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Research paper

Molecular cloning, expression and characterization of acylpeptide hydrolase in the silkworm, *Bombyx mori*

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ABSTRACT

Acylpeptide hydrolase (APH) can catalyze the release of the N-terminal amino acid from acetylated peptides. There were many documented examples of this enzyme in various prokaryotic and eukaryotic organisms. However, knowledge about APH in insects still remains unknown. In this study, we cloned and sequenced a putative silkworm *Bombyx mori* APH (*BmAPH*) gene. The *BmAPH* gene encodes a protein of 710 amino acids with a predicted molecular mass of 78.5 kDa. The putative BmAPH and mammal APHs share about 36% amino acid sequence identity, yet key catalytic residues are conserved (Ser566, Asp654, and His686). Expression and purification of the recombinant BmAPH in *Escherichia coli* showed that it has acylpeptide hydrolase activity toward the traditional substrate, Ac–Ala–pNA. Furthermore, organophosphorus (OP) insecticides, chlorpyrifos, phoxim, and malathion, significantly inhibited the activity of the APH both *in vitro* and *in vivo*. In addition, *BmAPH* was expressed in all tested tissues and developmental stages of the silkworm. Finally, immunohistochemistry analysis showed that BmAPH protein was localized in the basement membranes. These results suggested that BmAPH may be involved in enhancing silkworm tolerance to the OP insecticides. In a word, our results provide evidence for understanding of the biological function of APH in insects.

1. Introduction

Acylpeptide hydrolase (APH, EC.3.4.19.1), also known as acylaminoacyl-peptidases, acylamino-acid-releasing enzymes, or formylmethionine aminopeptidases, is a cytosolic enzyme belonging to the prolyl oligopeptidase family of serine peptidases (clan SC, family S9) (Polgar, 2002; Rea and Fulop, 2006). It catalyzes the removal of N-terminal blocked amino acid residues from peptides with diverse N-terminal acyl groups, such as formyl, acetyl, carbamyl, and chloroacetyl, producing an acylamino acid and a peptide with a free N-terminus (Jones et al., 1994). The characteristics and functions of this enzyme have been studied in many eukaryotes (Fujino et al., 2000; Gade and Brown, 1978; Kobayashi and Smith, 1987; Nakai et al., 2012; Quistad et al., 2005; Radhakrishna and Wold, 1989), bacteria (Brunialti et al., 2011; Parravicini et al., 2013), and archaea (Gogliettino et al., 2012; Ishikawa et al., 1998; Kiss et al., 2007).

Besides, many studies have demonstrated that APHs play important roles in organisms. First, some studies suggested that APHs might serve as a part of the secondary antioxidant defense system. For example, human erythrocyte APH, a cytosolic enzyme, is adherent to oxidized erythrocyte membranes and preferentially degrades oxidatively damaged proteins (Fujino et al., 1998; Fujino et al., 2000). Meanwhile, APH from *Trematomus bernacchii* shows remarkable oxidized protein hydrolase activity toward oxidized BSA (Gogliettino et al., 2014). Second, acylpeptide hydrolase can also clear cytotoxic denatured proteins (Shimizu et al., 2004). As APHs can degrade the proteins, it is considered as an important regulator in human cells. Third, acylpeptide hydrolase may be involved in regulation of neuropeptide turnover, such as amyloid-beta peptide, which is the pathogenic agent in Alzheimer's disease (Yamin et al., 2007). Finally, acylpeptide hydrolase is also thought to be a sensitive target for the organophosphorus (OP) compounds in mammals. APH was found to be more sensitive to dichlorvos and diazoxon than acetylcholinesterase (AChE) in rat brain (Richards et al., 1999). A similar result was obtained in porcine brain (Richards et al., 2000). Human and mouse APHs can also be inhibited by some OP compounds (Quistad et al., 2005).

Although APH has been identified in multiple species, the characterization and function of this enzyme in insects have not been reported so far. In this study, we identified a candidate gene (named *BmAPH*) that encodes acylpeptide hydrolase in the domestic silkworm, *Bombyx*

Abbreviations: APH, Acylpeptide hydrolase; AChE, Acetylcholinesterase; BMs, Basement membranes; BSA, Bovin serum albumin; DDP IV, Dipeptidyl peptidase IV; DTT, Dithiothreitol; DAPI, 4′, 6-diamidino-2-phenylindole; *E. coli*, *Escherichia coli*; EDTA, Ethylenediaminetetraacetic acid; FITC, Fluorescein isothiocyanate; Pi, Isoelectric point; IPTG, Isopropythio-β-D-galactoside; NJ, Neighbor-joining; OP, Organophosphorus; PMSF, Phenylmethylsulphonyl fluoride; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; PVDF, Polyvinylidene fluoride; POP, Prolyl oligopeptidase; RACE, Rapid-amplification of cDNA ends; RT-PCR, Reverse transcription-polymerase chain reaction; SDS-PAGE, Sodium dodecylsulfate–polyacylamide gel electrophoresis; TBST, Tris-buffered saline-Tween.

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mori. Then, we cloned and sequenced the gene. The heterogeneously expressed protein of *BmAPH* gene shows acylpeptide hydrolase activity toward Ac–Ala–pNA (AANA). The OP insecticides can inhibit BmAPH activity both *in vitro* and *in vivo*, suggesting that the silkworm acylpeptide hydrolase may be also a target for the OP compounds, and be involved in the OP insecticides tolerance. Immunohistochemistry analyses were performed to survey the distribution of the BmAPH protein. Our results provide evidence for understanding the function of APH in insects.

2. Materials and methods

2.1. Animals

Silkworm *DaZao* strain was reared under normal conditions at 25 °C, 65 ± 5% relative humidity (RH) and 12 h light (L):12 h dark (D) photoperiod with fresh mulberry leaves. The technical-grade insecticides chlorpyrifos, malathion, and phoxim were from Sigma–Aldrich (St. Louis, MO). Larvae of the 3rd day of the 5th instar silkworm were treated with selected compounds in acetone or carrier solvent alone as a control and maintained for 2 days. Mulberry leaves were dipped into the solutions of chlorpyrifos, malathion, and phoxim diluted with distilled water for 20 s. The organic solvent and water on the leaves were evaporated under the room temperature. These doses (0.375 and 0.75 mg/L) were chosen based on the tolerated level from previous investigations in this laboratory. The whole body and midgut of the larvae with insecticides treated for 48 h (whose peritrophic membranes were removed) were collected from each treatment and frozen at −80 °C for subsequent enzyme assay.

2.2. cDNA cloning and rapid-amplification of cDNA ends (RACE)

Several larvae of the 3rd day of the 5th instar silkworm removed from the mulberry leaves were dissected in a cold normal saline solution and grinded in liquid nitrogen to powders and stored in TransZolTM Up (TransGen Biotech, China). Total RNA was extracted using the TransZolTM Up Plus RNA Kit according to the manufacturer's instruction. The first strand of cDNA was synthesized using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix following the manufacturer's instructions (TransGen Biotech, China).

Based on the bioinformatics analyses, several specific primers were designed and used to amplify the DNA sequences of the putative *BmAPH* gene (Table S1). Reverse transcription–polymerase chain reaction (RT-PCR) was performed using S_1/A_1 primers under the following conditions: 98 °C for 30 s; 98 °C for 10 s, 60 °C for 30 s, and 72 °C for 2 min; 98 °C for 10 s, 59 °C for 30 s, and 72 °C for 2 min; 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 2 min; 28 cycles of 98 °C for 10 s, 57 °C for 30 s, 72 °C for 2 min, with a final extension at 72 °C for 10 min. In order to identify the full-length cDNA of the *BmAPH*, the RACE PCR procedures were carried out by SMARTer™ RACE cDNA Amplification kit (Clontech, USA). The specific primers for RACE are listed in Table S1. The following temperature program was used: 94 °C for 4 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. All the PCR products were purified with the EasyPure PCR Purification Kit (Transgen Biotech, China) and analyzed on 1% agarose gel, and then cloned into the pEASY-T1 Simple Cloning Vector (Transgen Biotech, China). The sequences of positive clones were determined with an automated DNA Analyzer (Applied Biosystems 3730xl, Shanghai, China). The sequences were edited and analyzed with the BioEdit (Hall, 1999) and deposited in the GenBank database under the accession number KR094958.

2.3. Multiple sequences alignment and phylogenetic analysis

In order to reconstruct the phylogenetic tree for APHs, the putative protein sequence of the *BmAPH* gene was used as a query sequence to search for the homologous sequences of other species in NCBI (http://www.ncbi.nlm.nih.gov/). Sixteen reported APH protein sequences and five putative APHs in insects (Table S2) were selected to construct the phylogenetic tree. All the sequences were aligned by the MUSCLE algorithm (Edgar, 2004). The phylogenetic tree was constructed by the neighbor-joining (NJ) method implemented in the MEGA6 program (Tamura et al., 2013). Bootstrap values were calculated with 1000 replications. Gaps were completely deleted. The serine protease DDP IV was used as the outgroup.

2.4. Expression and purification of recombinant BmAPH

The open reading frame of *BmAPH* was amplified by PCR using the primers S_4/A_4 containing the *BamH I* and *Not I* restriction sites, respectively. The amplified fragments and the prokaryotic expression vector (pET28a(+): six His-tagged vector) were then digested by the same restriction enzyme (*BamH I* and *Not I*) for 3 h and purified by gel extraction, followed by ligation for overnight (12 h) at 16 °C. The recombinant plasmid was sequenced and named pET28a(+)–*BmAPH*, transformed into the *E. coli* BL21 (*Transetta*, DE3). The positive clones were selected to be cultured in Luria–Bertani medium containing Kanamycin (30 μg/ml) with shaking at 37 °C until the A_{600} reached 0.6–1.0. The recombinant proteins with N-terminal His-tags (rBmAPH) were induced by isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM for 6 h at 30 °C. BL21 cells transformed with pET-28a (+) were incubated to produce control protein in the same culture condition. To purify the recombinant protein, the culture medium was centrifuged at 5000 × *g* for 10 min at 4 °C, and the pellet was collected. The pellet was washed and resuspended in binding buffer (20 mM phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole), lysed by freeze–thaw method, sonicated, and centrifuged at 6000 × *g* for 45 min at 4 °C. The supernatant was collected and the His-tagged fusion protein was purified by using nickel affinity chromatography. The filtered supernatant was loaded onto a HisTrap HP column (GE Healthcare, Buckinghamshire, UK). After washing with 20 ml wash buffer (20 mM phosphate buffer, pH 7.4, 500 mM NaCl, 40 mM imidazole), elution was performed using an elution buffer (20 mM phosphate buffer, pH 7.4, 500 mM NaCl, 300 mM imidazole). The eluate was dialyzed against a phosphate buffer (PBS: 10 mM Na_2HPO_4, 137 mM NaCl, 10 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4) and stored at −80 °C with 10% glycerol until use. Cell extracts of *Escherichia coli* and purified recombinant protein were analyzed by 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and followed staining with Coomassie Brilliant Blue G250. The Coomassie stained band was excised from the gel and *in-gel* digestion was performed with trypsin. The peptide mixture was used for Maldi–TOF–TOF analysis. MASCOT (MatrixScience, UK) was used for database searching against NCBInr database.

2.5. Assay of rBmAPH activity

Acylpeptide hydrolase activity of rBmAPH from *E. coli* was measured using AANA (Bachem, catalog number L-1640) as the substrate (Jones et al., 1994). The release of *p*-nitroanilide ($\varepsilon_{405\ nm} = 7530\ M^{-1}\cdot cm^{-1}$) was monitored at 405 nm with a Synergy™ HTX Multi-Mode Microplate Reader (Biotek). All experiments were carried out in triplicate in 96-well microplates (Corning). Data were fitted to the Michaelis–Menten equation by nonlinear regression (curve fit) with GraphPad Prism. The reaction mixture (200 μl), containing the appropriate amount of enzyme in 50 mM Tris/HCl (pH 7.5), was pre-incubated under the assay conditions for 5 min. Then different concentrations of substrate were added and the release of product was measured. Stock solutions of substrates were prepared in DMSO and the final assay concentration of DMSO was 1.0%. Calculated activities were based on the initial linear phase of release. One unit of rBmAPH activity (U) was defined as the amount of enzyme releasing 1 μM of substrate per minute under the assay conditions.