

*Full Length Research Paper*

# Purification and partial characterization of acetylcholinesterase from *Pardosa astrigera* L. Koch

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A simple and effective method was set up to purify acetylcholinesterase (AChE, EC3.1.1.7) from the head and appendage of *Pardosa astrigera*. The AChE was purified by salt fractionation, DEAE-52 ion-exchange chromatography and Superdex 200 methods. The purity and molecular weight of the acetylcholinesterase were detected with sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis. Biochemical properties were measured from the purified AChE by improves Ellmans method. The AChE was extracted from the head and appendage of *Pardosa astrigera*. SDS-PAGE electrophoresis showed a single band with relative molecular weight of 66.35 KDa. However, three bands resolved on PAGE gel electrophoresis, leading to the inference that native AChE exists in three forms. AChE purification fold can be reached to 229.60 when the yield was 13.88%. Significantly highest purified AChE activity was observed when the experiment conditions were 30°C, pH 7.5, 700  $\mu\text{mol.L}^{-1}$  ATChI.  $\text{IC}_{50}$ , the half maximal inhibitory concentration, represents the concentration of an inhibitor that is required for 50% inhibition of things. The  $\text{IC}_{50}$  values ratios of crude extract to purified AChE were 6.54, 7.52, 3.92 and 5.08 times respectively, which was caused by methomyl, phoxim, beta cypermethrin and chlorpyrifos. We got the AChE of the electrophoretically purity from the head and appendage of *Pardosa astrigera*, and the sensitive of purified enzyme was restrained more easily.

**Key words:** Salt fractionation, chromatography, characterization.

## INTRODUCTION

Acetylcholinesterase (AChE, EC3.1.1.7) is an enzyme that breaks down the neurotransmitter acetylcholine at the synaptic cleft (the space between two nerve cells) so the next nerve impulse can be transmitted across the synaptic gap. Thus it plays an important role in the transmission of nerve impulses in the nervous system (Melanson et al., 1985). Pesticides of the organophosphate and carbamate types act to paralyze and kill insects by inhibiting their acetylcholinesterase. The organophosphorus and carbamate are widely used as pesticides to control agricultural pests. As other pesticides, the toxicity to nontarget organisms is a key problem. They cause the accumulation of acetylcholine in

synapses which disrupts the nerve function, and finally results in respiratory paralysis and the death of the organism, the AChE are often used as a specific biomarker for pesticide contamination and the screening of new pesticides in agriculture, food, environmental, and military fields (Zhu et al., 2006). However the non-AChE factors in AChE crude extracts, such as carboxylesterase, can affect the measurement of AChE characteristics and thus affect the sensitivity of the rapid pesticide residues detection. The isolation and purification of AChE is the best way to solve this problem.

Since the first attempt to separate cholinesterase from the horse serum in 1953 (Stedman and Easson, 1932), the AChE have been successfully separate and purify from many animals and a few insect (Xie et al., 2003; Zhang, 2005; Fournier and Cuany, 1987; Wei et al., 2009; Zhu et al., 1992; Gao et al., 1998a; 1998b; Sharon and

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Ryan, 1999; Prabhakaran and Kamble, 1996; Siegfried et al., 1997; Peng et al., 2002; Li, 2003; Peng, 2007; Ma et al., 2004). But little is known the arthropod and this kind of AChE cannot be representative of arthropod arachnida of AChE. Because even different species of aphids, their AChE have significantly different responses to the same inhibitors (Gao, 1988). Here, we reported the purification of AChE from the head and appendage of *Pardosa astrigera* in which the high levels AChE contained, by Salt fractionation, DEAE-52 ion-exchange chromatography and Superdex 200 methods. And further measured the sensitivity of different insecticides on the purified and crude AChE activities.

## MATERIALS AND METHODS

### Reagents

DEAE-52 was purchased from Whatman company (BM0251). Superdex 200 was obtained from Shanghai Yeli Bioscience Company (17-1043-10). Standard proteins (PR1400) for SDS-PAGE were obtained from Shanghai Runcheng Bioscience Company.

SDS molecular weight markers (range 14,400–97,400 Da) was purchased from Sigma chemical company. Other chemicals were from Chinese chemical companies, all of analytical grade. 25 g.L<sup>-1</sup> cypermethrin original drug, from Tianjin Pesticide Company.

Phoxim 40% EC, Xingtai Pesticide Company. 48% chlorpyrifos, the U.S. company Dow Agrosiences. 93.8% methomyl powder, Henan agricultural chemical company.

### *Pardosa astrigera* and rearing conditions

*Pardosa astrigera* was collected from the Guiling Mountain of Taigu County in Shanxi province, China. Living spiders were reared in the tubes, and feed with no food except water supplied with a piece of wet sponge. After two days starvation, the spiders were used for the AChE extraction.

### Methods

#### AChE activity determination

AChE activity was determined as described by Gao et al. (1988) and Ellman et al. (1961), with minor modification that allowed the use of the kinetic assay with a molecular devices BioRad microplate reader. Twenty microL of each enzyme (crude or purified) and 20 uL, 0.6 mM ATChI were mixed, and then the mixture was incubated for 10 min in a water bath at 30°C. Reaction was initiated and monitored at 420 nm for 20 min after the addition of 0.18 mL, 0.4 mM DTNB. Each treatment was replicated 3 times.

#### Determination of protein concentration

Protein content was measured according to Bradford method using bovine serum albumin as the standard (Bradford, 1986).

#### Purification of *Pardosa astrigera* AChE

**Step 1: Preparation of crude extract.** About 1 g cephalothoraxes and appendage of spider adults was collected and homogenized in 15 mL phosphate buffer (pH 7.0, 0.1M, containing 1 mM EDTA, 1%

Triton X-100, 1M NaCl). After filtering via fiberglass, homogenates were centrifuged at 12,000×g for 30 min at 4°C. The supernatant were collected and then added solid ammonium sulfate to 45% saturation on ice with gentle stirring. After solids completely dissolved, let the suspension stand for 20 min at 4°C, and then centrifuged at 12,000×g for 30 min at 4°C. Pellets were collected and dissolved, loaded the dialysis bag, then put into the distilled water, at last condense the mixture by PEG 6000. The enzyme obtained from the above steps was further purified by passing through a DEAE-52 column.

**Step 2: Chromatography on DEAE-52.** DEAE-52 column previously was equilibrated with 0.1 M phosphate buffer (pH 7.0, containing 1% Triton X-100) and thoroughly washed with phosphate buffer. Then the supernatant about 8 mL from step1 was loaded onto the prepared DEAE-52 column and the gel being rinsed with phosphate buffer (containing 0~0.5M NaCl) to gradient elution. The fractions were collected at a constant flow rate (0.3 mL·min<sup>-1</sup>) with an automatic fraction collector at 4°C. Fractions containing the AChE activity were collected as the enzyme resource for the next step.

**Step 3: Chromatography on superdex 200.** The collected fractions from step 2 were loaded onto a Superdex 200 column (length 65 cm and diameter 1.1 cm) which was previously equilibrated with 0.1 M phosphate buffer (pH 7.0, containing 0.4 M NaCl). The gel was rinsed with 0.1 M phosphate buffer at 4°C at the flow rate of 0.3 mL·min<sup>-1</sup>. Fractions containing the highest enzyme activity were collected for the next step.

### Non-denaturing and denaturing polyacrylamide gel electrophoresis

For both non-denaturing (PAGE) and denaturing polyacrylamide gel electrophoresis (SDS-PAGE), 4% stacking gel was overlaid on a 12% resolving gel. Samples were run with constant voltage of 80 V at the stacking gel and 120 V at the resolving gel. PAGE were performed at 4°C whereas SDS-PAGE were at room temperature. Molecular weight markers for SDS-PAGE were a high molecular weight standard mixture including rabbit phosphorylase B (97,400 Da); bovine serum albumin (66,000 Da); rabbit action (43,000 Da); bovine carbonic anhydrase (31,000 Da); trypsin inhibitor (20,100 Da); hen egg white lysozyme (14,400 Da). The gel was stained with Coomassie Brilliant Blue R250.

### Enzymatic properties of crude extract and purified AChE

The effect of pH, temperature, and concentrations of ATChI on enzyme activity was measured. The concentrations of ATChI ranging from 250 to 1200 M, the pH from 5.0 to 9.0, and the temperature from 5 to 75°C.

### AChE activity and sensitivity to four different pesticides

For a sensitivity test, four different pesticides were used to determine the purified and crude AChE activity. The IC<sub>50</sub> ratios were tested by methomyl, phoxim, beta-cypermethrin and chlorpyrifos.

## RESULTS

### Purification of *pardosa astrigera* AChE

AChE from of *Pardosa astrigera* were purified by

ammonium sulfate precipitation, ion-exchange chromatography on DEAE-52 column followed by chromatography on Superdex 200 column. The yields of each step from *Pardosa astrigera* was presented in Table 1. The protease in  $(\text{NH}_4)_2\text{SO}_4$  precipitation was purified to 2.26 fold with a recovery of 52.09%. The enzyme was further purified to 100.81 fold with a recovery of 23.05% through DEAE-52 chromatography (Figure 1). The protease was finally purified by Superdex 200 chromatography, which was purified to 229.60 fold with a recovery of 13.88% (Figure 2).

The purified AChE was resolved as a single band by denaturing polyacrylamide electrophoresis. The relative molecular mass of purified AChE monomer in the presence of SDS was estimated to be 66,350 Da. Furthermore, the purified AChE was resolved into three bands by PAGE, which means AChE of *Pardosa astrigera* has three native forms (Figure 3).

### AChE activity under different reaction conditions

Enzyme activity of crude extract and purified AChE from *Pardosa astrigera* was measured under different conditions. The data were shown here to demonstrate the difference between AChE crude extract and purified enzyme. The changing trend of crude extract activity was similar with that of purified AChE at different temperature ranging from 5 to 75°C, its activity increased with the temperature from 5 to 30°C and the activity reached a maximum at 30°C. Experiments also reveal that enzyme activity of crude extract and purified decreased and temperature continued to climb (Figure 4). The results showed that the inhibition in enzyme activity may be produced by high temperature, which may cause the protein denaturation.

The influence of pH on the activities of crude and purified AChE from cephalothoraxes of *P. astrigera* were shown in Figure 5. Purified AChE showed a higher activity at different pH ranging from 7.5 to 8.0.

The activity of purified AChE decreased when the pH values below 7.5. And the optimal pH value of purified enzyme is 7.5. However, when the pH value was up to 7.0, the activity of crude AChE would be the maximum. The activity showed the variation tendency of falling after 7.0. So, the optimal pH value of crude enzyme is 7.0, however that of purified enzyme is 7.5.

To crude and purified AChE, the enzyme activity improves remarkably as the concentration of substrate was low. When the concentration of substrate was 600  $\mu\text{M}\cdot\text{L}^{-1}$ , the activity of crude AChE being maximum. However, the activity of purified AChE was maximum when the concentration of substrate was 700  $\mu\text{M}\cdot\text{L}^{-1}$ . After the enzyme activity reached the maximum value then has decreasing tendency with the concentration of substrate continue to increase. It illustrated that excess substrate had the inhibition to the AChE (Figure 6).

### The sensitivity of crude and purified AChE to four pesticides

The results indicated that the four pesticides had different inhibition action on the activity of crude and purified AChE (Table 2). The  $\text{IC}_{50}$  of purified AChE was greater than that of crude one. Similarly the sensitivity of purified AChE was also higher than that of crude AChE. The  $\text{IC}_{50}$  values ratios of crude extract to purified AChE were 6.54, 7.52, 3.92 and 5.08 times respectively, which is caused by methomyl, phoxim, beta cypermethrin and chlorpyrifos. The results showed that other esterases existed in the crude enzyme, they may protected the AChE (Gao et al., 1998a).

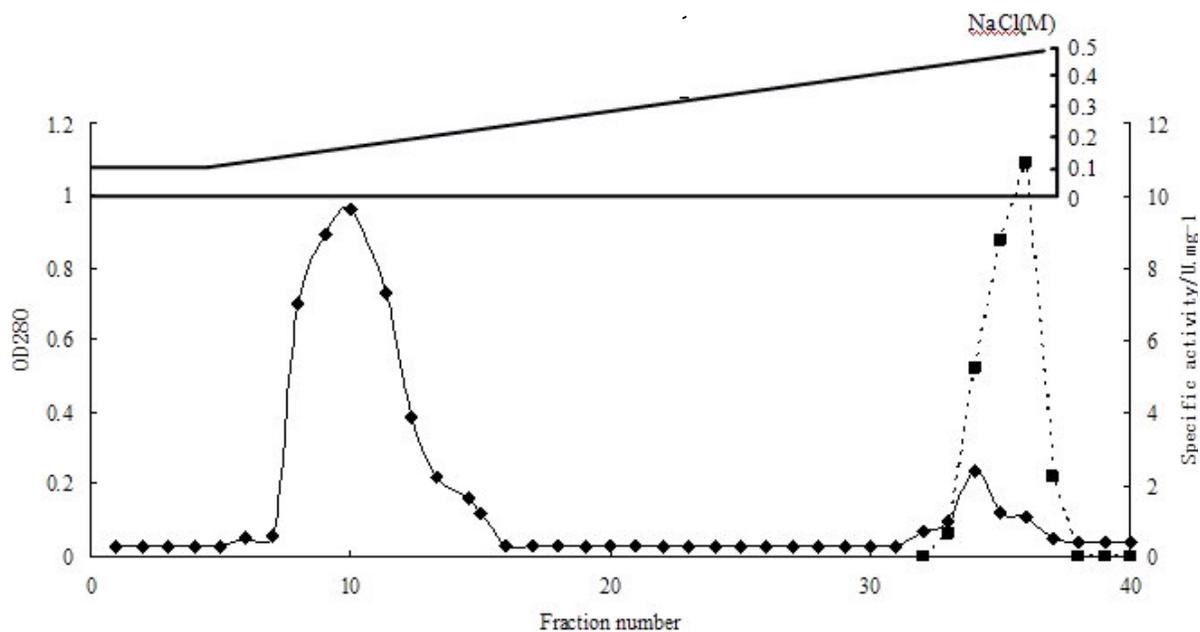
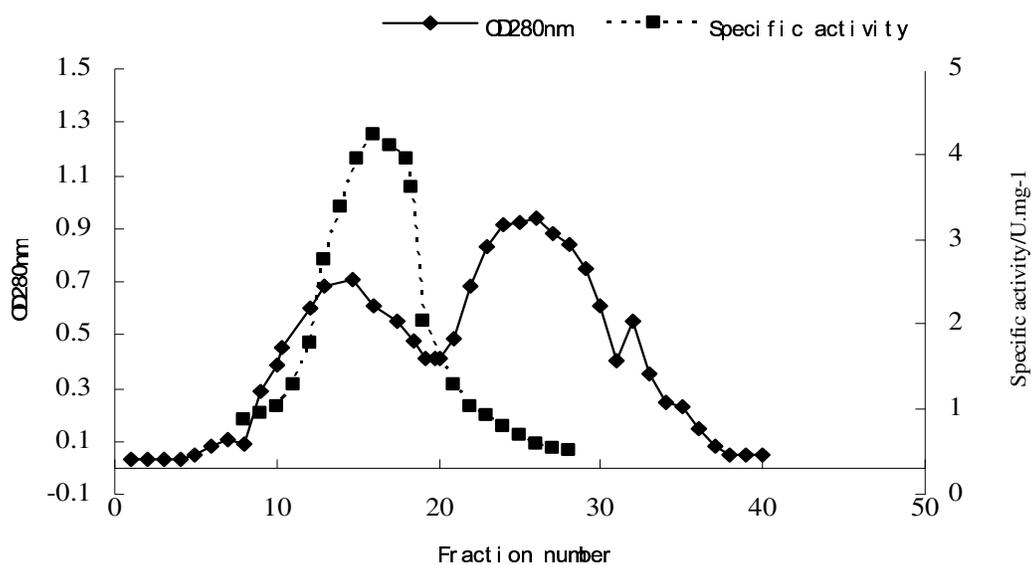
### DISCUSSION

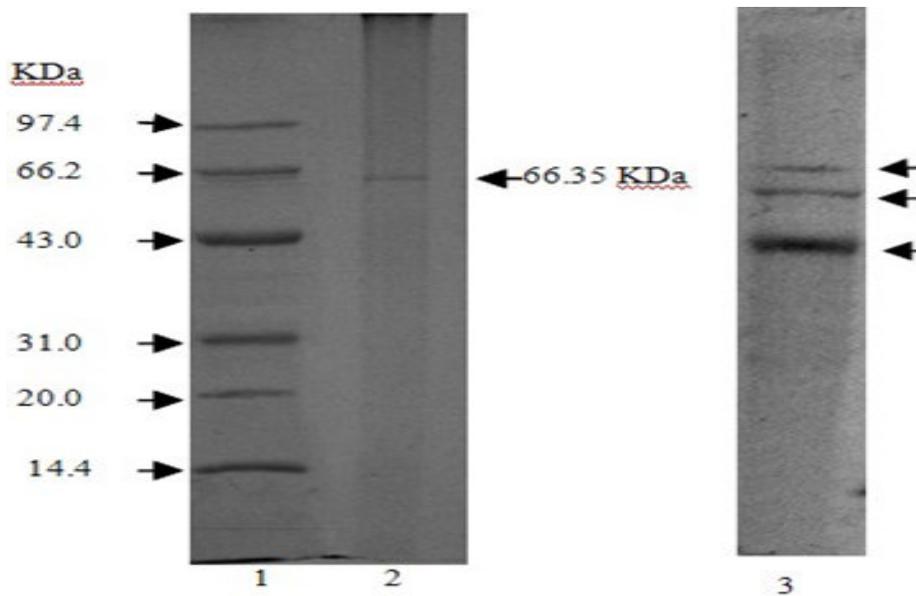
In order to exclude the non-AChE influence, the head and appendage of *Pardosa astrigera* was taken as the test material to purify the AChE by salt fractionation, DEAE-52 ion-exchange chromatography and Superdex 200 methods. Previous studies showed that there are different ammonium sulfate concentrations being used in different experiments, such as 36, 45 and 58% (Du and Dong, 1986). The ammonium sulfate concentration was determined by salting out step by step. When the mass concentration was 45%, the mass of the AChE were purified. When the mass concentration was 60%, few AChE existed in the crude homogenates, the determined optimal ammonium sulfate concentration was 45%. But the purified effect was not ideal, and the quality loss of protein is much, it may be high-protein content and many other proteins in the crude homogenates. All of these factors lead to the loss of protein. But by this method, other protein can be partially removed, and relieve the burden on chromatography. Small portions of the preparations resulting from the purification steps were desalted where possible by dialysis. This step was followed by DEAE-52 ion-exchange chromatography and Superdex 200 chromatography.

Insect AChE exists mostly in different native forms. Electrophoresis on polyacrylamide gradient gel revealed three different native forms of purified *M. domestica* AChE (Fournier, 1987). *L. hesperus* AChE was also reported with three forms (Zhu et al., 1992). In the Colorado potato beetle, *L. decemlineata* (Say), the purified native AChE consisted of two molecular forms (Zhu and Clark, 1994). In this paper, purified *Pardosa astrigera* AChE shows three bands on non-denaturing PAGE. The exact molecular weight for each was not determined. There could be two possible explanations. One was that native *P. astrigera* AChE exists in polymers, which consisted of similar or identical subunits of 66,350 Da that resolved as a single band in denaturing SDS-PAGE. The other is that the three bands in the PAGE gel may correspond to the amphiphilic dimer, the hydrophilic

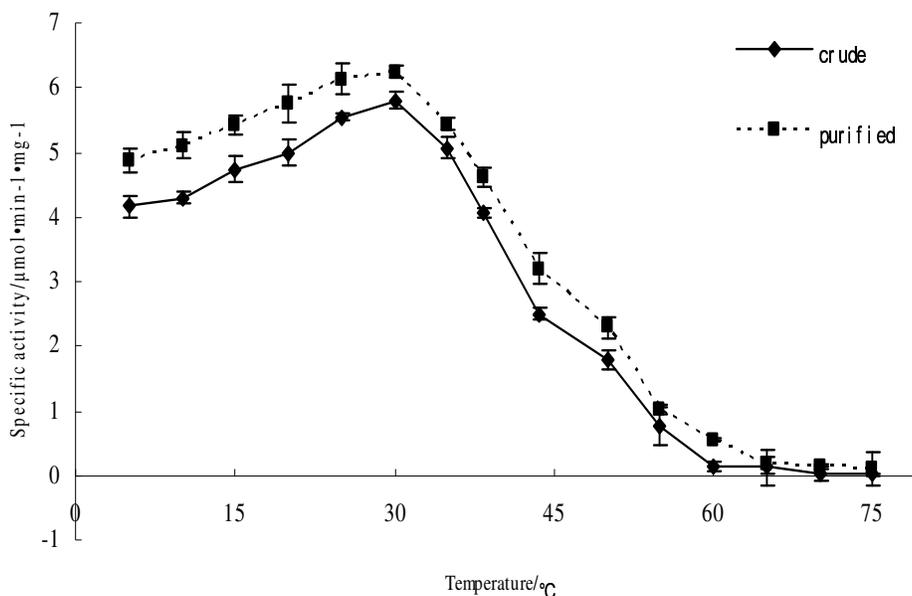
**Table 1.** Results of the purification of acetylcholinesterase from *P. astrigera*.

Purification step	Volume /mL	Total protein /mg	Specific activity/U.mg <sup>-1</sup>	Total activity /U	Purification (fold)	Recovery (%)
Crude extract	15	253.80	0.11	26.65	1.00	100.00
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation	8	58.57	0.24	13.88	2.26	52.09
Dialysis	10	43.07	0.24	10.40	2.30	39.03
DEAE-52	8	0.58	10.59	6.14	100.81	23.05
Superdex 200	3	0.15	24.11	3.70	229.60	13.88

**Figure 1.** Elution profiles of AChE from DEAE-52 at 280 nm absorbance and acetylcholinesterase activity.**Figure 2.** Elution profile of acetylcholinesterase by Superdex 200 chromatography at 280nm absorbance and acetylcholinesterase activity.



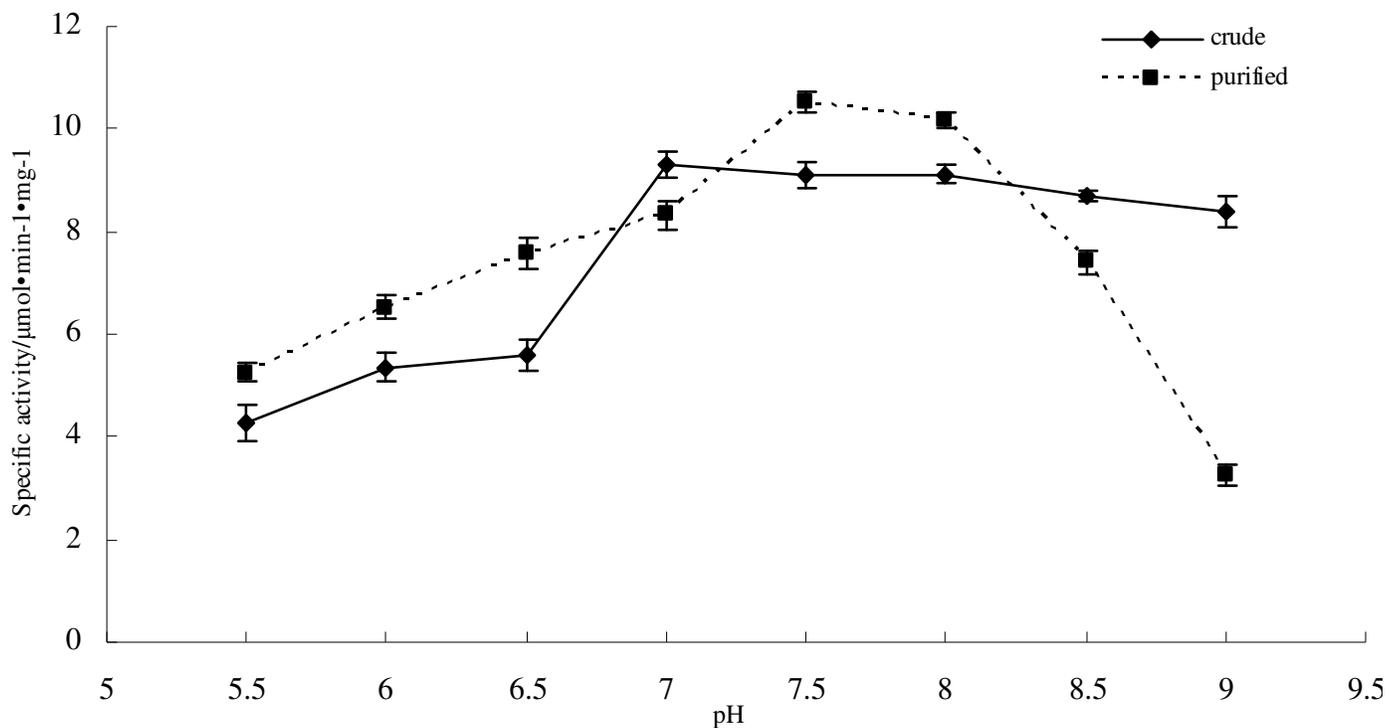
**Figure 3.** Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing polyacrylamide gel electrophoresis (PAGE) of purified AChE from *Pardosa astrigera*. All gels were stained with Coomassie brilliant blue R250. Lane 1: SDS-PAGE protein marker; Lane 2: purified AChE on SDS-PAGE gel, showing a single band. Lane 3: purified AChE on PAGE gel, showing three bands.



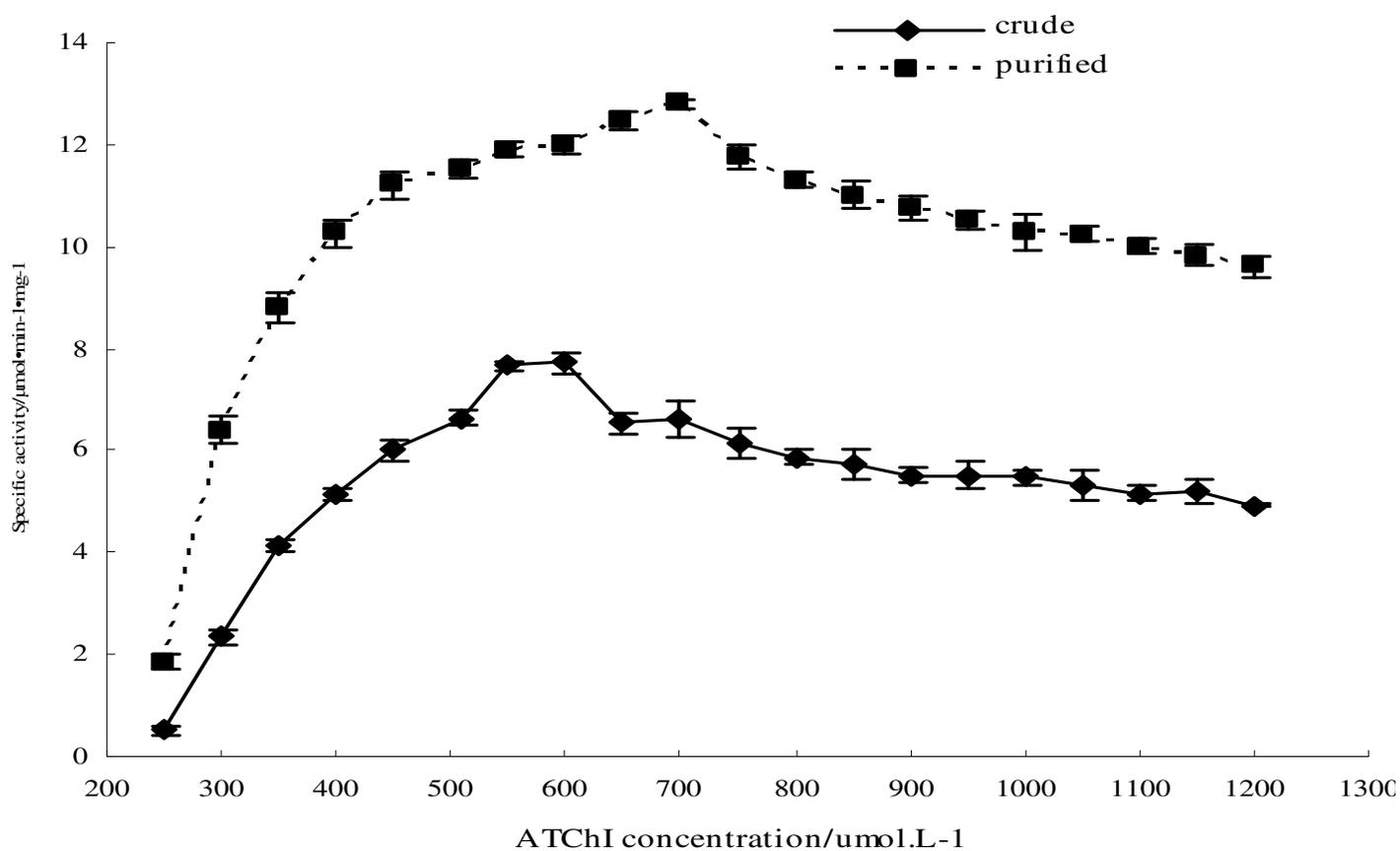
**Figure 4.** Effect of temperature on the activities of crude and purified AChEs from cephalothoraxes of *P. astriger*.

dimer, and the hydrophilic monomer as in some other insects, Gao et al. (1998b) get Schizaphis AChE with molecular weight of 72 KDa, Peng xia (2007) purified the head of silkworm AChE, its molecular weight of 77.8 KDa. In this study, AChE purification fold can be reached to 229.60 when the yield was 13.88%.

IC<sub>50</sub> ratio is often used to indicate the sensitivity of pesticide enzyme inhibitor. As Li shaonan et al. (Li and Fan, 1997) reported that the sensitive of AChE activity in the purified AChE is much higher than that of the crude AChE, and the sensitive of purified enzyme was restrained more easily. Our results showed that the behaviour



**Figure 5.** Influence of pH on the specific activities of crude and purified AChEs from cephalothoraxes of *P. astrigera*.



**Figure 6.** Influence of different substrate on the specific activities of crude and purified AChE from cephalothorax of *P. astrigera*.

**Table 2.** IC<sub>50</sub> values of four insecticides to crude and purified AChE activities from *P. astrigera*.

Insecticide	Crude AChE	Purified AChE	IC <sub>50</sub> ratio
	IC <sub>50</sub> /M.L <sup>-1</sup>	IC <sub>50</sub> /M.L <sup>-1</sup>	Crude/ Purified
Methomyl	7.76×10 <sup>-5</sup>	1.18×10 <sup>-5</sup>	6.54
Phoxim	1.76×10 <sup>-4</sup>	2.34×10 <sup>-5</sup>	7.52
Beta cypermethrin	4.12×10 <sup>-4</sup>	1.05×10 <sup>-4</sup>	3.92
chlorpyrifos	4.94×10 <sup>-4</sup>	9.72×10 <sup>-5</sup>	5.08

of crude extracts was dissimilar with that of purified AChE. And the results indicated that there might be some unknown factors in the extracts, which influence the measurement of AChE activity. AChE sensitivity to methomyl, phoxim, beta cypermethrin and chlorpyrifos, was also seriously affected. Moreover, the different insecticides have different IC<sub>50</sub> ratio and maybe that has to do with different kind of pesticides or living in different pesticide sensitivity. The purified AChE from the spider was 6.54, 7.52, 3.92 and 5.08 fold more sensitive to methomyl, phoxim, beta cypermethrin and chlorpyrifos, respectively. The dissimilarity of biochemical characteristics and the magnification of AChE insensitivity to inhibitors and pesticides have a close relation to the non-AChE protein, especially factors related to pesticide resistance, such as esterase, MFO, and glutathione S-transferase (Li, 2003). These non-AChE existed in the crude enzyme also significant impact on the result of biochemical properties of AChE.

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