Studies on the Cholesterol Ester Hydrolase of *Trogoderma* (Coleoptera)

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The enzyme cholesterol ester hydrolase (EC 3.1.1.13) was detected in the larvae of the khapra beetle, *Trogoderma granarium* (Everts). The pH and temperature optima for the enzyme were 6.6° and 37°C respectively. The mol.wt. of the enzyme was 76000–80000. The enzyme was equally effective in hydrolysing cholesteryl acetate, stearate and oleate. Cholesterol derivatives, namely the chloride and the methyl ether, inhibited the enzyme activity almost completely. It was also inhibited completely by *p*-hydroxymercuribenzoate. This inhibition was reversed by the addition of dithiothreitol, reduced glutathione or cysteine. The enzyme activity was associated predominantly with the 104000g fraction.

Insects in general require a dietary source of sterol, which they are unable to synthesize. As they can esterify free sterols and hydrolyse sterol esters, this requirement may be fulfilled by a suitable free sterol or its ester (Clayton, 1964). The hydrolysis of the sterol esters is catalysed by the enzyme sterol ester hydrolase (EC 3.1.1.13). This enzyme has been studied in higher organisms (Vahouny & Treadwell, 1968), but there are no such studies in insects, except that of Casida *et al.* (1957) of the American cockroach, *Periplaneta americana*. The sterol ester hydrolase is very active and is considered necessary during the absorption of sterols in the alimentary canal of higher organisms (Vahouny & Treadwell, 1968). Sterol derivatives such as cholesteryl chloride prevent the utilization of sterols in insects, and it has been suggested that this is due to the inhibition of cholesterol esterase (Noland, 1954). No direct evidence supporting this idea has been presented.

The khapra beetle, *Trogoderma granarium* (Everts), a serious pest of stored wheat, utilizes free cholesterol or its esters, such as the acetate and stearate, equally well (Agarwal, 1970), whereas esters such as the formate, propionate, butyrate etc. are unable to support the development of this insect (A. M. G. Nair & H. C. Agarwal, unpublished work). Cholesterol derivatives (chloride and methyl ether) inhibit the utilization of cholesterol in this insect (Sehgal & Agarwal, 1973), but the mechanism is not known. For these reasons a study of the cholesterol ester hydrolase in *T. granarium* was considered to be of interest.

Materials

The last-instar larvae of *T. granarium* were separated from the stock cultures maintained in the laboratory at $35 \pm 1^\circ\text{C}$ by the method of Rao & Agarwal (1969). Cholesteryl acetate, bovine serum

albumin, egg albumin (from chicken egg) and haemoglobin were from Vallabhbhai Patel Chest Institute, Delhi, India. The following cholesterol derivatives and enzymes were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.: formate, propionate, isobutyrate, hexanoate, benzoate, heptanoate, octanoate, cinnamate, pelargonate, decanoate, laurate, myristate, palmitate, stearate and oleate; phosphoglyceromutase and glyceraldehyde phosphate dehydrogenase. Cholesteryl chloride, cholesteryl methyl ether and 6-oxocholesterol were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-100 was from Pharmacia, Uppsala, Sweden. *N*-Ethylmaleimide and merthiolate (thiomersal), and Cleland's reagent (dithiothreitol) were from BDH Chemicals Ltd., Poole, Dorset, U.K., and Calbiochem, Los Angeles, CA, U.S.A., respectively.

Methods

The larvae were weighed and washed quickly with water to remove any extraneous matter and were homogenized in a glass homogenizer in 0.3 M-sucrose/0.15 M-sodium phosphate buffer, pH 6.6 (10 ml/g of larvae). The homogenate was centrifuged at 1000g for 10 min and the supernatant re-centrifuged at 11700g for 20 min. This second supernatant was centrifuged at 104000g for 1 h and the supernatant removed. The residue was suspended in sodium phosphate buffer, pH 6.6 (25 ml), and used for enzyme assays, as it was found to be the most active preparation.

The substrate for the enzyme reaction was prepared by the method of Casida *et al.* (1957). The reaction mixture contained 2 μmol of cholesteryl acetate, 2.1 μmol of sodium taurocholate, 50 μg of merthiolate and 5 mg of egg albumin in 1 ml of 0.15 M-sodium phosphate buffer, pH 6.6. Cholesteryl acetate was

first dissolved in diethyl ether and then added to the remaining components of the reaction mixture in a glass homogenizer. The reaction mixture was homogenized and then kept in a water bath at 40°C to evaporate the diethyl ether. It was used immediately afterwards. Initially [^3H]cholesteryl acetate was used as the substrate, but subsequently this was replaced by unlabelled cholesteryl acetate. The suspension of the 104000g residue was used as the source of enzyme and 0.1 ml of this, containing 0.107 mg of protein, was added to the substrate (1 ml). Incubation was for 3 h at 37°C. Zero-time incubations were used as enzyme blanks. All the experiments were carried out at least in triplicate. The effect of time, temperature, pH etc. was studied by modifying the conditions as shown in the Figures and Tables.

The enzyme reaction was stopped by the addition of chloroform/methanol (2:1, v/v; 6 ml) and the contents were mixed thoroughly and allowed to settle. After separation of the two phases the organic solvent layer was removed and the aqueous phase was further extracted twice with smaller quantities of chloroform/methanol. The chloroform/methanol extracts were combined, evaporated to dryness under

N_2 at 35–40°C and the residue was dissolved in a known volume of acetone/ethanol (1:1, v/v). The cholesterol released was determined by the method of Sperry & Webb (1950), directly from the chloroform/methanol extract. Proteins were precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid and the precipitate was dissolved in 0.1 M-NaOH. The method of Lowry *et al.* (1951) was used for protein determination.

The products of the enzyme reaction were analysed by t.l.c. of the above extract on silica gel G in the solvent system benzene/ethyl acetate (5:1, v/v). The spots were developed by spraying with Liebermann-Burchard reagent (Sperry & Webb, 1950). The products of the enzyme reaction were further analysed by g.l.c. of the total lipid fraction with and without silylation. The samples were silylated by using the 'Sil-prep Kit' of Applied Science Laboratories, State College, PA, U.S.A. Coiled glass columns (2 m $\times$ 2 mm internal diam.) were used. The columns were packed with 1% OV-17 on Chromosorb P 100/120 mesh or 1% SE-30 on Gas-Chrom P (100–120 mesh). Cholestane was used as internal standard, and the relative retention times were determined in

Fig. 1. G.l.c. separation of sterols from the chloroform/methanol extract of the incubation medium for cholesterol ester hydrolase of *Trogoderma*

The mixture of sterols was silylated with the 'Sil-prep Kit' from Applied Science Laboratories. A 2 m coiled-glass column (internal diam. 2 mm) packed with 1% OV-17 on Chromosorb P (100–120 mesh) was used at a temperature of 250°C. N_2 was used as the carrier gas at a flow rate of 40 ml/min. The chart speed was 2.5 cm/min. Cholestane was used as the internal standard. The peaks are: 1, cholestane; 2, cholesterol; 3, campesterol; 4, cholesteryl acetate and β -sitosterol. (a) Zero-time enzyme blank; (b) after enzyme action.

Fig. 2. Effect of enzyme concentration on the cholesterol ester hydrolase activity of *T. granarium* larvae. Conditions for incubation were as in Table 1.

relation to this. A Packard model A 7400 gas chromatograph with a H_2 flame ionization detector was used. The operating conditions used are given in the legend to Fig. 1.

The molecular weight of the enzyme was estimated by sucrose-density-gradient centrifugation (Martin & Ames, 1961) and by gel filtration (Andrews, 1965). Haemoglobin (mol.wt. 64000), bovine serum albumin (monomer, mol.wt. 69000; dimer, mol.wt. 138000), phosphoglyceromutase (mol.wt. 112000) and glyceraldehyde phosphate dehydrogenase (mol.wt. 122000) were used as standards.

Results

The combined chloroform/methanol extract from the enzyme incubations gave two spots on t.l.c. plates coinciding with cholesterol and cholesteryl acetate respectively, whereas the enzyme blank gave

Fig. 3. Sucrose-density-gradient centrifugation of cholesterol ester hydrolase of *T. granarium*

A linear gradient of 5–20% (w/v) sucrose in 0.15M-sodium phosphate buffer, pH 6.6, was used. After centrifugation at 175000g for 17 h a 0.5 mm-diam. hole was made at the bottom of each tube. Fractions (10 drops each) were collected and analysed for protein content and enzyme activity. Human γ -globulin (peak in tube 11) and bovine serum albumin (peak in tube 20) were used as marker proteins. O, Sample protein; Δ , marker protein; $\bullet$, enzyme activity.

only one spot with the same R_F value as that of the cholesteryl acetate. The g.l.c. analysis of the silyl derivatives of the total lipid fraction from the enzyme blank and enzyme reaction on 1% OV-17 is shown in Figs. 1(a) and 1(b). Three compounds were obtained in each case and were identified as cholesterol, campesterol and cholesteryl acetate respectively on the basis of their relative retention time (relative to cholestane). β -Sitosterol had the same relative retention time as cholesteryl acetate. In the enzyme blank experiment, the cholesterol peak accounted for about 3–4% of the total sterols and as a result of enzyme action it increased to about 24%, whereas the proportion of cholesteryl acetate declined. The g.l.c. of unsilylated total lipid extract gave three compounds on the OV-17 column. They were identified as cholesterol, cholesteryl acetate and β -sitosterol on the basis of their relative retention times, which were 2.7, 3.5 and 4.4 min respectively. Campesterol did not separate from cholesteryl acetate. In the enzyme-blank experiment, cholesterol and β -sitosterol peaks were insignificant, whereas in the fraction from the enzyme reaction the cholesterol peak increased greatly. The g.l.c. on the 1% SE-30 column of the

total lipid extract before and after silylation gave four compounds each. These were identified as cholesteryl acetate, cholesterol, campesterol and β -sitosterol. Their relative retention times before and after silylation were 1.3, 2.8, 3.8 and 4.5 min, and 1.1, 1.5, 2.0 and 2.5 min respectively. The peaks for campesterol and β -sitosterol were insignificant in both cases. The cholesterol peak was very small in the enzyme-blank experiment and it increased greatly as a result of enzyme action.

Properties of the enzyme

The optimum pH of the enzyme was 6.6. Below pH 5 and above pH 8 there was almost no activity. The maximum enzyme activity was obtained at 37°C and the peak for temperature effect was quite sharp. The effect of enzyme concentration is shown in Fig. 2. The enzyme activity exhibited a linear relationship up to 0.6 ml of enzyme (0.64 mg of protein) and any further increase in enzyme concentration did not result in a corresponding increase in activity. Enzyme activity was directly proportional to time up to 4 h. The results of sucrose-density-gradient centrifugation are presented in Fig. 3. The marker proteins, human γ -globulin and bovine serum albumin, gave peaks in tubes 11 and 20 respectively. From these results, the mol.wt. of cholesterol ester hydrolase was estimated to be about 77000. The mol.wt. of the enzyme measured by gel filtration was 76000–80000.

The enzyme hydrolysed cholesteryl acetate, stearate and oleate more readily than cholesteryl palmitate, myristate and hexanoate (Table 1). The remaining esters were hydrolysed only slightly. The cholesteryl chloride and methyl ether and 6-oxocholesterol inhibited the enzyme activity almost completely (Table 2). It was also inhibited by *p*-hydroxymercuribenzoate and partly by iodoacetate and azide. The

Table 1. *Substrate specificity of cholesterol ester hydrolase of T. granarium larvae*

The substrate mixture was prepared by the method of Casida *et al.* (1957); 1 ml of mixture contained 2 μ mol of cholesterol ester, 2.1 μ mol of sodium taurocholate, 50 μ g of merthiolate and 5 mg of egg albumin in 0.15 M-sodium phosphate buffer, pH 6.6. To this was added 0.1 ml of enzyme preparation from *T. granarium* larvae containing 0.107 mg of protein. Incubation was for 3 h at 37°C. The relative extent of hydrolysis was calculated by considering the hydrolysis of acetate as 100%.

Cholesterol ester	Cholesterol released		Relative extent of hydrolysis
	(μ mol)	(% hydrolysis)	
Formate	0.12 $\pm$ 0.04	6.1	10.7
Acetate	1.14 $\pm$ 0.16	57.0	100.0
Propionate	0.33 $\pm$ 0.06	16.3	28.6
Isobutyrate	0.49 $\pm$ 0.08	24.5	42.9
Hexanoate	0.82 $\pm$ 0.12	40.8	71.6
Benzoate	0.45 $\pm$ 0.08	22.4	39.3
Heptanoate	0.16 $\pm$ 0.04	8.2	14.4
Octanoate	0.33 $\pm$ 0.06	16.3	28.6
Cinnamate	0.24 $\pm$ 0.05	12.2	21.4
Pelargonate	0.33 $\pm$ 0.06	16.3	28.6
Decanoate	0.16 $\pm$ 0.04	8.2	14.4
Laurate	0.49 $\pm$ 0.08	24.5	42.9
Myristate	0.98 $\pm$ 0.14	48.9	85.8
Palmitate	0.98 $\pm$ 0.14	48.9	85.8
Stearate	1.14 $\pm$ 0.16	57.0	100.0
Oleate	1.06 $\pm$ 0.15	52.95	92.9
Chloride	0.08 $\pm$ 0.03	4.1	7.2
Methyl ether	0.16 $\pm$ 0.04	8.2	14.4

Table 2. *Effect of some cholesterol derivatives on the cholesterol ester hydrolase activity of T. granarium larvae*

For the assay, 2 μ mol each of cholesteryl acetate and/or cholesterol derivative was used. Other conditions were as in Table 1.

Cholesterol derivatives present	Cholesterol released	
	(μ mol)	(% hydrolysis)
Cholesteryl acetate	1.14	57.0
Cholesteryl chloride	0.08	4.1
Cholesteryl acetate +cholesteryl chloride	0.12	6.1
Cholesteryl methyl ether	0.16	8.2
Cholesteryl acetate +cholesteryl methyl ether	0.16	8.2
Cholesteryl acetate +6-oxocholesterol	0.08	4.1

Table 3. *Effect of various additives on the cholesterol ester hydrolase activity of T. granarium larvae*

Conditions for incubations were the same as in Table 1, except that various additives were present in the concentrations indicated; cholesteryl acetate was the cholesterol ester used.

Additive	Concentration (mm)	Cholesterol released (μ mol)	Activity (%)
—	—	1.14	100.0
<i>p</i> -Hydroxymercuribenzoate	1	0.0	0.0
<i>p</i> -Hydroxymercuribenzoate and dithiothreitol	5	0.98	85.8
<i>p</i> -Hydroxymercuribenzoate and cysteine	10	1.06	92.9
<i>p</i> -Hydroxymercuribenzoate and glutathione	10	1.06	92.9
Iodoacetate	1	0.16	14.3
NaN ₃	1	0.08	7.1
<i>N</i> -Ethylmaleimide	1	0.18	16.0

inhibition by *p*-hydroxymercuribenzoate was reversed by dithiothreitol (Cleland, 1964), cysteine and reduced glutathione (Table 3). The cholesterol ester hydrolase activity was found predominantly in the 104000g residue.

Discussion

Cholesteryl acetate was hydrolysed by an enzyme from *Trogoderma* to cholesterol as confirmed by t.l.c. and g.l.c. This showed that the enzyme cholesterol ester hydrolase was present in *T. granarium* larvae. It explains the utilization of cholesterol esters by *Trogoderma* as well as free cholesterol. Other insects are also able to use sterol esters, apparently owing to the presence of the hydrolase. This enzyme was also detected in the American cockroach *P. americana* (Casida *et al.*, 1957).

The optimum pH of *Trogoderma* enzyme, 6.6, was the same as that used by Casida *et al.* (1957) for the assay of the enzyme from *P. americana*. These workers did not investigate the optimum pH of the enzyme. The maximum activity of *Trogoderma* hydrolase was at 37°C, and this was the temperature used by Casida *et al.* (1957) for enzyme assays from the cockroach. The mol.wt. of *Trogoderma* cholesterol ester hydrolase was 76000–80000 on the basis of two methods of measurement. Such information is not known for this enzyme from any other insects. However, the pancreatic-juice cholesterol esterase monomer of the rat had a mol.wt. of 65000 (Hyun *et al.*, 1971).

Of the substrates tested, the cholesteryl acetate, stearate and oleate were hydrolysed by *Trogoderma* enzyme most rapidly. Dietary cholesteryl acetate and stearate were utilized as effectively as cholesterol by *Trogoderma* (Agarwal, 1970). The rate of hydrolysis of these and other esters by this enzyme was directly correlated with their utilization for growth in *Trogoderma* (A. M. G. Nair & H. C. Agarwal, un-

published work). A number of cholesterol esters were also shown to be quite effective for promoting growth of the cockroach, *Blatella germanica* (Noland, 1954). It appears that the cholesterol ester hydrolase of *Trogoderma* is an enzyme with low substrate specificity for the fatty acid moiety. The cholesterol derivatives, cholesteryl chloride and cholesteryl methyl ether, inhibited the *Trogoderma* enzyme almost completely. These compounds are known to prevent the utilization of dietary cholesterol by *Trogoderma* (Sehgal & Agarwal, 1973). These compounds, and several others, inhibit cholesterol utilization in several insect species (Clayton, 1964). Noland (1954) suggested that these effects are due to the inhibition of cholesterol esterases, which apparently have a role in the transport of cholesterol through the intestinal wall. The studies with *Trogoderma* enzyme show clearly that cholesterol ester hydrolase is, in fact, inhibited by cholesteryl chloride and cholesteryl methyl ether.

The *Trogoderma* enzyme was inhibited by *p*-hydroxymercuribenzoate, but inhibition was reversed completely by dithiothreitol, reduced glutathione and cysteine, suggesting a requirement for a free thiol group. The cholesterol ester hydrolase of *Trogoderma* was found predominantly in the 104000g residue.

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