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ACETYLCHOLINESTERASE (EC 3.1.1.7), A NEUROTRANSMITTER ENZYME IN SCORPION HEMOLYMPH

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Abstract—Acetylcholinesterase (AchE: EC 3.1.1.7) was identified and purified from the hemolymph of the scorpion *Heterometrus bengalensis*. The purity of the enzyme was determined by polyacrylamide gel electrophoresis (PAGE). The molecular weight of the enzyme, determined by sodium dodecyl sulfate-PAGE, was 80,000. The purified AchE hydrolysed acetylthiocholine iodide, but it did not react with butyrylthiocholine iodide. BW284C51, a specific inhibitor of AchE, strongly inhibited the enzyme. The known inhibitor (tetramonoisopropylpyrophosphortetramide) of pseudocholinesterase did not produce any inhibition of the enzyme activity. The purified AchE of scorpion hemolymph was vulnerable to high substrate concentration. The presence of Cu^{2+} and Ni^{2+} reduced the enzyme activity, whereas the metal ion, Sn^{2+}, enhanced AchE activity. Ca^{2+} produced neither inhibition nor activation. (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activities were greatly enhanced by the purified AchE.

Acetylcholinesterase (AchE; EC 3.1.1.7) plays a central role in neurotransmission. The regulation of its activity by various effectors has been studied extensively [1, 2]. The primary role of AchE in the cholinergic system is to hydrolyse the neurotransmitter, acetylcholine, at the post-synaptic junction. AchE is associated with membranes in all vertebrate tissues. Human serum contains a large excess of pseudocholinesterase (PseudochE, EC 3.1.1.8) but a negligible amount of AchE [1]. AchE has been found to occur freely in the hemolymph of the marine invertebrate *Aplysia* [3]. The AchE of *Aplysia* hemolymp is unusual, since it is a large enzyme complex that is not associated with membranes under physiological conditions. We report here the identification, purification and partial characterization of an AchE from the hemolymph of *Heterometrus bengalensis*.

MATERIALS AND METHODS

Acetylthiocholine iodide (AcThchI), butyrylthiocholine iodide (BuThchI), dithiobis nitrobenzoic acid (DTNB), choline chloride, alkaline phosphatase (*Escherichia coli*) (mol. wt 80,000), MW-SDS-70 molecular weight marker kit, adenosine triphosphate (ATP), pyridine-2-aldoxime methiodide, diisopropyl fluorophosphate (DFP), eserine, and tetramonoisopropylpyrophosphortetramide (ISO-OMPA) were from the Sigma Chemical Co., St. Louis, MO, U.S.A. Thymolphthaline was from BDH, Poole, England. Ammonium reineckate and ammonium molybdate were from E. Merck, Darmstadt, West Germany. 1,5-Bis (4-allyldimethylammoniumphenyl)-pentan-3-one-dibromide (BW 284C51) was from the Burrough's Wellcome Co., Kent, England. Biogel P-300 was from Bio-Rad, Richmond, CA, U.S.A. PBE-94 was from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade.

Animals and collection of hemolymph

The scorpions, *H. bengalensis*, were supplied by M/s Nazir Ali, licensed dealer of reptiles, Baruipur, 24 Parganas, West Bengal, India. The identification of the animal was facilitated by the Zoological Survey of India. Bleeding of the animals was done by amputation of the legs. An average of 0.5 ml of hemolymph per animal was collected in the cold under aseptic conditions. Pooled hemolymph was centrifuged at 500 *g* for 10 min at 10° in the Sorvall RC5B high speed centrifuge to remove the cells and other materials. The supernatant fraction thus obtained was dialysed overnight in the cold against 50 mM Tris–HCl buffer containing 100 mM NaCl, 10 mM $CaCl_2$ and 10 mM $MgCl_2$, pH 7.2.

Purification of acetylcholinesterase

The purification of AchE from the hemolymph was carried out in the following steps. All operations were performed between 0 and 10°.

Step. 1. The dialysed hemolymph (16,250 mg protein) was centrifuged at 120,000 *g* for 2 hr at 4° using a Sorval OTD 50B ultracentrifuge. This resulted in a clear supernatant fraction and a thick pellet. AchE activity was found in the pellet.

Step 2. The pellet was suspended in 50 mM Tris–HCl buffer, pH 7.2, containing 100 mM NaCl. The dissolved pellet solution was made 0–60% with respect to $(NH_4)_2SO_4$ saturation by the gradual addition of $(NH_4)_2SO_4$ and kept overnight in the cold. It was then centrifuged at 27,000 *g* for 60 min. The pellet obtained after centrifugation was resus-

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pended in 50 mM Tris–HCl buffer, pH 7.2, containing 100 mM NaCl. The clear supernatant fraction contained most of the enzyme activity.

Step 3. The gel filtration of the most active fraction was done on a Biogel P-300 (1.5 × 80 cm) column). The elution was carried out with 50 mM Tris–HCl buffer, pH 7.2, containing 100 mM NaCl. The fractions showing enzyme activity were pooled and concentrated respectively.

Step 4. The respective concentrated active fractions from step 3 were dialysed against 0.025 M ethanolamine acetic acid buffer, pH 9.4, with several changes. The fraction with the highest enzyme activity was then applied on a chromatofocusing column of PBE-94 for further purification. Prior to the application of the sample, the PBE-94 column of 25 ml packed volume was washed repeatedly and equilibrated with 0.025 M ethanolamine acetic acid buffer, pH 9.4. The active fractions were eluted from the column with a pH gradient of 9–6 using 0.025 M sodium acetate–acetic acid buffer, pH 6.0, at a flow rate of 5 ml/hr. The proteins, eluted at pH 6.4 from the column, possessed all the activity.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of the purified AchE was performed at pH 8.3 in 7.5% gel [4]. The protein staining was done with Coomassie Brilliant Blue. The molecular weight of the sodium dodecyl sulfate (SDS) treated enzyme in the presence of 2-mercaptoethanol was determined [5] using alkaline phosphatase (*E. coli*) (mol. wt 80,000), and an MW-SDS-70 molecular weight kit as a standard marker.

Estimation of protein

Protein was determined by the method of Lowry *et al.* [6], using bovine serum albumin as standard. The column eluents were monitored spectrophotometrically at 280 nm.

Estimation of cholinesterase activity

Cholinesterase activity was assayed according to the method of Ellman *et al.* [7]. The reaction mixture contained 3 ml of 0.1 M phosphate buffer (pH 8) 20 μl of 0.075 M substrate (AcThchI or BuThchI), 100 μl of 0.01 M DTNB and 50 μl of the enzyme protein (1.5 mg/ml); it was incubated for 30 min at 37°. The enzyme activity was calculated in units of μmol substrate hydrolysed per min. The inhibitors were incorporated into the reaction mixture and preincubated for 15 min before the substrate addition.

Estimation of ATPase activity

Rabbit erythrocyte membrane was prepared as the crude source of ATPase enzyme [8]. The incubation mixtures for (Na^+, K^+)-ATPase and for Mg^{2+}-ATPase were prepared according to the method of Bloj *et al.* [9]; the reaction mixture for Ca^{2+}-ATPase was made according to the method of Macleannan [10]. The inorganic phosphate was estimated by the method of Fiske and Subbarow [11]. The radiolabelled phosphate [^{32}P] was estimated [10] using [γ-^{32}P]ATP and 10 mM histidine–HCl buffer (pH 7.5) instead of ATP and Tris–HCl buffer (pH 8.0) respectively.

Choline extraction and estimation

Choline was extracted from the hemolymph [12], and the choline content was measured according to the method of Dittmer and Wells [13].

RESULTS AND DISCUSSION

Table 1 presents the purification of AchE with concomitant increases in specific activity using AcThchI as substrate; BuThchI was not hydrolysed by the enzyme. The pellet obtained after ultracentrifugation yielded 90% recovery of the initial activity, with 1.4-fold purification. In the second stage of purification, the supernatant of the 0–60% ammonium sulfate fraction showed about 5.4-fold purification. Gel filtration of this active supernatant fraction on a Bio-gel P-300 column yielded six fractions. AchE activities were observed in P_1, P_2 and P_4 fractions. The activity in fraction P_2 was higher than that in P_1 and P_4. Further resolution of fraction P_2 by chromatofocusing gave several peaks (Fig. 1). The peak eluted at pH 6.4 was the active peak, with 146-fold purification. PAGE of the active peak

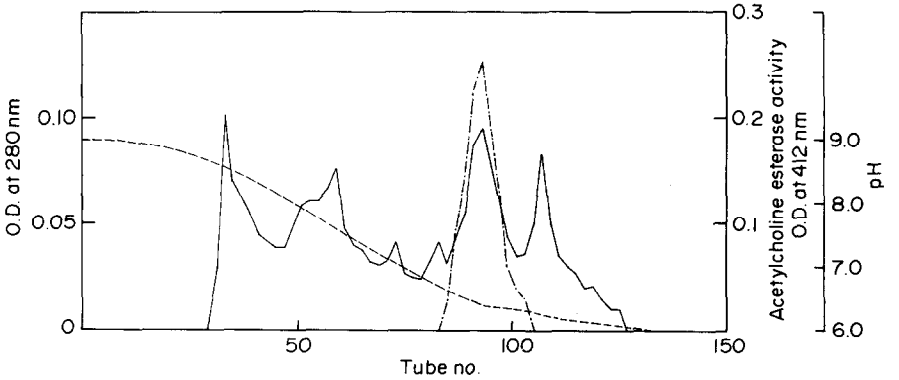

Fig. 1. Elution profile of acetylcholinesterase by chromatofocusing. Flow rate 5 ml/hr; 1.5 ml/tube. Amount of protein applied: 5 mg. Key: (----) pH gradient; (——) 280 reading; and (—·—·—·—) activity.

Table 1. Purification of hemolymph acetylcholinesterase

Purification steps	Total protein (mg)	Total activity (Units)		Specific activity (units/mg protein)	Purification fold
		AcThchI	BuThchI		
Crude hemolymph	16,250	373.8	Nil	0.02	1
Step 1*					
(a) Supernatant	3,995	Negligible	Nil		
(b) Pellet	10,976	340.2	Nil	0.03	1.4
Step 2†					
(a) Supernatant	1,869	230	Nil	0.12	5.4
(b) Precipitate	9,001	90	Nil	0.01	
Step 3‡					
Fraction P_1	400	45	Nil	0.11	
Fraction P_2	195	140	Nil	0.72	31
Fraction P_3	210	Nil	Nil		
Fraction P_4	305	30.2	Nil	0.10	
Fraction P_5	345	Nil	Nil		
Fraction P_6	355	Nil	Nil		
Step 4§	36	119.5	Nil	3.37	146

* Step 1: ultracentrifugation.
† Step 2: 0–60% $(NH_4)_2SO_4$ cut of dissolved pellet of step 1.
‡ Step 3: Bio-Gel P-300 column chromatography of Step 2.
§ Step 4: chromatofocusing of fraction P_2.

showed a single band which indicated homogeneity of the protein. SDS-PAGE showed it to be a single protomer. The molecular weight determined by SDS-PAGE, was 82,000 daltons for the marker proteins. The kinetic characterization of the enzyme showed that the V_{max} and the K_m for the purified AchE using AcThchI as substrate were 439 μmol/hr/mg protein and 1.29×10^{-4} M respectively (Fig. 2). A clear preference for AcThchI over BuThchI satisfied one of the most important criteria for AchE as a purified enzyme preparation from scorpion hemolymph. True acetylcholinesterase hydrolyses acetylcholine faster than butyrylcholine [1, 14, 15]. AchE activity was inhibited at high substrate concentration and was most active against a low concentration of AcThchI (Table 2). An important characteristic of AchE is its vulnerability to inhibition in the presence of excess substrate [1, 15]. The

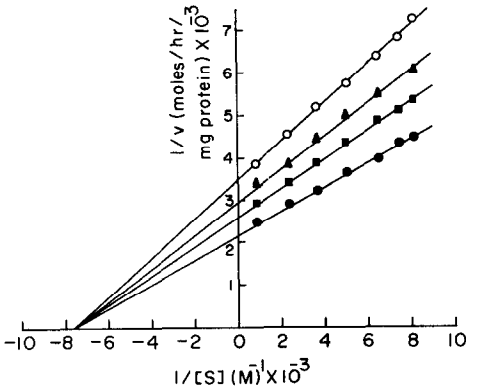

Fig. 2. Non-competitive inhibition of AchE. Key: (●—●) acetylthiocholine iodide; and (○—○) 1×10^{-3} M, (▲—▲) 1×10^{-4} M, and (■—■) 1×10^{-5} M BW284C51.

Table 2. Effects of substrate concentration, inhibitors and metal ions on acetylcholinesterase activity

A. Substrate (AcThchI) concentration, 0.075 M		Activity of purified enzyme
20 μl		0.362
40 μl		0.252
100 μl		0.098
B. Inhibitor	**Final concn (M)**	**% activity**
Control*		100 (0.369)†
+ Eserine	1×10^{-3}	15
+ DFP	1×10^{-3}	Nil
	1×10^{-4}	40
	1×10^{-5}	80
	1×10^{-6}	90
	1×10^{-7}	100
+ ISO-OMPA	1×10^{-3}	100
+ BW284C51	1×10^{-3}	Nil
	1×10^{-4}	Nil
	1×10^{-5}	29
C. Metal ions	**Final concn (mM)**	**% activity**
Control*		100 (0.369)
Na^+	10	130
K^+	10	125
Cd^{2+}	10	132
Ca^{2+}	10	100
Sn^{2+}	10	170
Mg^{2+}	10	120
Mn^{2+}	10	98
Zn^{2+}	10	74
Cu^{2+}	10	50
Ni^{2+}	10	55

* Control: as given in the text.
† Figure in parentheses indicates the O.D. value.

Table 3. ATPase activity in the presence of acetylcholinesterase (AchE)

Systems	ATPase activity	
	O.D.	cpm
Control A* (Ca^{2+}-ATPase)	0.329 ± 0.02	8.260 ± 150
Control A + AchE	0.375 ± 0.01	9,500 ± 80
Control B† (Mg^{2+}-ATPase)	0.275 ± 0.01	7,920 ± 110
Control B + AchE	0.446 ± 0.01	11,300 ± 210
Control C‡ [(Na^+, K^+)-ATPase]	0.345 ± 0.01	8,670 ± 75
Control C + AchE	0.472 ± 0.02	12,200 ± 250

AchE in each case was 50 μl of 1.5 mg protein/ml. Results are means ± SEM of six experiments.

* Control A: 25 mM Tris–HCl buffer, pH 8.0; 1 mM ATP; 50 μg enzyme protein; and 45 mM $CaCl_2$.

† Control B: 25 mM Tris–HCl buffer, pH. 8.0; 1 mM ATP; 50 μg enzyme protein; 33 mM KCl; 80 mM NaCl; 8 mM $MgCl_2$; and 10 mM ouabain.

‡ Control C: a§-Control B.

§ 25 mM Tris–HCl buffer pH 8.0; 1 mM ATP; 50 μg enzyme protein; 33 mM KCl; 80 mM NaCl; and 8 mM $MgCl_2$.

enzyme activity was inhibited by eserine, DFP and BW284C51, whereas ISO-OMPA had no inhibitory effect on the purified AchE (Table 2). DFP at the highest concentration produced a complete loss of enzyme activity but at lower concentrations the AchE activity remained unchanged. Differential inhibitors like DFP affect both types of enzyme but AchE requires a higher concentration of DFP to be effectively inhibited than does PseudochE [16]. The inhibition of AchE by BW284C51 was non-competitive (Fig. 2). BW284C51 is regarded as a specific inhibitor of AchE [17]. ISO-OMPA, in contrast, selectively inhibits PseudochE [18]. The results obtained with these compounds confirmed that the enzyme obtained from the scorpion hemolymph was AchE. Hemolymph AchE was strongly inhibited by Cu^{2+} and Ni^{2+}, respectively, whereas Sn^{2+} acted as a powerful activator of the enzyme activity. No effect was elicited by Ca^{2+} (Table 2). (Na^+, K^+) ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase activities of rabbit erythrocyte membrane were measured in the presence of AchE (Table 3). (Na^+, K^+)-ATPase and Mg^{2+}-ATPase activities were greatly enhanced by AchE. Ca^{2+}-ATPase activity did not show any marked increase in the presence of the enzyme. Maheswari *et al.* [19] obtained some evidence earlier that there may be an association between ATPase and AchE.

In conclusion, the three major criteria, viz. substrate specificity, proclivity for inhibition by substrate, and selective sensitivity to specific inhibitors, confirmed the existence of true cholinesterase (EC 3.1.1.7) in scorpion hemolymph. There are numerous aspects of neural control of arachnid muscle, and no neurotransmitter has been identified for any arachnid neuromuscular system [20]. The regulatory role of AchE in neurotransmission has been identified in almost all phyla. However, the presence and action of AchE in the hemolymph where it does not behave as a neurotransmitter are interesting.

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