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HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF PROTEINS ON A SERIES OF POLY(ALKYL ASPARTAMIDE)-SILICAS

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SUMMARY

A series of bonded poly(alkyl aspartamide) coatings was prepared on silica. The products showed a wide range of hydrophobicity, and were evaluated for high-performance hydrophobic-interaction chromatography (HPHIC) of proteins. Poly-(propyl aspartamide)-silica is a good general-purpose material for protein HPHIC; poly(ethyl aspartamide)-silica may be useful for more hydrophobic proteins.

Retention of proteins generally increased dramatically with ligand length in the coating, but this was not true of certain apolipoproteins with no well-defined tertiary structure. A correlation was also observed between peak width and conformational lability; the apolipoproteins (an extreme case) were eluted in exceptionally broad peaks. These principles can be used to obtain data on the physical characteristics of new proteins. This was demonstrated with *recA* protein.

INTRODUCTION

Hydrophobic-interaction chromatography (HIC) is a technique for purifying or analyzing proteins, which complements ion-exchange, steric-exclusion, and affinity methods[1–3]. Separation is based on differences in surface hydrophobicity of proteins. Column packings currently in use generally feature short hydrophobic ligands or poly(ethylene glycol) grafted to the surface. The protein mixture usually is applied in a buffer containing a high concentration of a salt high in the Hofmeister series (*e.g.*, citrate or sulfate)[4,5]. Under these conditions, proteins will be "salted out" on the surface of the packing. Subsequently, the column is eluted with a gradient of decreasing ionic strength and/or an increasing gradient of glycols, sucrose, or detergent. These conditions cause proteins to be eluted in the order of increasing hydrophobicity. The tertiary structure generally is retained under these conditions, and enzymes are usually recovered with high yields of applied activity. This stands in contrast with reversed-phase chromatography, which employs conditions that denature many enzymes[6,7].

Recently, a number of materials have been developed for high-performance hydrophobic-interaction chromatography (HPHIC) of proteins. TSK Phenyl-5PW is a polymeric material with pendant phenyl groups[8,9]. Fausnaugh *et al.*[10] made a

polyamine-coated silica react with a series of acid anhydrides or chlorides to produce materials with different ligands in the coating. Gooding et al.[11] also prepared a series of coated silicas in which various ligands were attached to a polymeric coating. Miller et al.[12] employed silica with a bonded polyether coating, while Horváth's group investigated the use of silica with a hydrophobic layer beneath a hydrophilic coat[4,13].

A process has recently been developed which produces silica-bonded polypeptide coatings[14]. The reactive polymer poly(succinimide) generates a reproducible, self-assembling coating on aminopropyl-silica. Succinimide rings in the coating can then be hydrolyzed to yield poly(aspartic acid)-silica or they can be allowed to react with alkyl amines to produce silica with poly(alkyl aspartamide) coatings. When applied to high-performance liquid chromatography (HPLC) of proteins, the performance of these materials is excellent, presumably due to the uniformity and polypeptide nature of the coatings. In this paper I describe the preparation of a series of poly(alkyl aspartamide)-silicas for HPHIC of proteins.

MATERIALS AND METHODS

HPLC apparatus and columns

HPLC was performed on a Beckman Model 110A gradient system (Beckman Instruments, Berkeley, CA, U.S.A.). Samples were applied with a Valco valve (Valco Instruments, Houston, TX, U.S.A.), equipped with a 25-μl loop. The detector was a SpectroMonitor II from Laboratory Data Control (Riviera Beach, FL, U.S.A.). The SynChropak PROPYL column (25 × 0.41 cm) was from SynChrom (Linden, IN). Poly(ethyl aspartamide)-silica and poly(propyl aspartamide)-silica were slurry-packed into 20 × 0.46 cm columns. Such columns are available from Custom LC (Houston, TX, U.S.A.) or PolyLC (Columbia, MD, U.S.A.) under the names PolyETHYL A and PolyPROPYL A.

Reagents

Vydac 101TP silica (particle diameter 5 μm; pore diameter 330 Å) was purchased from The Separations Group (Hesperia, CA, U.S.A.). The following were from Sigma (St. Louis, MO): cytochrome *c* (Type VI, from horse heart), myoglobin (type II, from sperm whale skeletal muscle), ribonuclease A (type III-A, from bovine pancreas), conalbumin (type I), α-chymotrypsin (type II, from bovine pancreas). Thiolase I (pig heart)[15] was a gift of H. F. Gilbert and E. Izbicka (Baylor College of Medicine, Houston, TX, U.S.A.); *recA* protein (*E. coli*) was a gift of K. L. Beattie and M. K. Brawn (Baylor College of Medicine); apolipoproteins were gifts of J. D. Morrisett, H. J. Pownall, and J. T. Sparrow (Baylor College of Medicine). Reagents and solvents were from Aldrich (Milwaukee, WI, U.S.A.) and were of the purest grades available. Methylamine was obtained as a 40% (w/w) solution in water, and ethylamine was obtained as a 70% (w/w) solution in water.

Preparation of poly(alkyl aspartamide)-silicas

Poly(succinimide)-silica was prepared essentially as described earlier[14].

Poly(succinimide)-silica was weighed into a flask and swirled in seven volumes of N,N-dimethylformamide (DMF). Swirling was continued for 1 min, while the material was degassed under vacuum. An alkylamine (3.6 mmol/g coated silica) in

an additional two volumes of DMF was added. The resulting mixture was left 24 h at room temperature with frequent swirling during the first 2 h and occasional swirling thereafter. The product was collected in a medium-porosity sintered-glass funnel and washed well with water, then 0.05 M hydrochloric acid, water, and acetone, and finally dried by continued suction.

Assay of protein cation-exchange capacity

This was performed with hemoglobin, as previously described[14]. Released hemoglobin was quantitated, using the value $A_{410} = 1.0$ a.u. for a solution of 0.5 mg hemoglobin per ml. (A recent study indicates that the true value is probably 0.126 mg hemoglobin per ml for $A_{410} = 1.0$ a.u., with sedimentable impurities in the hemoglobin preparation accounting for much of the difference[16].)

Assay of protein hydrophobic-interaction binding/release (HIB/R) capacity

This was performed with hemoglobin, using a published method[10,16]. The initial hemoglobin stock solution was filtered after particulate matter had been removed by centrifuging.

RESULTS

Preparation of poly(alkyl aspartamide)-silicas

Poly(propyl aspartamide)-silica and the other HPHIC column materials were prepared as shown in Fig. 1. Poly(succinimide) attaches itself to the surface of aminopropyl-silica through amide bonds. The remaining succinimide rings react with n-propylamine in DMF solution to form α,β-propyl aspartamide units.

Rates of reaction

In order to optimize the preparation of the HPHIC materials, the time course of addition of n-propylamine to the poly(succinimide) coating was followed. Samples of poly(succinimide)-silica were immersed in solutions of n-propylamine and worked up at various times. The products were assayed for their HIB/R capacity with hemoglobin. Since poly(succinimide)-silica has a modest cation-exchange capacity for hemoglobin[14], the change in this quantity was also followed to show the transformation of the original coating. Fig. 2 shows that the addition of n-propylamine to the coating is quite rapid, and is over 90% complete in 6 h under the reaction conditions employed. The increase in the HIB/R capacity was paralled by a corresponding decrease in the cation-exchange capacity. This suggests that hemoglobin does not interact with the product through a mixed-mode mechanism. Reaction was continued for a total of 24 h to insure thorough derivatization of the coating.

A sample of the same poly(succinimide)-silica was hydrolyzed to poly(aspartic acid)-silica, as previously described[14]. The hemoglobin cation-exchange capacity of the product was 382 mg hemoglobin per g packing. This compares with a figure of 427 mg hemoglobin per g packing for the most thoroughly derivatized sample of poly(propyl aspartamide)-silica when assayed in the HIB/R mode. Thus, though the two products sorb hemoglobin through completely different mechanisms, the ultimate degree of surface coverage by the protein is similar. In either mode, the capacity is quite high.
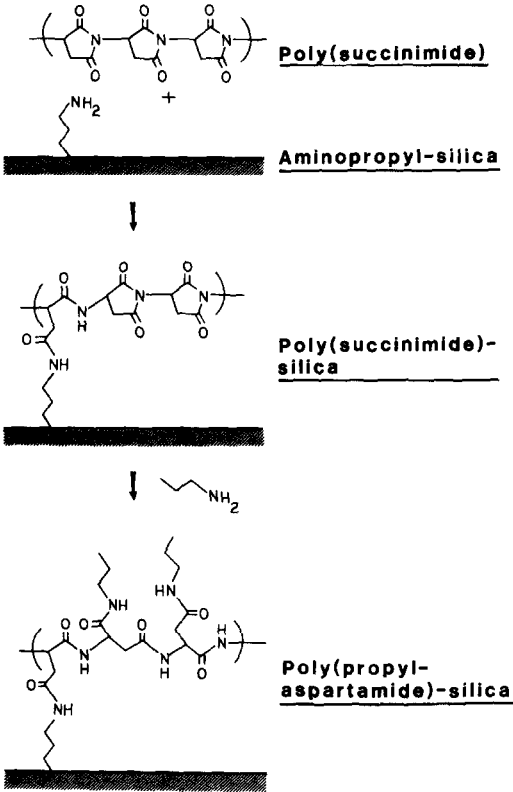
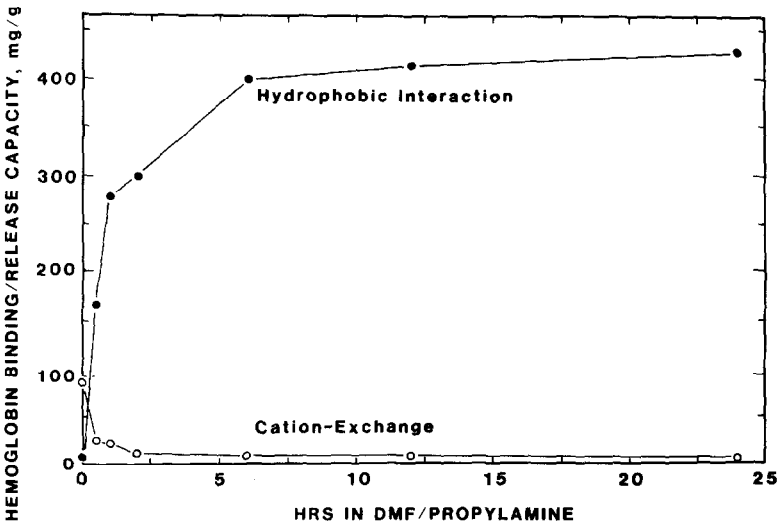Fig. 1. Reaction scheme for the preparation of poly(propyl aspartamide)-silica.

Fig. 2. Reaction of poly(succinimide)-silica with *n*-propylamine.

Properties of poly(alkyl aspartamide)-silicas

A series of poly(alkyl aspartamide)-silicas was prepared with the amines listed in Table I. The hemoglobin HIB/R capacity of the products is shown in Fig. 3. Poly(2-hydroxyethyl aspartamide)-silica binds virtually no hemoglobin in this mode. This lack of hydrophobicity contrasts with the modest degrees observed with the column materials currently in use for steric exclusion HPLC of proteins[17,18] and suggests that the new material may be useful for that purpose. The HIB/R capacity increases dramatically in the ligand series methyl < ethyl < propyl, but decreases with the butyl and pentyl ligands. A similar progression is evident in Table I of ref. 10. This decrease reflects incomplete release of bound hemoglobin rather than decreased binding; materials in the series through propyl released bound hemoglobin cleanly to yield off-white products, while products with longer ligands ranged in color from light brown (butyl, benzyl) to dark brown (pentyl, phenethyl, 4-hydroxyphenethyl). It is characteristic of HIC that small changes in ligand length lead to dramatic changes in the strength of hydrophobic binding[1,10,11], as seen here. This is in contrast with the minor changes such variation produces in reversed-phase chromatography[11,19].

The data indicate that coatings with ligands longer than propyl will not be useful in the general-purpose HPHIC of proteins. However, the more hydrophobic materials may be useful for the immobilization of enzymes in reactors through hydrophobic adsorption. Preliminary data indicate that enzymes can be adsorbed on poly(phenethyl aspartamide)-silica with preservation of activity (data not shown).

The reaction solutions of methylamine and ethylamine were prepared from the commercially available aqueous solutions of the amines. This raises the possibility of some base-catalyzed hydrolysis of the succinimide rings by the modest amounts of water thus introduced. It appears that nucleophilic reaction of the amines with the rings was much more rapid, though, since none of the HPHIC materials displayed more than a negligible hemoglobin cation-exchange capacity.

Chromatography performance

HPLC columns were packed with poly(ethyl aspartamide)-silica (PolyETHYL A) and poly(propyl aspartamide)-silica (PolyPROPYL A). A mixture of standard proteins was resolved on the two columns under identical conditions (Fig. 4). Most

TABLE I

AMINES USED TO PREPARE POLY(ALKYL ASPARTAMIDE) COATINGS

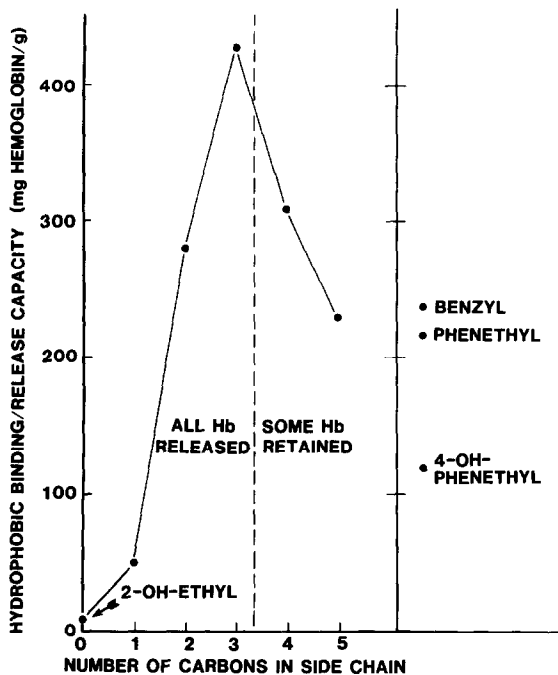| Amine	Resulting alkyl ligand
Ethanolamine	2-Hydroxyethyl
Methylamine	Methyl
Ethylamine	Ethyl
n-Propylamine	Propyl
n-Butylamine	Butyl
n-Pentylamine	Pentyl
Benzylamine	Benzyl
Phenethylamine	Phenethyl
Tyramine	4-Hydroxyphenethyl

Fig. 3. HIB/R capacity of poly(alkyl aspartamide)-silicas (curve) and poly(aryl aspartamide)-silicas (vertical points).

proteins were eluted in sharp, symmetrical peaks with little tailing. Presumably, this reflects the uniform, polypeptide nature of the coating. Retention times on Poly-PROPYL A were nearly twice as great as on PolyETHYL A, a difference in hydrophobic character which was also reflected in their HIB/R capacities. PolyPROPYL A appears to be a good column material for general-purpose HPHIC of proteins, while PolyETHYL A may be useful for more hydrophobic proteins and other natural polymers. Columns were reasonably durable, having a useful life of at least several hundred hours of operation. The retention times of the proteins in Fig. 4 varied within a range of ±5% on columns of PolyPROPYL A from four different lots, while the HIB/R capacity was 403 mg hemoglobin per g PolyPROPYL from five different lots, with a variation of ±5%. The good reproducibility of the material is in keeping with the self-assembling nature of the coating.

The relative movement of different proteins through a HIC column can vary with changes in elution conditions[8-11,20]. Complementary selectivity is also observed between different column materials used under the same conditions. For example, the relative positions of ribonuclease and myoglobin are reversed on PolyPROPYL A and PolyETHYL A columns (Fig. 4). Adsorption of a protein on a hydrophobic surface is through non-polar residues in "pockets" near the surface as well as on the surface of the tertiary structure[1,21]. If minor conformational changes ("breathing") in the tertiary structure are prevented by intramolecular crosslinks, then access to the pocket residues is denied, and the protein is eluted much more rapidly from a hydrophobic column[22]. In this case, myoglobin probably has a greater proportion of
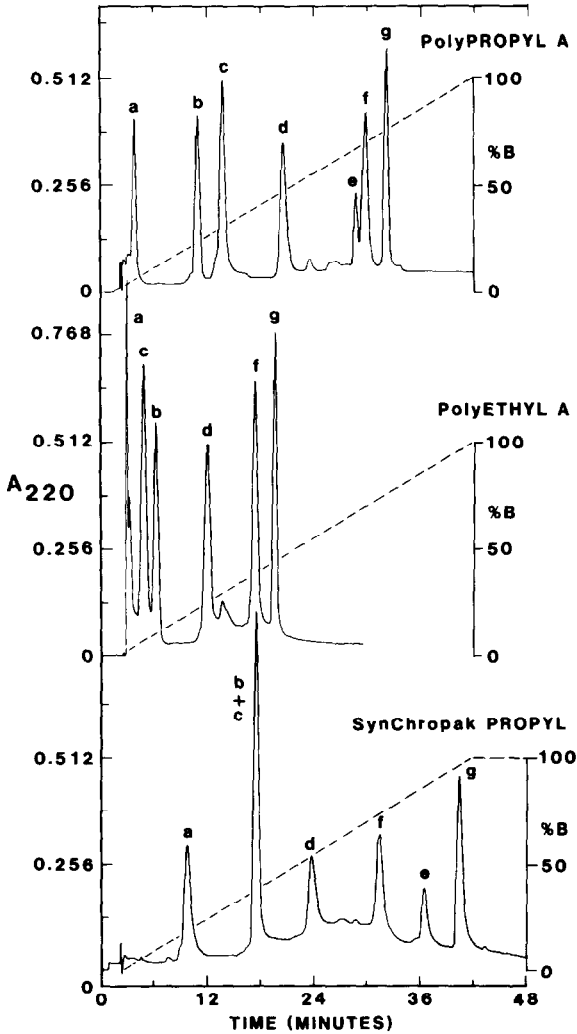
Fig. 4. HPHIC of standard proteins. The columns are described in Materials and methods. Sample: 25 μl containing 25 μg of each protein in buffer A. Buffer A: 1.8 M ammonium sulfate + 0.1 M potassium phosphate, pH 7.0. Buffer B: 0.1 M potassium phosphate, pH 7.0. Gradient: 40-min linear, 0–100% buffer B. Flow-rate: 1 ml/min. Detection: A_{220} = 1.28 a.u.f.s. Peaks: a = cytochrome c, b = ribonuclease A, c = myoglobin, d = conalbumin, e = neochymotrypsin, f = α-chymotrypsin, g = α-chymotrypsinogen A.

its (surface + pocket) non-polar residues in the pocket domain than does ribonuclease; increasing the ligand length by one methylene unit permits interaction with an appreciably greater number of the non-polar residues of myoglobin, with a much greater effect on its migration through the column. Unusual sensitivity to ligand length was also observed with human serum albumin and α-fetoprotein, and was related to the location of non-polar residues in pockets rather than on the surface of the protein[21].

The resolution of the same standard proteins was also performed with a Syn-Chropak PROPYL column in order to compare the selectivity of the polyaspart-amide-based coatings with that of one based on a different polymer (Fig. 4). Reten-tion times are somewhat longer on the SynChropak PROPYL column than on the PolyPROPYL A column. Since the hydrophobic ligand in both coatings has the same nominal length, this presumably reflects a greater ligand density on the surface of SynChropak PROPYL. In fact, the effective length of its ligand may be intermediate between those of the poly(ethyl aspartamide) and poly(propyl aspartamide) coatings, since myoglobin and ribonuclease coincide in their migration through the column.

Protein recovery

Recovery of protein applied to PolyPROPYL A and PolyETHYL A columns is generally in excess of 90%, as judged from A_{220} OD units. Most enzymes are eluted with preservation of full activity, but some exceptions were noted. Thiolase I (pig heart) was largely inactivated by HPHIC on PolyPROPYL A under the con-ditions given in Fig. 4; this proved to be due to the high ionic strength of the medium, in which this enzyme is unstable. Lower recoveries were also observed for the labile proteins interleukin-1 (from human leukemic monocytes)[23] and a cytolytic factor from BCG-stimulated murine peritoneal macrophages[24]. However, HPHIC is still useful for such applications, since some other modes of HPLC lead to even greater losses of activity of these proteins as well as lesser degrees of purification. Substitution of citrate for sulfate and inclusion of ethylene glycol or glycerol in the buffer are sometimes helpful in these cases. A 1.1 *M* citrate solution is about as effective as 1.8 *M* sulfate in promoting protein binding, but its use precludes the monitoring of the effluent at 220 nm. Another technique is the inclusion of detergents in the mobile phase. This may improve recoveries by promoting solubilization and may also in-crease selectivity[25].

Retention characteristics and conformational lability

Some proteins are eluted from chromatography columns in unusually broad peaks, or even in discrete multiple peaks. This has been ascribed to several causes: (1) microheterogeneity due to post-translational modification or proteolysis; (2) gra-dient artifacts[10]; (3) aggregation, as in the resolution of human serum albumin oli-gomers by steric exclusion[26]; (4) heterogeneity in the adsorption sites of the column material[3]; and (5) denaturation. The native and denatured forms can be resolved if conformational equilibrium is slow relative to the time scale of migration through the column[27–31]; otherwise, a single broad peak will be obtained which is the weighted average of the forms in equilibrium[28,29,32,33]. Karger and co-workers[30–32] have dealt extensively with factors influencing the denaturation of proteins on reversed-phase columns.

Štrop and co-workers[20,21] ascribe the broad or multiple HIC peaks of human serum albumin and α-fetoprotein to normal conformational variations in the tertiary structures. The implication is that a protein can assume different conformations well short of denaturation which can be adsorbed on the surface of the packing material with varying affinities and therefore be desorbed at varying times in gradient elution. Srinivasan and Ruckenstein[3] have further developed this idea, and Ingraham *et al.*[33] have obtained evidence of this process in HPHIC. Supporting this interpretation is

the observation that the peak for chymotrypsin is always broader than the peak for chymotrypsinogen, regardless of their elution sequence (see Fig. 4 and refs. 8 and 11). The peptide cleavages that convert chymotrypsinogen to chymotrypsin have little effect on the overall surface hydrophobicity[7] but probably increase the conformational lability of the protein.

To explore this issue, the retention characteristics of apolipoproteins were studied. In solution, the monomer forms of most apolipoproteins are loosely folded, and non-polar residues are not sequestered[34]. Fig. 5 shows the elution of rabbit apolipoprotein E (apoE) from PolyPROPYL A and PolyETHYL A columns. This apolipoprotein has no disulfide crosslinks and displays three isomorphic bands in isoelectric focussing. The mixture is resolved into three peaks by the PolyPROPYL A column, but the peaks are up to twenty times broader than those for the proteins in Fig. 4. The elution profile from the PolyETHYL A column is almost identical. This surprising result shows that there is no hydrophobic region in these proteins that is accessible to a propyl ligand but not an ethyl ligand. This is in keeping with the lack of defined tertiary structure for these proteins[35]. It also lends support to the idea that the extreme breadth of the peaks reflects the possible adsorption of the apolipoproteins in a wide variety of configurations.

Fig. 6 shows the resolution of human apoC-I and apoC-III-1. ApoC-I behaves like the apoE isomorphs in its insensitivity to ligand length, and it is eluted in an extremely broad peak. ApoC-III-1 has a very hydrophobic surface, judging from the marked retention on PolyPROPYL A. However, the peak is reasonably sharp, al-
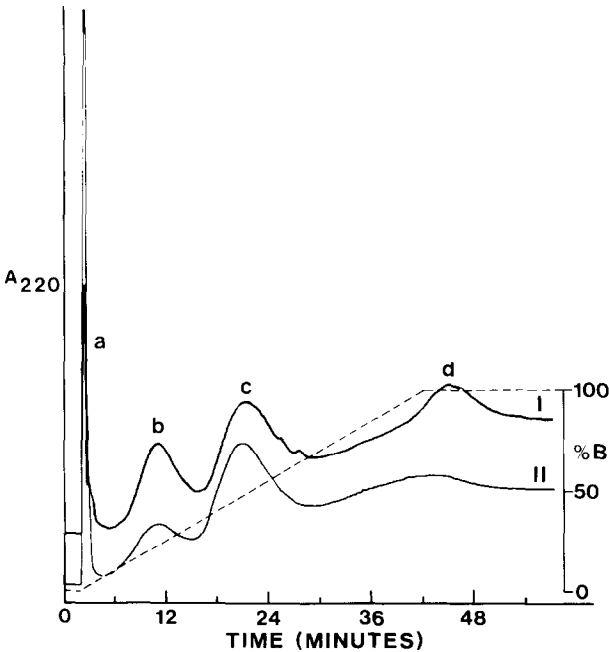

Fig. 5. HPHIC profile of rabbit apoE on PolyPROPYL A (I) and PolyETHYL A (II). Sample: 25 μl containing 0.45 mg protein per ml in buffer A. Detection: A_{220} = 0.32 a.u.f.s. Other conditions as in Fig. 4. Peaks: a, b, c = apoE peaks, d = gradient artifact.
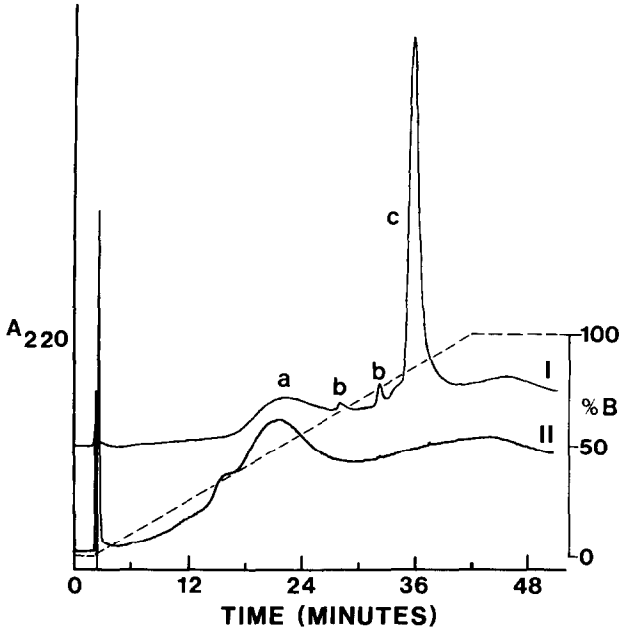
Fig. 6. HPHIC profile of human apoC-1 and apoC-III-1 on PolyPROPYL A (I) and PolyETHYL A (II). Sample: 25 μl containing 0.56 mg/ml apoC-I and 0.75 mg/ml apoC-III-1 in buffer A; sample prepared by mixing solutions of the pure proteins immediately before injection. Detection: A_{220} = 0.64 a.u.f.s. (I) and 0.32 a.u.f.s. (II). Other conditions as in Fig. 4. Peaks: a = apoC-I, b = aggregates of apoC-I and apo-C-III-1 (not observed with solutions of the pure apolipoproteins), c = apoC-III-1.
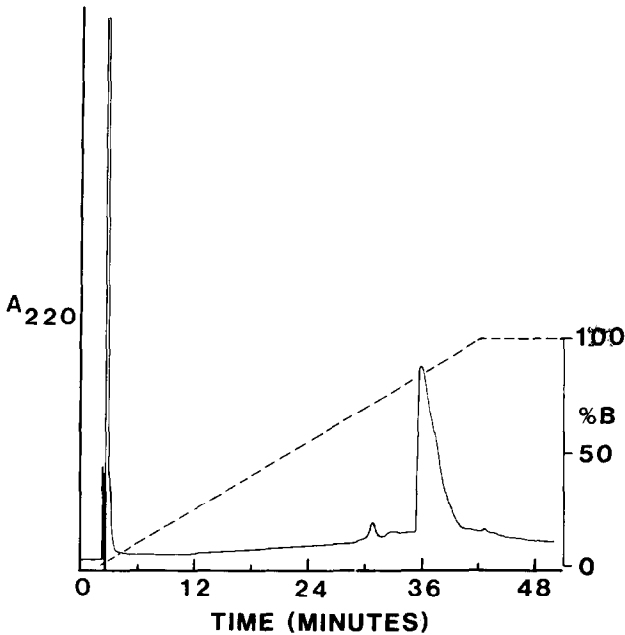

Fig 7. HPHIC profile of a *recA* protein sample on a PolyPROPYL A column. Sample: 25 μl containing 0.5 mg protein per ml in buffer A. Detection: A_{220} = 1.28 a.u.f.s. Other conditions as in Fig. 4.

though somewhat broader than those in Fig. 4. This apolipoprotein has a well-defined region for hydrophobic binding, and it assumes a more defined structure in the presence of a lipid or high salt concentration[36]. Evidently, these characteristics lead to adsorption in HIC in a more restricted range of configurations. The tertiary structure of adsorbed apoC-III-1 is sufficiently well defined for non-polar residues to be sequestered, since retention is affected by ligand length; apoC-III-1 is well-resolved from apoC-I on a PolyPROPYL A column but the two coincide on a PolyETHYL A column.

HPHIC was used to obtain data on physical characteristics of recA protein. This cytosolic protein displays a wide variety of enzymatic activities toward hydrophobic nucleic acid substrates, but very little is known about its structure[37]. Fig. 7 is a profile of a recA protein sample on a PolyPROPYL A column. The moderately broad peak suggests that recA protein is capable of assuming a variety of structural conformations in the presence of a hydrophobic surface, which is consistent with its multiple enzymatic activities. The strong retention shows that the surface of the protein is quite hydrophobic. This provides a plausible explanation for the recently reported association[38] between recA protein and the cellular membrane under some circumstances. It also suggests an explanation for the fairly specific affinity which a column of immobilized recA protein shows in adsorbing more recA protein from a crude homogenate of E. coli[39]; this may be a case of HIC adsorption of one of the most hydrophobic proteins in the homogenate rather than of a specific interaction between recA molecules.

DISCUSSION

The poly(alkyl aspartamide) coatings for silica are easily prepared and confer a wide range of hydrophobic properties. Proteins are generally eluted from columns of these materials in sharp peaks and with good recovery. A coating with the propyl ligand appears to be a good choice for general HPHIC of proteins, as has been noted with other coatings for silica-based packings[10,11]. The ethyl ligand coating is useful for studies of conformation and possible HPHIC of more hydrophobic species. The capacity of these materials is quite high, and this should make them useful for preparative chromatography.

HIC has been distinguished from reversed-phase chromatography by the marked increase in protein retention that is caused by small increases in the length of the hydrophobic ligand on the surface of the packing material. Here, the hemoglobin binding data and column comparisons show this generally to be the case. However, this sensitivity to ligand length requires that the protein possess enough secondary and tertiary structure to sequester non-polar residues, which would then be accessible to long ligands but not to short ones. In the extreme cases of apoC-I and apoE, there is so little defined secondary and tertiary structure that the sensitivity to ligand length is virtually abolished.

The Van der Waals forces which are involved in HIC[1,3] have a shorter range than do electrostatic effects. This suggests that access of a ligand to adsorption sites on a protein surface will be more sensitive to conformational variation in HIC than in ion-exchange. Thus, different conformations of a protein would display greater variations in binding affinity in HIC, and protein peaks in HIC would tend to be

broader than in ion exchange. The data in this study do show that when a protein is conformationally labile, the HIC peak is broad. This property is independent of the overall affinity for the sorbent surface, since apoC-I and apoE were eluted in extremely broad peaks but had appreciably shorter retention times than apoC-III-1 or chymotrypsinogen. The broad peaks observed with apoC-I and apoE could conceivably reflect some degree of aggregation. This seems unlikely, since protein concentrations were sufficiently low to favor the monomer forms somewhat[34], and apoE was eluted in three discrete peaks rather than a continuum. In cases where protein quaternary structure depends upon hydrophobic interactions, it has been suggested[33] that proximity to a hydrophobic surface could shift the equilibrium in the direction of the monomer form. In addition, the aggregation of apoC-I causes an increase in defined structure and sequestering of non-polar residues[34], and this would lead to a sensitivity to ligand length, but such a sensitivity was not observed here.

These principles indicate that HPHIC could be used to obtain data rapidly and conveniently about physical characteristics of proteins, some of which may be available only in limited quantity. This was demonstrated with *recA* protein, which appears to have a very hydrophobic surface and a fairly flexible conformation.

ACKNOWLEDGEMENTS

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