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Esterase Activity in the Greater Wax Moth Larvae

Daphne Lin Pai

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ESTERASE ACTIVITY IN THE GREATER WAX MOTH LARVAE

A Thesis
Presented to
the Faculty of the Department of Chemistry
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
of the Requirements of the Degree
Master of Science

by
Daphne I. Lin Pai
December 1974
ESERASE ACTIVITY IN THE GREATER WAX MOTH LARVAE

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ESTERASE ACTIVITY IN THE GREATER WAX MOH LARVAE

Daphne I. Lin Pai  December 1974  31 pages

Directed by: David R. Hartman and Larry C. Byrd

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Some properties of esterases of the greater wax moth larvae, Galleria mellonella (L.), were examined and the enzymes partially purified. Several buffer systems were tried and it was found that tris buffer was most suitable. The tris buffer resulted in less hydrolysis of the acetylsalicylic acid. Esterase activity was determined with acetylsalicylic acid in tris buffer, pH = 7.85, 0.05 M. The enzymes were partially purified by combination of acetone powder preparations, ammonium sulfate precipitations, and gel filtration.

The ratio of esterase activity to protein content was increased 1.03 fold by acetone powder preparation and 1.5 fold by 70% ammonium sulfate precipitation. The ammonium sulfate precipitate in 60-70% was very reproducible. The enzyme showed the greatest instability after 70% ammonium sulfate precipitation; this made further purification difficult. The acetone powder solution was found to be stable at room temperature for about 2 hours.

The pH optimum of esterases was found to be close to 8.0. The esterases were classified as all-esterases and cholinesterases on the basis of inhibition and activation studies. The enzyme was completely inhibited by 0.05 M veratrine sulfate; 0.001 M mercuric chloride resulted in a 45% inhibition. EDTA increased the activity of the esterases 195% at 0.001 M. This may indicate that there are metal ions in the other tissues of the greater wax moth larvae that are causing inhibition of gut esterases; or, it may indicate that the other tissues contain esterase activity which is sensitive to the presence of metal ions.
Esterases are enzymes which catalyze the hydrolysis of uncharged carboxylic esters:

\[
\text{Ester} + \text{water} \rightarrow \text{Esterase} \rightarrow \text{acid} + \text{alcohol}
\]

Esterases are widely distributed in nature having been detected in various mammalian tissues, insects (1), plants (2, 3), mycobacteria (4), and fungi (5). In vertebrate tissues, the highest activities are found in liver, kidney, duodenum, and in the brain. Esterase activity in liver and kidney is predominantly membrane-bound in the microsomal fraction (6).

Esterases that are inhibited by \(1.0 \times 10^{-5}\) M eserine, are classified as cholinesterases (7). Two of the most widely studied cholinesterases are acetylcholinesterase (I.U.B. 3.1.1.7) and butyryl or pseudo-cholinesterase (I.U.B. 3.1.1.8).

Esterases that are not sensitive to \(1.0 \times 10^{-5}\) M eserine are classified as A-, B-, or C- esterases (I.U.B. 3.1.1.-). These enzymes seem to have a very broad substrate specificity, therefore they are further classified on the basis of the effect of organophosphate inhibitors on their activity (18). Aryl- or A-esterases (I.U.B. 3.1.1.2) are not
inhibited by organophosphate but actually hydrolyze these compounds. Arylesterases hydrolyze aromatic esters and aliphatic esters containing double bond adjacent to the ester linkage in the alcohol moiety (8). Arylesterases are activated by calcium ions and are inhibited by chelating reagents and sulfhydryl inhibitors (9).

Another group of esterases is sensitive to low concentrations of organophosphates and is designated B- or ali-esterases. These esterases are reported to hydrolyze other derivatives of carboxylic acid such as amides (4), acid anhydrides (10), and thioesters (11).

C-esterases do not hydrolyze nor are they inhibited by organophosphates. However, they are activated by p-chloromercuribenzoic acid (12).

Esterases in extracts can be detected in vitro by disc electrophoresis in starch gel (13). Esters of naphthols and n-fatty acids are used as substrates; subsequently, the liberated α-naphthol is coupled with a diazonium salt yielding an insoluble azo dye. In rat livers and in rat kidneys as many as 13 and 11 electrophoretically different esterases have been found, respectively (14).

Column chromatography techniques have resulted in the successful separation of contaminating hemoproteins during purification of esterases. In 1963, Krisch (15) isolated an acetonilide—hydrolyzing amidase from pig liver microsomes which was identical with pig liver esterase. After extracting the microsomal fraction with glycerol the enzyme was subsequently purified by fractionation with ammonium sulfate, acid precipitation at pH 4.2, and column chromatography on DEAE-sephadex A-50.

In 1969, Horgan et al reported purification procedures of pig liver esterase starting from a chloroform—acetone powder (16). Gel electrophoresis showed only one band, and from ultracentrifugation the esterase
was estimated to be 88% pure. The crystalline products of Krisch and Horgan were compared and found to have similar crystal structures (16).

Kingsbury and Masters (13) have determined the molecular weights of electrophoretic variants of numerous vertebrate carboxylesterases by disc electrophoresis on polyacrylamide gels of different composition. They found two molecular weight ranges: (I) 145,000-160,000, probably a dimer, and (II) 80,000, probably a monomer. This indicated the main enzyme is in fact a dimer with two active sites which, under suitable conditions, may exist as an active monomer.

The pH optimum of most esterases from mammalian sources is 7.5-9.0 (17, 18). At pH 4.5, pig liver esterase reversibly dissociates to active half molecules. Below pH 4 the enzyme undergoes irreversible denaturation to inactive half-enzyme molecules of altered shape (19).

The enzyme used in this investigation was from the larvae of the greater wax moth, Galleria mellonella (L). The greater wax moth has the ability to metabolize beeswax. Beeswax contains 85% of monoesters with long chain acids and alcohols, 13% are free fatty acids, and the remainder is chiefly hydrocarbons. The natural diet of the wax moth is honeycomb, which contains 50-80% lipids (20). The insects convert the various acids of honeycomb into normal saturated and unsaturated fatty acids. The excess acids resulting from the high lipid diet are excreted (21).

Excreta of wax moth larvae contain a smaller amount of fatty acids and unsaponifiable substances than does beeswax. About 50% of the ingested wax was utilized and oxidized by the larvae (21). Niemerko (22) has reviewed the metabolism of wax by the wax moth. He suggested that after hydrolysis of esters in the gut, the alcoholic moiety is oxidized to fatty acids which are further broken down.
The nature of the enzymes that are responsible for the hydrolysis of carboxylic acid esters of beeswax in the wax moth is unknown. Pancreatic lipase is probably the only digestive enzyme which has been purified that hydrolyzes carboxylic acid esters (24). Desnuelle (25) has reviewed the properties of this enzyme. Pancreatic lipase apparently hydrolyzes only emulsified esters (26) and is separable from those enzymes (esterases) in the pancreas that hydrolyze esters in solution (24, 25).

The mechanism of esterase action is thought to be similar to that of proteolytic enzymes. The deacylation of esters by an enzyme is generally thought to proceed by a mechanism involving a serine and histidine residue on the enzyme and an unknown acid. The steps in deacylation are shown in Figure 1 (27).

In 1966, Jacobson and Young (28) isolated a number of esterases from the gut of Galleria mellonella (L). The media they used to grow the larvae consisted of infant cereal, honey, and glycerol. Purification methods that were employed included polyacrylamide gel electrophoresis on disc and vertical slabs and polyacrylamide gel column chromatography. About 10 esterases were found by column chromatography of midgut preparations. Six or seven different esterases were found by disc electrophoresis. Several organic and inorganic compounds were tested to determine their effect on the activity of the enzymes. Only p-chloromercuribenzoic acid and eserine were shown to cause inhibition of enzyme activity. The data indicates that the esterases of the wax moth gut should be classified as B- or all-esterases. The enzyme assay was conducted at 291 nm in a Bausch and Lomb Spectronic 505 recording spectrophotometer for 3 minutes at 35°, pH 7.85, in 0.05 M tris buffer with 0.01-0.02 M substrate concentration. Esters of m-hydroxybenzoic acid were used as
Figure 1. Possible Mechanism of Enzymatic Deacylation by an Esterase. The mechanism involves a Serine and a Histidine Residue in the Enzyme and a Weak Acid of Unknown Nature.
substrates. Optimum esterase activity for 3-carboxyphenyl-n-octanoate was found at pH 8.0.

The present investigation was initiated as an attempt to verify the work of Jacobson and Young (29). We isolated esterase activity from whole larvae instead of the gut alone. The larvae diet used by Jacobson and Young (29) was altered to include beeswax and vitamins. This new diet should stimulate growth and esterase activity in the greater wax moth larvae. The factors observed for their effect on esterase activity were: pH, activators, inhibitors, and storage conditions.
CHAPTER II
MATERIALS AND METHODS

Part I. Materials

A. Wax Moth Diet

The diet components were Gerber Mixed infant cereal, (Gerber Products Co., Fremont, Michigan), crude sucrose, U.S.P. glycerol (Matheson, Coleman and Bell, Norwood, Ohio), Poly Vi-Sol vitamin mixture (Mead Johnson Laboratories, Evansville, Indiana), crude honey, and crude beeswax.

B. Esterase Assay

Buffer reagents were tris (hydroxy methyl) amino methane, (Schwarz/Mann, Orangeburg, New York), sodium phosphate (Matheson, Coleman and Bell), and barbital sodium (Fisher Scientific Co., Fair Lawn, New York). The acetyl salicylic acid that was used as a substrate was purchased from Matheson, Coleman and Bell Company. The salicylic acid that was used to check the purity and spontaneous hydrolysis of the substrate was purchased from the same company. The bovine albumin was obtained from the Sigma Chemical Company (P.O. Box 14508, St. Louis, Missouri) and used as a standard in the biuret test for protein determination.

C. Purification

Reagent grade acetone and ether were obtained from Eastman Kodak Company (Rochester, New York). Grade A ammonium sulfate was obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.
D. Inhibitors and Activators

Mercuric chloride, N.F., (Fisher Scientific Co.) and veratrine sulfate (Nutritional Biochemicals Corp., Cleveland, Ohio) were used as inhibitors. The disodium salt of (ethylenedinitrilo)-tetraacetic acid (EDTA) was used as an activator and was purchased from Matheson, Coleman and Bell Company.

Part II. Methods

A. Insect Preparation

1. Culture of the Greater Wax Moths

Wax moths, Galleria mellonella, were reared in screen-covered 5-gallon buckets. The synthetic diet was prepared by boiling a mixture of 300 g of sucrose and 500 ml of a solution consisting of equal parts of glycerol and tap water. The Poly VI-Sol vitamin (2.5 ml) formula was added and the mixture poured over 24 oz of Gerber Mixed Cereal. Two hundred fifty-five (255) grams of melted beeswax with 25 ml of honey were then blended to a consistency of wet sand. Fifty adult moths were placed in the 5-gallon bucket with the diet mixture to allow the females to lay eggs. There was no need to replenish the food during the period of growth. In 4 to 6 weeks the larvae hatched from the eggs and entered the rapid growth phase which lasted 2 to 3 weeks. The greater wax moth larvae were found to grow best at 35°C and 50-60% relative humidity (28).

2. Larvae Harvest

After approximately 7 weeks of growth, the large, light colored larvae weighing about 173 mg were removed from the synthetic diet and placed in a beaker in an ice bath. These were stored in the freezer overnight and used to prepare the acetone powder.
B. Preparation of Acetone Powder

Fifty grams of fresh frozen larvae was homogenized with 5.0 l of reagent grade acetone (-30°C to -20°C) in Vir Tis '45' blender. The acetone was kept in a mixture of dry ice and isopropyl alcohol to maintain the temperature below -20°C. Five or six larvae were added each time to 300 ml cold acetone and blended 10 minutes. Subsequently the homogenate was filtered through a large Buchner funnel (24 cm, Whatman No. 542 paper). Fresh larvae were added and blended in this manner until 5.0 l of acetone was used.

The acetone-extracted paste was transferred to a 500 ml Vir Tis flask and extracted ten times with 250 ml of a mixture of equal parts of diethyl ether and acetone (-30°C to -20°C). The filter cake was then washed with 1.25 l of cold diethyl ether (-30°C to -20°C); the filtrate was colorless at this time. The finely divided cake was dried over Drierite with a water aspirator vacuum for four hours at 10°C. Final traces of acetone were removed by evacuating the powder with a vacuum pump (Precision Model 75) for another 5 hours (10°C). The thoroughly dried acetone powder was stored in air-tight bottles at 4°C.

C. Spectrophotometric Determination of Substrate Stability

Salicylic acid strongly absorbs ultraviolet light in the wavelength range of 290 nm - 300 nm, whereas acetyl-salicylic acid (substrate) does not absorb at all in this region. This principle provides a convenient and sensitive method for the determination of the purity and the influence of spontaneous hydrolysis on acetyl-salicylic acid.

The measurements were carried out in a Bausch and Lomb Spectronic 505 spectrophotometer. The reference cell contained buffer only, the control cells contained buffer, 10^{-4} M substrate and buffer, 10^{-4} M salicylic
acid. Different kinds of buffers were tried to determine which buffer had less hydrolytic effect on the acetylsalicylic acid.

Solutions of substrate were prepared according to the method of Hofstee (29) by warming the correct quantity of ester in a volumetric flask until it melted. Then a few drops of 95% ethanol were added and the slurry was gently swirled while adding buffer containing an amount of 1M NaOH, sufficient to neutralize the free carboxyl group. Solutions of the substrate were prepared immediately before each experiment or kept in the cold room for not longer than one day.

D. Protein Assay

The protein content of a sample was determined by the biuret test at 540 nm in the Spectronic 505 spectrophotometer. Bovine albumin was used to establish a standard curve. The reaction cuvette contained 1.0 ml of enzyme and buffer mixture with 4.0 ml biuret reagent. The reference cuvette contained 1.0 ml buffer instead of the enzyme-buffer mixture. When concentrations of protein were below 1.0 mg/ml, the absorbance was read at the 300 nm wavelength.

E. Enzyme Assay

The esterolytic activity of esterases was detected from both fresh larvae preparations and from an acetone powder preparation of the larvae. Two larvae were placed in one ml of cold tris buffer, 0.05 M, pH 7.85 and were blended in Vir Tis '45' homogenizer at high speed for 5 minutes. The mixture was then centrifuged in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge with a Sorvall 55-34 rotor at 34,760 x g for 2 hours. The supernatant was filtered through a glass fiber filter and B-12 membrane filter; the filtrate was then clear.
The acetone powder solution was brought into solution by adding 0.1 g acetone powder to 5 ml of cold tris buffer, 0.05 M, pH 7.85. The mixture was slowly stirred for 1 hour in the refrigerator. The solution was then centrifuged at 11,360 x g for 15 minutes and filtered in the same manner as described previously.

Activity tests on esterase solutions were performed at 28°C and pH 7.85 in 3 ml cuvettes in the Spectronic 505 spectrophotometer at 295.8 nm. Three ml of a freshly prepared 10⁻³ M solution of acetylsalicylic acid (prepared by the method of Hofstee) at 28°C was added to each cuvette, then 0.1 ml of the enzyme solution was added to the reaction cuvette. An equal amount of tris buffer was used in the blank in place of the enzyme solution. At the same time, another cuvette containing 0.1 ml of filtered, heated enzyme was used in order to observe the influence of spontaneous hydrolysis on the reaction rate. The reaction was usually followed for 10 minutes, although this was varied for more or less active preparations. That portion of the curve with the greatest slope was used to calculate the initial reaction rate.

A unit of esterase activity was defined as the amount of enzyme that produces one micromole of salicylic acid (at 295.8 nm) in one minute in a 10⁻³ M acetylsalicylic acid solution at 28°C and pH 7.85. Specific activity was expressed as the ratio of units of enzyme activity per milligram of protein in solution.

F. Determination of Optimal pH

The pH optimum for esterase activity was determined using 3 ml of a 10⁻³ M solution of the acetylsalicylic acid and 0.2 ml of the enzyme solution (acetone powder) containing 10.3 activity units in 1 ml of
buffer solution. The pH of the substrate was adjusted with concentrated HCl and 1M NaOH. The change in absorption for 10 minutes was recorded.

G. Ammonium Sulfate Fractionation

Acetone powder (0.2 g) was added to 10 ml cold tris buffer (0.05 M) at pH 7.85. The solution was slowly stirred with a magnetic stirrer at 4°C for 2 hours. When all the acetone powder was dissolved, the sample was centrifuged at 12,100 x g for 15 minutes (0°C). Cold saturated ammonium sulfate (prepared using distilled, deionized water) solution was added to the filtered supernatant to yield the desired percentage saturation. The solution was stirred slowly at 4°C for another 4 hours and centrifuged (12,100 x g, 15 min, 0°C). The solution prepared from the ammonium sulfate precipitate was assayed for esterase activity and protein concentration. The precipitate was redissolved in 2 ml cold tris buffer pH 7.85 with gentle stirring using a glass rod in an ice bath for 30 minutes.

H. Determination of Enzyme Stability

1. Spontaneous Inactivation

Solutions of fresh larvae preparation (2 larvae/ml), acetone powder preparation (0.1 g acetone powder/5 ml) and ammonium sulfate precipitate preparation (50% and 70%) were tested for esterase activity after 2 hrs and 24 hrs. The solutions were stored at 4°C at pH 7.85 with tris buffer.

2. Sensitivity to Organic and Inorganic Compounds

Enzyme solutions containing 5.30 esterase activity units were tested for sensitivity to various compounds without preincubation in the enzyme solution. The compounds were placed in solutions made up to a final concentration of 10⁻³ M acetylsalicylic acid and a final pH of 7.85.
3. Sensitivity to pH

The activity of solutions of esterase made from 50 percent and 70 percent ammonium sulfate precipitates was observed at several pH's without preincubation.
CHAPTER III

RESULTS AND DISCUSSION

A. Ultraviolet Absorption of Acetylsalicylic Acid

The absorption spectra of $10^{-4}$ M salicylic acid and acetylsalicylic acid in different buffer systems are shown in Figure 2 and 3. Veronal buffer (0.025 M, pH 8) and deionized water were also tested as solvents, the absorption spectra were similar to that obtained in tris buffer (0.05 M, pH 7.85) shown in Figure 2. From Figure 3 it can be seen that the absorption spectrum for acetylsalicylic acid in phosphate buffer (0.05 M, pH 8.0) is strongly absorbing in the region of 280-300 nm. Therefore, acetylsalicylic acid underwent considerable spontaneous hydrolysis and was less stable in phosphate buffer. Veronal buffer developed a precipitate at the 0.025 M (pH 8.0) concentration after a period of time. Hence tris buffer was considered to be more satisfactory than the other two buffer systems. The maximum absorption was found to be at 295.8 nm.

The use of the absorption of salicylic acid at 300 nm according to the method of Hofstee (30) was found to be somewhat less sensitive than the absorption at 295.8 nm. Figure 4 illustrates the linear increase in absorption with increasing concentrations of salicylic acid at 295.8 nm at 28°C and pH 7.85 with a 1 cm light path.
Figure 2. Ultraviolet Spectrum of salicylic acid (closed circles) and acetylsalicylic acid (open circles) in tris buffer (0.05 M, pH 7.85). Both concentrations of salicylic acid and acetylsalicylic acid are $10^{-4}$ M. For conditions, see text.
Figure 3. Ultraviolet Spectrum of salicylic acid (closed circles) and acetylsalicylic acid (open circles) in phosphate buffer (0.05 M, pH 8.0). Both concentrations of salicylic acid and acetylsalicylic acid are 10⁻⁴ M. For conditions, see text.
Figure 4. The absorption of salicylic acid at 295.8 nm. Concentrations are $2 \times 10^{-4}$ M of salicylic acid and dilutions of it. For conditions, see text.
ABSORBANCE (295.8 nm)

SALICYLIC ACID (MICROMOLES)
B. Esterase Activity of the Greater Wax Moth Larvae

A series of solutions containing 0.1 ml of 2, 4 and 6 greater wax moth larvae per ml of buffer and acetone powder solutions were assayed for protein concentrations and esterase activity.

Figure 5 shows that the amount of protein which dissolved from the fresh larvae was similar for 2, 4 and 6 larvae per ml preparations on a per larva basis. It can be seen from Table 1 that the esterase specific activity of acetone powder solutions was 1.09 for one larva, which was 44% higher than the fresh larvae preparations. Solutions of the fresh larvae preparation (2 larvae/ml) contained 16.6 activity units per larva at pH 7.85 in 0.05 M tris buffer, and the acetone powder preparation contained 22.5 activity units at pH 8.12 in 0.05 M tris buffer on a per larva basis.

The above enzyme preparations were stored at 28°C for 2 hrs to determine the stability of both samples. Figure 6 shows that esterolytic activity is linear for a short period of time for both the acetone powder preparation and for the fresh larvae preparation. It was found that this relationship held for 30 minutes for the fresh larvae preparations and for 110 minutes for acetone powder solutions. Acetone powder preparation was more highly purified; this apparently removed some lipid material that was causing esterase inhibition.

The acetone powder is more active and stable than the fresh larvae preparation. The dry active powder can be stored at 4°C for at least 3 months, therefore, it is a good starting material from which the esterases can be extracted with buffer solution.
Figure 5. Protein assay of fresh larvae preparation by biuret test at 540 nm. A series of solutions containing 0.1 ml of 2, 4 and 6 larvae per ml were tested. For conditions, see text.
TABLE 1
ESTERASE ACTIVITY OF THE GREATER WAX MoTH LARVA

The protein concentration and specific activity are all expressed per one larva.

<table>
<thead>
<tr>
<th>Sample Content</th>
<th>mg prot/ml</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Fresh larvae preparation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 2 larvae/ml</td>
<td>15.0 ± 0.3</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>b. 4 larvae/ml</td>
<td>13.7 ± 1.4</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td>c. 6 larvae/ml</td>
<td>12.4 ± 1.3</td>
<td>0.79 ± 0.08</td>
</tr>
<tr>
<td>Ave.</td>
<td>13.7 ± 1.0</td>
<td>0.75 ± 0.05</td>
</tr>
<tr>
<td>B. Acetone Powder Solution</td>
<td>6.7 ± 0.1</td>
<td>1.07 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 6. The rate of enzyme catalyzed hydrolysis of $10^{-2}$ M acetylsalicylic acid at 295.8 nm. The system consisted of a fresh larvae preparation at pH 7.85 (closed circles) and acetone powder solution at pH 8.12 (open circles), both samples were kept at 28°C.
C. pH Optimum

The pH optimum for enzyme activity was found to be near 8.0 for tris buffer and 7.9 for veronal buffer as shown in Figure 7. The pH that was used in the succeeding experiments was 7.85. One of the several enzymes with esterolytic activity present in the gut of the greater wax moth larvae (28) may account for the increased activity at pH 6.19 shown in the curve of Figure 7.

D. Effect of Organic and Inorganic Compounds

Mercuric chloride, veratrine sulfate, and sodium ethylene-1,2-diamine-N,N,N',N'-tetraacetate (EDTA) were tested to determine if they influenced the activity of the esterolytic enzymes of the greater wax moth larvae. The results are shown in Table 2. Mercuric chloride and veratrine sulfate had a slight inhibitory effect on the enzyme activity at low concentrations. This inhibition increased at higher concentrations, especially for veratrine sulfate which completely inhibited the enzyme activity at 0.05 M.

The chelating ligand reagent, sodium ethylene-1,2-diamine-N,N,N',N'-tetraacetate (EDTA), was used in order to find ions associated with the enzyme or in the enzyme solution. Since this compound increased the activity of the enzyme, it is possible that calcium or magnesium ions are not required by the enzyme. Possibly, some heavy naturally occurring metal ions interfere in some manner with the active site of the enzyme and are chelated by EDTA resulting in increased activity.

According to the definitions used for esterases, the esterases of the greater wax moth larvae are not aryl-esterases, because the greater wax moth larvae esterases are not activated by calcium ions and inhibited by chelating agents. Also, veratrine sulfate is a nerve inhibitor which
Figure 7. pH Optimum for esterolytic activity. The pH ranges to be tested were 6.19-9.15 for tris buffer (open circles) and 6.37-9.22 for veronal buffer (closed circles).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercuric Chloride</td>
<td>0.000003 M</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.00001 M</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>0.0001 M</td>
<td>55</td>
</tr>
<tr>
<td>Veratrine Sulfate</td>
<td>0.0001 M</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>0.005 M</td>
<td>0</td>
</tr>
<tr>
<td>EDTA $^\dagger$</td>
<td>0.001 M</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>0.01 M</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>0.1 M</td>
<td>266</td>
</tr>
</tbody>
</table>

$^\dagger$ Sodium ethylene-1,2-diamine-N,N,N',N'-Tetraacetate
normally inhibits the esterase activity of cholinesterases. So the esterases in this crude preparation probably have the properties of both cholinesterases and ali-esterases.

E. Ammonium Sulfate Fractionation

The esterase activity of the greater wax moth larvae was partially purified by ammonium sulfate fractionation. The acetone powder was used as starting material. The activity tests were carried out on the redissolved ammonium sulfate precipitates rather than on the ammonium sulfate solutions because of the high salt concentration in the solutions. Table 3 shows that the specific activity increased when the higher percent saturation was employed. With the 70% ammonium sulfate precipitate preparation, the percent recovery was more than 100%. This might be caused by most of the esterolytic enzymes precipitating and the metal ion inhibitors remaining in the solution.

F. Spontaneous Inactivation

Solutions of different larvae preparations were tested for esterase activity after certain periods of time. The solutions were all stored at 4°C in tris buffer, pH = 7.85, 0.05 M. It can be seen from Table 4 that the stability of the esterase activity decreased with each additional purification step. The fresh larvae preparation was stored at 4°C for 24 hrs and lost 26% of its activity. Solutions of the acetone powder preparation lost 46% of its activity after 24 hours at 4°C. After 70% ammonium sulfate precipitation, storage of the solution at 4°C for 24 hrs resulted in complete loss of activity. This is probably because the enzyme has been protected by a coat of smaller proteins. Removal of these proteins by purification procedures would make the enzyme increasingly vulnerable to denaturation.
<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (μmoles/min)</th>
<th>Specific Activity (μmoles/min mg prot)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone Powder</td>
<td>66.5 ± 1.4</td>
<td>20.6 ± 0.6</td>
<td>0.308 ± 0.015</td>
<td>100%</td>
</tr>
<tr>
<td>50% Saturated Ammonium Sulfate</td>
<td>38.2 ± 0.0</td>
<td>10.7 ± 0.7</td>
<td>0.280 ± 0.018</td>
<td>50.5%</td>
</tr>
<tr>
<td>60% Saturated Ammonium Sulfate</td>
<td>41.1 ± 1.1</td>
<td>18.4 ± 1.6</td>
<td>0.444 ± 0.025</td>
<td>88.9%</td>
</tr>
<tr>
<td>70% Saturated Ammonium Sulfate</td>
<td>51.3 ± 0.0</td>
<td>23.1 ± 1.9</td>
<td>0.451 ± 0.082</td>
<td>112%</td>
</tr>
</tbody>
</table>
TABLE 2
SPONTANEOUS INACTIVATION OF ESTERASES

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Time</th>
<th>% Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh larvae (2 larvae/ml)</td>
<td>2 hrs</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>24 hrs</td>
<td>74</td>
</tr>
<tr>
<td>Acetone Powder</td>
<td>24 hrs</td>
<td>54</td>
</tr>
<tr>
<td>50% Ammonium Precipitate</td>
<td>2 hrs</td>
<td>69</td>
</tr>
<tr>
<td>70% Ammonium Precipitate</td>
<td>24 hrs</td>
<td>0</td>
</tr>
</tbody>
</table>
G. Sensitivity to pH

In Figure 8 the esterase activity in 50% and 70% ammonium sulfate precipitates is shown at various pH's. The pH optimum for the 50% ammonium sulfate precipitate was found to be 7.92, which was close to that of the acetone powder preparation. Unexpected results were obtained with the 70% ammonium sulfate precipitate. Throughout the whole pH range, the specific activity increases with increasing pH. It is possible that the high concentration of ammonium sulfate with a basic pH exerts an activating effect on the enzyme reaction, or that the 70% ammonium sulfate precipitates some esterolytic enzymes that have activity at basic pH.
Figure 8. Stability of Esterases with changes in pH. A buffer solution of esterases precipitated with 50% ammonium sulfate (closed circles). A buffer solution of esterases precipitated with 70% ammonium sulfate (open circles).
BIBLIOGRAPHY


15. Krisch, K., l. Isolation of an esterase from hog liver microsomes.  


