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NEURAL AND CIRCULATING CHOLINESTERASES OF THE MARINE MOLLUSK *APLYSIA CALIFORNICA*

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Gastropod mollusk *Aplysia californica* has significant levels of cholinesterase in its circulation. Hemolymph (blood) cholinesterase of *Aplysia* has both cholinergic and non-cholinergic functions, which include promoting neurite growth. In order to understand the structure-function relationship of hemolymph cholinesterase, we purified and characterized this enzyme and compared it to the cholinesterase present in the ganglia of the central nervous system of *Aplysia*. Our results show that the cholinesterases from the CNS and hemolymph of *Aplysia* are acetylcholinesterases, which differ from that of the vertebrates in their unique heavy molecular form. The enzyme from the CNS differs from that of the hemolymph in its rate of hydrolysis, substrate preference profiles and molecular forms, suggesting the presence of two kinds of cholinesterases in *Aplysia*.

The enzyme acetylcholinesterase (AChE) which catalyzes the hydrolysis of acetylcholine (ACh) is ubiquitous in the animal kingdom [1]. It is a well-characterized enzyme in the vertebrates due to its critical catalytic function at the cholinergic synapses. AChE is also present in blood, cerebrospinal fluid and non-cholinergic cells and the significance of its presence at these sites is poorly understood.

In *Aplysia,* a marine gastropod, it is present in the gill [2], in the blood (hemolymph) and in the cholinergic as well as non-cholinergic neurons in the central ganglia of the nervous system [3, 4]. AChE activity in the hemolymph of *Aplysia* is of a greater magnitude than in other tissues and its activity levels change with age [4]. AChE from the CNS and the hemolymph of *Aplysia* has been described before [3, 5]. A high level of AChE activity in the hemolymph and its cholinergic and non-cholinergic effects on neurons [6–8] prompted us to further characterize this enzyme from these two sources as an initial step towards understanding the structure-function relationship of this molecule.

We purified the AChE in the hemolymph by affinity column chromatography and extracted the enzyme from the central ganglia of *Aplysia* sequentially using low salt, detergent and high salt buffers. Analysis of the properties of the enzyme from the two sources show that the cholinesterase from the CNS and hemolymph of *Aplysia* is true acetylcholinesterase . However the enzyme from the CNS differs from that of the hemolymph in its rate of hydrolysis and substrate preference profiles. The details on the methods and the results are reported here.

METHODS

Enzyme preparation for assays. Hemolymph and the central ganglia (CNS) of sexually mature adult *Aplysia* supplied by Marinus (Long Beach, CA) were used in this study. Hemolymph was drawn from the hemocoel using sterile needle and syringe and was centrifuged filtered through Filtron microsep tubes (membrane molecular weight cut off was at 100 kd). The concentrated hemolymph was used to purify AChE. Epoxy-activated Sepharose 6B (Pharmacia) was coupled to 0.2 M edrophonium chloride (Sigma) following the procedure described by Hodgson and Chubb [9]. Hemolymph was mixed with the gel at a ratio of 10:1 and packed carefully in a Bio Rad Econo-column (1.0×20 cm) avoiding air being trapped. The column was washed with 10 column volumes each of 50 mM sodium phosphate buffer, pH 8.0, followed by 50 mM sodium phosphate containing 0.5M sodium chloride. AChE was eluted with 12 mM of edrophonium chloride in phosphate buffer (50 mM, pH 8.0) containing 0.5 M sodium chloride. The eluted AChE was concentrated and desalted using Amicon Centricon centrifugal filter devices (MW 10,000) and Sephadex J PD-10 (Pharmacia) columns.

For isolating the ganglia, the animals were anesthetized by injecting isotonic $MgCl_2$ the volume of which was 1/3 of the animal's body weight (vol/wt). They were perfused with several volumes of cold , filtered artificial sea water (ASW) directly through the heart to remove traces of hemolymph. The ganglia were dissected out, rinsed in several changes of ASW and were homogenized as follows: CNS from twenty animals were homogenized in 0.01M Tris-HCl, pH 7.4 containing 1M NaCl and 0.5% Triton X- 100. This homogenate was centrifuged at 30.000 rpm for 30 minutes and the supernatant was used for enzyme-substrate and enzyme-inhibitor interactions. CNS from another group of six *Aplysia* were homogenized sequentially in low salt buffer (LSS), low salt buffer containing detergent Triton X-100 (DSS) and high salt buffer containing Triton X-100 (HSS) as described by Bon and Massoulie [10]. Each time the homogenate was centrifuged as before and the supernatant was used to determine the solubility

characteristics of *Aplysia* cholinesterase. Protein concentrations of purified AChE from hemolymph and CNS homogenate were determined using Pierce BCA / micro BCA protein assay reagents (Pierce, Rockford, III).

Enzyme activity measurement. Cholinesterase activity was assayed by the spectrophotometric procedure of Ellman et al. [11]. Acetyl, butyryl and propionyl thiocholine iodides (Sigma) were used as substrates and BW284c51 (1,5-bis(4 -allyldimethyl ammoniumphenyl)-pentan 3-one dibromide, *Sigma*), *iso*-OMPA (*Sigma*) and ethopropazine (*Sigma*) were used as inhibitors.

Density gradient centrifugation. Hemolymph and CNS homogenate were layered on separate 5–20% sucrose gradients in a high salt buffer (NaCl, 1M; $MgCl_2$, 0.05M; Tris-HCl, 0.01M, pH 7.4) and centrifuged with a Beckman TLS55 rotor at 45,000 rpm, for 16 hours at 4°C. At the end of the run, fractions were collected by puncturing the bottom of the tubes. Enzyme activity was assayed in these. The standards used were *E. Coli* β- galactosidase (16S) and beef liver catalase (11.4S).

All values given are averages of three or more repeats.

RESULTS AND DISCUSSION

Substrate specificity. The hemolymph cholinesterase as well as the enzyme in the CNS homogenate showed a preference for acetylcholine over butyryl or propionylcholine as their substrate. As Fig. 1 shows when assayed for cholinesterase activity using 1mM of acetyl, butyryl and propionyl thiocholine iodide, the ratio of the rates of hydrolysis of these substrates by CNS enzyme was 178:26:19. Thus CNS cholinesterase hydrolyzed acetylthiocholine iodide 7 times faster than butyrylcholine and 9 times faster than propionyl choline and hence is acetylcholinesterase. However this enzyme also exhibited a modest rate of hydrolysis with increasing concentrations of butyrylcholine suggesting that the CNS enzyme may have a mixture of or properties of both acetyl and butyrylcholinesterases. In a similar assay of hemolymph cholinesterase, the ratio of the rates of hydrolysis of these respective substrates was 619:26:7. Hemolymph cholinesterase hydrolyzed acetylthiocholine iodide 24 times faster than butyrylcholine and 88 times faster than propionylcholine and hence is acetylcholinesterase (Fig. 1). Though the enzyme from hemolymph as well as from CNS were acetylcholinesterases, they differed in their rates of hydrolysis of acetylcholine since hemolymph acetylcholinesterase had a preference for acetylcholine 3.5 times greater than CNS acetylcholinesterase.

Inhibition profile of hemolymph and CNS cholinesterases of Aplysia. Aliquots of CNS homogenate and the enzyme purified from hemolymph were pre-incubated with increasing concentrations of (*a*) BW284c51 which inhibits vertebrate acetylcholinesterase at μM concentration, (*b*) ethopropazine and (*c*) *iso*-OMPA , both of which inhibit pseudocholinesterase at μM concentrations in vertebrates

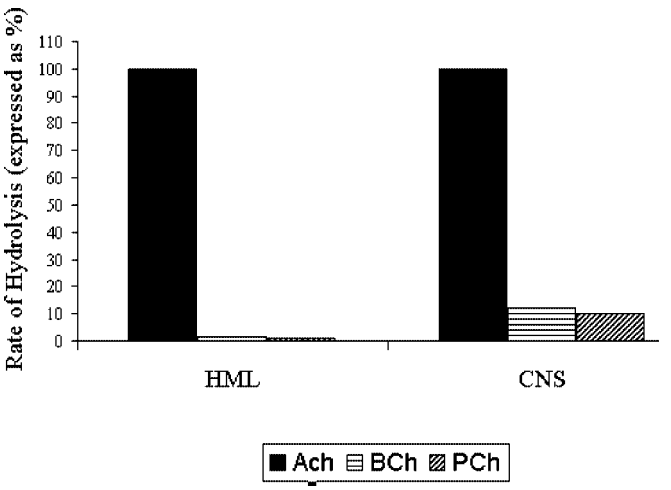

Fig. 1. *Aplysia* CNS homogenate and purified enzyme from hemolymph were assayed for cholinesterase activity using 1 mM of acetyl, butyryl and propionyl thiocholine iodide. The rate of hydrolysis is expressed setting the hydrolysis of acetylcholine as 100%. The two enzymes clearly showed a preference for acetylcholine as substrate

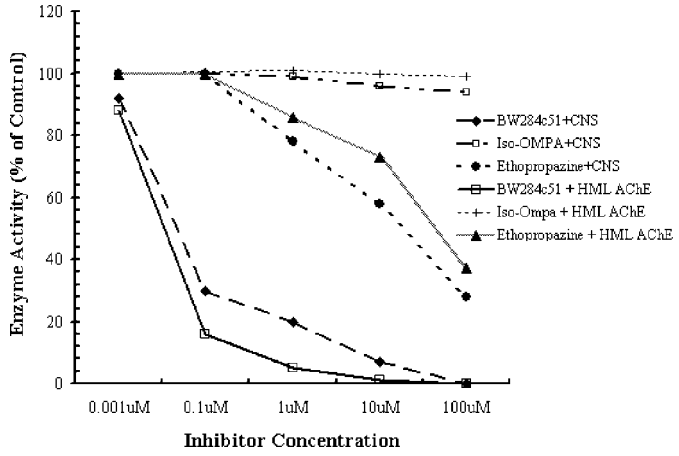

Fig. 2. Aliquots of CNS homogenate and purified enzyme from hemolymph were pre-incubated with increasing concentrations of (a) BW284c51 which inhibits vertebrate acetylcholinesterase at μM concentration, (b) ethopropazine and (c) iso-OMPA , both of which inhibit pseudocholinesterase at μM concentrations in vertebrates and assayed for enzyme activity. Since BW284c51 inhibited >75% of activity at 0.1 μM concentration and iso-OMPA did not inhibit even at a high concentration, the enzyme from the two sources are acetylcholinesterases

[12] and assayed for enzyme activity. Since BW284c51 inhibited 75% of CNS enzyme activity at 0.1 μM concentration and ethopropazine inhibited most of the CNS enzyme activity only at 1mM concentration, the enzyme present in CNS is confirmed to be acetylcholinesterase (Fig. 2). However unlike ethopropazine, iso-OMPA did not inhibit the enzyme activity even at 1mM concentration indicating that this enzyme has a unique inhibition profile. The enzyme in hemolymph turned out to be a more of a true acetylcholinesterase than that of the CNS since BW284c51 inhibited 85% of hemolymph enzyme activity at 0.1 μM concentration and ethopropazine inhibited only 65% of the activity even at 1mM concentration (Fig. 2). However higher concentrations of ethopropazine (>1 mM)

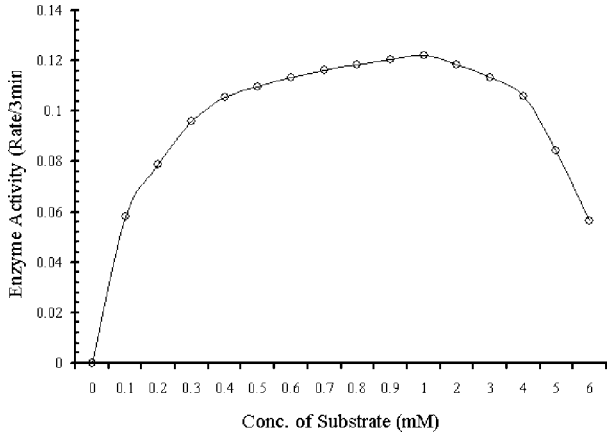

Fig. 3. Purified hemolymph cholinesterase activity was assayed with increasing concentrations of acetylthiocholine iodides as substrate. Enzyme activity started to decline at substrate concentrations > 3 mM. Concentrations of acetylcholine higher than 10 mM inhibited the activity of the enzyme showing that the hemolymph cholinesterase is inhibited by the presence of excess substrate

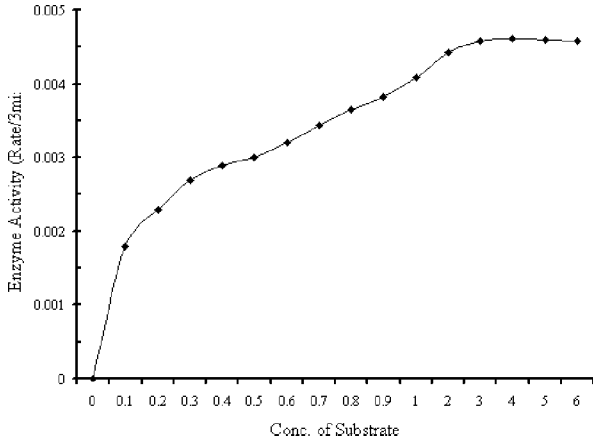

Fig. 4. CNS cholinesterase activity was assayed with increasing concentrations of acetyl thiocholineiodide as substrate. At concentrations of acetylcholine higher than 3 mM, the rate of 9 hydrolysis reached a plateau indicating that this enzyme was not inhibited by excess substrate

did not inhibit the enzyme 4 activity any further indicating that the hemolymph enzyme behaves differently compared to that of CNS in binding with ethopropazine as well as with BW284c51. Giller and Schwartz [3] suggested that the hemolymph enzyme might not be a true acetylcholinesterase based on their comparison of its inhibition by eserine to that of the CNS enzyme. Eserine sulphate is a non-specific cholinesterase inhibitor [12]. Further we used specific acetylcholinesterase and pseudocholinesterase inhibitors and determined the dose response of the enzyme in hemolymph and CNS in this study. Hence our results clearly show that the hemolymph enzyme is indeed a true acetylcholinesterase.

Michaelis-Menten Constant (K_m). In order to determine the K_m of the two enzymes, cholinesterase activity of the two samples was assayed with increasing concentrations of acetylthiocholine iodides as substrate. As shown in Fig. 3, at concentrations of acetylcholine higher than 3mM the rate of hydrolysis of hemolymph enzyme started to decline and reached total inhibition at 10mM. Hence this enzyme exhibits excess substrate inhibition, a characteristic of most of the vertebrate acetylcholinesterases showing that with regards to this feature, the hemolymph cholinesterase is similar to vertebrate acetylcholinesterase. A similar result was reported by Giller and Schwartz [3]. The rate of hydrolysis of CNS cholinesterase with increasing concentrations of acetylthiocholine was different from that of hemolymph cholinesterase. The rate showed a steady increase up to 4mM of acetylthiocholine and then reached a plateau (Fig. 4). Thus the CNS enzyme failed to show inhibition by excess substrate. The differences in the kinetic behavior between the CNS enzyme and the hemolymph enzyme are reflected in the Michaelis-Menten Constant (K_m) of CNS AChE being 0.2201Mm and of hemolymph AChE being 0.1219Mm based on their respective Lineweaver-Burke plots.

Molecular forms of hemolymph and CNS AChE of Aplysia. The sequential extraction of CNS 5enzyme using low salt, detergent and high salt buffers showed that the CNS enzyme exists primarily as a less salt soluble (58.6%) and a detergent soluble form (38.8%) with a very minor fraction of high salt soluble form (2.6%). Sucrose density gradient centrifugation of purified AChE from the hemolymph and CNS homogenates revealed similarity as well as differences in the molecular forms between the enzymes from the two sources. CNS homogenate showed the

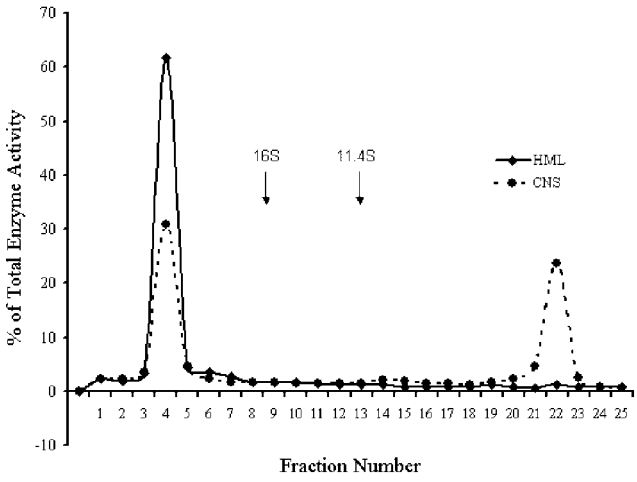

Fig. 5. CNS homogenate and purified enzyme from hemolymph were layered on separate 5–20% sucrose gradients in a high salt buffer (NaCl, 1M; $MgCl_2$, 0.05M; Tris-HCl, 0.01 M, pH 7.4) and centrifuged with a Beckman TLS55 rotor at 45,000 rpm, for 16 hours at 4°C. Fractions were collected by puncturing the bottom of the centrifuge tubes and assayed for acetylcholinesterase activity. CNS homogenate showed the presence of a 4.5 S and a heavier molecular form of acetylcholinesterase. Hemolymph enzyme showed the presence of a single heavier molecular form of acetylcholinesterase. The standards were *E. Coli* β-galactosidase (arrow 1, 16S) and beef liver catalase (arrow 2, 11.4S)

presence of a 4.5S, and a heavier molecular form of acetylcholinesterase (Fig. 5) based on the standards *E. Coli* β-galactosidase (16S) and beef liver catalase (11.4S). Unlike the CNS enzyme hemolymph enzyme showed the presence of only a single heavier molecular form of acetylcholinesterase (Fig. 5) and this is not due to its aggregation in a low ionic strength buffer or due to its presence as an amphiphilic particulate protein since centrifugation in a high salt gradient containing Triton X-100 did not alter its sedimentation profile. The molecular forms of *Aplysia* acetylcholinesterase differs from the dimeric and tetrameric forms of acetylcholinesterase reported to be present in the gastropod mollusk, *Murex* brandaris [13]. Thus the presence of a very heavy molecular form (>18S) of acetylcholinesterase appears to be unique to *Aplysia.*

Our results show that the cholinesterase present in the hemolymph and CNS of *Aplysia* is acetylcholinesterase. The hydrolysis and the inhibition profiles of the enzyme from the CNS and hemolymph indicate that the hemolymph enzyme is more of a true acetylcholinesterase than the enzyme from the CNS. The CNS may contain a mixture of acetyl and butyryl cholinesterases or an acetylcholinesterase, which also has characteristics of butyryl cholinesterase. The CNS 6 enzyme exists primarily as a less salt soluble and a detergent soluble form with a very minor fraction of high salt soluble form. Acetylcholinesterase present in the hemolymph exists as a very heavy molecular form while the enzyme from the CNS appears to exist as a monomeric globular form in addition to having the heavy molecular form. *Aplysia* hemolymph consists of a considerable amount of acetylcholinesterase with a unique molecular form and thus provides an excellent source to investigate the molecular evolution of this enzyme.

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НЕЙРОНАЛЬНЫЕ И ПЕРИФИРИЧЕСКИЕ ЭСТЕРАЗЫ ИЗ МОРСКОГО МОЛЮСКА *APLYSIA CALIFORNICA*

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Брюхоногий моллюск *Aplysia californica* имеет высокий уровень холинэстеразы в циркулирующей гемолимфе. Холинэстераза гемолимфы (крови) выполняет как холинэргическую, так и нехолинэргическую функции, заключающиеся в стимуляции роста нейритов. С целью изучения структурно-функциональной взаимосвязи гемолимфоцитарной холинэстеразы, этот фермент был получен в чистом виде и охарактеризован. Его сравнили с холинэстеразой, присутствующей в ганглиях центральной нервной системы (ЦНС) *Aplysia*. Было показано, что холинэстеразы из ЦНС и гемолимфы *Aplysia* отличаются от холинэстераз позвоночных наличием уникальных тяжелых молекулярных форм. Ферменты, присутствующие в гемолимфе и ЦНС, отличаются по скорости гидролиза, профилям субстратной специфичности, а также по молекулярной форме, что предполагает присутствие двух типов холинэстераз в организме *Aplysia*.