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CHROMATOGRAPHIC PURIFICATION OF A MAMMALIAN HISTIDINE DECARBOXYLASE ON CHARGED AND NON-CHARGED ALKYL DERIVATIVES OF AGAROSE

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Summary

Histidine decarboxylase (EC 4.1.1.22) from a mouse mastocytoma has been purified by chromatography on charged and non-charged *n*-alkyl derivatives of agarose. The former was represented by the coupling product of CNBr-activated agarose and alkylmonoamines (alkylamino-agarose), the latter by the coupling product of agarose and alkylglycidyl ethers (alkyl agarose). The choice of fractionation medium was restricted by the enzyme stability; excessively high ionic strength media could not be used. Under the conditions investigated, the best result was obtained with the non-charged octyl agarose. The enzyme was adsorbed to this gel at a relatively high ionic strength, and on stepwise decrease in ionic strength of the eluting buffer it was desorbed with a total recovery of 80%. There was an approx. 10-fold increase in specific activity. The histidine decarboxylase, thus purified, retained 90—100% of its activity for 10 days or more at 6—8° C.

Some general comments on protein fractionation on charged and non-charged alkyl derivatives of agarose are given. The complexity of protein interaction with the charged alkyl derivatives is illustrated by experiments with a colored protein, phycoerythrin.

Introduction

The formation of nascent histamine is of importance in the inflammatory response, wound healing, microcirculation, gastric secretion etc., as reviewed for instance by Kahlson and Rosengren [1], Schayer [2] and Morris and Fillingame [3]. The high concentration of free histamine, seen in allergic reactions, reflects probably not only an increased liberation of histamine from granule, but also an increased histamine synthesis [4].

To explain the mechanisms involved in these phenomena in molecular terms, we need more information on the molecular properties of the enzyme, histidine decarboxylase, responsible for histamine formation.

In a series of papers of which this is the first, we shall present our experiments on the stability, purification and characterization of this enzyme. This study was primarily undertaken to evaluate the possibility of using "hydrophobic interaction chromatography" for the isolation of histidine decarboxylase from a mouse mastocytoma. Furthermore, some methodological investigations on this separation technique were also performed.

Recently alkyl and aryl derivatives of agarose have been introduced as "hydrophobically interacting" matrices for macromolecular fractionation [5—11]. However, the CNBr method [12], which has been the most commonly used coupling method [5—8], gives rise to charged structures [8,11,13]. The presence of ionic interactions due to these structures may be expected to cause complications in the interpretation of the fractionation mechanism or even to obscure the effects of hydrophobic interactions [8,11]. Non-charged alkyl or aryl derivatives of agarose have been designed by Porath et al. [9], Hjertén et al. [10] and Jost et al. [11].

Materials and Methods

Preparation of alkyl derivatives of agarose

The agarose derivatives were prepared from non-cross-linked agarose (Sepharose 4B, Pharmacia Fine Chemicals AB, Uppsala, Sweden) and alkylamines or alkylglycidyl ethers of different chain lengths. The structures and denotations of the two coupling products are shown in Fig. 1.

(a) Alkylamino-agarose. *n*-Alkylmonoamines (Fluka) were coupled to agarose by the CNBr method of Axén et al. [12] (1 mmol CNBr and 4 mmol ligand were used per g wet gel); for the octylamine this procedure was performed in a mixture of water and dioxane, for the propyl-, butyl- and pentylamines, in water.

(b) Alkyl agarose. *n*-Alkylglycidyl ethers were synthesized as described by Ulbrich et al. [14]. The coupling method of Ellingboe et al. [15] was applied to the alkylglycidyl ethers and agarose in dioxane as has been described by Hjertén et al. [10].

Preparation of the crude extract of histidine decarboxylase

A crude extract of histidine decarboxylase was prepared from a mouse

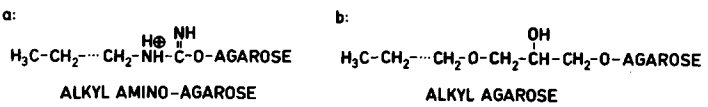

Fig. 1. General structures and denotations of the agarose derivatives used in the experiments presented in this paper. The isourea derivative (a) is shown by Broström et al. [13] in model experiments to be the main derivative formed in the CNBr coupling method of Axén et al. [12]. "Alkylamino-agarose" has been previously used by Jost et al. [11] to denote the derivatives formed in the CNBr coupling reaction from alkylmonoamine and agarose. The hydroxy-alkoxypropyl agarose (b) is in this and a previous paper [10] denoted simply "alkyl agarose".

mastocytoma [16] maintained as a solid tumour in $(A/SnxLeaden)_{F1}$ mice by intramuscular transplantation in the hind legs every 10—14 days.

The tumours were frozen in liquid nitrogen immediately after removal. 3 g of frozen material was homogenized in 15 ml of cold distilled water and centrifuged at 25 000 × *g* for 15 min (Spinco, Rotor SW 50). The pellet was removed and the supernatant was further centrifuged at 150 000 × *g* for 120 min. The clear supernatant was used as the sample for the chromatographic experiments. This crude extract could be stored at −22° C for more than 3 weeks, or in the standard buffer (for composition see below) at 6—8° C for about 10 days, with a loss in activity of less than 10%.

Determination of the histidine decarboxylase activity

Fractions from the chromatographic experiments were analyzed for protein by absorption measurements (A_{280nm}) and after dialysis against the standard buffer (see below), for histidine decarboxylase activity, determined as follows.

The activity of histidine decarboxylase was determined as the amount of $^{14}CO_2$ developed from L-[*carboxy*-^{14}C]histidine. The method used was modified from Aures et al. [17]. A scintillator vial, sealed by a rubber cup was used as incubation chamber and a small glass beaker containing the incubation mixture (100 µl) was suspended in the middle of the vial [18].

Incubation. The enzyme was incubated under N_2 for 120 min at 37° C in 100 µl of 0.15 M potassium phosphate buffer (pH 6.8), containing 0.8 mM L-histidine, 40 nCi L-[1-^{14}C]histidine (New England Nuclear), 10 µM pyridoxal phosphate, and 50 µM glutathione (reduced) or 1 mM 2-mercaptoethanol. The reaction was stopped by the addition of 100 µl of 10% trichloroacetic acid to the incubation mixture. The $^{14}CO_2$ developed in the reaction was adsorbed in 0.2 ml of 1 M hyaminehydroxide (Nuclear Chicago), or 0.6 M NCS (Amersham/Searle) at the bottom of the vial. After post-incubation for 1 h, the cup with the glass beaker was removed and 5 ml of the liquid scintillator was added to the vials. ^{14}C was measured in a Nuclear Chicago Mark I scintillation counter.

Liquid scintillator. 4 g of PPO (Nuclear Chicago) and 50 mg of POPOP (Packard) in 1 l of toluene.

Standard buffer etc.

Standard buffer: 0.15 M potassium phosphate, containing 10 µM pyridoxal phosphate, 3 mM NaN_3, 1 mM 2-mercaptoethanol (pH 6.8, I = 0.3).

The ionic strength, I, is given as mol per l. The pH of the buffer solutions mentioned in this paper refers to measurements made on the final solutions at room temperature. (Apparent pH.)

Results

Fractionation pattern of the crude extract of histidine decarboxylase on alkylamino-agarose

A crude extract of histidine decarboxylase was fractionated on propyl-, butyl- and pentylamino-agarose at 6—8° C (Fig. 2). After adsorption to the gels

(10 mM potassium phosphate, pH 6.8, I = 0.02), elution was performed by increasing the ionic strength (I = 0.6), by increasing the pH to 9.8 and finally by lowering the ionic strength (pH 9.8, I = 0.008) (Fig. 2).

When the carbon-chain length was increased, more protein material was adsorbed. The enzyme applied was not retained on the propylamino-agarose, was partly retained on the butylamino-agarose and was completely adsorbed on the pentylamino-agarose. Adsorbed histidine decarboxylase, as well as most of the adsorbed protein material, was desorbed by increasing the ionic strength of the eluting buffer (buffer II, Fig. 2). The protein material still remaining on the gels (<1, 2 and 9% of the applied material for propyl-, butyl- and pentylamino-

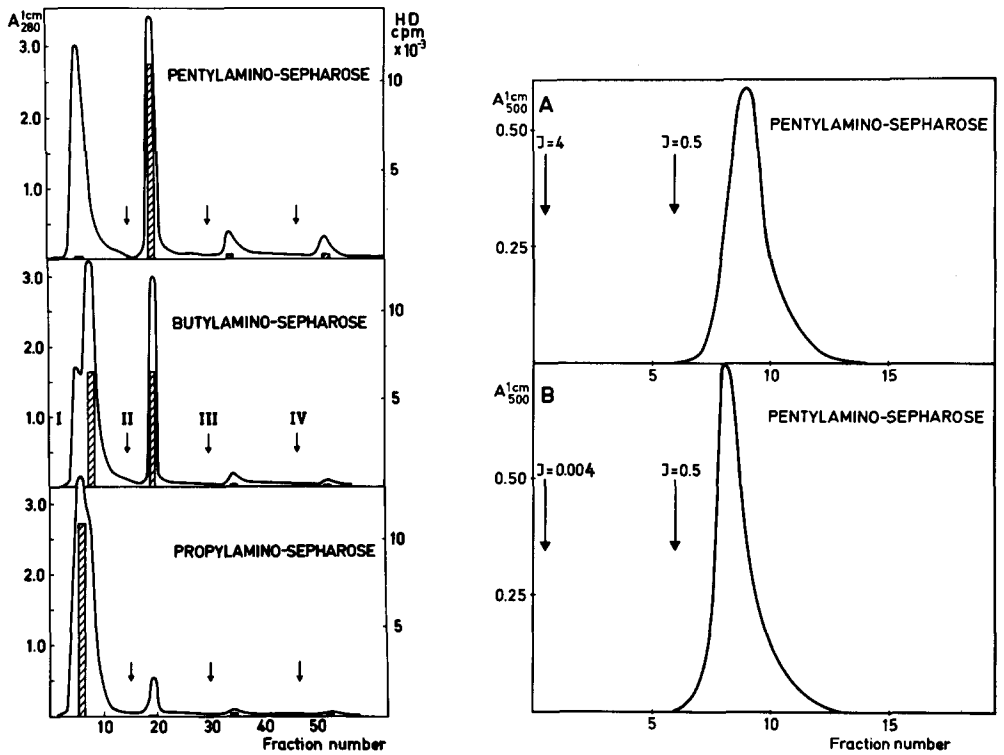

Fig. 2. Fractionation pattern of a crude extract of histidine decarboxylase on propyl-, butyl- and pentylamino-Sepharose 4B. Sample: 10 mg of a crude extract in 1 ml, with a total histidine decarboxylase activity of 158 000 cpm, were applied to each column. Gel beds: 0.8 ml in 1 cm wide perspex columns. Flow rate: 6 ml/h. Fraction volume: 0.5 ml. Temperature: 6—8° C. The gels and the samples were equilibrated with buffer I prior to chromatography. Elution: I, 10 mM potassium phosphate, 3 mM NaN_3 (pH 6.8, I = 0.02); II, 0.3 M potassium phosphate, 3 mM NaN_3 (pH 6.8, I = 0.6); III, 1.2 M glycine/NaOH, 3 mM NaN_3 (pH 9.8, I = 0.6); IV, 10 mM glycine/NaOH, 3 mM NaN_3 (pH 9.8, I = 0.008). Only the main protein fractions were analysed for histidine decarboxylase activity. The activity, in cpm/100 µl, is indicated as shaded bars.

Fig. 3. Demonstration of the adsorption of phycoerythrin to pentylamino-Sepharose 4B both at high and at low ionic strength, indicating hydrophobic as well as electrostatic interaction. Sample: phycoerythrin (its concentration is indicated as $A^{1\,cm}_{500\,nm}$ in the diagram). Gel beds: pentylamino-Sepharose 4B, 1 ml. Temperature: 20° C. Adsorption: A, 4 M NaCl, 2 mM potassium phosphate (pH 6.8, I = 4); B, 2 mM potassium phosphate (pH 6.8, I = 0.004). Elution A and B: 0.5 M NaCl, 2 mM potassium phosphate (pH 6.8, I = 0.5).

agarose, respectively) was desorbed by buffers III and IV (Fig. 2), the composition of which are given in the legend to the figure. The maximum increase in specific activity obtained was three times.

Model experiment with phycoerythrin

The results shown in Fig. 2 (histidine decarboxylase crude extract fractionated on alkylamine derivatives of agarose) suggested superimposed electrostatic and hydrophobic interactions. The following experiments with phycoerythrin, prepared from *Ceranium rubrum*, a red alga [19], were made to further illustrate the two effects.

Phycoerythrin was adsorbed to pentylamino-agarose both at high ionic strength (I = 4, Fig. 3A) and low ionic strength (I = 0.004, Fig. 3B). The protein was desorbed in both cases with a buffer of an intermediate ionic strength (I = 0.5). When, under the same conditions, the experiment was performed on pentyl agarose, the phycoerythrin was adsorbed only at high ionic strength. In similar experiments, octyl gels were loaded at high ionic strength with the protein. Decrease in the ionic strength of the eluting buffer desorbed the protein from the neutral octyl agarose, but not from the charged octylamino-agarose. Changes in ionic strength could not completely desorb the phycoerythrin adsorbed to octylamino-agarose.

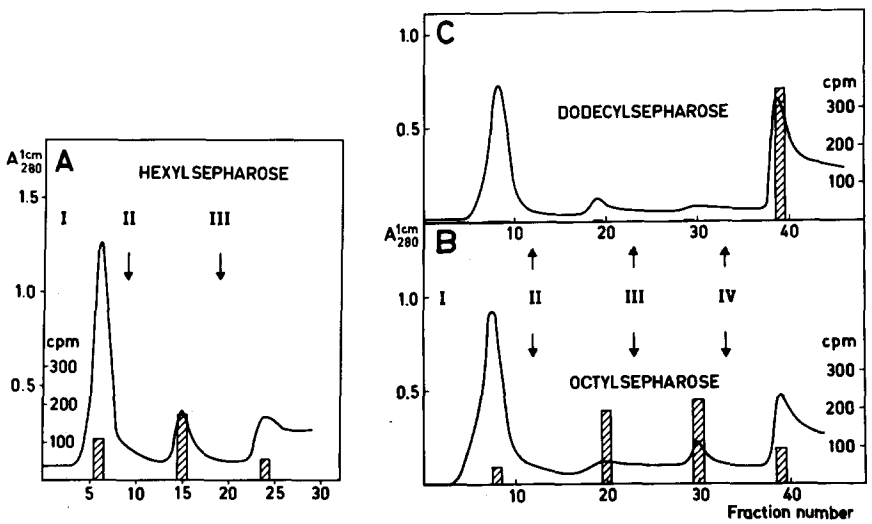

Fig. 4. Fractionation pattern of a crude extract of histidine decarboxylase on alkyl ethers of Sepharose 4B. Sample: 2.1 mg of a crude extract in 0.5 ml. Gel volume: 4 ml in 1-cm wide perspex columns. Flow rate: 2 ml/h. Fraction volume: 0.5 ml. Temperature: 6—8° C. The gels and the samples were equilibrated with buffer I prior to chromatography. Buffers mentioned below, contain 10 μM pyridoxal phosphate, 3 mM NaN_3 and 1 mM 2-mercaptoethanol. Only the main protein fractions were assayed for histidine decarboxylase. The activity, in cpm/100 μl, is indicated as shaded bars. Elution: (A) Hexyl Sepharose 4B; I, 0.3 M potassium phosphate, 0.5 M NaCl (pH 6.8, I = 1.1); II, 10 mM potassium phosphate (pH 6.8, I = 0.02); III, 10 mM potassium phosphate, 50% ethylene glycol (pH 6.8, I = 0.02). (B) Octyl and (C) Dodecyl Sepharose 4B: I, as A:I above; II, 0.3 M potassium phosphate (pH 6.8, I = 0.6); III, as A:II above; IV, 10 mM glycine/NaOH, 50% ethylene glycol (pH 9.8, I = 0.008).

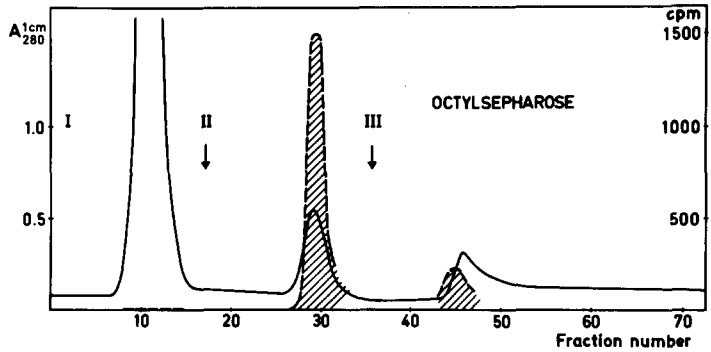

Fig. 5. Fractionation pattern of a crude extract of histidine decarboxylase on octyl Sepharose 4B. Sample: 7.8 mg of a crude extract in 0.7 ml (28 000 cpm). Gel bed: Octyl Sepharose 4B, 3.6 ml in a 1-cm wide perspex column. Flow rate: 3.4 ml/h. Fraction volume: 0.6 ml. Temperature: 6—8°C. Elution: I, 0.6 M potassium phosphate (pH 6.8, I = 1.2); II, 1 mM potassium phosphate (pH 6.8, I = 0.005); III, 4 mM glycine/NaOH, 50% ethylene glycol (pH 9.6, I = 0.005). All buffers contained 3 mM NaN_3, 10 μM pyridoxal phosphate and 1 mM 2-mercaptoethanol. The gels and the samples were equilibrated with buffer I prior to chromatography. The histidine decarboxylase activity, in cpm/100 μl, is indicated as shaded areas in the chromatogram. The gel bed and eluting buffers were selected from the results shov n in Fig. 4, to get the enzymic activity localized in one main fraction and yet obtain a considerable purification. Furthermore, we wanted to investigate if a higher recovery of activity could be obtained when NaCl was replaced by potassium phosphate in the high ionic strength adsorption buffer.

Fractionation pattern of the crude extract of histidine decarboxylase on alkyl agarose

The alkyl agarose chromatograms in Figs 4A—4C were developed by decreasing the ionic strength of the eluting buffer, increasing the pH and finally applying a buffer containing 50% ethyiene glycol. Adsorption of protein depended on the alkyl chain length; 26, 32 and 47% of the applied protein was retained on the hexyl, octyl and dodecyl gels, respectively. The strength of the adsorption was influenced too, since more drastic changes in the composition of the eluting buffer were necessary for the desorption of proteins from the derivatives with longer alkyl chains than from those with shorter ones; 6, 12 and 27%, respectively, of applied protein, were not displaced until ethylene glycol was added to the eluting buffer (Figs 4A—4C).

The total recovery of the enzymic activity, i.e. the sum of the enzymic activities in the different chromatographic fractions as a percentage of the total enzymic activity in the applied sample, was calculated to be about 60% (experiments in Fig. 4). The material eluted by buffer II in Fig. 4B (octyl-Sepharose) represents a 30% recovery of the enzymic activity and has 11 times the specific activity of the applied sample.

Histidine decarboxylase is inactivated by high salt concentrations; 30—35% of the enzymic activity was lost when the enzyme was exposed to 0.5 M NaCl, 0.3 M phosphate buffer (buffer I, Fig. 4, I = 1.1) prior to dialysis against the standard buffer. Potassium phosphate, being less harmful to the enzyme than NaCl of the same ionic strength, appeared, however, to be equally suitable for adsorption (Fig. 5). Fractionation on octyl agarose as outlined in Fig. 5 gave a 7—9-fold purification in fraction II and the total recovery was 80%, i.e. higher than in the experiments illustrated in Fig. 4.

Discussion

Fractionation of proteins on charged and non-charged alkyl derivatives of agarose

Factors affecting hydrophobic interaction on gels with non-polar substituents have recently been discussed by Hjertén [8].

A characteristic property of the hydrophobic interaction is that it is reinforced by an increase and weakened by a decrease in the ionic strength of the medium. Adsorption of proteins to gels with non-polar substituents can accordingly be achieved at high ionic strength and desorption at a lower ionic strength of the eluting buffer, as is evident from our experiments (Fig. 2, buffer IV and Figs 3A, 4 and 5) and those of others [8—10,20].

When, as in this work, the use of high ionic strength is limited by enzyme stability, a comparatively long alkyl chain must be introduced in order to obtain sufficient capacity (Fig. 4). However, with excessively long carbon chains protein adsorption may also be significant in a very low ionic strength medium (Fig. 4C). Stronger elution media must then be used for desorption, for instance ethylene glycol [7].

Unlike the case with hydrophobic interactions, electrostatic interactions are facilitated at low ionic strength and weakened by increasing the ionic strength of the medium, a property utilized for fractionation on ionic exchangers. The separation mechanism, therefore, is more complex when both charged and non-polar structures are present in the bed materials, as is the case in CNBr-coupled alkylamine derivatives of agarose [8,11,13], since both hydrophobic [8,20] and electrostatic [5—8] interactions may occur. For instance, in a 10 mM potassium phosphate buffer (pH 6.8, I = 0.02) the enzyme histidine decarboxylase is not adsorbed to the three-carbon amine derivatives of agarose (Fig. 2), suggesting that the charges in the derivatives alone are not sufficient for adsorption of the enzyme. The length of the carbon chain might affect the pK_a of the amine structures in the derivatives, positively charged at neutral pH. In butyl amino-agarose Jost et al. [11] found an apparent pK_a of 10.5. This is more than three pH units above the pH used for adsorption in our experiments. Only a marked decrease in this pK_a with increasing chain length could therefore give an effect on the electrostatic adsorption of this anionic enzyme provided the degree of substitution is roughly the same. The effect would be a decreased adsorption capacity. Since we observed an increase (Fig. 2), other factors must be involved in the adsorption process. That the electrostatic effects play an important role is, however, evident from the fact that desorption can be performed by a moderate increase in the ionic strength of the medium (step II in Fig. 2). This complexity is also illustrated in Fig. 3, which shows adsorption of phycoerythrin to pentylamino-agarose at both high and low ionic strength, and desorption by an intermediate ionic strength. When a longer alkylamine derivative, octylamino-agarose, was used complete desorption was not achieved by any changes in the ionic strength of the medium. Thus, on this (and other) charged alkyl derivatives factors diminishing the hydrophobic interaction will promote the electrostatic one and vice versa. On the non-charged pentyl and octyl agarose on the other hand, the protein was adsorbed only at high ionic strength.

Stability, recovery and purification of animal histidine decarboxylase

A limiting factor in the study of the histidine decarboxylase molecule is its lability upon fractionation [21,22]. The methods hitherto developed, $(NH_4)_2SO_4$ precipitation by Håkansson [21] and $(NH_4)_2SO_4$ precipitation combined with isoelectric precipitation by Aures and Håkansson [22] have yielded purified products stable only for "a few days". We have found the enzyme extremely labile after treatment with high concentrations of salts. This effect seems to be time dependent and is more pronounced at high than at neutral pH (Hammar, L., unpublished). Potassium phosphate, however, was less harmful than NaCl at the same ionic strength, and could alternatively be used for the adsorption of the enzyme (Fig. 5). Consequently there was a higher yield when the buffer was made up from potassium phosphate only. On the octyl agarose, the total recovery of the enzymic activity was 80% (Fig. 5), compared with 60% when about half of the ionic strength was made up from NaCl (Fig. 4B). An approx. 10-fold purification was achieved.

By introducing extremely long alkyl chains, the need for high ionic strengths for adsorption diminished (Fig. 4C), as did the possibility of desorbing the enzyme simply by decreasing the ionic strength. Increasing the concentration of ethylene glycol might be a suitable desorption method for such strongly interacting gel beds.

Restricted to the ionic strength range allowed by the enzyme stability, there was no advantage in using the alkylamine derivatives of agarose compared to the alkyl glycidyl ether derivatives for fractionation of histidine decarboxylase. We want to stress, however, that the alkylamine derivatives are powerful separation tools [5—8].

The chromatographic purification methods used in this work gave an enzyme with the same stability as that of the crude extract. A drawback was the indistinct elution of the enzyme upon small changes in the elution conditions, a phenomenon, however, often encountered also in ion-exchange and hydroxyapatite chromatography.

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