Pesticide Biochemistry and Physiology 98 (2010) 175–182

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Comparison of catalytic properties and inhibition kinetics of two acetylcholinesterases from a lepidopteran insect

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ARTICLE INFO

Article history:
Received 25 January 2010
Accepted 8 June 2010
Available online 11 June 2010

Keywords:
Acetylcholinesterase
Catalytic properties
Inhibition kinetics
Insecticides
Bombyx mandarina

ABSTRACT

Acetylcholinesterase (AChE) is the primary target of organophosphate (OP) and carbamate (CB) insecticides. Many insect species have been shown to have two different AChE genes. The amino acid identity between the two lepidopteran AChEs is lower than 40%, and potential differences in enzymatic function have not been characterized. In this study, the cDNAs encoding two AChEs (Boma-AChE1 and Boma-AChE2) from *Bombyx mandarina* were sequenced, and the corresponding proteins were heterologously expressed to compare their enzymatic properties and interactions with insecticides *in vitro*. Both of these enzymes had high specific activities for acetylthiocholine iodide. Studies on substrate and inhibitor specificities confirmed that both enzymes belong to AChE. Insecticide inhibition assays indicated that Boma-AChE1 was more sensitive than Boma-AChE2 to eight of the nine insecticides tested. However, Boma-AChE2 was more sensitive than Boma-AChE1 to one of the OP insecticides, heptenophos. The results suggested that two AChEs from a lepidopteran insect have distinct catalytic properties and responses to different inhibitors.

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1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) is a key enzyme in the cholinergic system that regulates the levels of the neurotransmitter acetylcholine (ACh) and terminates nerve impulses by catalyzing the hydrolysis of ACh in the synaptic cleft. The enzyme is encoded by the acetylcholinesterase gene (*ace*) and represents the primary target of organophosphate (OP) and carbamate (CB) insecticides.

Since *ace* was first cloned from *Drosophila melanogaster* [1], this gene has been widely studied in various insect species. A second *ace* locus (an *ace* paralogous), however, was implicated in a different linkage group in *Culex pipiens* [2]. Nevertheless, the first cDNA encoding the paralogous AChE was first sequenced in the greenbug (*Schizaphis graminum*) in 2002 [3]. To date, many insect species have been found to possess two *ace* genes [3–8]. A focus of interest in insect toxicology therefore has been to elucidate the functions of the insect AChE genes. Previously, the enzyme kinetics and inhibitor properties of purified AChE have been characterized in several insect species [6,9]. The purified AChEs can help us understand their functions, however, the mixture of two types of AChEs isolated from the insect bodies made it difficult to understand their difference in catalytic properties and inhibition kinetics. The biochemical properties of *Plutella xylostella* AChE were studied using an enzyme expressed from a cDNA in Sf9 cells cultured in a medium supplemented with 10% FBS [10]. In addition, the *Ace1* cDNA (with and without a Phe455Trp substitution) and the *ace2* cDNA from *Culex tritaeniorhynchus* were expressed in a baculovirus–insect cell system from which the biochemical properties of AChEs were determined [11]. Jiang et al. [12] expressed the catalytic domain of *Anopheles gambiae* AChE1 (r-AgAChE1) using the baculovirus system and examined kinetic parameters of recombinant *A. gambiae* AChE1. Alout et al. [13] compared the AChE1 biochemical properties of *A. gambiae and C. pipiens*. All of these studies have deepened our understanding of the properties of AChE. The difference of two AChEs may be used to develop new selective insecticides [14,15].

Although much work has been carried out on this gene, many questions remain unanswered. For instance, why do fruit flies and houseflies have only a single *ace* in their genome, whereas many other insect species have two? Do they perform overlapping or non-overlapping functions? Are both types of AChEs targeted by OP and CB insecticides? When an insecticide is designed, should the different inhibition kinetics of the two types of AChEs be considered? We believe that studying the expression and properties of the two *ace* gene products separately is one of the best strategies to answer these questions.

The wild silkworm *Bombyx mandarina* is an insect pest of the mulberry plant. In this study, two AChE cDNAs were sequenced

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doi:10.1016/j.pestbp.2010.06.003

from *B. mandarina* and functionally expressed in insect cells cultured in serum-free medium. The enzymatic properties and inhibition kinetics of the two AChEs with respect to nine selected insecticides were compared to understand the differences between two types of AChEs in a single insect species.

2. Materials and methods

2.1. Insects

Bombyx mandarina (Zhejiang) larvae were collected in mulberry gardens in Zhejiang Province in 2007. The samples were frozen immediately and stored at −80 °C until mRNA extraction.

2.2. Chemicals

Acetylthiocholine iodide (ATC), propionylthiocholine iodide (PTC), butyrylthiocholine iodide (BTC), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), BW254c51, eserine, and Iso-OMPA were purchased from Sigma Chemical Co. (USA).

Arprocarb, methomyl, isoprocarb-1, carbaryl, dichlorvos, paraoxon, paraoxon-methyl, omethoate, and heptenophos were kindly provided by the Institute of Plant Physiology and Ecology at Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences in Shanghai, and Institute of Quality Standards for Agricultural Products at the Zhejiang Academy of Agricultural Sciences in Hangzhou.

2.3. PCR amplification and cloning of Boma-ace1 and Boma-ace2

Total RNA was extracted from the whole bodies of 4th-instar larvae using TRIzol according to the manufacturer's protocol (Shanghai Sangon Co., China). First strand cDNA was synthesized from total RNA using reverse transcriptase with an oligo dT primer. Polymerase chain reaction (PCR) primers were designed based on the two *ace* sequences of *Bombyx mori* (GenBank Accession Nos. DQ186605 and DQ115792), which is very closely related to *B. mandarina*.

We used the following primer sets for amplification: *ace1* coding region: Boma-*ace*1F1 (5′-CGG TAT GCG CGT GGT GTT GGC-3′) and Boma-*ace*1R1 (5′-TTA TAT GGT GTA TTT GAA CAG TGC-3′); *ace2* coding region: Boma-*ace*2F1 (5′-TCA CAA TGA TCA ACT ACG GCA AG-3′) and Boma-*ace*2R1 (5′-TTA CAA AGC AAT AGT GAT TGC CA-3′). The primers Boma-*ace*1F2 5′-(<u>CTC GAG</u> ATG CGC GTG GTG TTG GCA GC-3′) (XhoI site underlined) and Boma-*ace*1R2 (5′-<u>AAG CTT</u> TTA ATT CGT ACA ATT CTT CGGC-3′) (HindIII site underlined) were used to amplify a C-terminally truncated fragment of Boma-*ace*1 for the construction of Boma-*ace*1 expression vectors. The primers Boma-*ace*2F2 (5′-<u>AGA TCT</u> ATG ATC AAC TAC GGC AAG-3′) (BglII site underlined) and Boma-*ace*2R2 (5′-<u>CTC GAG</u> TTA CCC GTC ACA CGG TAC AC-3′) (XhoI site underlined) were used to amplify a C-terminally truncated fragment of Boma-*ace*2 for the construction of Boma-*ace*2 expression vectors.

PCR was initiated by an initial denaturation step of 3 min at 94 °C, followed by 31 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 2 min, with a final extension step at 72 °C for 10 min. PCR of Boma-*ace*1 was performed with a buffer specifically designed for GC-rich sequences (Takara Co., China). PCR products were purified, subcloned into the pMD-18T vector (Takara Co., China), and sequenced.

2.4. Generation of recombinant baculoviruses and protein expression in Tn-5B1-4 cells

The C-terminally truncated fragments of the two *ace* genes were inserted into pFastBac1, and the recombinant plasmids were subsequently transformed into competent DH10Bac to obtain recombinant Bacmid DNA. The constructed Bacmid DNAs were transfected into Tn-5B1-4 cells using lipofectin to obtain recombinant virions. AChEs were expressed by infecting Tn-5B1-4 cells with the recombinant virus as our previous report [16]; Tn-5B1-4 cells infected with non-recombinant Bacmids were used as a control.

2.5. Purification of AChEs

AChE was purified by Sepharose affinity chromatography on a computer-monitored liquid chromatography system (AKTAbasic UPC 100 system with Frac 920, GE Healthcare). The affinity column used in this study was prepared using a modified version of a previously described method with ECH Sepharose 4B as a matrix and procainamide as a ligand [17].

The supernatant containing the secreted expression products of the cell culture was harvested by centrifugation at 5000*g* for 15 min to remove cells and then subjected to 50% ammonium sulfate fractionation. The pellets obtained after ammonium sulfate fractionation were resuspended in 25 mM phosphate-buffered solution (pH 7.0 with 0.02% NaN_3) and dialyzed. The dialyzed fraction was then loaded onto a previously prepared procainamide affinity column. After loading the sample, non-specifically bound proteins were removed by washing the column with Buffer A (0.05 M sodium phosphate buffer, pH 7.0, containing 0.05 M NaCl) until no protein was detected in the eluate. The bound AChE was then eluted with Buffer A containing 0.1 M procainamide. Selected fractions were analyzed using AChE activity assays and SDS–PAGE. Fractions containing AChE activity were pooled and dialyzed for 72 h, with a change of distilled water every 12 h; the purified AChE was then concentrated by freeze drying. All samples were stored at −80 °C prior to further analysis. Protein concentration in this study was determined by the Bradford method with a microplate reader at 595 nm with bovine serum albumin as the standard.

The predicted molecular weights of Boma-AChE1 and Boma-AChE2 were calculated with DNA Star software. SDS–PAGE gels were analyzed with a GS-800 Calibrated Densitometer, Quantity One-4.4.0 software. Protein concentration was determined with a microplate reader at 595 nm by the Bradford method, using bovine serum albumin as the standard.

2.6. AChE activity assays

AChE activity was evaluated by measuring the product of an enzymatic reaction with the substrate ATC. The product, thiocholine, which is produced enzymatically by AChE, was evaluated by the method of Ellman with some modifications [18]. The reaction mixture contained 180 μl of 0.1 M phosphate buffer (pH 8.0), 1 mM ATC, 0.1 mM DTNB, and 20 μl of enzyme. The reaction mixture was incubated at room temperature for 5 min, and AChE activity was determined by measuring the OD at 405 nm with a multifunctional microplate reader (Tecan, Genios).

2.7. Determinations of substrate specificity and kinetic parameters

The Michaelis–Menten constant (K_m) and maximal reaction velocity (V_{max}), were determined for the three substrates using Enzyme Kinetics demonstration software (SPSS Enzyme Kinetics module 1.10 Demo). Eleven substrate concentrations of ATC, BTC, and PTC, ranging from 7.8 to 8000 μM, were used. Because both the enzymes were inhibited by high concentration of substrates, we chose substrate inhibition methods to analyze the substrate specificity and kinetic parameters. A non-linear fit to the plot of v against S was performed using the following equation:

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