Int. J. Indust. Entomol. Vol. 1, No. 1, 2000, pp. 73~78

International Journal of *Industrial Entomology*

Biochemical Properties of Acetylcholinesterase from the Larval Head of *Bombyx mori*

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(Received 27 July 2000; Accepted 10 August 2000)

We investigated some biochemical properties of acetylcholinesterase (AChE) in the *Bombyx mori* larval head. 1% Triton X-100 (v/v) was suitable for extracting AChE from the silkworm larval head but 1 M NaCl was not suitable. PAGE analysis showed a single band of AChE that was detected by histochemical staining using acetylthiocholine as a substrate. AChE was also partially purified with Sepharose 6B and DEAE-cellulose column. Finally, the specific activity of partially purified enzyme solution was 7.6. The study on inhibitor specificity indicated that the enzyme un- der study was a true cholinesterase (ChE) or AChE. AChE activity was maximum at the substrate concentration of 5×10^{-4} M and the excess substrate inhibited the AChE activity. The optimal pH and temperature were pH 7.0 - 9.0 and 30 - 35°C.

Key words : Silkworm, *Bombyx mori*, Acetylcholinesterase, Purification, Inhibitor specificity, Substrate inhibition

Introduction

Acetylcholinesterase (AChE, E.C. 3.1.1.7) plays an important role in the function of nerve impulse transmission. The main action of AChE is the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapse (O'berion, 1978).

Biochemical properties of AChE have been studied in detail in several insects, such as *Musca domestica* (Huang and Dauterman., 1972), *Drosophila melanogaster* (Gnagey *et al.*, 1987), *Lygus hesperus* (Zhu *et al.*, 1991), *Haematobia irritans* (Xu and Bull, 1994), etc. Specially, there are many researches on changes in AChE activity following insecticide exposure, because this enzyme is a target site of organophosphate and carbamate insecticides (Siegfried *et al.*, 1990; Zhu and Brindley, 1990; Rumpf *et al.*, 1997; Francois *et al.*, 1998). *Bombyx mori* is one of most important industrial insect and is extreamly sensitive to insecticides than other insect species (Watanabe, 1978). But there is no research on the biochemical properties of AChE in the silkworm larval head.

In this paper, a method for the partial purification of AChE from *B. mori* was described, and some biochemical properties of the enzyme were also examined.

Materials and Methods

Insects

The domestic silkworm (*Bombyx mori*) strain used in this study was Baeokjam (Jam 123×Jam 124). The silkworm larvae were reared under the standard condition. The 5th instar 3 day old larval heads were cut off and collected.

Chemicals

All electrophoresis reagents such as acetylthiocholine iodide (ATC), Triton X-100, α-naphthyl acetate, Sepharose 6B, DEAE cellulose, bovine serum albumin (BSA), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), eserine, 1,5-bis (4-allyldimethylammonium phenyl)-pentan-3-one dibromide (BW284C51) and ethopropazine were purchased from Sigma Chemical Co. All other chemicals were of analytical qualities and were purchased from commercial suppliers.

Extraction with Triton X-100 and NaCl

Larval head (0.2 g) was homogenized in 1 ml of 0.1 M

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sodium phosphate buffer (pH 7.0) containing 0, 0.5, 1.0 or 1.5% (v/v) of Triton X-100. The homogenate was centrifuged at 10,000 ×g for 1 hr at 4°C, and AChE activity of supernatant was measured. At the same time, a parallel study in which 1 M NaCl was additionally added was also conducted. For the purification purpose, one gram of head was homogenized in 5 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 1.0% Triton X-100 (v/v). The homogenate was centrifuged at 10,000 ×g for 1 hr at 4°C, and the supernatant was subjected to the next step for purification.

Polyacrylamide gel electrophoresis (PAGE)

PAGE under native condition was performed by a modification of the methods of Davis (1964) using 7.5% acrylamide gel. Triton X-100 was added to gel and buffer to obtain a final concentration of 1% (v/v). The general esterases were detected using α-naphtyl acetate as substrate (Kambysellis *et al.*, 1968) and AChE was detected using ATC as a substrate (Karnovsky and Roots, 1964)

Gel filtration chromatography

Sepharose 6B column (1.7×35 cm) was equilibrated in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.5 % Triton X-100 (v/v). Five mililiter of sample was applied to the column and fractions (4 ml per fraction) were collected at a constant flow rate (0.3 ml/min). The fractions containing high AChE activity were combined for the next step of purification.

Ion exchange chromatography

DEAE-cellulose column (1.5×25 cm) was equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.5% Triton X-100 (v/v). Elution was performed with same buffer, which contained a linear gradient of NaCl from 0 to 1.0 M. Fractions (5 ml per fraction) were collected at a constant flow rate (0.3 ml/min). The fractions containing high AChE activity were combined for enzyme property analysis.

Enzyme assay

AChE activity was measured according to the method of Ellman *et al.* (1961) using ATC as a substrate. The reaction mixture (1.16 ml) containing appropriate amount of partially purified AChE, 0.5 mM substrate and 0.32 nM DTNB in 0.1 M sodium phosphate buffer (pH 8.0) was incubated at 25°C for 15 min. The absorbance at 412 nm was determined against the control that lacked enzyme. One unit of AChE is defined as the amount which will catalyze 1 μM of ATC per min at 25°C and pH 8.0.

Protein assay

Protein concentrations were determined with the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Results

Enzyme extraction with Triton X-100 and NaCl

Fig. 1 showed the results of enzyme extraction with the buffer of different concentrations of Triton X-100 and NaCl. When 0.5% Triton X-100 (v/v) was incorporated in buffer, AChE acitivity were 1.8 fold greater than that of the control which lacks Triton X-100 and NaCl. And when each 1.0% and 1.5% of Triton X-100 (v/v) was incorporated respectively, we could gain about 2.3 fold increased activity of AChE. However, when 1 M NaCl was incorporated in the buffer at the same time, we gained only about 1.2 - 1.3 fold increased activity of AChE compared with that of the control. Thus, we used the buffer containing 1% Triton X-100 for AChE extraction for futher study.

PAGE analysis of AChE in silkworm larval head

PAGE analysis showed a single band of AChE that was detected by the histochemical staining method for AChE. AChE band was also stained by the general esterase staining method as indicated by the arrow in Fig. 2. When the specific inhibitor BW284C51 was incorporated in staining buffer, no band was detected with AChE staining method, indicating that the band in Fig. 2B represents AChE.

Partial purification of AChE from silkworm larval head

The profile of gel filtration of AChE in *B. mori* head are shown in Fig. 3. We could gain the AChE activity from

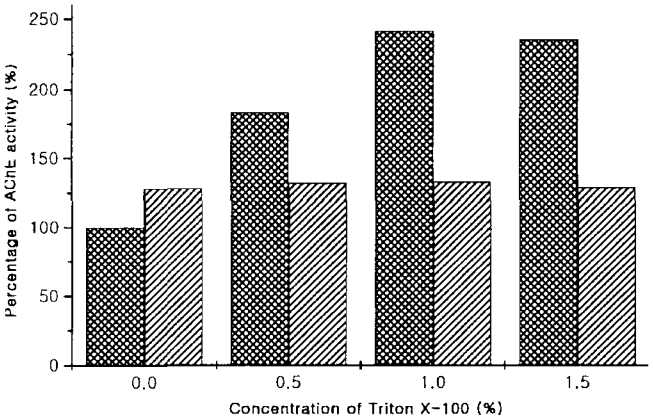

Fig. 1. Solubilization of AChE in the silkworm larval head. Activity in the absence of Triton X-100 and NaCl was considered to be 100%. Data were mean of four determinations. ■ ; 0 M NaCl, ■ ; 1 M NaCl.

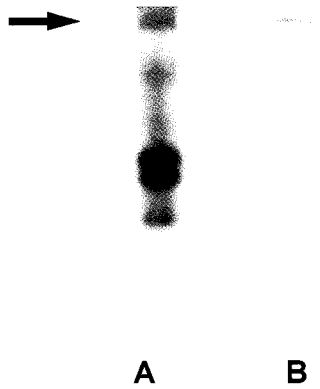

Fig. 2. PAGE analysis of general esterase and acetylcholinesterase in the larval head of the silkworm. A, Staining of general esterase; B, Staining of AChE; C, Stainning of AChE in the presence of BW284C51 in staining buffer. The arrow denotes AChE band.

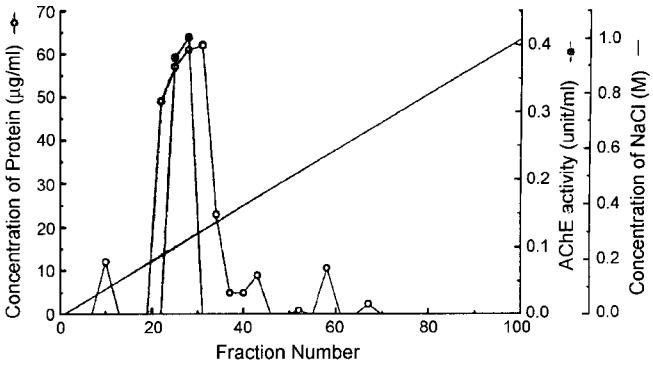

Fig. 4. DEAE-cellulose ion exchange chromatography of AChE in the silkworm larval head. column (1.5×25 cm) was equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5% (v/v) Triton X-100. Elution was with the same buffer, which contained a linear gradient of NaCl from 0 to 1 M. Flow rate was 0.3 ml per min. Five mililiter fractions were collected.

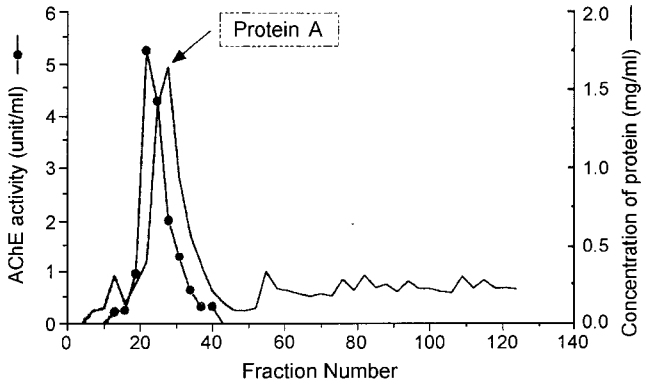

Fig. 3. Sepharose 6B gel filtration of AChE in the silkworm larval head. Column (1.7×35 cm) was equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.5% Triton X-100. Flow rate was 0.3 ml per min. Four mililiter fractions were collected at 4°C. One unit of AChE was defined as that amount which will catalyze 1 μM of acetylthiocholine per min at 25°C and pH 7.5.

fraction No. 25 to 30, and these fractions were combined for DEAE-cellulose chromatography. The profile of ion-exchange chromatography of AChE was shown in Fig. 4 and AChE was eluted at the 0.1 - 0.3 M NaCl concentrations ranged between 0.1 and 0.3 M. As the result, we could get the partially purified AChE solution with the specific activity of 7.6 and the yield of 8.5% (Table 1).

Properties of AChE in silkworm larval head

When ATC was used as a substrate for the enzyme activity of the partially purified AChE, it was noted that the enzyme activity was maximized at the concentration of 5 $\times 10^{-4}$ M and inhibited by the excess substrate (Fig. 5). This result was a typical phenomenon of AChE by substrate inhibition. The Km value of AChE determined by Hanes plot was 3.77×10^{-5} M.

The inhibitor specificity of AChE from the silkworm larval head are shown in Fig. 6. AChE activity was totally inhibited by a non-specific Cholinesterase inhibitor, eserine and a specific AChE inhibitor BW284C51 at 1×10^{-4} M. A specific pseudo ChE inhibitor, ethopropazine, did inhibit the AChE activity but it was much less effective than eserine and BW284C51. The I_{50} value was 0.075 μM for eserine, 0.1 μM for BW284C51 and 0.1 mM for ethopropazine.

The AChE activity was conducted at five pH value ranging from 5.0 to 9.0, with the constant temperature of 25°C (Fig. 7). The maximum AChE activity was measured over a broad pH range from 7.0 to 9.0. Effect of temperature on AChE activity was studied at seven temperatures between 20 - 50°C (Fig. 8). Thus, the optimal

Table 1. Summary of purification procedure for AChE from heda of the silkworm

Procedure	Total volume (ml)	Total protein (mg)	Protein (mg/ml)	Total activity (unit*)	Activity (unit/ml)	Yield (%)	Specific activity (unit/mg)
Crude extract	5	120.5	24.1	157.6	31.52	100	1.3
Sepharose 6B	24	12.09	0.5	62.4	2.6	39.5	5.2
DEAE-cellulose	30	1.65	0.055	12.6	0.42	8.0	7.6

* One unit of AChE is defined as that amount which will catalyze the 1 μM of acetylthiocholine per min at 25°C and pH 8.0.

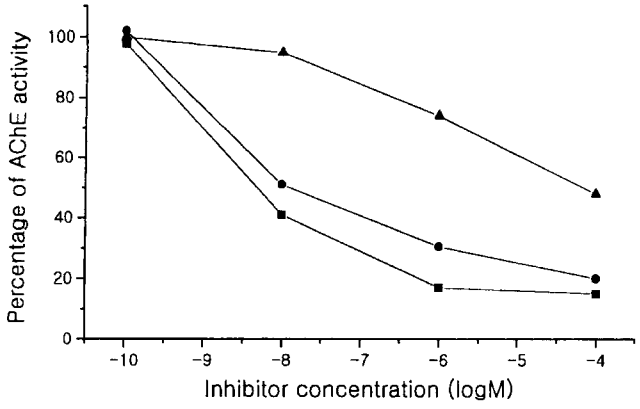

Fig. 5. Effect of inhibitor on AChE activity. Reaction mixture were incubated at 25°C, pH 8.0, 0.5 mM ATC. Data were the mean value of three determination. ▲, ethopropazine; ●, BW284C51; ■, eserine.

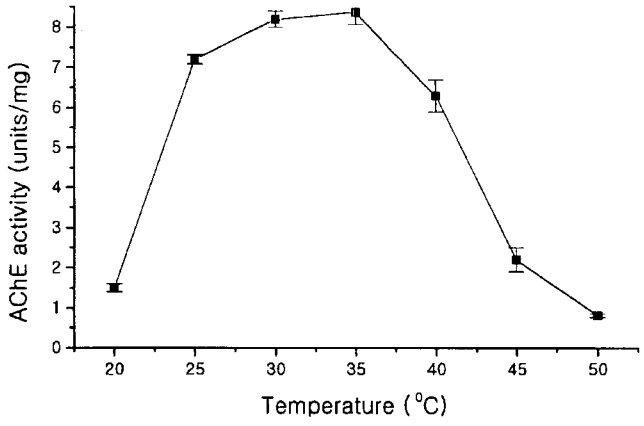

Fig. 7. Effect of temperature on AChE activity. Reaction mixture were incubated at pH 8.0, 0.5 mM ATC. Data were the mean value±SE of three determinations.

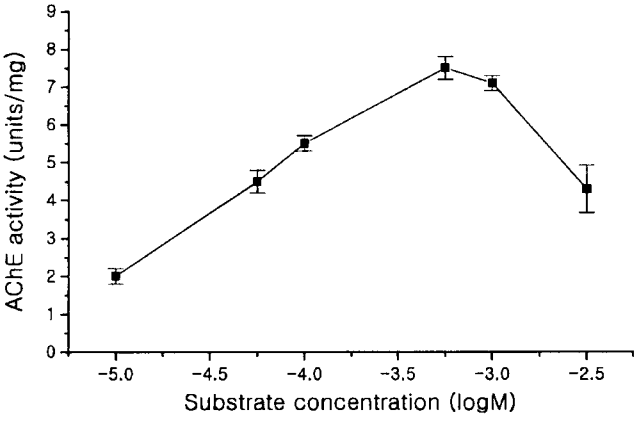

Fig. 6. Effect of ATC concentration on AChE activity. Reaction mixture were incubatedd at pH 8.0, 25°C. Data were the mean value±SE of three determinations.

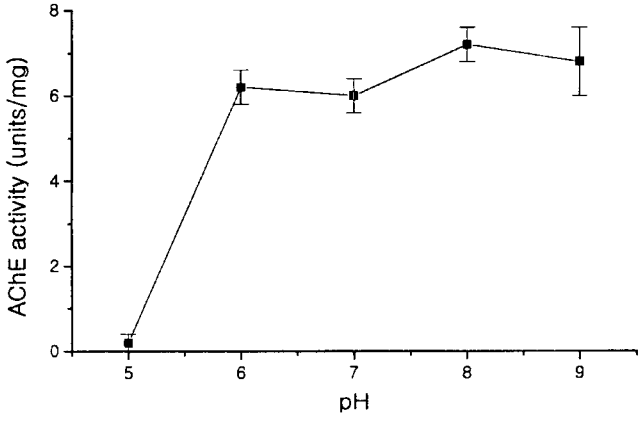

Fig. 8. Effect of pH on AChE activity. Reaction mixture were incubated at 25°C, 0.5 mM ATC. Data were the mean value± SE of three determinations.

temperature was 30 - 35°C, and AChE activity was rapidly decreased above 40.

Discussion

Generally, AChE in insects is membrane-bounded enzyme, so we added Triton X-100 and NaCl to extraction buffer in order to gain more AChE activity (Eldefrawi, 1985). Fig. 1 showed that 1% Triton X-100 (v/v) was suitable for extracting AChE from silkworm larval head but 1 M NaCl was not suitable. So, we could suggest that the Triton X-100 enhanced the solubility of AChE.

The PAGE analysis showed that there was only one AChE isozyme in silkworm larval head like other insects such as house fly (Davis *et al.*, 1987) and tobacco hornworm (Huang *et al.*, 1972). But recently AChE isozymes have founded in some insect although the quantity of that is very small (Xu *et al.*, 1994).

We could partially purified AChE from silkworm by gel filtration and ion exchange chromatography. The specific activity was 7.6 unit/mg and this estimates was lower than what we expected. This result maybe was due to protein A (in Fig. 3) whose size was very similar to AChE. But there is no general esterase activity except AChE activity in partially purified enzyme solution (data not shown). So our partially purified AChE is more profitable than the crude extract because the general esterases disturb studying the biochemical properties of AChE (Motoyama *et al.*, 1984)

The partially purified AChE was totally inhibited by eserine or BW284C51 at a concentration of 1.0 mM but by ethopropazine at the same concentration. This inhibitor specificity was the typical charateristic of the true ChE or AChE (Eldefrawi, 1985).

Our study on the effect of substrate concentration on AChE activity showed that AChE activity was inhibited by the excess substrate (ATC). This phenomenon can be due to the peripheral regulatory site (Cohen *et al.*, 1991)

Table 2. Comparison of characteristics of acetylcholinesterase from various neuronal sources

Source	Km (M)	Optimal pH	Optimal tmeperature °C	Reference
Silkworm head	3.77×10^{-5} (ATC*)	7.0-9.0	30-35	Present
Aphid	6.8×10^{-5} (ATC)	7	-	Breskin *et al.* (1985)
Lygus hesperus Knight	6.9×10^{-5} (ATC)	7.5-8.0	35-40	Zhu *et al.* (1992)
House Criket	1.67×10^{-4} (ATC)	8.0	-	Lee *et al.* (1974)
Housefly head	6.3×10^{-4} (ACh**)	8-9	30-35	Silver (1974)
Manduca brain	2.3×10^{-5} (ACh)	8.5	37	Lester *et al.* (1987)
Mammalian	1.7×10^{-4} (ACh, ATC)	8.0-8.5	37-40	Silver (1974)

*acetylthocholine, **acetylcholine

and is also the typical characteristic of AChE (Eldefrawi, 1985). Km value of AChE from the silkworm larval head was $3.7 \times 7\ 10^{-5}$ M at pH 7.0 using ATC as substrate and it was within ranges typical for insects (Table 2).

The optimal pH and temperature of silkworm AChE was pH 7.0 - 9.0 and 30 - 35°C which is much broader. Like silkworm AChE, *Boophilus microplus* AChE showed no sign of diminishing activity from pH 7.0 to 10 (Nolan and Schnitzerling, 1975) and *Triatoma infestant* AChE activity was gradually increased up to pH 9.0 (Wood *et al.*, 1979). But pH and temperature optimum of AChE in silkworm larval head was broader than other insects (Table 2).

Acknowlegments

This study was supported by special reseach grant (No. 95-45) from Agriculture and Forest Technical Center of the Ministry of Agriculture and Forest.

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