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Acetylcholinesterase in the sea urchin *Lytechinus variegatus*: Characterization and developmental expression in larvae

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Abstract

Acetylcholinesterase (AChE) in the echinoid *Lytechinus variegatus* has been characterized. Kinetic parameters V_{max}, K_m, K_{ss}, and b were 2594±1048 nmol ATCh hydrolyzed/min/mg tissue wet weight, 185±11 μM, 308±100 mM, and 0.2, respectively for the substrate ATCh and 17.8±6.87 nmol BTCh hydrolyzed/min/mg tissue wet weight, 654±424 μM, 36±31 mM, and 0.6, respectively for BTCh. Pharmacologic analyses were performed with four inhibitors of cholinesterases, physostigmine, BW284c51, ethopropazine, and iso-OMPA, and yielded IC_{50} values of 106±4 nM, 718±118 nM, 2.57±0.6 mM, and >0.0300 M, respectively. Both kinetic and pharmacologic results confirmed the existence of AChE in larval *L. variegatus*. Dimeric and tetrameric globular forms (determined by velocity sedimentation on sucrose gradients) were present in *L. variegatus* larvae. Activity of AChE increased significantly as larvae progressed in development from embryos to eight-arm larvae. Acetylcholinesterase activity of F1 larvae derived from sea urchins collected from wild populations and of F1 larvae derived from sea urchins cultured in the laboratory and fed two different diets suggest that the nutritional and/or environmental history of the adult sea urchin affect the developmental progression of AChE activity in the F1 offspring.

Keywords: Acetylcholinesterase; Nutrition; Sea urchin; *Lytechinus variegatus*; Larvae; Acetylcholine

1. Introduction

Acetylcholinesterase (EC 3.1.1.7) is a cholinesterase found in vertebrates as well as invertebrates. It is a serine hydrolase that rapidly and efficiently hydrolyzes the neurotransmitter acetylcholine (ACh) into choline and acetic acid (Massoulié et al., 1993) within the cholinergic synapses of neuromuscular junctions of the nervous system and is found throughout the central and peripheral nervous systems. Cholinergic molecules are also found in non-neuromuscular tissues and various pre-nervous structures of developing vertebrates and invertebrates (Harrison et al., 2002).

There are two classes of the quaternary structure of AChE: the asymmetric and the globular molecular forms (Massoulié and Rieger, 1969). Asymmetric forms interact with the extracellular matrix by way of the triple-helical-collagen tail. Globular forms differ from the asymmetric forms in both quaternary structure and method of attachment. Globular forms can exist as monomers (G_1), dimers (G_2), or tetramers (G_4). The globular forms do not contain collagen tails; however, globular forms can exist as either membrane-anchored or soluble forms (Gibney and Taylor, 1990; Duval et al., 1992). These various molecular forms are generally identified by their sedimentation coefficients, various structural elements, and solubility characteristics. Vertebrates possess both globular and asymmetric forms of the enzyme; however, asymmetric forms have not been found in invertebrates, suggesting that the collagen tail arose in early vertebrate phylogenetic divergence (Massoulié et al., 1993).

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The presence of cholinergic molecules and receptors has been reported in several sea urchin species. ACh and ACh receptors are present in unfertilized sea urchin eggs and other pre-nervous developmental stages (Harrison et al., 2002; Qiao et al., 2003). Augustinsson and Gustafson, pioneers in the study of AChE in sea urchins, observed that cholinesterase (later determined to be AChE) activity is not observed in the unfertilized eggs of sea urchins (Augustinsson and Gustafson, 1949); however, with a more sensitive enzymatic assay (the Ellman assay, Ellman et al. (1961)), Ozaki (1974) has shown that AChE activity is present even in unfertilized *Pseudocentrotus depressus* eggs. The same assay method was used to obtain similar results revealing AChE activity associated with *Strongylocentrotus purpuratus* egg ghosts (Barber and Foy, 1973).

AChE activity has been traced throughout the development of several species of sea urchins. These species include *P. depressus* (Ozaki, 1974), *S. purpuratus* (Ozaki, 1976), and *Hemicentrotus pulcherrimus* (Akasaka et al., 1986). Not only is AChE activity present in sea urchin embryos and larvae, a characteristic trend of increasing activity throughout development is observed in various species reported in the literature. ACh and AChE are present in early cleavages of the developing embryo, but sustained increases in the levels of ACh and AChE activity are observed during gastrulation (Falugi et al., 2002; Akasaka et al., 1986) and post-gastrulation (Augustinsson and Gustafson, 1949; Ozaki, 1974; 1976). This rapid increase in AChE activity is thought to be the possible beginnings of neuronal differentiation (Akasaka et al., 1986). Through the use of an AChE staining method, the Cu-thiocholine method of Karnovsky and Roots (1964), Ozaki (1974, 1976) determined that AChE is localized in the mesenchyme cells of sea urchin larvae. The mesenchyme cells are associated with the larval skeleton, oral lobe, and arms (Ozaki, 1974, 1976).

The sea urchin has been proposed as a model organism for neurotoxicity (Buznikov et al., 2001; Qiao et al., 2003; Cunha et al., 2005). Qiao et al. (2003) used the embryos of the sea urchins *S. purpuratus* and *Strongylocentrotus droebachiensis* as invertebrate models for developmental neurotoxicity in mammals, focusing on the high-affinity choline transporter, and suggested that the sea urchin has cholinergic structures and activity similar to that found in a mammalian brain. It has also been proposed that the sea urchin be used as a model to test the effects of various pesticides and organic compounds on early development (Buznikov et al., 2001), and the AChE of *Paracentrotus lividus* has recently been considered as a biomarker of environmental contamination (Cunha et al., 2005).

The primary goals of this research were to characterize the enzyme AChE in the sea urchin *Lytechinus variegatus* and to evaluate its activity in developing larvae. The goals were accomplished by a kinetic analysis of the enzyme's substrate specificity and pharmacological inhibition, as well as a determination of the various molecular forms present. Following the characterization, developmental progressions of AChE activity were evaluated in F1 embryos and larvae derived from adult sea urchins either collected from wild populations or cultured in the laboratory on nutritionally-different diets. There have been no studies that investigate the effect of parental nutrition on AChE expression of developing sea urchins F1 embryos and larvae. Although developmental progressions in at least three other cold water sea urchin species have been constructed, *L. variegatus* is a warm water species that is more readily adapted as an experimental animal model. Thus, a final goal was to evaluate the suitability of AChE in *L. variegatus* as a biomarker for determining the well-being of developing organisms.

2. Materials and methods

2.1. Collection and culture of sea urchins for the characterization of AChE

Adult *L. variegatus* sea urchins were collected from St. Joseph Bay, Florida in May of 2006 and transported to the University of Alabama at Birmingham (UAB). Individuals were held in recirculating seawater systems containing synthetic seawater (Instant Ocean, 32 ppt; 22–24 °C) and fed a formulated feed (Hammer, 2006) until analysis. Adult sea urchins were spawned by injection of approximately 1 mL of 0.1 M ACh. Gametes were collected by inverting females over a beaker while sperm was collected dry by removing expressed sperm by pipette. Fertilization tests were performed by obtaining a sample of eggs and fertilizing with a sample of diluted sperm on a microscope slide. Eggs were fertilized with diluted sperm to reduce the possibility for polyspermy. After fertilization, zygotes were placed in a shallow glass fingerbowl in synthetic seawater (32 ± 1 ppt). After the first cell divisions were complete, embryos were placed into a larger volume of aerated synthetic seawater and were fed twice daily combined mixtures of the algae *Dunaliella tertiolecta*, *Isochrysis galbana*, and *Rhodomonas salina* (obtained from the University of Texas, Port Aransas, TX) to apparent satiation (stomachs were observed to be full).

At eight days post-fertilization, a subsample of eight-arm larvae was collected by siphoning excess culture seawater through a Nitex screen (mesh size 75 μm), while retaining the larvae and concentrating the organisms. Once concentrated, the larvae were transferred to several 50 mL centrifuge tubes to further concentrate the sample. Samples were centrifuged at ≤ 470 *g* (Beckman TJ-6R Tabletop Centrifuge, TH-4 rotor), depending upon the developmental stage, at 4 °C for 10 min. After centrifugation, the supernatant was removed by aspiration and remaining pellets were combined. Synthetic seawater was added to resuspend the combined pellets to a final volume of 25 mL. Organisms in three 50 μL aliquots were counted on a microscope slide to obtain the number of organisms in each sample. Also, a 1 mL aliquot from each sample was preserved in 10% buffered formalin to confirm developmental stage. Samples were re-centrifuged, the supernatant removed by aspiration, and the pellet homogenized with 5 mL of high-ionic strength (HIS) buffer (10 mM $NaHPO_4$, 1 M NaCl, 1 mM EDTA, 1% Triton X-100, pH 7). Samples were then frozen at −30 °C until analysis.