RP-8 column exhibited better resolution of these six proteins (Table IV). This is not surprising, since the selectivity of the reversed-phase column is completely different from that of the HIC columns. Proteins that were nearest neighbors in the RPC chromatogram were better resolved by the HIC column.

TABLE III

PROTEIN RETENTION AND RESOLUTION BY HIC

Fraction phenyl	*CYT c* t_R *(min)*	R_s	*MYO* t_R *(min)*	R_s	*RNAse* t_R *(min)*	R_s	*OVA* t_R *(min)*	R_s	*LYZ* t_R *(min)*	R_s	*β-GLU* t_R *(min)*
1	0.9	1.7	3.5	1.1	7.0	2.6	15.0	1.5	19.2	1.1	22.0
0.72	0.6	0.4	0.8	1.2	1.9	2.8	7.3	0.9	9.6	2.3	14.3
0.65	0.6	0	0.6	0.7	1.2	1.4	4.0	0.7	5.8	2.9	12.2
0.48	0.6	0	0.6	0.7	1.1	1.8	4.0	0.4	5.0	3.8	13.2
0.37	0.6	0	0.6	0.6	1.0	0.8	2.2	1.1	4.8	3.3	12.3
0.12	0.7	0	0.7	0.6	1.6	1.4	4.9	0	4.9	2.8	10.8
0	0.6	0	0.6	0.6	1.0	1.3	2.9	0.8	4.7	3.6	12.4
TSK-phenyl	2.8	1.6	3.8	3.1	6.9	3.3	11.7	1.1	13.2	6.5	19.2

Recovery of enzyme activity

Sample sizes ranging from 10–200 μg were chromatographed on a phenyl–acetyl and the Synchropak RP-8 columns, collected and assayed for enzymatic activity. Control samples of the same size were injected into empty capillary tubing. Recovery of lactic dehydrogenase (LDH) and α-CHY by HIC are shown in Table V. Enzyme recovery exceeded 90% for LDH and 86% for α-CHY in all trials. When β-GLU was assayed after reversed-phase chromatography, no detectable activity was found in either the control or the column sample. Recovery of α-CHY by RPC as shown in Table VI ranged from 54–91%. The enzymatic activity was significantly reduced after elution with 2-propanol for both the column sample and the control when compared to the activity recovered from the HIC column. The average activity recovered from the reversed-phase column for the five protein samples was 12.8 units/mg enzyme, while the average activity recovered from the HIC column was 36.6 units/mg enzyme. A second control for the reversed-phase samples was prepared by hydrating α-CHY in the initial reversed-phase buffer, 0.1% TFA in water. There-

TABLE IV

HIC AND RPC RESOLUTION

		R_s				
		MYO	*RNAse*	*OVA*	*LYZ*	*β-GLU*
CYT c	HIC	1.7	2.8	9.7	9.6	16.9
	RPC	3.0	1.3	4.3	2.0	5.4
MYO	HIC		1.1	4.8	5.5	8.4
	RPC		4.4	1.6	1.3	8.2
RNAse	HIC			2.6	3.5	5.3
	RPC			5.6	3.6	4.5
OVA	HIC				1.5	3.3
	RPC				3.0	9.0
LYZ	HIC					1.1
	RPC					8.2

TABLE V

RECOVERY OF ENZYME ACTIVITY AFTER HIC

Amount of protein injected (μg)	*Lactic dehydrogenase*			*α-Chymotrypsin*		
	Units recovered/mg enzyme		*Recovery (%)*	*Units recovered/mg enzyme*		*Recovery (%)*
	Sample	*Control*		*Sample*	*Control*	
10	39.8	39.8	100	58.0	51.0	114
25	54.8	51.3	107	41.4	47.8	86.6
50	44.2	35.4	125	25.2	28.2	89.4
100	20.3	21.7	93.5	35.6	33.2	107
200	24.1	21.2	114	32.6	25.4	128

TABLE VI

RECOVERY OF ENZYME ACTIVITY (α-CHY) AFTER RPC

Amount of protein injected (μg)	*Units recovered/mg enzyme*		*Recovery (%) Blank**		*Percentage of blank*
	Sample	*Control*			
10	26.4	29.0	91.0	21.8	121
25	10.9	19.4	56.2	16.0	68.1
50	9.8	14.5	67.6	9.8	100
100	4.9	9.1	54	7.3	68
200	11.8	13.6	86.8	27.2	43.4

* Blank assays contained protein in a 0.1% TFA without propanol.

fore, the protein was not exposed to 2-propanol. This sample was assayed in the same manner as the column sample and the control and is referred to as the blank in Table VI. In three of the five trials the column sample had less activity than the blank which had less activity than the control. In one trial the sample activity was equal to the blank activity, and in the 10-μg trial the sample activity was greater than the blank. Therefore, it is not only the organic solvent that is denaturing the protein and causing a loss of activity, but the interaction with the column as well.

Load capacity

Load capacities of the butyrate and the TSK-phenyl columns were determined by measuring the change in resolution between two standard proteins as a function of protein load. Resolution on the butyrate column declined significantly as the protein load increased above 4 mg. Column dimensions of the butyrate column were 4 cm × 0.41 cm I.D., giving an internal volume of 0.53 cm^3, and a load capacity of 7.5 mg/cm^3. Resolution on the TSK-phenyl column also delcined significantly as the protein load increased above 4 mg. However, the dimensions of the TSK-phenyl column were 7.5 cm × 0.75 cm I.D., giving an internal volume of 3.3 cm^3 and a load capacity of 1.2 mg/cm^3. The load capacity on an RP-8 RPC column as calculated from data by Pearson *et al.*[30] was 9.1 mg/cm^3.

CONCLUSIONS

Protein retention on a HIC column can be easily manipulated through mobile phase variables, which include salt concentration, salt type, and pH. In optimizing the resolution of a protein from a mixture, initial experiments should be performed with a salt of intermediate salting-out ability at a concentration of at least 1 *M*. An increase in the initial salt concentration or the use of a neutral salt with a greater salting-out ability can increase protein retention, while a decrease in the initial salt concentration or the use of a neutral salt with a lesser salting-out ability can decrease protein retention. For support materials synthesized using the covalently bound polyamine chemistry, a decrease in pH from neutrality caused an increase in retention. Another method for manipulating protein retention is through matrix surface variables, such as alkyl chain length and ligand density. As previously shown[6], an increase in the alkyl chain length causes an increase in protein retention. In general, decreasing the ligand density decreases protein retention. The extent of this effect is apparently dependent upon the size of the protein. Small proteins with less surface area in contact with the column material exhibited a decrease in retention which leveled-off at lower ligand densities. Larger proteins which have a greter contact surface, exhibited a continuous decrease in protein retention with decreasing ligand density. This effect was most dramatic at high ligand densities where the largest proteins were irreversibly bound to the column material. Surface hydrophobicity is apparently the most important factor in the extent of protein retention, although size does contribute.

Retention and resolution both decreased concomitantly with ligand density. The only major exception to this observation was the resolution between LYZ and β-GLU, where the value increased by a factor of three as the phenyl ligand density decreased to zero. This increased resolution was due to the lesser rate at which the

retention of β-GLU decreased with decreasing ligand density.

A comparison of the selectivity of the HIC and RPC columns was made for twelve proteins of differing molecular weights and isoelectric points. Several proteins that were only weakly retained on the HIC column were strongly retained on the RPC column, while the converse was found for other proteins.

Recovery of enzymatic activity was measured for proteins eluted from both the HIC and RPC columns. The recovery of LDH exceeded 90%, while the recovery of α-CHY exceeded 86% from the HIC column. From the RPC column only 54–91% of the activity of α-CHY and none of the activity of β-GLU was recovered. This further supports the supposition that protein separation on a HIC column is based on native hydrophobicity, while that on a RPC column is based on the hydrophobicity of the denatured protein. When the activity of the enzyme after it is eluted from the column was compared to a control sample and a blank sample in which no 2-propanol was added, it was observed that the interaction with the column contributed to the denaturation of the enzyme.

Load capacity of the butyrate column as determined by the decrease in resolution with increasing protein load was found to be 7.5 mg/cm^3 of column material. This value was slightly less than the 9.1 mg/cm^3 of the RPC column[30].

Although both RPC and HIC separate proteins on the basis of hydrophobicity, there are major differences between the two processes. HIC does not alter the native structure of the protein through interaction with the column or mobile phase. In RPC, the proteins are at least partially denatured through interaction with the mobile phase and the column material. Further experimentation is required to understand the mechanism of both RPC and HIC separations.

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REFERENCES

1 S. Shaltiel and Z. Er-el, *Proc. Nat. Acad. Sci. U.S.*, 70 (1973) 778.
2 Z. Er-el, Y. Zaidenzaig and S. Shaltiel, *Biochem. Biophys. Res. Commun.*, 49 (1972) 383.
3 H. A. Arfmann and S. Shaltiel, *Eur. J. Biochem.*, 70 (1976) 269.
4 S. Hjertén, *J. Chromatogr.*, 87 (1973) 325.
5 T. Fujita, Y. Suzuki, J. Yamauti, I. Takagahara, K. Fuji, J. Yamashita and T. Horio, *J. Biochem.*, 87 (1980) 89.
6 J. L. Fausnaugh, E. Pfannkoch, S. Gupta and F. E. Regnier, *Anal. Biochem.*, 137 (1984) 464.
7 G. E. Berendsen, K. A. Pikaart and L. de Galan, *J. Liquid Chromatogr.*, 3 (1980) 1437.
8 J. D. Pearson, W. C. Mahoney, M. A. Hermodson and F. E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
9 H. P. J. Bennett, *J. Chromatogr.*, 266 (1983) 501.
10 M. J. O'Hare, M. W. Capp, E. C. Nice, N. H. C. Cooke and B. G. Archer, *Anal. Biochem.*, 126 (1982) 17.
11 J. Luiken, R. van der Zee and G. W. Welling, *J. Chromatogr.*, 284 (1984) 482.
12 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 120 (1976) 321.
13 H. Engelhardt and D. Mathes, *J. Chromatogr.*, 142 (1977) 311.

14 E. Pfannkoch, K. C. Lu, F. E. Regnier and H. G. Barth, *J. Chromatogr. Sci.*, 18 (1980) 430.
15 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 49.
16 S. Gupta, E. Pfannkoch and F. E. Regnier, *Anal. Biochem.*, 128 (1983) 196.
17 S. Gupta and F. E. Regnier, *U.S. Patent application* 441,057 (1984).
18 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1979) 375.
19 B. C. W. Hummel, *Can. J. Biochem. Physiol.*, 37 (1959) 1393.
20 S. Morgenstern, R. Flor, G. Kessler and B. Klein, *Anal. Biochem.*, 13 (1965) 149.
21 P. Om. Bahl and K. M. L. Agrawal, *J. Biol. Chem.*, 243 (1968) 98.
22 W. Melander and Cs. Horváth, *Arch. Biochem. Biophys.*, 183 (1977) 200.
23 M. Dixon and E. C. Webb, *Advan. Prot. Chem.*, 16 (1961) 197.
24 W. Kopaciewicz and F. E. Regnier, *Anal. Biochem.*, 126 (1982) 8.
25 P. G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1.
26 P. G. Righetti, G. Tudor and K. Ek, *J. Chromatogr.*, 220 (1981) 115.
27 Y. Kato, T. Kitamura and T. Hashimoto, *Third Int. Symp. on HPLC of Proteins, Peptides and Polynucleotides, Monte Carlo, 1983*, Paper No. 308.
28 H. P. J. Bennett, C. A. Browne and S. Soloman, *J. Liquid Chromatogr.*, 3 (9) (1980) 1353.
29 S. T. Freer, J. Kraut, J. D. Robertus, H. T. Wright and Ng. H. Xuong, *Biochemistry*, 9 (1970) 1997.
30 J. D. Pearson, N. T. Lin and F. E. Regnier, *Anal. Biochem.*, 124 (1982) 217.