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PURIFICATION AND CHARACTERIZATION OF AN ASCIDIAN LARVAL ACETYLCHOLINESTERASE

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Summary

Larval acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) of the ascidian *Ciona intestinalis* (L.) was purified by a two-step affinity chromatography procedure. Concanavalin A-Sepharose chromatography in batches provided the initial purification and was followed by chromatography on columns to which competitive inhibitors of acetylcholinesterase had been attached. The most efficient of these used *m*-carboxyphenylmethylammonium iodide coupled to Sepharose 4B via a hydrophobic 6-carbon spacer. In combination with the concanavalin A-Sepharose step, this affinity resin yielded recoveries of 30—39% with specific activities ranging from 580—730 units/mg protein, a total purification of 5000—7000-fold. Analysis of this product by polyacrylamide gel electrophoresis in the presence of SDS and β-mercaptoethanol revealed a single major polypeptide of M_r 65 000—70 000. This protein was identified as the basic catalytic subunit of acetylcholinesterase by its coelectrophoresis with [^{3}H]diisopropyl fluorophosphate-labeled enzyme. Sucrose density gradient studies demonstrated that the purified enzyme consisted of three distinct species that appeared to be qualitatively the same as those seen in crude extracts. The largest species (11 S) is possibly a tetramer of the basic catalytic subunit and the two smaller forms, the monomer and dimer. Purified enzyme was also used to produce anti-acetylcholinesterase antibody in rabbits. IgG prepared from the sera of immunized rabbits was shown to react completely (greater than 98%) with acetylcholinesterase from crude larval homogenates. This result also supports the conclusions that no qualitative selection occurred during the purification procedure and that the basic catalytic subunit is a fundamental component of all the larval acetylcholinesterases.

Abbreviations: Dip-F^3, diisopropyl fluorophosphate; mCPA, *m*-carboxyphenyldimethylammonium iodide; MAP, *n*-methyl-3-aminopyridinium iodide; IgG, immunoglobulin G.

Introduction

One approach to understanding the events of embryogenesis is to study the expression of specific proteins that serve as markers of phenotypic differentiation. Ascidians (subphylum Urochordata; class Ascidiacea) are particularly suited to this approach since several histospecific enzyme activities such as acetylcholinesterase, alkaline phosphatase and tyrosinase appear during larval development [1]. Acetylcholinesterase activity is localized in the tail muscle cells of the developing larva [2—5] and quantitative estimates have demonstrated a 35—40-fold increase in this activity during the 12 h period from gastrulation to hatching [5]. Such a rapid and extensive increase in the activity of this enzyme offers an opportunity to investigate the regulation of a marker of muscle cell differentiation at a biochemical level.

Initial attempts to study acetylcholinesterase expression using metabolic inhibitors such as puromycin and actinomycin D suggested that changes in enzyme activity required both protein and RNA synthesis [3,5]. Further investigation of the role these processes play in enzyme expression demands a more direct approach. For example, it would be possible to study the requirement for de novo enzyme synthesis by radioimmunoassay or to determine the levels of translatable acetylcholinesterase mRNA at various stages of development in an mRNA-dependent in vitro protein synthesis system. However, before such assays can be developed, the biochemical nature of acetylcholinesterase must be established, and the enzyme must be isolated in sufficient quantity to be used as an immunogen.

We describe here the isolation and biochemical characteristics of the larval acetylcholinesterase of *Ciona intestinalis*, and the preparation of antibody raised in rabbits to this enzyme. These findings represent a first step toward understanding the mechanisms controlling the expression of this enzyme.

Materials and Methods

Larvae. Eggs were collected and fertilized as described by Whittaker [6]. Since enzyme activity reaches a plateau 30—36 h after fertilization [5], larvae were raised until this time then harvested and washed by centrifugation several times in ice-cold sea water containing 1 mM phenylmethylsulfonylfluoride.

Assay methods. Acetylcholinesterase activity was measured by the radiometric assay of Schrier et al. [7] as previously described [5]. 1 unit enzyme activity equals 1 μmol product formed per min in this assay. Protein concentrations were assayed by the method of Lowry et al. [8] with bovine serum albumin as the standard.

Larval homogenates. 10% (w/v) embryo extracts in 0.1 M potassium phosphate buffer, pH 7.6/1.0 M NaCl/2% Triton X-100 were prepared using a Virtis '23' tissue homogenizer. This extract was stirred vigorously for 2 h at 4°C on a magnetic stirrer and then centrifuged for 30 min at 20 000 $\times g$. The resulting supernatant, which contained at least 95% of the enzyme activity, was used for the isolation of acetylcholinesterase.

Concanavalin A-Sepharose chromatography. The 20 000 $\times g$ supernatant fraction was titrated with concanavalin A-Sepharose 4B (Sigma) on a rocking

platform at 4°C until at least 95% of the enzyme activity was removed from solution. The bound enzyme was washed repeatedly in homogenizing buffer and then eluted in this buffer containing 0.1% Triton X-100 and 20% (w/v) α-methyl-(+)-mannoside. After separation of the eluted enzyme from the concanavalin A-Sepharose, this procedure was repeated. The first eluate contained 40—45% of the applied activity and the second about half this amount. Recovered enzyme was dialyzed twice against 10—20 vols. of the appropriate buffer before application to the second affinity column.

Preparation of affinity resins. All the reagents for preparing the affinity resins were obtained from Aldrich unless otherwise specified.

N-methyl-3-aminopyridinium iodide (MAP)-Affi-Gel 202. A competitive inhibitor of acetylcholinesterase, MAP, was synthesised from 3-aminopyridine and iodomethane as described by Goodkin and Howard [9]. MAP was attached to Affi-Gel 202 (BioRad) by reaction with 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (BioRad) at pH 4.7.

MAP-Sepharose. Cyanogen bromide-activated Sepharose 4B (Pharmacia) was washed with 1 mM HCl, dispersed in $NaHCO_3$ at pH 10, and incubated overnight at 4°C with 1,6-hexanediamine. After thorough washing, the aminated gel was dispersed in distilled water and succinylated with succinic anhydride for 5 h at 4°C while a pH of 6.0 was maintained by titration with 5 N NaOH. Excess reagents were removed by filtration, and the succinylated gel was incubated with 0.1 N NaOH for 30 min at room temperature, then washed. Another molecule of 1,6-hexanediamine was added by the carbodiimide-coupling procedure, and a second succinylation completed the spacer. MAP was attached to the free carboxyl of this sidearm by reaction with carbodiimide.

m-Carboxyphenyldimethylethyl ammonium iodide (mCPA)-Sepharose. mCPA was synthesized from *m*-(dimethylamino)benzoic acid and iodoethane as described by Massoulié and Bon [10]. 1,6-Hexanediamine was attached to cyanogen bromide-activated Sepharose 4B followed by carbodiimide coupling of the ligand to the amino terminus of the spacer arm.

All of the columns were operated in 0.01 M Na_2HPO_4 buffer, pH 7.0, 0.1% Triton X-100 and 1.0 mM phenylmethylsulfonylfluoride. NaCl was added to this buffer to achieve the desired ionic strength.

[^{3}H]Diisopropyl fluorophosphate labeling. Approx. 1.5 μg purified acetylcholinesterase in 0.01 M Na_2HPO_4, pH 7.0, 0.4 M NaCl and 0.1% Triton X-100 (containing 100 μg ovalbumin to stabilize the enzyme) were incubated at 25°C with [^{3}H]Dip-F^3 (Amersham) until no enzyme activity remained. This preparation was then dialyzed against three changes of the same buffer, once against 0.1% SDS and lyophilized.

Polyacrylamide gel electrophoresis. Samples were analyzed electrophoretically using the method of Laemmli [11]. The acrylamide concentration in the stacking gel was 3%, and in the separating gel, 10%. Samples in 0.0625 M Tris-HCl, pH 6.8, containing 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 2% (w/v) SDS were denatured before electrophoresis by heating for 2 min at 100°C. After electrophoresis gels containing [^{3}H]Dip-F^3-labeled enzyme were frozen, sliced and eluted overnight at 37°C in freshly prepared 3% (v/v) Protosol/Econofluor (New England Nuclear). Radioactivity was assayed by liquid scintillation counting. Protein was detected in gels by staining with 0.2% (w/v)

Coomassie blue. Molecular weights of unknowns were established by comparison with the relative mobilities of standard proteins as described by Weber and Osborn [12]. The standards used (molecular weights in parentheses) were phosphorylase *A* (92 500), bovine serum albumin (68 000), human gamma globulin heavy chain (55 000), and ovalbumin (43 000).

Sucrose density gradient analysis. Enzyme-containing solution (250 μl) was layered on 13.5 ml preformed gradients consisting of 5—20% (w/v) sucrose in 0.1 M potassium phosphate, pH 7.6/1.0 M NaCl/0.1% Triton X-100 and centrifuged in an SW40 rotor at 40 000 rev./min for 18 h at 4°C. Enzyme activity was measured in aliquots of the fractionated gradients by the standard radiometric assay. Catalase, included as a size marker, was detected by measuring absorbance at 410 nm.

Immunological procedures. Acetylcholinesterase purified by affinity chromatography was used as the immunogen. Approx. 11 units (15 μg protein) were emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into three New Zealand white female rabbits; 2 weeks later, the procedure was repeated. 1 month after the initial injection, rabbits were bled, and antibody to acetylcholinesterase was found in the three animals. Antibody titer was increased and maintained at higher levels by repeated injections with antigen in Freund's incomplete adjuvant. All the experiments reported in this communication were done with antibody obtained from a single rabbit bled 7 weeks after the initial injection.

Before testing sera for anti-acetylcholinesterase activity, it was necessary to remove the serum cholinesterases that interfered with the assay. This was done by purifying the IgG-fraction on DE-52 cellulose (Whatman) at pH 6.3 as described by Levy and Sober [13]. Antibody activity was detected by incubation of this preparation with crude larval acetylcholinesterase in 0.145 M NaCl/0.05 M Tris-HCl pH 7.2/0.5% Triton X-100 for 1 h at room temperature. Enough *Staphylococcus aureus* protein A [14] coupled to Sepharose 4B-CL (Pharmacia) to bind all of the rabbit IgG was then added and the incubation continued for 2 h at 4°C while stirring intermittently with a Vortex mixer. The complexes were collected by centrifugation and the enzyme activity in the supernatant was measured.

Results

Purification of acetylcholinesterase

Concanavalin A-Sepharose chromatography, previously used in the purification of acetylcholinesterase from a variety of sources [15,16], served as the initial step in isolating the *Ciona* enzyme. Enzyme in the 20 000 × *g* supernatant of crude embryonic homogenates was adsorbed to concanavalin A-Sepharose, then eluted with α-methyl-(+)-mannoside. This procedure yielded a 3- to 5-fold purification and a recovery of 55—70% of the original activity. In addition to achieving a partial purification, the larval acetylcholinesterase was shown to be a glycoprotein.

Three affinity columns, employing two different competitive inhibitors of acetylcholinesterase as ligands, were used to further purify the enzyme (Table I). The inhibitor MAP, attached to Affi-Gel 202 (MAP-Affi-Gel 202) has

TABLE I

PURIFICATION OF ACETYLCHOLINESTERASE

Con A-Sepharose, concanavalin A-Sepharose.

Purification	Total (mg)	Total (Units)	Specific (Units/mg)	Purification	Yield
Crude homogenate	2130	220	0.103	1.00	100
20 000 × *g* supernatant	2025	217	0.107	1.04	99
Con A-Sepharose	252	121	0.480	4.66	55
mCPA-Sepharose	0.090	66	733	7120	30

been used to purify acetylcholinesterase from several sources [17,18], however, attempts to purify *Ciona* acetylcholinesterase using this column were unsuccessful. Since the presence of hydrophobic regions in the column spacer arm were necessary to purify eel acetylcholinesterase when employing certain ligands [10], a column was prepared with a sidearm that contained two hydrophobic regions of six carbons each and MAP as a ligand (MAP-Sepharose). Enzyme applied to this column in buffer containing 0.1 M NaCl was retained and could be displaced with decamethonium bromide. This resulted in significant purification, however, SDS-polyacrylamide gel analysis revealed that this preparation contained a number of proteins. Attempts to remove nonspecifically-bound protein from this column by increasing the ionic strength were unsuccessful, as this resulted in the elution of acetylcholinesterase. This finding suggests that hydrophobic interactions were at least partly responsible for enzyme binding.

A third column, also with a hydrophobic spacer arm, but employing mCPA as a ligand (mCPA-Sepharose) was prepared. Enzyme applied in buffer of moderate ionic strength (0.2 M NaCl) was retained by this column and remained bound during extensive (20—25 column vols.) washing with buffer containing 0.4 M NaCl (Fig. 4). In four separate experiments, 70—80% of the applied activity remained bound to the column during the entire sample application and washing procedure. Displacement of enzyme from the column, with decamethonium bromide, was carried out in a buffer of lower ionic strength (0.3 M NaCl) than the washing medium, in an attempt to maximize the specificity of elution. This method resulted in the recovery of 50—65% of the applied activity and a single-step purification of 1400—1800-fold.

The sequential use of concanavalin A-Sepharose and mCPA-Sepharose affinity chromatography resulted in a 5000—7000-fold purification with recoveries ranging from 30—39% of the original activity and enzyme-specific activities of 580—733 units/mg protein. The results of a single purification run using these procedures are shown in Table I. All preparations of purified enzyme described in this report were isolated in this manner.

Analysis of purified acetylcholinesterase

Affinity-purified acetylcholinesterase was analyzed by SDS-polyacrylamide gel electrophoresis after complete reduction with β-mercaptoethanol. Staining of the proteins with Coomassie blue revealed a single major polypeptide with a

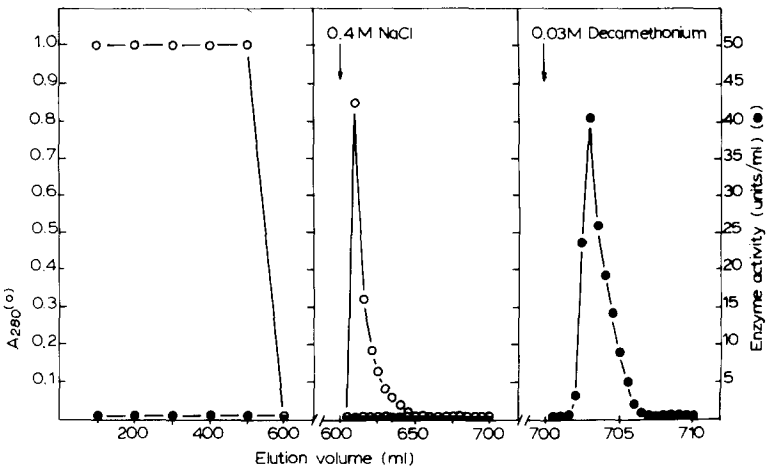

Fig. 1. Affinity chromatography of acetylcholinesterase on mCPA-Sepharose. Enzyme purified by concanavalin A-Sepharose chromatography was dialyzed against buffer containing 0.2 M NaCl and applied to a 5 ml column of mCPA-Sepharose at a rate of 18 ml/h. In this experiment, the sample vol. was 550 ml and 100-ml fractions were collected. After application, the column was washed with 100 ml of buffer containing 0.4 M NaCl (5 ml fractions) and the enzyme was eluted in 0.3 M NaCl buffer with 30 mM decamethonium bromide (0.5 ml fractions). Of the 120 units applied, approx. 20 were not bound, 20 were eluted during the washing procedure and 67 were eluted with the inhibitor.

molecular weight of approx. 65 000—70 000 (Fig. 2) and a few minor bands. Because the serine at the active site of acetylcholinesterase can be irreversibly labeled with [^{3}H]Dip-F^3 [19], it was possible to examine the distribution of the polypeptide containing the active site even after complete reduction and denaturation [19]. When this was done, a single peak of radioactivity with the same mobility as the major Coomassie blue-stained polypeptide was seen (Fig. 3). These results demonstrate that the purified enzyme has a single catalytic subunit with a molecular weight of 65 000—70 000.

The distribution of the forms of embryonic acetylcholinesterase occurring in crude homogenates was compared with those found in the purified preparation by sucrose gradient analysis. All three forms present in the crude homogenate (Fig. 4b) were also found in the purified sample (Fig. 4a). Although quantitative differences were noted, these results indicate that no qualitative selection occurred during the isolation procedure. Comparison with the catalase marker (arrow) suggests an approx. molecular weight of 240 000—250 000 for the largest form. It is possible that this represents a tetramer of the 65 000-dalton subunit, with the smaller forms being the dimer and monomer. More detailed studies are necessary to confirm these speculations.

Production and detection of anti-acetylcholinesterase antibody

An indirect immunoprecipitation procedure was used to test for the presence of anti-acetylcholinesterase activity in the sera of rabbits immunized with affinity-purified enzyme (Materials and Methods). In this assay, the amount of enzyme remaining in the supernatant is expected to decrease in the presence of anti-acetylcholinesterase antibody. The level of enzyme activity detected in the supernatant was found to be inversely related to the amount of IgG from the

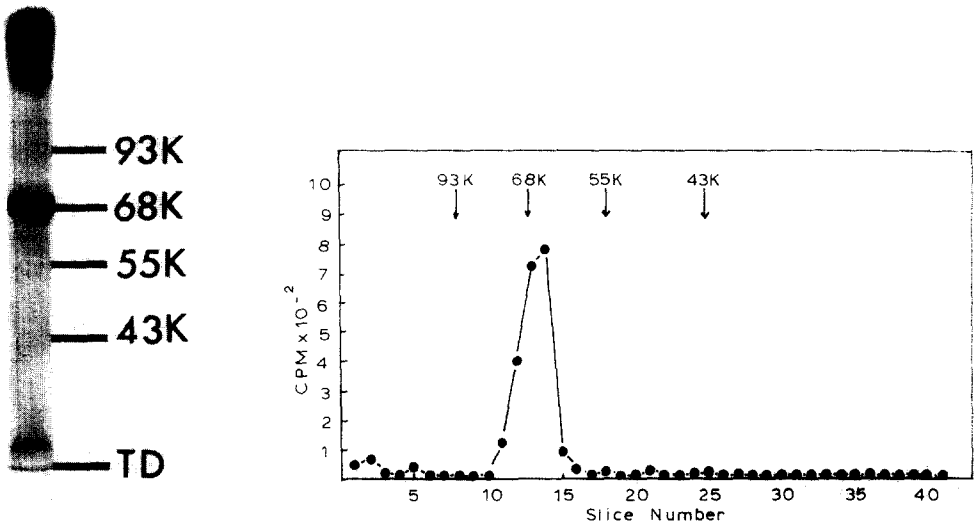

Fig. 2. SDS-polyacrylamide gel analysis of mCPA-Sepharose-purified acetylcholinesterase. Purified enzyme was electrophoresed on SDS-polyacrylamide gels, and the distribution of Coomassie blue-stained material was obtained as described in Materials and Methods. Positions of the molecular weight standards were established by their mobilities in parallel gels. TD designates the position of the Bromophenol blue tracking dye.

Fig. 3. SDS-polyacrylamide gel analysis of [^{3}H]Dip-F^3-labeled acetylcholinesterase. Purified acetylcholinesterase was labeled with [^{3}H]Dip-F^3 and analyzed on SDS-polyacrylamide gels. The positions of the molecular weight standards were established by their mobilities in parallel gels. Only the separating gel is shown; the stacking gel contained no radioactivity.

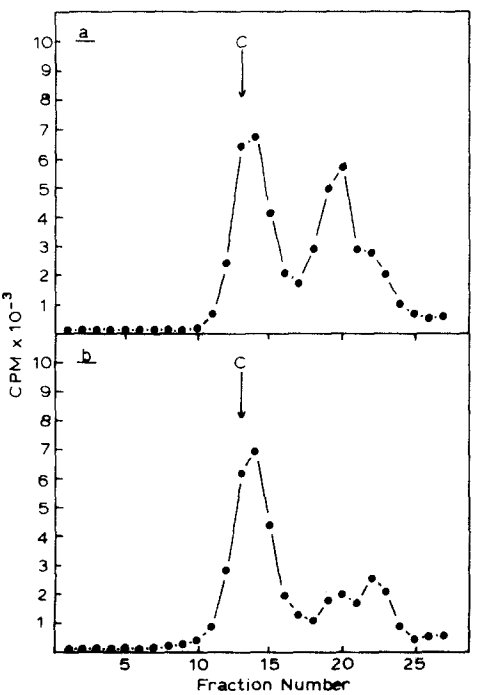

Fig. 4. Sucrose density gradient analysis of acetylcholinesterase. Enzyme distribution on sucrose gradients was determined by radiometric assay. a. mCPA-Sepharose-purified enzyme. b. Crude enzyme from the 20 000 X *g* supernatant fraction of 30-h embryo homogenates. Sedimentation was from right to left. The arrows designate the position of catalase.

immune sera added, demonstrating the presence of anti-acetylcholinesterase antibodies. It should also be mentioned that although antiserum was prepared to purified acetylcholinesterase, it precipitated essentially all (greater than 98%) of the enzyme in crude homogenates. No enzyme was removed from supernatants when IgG obtained from rabbits, before immunization, was used.

The frequency of successful immunization (all three rabbits injected produced antibody) and the relatively small amount of enzyme used (approx. 15 μg/injection) indicated that the purified enzyme was highly antigenic, at least in rabbits. Interestingly, antibody raised to the enzyme from *E. electricus* did not cross-react with that from *Ciona*, and no antibody was produced (at least to active enzyme) when an affinity purified preparation extracted from SDS-containing gels was used as an immunogen.

Discussion

A simple, two-step affinity chromatography method has been developed for the purification of acetylcholinesterase from larvae of *C. intestinalis*. The initial step of concanavalin A-Sepharose chromatography yielded a purification (3—5-fold) that was somewhat less than reported for other acetylcholinesterase purified by this technique [15,16]. Nonetheless, this step was important because the efficiency of the second affinity column was greatly reduced when it was omitted. Of the three affinity columns prepared from competitive inhibitors of acetylcholinesterase, two (MAP-Sepharose and mCPA-Sepharose) provided an extensive purification, but only mCPA-Sepharose yielded enzyme that was significantly free of contaminating protein. This difference may be accounted for by the salt concentrations at which the two columns could be run while still retaining the enzyme (0.4 M, mCPA-Sepharose; 0.1 M, MAP-Sepharose), since at higher concentrations, nonspecific interactions should be reduced.

The affinity-purified product was characterized by SDS-polyacrylamide gel electrophoresis after complete reduction. Protein staining with Coomassie blue revealed a single major polypeptide with a molecular weight of 65 000—70 000 that was identified as acetylcholinesterase by its coelectrophoresis with [^{3}H]-Dip-F^3-labeled enzyme. This value was less than the 75 000—90 000 calculated for the catalytic subunit of acetylcholinesterase from a variety of sources [16, 17,19—23]. Purified *Ciona* enzyme had a specific activity of 580—730 units/mg protein, a value comparable to that reported for acetylcholinesterase of the house-fly head [16,24] and 5—10-times less than that for the enzyme from the electric organ of fish [18,19,25,26].

Three molecular species were found by sucrose density gradient analysis of the crude enzyme. The largest sedimented at about 11 S and may correspond to the globular (G or 11 S) form, composed of four catalytic subunits, present in the electric organ of fishes [19,21,25,27]. This form is derived by proteolysis or autolysis of much larger asymmetric species that are composed of various numbers of tetramers linked to a 'collagen-like' tail [19,22,23,28]. Since the *Ciona* enzyme was extracted under conditions that limit such degradation, it seems likely that such asymmetric forms were absent from larvae. If this is true, the three species of enzyme most probably represent monomers, dimers, and tetramers of the catalytic subunit. However, the possi-

bility of another polypeptide(s) that serves to 'glue' together catalytic subunits can not be excluded. In its sedimentation properties, the ascidian acetylcholinesterase resembles that from sea urchin embryos that was shown to have two enzyme species [7.6 S and 10.6 S Ref. 29]. Although these were described as a monomer and dimer with molecular weights of 190 000 and 380 000, respectively, it is possible that they represent the dimer and tetramer of a smaller, undetected subunit. This interpretation would be more consistent with the size determined for the catalytic subunit of acetylcholinesterase from most sources.

Sucrose density gradient analysis of purified enzyme also revealed the three molecular forms in crude homogenates, suggesting that no qualitative selection occurred during the isolation procedure. If this is true, and since the purified enzyme has only the single M_r 65 000—70 000 catalytic subunit, all of the larval acetylcholinesterase must also be made up of this basic subunit. This hypothesis was supported by studies with antibody raised against affinity-purified acetylcholinesterase showing that IgG from immunized rabbits precipitated essentially all (greater than 98%) of the enzyme in crude homogenates.

The findings presented in this communication provide a basis for further study of the expression and properties of ascidian larval acetylcholinesterase. The demonstration that the catalytic subunit of this enzyme was composed of a single polypeptide should greatly facilitate such work, particularly in assaying the level of translatable acetylcholinesterase mRNA by in vitro protein synthesis. In addition, the highly purified enzyme and anti-acetylcholinesterase antibody obtained will be indispensible in developing a radioimmunoassay, as well as in analyzing protein products synthesized in vitro.

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