ON THE MODE OF ADSORPTION OF PROTEINS TO "HYDROPHOBIC COLUMNS"

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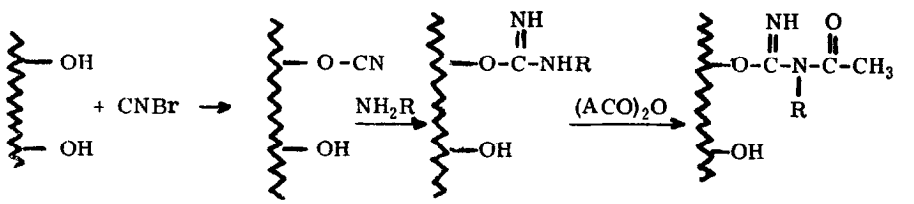
SUMMARY: α-Lactalbumin and ovalbumin were found to bind tightly to alkyl amino agaroses with hydrocarbon chains of 4-6 carbon atoms. These proteins were not adsorbed when the columns were acetylated. The binding of protein to the hydrophobic column appears to occur in two phases. The first of these is suggested to involve binding of the protein anions to the cationic site on the columns. The second phase is suggested to involve binding of the hydrophobic portions of the column with the hydrophobic amino acid residues of the protein.

INTRODUCTION

The purification of proteins on a series of hydrocarbon coated agaroses varying in the length of their alkyl side chain is called "hydrophobic chromatography" (1). Most adsorbents in use have been derived by the cyanogen bromide procedure which is known to introduce positively charged groups into the absorbent matrix (2,3). Recently we have shown that proteins, which were adsorbed to the alkyl amino agaroses, were not adsorbed under the same conditions by the corresponding or longer alkyl hydrazide derivatives of agarose which are devoid of charge (4). This effect of charge shows that electrostatic interactions seem to play a key role in the resolution of proteins by this method. Since the hydrazides are not direct analogs of alkyl amines we looked for another method to prove the role of charge in these interactions.

Recently we have established that coupling of amines to cyanogen bromide activated agarose results mainly in the formation of N-substituted isoureas (5). These isoureas are good nucleophiles and can be acetylated with acetic anhydride (6,7) to give uncharged columns according to the following scheme.



The experiments presented in this paper described the adsorption of ovalbumin, α-lactalbumin and bovine serum albumin to hexylamino- and butylamino-agarose, and demonstrate the abolishment of such binding on the acetylated uncharged alkyl amino agaroses prepared by acetylation of the parent alkyl amino agarose.

MATERIALS AND METHODS

Bovine serum albumin, and ovalbumin were obtained from Schwarz Mann; α-lactalbumin was a gift from Dr. Y. Shechter; butylamine hexylamine and cyanogen bromide were purchased from Fluka. Sepharose 4B was a product of Pharmacia. The activation of Sepharose by cyanogen bromide was performed by two different methods: the pH stat method or the buffer method (8,9). The coupling of alkyl amines to activated Sepharose was performed as described elsewhere (4). Potentiometric titrations of the agarose derivatives were carried out in a Radiometer titration assembly (automatic setup) (4).

Acetylation of Alkyl Amino Agarose was performed as follows: 1 Gram of settled alkyl amino agarose was suspended in 2 ml of 50% dioxane and potassium carbonate pH 8.5 and cooled to 4^0. To this suspension 1 ml of acetic anhydride was added gradually with stirring for 30 min. The pH was maintained by adding potassium carbonate. After 1 hr the column was washed and reused.

RESULTS

In order to check whether upon acetylation alkyl amino agaroses lose their charge, the potentiometric titration profiles of hexylamino-agarose and acetylhexylamino-agarose were compared with unmodified agarose. As can be seen from Fig. 1, unmodified agarose and the acetylated hexylamino-agarose have a negligible number of titratable groups between pH 5.0 and 11.0. The hexylamino-agarose showed a major

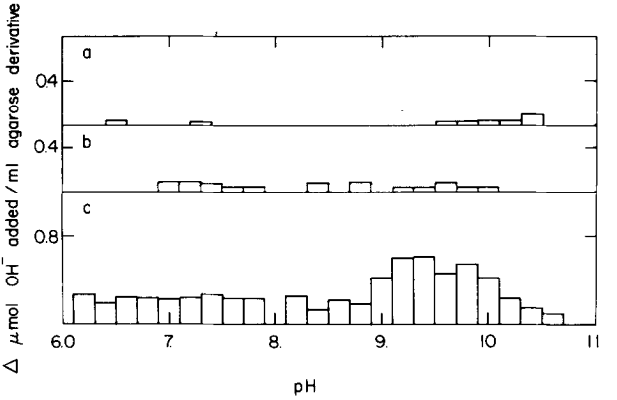


Fig. 1. Differential titration of (a) agarose or cyanogen bromide activated agarose; (b) acetylated hexylamino agarose; (c) hexylamino agarose.

titration peak corresponding to a pK_a of 9.6, and about 25 μmoles were titrated per g of wet gel.

Adsorption of Protein to Alkyl Amino Agarose. In order to compare the behavior of proteins on the charged and non-charged agarose derivatives, α-lactalbumin, ovalbumin and bovine serum albumin were applied to the alkyl amino agarose columns in 0.05 M Tris-HCl buffer (pH 8.0). All the three proteins were adsorbed to the column. None of the protein was adsorbed by agarose or by CNBr activated agarose alone. The partial ionic nature of this adsorption was established by desorption of bound proteins with high salt concentration (1 M NaCl) (Table 1).

If binding of the proteins occurred by hydrophobic forces only, no change in the behavior of the protein on the acetylated uncharged alkyl amino agarose would be anticipated. As shown in Table 1, α-lactalbumin and ovalbumin were not adsorbed to the acetylated column. The absence of the positive charge on the matrix resulted in the abolishment of binding. Consequently the adsorption of the two proteins to alkyl amino agarose must be primarily due to ionic interactions, whereas the hydrocarbon moiety is only assisting the binding. Binding of bovine serum albumin to these columns is not surprising since this protein has binding sites for hydrophobic groups.

TABLE 1. Adsorption of proteins to alkyl amino agarose and acetyl alkyl amino agarose

Agarose derivative	α-Lactalbumin		Ovalbumin		Bovine serum albumin	
	% Adsorbed	% Eluted	% Adsorbed	% Eluted	% Adsorbed	% Eluted
Activated agarose	0	-	0	-	0	-
Hexylamino agarose	100	80	100	100	100	34
Butylamino agarose	100	100	100	100	100	47
Acetyl hexylamino agarose	13	100	0	-	100	33
Acetyl butylamino agarose	0	-	0	-	nt	

1 Gram of each adsorbent was filled into a Pasteur pipette. The column was equilibrated with 0.05 M Tris-HCl buffer pH 8.0. 1 mg of each protein was applied and 10 fractions of 2.0 ml collected. The added absorbances of the fractions at 280 nm were used to calculate the % of adsorbed protein. Desorption was tried by adding 1 M NaCl to the same buffer and another 10 fractions were collected. The total of the A_{280} nm gives the % of eluted protein, nt, not tested.

TABLE 2. Affinity chromatography of α-chymotrypsin

Sepharose derivative	% Adsorbed	% Eluted	Specific activity (units/mg protein)	Purification factor
Agarose	0	-	33	1
Agarose-ε-aminocaproyl D-tryptophan methyl ester	90	100	46	1.4
Acetyl-agarose-ε-caproyl D-tryptophan methyl ester	70	100	63	1.9

1 Gram of each adsorbent was filled into a Pasteur pipette. The column was equilibrated with 0.05 M Tris-HCl buffer pH 8.0. 1 mg of α-chymotrypsin was applied in the same buffer. One-milliliter fractions were collected and assayed. α-Chymotrypsin was eluted with 0.1 M acetic acid.

Acetylation of the isourea group may also help in the specific purification of protein by affinity chromatography by decreasing the possibility of non-specific interaction with the charged matrix. Thus, commercial chymotrypsin was purified two-fold on acetylated ε-amino caproyl-D-tryptophan methyl ester agarose column, but only 1.4-fold on the non-acetylated column (Table 2).

DISCUSSION

The results of this study indicate that the adsorption of proteins to alkyl amino agarose may occur in two phases. The first of these is suggested to involve binding of the protein's anions to the cationic sites on the column. The second phase is suggested to involve binding of the hydrophobic portions of the column to the hydrophobic amino acid residues of the protein. This is the reason that after long incubation of protein on these columns, elution can be achieved only with detergents. This two phase binding is similar to the suggested binding of sodium dodecyl sulfate to proteins (10) and is in contrast to the two phase interaction we suggested previously in which the hydrophobic interaction was considered as the first phase (4). Our previous assumption was based on the arguments in other studies (1,3) in which a homologous series of alkyl amino agarose varying in the length of their alkyl side chain were used. It was reasoned that hydrophobic interactions are the major forces involved, since within the homologous series, all the members are identical in all respects including

charge except for the length of the hydrocarbon chains, yet they differ in their adsorption power. An alternative explanation for this phenomenon could be that the hydrophobic side chains markedly enhance ion pair formation between carboxyls on the protein and the isourea on the column. The enhancement of ion-pair formation by hydrophobic chains is a known phenomenon: e.g. the equilibrium constant for ion-pair formation in water between benzene sulfonic acid and N-trimethyl-aminodecane is $K_{ass} = 7.1$ ℓ/g ion, compared with $K_{ass} = 42,000$ ℓ/g ion for anthracene sulfonic acid and the same cation (11).

In connection with this study it is worthwhile to comment on the new hydrophobic columns for protein separation continually appearing in the literature (12, 13). Upon careful analysis of these columns it seems that the hydrophobic interaction between the column and the protein are of secondary importance. The major contributions are due to other kinds of interactions. In the example presented in this report, where alkyl-amines are coupled to agarose and low salts are being used for adsorption of the protein, the major interaction is ionic. In cases where neutral carriers are being used, proteins are adsorbed only in the presence of high salt concentration. The reason for the efficiency of these columns has been suggested by Van der Haar and others (14, 15), and has also been observed by us. The protein precipitates on the column, since the carrier reduces the concentration of salt required for precipitation. Another reason for the requirement of high concentrations of $(NH_4)_2SO_4$ may be that the salt is increasing the hydrophobic interaction inside the protein and thus exposing additional hydrophilic groups on the surface of the protein. The protein is now susceptive to absorption by the "hydrophilic-columns" (previously referred to as hydrophobic matrices in the literature).

Finally, the forces involved in the so-called "hydrophobic chromatography" are not yet completely understood. These forces are, however, a serious interference to specific affinity chromatography. Partial elimination of such interference can be achieved by omitting the charge from the isourea bond either by using hydrazides (4) or by acetylation. Although the latter procedure has enabled better purification of chymo-trypsin, achieved after acetylation of the tryptophan column, this is not always a possible solution since ligands containing amine, phenol and other nucleophiles will also be acetylated. Another undesirable side effect of acetylation is that we have observed a much higher incidence of ligand leakage from acetylated columns than from non-acetylated ones.

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REFERENCES

1. Shaltiel, S., and Er-el, Z. (1973) Proc. Natl. Acad. Sci. USA, 70, 778-781.
2. Wilchek, M. (1974) Adv. Exptl. Med. and Biol. 42, 15-31.
3. Hofstee, B.H.J. (1973) Biochem. Biophys. Res. Commun. 50, 751-757.
4. Jost, R., Miron, T., and Wilchek, M. (1974) Biochim. Biophys. Acta, 362, 75-82.
5. Wilchek, M., Oka, T., and Topper, Y.J. (1975) Proc. Natl. Acad. Sci. USA, 72, 1055-1058.
6. Bruce, W.M. (1904) J. Amer. Chem. Soc. 26, 419-436.
7. Werber, M.M. (1975) Anal. Biochem. (in press).
8. Axen, R., Porath, J., and Ernback, S. (1967) Nature, 214, 1302-1304.
9. March, S.C., Parikh, I., and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149-152.
10. Bukhard, R.K, and Stolzenberg, G.E. (1972) Biochemistry, 11, 1672-1677.
11. Packter, A., and Denbrow, M. (1962) Proc. Chem. Soc. (London), 220-221.
12. Hjerten, S., Rosengren, J., and Pahlman, S. (1974) J. Chromatogr. 101, 281-288.
13. Mevarech, M., Leicht, W., and Werber, M.M. (1976) Biochemistry, 15, 2383-2387.
14. Van der Haar, F. (1976) Biochem. Biophys. Res. Commun. 70, 1009-1013.
15. King, T.P. (1972) Biochemistry, 11, 367-371.