Journal of Solid-Phase Biochemistry, Vol. 1, No. 1, 1976

AFFINITY CHROMATOGRAPHY OF FROG EPIDERMIS DOPA-OXIDASE

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Accepted December 17, 1975

Benzoic acid is a competitive inhibitor of the enzyme dopa-oxidase. *p*-Aminobenzoic acid was coupled by a single-step reaction of the amino group to: (1) CNBr-activated Sepharose 4B: (2) Enzacryl AA, by reaction with thiophosgene (pH 10.0); and (3) CM-Sephadex G-50, modified to azide by the Curtis procedure. These three solid supports were used as affinity adsorbents in the dopa-oxidase purification. The enzyme was obtained from frog epidermis, and was retained at pH 4.7 with 0.1 M acetate buffer. The enzyme elution was carried out using a linear pH gradient with 0.1 M phosphate buffer pH 8.0. We have examined the interaction of the enzyme with the immobilized PABA in relation to the nature of the support, the lengthening of the "arm" of the ligand, bathwise adsorption, and the enzyme activation by immobilized trypsin.

INTRODUCTION

Affinity chromatography is a term that describes a unique property of many proteins and polypeptides (1). Many proteins will bind specifically and reversibly to a ligand. The use of this unique binding property permits the selective isolation and purification of enzymes and other biologically important macromolecules (2).

In 1953, Lerman (3) described the first example of affinity chromatography. He purified mushroom tyrosinase on an adsorbent prepared by reacting diazotized aminophenol to cellulose that contained resorcinol residues in ether linkage. Elution of the enzyme was achieved with buffers having a pH of about 9.5.

O'Neill et al. (4) have examined different insoluble supports with ligands of phenolic and benzoic types for mushroom tyrosinase purification. They concluded that dopamine attached covalently to Sepharose 4B by its amino group was the most effective adsorbent. With that ligand as substrate, they obtained a 10-fold purification and recovered 80% of the initial activity.

In this report, we have demonstrated that benzoic acid, an inhibitor of the dopa-oxidase system (E.C. 1.14.18.1) (5), is a good ligand for purification of this enzyme from frog epidermis. The adsorbents were prepared by immobilization as *p*-aminobenzoic acid (PABA) on different solid supports. This study also compares the supports studied and the conditions best suited for enzyme purification.

MATERIALS AND METHODS

Materials

Trypsin (E.C. 3.4.4.4) (5) was purchased from Sigma Chemical Co., U.S.A. (12,500 BAEE U/mg type III): CNBr-Sepharose 4B and CM-Sephadex G-50 from Pharmacia Chem., Sweden; Enzacryl AA from Koch–Light Lab., England; and ω-aminoalkyl agarose from Miles Yeda Ltd., Israel. The 3,4-dihydroxy-L-phenylalanine (L-dopa), *p*-aminobenzoic acid (PABA), hydrazine hydrate, and all the other reagents were Merck Company, analytical grade. They were used without further purification, except PABA, which was purified 3 times with active charcoal on hot water and recrystallized with cold water.

Methods

Preparation of Supports. The CM-Sephadex azide was obtained by a modification of Warton's procedure (6) for CM-Cellulose.

Enzacryl AA activation was accomplished with thiophosgene at pH 10 by the Koch–Light Laboratories method (7), which was also utilized for PABA coupling.

The coupling of PABA to CNBr-activated Sepharose 4B and succinyl aminohexyl agarose was carried out by the method of Cuatrecasas and Anfinsen (8).

The quantity of PABA coupled to each support was evaluated by two different procedures: (a) by measuring the absorbance, in a Perkin–Elmer 402 spectrophotometer, of PABA at 267 nm, before and after the coupling process; (b) by complexometric titration, with EDTA and Erio T as indicator, of Ca^{2+} ion retained by the carboxylate group of the gel (9). Both procedures gave the same results.

The retained water was determined by the weight difference between wet and dry gel after exposure to P_2O_5 in a vacuum desiccator.

Dopa-Oxidase Extraction Method. Frogs (*Rana esculenta rudibunda*) were obtained from local suppliers. Epidermis was separated from dermis

after incubation at 0–4°C with 2 M NaBr solution for 24 h. After being washed several times with distilled water, epidermis was lyophilized and retained at −30°C until used.

A quantity of 150–200 mg lyophilized epidermis was ground in a mortar with purified sand sea and 0.1 M phosphate buffer pH 7.0 for 10 min. The suspension was centrifuged at 18,000*g* for 30 min at 0–5°C in a Sorvall SS-1.

Determination of Protein and Enzymatic Activity. Protein was determined by a modification of the Lowry method (10).

Frog-epidermis extract did not show dopa-oxidase activity, and previous activation of the proenzyme was necessary (11). The activation of dopa-oxidase was performed in the following way: To 100 μl enzyme solution and 10 μl trypsin solution (1 mg/ml), 0.1 M phosphate buffer, pH 7.0, was added to a total volume of 0.5 ml. The mixture was incubated for 5 min at 37°C to activate the enzyme.

For the activity measure, 2.5 ml 1.06×10^{-2} M L-dopa in 0.1 M phosphate buffer, pH 7.0, was added to 0.5 ml of the activated enzyme solution, and the dopachrome formation was measured at 475 nm.

One unit of dopa-oxidase activity represents the amount of enzyme that catalyzes the formation of 1 μmol dopachrome/min at 25°C and pH 7.0 (12).

Enzyme Activation with Immobilized Trypsin. The activation of the enzyme was realized by means of the action of immobilized trypsin on CNBr-Sepharose 4B, coupled by the procedure described by Axen and Ernback (13).

The activation of dopa-oxidase was performed in the following way: Trypsin-Sepharose, 1 g, was placed in a Pharmacia K 10/20 column and washed with 0.1 M phosphate buffer, pH 7.0. A known volume of enzyme solution was then passed through the column at a rate of 20 ml/h at 25°C. After being passed through the column, the enzyme could not be further activated by the addition of a trypsin solution and incubation at 37°C. The use of the immobilized trypsin was advantageous because there was no contamination of the enzymatic system and the trypsin did not continue its proteolytic action.

Affinity Chromatography. About 1 g dry gel containing immobilized PABA was swollen in 0.1 M acetate buffer, pH 4.7, and poured into a Pharmacia K 16/20 or Whatman 10/20 column. A known volume of extract solution was pumped through the column with an LKB type 10,200 peristaltic pump at a flow rate of 20 ml/h. The column was then washed with 0.1 M acetate buffer, pH 4.7, and fractions were collected until no protein was detected eluting from the column. Next, the gel was eluted with 0.1 M phosphate buffer, pH 7.0, and fractions were collected. These fractions

contained the dopa-oxidase activity. Finally, 0.1 M phosphate buffer, pH 8.0, was used for elution of any remaining protein.

Protein concentration and enzymatic activity were determined in the extract and all fractions by previously described methods.

Batch Extraction. The batch technique was accomplished using PABA–Enzacryl AA support, by assuming that the equilibrium among enzyme, inhibitor, and enzyme–inhibitor complex is quickly reached. To 1 g wet gel was added, with mild stirring, 10 ml extract solution of previously determined protein concentration and dopa-oxidase activity (either in proenzyme or enzyme form). The binding kinetics of the enzyme to insoluble inhibitor were determined by the removal of aliquots at known times. The dopa-oxidase activity was assayed. After 10 min, no dopa-oxidase activity was detected in the extract. The gel was collected on a glass filter, and dopa-oxidase enzyme was desorbed with 0.1 M phosphate buffer, pH 7.0, followed by treatment with 0.1 M phosphate buffer, pH 8.0.

Hydrophobic Chromatography. A series of 6 small-columns were each filled with 1.0 ml of one of 6 ω-amino alkyl agarose gels, each containing a different number of carbon atoms in the alkyl chain.

The procedure for hydrophobic chromatography was as follows: All columns were thoroughly washed with 0.1 M acetate buffer, pH 4.7. A small aliquot (200 μl) of the protein extract was applied to each of the columns. The columns were eluted with 2 ml of the same buffer, and the activity in each eluate was measured. The activity percentage eluted in the 2 ml, relative to the total originally applied, was calculated.

The activity percentages versus the number of carbon atoms in the ω-amino alkyl chain of the columns were plotted.

RESULTS

Figure 1 shows the possible structures of the supports utilized. The properties of the attached ligands and their retained water quantities are indicated in Table 1.

The most active support was PABA–Sepharose 4B. We noted the stability of ligand–matrix union. Supports 2 and 3 lose their purification capacity with time, support 3 at a greater rate then support 2, possibly by loss of ligand due to instability of the PABA–matrix bond. In the second place, we also noted the best flow characteristics of CNBr-Sepharose–activated gel. Enzacryl AA has low porosity, and its behavior was different when some properties such as pH and ionic strength were changed. It was therefore not used for column techniques; it was suitable for the batch method. Likewise, the modified CM-Sephadex had poor flow characteristics. Finally, the

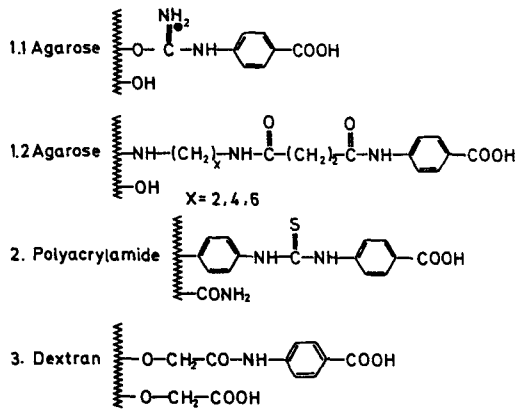

FIG. 1. Possible structures of assayed supports.

purification and recovery yields for the supports were different, the highest recoveries being obtained on PABA–Sepharose.

The elution pattern of a frog-epidermis extract in the proenzyme and enzyme forms, using PABA–Sepharose as support, is indicated in Figs. 2 and 3, respectively. The activation process was achieved by passing the proenzyme through an immobilized trypsin column.

Adsorption of the enzyme onto the derivative was obtained by complexing benzoic acid, in protonated form, on the enzyme system ($K_i = 1.4 \times 10^{-4}$ M) (11). The uncharged form of the carboxyl group was attained by lowering the pH to 4.7 with 0.1 M acetate buffer.

Elution of the enzyme was realized by raising ionic strength and bringing to pH 7.0 with 0.1 M phosphate buffer. At this pH, benzoic acid was in the carboxyl form, which does not complex the enzyme.

Figures 2 and 3 both indicate, depending on the dopa-oxidase conformational state, 2 or 3 protein peaks in the proenzyme or enzyme form. The activities and elution patterns were different: The major protein peaks,

TABLE 1. **Quantities of PABA Immobilized and Water Retained by the Assayed Supports**

Support	PABA attached (mg/g dry gel)	Water retained (g/g dry gel)
1	12.2	21.5
2	73.8	10.1
3	21.5	14.5

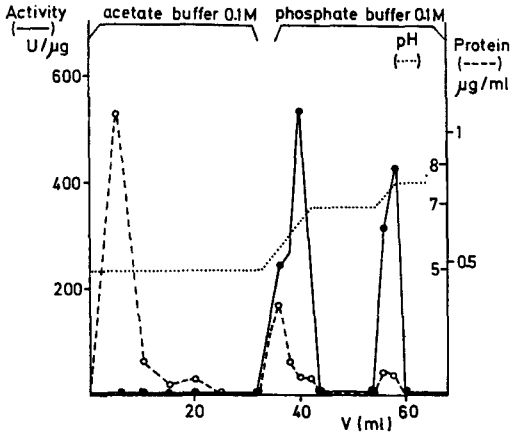

FIG. 2. Elution patterns of frog-epidermis dopa-oxidase proenzyme from a PABA–Sepharose column. The solid line represents enzymatic activity; the broken line, the protein concentration.

which eluted at pH 6.0, did not overlap with the major activity peak, which was shifted to a higher pH, both when the extract was in proenzyme form and when it was in enzyme form.

If water-soluble proteins were removed before the standard extraction procedure, the elution behavior of the new extract was different. The peak that eluted at pH 8.0 did not appear, even though the aqueous solution showed no dopa-oxidase activity.

Observed purifications were dependent on the PABA microenvironment. As Table 2 shows, when PABA–Sepharose support was used, a 15-fold purification was obtained. This ratio became higher when the enzyme was activated previously with immobilized trypsin at pH 7.0, and fell to nearly zero when the activation was achieved at pH 4.7, because of the low activity of both enzymes at this pH.

Figure 4 shows the dependence of the percentage activity on the number of carbon atoms in the ω-amino alkyl chain. As the number of carbon atoms became greater, a lower percentage of activity was eluted. These results confirm the values shown in Table 2 when PABA was coupled to succinyl amino hexyl agarose.

When Enzacryl AA was used as the support, an 11-fold purification was obtained, but only 65% of the activity introduced into the column was recovered. Using PABA–CM-Sephadex as support, very low purification was obtained.

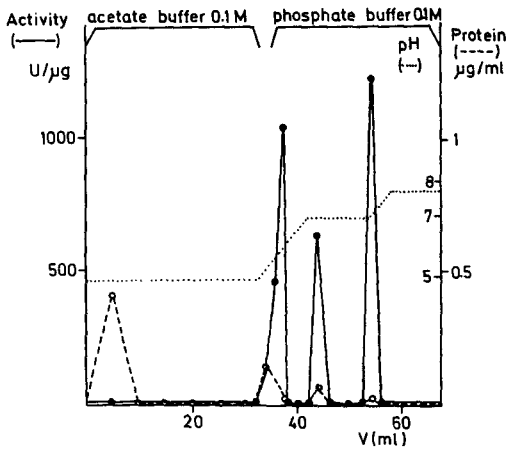

FIG. 3. Elution patterns of frog-epidermis dopa-oxidase enzyme from a PABA–Sepharose column. The solid line represents enzymatic activity; the broken line, the protein concentration.

TABLE 2. **Purification of Frog-Epidermis Dopa-Oxidase on Different PABA Supports**

Support	Enzyme form	Specific activity (U/μg)	Fraction pH	Purification factor	Enzyme recovered (%)
1.1	Proenzyme	40	6	13.5	
			7.5	10.7	110
1.1.	Activated (pH 7.0)	70	6	14.0	
			7	4.7	
			8	18.2	127
1.1.	Activated (pH 4.8)	40	6	2	
			7	3	
			8	0	90
1.2. ($X=6$)	Proenzyme	170	6	~0.2	
			7.5	0	15
1.2.	Activated (pH 7.0)	41	6	1	
			7	0	
			8	0	33
2	Proenzyme	30.3	8	11	65
3	Proenzyme	89.2	7	6.1	47.6
3	Activated (pH 7.0)	61.6	7	1.8	18.8

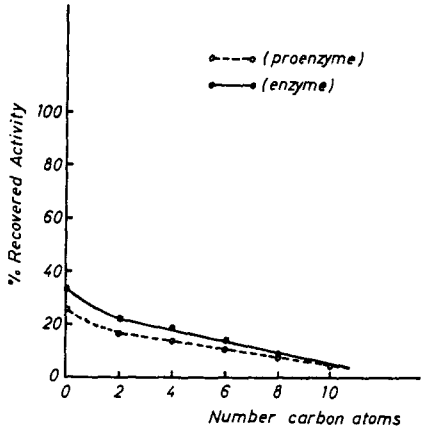

FIG. 4. Hydrophobic chromatography on ω-amino alkyl Sepharose of frog-epidermis dopa-oxidase on 0.1 M acetate buffer, pH 4.7.

Figure 5 shows the rate constant values for the proenzyme and enzyme union to support 2. The values were determined by plotting log activity versus time. It should be noted that the result was a straight-line plot that involves first-order kinetics. These results indicate that enzyme union to ligand was 10-fold faster than that of proenzyme.

DISCUSSION

The affinity chromatography on immobilized PABA can be used for purification of dopa-oxidase in both proenzyme and enzyme form. This procedure also permits analysis of multiple forms of the enzyme the existence of which has recently been postulated (14).

The different major protein peaks have been assigned to several enzyme forms because of diverse interaction with ligand. These differences are reflected in the elution patterns. Polyacrylamide gel electrophoresis of active fractions is now underway.

The fluorescence spectra of these same peaks were markedly unequal. This inequality could involve different conformational states that can be identified, or it could be caused by postulated multiple forms of the enzyme (unpublished results).

A suitable interpretation of the peak that elutes at pH 8.0, which did not appear when water, soluble proteins were removed, could be related to the existence of natural activatory substances in addition to proteolytic enzymes, as has been described (15).

The enzyme shows a greater affinity than the proenzyme for the immobilized inhibitor, as can be seen in Fig. 5 and Table 2; this finding

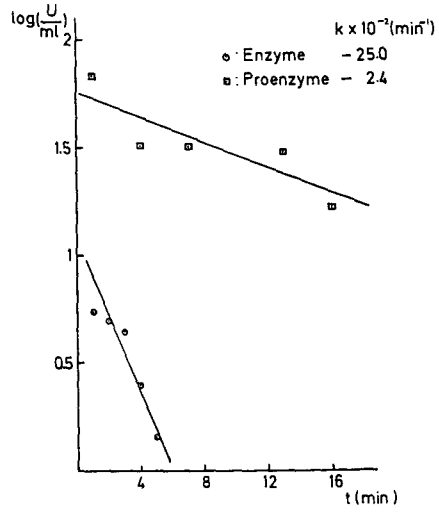

FIG. 5. First-order rate constants of extraction of dopa-oxidase from solution when using support 2. Quantity of wet gel, 1.27 g; extract volume, 10.0 ml; protein concentration, 11.9 μg/ml.

confirms Lozano's interpretation (16) that the enzyme is unfolded, as compared with the proenzyme form. The enzyme is therefore more easily accessible to interaction with ligand. These results also coincide with Fig. 4, which shows the major hydrophobic affinity of the proenzyme and the enzyme.

It was concluded that affinity chromatography on immobilized PABA is a suitable method for purification of frog epidermis dopa-oxidase. The insolubilized inhibitor can be reused, unlike the results of O'Neill et al. (4), in which the use of dopamine as ligand permitted only a single use of the support. This was caused by changes in the structure of the ligand by reaction with the enzyme, which becomes denatured by its performance with substrates.

ACKNOWLEDGMENT

We sincerely thank Professor J. A. Lozano for the critical reading of the manuscript and for his interest in this work.

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