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PURIFICATION OF ACETYLCHOLINESTERASE FROM HOUSE FLY BRAIN BY AFFINITY CHROMATOGRAPHY

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Summary

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from the heads of house flies (*Musca domestica* L.) was purified by affinity chromatography. The enzyme was adsorbed from the crude extracts on an affinity column containing trimethyl(*p*-aminophenyl) ammonium chloride hydrochloride ($K_i \approx 1.7 \cdot 10^{-4}$ M), covalently linked to Sepharose 4B, then eluted with a solution of a selective reversible inhibitor, 1,5-bis (4-allyl dimethyl ammoniumphenyl)-pentan-3-one dibromide (BW 284C51; $K_i \approx 1 \cdot 10^{-7}$ M). The enzyme was purified 1223 times in one step and had a specific activity of 752 units/mg protein. Disc gel electrophoresis in polyacrylamide gel revealed five protein bands, four corresponding to the enzyme activity bands and one devoid of enzyme activity. On the basis of periodic acid-Schiff stain intensity, the slower moving isozyme I and the contaminating band appear to be rich in carbohydrate. The purity of the enzyme estimated by disc gel electrophoresis was 94%. Density gradient centrifugation in sucrose showed two major species each of which ran as a single band on disc gel electrophoresis. The average molecular weights were 306 000 (±11 150) for heavy ($s_{20,w}$ = 11.5 S) form and 143 000 (±4700) for light ($s_{20,w}$ = 6.9 S) form.

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is widely distributed in vertebrate and invertebrate tissues and occurs as multiple molecular forms [1—9]. Its molecular properties are of great interest because of its involvement in synaptic transmission and its role in organophosphate and carbamate poisoning [10]. The enzyme from vertebrates has so far been extensively purified by using the technique of affinity chromatography from a variety of vertebrate tissues [11—19]. However, acetylcholinesterase from insect sources has only been purified using the multi-step conventional procedures based on physicochemical properties [20—22]. These methods are usually laborious and yields are frequently low (e.g. 16%) primarily because of the many steps required for purification.

In the present investigation, we describe a simple, one-step procedure for almost complete purification of acetylcholinesterase from house fly brain (*Musca domestica* L.). The procedure involved linking a reversible competitive inhibitor, trimethyl (*p*-aminophenyl) ammonium chloride hydrochloride through an extended side-arm to Sepharose 4B and then specifically eluting the enzyme from the column with a high affinity inhibitor, BW 284C51 (1,5-bis(4-allyl dimethyl ammoniumphenyl)-pentan-3-one dibromide).

Materials and Methods

Materials. Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden). Cyanogen bromide, 1,5-diaminopentan, succinic anhydride and "carbodiimide" (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene-sulfonate) were obtained from Aldrich Chemical Company (Milwaukee, Wisc.); acetylthiocholine chloride, and 5,5′-dithiobis-(2-nitrobenzoic acid) from Nutritional Biochemicals Corporation (Cleveland, Ohio); 1,5-bis(4-allyl dimethyl ammnium phenyl)-pentan-3-one dibromide (BW 284C51) from Burroughs Wellcome Company, North Carolina. The chemicals used in polyacrylamide gel electrophoresis were obtained from Eastman Organic Chemicals (Rochester, N.Y.). The Coomassie Brilliant Blue R-250 was obtained from Bio Rad (Richmond, California). All reagents used were of analytical grade.

Preparation of affinity gel. Trimethyl (*p*-aminophenyl) ammonium chloride hydrochloride was synthesized by the method of Traylor and Singer [23]. The preparation of affinity gel was essentially done according to the procedure described by Cuatrecasas and Anfinsen [24]. The following example is typical of coupling procedure. 50 ml of washed and settled Sepharose 4B was mixed with 50 ml of distilled water and 5.3 g of cyanogen bromide was added with constant stirring in a ventilated hood. The pH of this solution was immediately raised and maintained at 11.0 with cold 4 M NaOH and the temperature was maintained at 20°C by adding pieces of ice. The reaction was completed in about 7—8 min. The suspension was quickly transferred to a sintered-glass Buchner funnel and washed under suction with cold buffer (0.1 M Na_2CO_3, pH 10.0) using 2 liters. The washed, activated gel was then quickly added to a solution containing 2.55 g of 1,5-diaminopentan in 50 ml of cold distilled water previously adjusted to pH 10.0 with 6 M HCl. This solution was stirred at 4°C for 16 h and then excess of amine was removed by washing with 500 ml of distilled water followed with 500 ml of 1 M NaCl and one liter of distilled water. This aminated gel was dispersed in an equal volume of distilled water and succinylated with 5 g of succinic anhydride. The pH was raised to and maintained at 6.0 by titrating with 20% cold NaOH for at least 30 min at 4°C. The solution was stirred for 5 h at 4°C and then washed as described above. A second molecule of 1,5-diaminopentan was added by the aid of carbodiimide and followed by a second coupling of succinic anhydride to form an adequate "spacer". At the end of this spacer, with the aid of carbodiimide, the trimethyl (*p*-aminophenyl) ammonium chloride hydrochloride was covalently attached.

Extraction. Heads of house flies (*Musca domestica* L., tetrachlorvinphos resistant strain) were separated by the method of Moorefield [25] and a 20% (w/v) homogenate was prepared as described previously [26]. The homogenate

was filtered through six layers of cheesecloth and centrifuged at 100 000 × *g* for 60 min at 4°C. The supernatant fluid fraction was used as an enzyme source. The soluble acetylcholinesterase used in these experiments represents 24—34% of the total activity [27,28].

Enzyme assay. The enzyme activity was measured at 25°C by the colorimetric method of Ellman et al. [29]. The usual assay mixture contained 1 mM acetylthiocholine, 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic) acid, 75 mM NaCl, and 40 mM $MgCl_2$ dissolved in 50 mM sodium phosphate buffer (pH 7.4). The reaction was followed spectrophotometrically by the increase in absorbance at 412 nm on the Beckman Acta III spectrophotometer. One unit of activity is defined as that amount of enzyme required to hydrolyze 1 μmole of acetylthiocholine per minute under these conditions.

Protein determinations. Protein was determined according to the method of Lowry et al. [30] using bovine serum albumin as the standard.

Acrylamide disc gel electrophoresis. Disc gel electrophoresis was performed on 7% acrylamide gels in Tris/glycine buffer (pH 8.3) according to the procedure of Davis [31]. The gels were run at a constant current of 3 mA/gel tube for 1 h at 4°C and were stained with 0.2% Coomassie Brilliant Blue R-250 in ethanol/water/acetic acid (45 : 45 : 10, v/v) at 65°C for 30 min and then destained in ethanol : water : acetic acid (25 : 65 : 10, v/v) at 37°C overnight on a shaking waterbath. The acetylcholinesterase activity was located on the gels using an acetylthiocholine method described earlier [32]. The basis of the method is that thiocholine released after enzymatic hydrolysis reduces Cu^{2+} to form insoluble copper-ferrocyanide in presence of citrate ions. The total reaction mixture of 25 ml consisted of 1 mM acetylthiocholine; 18 ml of 0.1 M sodium phosphate buffer, pH 6.0; 1 ml of 0.1 M sodium citrate; 2 ml of 30 mM $CuSO_4$ and 4 ml of 5 mM potassium ferric cyanide. The gels were scanned in Beckman Acta III recording spectrophotometer equipped with a gel scanner at 0.2 mm slit width. The gels for carbohydrate were stained with periodic acid-Schiff according to the method of Kapitany and Zebrowski [33].

Sucrose density gradient centrifugation. Linear sucrose density gradients (5—20%) in 50 mM sodium phosphate buffer, pH 7.4 were prepared in nitrocellulose tubes (12 ml volume) with a Beckman gradient former.The marker protein (aldolase) and 0.2 ml of the purified acetylcholinesterase were layered on the gradients immediately prior to centrifuging at 39 000 rev./min for 18 h at 4°C using an SW40 swing-out rotor in a L-265B Beckman ultracentrifuge. Ten-drop fractions (0.2 ml) were collected from a hole punctured in the bottom of each tube. Aldolase peaks were determined by protein assay.

In the calculation of sedimentation coefficients, it was assumed that the partial specific volume, $\overline{v}$, of the acetylcholinesterase was the same as the standard, aldolase, for which values of $\overline{v}$ = 0.742 and S = 7.35 were accepted [34]. Reported values of $\overline{v}$ for acetylcholinesterase have appeared only for eel enzyme and have been variously calculated as 0.731 [35] or 0.72 [2] and measured as 0.74 to 0.75 [36]. For the calculation of molecular weights, the simple ratio method of Martin and Ames [37] was used, which assumed that the molecular weights are proporional to $s^{3/2}$; aldolase was used as standard, with a molecular weight taken as 156 000 [34].

Procedures for purification of acetylcholinesterase. 150 ml of affinity gel

was placed in a K 16/100 Pharmacia column. All subsequent steps were carried out at 4°C. The column was washed thoroughly with 50 mM sodium phosphate buffer (pH 7.4) containing 50 mM NaCl. Typically 100 ml of the crude enzyme was applied at a rate of 20—30 ml/h and about 95% of the enzyme activity was bound to the column. The column was washed with the same buffer until the effluent solution was free of contaminating proteins and eye pigments. The acetylcholinesterase was then eluted at 20—30 ml/h with 300 ml of a solution of 1 mM BW 284C51 in the same buffer. The BW 284C51 was then removed by dialysis or Sephadex treatment; originally, batch dialysis against 3 liters of 50 mM sodium phosphate (pH 7.4) with 150 mM NaCl and 10 mM $MgCl_2$ with 6 to 8 changes was used. Subsequently, dialysis was replaced by passage through a Sephadex G-50 column (Pharmacia K 50/100, with a bed volume of 1800 ml), the eluant being sampled for enzyme activity, and active fractions were pooled and concentrated at 2—5 ml by a vacuum dialysis procedure which uses a collodion membrane bag (Schleicher and Schuell, Inc.) for subsequent characterization. The column was then regenerated with 6 M guanidine HCl, followed by washing with four liters of 1 M NaCl and 2 liters of 50 mM sodium phosphate buffer (pH 7.4) containing 50 mM NaCl before reutilization.

Results and Discussion

A Sepharose-PTA affinity column is known to bind acetylcholinesterase specifically as well as other negatively charged proteins nonspecifically [11, 38—41]. Therefore the problem is to choose a ligand to elute acetylcholinesterase selectively. In the purification of vertebrate acetylcholinesterase by affinity chromatography, displacing ligands which have been successfully used include tetramethylammonium [18], edrophonium [12], and decamethonium [13]. However, the present studies (Table I) indicated that although the yields with these ligands were sometimes adequate, the specific activities were not very high. In addition, electrophoresis of the eluted enzyme showed 3 to 5 protein bands in addition to bands of acetylcholinesterase. In another study (Tripathi, R.K. and O'Brien, R.D., unpublished) it was found that the dissociation constants for these three ligands for house fly acetylcholinesterase were respectively 2.8, 0.06, and 0.03 mM. A search for a more potent and selective ligand led to the compound BW 284C51 [42,43]. It proved to have a dissociation constant for the enzyme of 0.097 μM. This became the eluant of choice in the remainder of this work.

TABLE I

ELUTING LIGANDS USED IN INITIAL ATTEMPTS AT PURIFICATION

Ligands	Specific activity (units/mg protein)	Yield (%)
Tetramethylammonium, 50 mM	83	5
Gallamine, 20 mM	259	42
Decamethonium, 50 mM	355	23
Edrophonium, 100 mM	399	34

Table II presents the results of a typical purification of acetylcholinesterase from house fly brain. The high specific activity and good recovery of enzyme activity in a single step makes the affinity chromatography a very useful method for purification of acetylcholinesterase from insect sources where the amount of material available is limited. The recovery of the enzyme activity from three separate runs varied in different enzyme preparations from 26—47% and the enzyme had a specific activity from 620 to 1014 units/mg protein. When specific activity described in this paper is compared to the specific activity of 739 units/mg reported by Huang and Dauterman [20], it appears that the affinity column method gives, in a single step, as much purification as a multistep conventional procedure. The yields also compare favorably with their values of 16%.

Previous electrophoretic studies with crude house fly head enzyme had shown four molecular forms [27,44]. The purified enzyme showed (Fig. 1) precisely the same bands; this contrasts with the two forms resulting from conventional purification [20]. When the gels were stained for protein, four protein bands were observed which corresponded with enzyme activity, but the ratios of peak heights (i.e. enzyme peak/protein peak) varied considerably, being 1.4, 3.0, 6.1 and 1.0 for enzyme bands I through IV. A clear small peak of protein, presumably an impurity, was observed of relative mobility 0.34, representing 6% of the total protein. At band IV of acetylcholinesterase activity, a doublet peak occurred. Because the resolution of protein bands is considerably sharper than that of acetylcholinesterase activity, it is presently uncertain whether the protein doublet represents two isozymic bands, or an impurity plus an isozyme.

Staining of the electrophoretic gels with periodic acid-Schiff stain was performed (Fig. 2). The results showed one clear peak of carbohydrate activity corresponding to isozyme I, which is thus almost certainly a glycoprotein, as shown for *Torpedo* [19] and *Electrophorus* [45]. As for the other isozymes, they are either not glycoproteins or their sugar content is too low, in comparison with the background, to permit a conclusion. It is an intriguing possibility that one difference in isozyme composition may be the sugar content.

The highly purified preparation on sucrose density gradient centrifugation showed two major peaks exactly like the crude preparation (Fig. 3). Polyacrylamide disc gel electrophoresis showed that the heavy peak corresponded to the slowest moving isozyme I, and the light peak to the fast moving isozyme III (Fig. 2). The molecular weights and sedimentation coefficients of these two major species were 306 000 (±11 150) for heavy ($s_{20,w}$ = 11.5 ± 0.28 S) form

TABLE II

TYPICAL PURIFICATION BY AFFINITY CHROMATOGRAPHY WITH ELUTION BY BW 284 C51

Stage	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification factor
Crude extract	99.22	620	0.614	100	1
Purified acetylcholineesterase	33.10	0.044	752	34	1223

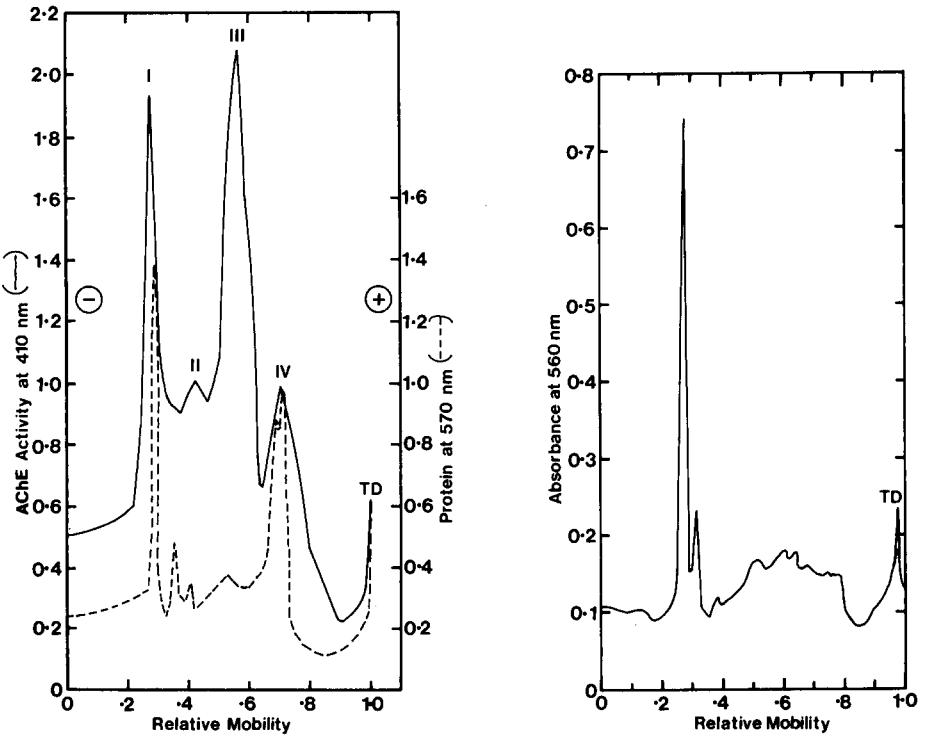

Fig. 1. Electrophoretograms of purified house fly brain acetylcholinesterase. Scan of gel stained for protein with Coomassie Blue (- - - - - -); scan of gel stained for acetylcholinesterase (———). TD denotes the portion of tracking dye. Acetylcholinesterase isozymes are numbered I through IV in order of increasing anodic mobility in the separating gel.

Fig. 2. Electrophoretogram of purified house fly brain acetylcholinesterase. Scan of gel stained for carbohydrate with period acid-Schiff reagent.

and 143 000 (±4700) for light ($s_{20,w}$ = 6.9 ± 0.15 S) form (Table III).

Highly-purified enzyme was moderaly stable in that it lost 31% of its activity after 4 months at 0°C, and 30—50% of its activity after dialysis and ultrafiltration.

TABLE III

SEDIMENTATION COEFFICIENTS AND MOLECULAR WEIGHTS OF THE PRINCIPLE MOLECULAR FORMS

Values are means ± S.E.M. The numbers of replicates were 5 for crude, and 4 for pure. The difference between the molecular weights of I and III in crude and pure were not significant (t test, P = 0.05).

	Crude		Pure	
	$s_{20,w}$	Mol. wt. (X 10^{-3})	$s_{20,w}$	Mol. wt. (X 10^{-3})
Isozyme I	11.5 ± 0.47	307 ± 8.3	11.5 ± 0.28	306 ± 11.15
Isozyme III	7.2 ± 0.30	152 ± 9.8	6.9 ± 0.15	143 ± 4.7

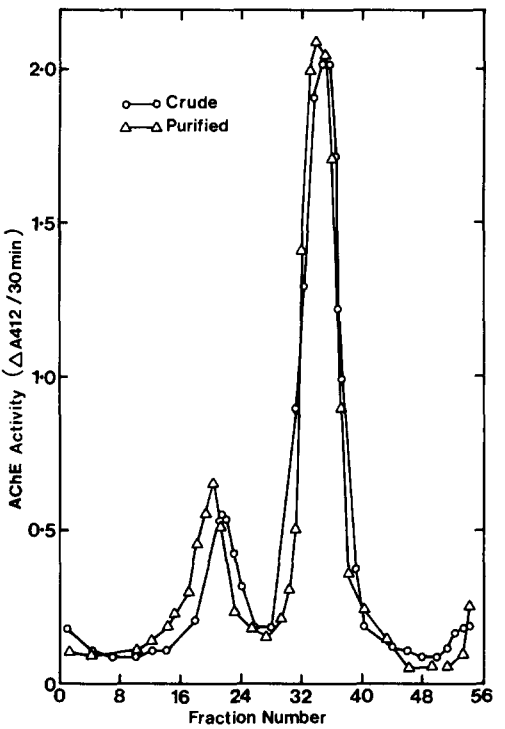

Fig. 3. Sucrose density gradient pattern of acetylcholinesterase. Crude (○———○); purified (△———△).

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