ACETYLCOLINESTERASE IN THE SEA URCHIN *LYTECHINUS VARIEGATUS*: CHARACTERIZATION AND DEVELOPMENTAL EXPRESSION IN LARVAE

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ACETYLCHOLINESTERASE IN THE SEA URCHIN *LYTECHINUS VARIEGATUS*: CHARACTERIZATION AND DEVELOPMENTAL EXPRESSION IN LARVAE

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BIOLOGY

ABSTRACT

Acetylcholinesterase (AChE) in a deuterostome invertebrate, the echinoid *Lytechinus variegatus*, has been characterized. Kinetic parameters $V_{\text{max}}$, $K_m$, $K_{ss}$, and $b$ were $453 \pm 183$ µM ATCh hydrolyzed/minute, $185 \pm 11$ µM, $308 \pm 100$ mM, and 0.2, respectively for the substrate acetylthiocholine (ATCh) and $3.1 \pm 1.2$ µM BTCh hydrolyzed/minute, $654 \pm 424$ µM, $36 \pm 31$ mM, and 0.6, respectively for substrate butyrylthiocholine (BTCh). Pharmacologic analyses were performed with four inhibitors of cholinesterases, physostigmine, BW284c51, ethopropazine, and iso-OMPA, and yielded IC$_{50}$ values of $106 \pm 4$ nM, $718 \pm 118$ nM, $2.57 \pm 0.6$ mM, and $> 0.0300$ M, respectively. Both kinetic and pharmacologic results confirmed the existence of AChE in larval *L. variegatus*. Molecular forms were determined by velocity sedimentation on sucrose gradients. Dimeric (G$_2$) and tetrameric (G$_4$) globular forms were found to be present in *L. variegatus* larvae. Activity of AChE increased significantly as larvae progressed in development from embryos to 8-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. Acetylcholinesterase activity was detected in wild adult sea urchin tissues. Activity was significantly higher in
the gonad than the esophagus, stomach, and intestine. The functional consequences of differential AChE activities among tissues are unknown.
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INTRODUCTION

Specific Goals

The primary goals of this research are to characterize the enzyme acetylcholinesterase (AChE; EC 3.1.1.7) in the sea urchin *Lytechinus variegatus* and to evaluate its activity in larval and adult tissues and organs. The goals will be 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 will be evaluated in F1 embryos and larvae derived from adult sea urchins either collected from wild populations or cultured in the laboratory on different diets. 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. Also, there have been no studies that investigate the effect of parental nutrition on AChE expression of developing sea urchins. Finally, a survey of AChE activity in adult sea urchin tissues will be determined. The digestive and reproductive tissues will be collected from adult sea urchins because these are the major internal organs and the growth of these tissues is influenced by the nutrition history of the organism.
Characteristics of Acetylcholinesterase

In the “cholinergic system” of vertebrates and invertebrates, acetylcholine (ACh) is a signaling molecule or neurotransmitter (Harrison et al., 2002). Choline acetyltransferase (ChAT) is the “biosynthetic enzyme” that synthesizes acetylcholine, and acetylcholinesterase is the degradative enzyme in this system. There are two common types of receptors found in the cholinergic system: muscarinic and nicotinic, differing in position within the nervous system and with respect to chemical agonists and antagonists.

Acetylcholinesterase is a cholinesterase found in vertebrates as well as invertebrates. It is a serine hydrolase that rapidly and efficiently hydrolyzes the neurotransmitter acetylcholine 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). The common catalytic domain of AChE, containing 535 amino acids, is highly homologous in all cholinesterases, from invertebrates and vertebrates (Massoulié et al., 1993).

There are two classes of the quaternary structure of AChE: the asymmetric and the globular molecular forms (Massoulié et al., 1969). Asymmetric forms contain tetrameric catalytic subunits, and colQ, a triple-helical-collagen tail (Fig. 1). Each catalytic subunit can attach to one collagogenic tail; thus, asymmetric forms can have one, two, or three catalytic tetramers and are named A₄, A₈, and A₁₂, respectively. These 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 (Fig. 1). Globular forms can exist as monomers (G₁),
dimers (G₂), or tetramers (G₄). 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). The monomer (G₁), exists as a Type II
AChE that is attached via an amphiphilic anchor. Dimers (G₂) can exist as either Type I
AChE, containing a glycophosphatidylinositol (GPI) membrane anchor, or Type II
AChE, containing an amphiphilic membrane anchor (Massoulié et al., 1993). Tetramers
(G₄) can exist as a soluble form or as a hydrophobically-tailed form. The
hydrophobically-tailed G₄ form contains the 20 kDa non-catalytic hydrophobic subunit,
P. This P subunit allows for membrane attachment.

Fig. 1. Quaternary structure of the various molecular forms of acetylcholinesterase (from
Massoulié et al., 1993).

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).
Acetylcholinesterase Kinetics

Quinn (1987) considers AChE to be a “perfect enzyme” because of its unique kinetic characteristics. Acetylcholinesterase is one of the most catalytically efficient enzymes known, reaching its theoretical maximal velocity and limited only by substrate diffusion (Massoulié et al., 1993). Radić et al. (1993) indicated that AChE does not follow typical Michaelis-Menten enzyme kinetics. There are multiple subsites within the catalytic gorge of AChE: the catalytic triad, the choline binding site, peripheral site, the hydrophobic site, the oxyanion hole, and the acyl pocket (Sussman et al., 1991). There are two substrate binding sites within this gorge: the choline binding site of the active site and the peripheral site. Thus, AChE kinetics are more complicated because the peripheral subsite serves as a secondary substrate binding site and allows potential substrate inhibition (Radić et al., 1993).

To determine the catalytic parameters of AChE, the reaction scheme described by Radić et al. (1993) was assumed (Fig. 2). Not only can a maximal velocity ($V_{\text{max}}$) and the Michaelis constant ($K_m$) be calculated for AChE, but $K_{ss}$ (the dissociation constant for binding of the substrate to the secondary site) and $b$ (a measure of the fractional catalytic activity of the ternary complex of substrate-enzyme-substrate compared to the substrate-enzyme complex) can also be calculated (Taylor et al., 1994). By determining the $b$ value for a cholinesterase, one can identify the enzyme source. When $b$ is greater than 1, the enzyme experiences substrate activation; when $b$ is less than 1, the enzyme experiences substrate inhibition; and when $b$ is equal to 1, the enzyme is characteristic of typical Michaelis-Menten enzyme kinetics. At high levels of substrate, AChE
experiences substrate inhibition, while butyrylcholinesterase (BuChE, EC 3.1.1.8) experiences substrate activation.

![Enzymatic scheme of acetylcholinesterase](image)

**Fig. 2.** Enzymatic scheme of acetylcholinesterase (from Radić et al., 1993). Substrate (S) can bind to enzyme (E) at two distinct sites: the active site and the peripheral site. Depending upon substrate concentration, substrate could be either directly converted into product as a result of substrate binding to only the active site or production of product could be inhibited by the development of the ternary complex (SES) in which substrate binds to both the active and peripheral sites.

The following equation is derived from the scheme from Radić et al. (1993).

\[
v = \left[ \frac{1 + b[S]/K_{ss}}{1 + [S]/K_{ss}} \right] \left( \frac{V_{max}}{1 + K_{m}/[S]} \right)
\]

**Acetylcholinesterase in Vertebrates**

Cholinesterases have been studied in vertebrates for much of the last century. In 1914, H. H. Dale proposed the existence of such enzymes. Twelve years later the existence of cholinesterases was confirmed by Loewi and Navratil (1926).

Acetylcholinesterase was found in high concentrations in the electric organs of the electric ray *Torpedo* and the electric eel *Electrophorus* (Marnay, 1937; Marnay et al., 1937; Marnay et al., 1938). The first purification and crystallization of the enzyme was similarly accomplished with electric eel AChE (Leuzinger et al., 1967). The three-
dimensional structure of this enzyme was first determined by Sussman et al. (1991) in the Pacific electric ray.

Vertebrates possess two genes that code for the cholinergic enzymes AChE and BuChE (Massoulié et al., 1993). The predominant molecular form of AChE, within mammalian central nervous systems, is the amphiphilic, membrane-bound, tetrameric $G_4$ form (Massoulié et al., 1993). The chromosome placement of the gene coding for AChE varies in vertebrates. In *Homo sapiens* the gene is found on chromosome 7q22 (Getman et al., 1992), and in the mouse it is located on chromosome 5 (Rachinsky et al., 1992). The coding regions of the cholinesterase genes vary in length among vertebrates; however, both the BuChE and AChE intron placement within the genes have been highly conserved throughout vertebrate genomes (Massoulié et al., 1993).

**Acetylcholinesterase in Invertebrates**

While many vertebrates appear to have only one gene for AChE, the number of genes coding for AChE in invertebrates can vary depending on the organism (Massoulié et al., 1993). For example, the *Drosophila* genome contains one gene that codes for AChE (Hall et al., 1986), *Caenorhabditis elegans* contains four genes (Combes et al., 2000), and the sea urchin *Strongylocentrotus purpuratus* contains several putative genes (Burke et al., 2006). The structure of the *Drosophila* AChE gene differs from the vertebrate AChE gene in that it is divided into seven coding sequences (Fournier et al., 1989) compared to the four coding exons found in *Torpedo* (Maulet et al., 1990). However, the AChE gene structure is quite variable among invertebrates.
Acetylcholinesterase in Sea Urchins

The presence of cholinergic molecules and receptors has been reported in several sea urchin species. Acetylcholine 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), 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 in *Strongylocentrotus purpuratus* egg ghosts (Barber and Foy, 1973).

Acetylcholinesterase activity has been traced throughout the development of several species of sea urchins. These species include *Pseudocentrotus depressus* (Ozaki, 1974), *Strongylocentrotus 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 (Figures 3, 4, and 5, respectively). Acetylcholine 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 and 1976). This sudden increase in AChE activity is thought to be the possible beginnings of neuronal differentiation.
(Akasaka et al., 1986). It has also been suggested that ACh is involved in the mechanism of triggering cleavage divisions (Buznikov et al., 1970).

Fig. 3. Developmental progression of AChE activity in *Pseudocentrotus depressus* (from Ozaki, 1974).
Fig. 4. Developmental progression of AChE activity in *Strongylocentrotus purpuratus* (from Ozaki, 1976). Hours of development versus AChE activity in absorbance per milligram protein.

Fig. 5. Developmental progression of AChE activity in *Hemicentrotus pulcherrimus* (from Akasaka et al., 1986).

The localization of AChE activity has also been investigated. Through the use of an AChE staining method, the Cu-thiocholine method of Karnovsky and Roots (1964),
Ozaki (1974 and 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 and 1976).

Acetylcholinesterase of the sea urchin *Strongylocentrotus purpuratus* is heat labile and loses stability between 30 and 35°C (Ozaki, 1976). Ozaki (1976) also tested the stability of *S. purpuratus* AChE in response to various concentrations of acetic acid. The enzyme lost stability below pH 5.5.

The sea urchin has been proposed as a model organism for neurotoxicity. Qiao et al. (2003) used the sea urchin embryo as a model for developmental neurotoxicity in mammals. They suggested that the sea urchin has cholinergic structures and activity similar to that found in a mammalian brain. Numerous investigators have also 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).

**Acetylcholinesterase as a Biomarker**

Acetylcholinesterase has been used as a biomarker for various environmental contaminants and neurotoxic agents because it is the target of many pesticides and nerve gases (Main, 1979). Acetylcholinesterase activity and AChE inhibition in numerous bioindicator-invertebrate species have been monitored in several geographical regions. For example, AChE in zebra mussel was used to monitor the presence of various pollutants in Lake Maggiore in Italy as well as the Italian Great Lakes (Binelli et al., 2005; Ricciardi et al., 2006). Acetylcholinesterase activity has also been used as a successful biomarker for metal toxicities, except nickel (Frasco et al., 2005). Significant
differences in AChE activity were found in the mussel *Mytilus galloprovincialis* at various testing sites exposed to industrial pollutants and pesticides along the coast of Portugal (Moreira et al., 2005). Acetylcholinesterase levels in grass shrimp, *Palaemonetes intermedius*, in southern Florida were lower in shrimp exposed to herbicides and pesticides including atrazine, chlorpyrifos, and diazinon as compared to the control group (Key et al., 2003). Acetylcholinesterase has also been used as a biomarker for neurotoxicity. Acetylcholinesterase was utilized as a biomarker of neurotoxic compounds from an industrial zone in the Lagoon of Venice (Matozzo et al., 2005). Acetylcholinesterase toxicity studies in invertebrates have significant potential for modeling effects in many organisms, including humans.
MATERIALS AND METHODS

Collection and Culture of Sea Urchins for the Characterization of AChE

Adult *Lytechinus 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, 32ppt; 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. 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 micron), 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 minutes. 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 HIS buffer (10 mM NaHPO₄, 1 M NaCl, 1 mM EDTA, 1% Triton X-100, pH 7). Samples were then frozen at -30°C until analysis.

Characterization of Acetylcholinesterase (AChE)

*Determination of AChE Activity*

Acetylcholinesterase (EC 3.1.1.7) specific activity was determined by a colorimetric assay as described by Ellman et al. (1961). In this assay, two reactions are coupled to determine AChE activity. Acetylcholinesterase hydrolyzes the substrate acetylthiocholine (ATCh) into thiocholine and acetate. Thiocholine then reacts with dithiobisnitrobenzoate (DTNB) to produce a yellow color. The rate of the reaction is determined colorimetrically. This assay is sensitive to low concentrations of AChE and allows for kinetic analysis of the enzyme (Ellman et al., 1961). Samples were homogenized by Polytron homogenizer and centrifuged at 17,000 G at 4°C for 20 minutes. In this assay, 50 µL of each supernatant were transferred into individual wells of a 96-well microtitre plate. To each well, 250 µL of Ellman’s solution (100 mM NaHPO₄, 2.5 mM DTNB, and 0.833 mM ATCh, final concentrations) were added. The
colorimetric determination was performed using an ELx808 Ultra Microplate Reader (Biotek Instruments, Inc. Winooski, Vermont).

Similar concentrations of the algae *Dunaliella tertiolecta, Isochrysis galbana*, and *Rhodomonas salina* were analyzed for the presence of AChE activity. No significant enzyme activity was detected in these samples.

**AChE Kinetic Analysis: Substrate Specificity**

Acetylcholinesterase is generally identified by the substrate it will hydrolyze (Augustinsson, 1948). Acetylthiocholine is hydrolyzed by AChE; however, butyrylthiocholine (BTCh) is not hydrolyzed by AChE. To determine the kinetics of AChE, Ellman’s colorimetric assay was performed separately with various concentrations of the substrates acetylthiocholine iodide (minimum 98% TLC, Sigma-Aldrich) and S-butyrylthiocholine iodide (minimum 98%, Sigma-Aldrich). A serial dilution was performed for each substrate with final concentrations ranging from $3.16 \times 10^{-7}$ M to $1 \times 10^{-1}$ M. The kinetic parameters $V_{max}$, $K_m$, $K_{ss}$, and $b$ were calculated. AChE activity versus substrate concentration curves were constructed with Sigma Plot software using the scheme described by Radić et al. (1993) to determine kinetic parameters.

**AChE Kinetic Analysis: Pharmacological Inhibition**

Inhibitors of cholinesterases including physostigmine (eserine), an inhibitor of all cholinesterases, BW284c51, a preferential inhibitor of AChE, and iso-OMPA and ethopropazine, preferential inhibitors of BuChE, were tested (Silver, 1974). A dilution series of each inhibitor was assayed to determine inhibition of enzyme activity. Inhibitor
concentrations varied from $1 \times 10^{-9}$ M to $3 \times 10^{-2}$ M. After a 20 minute incubation of the enzyme and inhibitor at room temperature, 200 µL of Ellman’s solution were added to initiate the reaction. Ellman’s colorimetric enzyme assay was performed and plots of inhibitor concentration versus the fractional AChE activity for each inhibitor were constructed with Sigma Plot software. IC$_{50}$ values were also calculated with Sigma Plot.

*Determinations of Larval Molecular Forms of AChE*

The determination of the molecular forms of AChE was completed by velocity sedimentation of the enzyme sample on sucrose gradients as described by Massoulié and Toutant (1988). Homogenized samples and a catalase marker (11.3 S, as described by Rosenberry and Scoggin (1984)) were loaded onto sucrose gradients (5-25% sucrose) in the presence and absence of the detergent, Triton X-100. Samples were centrifuged for 20 hours at 35,000 rpm and 4°C (Beckman L7-55 Ultracentrifuge, SW 41 rotor). After centrifugation, each gradient was fractionated from the bottom using an LKB Ultrarac Fraction Collector 7000. Each fraction (100 µL) was assayed for catalase activity to determine which fraction contained the catalase marker. Next, 200 µL of Ellman’s solution were added to each well containing the fractions. The fractions were assayed for AChE activity as described previously. Data were analyzed with Sigma Plot to determine the sedimentation coefficients of the various molecular forms. One-way t-tests using SPSS statistical software version 12.0 for Windows (2006; Chicago, Illinois) were performed to determine the significance of the sedimentation coefficients calculated in the presence and absence of Triton X-100.
Developmental Progression of AChE Activity in Larval *Lytechinus variegatus*

Adult *Lytechinus variegatus* (ca. 40 mm diameter) were collected from St. Joseph Bay, Florida in December of 2006. Sea urchins were transported to UAB where they were held as described previously and starved for approximately three months. Sea urchins (n = 32 per treatment) were randomly assigned a diet treatment of either organic romaine lettuce and organic carrots (EPA reference diet (USEPA, 1995; USGS, 2000)) or a nutritionally-complete formulated feed (Hammer, 2006). Sea urchins were photographed and weighed at the beginning and end of the experiment. Sea urchins were placed into mesh baskets partitioned into four individual cages (ca. 11.5 x 11.5 x 15.5 cm). Eight baskets (32 individual cages), elevated by a piece of 3” PVC pipe split length-wise, were dispersed among four recirculating raceway systems (Fig. 6). Sea urchins were maintained in synthetic sea water (Instant Ocean Sea Salt, 32 ± 2 ppt, 23 ± 2°C) with a 12 hour light:12 hour dark photoperiod. Sea urchins were fed daily *ad libitum* for ca. 12 weeks (n = 32 individuals per diet treatment; additional diets were tested during this experiment but the results will not be presented here). Water chemistry parameters including ammonia, nitrate, nitrite, pH, and alkalinity, were monitored biweekly and maintained at appropriate levels.

At week 12, lab-cultured sea urchins fed the lettuce and carrot diet or the formulated feed were spawned as described previously. A fertilization test was performed to ensure gametes were viable. For each diet treatment, gametes of 3+ females and 3+ males were pooled and fertilized. Various stages of embryological and larval development (1.5, 3, 24, 48, 96, and 192 hours post-fertilization) were collected as described previously.
Adult *L. variegatus* were collected from wild populations at approximately the same time as cultured urchins were analyzed. The adult urchins were spawned, and gametes from one male and one female from the wild were fertilized. Various stages of embryological and larval development were collected as described previously. Homogenates of embryos and larvae from both cultured and wild sea urchins were stored at -30°C until analysis.

![Schematic diagram of the recirculating raceway system](image-url)

Fig. 6. Schematic diagram of the recirculating raceway system. Each raceway contains 8 cages which hold 4 individual urchins each. A. Side view of one raceway. B. End view of one raceway. (Modified version of original picture (from Taylor (2006))).
At the time of analysis, previously-collected samples were thawed and evaluated to ensure cell lysis. Advanced larval stages were further homogenized with the Polytron homogenizer. Five 50 µL aliquots of each homogenate were assayed for AChE activity in the presence of 250 µL of Ellman’s solution. AChE activity values (mAb/min) for each developmental stage were normalized to nmoles ATCh hydrolyzed per minute per 10^4 organisms. Mean values of specific AChE activity and standard errors for each developmental stage were determined. Specific AChE activity from wild and lab-cultured larvae were compared statistically with SPSS statistical software version 12.0 for Windows (2006; Chicago, Illinois). An analysis of variance (ANOVA) was performed to evaluate the effect of feed treatment on AChE activity at specific post-fertilization times. Significant differences were determined at P < 0.05 and were compared by the Tukey HSD post-hoc test.

_Determination of AChE Activity in Wild Adult Tissues_

Adult _L. variegatus_ sea urchins were collected from the wild as described previously. Four adult sea urchins were dissected to obtain esophagus, stomach, intestine, and gonad tissues. Other samples collected included sperm, eggs, and coelomic fluid. Adult tissue samples were homogenized (1:4, wet weight:volume) with HIS buffer and frozen at -30°C. To determine AChE activity, samples were thawed, further homogenized with a Polytron, and centrifuged at approximately 17,000 G at 4°C for 20 minutes (Sorvall RC 5C). Five 50 µL aliquots of each tissue sample were assayed for AChE activity in the presence of 250 µL of Ellman’s solution. AChE activity was normalized to nmoles ATCh hydrolyzed per minute per milligram wet weight, and
standard errors for each adult tissue were calculated. Significant differences among tissues were determined by ANOVA ($P < 0.05$) and Tukey HSD post-hoc test.
RESULTS

Acetylcholinesterase Kinetics: Substrate Specificity

Acetylthiocholine was the preferred substrate for larval *L. variegatus* cholinesterase; butyrylthiocholine was not hydrolyzed (Fig. 7). The low $\frac{V_{\text{max}}^{\text{BTCh}}}{V_{\text{max}}^{\text{ATCh}}}$ ratio (Table 1) confirmed that only ATCh is hydrolyzed significantly, indicating the homogenate contained AChE. Acetylcholinesterase also exhibits substrate inhibition at high substrate concentrations, indicated by a $b$ value of less than one (Fig. 7, Table 1). The $K_m$ values for ATCh and BTCh were 185 ± 11 µM (mean ± SE) and 654 ± 424 µM, respectively (Table 1). The $K_{ss}$ values for ATCh and BTCh were 308 ± 100 mM (mean ± SE) and 36 ± 31 mM, respectively (Table 1).
Fig. 7. Substrate specificity of acetylcholinesterase. AChE was extracted from *Lytechinus variegatus* larvae with HIS buffer and assayed with acetylthiocholine (●) or butyrylthiocholine (○).

Table 1
Kinetic analysis of acetylcholinesterase. Kinetic analysis was performed on samples of *Lytechinus variegatus* larval homogenates. Values are means ± SE (n = 4 determinations).

<table>
<thead>
<tr>
<th></th>
<th>ATCh</th>
<th>BTCh</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}^{ATCh}$ (µM ATCh hydrolyzed/min)</td>
<td>453 ± 183</td>
<td>3.1 ± 1.2</td>
</tr>
<tr>
<td>$K_m^{ATCh}$ (µM)</td>
<td>185 ± 11</td>
<td>654 ± 424</td>
</tr>
<tr>
<td>$K_{ss}^{ATCh}$ (mM)</td>
<td>308 ± 100</td>
<td>36 ± 31</td>
</tr>
<tr>
<td>$b^{ATCh}$</td>
<td>0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>$V_{max}^{BTCh}$ (µM BTCh hydrolyzed/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m^{BTCh}$ (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_{ss}^{BTCh}$ (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$b^{BTCh}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{max}^{BTCh} / V_{max}^{ATCh}$</td>
<td>0.007 ± 0.007</td>
<td></td>
</tr>
</tbody>
</table>
Physostigmine (an inhibitor of all cholinesterases) and BW284c51 (a specific inhibitor of AChE) inhibited *L. variegatus* AChE activity at lower inhibitor concentrations than ethopropazine and iso-OMPA (specific inhibitors of BuChE) (Fig. 8). The enzyme sample assayed independently with physostigmine and BW284c51 exhibited monophasic dose response curves. IC₅₀ values ranged from a low of 106 ± 4 nM for physostigmine to a high of > 0.03 M for iso-OMPA (Table 2).

![Graph showing the pharmacological inhibition of acetylcholinesterase](image)

**Fig. 8.** Pharmacological inhibition of acetylcholinesterase. Four inhibitors of cholinesterases were individually incubated with samples of *Lytechinus variegatus* larval homogenates for 20 minutes. Substrate ATCh was added, and Ellman’s assay was performed (□ = physostigmine, ■ = BW284c51, ○ = ethopropazine, ● = iso-OMPA).
Table 2  
IC₅₀ values of four inhibitors of cholinesterases. The inhibitors physostigmine, BW284c51, ethopropazine, and iso-OMPA were individually incubated with samples of *Lytechinus variegatus* larval homogenates for 20 minutes. Substrate ATCh was added and Ellman’s assay was performed. Values represent mean ± SE (n = 3 determinations).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physostigmine</td>
<td>106 ± 4 nM</td>
</tr>
<tr>
<td>BW284c51</td>
<td>718 ± 118 nM</td>
</tr>
<tr>
<td>Ethopropazine</td>
<td>2.57 ± 0.6 mM</td>
</tr>
<tr>
<td>Iso-OMPA</td>
<td>&gt; 0.0300 M</td>
</tr>
</tbody>
</table>

Characterization of the Molecular Forms of AChE in *Lytechinus variegatus*

Two peaks of approximately 7.94 S and 10.38 S suggested that G₂ and G₄ molecular forms are present in *L. variegatus* larvae (Fig. 9). Average sedimentation coefficients (S) of 7.62 ± 0.17 SE and 10.42 ± 0.02 SE in the presence of Triton X-100 and 8.80 ± 0.31 SE and 11.46 ± 0.40 SE in the absence of Triton X-100 for all gradients also suggested that putative G₂ and G₄ molecular forms are present (Table 3).
Fig. 9. Determination of larval molecular forms by velocity sedimentation in the presence of Triton X-100. *Lytechinus variegatus* larvae (8-arm stage) were collected and homogenized in HIS buffer. The larval homogenate and a catalase marker (11.3 S) were loaded onto sucrose gradients (5-25% sucrose) in the presence of Triton X-100. Sucrose gradients were centrifuged at 35,000 rpm for 20 hours at 4°C. The gradients were fractionated and assayed for catalase and AChE activity.

Table 3

Sedimentation coefficients (S) of the AChE molecular forms identified in *Lytechinus variegatus* 8-arm stage larvae. Sedimentation coefficients were determined by velocity sedimentation of larval homogenates in the presence of HIS buffer. Values represent mean ± SE (n = 3 determinations). Means with different superscripts are significantly different (One sample t-test, *P* < 0.05).

<table>
<thead>
<tr>
<th></th>
<th><em>G</em>₂ (-Triton X-100)</th>
<th><em>G</em>₂ (+Triton X-100)</th>
<th><em>G</em>₄ (-Triton X-100)</th>
<th><em>G</em>₄ (+Triton X-100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8.80 ± 0.31 (S)</td>
<td>7.62 ± 0.17 (S)</td>
<td>11.46 ± 0.40 (S)</td>
<td>10.42 ± 0.02 (S)</td>
</tr>
<tr>
<td>Superscript</td>
<td>^A</td>
<td>^B</td>
<td>^C</td>
<td>^D</td>
</tr>
</tbody>
</table>
Developmental Progression of Acetylcholinesterase Activity

Very little AChE activity was observed in pre-gastrulation stages of development. However, there was a substantial increase in AChE activity post-gastrulation, and AChE activity continued to increase exponentially throughout larval development in all groups (Fig. 10). Significant differences between groups were observed throughout all developmental stages.

![Bar chart showing developmental progression of AChE activity](image)

**Fig. 10.** Developmental progression of AChE activity in embryos and larvae obtained from adults collected from wild populations or cultured in the laboratory and fed lettuce/carrots or a formulated feed. Various stages of the same cohort of *Lytechinus variegatus* embryological and larvae were collected and assayed for AChE activity. AChE activity was standardized per $10^4$ organisms for each developmental stage. Values presented are means ($n = 4+$ determinations). Same letters represent no significant difference in AChE activity among treatments at the various post-fertilization times (ANOVA, $P < 0.05$; Tukey HSD post-hoc test).
Acetylcholinesterase Activity in Adult *Lytechinus variegatus* Tissues

Adult *L. variegatus* tissues exhibited substantial AChE activity (Fig. 11).

Gonadal tissue exhibited significantly higher enzymatic activity than the other gut tissues. Specific AChE activities of the gut tissues were not significantly different from one another.

![Graph showing AChE activity of adult *Lytechinus variegatus* tissue homogenates](image)

Fig. 11. AChE activity of adult *Lytechinus variegatus* tissue homogenates (n = 4 determinations, mean ± SE). Homogenates include, esophagus, stomach, intestine, and gonad (male and female) samples. Same letters represent no significant difference in AChE activity (ANOVA, *P* < 0.05; Tukey HSD post-hoc test).
DISCUSSION

Characterization of Acetylcholinesterase

Results from the kinetic analysis, including substrate specificity and pharmacology, indicated that *L. variegatus* express acetylcholinesterase. Analysis of the substrate specificity of larval *L. variegatus* enzyme homogenates resulted in the preferential hydrolysis of ATCh and no significant hydrolysis of BTCh. Because AChE is specific for substrate ACh while BuChE hydrolyzes butyrylcholine more efficiently than ACh (Alles and Hawes, 1940; Augustinsson, 1948), the results indicated that AChE is present in both larval and adult tissues. X-ray analysis of the Pacific electric ray, *Torpedo californica*, AChE revealed that the enzyme has a very narrow catalytic gorge that is lined with 14 aromatic residues and is approximately 20 Å deep (Sussman et al., 1991). Because AChE has such a narrow catalytic gorge, only substrates similar to acetylcholine are efficiently hydrolyzed. Butyrylcholine would not be substantially hydrolyzed by AChE because of its inability to penetrate its catalytic gorge. Fromson and Whittaker (1970) confirmed that cholinesterase from larval homogenates of the ascidian, *Ciona intestinalis*, preferentially hydrolyzed substrate acetylthiocholine iodide (100% hydrolysis) while only hydrolyzing 4.5% of the substrate BTCh, resulting in a low $V_{max}^{\text{BTCh}}/V_{max}^{\text{ATCh}}$ ratio that is indicative of AChE. Substrate-specificity analysis further indicated that inhibition of *L. variegatus* AChE occurred at high ATCh concentrations, which is also characteristic of AChE. Substrate inhibition of AChE activity at high substrate concentrations was also shown in the sea urchin *Paracentrotus lividus*. 
Augustinsson and Gustafson, 1949) and in *C. intestinalis* (Mعدل and Whittaker, 1979). Because of a secondary substrate binding site, the peripheral site, located near the entrance of the catalytic gorge, AChE has the potential for substrate inhibition (Sussman et al., 1991; Massoulié et al., 1993). Substrate inhibition of the enzyme sample was also indicated by a $b$ value of less than one. Furthermore, the kinetic parameters, $K_m$ and $K_{ss}$ calculated are similar to those reported by معدل and Whittaker (1979). These results are indicative that the cholinesterase in the *L. variegatus* larval homogenate was AChE.

Physostigmine, an inhibitor of all cholinesterases, and BW284c51, a preferential inhibitor of AChE, inhibited the enzyme sample at low inhibitor concentrations, while ethopropazine as well as iso-OMPA, both preferential inhibitors of BuChE, only inhibited enzyme activity at much higher inhibitor concentrations. Comparative studies indicated that the observed inhibition was characteristic of AChE (Fromson and Whittaker, 1970, Silver, 1974). Bisquaternary inhibitors, such as BW284c51, are able to penetrate the deep and narrow catalytic gorge of AChE and in doing so can block both the active and peripheral sites, therefore resulting in significant inhibition of AChE (Sussman et al., 1991; Radić et al., 1993). Inhibitors of BuChE are too bulky to penetrate the catalytic gorge, and therefore result in insignificant inhibition. Monophasic dose response curves also suggested that the larval sample contained only one cholinesterase.

Once *L. variegatus* cholinesterase was confirmed as AChE, the molecular forms of AChE in larvae were determined. Velocity sedimentation of larval *L. variegatus* AChE samples on sucrose gradients suggested that dimeric ($G_2$) and tetrameric ($G_4$) globular molecular forms are present. Average sedimentation coefficients ($S$) of *L. variegatus* AChE were similar to $G_2$ and $G_4$ sedimentation coefficients of *Electrophorus*
(7.7 and 11.8 S, respectively; Bon et al., 1976), *Torpedo* (7.8 and 11.1 S, respectively; Bon and Massoulié, 1980), bovine (7.2 and 10.9 S, respectively; Bon et al., 1979), and chicken (7.9 and 11.8 S, respectively; Allemand et al., 1981). The change in sedimentation coefficients of both globular forms suggested that the G₂ and G₄ forms were amphiphilic and interacted with the detergent Triton X-100. The amphiphilic G₂ and G₄ forms interacted with detergent and thus lowered the density and sedimentation coefficient of the molecular forms.

Molecular forms of AChE have been identified in other sea urchin species. Ozaki (1976) reported the presence of a monomer (7.6 S) and dimer (10.6 S) in *S. purpuratus*. Ozaki (1974) reported the presence of multiple forms in *P. depressus*. However, only one form was detected in embryos of *H. pulcherrimus* (Akasaka et al., 1986). Thus, the number of molecular forms of AChE can vary depending upon species. Akasaka et al. (1986) suggested that the production of the various molecular forms of AChE in sea urchin species is controlled by different mechanisms.

Based on previous reports, only globular molecular forms of AChE were predicted in *L. variegatus* larval homogenates. Massoulié et al. (1993) suggested that the triple-helical-collagen tail, which can bind 4, 8, or 12 catalytic subunits forming the A₄, A₈, and A₁₂ asymmetric forms, is only found in vertebrates. Therefore, the existence of asymmetric molecular forms probably arose in the early phylogenetic divergence of the vertebrates. However, additional unidentified peaks at approximately 12 and 17 S, suggested the possibility for asymmetric forms. A sequential extraction for the various AChE molecular forms of adult gonad tissue revealed no asymmetric forms (Appendix B), but larval tissues were not examined. The possibility of asymmetric forms in larval *L.*
variegatus cannot be completely discounted. Further analyses are required to confirm the presence of asymmetric forms in larval L. variegatus.

Effect of Parental Nutrition on AChE Activity throughout Sea Urchin Development

AChE activity of F1 embryos and larvae, from adults (F0) obtained from wild populations or from cultured adults fed either a diet of organic lettuce and carrots, or a nutritionally-complete formulated feed, exhibited similar developmental progressions of AChE activity as reported previously in four other sea urchin species (Augustinsson and Gustafson, 1949; Ozaki, 1974; Ozaki, 1976; Akasaka et al., 1986). Unfertilized L. variegatus eggs and pre-gastrulation stages expressed minimal AChE activity. Augustinsson and Gustafson (1949) have shown that AChE activity in unfertilized eggs and other pre-gastrula stages of the sea urchin, P. lividus, is insignificant when compared to advanced plutei. Ozaki (1974), Ozaki (1976), and Akasaka et al. (1986) have all reported very little AChE activity in pre-gastrula stages in P. depressus, S. purpuratus, and H. pulcherrimus, respectively. A significant increase in AChE activity occurred post-gastrulation in all experimental groups of L. variegatus as well as the three previously reported sea urchin species (Ozaki, 1974; Ozaki, 1976; Akasaka et al., 1986). We suggest that the increase in AChE activity at this developmental stage is related to increased motility. Development of the larval arms occurs post-gastrulation and is the stage at which the larval begin to feed by swimming up through the water column. The exponential increase in AChE activity post-gastrulation would suggest that increased AChE activity is required for the locomotion of the ciliated band, which allows the larvae to swim through the water column and feed (McEdward and Miner, 2007). Nerves have
been reported to be associated with the ciliated band and the larval esophagus (Burke, 1978). Histochemical stains of sea urchin larvae have indicated the presence of AChE in regions specialized for locomotion and feeding such as the mesenchyme cells associated with the larval skeleton, the oral lobe, and the anal arms (Ozaki, 1974; Akasaka et al., 1986). Augustinsson and Gustafson (1949) indicated that at 48 hours of development, larval *P. lividus* had a moving intestine and exhibited high AChE activity. The larval intestine has complex ciliary activity and muscular movement which is highly coordinated. They suggested that increased AChE activity may be needed for digestion.

The level of AChE activity throughout larval development in *L. variegatus* is influenced by the nutritional history of the parents. Larvae of individuals collected from the wild may have different nutrient reserves than offspring of cultured sea urchins, possibly requiring metabolic adjustments. As such, the significantly higher AChE activity levels seen in the wild organisms might be a result of differential locomotion and feeding patterns necessary for survival. In addition, individuals were collected from the same population for both wild and cultured sea urchins, but collections were at different times of the year. Consequently, observed differences may be due to genetic differences between the collected populations. Diet-induced epigenetic regulation is another alternative hypothesis for observed differences during the developmental progression of AChE activity.

Environmental factors may also affect differential expression of AChE activity. Adult sea urchins were obtained from the wild only five days before spawning. These individuals had been exposed to changing temperatures, salinities, and/or other seasonal or cyclical factors, resulting in a variable environmental history. Cultured sea urchins
were held in the laboratory for six months before spawning in defined and constant conditions of temperature, salinity, and photoperiod. The effects of the environmental history on enzyme expression patterns have not been described and will require further study.

Regardless of the mechanism(s) inducing differential developmental expression of AChE activity, these data suggest a significant plasticity in activity expression patterns. The consequence of plasticity may be adaptive, allowing larvae of different environmental or nutritional histories to adapt, thereby increasing survival and fitness. Expression plasticity may also represent a physiological difference in these offspring, with consequence to the developing organisms. To determine whether this plasticity confers an adaptive advantage or physiological consequence would require further culture and evaluation of the individuals.

**AChE Activity in the Gut and Gonad of Adult *L. variegatus***

Neuromuscular coordination of gut function has not been examined in sea urchins. The presence of activity in the esophagus, stomach, intestine, and gonad suggest that there are cholinergic innervations of the musculature of each structure, particularly within the gonad. The gonads of sea urchins have been shown to be innervated. Pearse and Cameron (1991) have shown that the gonadal lobes of the sea urchin overlay the aboral nerve ring. Both the muscular epithelium and the peritoneum of the gonad are infiltrated with neurons probably stemming from the aboral nerve ring. Also, the genital hemal sinus layer of the gonads of sea urchins and sea stars have ciliated myoepithelial cells that could also serve as a source of the AChE activity observed in the gonad.
Hinegardner (1961) recognized the importance of cholinergic innervation in sea urchin gonads and used acetylcholine to stimulate gonad contraction and express gametes for embryological studies.

Newman et al. (1995) have mapped the innervations of the gut of the sea star *Asterias rubens* using monoclonal and polyclonal antibodies and demonstrated extensive neuronal localization within the gut. Extensions of the basi-epithelial nerve plexus in *A. rubens* have been shown to infiltrate the connective tissue layer of portions of the esophagus, cardiac stomach, pyloric stomach, and pyloric caeca. Because AChE and other components of the cholinergic nervous systems are most readily associated with neuromuscular junctions, AChE activity in each of these adult *L. variegatus* tissue homogenates was not surprising. However, factors affecting expression patterns in these organs may ultimately affect organ function and, as a consequence, nutrient allocation and growth.
LIST OF REFERENCES


APPENDIX A

IACUC APPROVAL FORM
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: May 1, 2007

TO: Stephen A. Watts, M.S., Ph.D.
CH-375 1170
FAX: 975-6097

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Sea Urchin Culture for Improved Biomedical/Ecotoxicological Testing
Sponsor: Mississippi-Alabama Sea Grant Consortium
Animal Project Number: 070508135

On May 1, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invertebrates</td>
<td>A</td>
<td>1000</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from May 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 070508135 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.
APPENDIX B

ADULT ACETYLCHOLINESTERASE MOLECULAR FORMS
A sequential extraction of adult *L. variegatus* (obtained from the wild, June 2006) gonad tissue followed by velocity sedimentation on sucrose gradients (5-25% sucrose) was performed. Adult *L. variegatus* were dissected to obtain gonad tissue. Gonad tissue was stored in synthetic seawater and frozen at -30°C until analysis. The gonad tissue was centrifuged at 17,000 G at 4°C for 20 minutes (Sorvall RC 5C). The supernatant was discarded. Approximately 2.5 mL of low ionic strength (LIS) buffer with protease inhibitors were added to the pellet. The sample was homogenized with the Polytron homogenizer and centrifuged at 17,000 G at 4°C for 20 minutes to extract the globular molecular forms of AChE. The supernatant was saved as supernatant 1. Another 2.5 mL of LIS buffer were added to the pellet to serve as a wash for any remaining globular forms. The sample was homogenized and centrifuged as before, and the supernatant was saved as supernatant 2. Another 2.5 mL of high ionic strength (HIS) buffer with protease inhibitors were added to the pellet to extract any asymmetric molecular forms. The sample was homogenized and centrifuged as before, and the supernatant was saved as supernatant 3. Supernatants 1 and 3 were assayed for AChE activity.

Six sucrose gradients (5-25% sucrose) containing Triton X-100 were made. Thirty µL of a catalase marker (11.3 S) were loaded onto the gradients. LIS extracts (435 to 565 µL) were loaded on 3 gradients, while HIS extracts (755 to 1,510 µL) were loaded on the three remaining gradients. Gradients were centrifuged at 35,000 rpm at 4°C for 19 hours (Beckman L7-55 Ultracentrifuge). Following centrifugation, sucrose gradients were fractionated with the Teledyne ISCO Foxy, Jr. fraction collector (Teledyne ISCO, Lincoln, Nebraska) and assayed for catalase and AChE activity. Sedimentation coefficients were determined by Sigma Plot. Analysis of an LIS extract yielded two
peaks at 6.20 S and 14.22 S suggesting that the $G_1$ and possibly $G_4$ globular forms exist in adult gonad tissue (Fig.1). Average sedimentation coefficients of all LIS extracts, 6.43 ± 0.43 S and 14.73 ± 0.51 S (mean ± SE) also suggest that $G_1$ and $G_4$ globular forms exist in adult gonad tissue. When HIS extracts were analyzed, there was insufficient AChE activity in each fraction suggesting the absence of asymmetric forms. A peak near 14 S might represent a modified $G_4$ form.

![Graph]

**Fig. 1.** A sequential extraction of the acetylcholinesterase molecular forms in adult *Lytechinus variegatus* gonad tissue. A series of extractions in first LIS buffer, followed by a wash in LIS buffer, followed by another extraction in HIS buffer. Extracts and a catalase marker (11.3 S) were loaded onto sucrose gradients (5-25% sucrose) and centrifuged for 19 hours at 35,000 rpm at 4°C. Sucrose gradients were fractionated and assayed for catalase and AChE activity. The data presented are representative of a LIS extract.