PURIFICATION OF *E. COLI* ENZYMES BY CHROMATOGRAPHY ON AMPHIPHILIC GELS

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1. Introduction

Several authors have pointed out the interest in obtaining a method for protein separation based on another physical property than those generally employed (charge, size, solubility, . . .). Sepharose columns carrying hydrophobic groups have been examined for this purpose ('hydrophobic chromatography' [1], 'phosphate induced protein chromatography' [2], 'hydrophobic salting-out chromatography' [3]). Unfortunately, this method has been successfully applied to a too small number of proteins to be considered as general. Moreover the pattern of elution and the nature of the hydrophobic groups attached to the column vary considerably throughout the literature. The study reported here was aimed at designing a general method for using this type of chromatography. A general strategy is proposed for finding the proper adsorption and elution conditions, and is illustrated by the purification of some enzymes studied in our laboratory. The results obtained lead us to discuss the mechanism of the interactions responsible for this type of chromatography.

2. Materials and methods

2.1. *Synthesis of alkyl-agarose*

Sepharose 4B (Pharmacia) was activated and coupled to alkylamine as described by Er-el et al. [4]. For the coupling reaction 0.1 g of CNBr and 5 mmol of α-aminoalkane were used per ml of packed Sepharose.

2.2. *Tryptophanase: preliminary purification and enzyme assay*

The buffer used was 0.1 M potassium phosphate, pH 7.8, containing 5.10^{-5} M pyridoxal-5'-phosphate, 2.10^{-3} M EDTA and 5.10^{-3} M β-mercaptoethanol (Buffer A). The crude extract was obtained from *E. coli* K12, induced for tryptophanase. It was treated with protamine sulfate as previously described [5] and precipitated with 1.65 M ammonium sulfate (final pH = 7.0). The supernatant was diluted to 1.2 M ammonium sulfate with buffer A and applied to the column. The enzyme activity was measured as described by Newton and Snell [6].

2.3. *Aspartokinase I-homoserine dehydrogenase I: preliminary purification and enzyme assay*

The buffer used was 0.02 M potassium phosphate buffer, pH 7.2, containing 0.15 M KCl, 2.10^{-3} M Mg–Titriplex (Merck) 2.10^{-3} M L-threonine and 10^{-3} M dithiothreitol (Buffer B). The crude extract was obtained from *E. coli* K12, strain Tir 8, constitutive for this enzyme. It was brought to 1.65 M ammonium sulfate (final pH = 7.2) and left overnight at room temperature. The precipitate was dissolved in Buffer B supplemented with 0.5 M KCl and 0.5 M ammonium sulfate (final pH = 7.2), and applied to the column. The enzyme assay was performed as described by Patte et al. [7].

2.4. *β-Galactosidase: preliminary purification and enzyme assay*

The buffer used was 0.01 M Tris–acetate, pH 7.1, containing 0.1 M NaCl, 0.01 M $MgCl_2$ and 0.01 M

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β-mercaptoethanol (Buffer C). The crude extract was obtained from *E. coli* K12, strain 3000, induced for β-galactosidase with 5.10^{-4} M IPTG*. It was brought to 1.4 M in ammonium sulfate (final pH = 7.1) and left over-night at 5°C. The precipitate was dissolved in Buffer C, supplemented with 0.5 M NaCl and 0.4 M ammonium sulfate (final pH = 7.1) and applied to the column. The enzyme activity was measured as described by Ullmann et al. [8].

2.5. *Homoserine-kinase*

The buffer used was Buffer C except for the L-threonine which was replaced by L-homoserine (Buffer D). The enzyme assay was performed as described by Thèze et al. [9].

2.6. *Other assays*

Protein concentrations were measured by the procedure of Lowry et al. [10]. The concentration of ammonium sulfate was determined with the Nessler reagent.

3. Results

3.1. *Factors affecting the adsorption of proteins on alkyl-Sepharose*

Two factors have a great influence on the adsorption properties of a protein on the alkyl-sepharose column: the length of the aliphatic chain [1,11,12] and the concentration and nature of the salts present in the medium [1–3, 11–13]. The effect of these two factors was studied, each of them separately. Fig.1 shows the influence of the length of the aliphatic chain on the adsorption of tryptophanase and homoserine-kinase: the longer the carbon chain, the more retarded are these two enzymes. The β-galactosidase and aspartokinase I-homoserine dehydrogenase I (not shown in fig.1) were adsorbed in their respective buffers already on the seph-C_3. Fig.2 shows the effect of the concentration of various anions on the adsorption of β-galactosidase on seph-C_3. The effect is very different accord-

Abbreviations: *IPTG: isopropyl-β-D-thiogalactopyranoside; seph-Cn: sepharose 4B activated with CNBr and coupled to an α-aminoalkane (n = length of the aliphatic chain).

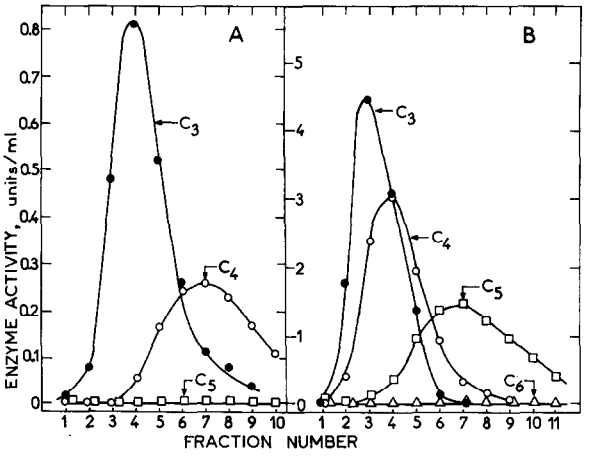


Fig.1. Influence of the length of the aliphatic chain on the adsorption. On a column containing 1 ml of Seph-C_n (abbreviated C_n in the fig.), equilibrated with Buffer A (fig.1A) or D (fig.1B), were applied respectively 3 units of tryptophanase (fig.1A) or 10.5 units of homoserine kinase (fig.1B). The columns were then eluted with the same buffers and fractions of 1 ml were collected.

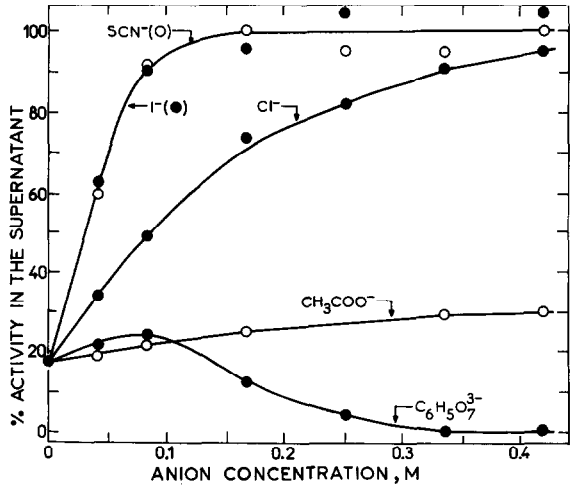


Fig.2. Influence of neutral salts on the adsorption. 100 μl of a suspension of Seph-C_3, having adsorbed 0.24 units of β-galactosidase per ml, were added to 0.5 ml of buffer C supplemented with 0.1 M NaCl and different concentrations of neutral salts (pH adjusted to 7.1, if necessary). All the neutral salts employed were potassium salts; only the anion is indicated in the fig. After 15 min incubation at room temperature, the supernatant obtained by filtration was assayed for enzyme activity. The activities are indicated in percentage of the initial adsorbed activity.

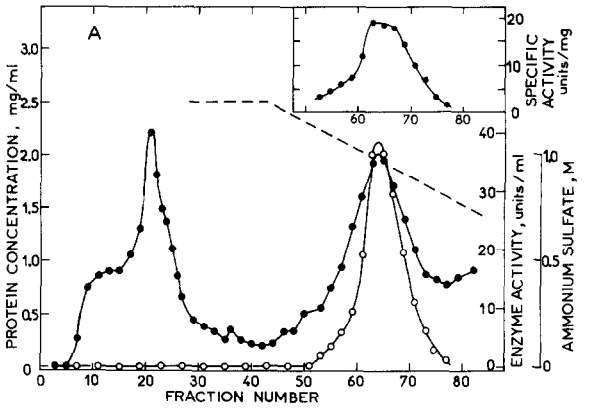
ing to the anion, and these could be ranged in the following order of decreasing adsorption:

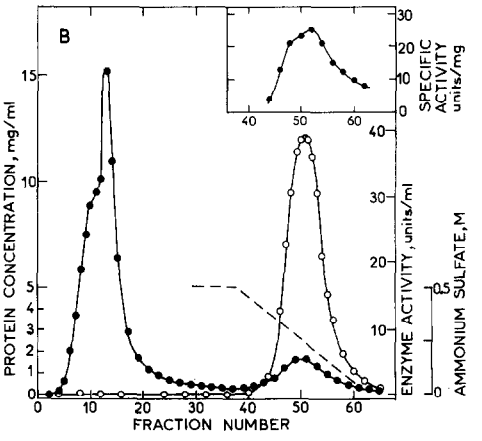
Thiocyanate = iodide > chloride > acetate > citrate

It should be pointed out that this order is identical to the Hofmeister series of neutral salts.

3.2. *Purification of some enzymes on Seph-C_3*

In the light of the preceding observations, the following strategy was chosen to generalize a protein purification on Seph-C_3: 1) Search for a buffer in which the enzyme is not adsorbed on Seph-C_3 (by the addition of sodium- or potassium chloride); 2) Search for the concentration of ammonium sulfate to be added to this buffer for assuring adsorption of the enzyme (the sulfate is near the citrate in the Hofmeister series and thus should induce the same adsorption effect); 3) Elution of the adsorbed enzyme by a gradient of a decreasing concentration of ammonium sulfate. The application of this technique to the purification of three enzymes is shown in table 1 and fig. 3.





4. Discussion

Many authors have tried to explain the mechanism of adsorption of proteins on alkyl-Sepharose columns, either by electrostatic interactions or by hydrophobic

Table 1
Purification of three enzymes on Seph-C_3

Enzyme	Buffer for adsorption	Buffer	Specific activity (μ/mg) of the enzyme applied to the column	Specific activity (μ/mg) of pooled fractions*	Yield in activity*	Specific activity (μ/mg) of pure enzyme
Tryptophanase	A + 1.2 M ammonium sulfate	A	5.4	14.5	70%	24–27 [14]
Aspartokinase I-homoserine dehydrogenase I	B + 0.5 M KCl + 0.5 M ammonium sulfate	B + 0.5 M KCl	2.6	22.5	76%	47 [15]
β-Galactosidase	C + 0.5 M NaCl + 0.4 M ammonium sulfate	C + 0.5 M NaCl	100 X 10^3	490 X 10^3	60%	800 X 10^3 [8]

* The specific activity and the yield in activity were measured on the pooled fractions, as indicated in the legend to fig. 3.

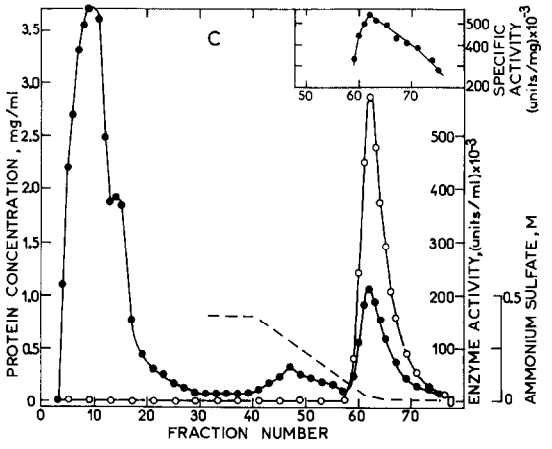


Fig.3. Chromatography of three enzymes on Seph-C_3. The partially purified extracts were applied to a column (16 × 1.3 cm) containing 20 ml of Seph-C_3. All the elutions were made at room temperature with a flow rate of 0.6 ml per min. 4 ml Fractions were collected. The elution was made with a linear gradient of a decreasing concentration of ammonium sulfate. A) Purification of tryptophanase: the applied extract contained 320 mg of proteins. The buffer used was Buffer A. Fractions 59 to 71 were pooled. B) Purification of aspartokinase I-homoserine dehydrogenase I: the applied extract contained 550 mg of proteins. The buffer used was Buffer B + 0.5 M KCl. Fractions 47 to 55 were pooled. C) Purification of β-galactosidase: the applied extract contained 250 mg of proteins. The buffer used was Buffer C + 0.5 M NaCl. Fractions 60 to 68 were pooled. (●——●——●) protein concentration, (○——○——○) enzymatic activity, (—— —— ——) ammonium sulfate concentration.

interactions, or by reconciling them in a 'detergent protein interaction' type mechanism [1,11,12]. However, the arguments employed for these conclusions are weak and even misleading. As shown in fig.2, the effect of ions, at constant ionic strength, is strongly dependent on the nature of these ions; some of them even increase the adsorption on the column. Their influence on the adsorption of proteins on alkyl-Sepharose is very similar to a lyotropic rather than to a pure electrostatic effect. Likewise, it is not obvious that the effect of increasing adsorption with increasing length of the aliphatic chain implies the participation of interactions of essentially hydrophobic character: the more or less extended aliphatic chain may have a local effect on the dielectric constant of the solvent, therefore changing the electrostatic interactions between the positive charge carried by the Sepharose, [11], and the protein. One less misleading way of looking at the phenomenon of adsorption is to take into consideration the interactions between three components: the alkyl-Sepharose (composed of the apolar part of the chain and the positive charge carried by the alkyl-Sepharose), the protein and the molecules of the solvent. The adsorption phenomenon is in a certain respect (i.e. the influence of the nature of the neutral salts present) very close to the salting-out process of proteins and to the several phenomena, where water molecules interfere as mediators of interactions between hydrophobic groups [16].

The adsorption of proteins on propyl-Sepharose has been used for the purification of three enzymes of *E. coli*. The technique chosen, already successfully used by Rimerman and Hatfield [2] and Doellgast and Fishman [17] consists in adsorbing the protein in an increased concentration of ammonium sulfate and the elution by a gradient of decreasing concentration of this salt. Under these conditions the sepharose does not function as a simple ion exchange column, leaving the hope that an ion exchange chromatography should constitute a satisfactory additional purification step. The advantages of this method are numerous: the high flow-rate and the high capacity of the column; the possibility of performing the purification in the presence of important concentrations of ammonium sulfate (known for its stabilizing effect on the native structure of many proteins [16], and in particular of tryptophanase [5]). Moreover, these columns can be used to obtain insolubilized active enzymes [13].

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