Pesticide Biochemistry and Physiology 129 (2016) 1–6

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Effects of mutations on the structure and function of silkworm type 1 acetylcholinesterase

B.B. Wang [a,1], F.C. Li [a,1], K.Z. Xu [a,1], M. Ni [a], J.S. Hu [a], J.H. Tian [a], Y.Y. Li [a], W.D. Shen [a,b], B. Li [a,b,*]

[a] School of Basic Medicine and Biological Sciences, Soochow University, Suzhou, Jiangsu 215123, PR China
[b] National Engineering Laboratory for Modern Silk, Soochow University, Suzhou, Jiangsu 215123, PR China

ARTICLE INFO

Article history:
Received 27 August 2015
Received in revised form 9 November 2015
Accepted 9 November 2015
Available online 10 November 2015

Keywords:
Silkworm
Acetylcholinesterase
Mutations
3D structure
Physostigmine
Phoxim

ABSTRACT

AChE is the target of organophosphate (OP) and carbamate (CB) pesticides, and mutations in the gene can significantly reduce insects' sensitivity to these pesticides. *Bombyx mori* is highly sensitive to pesticides. To investigate the effects of mutations on AChE1 structure and function, we used a prokaryotic system to express *B. mori* wild type AChE1 (wAChE1) and mutant AChE1 (mAChE1) in this study. Active AChE1 proteins were obtained after refolding and purification, and wAChE1 and mAChE1 had similar activities. After incubation with 10^{-6} M physostigmine and 10^{-3} mg/mL phoxim, the remaining enzyme activity of mAChE1 was 4.42% and 8.86% higher than that of wAChE1's, respectively. Three-dimensional analysis of mutation AChE1 (mAChE1) revealed that the Ser and Ala side chains extended toward the central part of S285 with distances of just 2.80 Å and 3.68 Å, respectively, which changed the spatial structure of the active center and reduced its sensitivity to pesticides. These results indicated that the mutations altered the 3D structure of AChE1, which may affect the binding of physostigmine and phoxim to the serine residue at the active center, leading to reduced sensitivity. Our study helps understand the relationship between AChE1 mutations and pesticide resistance and provides a new direction for the cultivation of new pesticide-resistant varieties of *B. mori*.

1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7), mainly present in neurons and neuromuscular junctions, catalyzes and hydrolyzes the neurotransmitter acetylcholine (ACh) to maintain normal nerve impulses [1]. AChE is an ellipsoid according to the resolved crystal structure of *Torpedo californica* AChE with a deep and narrow valley on the surface [2]; Y70, Y120, W279, and D72 constitute AChE's peripheral anionic site (PAS) that can quickly combine ACh and guide it into the valley to improve the catalytic efficiency of the enzyme; the valley's inner wall contains F288 and F290 that form the acyl pocket, the side chain of which is stretched into the valley to restrict the space of the active center and improve the specificity of the enzyme; W84, Y330, Y442, and E199 form the choline binding site, and the hydrolysis of ACh is catalyzed by S200, E327 and H440 located at the bottom [3,4]. In order to investigate the relationship between AChE's structure and function, AChE from multiple species was isolated, purified and crystallized. Due to AChE's extremely low expression and high purification difficulty, only *T. californica*, human, *Drosophila melanogaster* [5], and mouse [6] AChE's structures have been resolved.

AChE is the target of organophosphate (OP) and carbamate (CB) pesticides. These pesticides irreversibly bind to the serine of AChE's active center to inhibit its activity of ACh hydrolysis [7], which leads to sustained muscle impulses, insect convulsion, and even death [8]. In recent years, pesticide resistance has been significantly increased due to the widespread use of pesticides in agriculture and forestry [9]. The development of new specific pesticides becomes an important goal of researchers; the mechanisms of pesticide resistance need further study. It is currently accepted that changes in insect AChE are one of the causes of pesticide resistance, which include increased expression of ace [10,11], increased anchoring of *ace* on membrane [12], and *ace* mutations. Among them, *ace* mutations have attracted the most attention and are considered the most important cause of pesticide resistance. In *Bemisia tabaci*, the F392W mutation increases pesticide resistance [13], while *Musca domestica*'s G262V mutation can increase the pesticide resistance by 100 times [14]. Multiple mutations have also been shown to increase the pesticide resistance of insects, e.g. *Bactrocera oleae* with I214V and G488S mutations had 16-fold increase in pesticide resistance [15], and the 3 *ace*1 mutations (A201S, G227A, A441G) were most likely the cause of *Plutella xylostella*'s resistance to OPs [16]. The A441G mutation was also found in *Blattella germanica ace*1, but Kim et al. reported that *B. germanica* is not resistant to OPs, indicating that this mutation is not

* Corresponding author at: School of Basic Medicine and Biological Sciences, Soochow University, Suzhou, Jiangsu 215123, PR China.
E-mail address: lib@suda.edu.cn (B. Li).
[1] These authors contributed equally to this study.

http://dx.doi.org/10.1016/j.pestbp.2015.11.003
0048-3575/

related to pesticide resistance [17]. Li et al. performed structural analysis of *ace*1 and found that the L452G mutation was independently present in resistant *P. xylostella*, suggesting that this mutation may be related to pesticide resistance [18].

B. mori is an important economic insect in China, India, and other developing countries and also a model species of Lepidoptera. Lepidopteran insects are main agriculture and forestry pests, causing billions of dollars of losses each year. *B. mori* can be used to investigate pesticide toxicity and insect resistance, which may help develop new pesticides. *Bmace*1's expression level is higher than *Bmace*2's, and it is inducible by Ops, suggesting its relevance to pesticide resistance [19]. In order to investigate the relationship between *Bmace*1 mutations and pesticide resistance, we used mutations A303S, G329A and L554S and preliminarily analyzed their effects on pesticide sensitivity [20].

In order to further study the effects of some key sites mutant on the functions and structure of AChE1, the mutant *Bmace*1 proteins were expressed in *Escherichia coli* and purified for enzyme activity measurement and inhibition dynamics analysis. The 3D structures of AChE were also obtained before and after mutation to further explore the relationship between *B. mori* AChE structure and function.

2. Materials and methods

2.1. Materials

E. coli strains, Top10 and BL21, plasmid, pET-28a$^{(+)}$, were preserved in the laboratory. *Nde*I and *Xho*I restriction enzymes were purchased from Takara Biotechnology (Dalian) Co. Prestained protein ladder and T4 ligase were purchased from Fermentas. Other reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd.

2.2. Plasmid construction

Primers were designed based on *B. mori ace*1 gene sequence. *Ace*1 signal peptide sequence was eliminated from the primer design, and *Nde*I and *Xho*I restriction sites were added into the upstream and downstream primers, the sequence of primers are *ace*1-*Nde*I 5′- GGAATTC CATATGGGTCCGCACGAGCACCG -3′, *ace*1-*Xho*I 5′- CCGCTCGAGTTATAT GGTGTATTTGAACAGTGC -3′. The wild type *ace*1 (*wace*1) and mutant *ace*1 (*mace*1) were ligated into pET-28a$^{(+)}$, followed by restriction enzyme digestion and sequencing analysis.

2.3. Protein induction, expression, and identification

The constructed pET28-*mace*1 and pET28-*wace*1 were transformed into *E. coli* BL21. A final concentration of 0.1 mM of IPTG was used to induce protein expression, and cells were sonicated and lysed for protein extraction, followed by SDS-PAGE and Western blot analyses.

2.4. Protein extraction, denaturation and refolding

Bacterial cells were collected and suspended in lysis buffer (50 mM Tris, 100 mM NaCl, 0.1 mM PMSF, pH 8.5) with 1 mg/mL lysozyme and incubated in ice bath for 1 h, followed by sonication for 30 min. Inclusion bodies were collected by centrifugation and washed twice with the inclusion body washing buffer (50 mM Tris, 100 mM NaCl, 2 M urea, 10 mM EDTA, 0.5% (w/v) Triton X-100, pH 8.5), before being solubilized in the inclusion body solubilization buffer (50 mM Tris, 100 mM NaCl, 0.5 M L-arginine, 1 M tetramethylammonium chloride, 0.3% (w/v) PEG4000, 6 M guanidine hydrochloride, pH 8.5) at room temperature for 2 h. The supernatant was centrifuged, and renaturation buffer (50 mM Tris, 100 mM NaCl, 0.5 M L-arhinine, 1 M tetramethylammonium chloride, 0.3% (w/v) PEG4000, pH 8.5) was added until 0.5 M was reached for guanidine hydrochloride. Overnight dialysis was done to obtain refolded AChE1 protein.

2.5. Nickel column purification of proteins

One milliliter of Ni–NTA agarose was added to the refolding proteins and mixed evenly at 4 °C for 60 min to adsorb proteins onto the resin. The resin was precipitated and transferred to the column, which was washed three times with wash buffer to remove contaminating proteins. The eluted proteins were analyzed by SDS-PAGE and Western blot.

2.6. AChE1 activity test and inhibition effects analysis

AChE activity was determined using the kit (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instruction. In order to investigate the effects of AChE inhibitors physostigmine and phoxim on the activity of AChE, 20 μL 10^{-6} M of physostigmine and 10^{-3} mg/mL of phoxim were incubated respectively with the 20 μL enzyme solution at 37 °C for 10 min to determine the remaining enzyme activity of AChE.

2.7. Structural analysis of wAChE and mAChE

wAChE and mAChE amino acid sequences (signal peptide sequence eliminated) were uploaded to Swiss-model (http://www.swissmodel.expasy.org/) for structure prediction. Vector NTI was used to analyze the predicted protein structures.

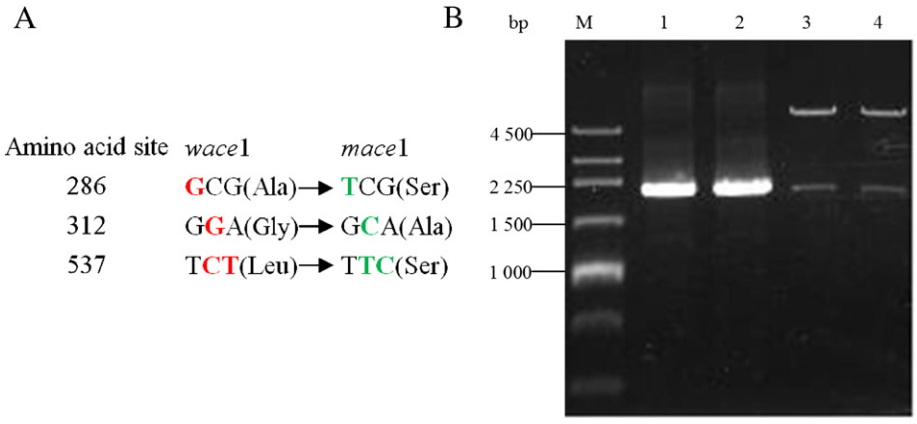

Fig. 1. Mutant nucleotides compare *wace*1 with *mace*1 and plasmid construction. A. Mutant nucleotides and amino acid compare *wace*1 with *mace*1, red and blue fonts indicate wild type and mutated nucleotides, respectively. B. PCR and restriction analyses of prokaryotic expression vectors. M. Marker; 1 and 2. *wace*1 and *mace*1 PCR products, respectively; 3 and 4. pET28a-*wace*1 and pET28a-*mace*1 digestion results, respectively.