Chapter 4

PROPERTIES OF INSECT FAT BODY LIPASE

I. KINETICS AND SUBSTRATE SPECIFICITY

Lipase activity has been studied extensively in materials from various sources like hog pancreas, (Willstatter and Waldschmidt-Leitz, 1923), wheat germ (Singer and Hofstee, 1948), Fusarium (Fiore and Nord, 1950), oat (Martin and Peers, 1953), pigeon pancreas (Scaria, 1958) pigeon breast muscle (George and Scaria, 1959 b), pigeon adipose tissue (George and Eapen, 1960), hog intestine (DiNella et al, 1960), pig adipose tissue, (Lynn and Perryman, 1960), insect flight muscle (Bhakthan, 1961) etc. It is known that lipases from different sources differ greatly in their properties such as substrate specificity, steriochemical specificity, optima for temperature and pH and behaviour towards activators and inhibitors.

The insect fat body lipase was reported by George and Eapen (1959 a, b) in the fat body of locust, Schistocerca gregaria but no detailed studies have been made on the nature of the lipase. In the present study, therefore, some properties of the cockroach fat body lipase have been investigated.

MATERIALS AND METHODS

1. Preparation of the fat body tissue homogenate

The fat body of the laboratory reared female cockroaches, Periplaneta americana, were used. The insects were anaesthetized by chilling for 15 minutes and then dissected. The perivisceral fat body tissue was carefully separated out from the tracheal branches, blotted with filter paper and weighed on a precision balance. The tissue was homogenized in a chilled mortar in ice cold distilled water and the enzyme was extracted for 20 minutes in cold. Usually a 2.5% or 5.0% fat body homogenate was prepared in this way. The homogenate was centrifuged at 2500 r.p.m. for 3 minutes and the supernatant white lipid scum and the sediment were discarded. The infranatant solution was pippetted out and mixed thoroughly and saved for the study of lipase actvity. This infranatant solution is referred to hereafter as the fat body homogenate.

2. Measurement of lipase activity

Method employed for the estimation of lipase activity was a manometric one, using the conventional Warburg apparatus and a bicarbonate-carbondioxide buffer system. The enzyme action was denoted by the production of fatty acid which in turn liberated carbondioxide from the sodium bicarbonate buffer.

Each reaction flask contained 1.5 ml of sodium bicarbonate solution in appropriate molar concentration to give the desired pH, 0.5 ml of enzyme solution and 0.5 ml of distilled water in the main chamber and 0.5 ml of substrate in the side arm, in a total volume of 3.0 ml.

The flasks and manometers were gassed for 3 minutes with a mixture of 5% cabondioxide and 95% Nitrogen. After 10 minutes of equilibration in the constant temperature water bath at 37°C, the substrate was tipped in and the mixture was allowed to equilibrate for a further period of 3 minutes. Throughout the experiments, the manometers were shaken horizontally at the rate of 120 oscillations per minute and an amplitude of 2.5 cms.

Readings were taken at regular intervals for one hour and the results are expressed as µl of CO_2 evolved during the initial 30 minutes time.

3. Preparation of substrates

For routine assay of lipase activity, tributyrin in sodium bicarbonate buffer was employed as substrate. Since tributyrin is not completely soluble in the bicarbonate buffer solution, it was emulsified with a small drop of Tween 80. The emulsion was quite stable and the emulsifier had no effect on the enzyme activity. Castor oil,

olive oil and triolein were treated the same way as tributyrin.

4. Localization of enzyme activity

The following proceedure was adopted based on those of Pearse and Scarpelli (1958), Hearfield and Kilby (1958) and Fenwick (1958). The working temperature was kept close to 0°C throughout the period of separation of the different fractions of the homogenate. A 15% fat body tissue homogenate was prepared in ice cold 0.25 M sucrose solution. The tissue was homogenized in a 'MSE' homogenizer for 1.5 minutes. The homogenate was centrifuged at 2500 r.p.m. for 3 minutes and the floating lipid scum and the sediment were discarded. The infranatant solution was used for the differntial fractionation using a 'MSE' super speed '25' refrigerated centrifuge. The rotor with an radius of 8.1254 cms., was adopted. After saving an aliquot of homogenate as fraction I, the homogenate solution was centrifuged at 36000 x g for 45 minutes and the supernatant, after removing the floating white lipid scum, was saved as fraction V. The sediment was resuspended in 0.25 M sucrose solution and the nuclear, mitochondrial and submicroscopic particle fractions were separated by conventional methods of differential centrifugation. The nuclear fraction (fraction II) was separated by centrifuging twice at 800 x g for ten minutes each. The supernatant was spun

at 7000 x g, for 15 minutes and the sediment was washed and resuspended and centrifuged at 7000 x g for the same length of time. The sediment saved as fraction III (mitochondria). A thick viscous submicroscopic particle fraction was separated from the supernatant solution by centrifuging at 36000 x g for 45 minutes (fraction IV). The supernatant from this was added to fraction V separated earlier.

Protein was estimated by the micro-Kjeldhal method (Hawk et al, 1954).

RESULTS

1. Effect of pH on the activity of the fat body lipase

The lipolytic activity of the enzyme preparation (5% fat body homogenate) was studied at different pH ranging from 6.0 to 8.0 using 4% (w/v) tributyrin as substrate. By adjusting the concentration of sodium bicarbonate solution, the pH of the mixture was varied, the precise concentration of sodium bicarbonate necessary to give the required pH with a gas phase of 5% CO_2 and 95% N_2 was calculated by means of the Henderson-Hasselbach equation (Umbreit et al, 1957).

It is seen from the graph that the optimal pH for the activity of the fat body lipase is 7.0. At

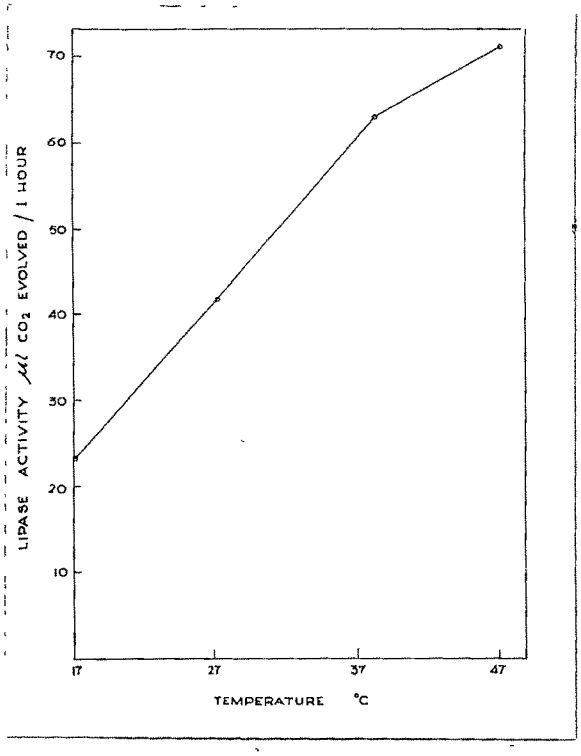

FIG.1.

Graph showing the effect of pH on the rate of hydrolysis of tributyrin and triacetin by the fat body lipase.

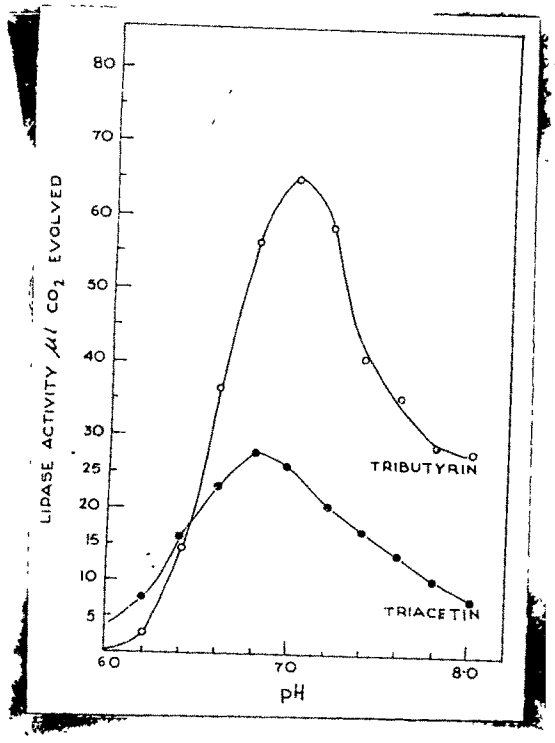

FIG.2.

Graph showing the effect of temperature on the rate of hydrolysis of tributyrin by the fat body lipase.

lower pH the fall in the activity of the enzyme was rapid while at higher pH the activity decreased gradually. At pH 6.0 practically no activity could be seen. (Fig.1). The activity is expressed as µl of CO_2 evolved per 0.5 ml of 5% homogenate enzyme solution, for the initial 30 minutes time.

With triacetin as substrate the pH optimum shifted to 6.8 at the same substrate concentration as that of tributyrin. (Fig.1.)

Studies at higher pH could not be carried out due to the limitations of the manometric techniques.

2. The effect of temperature on the activity of the fat body lipase.

The hydrolysis of tributyrin (4% in bicarbonate buffer) was studied at temperatures varying from 17°C to 47°C. The results are shown in figure 2. The rate of hydrolysis increased over the whole range of temperatures employed. The reaction rate however, fell at higher temperatures. The temperature coefficients for lipolysis were 1.8 in the range of 17-27°C, 1.5 between 27-37°C and 1.1 between 37-47°C.

3. The effect of enzyme concentration on the activity of the fat body lipase

A 10% fat body homogenate was prepared as described

under 'Materials and Methods' and was diluted to various concentrations and the lipase activity of each was determined. Protein content was estimated of each equivalent aliquots of enzyme solution used for the estimation of the activity. The lipase activity is expressed as µl of CO_2 per mg., protein during the initial 30 minutes time. The results are shown in figure 3. The activity was found to be proportional to the enzyme concentration except at very high concentrations(e.g. 10 mg protein/ml enzyme solution) and at very low concentrations (e.g. 0.4 mg. protein/ml enzyme solution, where the activity tended to fall.

4. The effect of substrate concentration on the activity of the fat body lipase

The rate of hydrolysis of tributyrin at concentrations varying from 0.002 M to 0.2 M was studied, using an enzyme solution of 7.5% fat body homogenate. The lipase activity was measured at intervals of 10 minutes each for a period of 60 minutes. The results are shown in figure 4. It is seen that at a low concentration of tributyrin (0.002 M) the activity was linear for about 20 minutes time and fell rapidly during the subsequent intervals. At all the concentrations used the activity was found to fall during the later period of the experiment. The activity was maximum and linear for a longer period (about 50 minutes) at 0.02 M

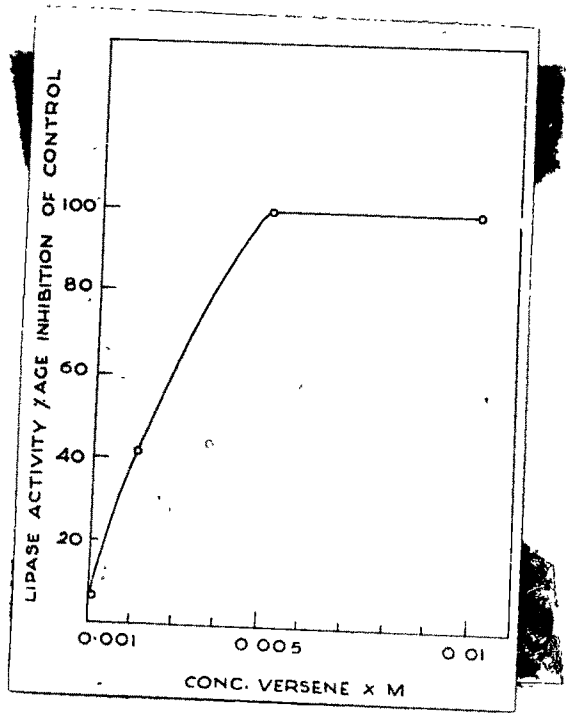

FIG.3.

Graph showing the effect of enzyme concentration on the activity of the fat body lipase

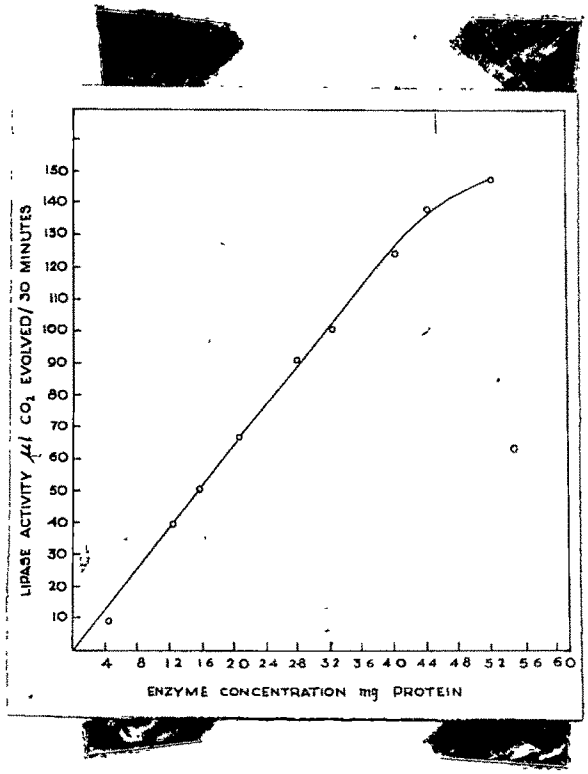

FIG.4.

Graph showing the effect of substrate concentration on the rate of hydrolysis by the fat body lipase.

tributyrin concentration.

5. The effect of different substrates on the activity of the fat body lipase.

Triacetin, tributyrin, triolein, castor oil and olive oil were tried as substrates at a concentration of 0.02 M and at pH 7.0 and temperature 37°C. Triolein, castor oil and olive oil were not attacked by the enzyme solution to any appreciable extent. The rate of hydrolysis of tributyrin was the maximum. Triacetin was acted upon quite slowly. It may be noted here that triacetin was soluble at the concentration used while tributyrin was not completely soluble.

6. The stability of the enzyme solution

A 5% fat body homogenate was prepared as described earlier. The lipase activity of the enzyme solution was measured, which was stored for various periods and at different temperatures as shown in table 1.

The enzyme solution was stable even when heated at 60°C for 30 minutes but the activity was reduced more than half when the heating was continued for one hour. At 37°C the enzyme solution deteriorated very rapidly after 18 hours time. However, at -4°C it was quite stable for 3 days.

TABLE.1.

Table showing lipase activity (%) of the fat body homogenate stored for various periods and at different temperatures.

Control	Homogenate incubated at 60°C for			Homogenate incubated at 37°C for			Homogenate kept frozen at -4°C for		
	15 min.	30 min.	60 min.	18 hrs.	36 hrs.	72 hrs.	18 hrs.	36 hrs.	72 hrs.
100.0	100.0	96.0	42.5	92.0	38.0	14.6	93.0	81.5	68.5

7. Distribution of lipase activity in different fractions of the fat body homogenate.

Lipase activity was estmated in four different fractions of the fat body homogenate as described under 'Materials and Methods' and the results are shown in table 2.

Part of the loss in the total yield of sum of all the fractions is attributed to the removal of the white lipid layer during the beginning of fractionation.

TABLE 2.

Table showing the distribution of lipase activity in the different fractions of the fat body homogenate.

Fraction	Activity units µl CO_2/hour	Yield (%)
Homogenate	390.0	100.0
Nuclei (800 x g)	39.0	10.0
Mitochondria (7000 x g)	45.0	11.5
Submicroscopic particle (36000 x g)	88.0	22.5
Soluble	190.0	48.7
Sum of fractions	362.0	92.7

DISCUSSION

One of the enzymes of the group of carboxylic esterases is a lipase for which the substrate is the ester of a higher fatty acid with glycerol or some other alcohol. The non specific esterases also act on fatty acid esters, though to a different degree, so that there is some degree

of over lapping between the two. Lipases are both hydrolytic and synthetic in action.

Various methods have been used for the determination of lipase activity which include manometric (Martin and Peers, 1953; Wills, 1954) as well as titrimetric (Balls et al ,1937; Fiore and Nord, 1949; Wills, 1954) ones. The manometric method after Martin and Peers (1953) was adopted in the present study, using tributyrin as substrate for routine assay work. It is generally agreed that the hydrolysis of tributyrin is effected by 'true' lipase' (Martin and Peers, 1953). Chemically, tributyrin meets the definition of a fat even though it is a smaller molecule (Dunkley and Smith, 1951). Dunkley and Smith (1951) studied 'tributyrinase' and 'Lipase' activity of milk and concluded that tributyrinase determination can be used as a measure of lipase activity.

Of the lipases known, much work has been done on pancreatic lipase which is regarded as a prototype of lipases having certain basic properties as regards pH and temperature optima, substrate specificity and behaviour towards activators and inhibitors.

The optimal conditions of pH and temperatures depend on the substrates used, state of purity of the enzyme, buffers used, the method of assay etc. For pancreatic lipase optimum pH for hydrolysis of triglycerides is around pH 7.0 while for higher triglycerides it is shifted to 8.8 (Schonhyder, 1945).

For intestinal lipase it was about 9.0 in Tris buffer with olive oil as substrate (DiNella et al, 1960). An acid lipase witha pH optimum of 4.3 has been recently found in the fatty layer obtained by centrifuging a homogenate of castor bean (Oryyet al, 1962). For the pigeon breast muscle lipase the pH optimum is 8.0 with McIlvan buffer and tributyrin as substrate (George and Scaria, 1959 b). In the present study for the fat body lipase in a bicarbonate carbondioxide buffer system the optimum pH has been found to be 7.0 for tributyrin and 6.8 for triacetin as substrates. It is seen therefore, the optimum pH for lipase from different sources varies from 4 to 9 and it is most often dependent on the substrates used and buffers chosen for the assay method.

The observation that the rate of hydrolysis increased with temperature over the whole range employed seems to be a common character of poikilothermic animals.

Lipases show preferential action on certain esters of fatty acids. There is general agreement that the unsaturated fatty acids glycerides are more easily split than their saturated analogues by most lipases. A summary of relative velocity rates for various substrates for lipases from different sources is given by Bier (1955). It is often found that the hydrolysis of the higher but not the lower

saturated triglycerides is largely dependent on the temperature. For example Balls et al, (1937) found that at low temperatures the pancreatic lipase would appear to be an esterase and with the increase in temperature the maximum rate of hydrolysis shifts towards longer chain fatty acids (C_7-cC_{10}). With increase in chain length they also found that the pH optimum shifted from 7.0 to 8.8.

In the present study it may be noted that the rate of hydrolysis of tributyrin was maximum under the experimental conditions described. Triacetin was acted upon but to a lesser extent. Triolein, castor oil and olive oil were not attacked to any appreciable extent.

The action of long chain fatty acids requires the substrate to be emulsified. And for this purpose various workers have used different substances like gelatin gum arabic (Wills, 1960), sodium taurocholate (Constantin, 1960), a mixture containing mono-fatty acid ester of poly ethylene glycol, soybean phosphatides, sorbitan monolaurate, sodium cholate and polyglycerolester of fatty acids (DiNella, et al, 1960). The substrate specificity is influenced by the nature of the emulsifying system (Lynn and Perryman, 1960). Recently Wood (1959) in a study of lipase activity in lingcod muscle preparations found that it is rather the insolubility of the substrate used (olive oil) which prevented the attack by lipase. Martin and Peers (1953) and George and Scaria, (1959) explained the non hydrolysis of olive oil in the

manometric system as due to the predominence of water in the system.

From results presented regarding the stability of the enzyme solution of the fat body homogenate it may be noted that the enzyme activity deteriorated rapidly after 18 hours of storage at 37°C and after 30 minutes of storage at 60°C. This instability in solution is often encountered in lipase studies. Fiore and Nord (1949) attributed such a distruction of Fusarium lipase to proteolysis. Singer and Hofstee (1948) observed a loss in the activity of wheat germ lipase during overnight storage at 3°C and this inactivation they thought to be due to the oxidation of essential -SH groups since they found that the inactivation was reduced by the presence of glutathione.

Optimal conditions for the actvity of the insect fat body lipase

With the manometric method of assay of enzyme activity in a bicarbonate-carbondioxide buffer system, the insect fat body lipase has the maximum activity at pH 7.0 with tributyrin as substrate at a concentration of 0.02 M. The temperature most suitable is between 17°C to 27°C. The rate of hydrolysis is linear over an initial period of 50 minutes.