Chapter IV

PROPERTIES OF CARBOXYLESTERASE FROM MIDGUT OF H.ARMIGERA
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INTRODUCTION

Enzymes are responsible for virtually all of the biochemical reactions proceeding in cells. The existence and the power of enzymes first were revealed in the nineteenth century when reactions that had been considered to occur only in the presence of cells and were found also to be mediated by cell free extracts.

Enzyme increases the rates of biochemical reactions by lowering the activation energy. In comparison with most catalyst, enzymes are especially effective. They are efficient in catalyzing reactions at high rates, at moderate pH in presence of substrate, of coenzymes and cofactors, etc. However, most of the time, being protein in nature, they are highly susceptible to change in their surrounding environment like extreme pH, temperature, enzyme concentration, substrate concentration etc. The rate of enzyme catalysed reactions respond to the subtle shift in pH. Enzyme shows minor or no activity at extreme pH and hence when enzymatic activity is observed in between pH 5-9, most of the time it is optimum. The rate of enzyme catalysed reactions is effected by temperature, rise in temperature increases the rate of reaction but this holds true only over a strictly limited range of temperature, owing to increase in kinetic energy of the reacting molecules; eventually, however, the kinetic energy of the enzyme exceeds the energy barrier for breaking hydrogen bonds and weak van der waals interactions which maintains its structure is crossed and enzyme lost its activity.

Substrate concentration also plays a key role for the optimum activity of an enzyme. If the concentration of substrate is increased, while all other conditions are kept constant, the initial velocity (the velocity when very little substrate is acted)
increases to a maximum value $V_{\text{max}}$ and then remain constant. The same is true for the enzyme concentration.

A large number of insecticides to which insects are exposed are esters of phosphoric and carbamic acids such as the organophosphates and carbamates. These esters are split by hydrolysis into their parent compounds, namely, the alcohols and the acids. Carboxylester hydrolase is associated with organophosphate and carbamate metabolism (Vishnu Mittre, 1967).

The metabolism of carbaryl has been extensively studied in insects and mammals. The following scheme shows the metabolic pathways of carbaryl as determined in vivo in houseflies, cockroaches and rabbits and also in vitro in some other animals.
Present study deals with effect of substrate concentration, enzyme concentration, pH, temperature and inhibitors on carboxylesterase.

**MATERIAL AND METHODS**

**Enzyme Source**

Purified carboxylesterase obtained by ion exchange chromatography.

**Chemicals**

α - naphthol, α - naphthyl acetate, bovine serum albumin fraction V, copper sulphate, CDNB, fast blue BB salt, folin's reagent, sodium azide, sodium carbonate, sodium phosphate diatomic. All chemicals were of high quality and were procured from reputed dealers or manufacturers in the country and abroad.

**Reagent Preparations**

1) **100 mM phosphate buffer pH - 7**: 1.184 gm of sodium phosphate dibasic and 0.52 gm of sodium phosphate monobasic were dissolved in 100 ml distilled water.

2) **Substrate stock solution**: (30 mM α-naphthyl acetate) 0.558 gm α-naphthyl acetate was dissolved in 100ml acetone.

3) **Working solution**: 0.33, 0.66, 1.0, 1.33, 1.66 ml of substrate stock solution were added to 100 ml phosphate buffer separately containing 0.10, 0.20, 0.30, 0.40, 0.50 mM α-naphthyl acetate respectively.

4) **Staining solution (prepare fresh)**: 1% Fast blue BB salt w/v in phosphate buffer (40mM, pH 7.0) and 5% SDS w/v in distilled water were prepared. The quantity of staining solution needed was determined and 2 parts of 1% Fast Blue BB solution was added to 5 parts of 5% SDS.
5) **Preparation of Phosphate buffer ranging from pH 6.0 to 8.8:**

**Stock solution A:** 0.1M sodium phosphate monobasic.

14.196 gm sodium phosphate monobasic was dissolved in 1000 ml distilled water.

**Stock solution B:** 0.1 M sodium phosphate dibasic.

15.601gm sodium phosphate dibasic was dissolved in 1000 ml distilled water.

i) **0.1 M SPB pH 6.0:** 12.0 ml stock A was added to 88.0 ml stock B to make 100 ml buffer.

ii) **0.1 M SPB pH 6.4:** 25.5 ml stock A was added to 74.5 ml stock B to make 100 ml buffer.

iii) **0.1 M SPB pH 6.8:** 46.3 ml stock A was added to 53.7 ml stock B to make 100 ml buffer.

iv) **0.1 M SPB pH 7.2:** 68.4 ml stock A was added to 31.6 ml stock B to make 100 ml buffer.

v) **0.1 M SPB pH 7.4:** 84.5 ml stock A was added to 15.5 ml stock B to make 100 ml buffer.

vi) **0.1 M SPB pH 8.0:** 93.2 ml stock A was added to 6.8 ml stock B to make 100 ml buffer.

vii) **0.1 M SPB pH 8.4:** 97.86 ml stock A was added to 7.14 ml stock B to make 100 ml buffer.

viii) **0.1 M SPB pH 8.8:** 102.52 ml stock A was added to 7.48 ml stock B to make 100 ml buffer.

6) **Enzyme concentrations:** 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 µl of the enzyme solution were added to 995, 994, 993, 992, 991, 990, 989, 988, 987, 986, 985 µl phosphate buffer (40mM, pH - 7.0) respectively.
Methods of Determination of Properties of Carboxylesterase

All assays were performed by the method of Gomori et al., (1953) with slight modifications.

1. **Effect of Temperature and Thermal Stability**: The enzyme was incubated at different temperature between 10°C to 90°C for 20 min and assayed each time at the same temperature using α-naphthyl acetate. The temperature stability was studied by incubating the enzyme at different temperature between 45 to 50°C for different intervals of time followed by rapid cooling to 0°C.

2. **Effect of pH**: To check the stability of enzyme towards pH variations, the enzyme was incubated at 0°C in buffers of different pH from 6.0 to 8.8 for 24 hrs. then each tube was kept at room temperature for 30 min. and the assays were performed at 37° C for each tube seperately.

3. **Effect of Substrate Concentration**: The enzyme was incubated at different substrate concentration between 0.1 mM to 0.5 mM for 20 minutes and assayed at each concentration using 0.1M sodium phosphate buffer pH 7.0 for esterase activity at 37° C.

4. **Effect of Enzyme Concentration**: The different enzyme concentrations between 5 µl to 50 µl were incubated at its optimum pH and temperature and assayed by using α-naphthyl acetate as a substrate.

5. **Effect of inhibitor**: The enzyme was incubated at different concentrations of inhibitors between 2.5 µM to 15 µM for carbaryl and 0.25 µM to 1.5 µM for paraoxon separately for 20 minute. The activity was assayed for carboxylesterase.

6. **Effect of SDS and 2 Mercaptoethanol**: The enzyme was incubated with SDS and 2 mercaptoethanol and then assayed for esterase activity along with untreated enzyme as a control.
RESULTS

1) **Effect of Temperature on Carboxylesterase Activity**: When the purified carboxylesterase was analysed under different temperatures ranging from 10°C to 90°C, highest carboxylesterase activity was found to be present at 47°C (Fig. 1) and remains stable in between 45° to 50°C.

2) **Effect of pH on Carboxylesterase Activity**: When the purified carboxylesterase was analysed under different pH range from 6.0 to 8.8, it was found to be optimally active at pH 7.6. The carboxylesterase enzyme was stable between pH 7.4 to 7.8 (Fig. 2).

3) **Effect of Substrate Concentration on Carboxylesterase Activity**: When the purified carboxylesterase was reacted under optimum conditions with different concentrations of substrate (0.1 to 0.6 mM), the purified enzyme showed highest carboxylesterase activity in 0.3 mM of α-naphthyl acetate concentration (Fig. 3) which remain unchanged by increasing upto 0.6 mM.

4) **Effect of Enzyme Concentration on Carboxylesterase Activity**: When the purified carboxylesterase (35 μg/μl protein) was analysed under different enzyme concentrations ranging from 5 to 15 μl, the purified enzyme showed highest carboxylesterase activity in 10 μl concentrations with the substrate α-naphthyl acetate (Fig. 4), in optimum concentrations and all optimum parameters.

5) **Effect of Carbaryl and Paraoxon Inhibitor on Carboxylesterase Activity**: When the purified carboxylesterase was analysed under different concentrations of carbaryl and paraoxon, the complete inhibition of carboxylesterase was found to be at 10 μM and 1 μM concentration of carbaryl and paraoxon respectively (Fig. 5 and 6).
Effect of Temperature on Carboxylesterase

Temperature °C

Unit/min/mg

Carboxylesterase activity

Fig. 1
Effect of Substrate Concentration on Carboxylesterase Activity

Fig. 3
Effect of Carbaryl on Carboxylesterase Activity

Concentration μM

2.5  5  7.5  10  12.5  15

μM/min/mg

<--- Carboxylesterase activity

Fig. 5
Effect of Paraaxon on Carboxylesterase

Fig. 6

Carboxylesterase activity

µM

0.25 0.50 0.75 1.0 1.25 1.5

1µM/min/mg

0 0.5 1 1.5 2 2.5 3
6) **Effect of SDS and 2 Mercaptoethanol on Carboxylesterase**:

The midgut homogenate was treated with SDS and 2 mercaptoethanol, to check inhibitory effect on carboxylesterase activity. It was found that when 5% SDS (10 μl) was added to homogenate (0.041 mg/ml protein), it showed negligible inhibitory effect as compare to control (0.96 μM/min/mg). On the contrary, the addition of 2 mercaptoethanol (10 μl) to homogenate (0.28 mg/ml protein), it showed significant inhibition in carboxylesterase activity (0.96 to 0.016 μM/min/mg). However 1 mM 2 mercaptoethanol have showed negligible inhibitory effect. When the combination of 5% SDS and 2 mercaptoethanol was treated with homogenate, it showed inhibitory effect (0.96 to 0.28). However 5% SDS along with 1 mM 2 mercaptoethanol was not having any inhibitory effect (Table 1).

**Table 1 : Effect of SDS and 2 Mercaptoethanol (ME) on Carboxylesterase Activity in H.armigera**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Sample</th>
<th>Total protein (mg/ml)</th>
<th>Carboxylesterase activity (μM/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Gut homogenate</td>
<td>0.048</td>
<td>0.96</td>
</tr>
<tr>
<td>2)</td>
<td>5% SDS + homogenate</td>
<td>0.041</td>
<td>0.90</td>
</tr>
<tr>
<td>3)</td>
<td>2ME + homogenate</td>
<td>0.28</td>
<td>0.016</td>
</tr>
<tr>
<td>4)</td>
<td>1mM 2ME + homogenate</td>
<td>0.042</td>
<td>0.89</td>
</tr>
<tr>
<td>5)</td>
<td>5% SDS + 2ME + homogenate</td>
<td>0.016</td>
<td>0.28</td>
</tr>
<tr>
<td>6)</td>
<td>5% SDS + 1mM 2ME + homogenate</td>
<td>0.043</td>
<td>0.94</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Many studies have revealed that carboxylesterases are a heterogeneous group of enzymes with broad and overlapping substrate specificities as well as different
inhibitor sensitivities and molecular properties (Hosokawa et al., 1990, Heymann, 1980; Brandit et al., 1982). In termites, midgut revealed the presence of two carboxylesterases based on their substrate specificity and inhibitor sensitivity (Morikawa et al., 1976; Holmes and masters, 1967). The same criteria were used to designate enzymes as carboxylesterase in the beetle H. cearulae (Veerbhadrapa et al., 1980). The rat plasma carboxylesterase activity was found to be highest with substrate α-naphthyl acetate (Klas Benth, 1968). This enzyme has highest affinity for substrate α-naphthyl acetate. The enzyme and substrate concentration required to form product α-naphthol in our studies are same as described by Kranthi et al., (1997). Parker et al., (1991) tested esterases from Australian sheep blowfly, L. cuprina for their ability to hydrolyze a series of esters which differed in the length of their acid side chains by using α- and β-napththyl acetate, α- and β-napththyl propioniate, α - and β - napththyl butyrate and β-naphthylol erate as these studies revealed that all the esterases were able to use either α- or β - napththyl acetate alone, but most of them utilized α-naphthyl acetate.

Our studies on carboxylesterase from midgut of H.armigera revealed that the carboxylesterase is active upto 70°C and optimum temperature for its activity is 47°C. Similar results were obtained in O.horni showing optimum carboxylesterase activity in between 45°C to 50°C and complete inactivation at 70°C (Sreerama et al., 1991). These results of H.armigera are similar to Kapin and Ahmad (1980) who reported that midgut esterase activity was linear upto 50°C in L.dispar with α-napththyl acetate as substrate. The decrease in activity may be due to denaturation of the enzyme. Similar results were reported with Flyhead cholinesterases (Chadwick and Lovell 1958) 14 C-deltamethrin hydrolysing activity was increasing upto 50°C when 10,000 x g supernatant was used as enzyme source and had considered 32°C as the optimum temperature (Verma, 1988).
Ishaaya and Casida (1980) reported 32°C as temperature optima for trans-permethrin hydrolysis though the reaction was linear up to 40°C. The metabolizing activity by the microsomal fraction was at its peak at 30°C. In contrast to our studies Hansen and Hodgson (1970) reported that the peak activity of insect microsomes is between 30°C and 33°C. This compares with 30°C for DDT hydroxylation in T. infestang microsomes (Agosin et al., 1969). In house fly, the temperature optima for NADPH - Cytochrome C-reductase was reported to be 40°C. After that with increase in temperature there was denaturation of enzyme (Wilson and Hodgson, 1971).

In our studies carboxylesterase from midgut of H. armigera was found to be optimally active at pH 7.6 and remained stable in the range of pH 7.4 to 7.8. This may be because of alkaline pH of midgut of this insect pest. In the termite O. horni, carboxylesterase is optimally active in between 6.5 to 7.0 pH (Sreerama 1991). According to Yu (1990), the optimum pH was 8.3 for esterase from tobacco budworm, corn earworm, cabbage looper, and spined soldier bug. At alkaline pH of 9.0 to 9.6, the maximum esterase activity was found in C. compactiuscula. The pH optima of most carboxylesterases from mammalian sources have been consistently reported to be in the range of pH 7.5 - 9.0 (Krisch, 1971). Similar results were also reported with various substrates in other species; pH 7.6 for C. tarsalis, pH 7.5 for Musca domestica (Van Asperen, 1962), a pH range of 6.5 to 8.5 for P. americana (Cook and Forgash, 1965) and for transpermethrin hydrolysis, it is between pH 8-9 in T. ni (Ishaaya and Caside, 1980). Esterase activity by the gut mohogenate of tobacco budworm was optimum at pH 7.8 (Shang and Soderlund, 1984). Hansen and Hodgson (1971) reported pH optimum between 7.7 and 8.1 for microsomal demethylation in insects. It has also been stated that pH between 7.0 and 8.0 would not matter much if the ionic strength of the buffer used is low.
In present studies, there is no effect of 5% SDS and 1 mM 2-mercaptoethanol on carboxylesterase activity in *H. armigera*. 2 mercaptoethanol showed significant inhibitory effect on carboxylesterase activity when treated alone but the same concentration of 2 mercaptoethanol added along with 5% SDS, the inhibitory effect was not that much. It showed that 5% SDS is having protecting effect over inhibitory effect of 2 mercaptoethanol. For general esterase, the enzyme activity remains unaffected upto 1.2 to 2 mM mercaptoethanol (Sadashivam, 1992). These findings confirms the absence of cystein in the active site of carboxylesterase. Native esterase enzyme from ox liver traeated with 0.72 M mercaptoethanol for 1 hr. at room temperature showed no loss of activity. Further, no free - SH groups could be detected after removal of the mercaptoethanol by dialysis. Similarly 6.5 M urea showed measurable free - SH contents on dialysis in nitrogen (Runnegar et al., 1969).

Our studies on inhibitors showed that carbaryl and paraoxon are the best inhibitors. Similar inhibitory response of the carboxylesterase towards sumioxon and carbaryl was best observed in the *H. armigera* populations collected from various regions of the country (Mehrotra and Phokela, 1986). This is because, they have been implicated in detoxification of carbamate and organophosphate. The presence of serine containing esteratic site was confirmed by the fact that the esterase activity was inhibited by arganophosphate compounds (Ecobicon 1979). In germinated finemillet, studies with different organophosphate and carbamate inhibitors showed that this enzyme was more sensitive to organophosphate inhibitor than carbamates (Upadhaya et al., 1985). Thus the studies revealed that carboxylesterase from midgut of *H. armigera* is heat stable, chemical stable with optimum pH of 7.6. Carbaryl and paraoxon are formed to be good inhibitors.