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# 9-(5-CARBOXYPENTYLAMINO)-ACRIDINE

# AN AFFINITY ADSORBENT LIGAND FOR *ELECTROPHORUS* ACETYLCHOLINESTERASE

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## Summary

We have developed a simple method for the synthesis of the ligand 9-(5-carboxypentylamino)-acridine and the resulting affinity adsorbent using Sepharose CL-4B. This affinity adsorbent is efficient and specific for purification of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) from *Electrophorus electricus* electric organ.

## Introduction

Purification of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) by affinity chromatography has been reported using a wide variety of ligands: phenyltrimethylammonium [1,2], *N*-methylpyridinium [3], acridine derivatives [4—6] and the covalent ligand phenylmethylphosphonate [7]. Previously reported methods of acridine ligand synthesis require numerous steps and purification schemes with low yields. In this paper, we report the synthesis of a similar affinity adsorbent starting with a two-step synthesis of the acridine ligand 9-(5-carboxypentylamino)-acridine (I). Its mixed anhydride (II) was then formed with isobutylchloroformate and coupled to Sepharose 1,3-diaminopropane to produce III (Fig. 1).

## Methods

Sepharose CL-4B was purchased from Pharmacia Fine Chemicals; 9-chloracridine was obtained from Eastman Organic Chemicals; 1,3-diaminopropane,

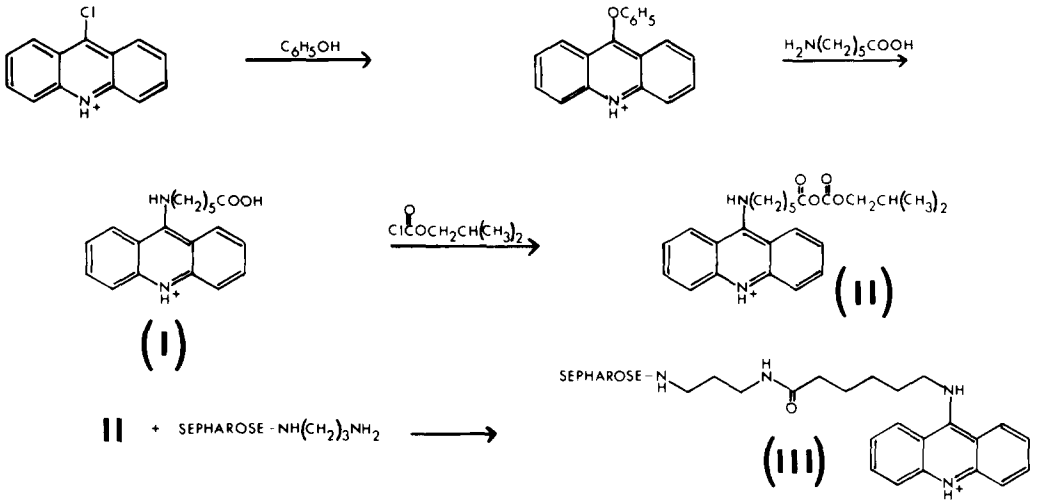


Fig. 1. Synthesis of affinity resin.

6-aminocaproic acid and triethylamine were obtained from Aldrich Chemical Company. Cyanogen bromide and isobutylchloroformate were obtained from Sigma Chemical Company. All other chemicals were of the highest purity available. *Electrophorus electricus* was obtained from Paramount Aquarium, Ardsley, New York.

Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN.

Acetylcholinesterase activity was assayed according to the method of Ellman et al. [8] at pH 8.0.

Protein was estimated by the method of Lowry et al. [16] as described by Ji [9].

*Preparation of I.* 3.08 g (11 mmol) 9-phenoxyacridine [10] and 1.49 g (11 mmol) 6-aminocaproic acid were refluxed in 120 ml methanol for 5 h. The yellow crystals were collected, washed with ice-cold methanol and recrystallized from methanol. Yield, 2.4 g (70%); m.p., 198–199°C (uncorrected).
Analysis: calcd. for $C_{19}H_{20}N_2O_2$:

Calcd: C, 74.00; H, 6.54; N, 9.09

Found: C, 73.84; H, 6.69; N, 8.98

$\lambda_{max}$ 411 nm, $\epsilon$ = 10 100 (10 mM sodium phosphate buffer, pH 7.0).

*Preparation of II.* The mixed anhydride (II) was prepared by adding stoichiometric amounts of I, isobutylchloroformate and triethylamine in anhydrous tetrahydrofuran. In a typical reaction mixture to prepare 100 ml resin III, 780 μmol of each of the three reactants were added to 200 ml tetrahydrofuran. The reaction flask was stoppered and vigorously stirred with a magnetic stirrer at room temperature for 20 min. The product was not isolated but used immediately after preparation, care being taken to protect it from moisture.

*Preparation of III.* Sepharose CL-4B was activated with CNBr and coupled with 1,3-diaminopropane, 30 μmol/ml packed gel, according to the method of Cuatrecasas et al. [14]. After the last wash with 0.1 M $NaHCO_3$/0.5 M NaCl,

pH 10, the amino-coupled Sepharose was washed with 10 vols. distilled water, 5 vols. of tetrahydrofuran and filtered.

The anhydrous Sepharose 1,3-diaminopropane was added to the above solution of II using a minimal amount of tetrahydrofuran to facilitate transfer. The flask was stoppered and placed in a shaker bath at room temperature for 4 h. The resulting affinity adsorbent was washed successively with 5 vols. of tetrahydrofuran, followed by 10 vols. each of methanol, distilled water and 0.1 M $NaHCO_3$, pH 10. Determination of bound ligand was performed by difference spectroscopy at 411 nm, using a 1 : 10 dilution of affinity resin with 0.1 M sodium phosphate buffer (pH 7.0).

*Purification of eel acetylcholinesterase.* Affinity chromatography of *Electrophorus* acetylcholinesterase was performed by a modification of the procedure of Rosenberry [5]. All steps were carried out at 0—4°C. Homogenates were de-aerated by vacuum prior to centrifugation. Freshly dissected electric organ was homogenized in 3 vols. 10 mM sodium phosphate buffer (pH 7.0) and centrifuged at $14\,000 \times g$ for 30 min. The supernatant was discarded, and the procedure was repeated twice. The pellet was then homogenized in 0.8 vols 20 mM sodium phosphate buffer/1.4 M NaCl (pH 7.0) and centrifuged at $48\,200 \times g$ for 30 min. The supernatant was collected, and the pellet was rehomogenized in 0.4 vols. 20 mM sodium phosphate buffer/1.0 M NaCl (pH 7.0) (column buffer). This supernatant was combined with the 1.4 M NaCl supernatant and applied to a 2.5 × 51 cm column packed with resin III, previously equilibrated with column buffer, at a flow rate of 0.5 ml/min. The column was washed with 20—25 column vols. of column buffer, or until the $A_{280}$ of the eluent was less than 0.05. The enzyme was then eluted with column buffer containing 10 mM decamethonium bromide at 0.5 ml/min and 3 ml fractions were taken. After assaying, the fractions with maximum activity were pooled, dialyzed extensively against column buffer and stored frozen in siliconized glass tubes.

## Results

*Synthesis of resin III*

A summary of several preparations of resin III is shown in Table I. From the data shown it appears that 30 μmol/ml gel of 1,3-diaminopropane was sufficient to bind the desired amount of acridine to the resin, since higher concentrations of amine did not result in significantly increased binding of acridine. This concentration of amine was probably sufficient to saturate all the CNBr-activated sites on the resin, generated under conditions as described in Methods. A ligand concentration of 0.7 μmol/ml gel was found to be most efficient in purifying eel acetylcholinesterase.

*Purification of eel acetylcholinesterase*

A typical elution profile of eel acetylcholinesterase is shown in Fig. 2. Peak enzyme activity corresponded to peak $A_{280}$. The purified enzyme obtained had a specific activity of approx. 5000 units/mg protein, as indicated in Table II. Specific elution of acetylcholinesterase amounted to 90% of column onput and overall yield was approx. 80%. The $K_i$ for I is 6.2 μM (0.1 M phosphate, pH

TABLE I

PREPARATION OF RESIN III

DAP, amount of 1,3-diaminopropane added to CNBr-activated Sepharose as described in Methods. Amount of II added to Sepharose 1,3-diaminopropane as described in Methods.

| 1,3-DAP (μmol/ml gel) | II (μmol/ml gel) | Bound acridine (μmol/ml gel) |
|---|---|---|
| 2000 | 5 | 1.1 |
| 400 | 32 | 2.8 |
| 300 | 7.8 | 1.4 |
| 30 | 7.8 | 0.7 * |

* Identical results were achieved with three different preparations at the concentrations listed.

7.0).

The enzyme exhibited a $A_{280}/A_{260}$ ratio of 1.7, which corresponds exactly to previously reported results [4]. When the enzyme was analyzed by continuous density gradient centrifugation, three molecular forms were evident corresponding to 18, 14 and 9 S in the ratio 55 : 35 : 10. No appreciable 11 S was found, and no additional protein bands were found by SDS-polyacrylamide gel electrophoresis (Struve, W.G. and Hitt, A.S., unpublished results).

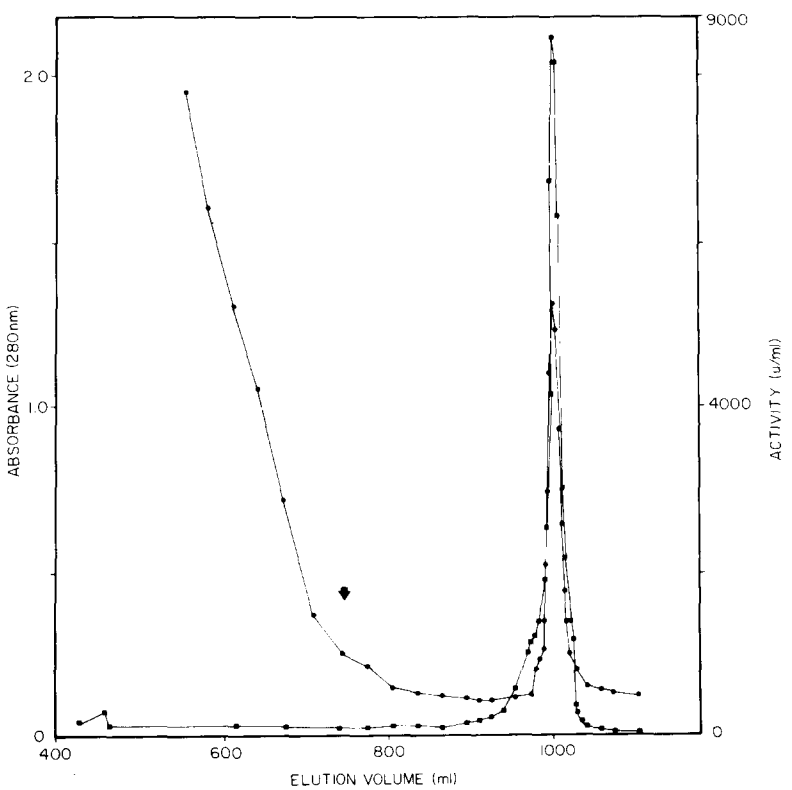


Fig. 2. Elution profile of Electrophorus acetylcholinesterase on resin III ●———●, absorbance (280 nm). ■———■, enzyme activity (units/ml). ↓ elution with 10 mM decamethonium bromide.

TABLE II

TYPICAL PURIFICATION OF EEL ACETYLCHOLINESTERASE

| Step | Vol. (ml) | Total units (μmol/min) | Total protein (mg) | Specific activity (μmol/min per mg) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|---|
| 1st homogenate | 1600 | 120 480 | 5984 | 20.1 | 1 | 100 |
| 1.4 M Supernatant | 370 | 93 600 | 425 | 220 | 8.1 | 89 |
| 1.0 M Supernatant | 150 | 14 120 | 748 | 18.9 | | |
| Affinity chromatography * | 6.6 | 77 107 | 15 ** | 5130 | 225 | 64 *** |

* Two peak fractions.
** Based on $\epsilon_{280}^{1\%}$ of 18.0 [5].
*** The activity in these two fractions represented 80% of the total activity eluted by decamethonium.

## Discussion

The irreproducibility of the original acridine ligand synthesis of Dudai et al. [4] has led to modifications of this procedure by other laboratories. Rosenberry and Richardson [5] reported one such modification, wherein half the spacer arm, 6-aminohexanoic acid, was initially linked to the Sepharose matrix. Subsequently, the ligand 9-γ-aminopropylaminoacridine dihydrobromide was linked to the substituted Sepharose. Synthesis of the ligand as reported required five steps and repeated isolation and recrystallization of products. Additionally, any free aminohexanoate residues remaining on the resin are capable of retaining protein through nonspecific binding, at least at low salt concentrations [11]. Massoulie and Bon [12] have reported retention of acetylcholinesterase and other proteins on hexyl- and acetamidohexyl-Sepharose at moderate (0.4 M) salt concentrations. The modification by Webb and Clark [13] required five steps with yields ranging from 16—86%, and included one step resulting in a mixture of isomers.

The method reported in this paper utilizes the same spacer arm as Rosenberry and Richardson, but in a reversed orientation. Diaminopropane, rather than 6-aminohexanoic acid, is initially linked to Sepharose, which reduces the possibility of nonspecific binding to unreacted spacer arms. Synthesis of I

TABLE III

COMPARISON OF VARIOUS ACRIDINE RESINS FOR PURIFICATION OF EEL ACETYLCHOLINESTERASE

All calculations for eluted enzyme are based on $\epsilon_{280}^{1\%}$ of 18.0 [5].

| Method | Specific activity of eluted enzyme (μmol/min per mg protein) | Yield from column (%) |
|---|---|---|
| Dudai and Silman [4] | 5400 | 50 |
| Rosenberry and Richardson [5] | 7200 | 60 |
| Webb and Clark [13] | 4200—7900 * | 43—88 * |
| Brooks, Tiller and Struve | 5130 | 90 |

* Depending on ligand concentration.

requires two steps from 9-chloroacridine and is obtained in high yield. A comparison of the various modifications is presented in Table III.

Resin III did not prove to be an efficient resin for purifying detergent-solubilized human erythrocyte acetylcholinesterase, despite the low $K_i$ (17.9 $\mu$M) of I for the erythrocyte enzyme. This enzyme bound weakly to the resin and could be eluted by 1 M NaCl. By extending the spacer arm [14,15] using two molecules of 1,12-diaminododecane, 90% of the erythrocyte enzyme bound irreversibly to the resin. Although an acridine ligand has been reported for purifying detergent-solubilized pig-brain acetylcholinesterase [6], the most efficient ligand reported for purifying the human erythrocyte enzyme is a phenyltrimethylammonium derivative [15].

## Acknowledgement

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