Lipase Activity In Some *Cucubitaceae* Species

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Abstract

Lipase activity was demonstrated in the endosperm of three varieties of both germinated and ungerminated seeds of Cucubitaceae: white melon (*Cucumeropsis manii Naud*); yellow melon (*Colocynthis vulgaris.*); and pumpkin (*Cucurbita moschata*). Fractionation of homogenates by centrifugation yielded the fat layer (lipid bodies), supernatant (water soluble fraction), and particulate fraction (pellets) with lipase located in all the fractions. The highest activity was determined in the lipid body fraction. However, a mixture of the lipid bodies and supernatant gave a much higher activity than the lipid body alone. Preparation of the supernatant at different pH's (4.0 – 9.0) and treating the supernatants with different concentrations of $(NH_4)_2SO_4$, further separated the lipid bodies with separation increasing as pH increases from 6.0 – 9.0. Separation of lipid bodies did not occur below pH 4. No enzyme activity was detected in the supernatant after precipitation with 90% $(NH_4)_2SO_4$. Lipase was tightly bound to the lipid body membranes as it resisted solubilization by repeated washing with buffers and NaCl solutions. Ultrasonnication of the lipid bodies and treatment with diethylether extracted triacylglycerols from the lipid bodies with lipase activity being recovered in the membrane fractions.

Keywords: Cucubitaceae, Lipase, Lipid Bodies, Homogenate Fractions.

Introduction

Lipases (Triacylglycerol acyl hydrolase EC 3.1.1.3) have been detected in food reserve tissues of growing seedlings of many plants especially in those that contain large amounts of triacylglycerol (Beevers and Hills,1987). During seedling growth of oil seeds, the reserve triacylglycerols in the storage tissues are rapidly mobilized (Huang and Lin, 1983). These triacylglycerols are hydrolyzed to glycerol and free fatty acids by lipase. This has been observed in germinating oil seeds of the pea (*pisum sativum L. cv alaskan*) 4d, kidney bean (*phaseolus vulgaris L.)* 4d, Lima bean (*phaseolus Lunatus L.*), 4d, Corn (*zea mays*) 6d, Cotton (*Gossipium hirsutum*) 4d, Peanut (*Arachis hypogea L.*) 4d, Sunflower (*Helianthus annus L.*) 7d, Garbanzo bean (*Cicer arietinum*) 4d, Soybean (*Glycine max L.)* 5d. ((Beevers and Hills, 1987), Tomato *(Lycopersicon esculentum mill*) 3d, and Cucumber (*Cucumis sativus)* 3d, (Huang and Moreau, 1978, Huang *et. al.,* 1983). It is only in castor seed that the lipase activity is at its peak in the dry seed, (Ory *et. al.,* 1968) since in many seeds, lipase activity is not present in dry seeds but appears rapidly aftergermination(Huang and Moreau,1978)..

The location of the enzymes that catalyse the initial triacyglycerol hydrolysis in germinating oil seeds has not been well documented. The only well studied oil seed lipase is that from castor bean. This enzyme is found in the membrane of the lipid bodies and is active in ungerminated seeds (Ory *et. al.,* 1968). However, lipid bodies isolated from germinated and ungerminated seeds of peanut, pine and soybean, did not contain lipase activity. Lipase activity was detected only in the soluble fraction of pine megagametophyte and the glyoxysomes of soybean (Ching, 1968, Jacks and Yatsu,1972, Huang and Lin,1983).

Previous studies on lipase activity in germinating seeds are few especially in *Cucubitaceae*, and are restricted to few plant species such as barley, and wheat (Laidman and Tavaner, 1972). Furthermore, some of these studies have been complicated by problems associated with the determination of lipase activity in oil storage plant tissues because of the presence of endogenous triacylglycerol. We report the activity and localization of lipase in melon seeds employing a modified Ducombe (1963) method.

Table 1: Lipase specific activity in the different fractions of germinated and ungerminated seeds (decoated and undecoated) of three species of *Cucubitaceae*

TYPES OF SEEDS		LIPASE ACTIVITY (units/mg protein)					
		Days of germination	Cucubitaceae seed	Lipid bodies	Supernatant	Pellets	Mixture lipid bodies and supernatant (M)
UNGERMINATED		Dry	White melon	11.04	13.05	0.92	16.25
UNGERMINATED		Dry	Yellow melon	9.99	12.65	0.82	13.08
UNGERMINATED		Dry	pumpkin	10.05	12.50	0.81	12.65
GERMINATED	UNDECOATED	Day 3	White melon	13.39	11.88	3.00	28.47
GERMINATED	UNDECOATED	Day 3	Yellow melon	12.92	11.33	2.54	15.33
GERMINATED	UNDECOATED	Day 2	pumpkin	12.00	11.11	1.37	14.69
GERMINATED	DECOATED	Day 3	White melon	13.94	15.38	2.75	34.00
GERMINATED	DECOATED	Day 3	Yellow melon	12.82	12.07	2.59	15.68
GERMINATED	DECOATED	Day 2	pumpkin	11.9	14.05	2.09	14.95

Table 2: Enzyme activity in different fractions of white melon seeds at different pH

Fractionation	Enzyme activities(units/mg protein) at different pH					
pH	4.0	5.0	6.0	7.0	8.0	9.0
Homogenate	4.40±0.01	4.90±0.09	5.35±0.10	5.50±0.03	6.0±0.00	6.20±0.10
Lipid Bodies*	9.60±0.00	10.40±0.21	11.90±0.11	12.80±0.01	13.65±0.03	13.30±0.02
Water soluble fraction	10.40±0.10	13.70±0.05	14.15±0.10	15.01±0.10	15.22±0.01	14.90±0.15

**After washing with diethylether. Values are average of three determinations.*

Materials and Methods

Reagents: BSA was obtained from BDH England and ß-mecapthoethanol was from Merk Darmsar – Germany. Other reagents and solvents were of analytical grade.

Fresh, mature and viable seeds of three varieties of *Cucubitaceae* namely, white melon (*Cucumeropsis manii* Naud*)*, yellow melon (*Colocynthis vulgaris*) and pumpkin (*Cucubita moschata*), were bought from a local market at Nsukka, Enugu state Nigeria.

Enzyme isolation and assay: The three varieties of oil seeds (*Cucumeropsis manii, Colocynthis vulgaris* and *Cucurbita moschata*) were decoated, washed in a solution of water containing 0.1% HgI (to prevent fungal growth) and steeped for 48h. The end of imbibition period was designated day zero of germination. Germination was carried out on moist jute bag in darkness at room temperature. Also some undecoated seeds from the three varieties were germinated for the same period as the decoated ones. They were harvested and the endosperm homogenized with pestle and mortar in a grinding buffer containing 0.6M sucrose, 1mM EDTA, 10mM KCl, 1mM $MgCl_2$, 1mM β-mercaptoethanol, all dissolved in 0.15M glycine solution, and the pH adjusted to 7.5 with KOH. The homogenate was filtered with about 25ml of the grinding buffer. 25ml of a grinding buffer containing 0.5M sucrose (instead of 0.6M sucrose), was layered on the filtrate giving a total volumes of 50ml. This was centrifuged at 10,000 x g for 15 min Germination studies and enzyme isolation were as in Huang, *et. al.* (1983).The fat layer (Lipid bodies) was combined with the water soluble fraction and used as the crude enzyme. The modified method of Ducombe (1963), in which the free fatty acids released were converted to copper soaps and quantitated by sodium diethyldithio-carbamate as a colour reagent was used. The reaction mixture in a total volume of 1.0ml contained 0.10ml 10mM deoxycholate solution, 0.4ml 1M triethanolamine buffer pH 7.5 and 0.5ml substrate emulsion (0.2ml of white melon seed oil + 0.2ml Gum Arabic + 0.1ml of distilled water vortexed at 100rev/sec for 30sec). To the 1.0ml reaction mixture, 0.1ml of enzyme was added and the mixture incubated at room temperature.

Table 3: Lipase specific activities (units/mg protein) in the water soluble fraction and Lipid bodies after $(NH_4)_2SO_4$ precipitation

pH	Lipase activities (units/mg protein) 10% $(NH_4)_2SO_4$ precipitation Water soluble fraction	10% $(NH_4)_2SO_4$ precipitation Lipid bodies*	65% $(NH_4)_2SO_4$ precipitation Water soluble fraction	65% $(NH_4)_2SO_4$ precipitation Lipid bodies*
4.0	8.20±0.13	4.55±0.12	3.95±0.08	5.68±0.10
5.0	8.85±0.03	4.55±0.20	4.35±0.05	6.82±0.15
6.0	9.25±0.15	6.86±0.04	5.60±0.11	23.86±0.20
7.0	11.50±0.10	10.23±0.05	6.40±0.04	26.13±0.25
8.0	11.80±0.30	19.54±0.15	6.55±0.04	29.54±0.04
9.0	11.55±0.25	15.00±0.07	5.78±0.11	25.00±0.13

*After washing with diethylether . Values are average of three determinations.

After exactly 20min of incubation, the tube was immersed in a water bath at 80°C for 1min to stop the reaction. 5ml of chloroform was added followed by addition of 2.5ml of copper reagent (1M triethanolamine, 1M acetic acid, 0.45% $Cu(NO_3)_2.3H_2O$, 9:1:10,v/v),respectively. The tube was vortexed for 5min and centrifuged at 10,000 x g for 5 min to separate the phases. The aqueous colored phase was carefully removed with a pipette. 2.0ml of the clear chloroform layer was transferred to a clean dry tube and 0.20ml of the colour reagent(0.1% sodium diethyldithiocarbamte in butan-2-ol,w/v) was added and the absorbance at 440nm was measured after 30min. The sample blank was prepared as in above without the 20min incubation. A standard solution, which contains 50µM stearic acid in 1.1ml solution and 2.5ml copper regents was included in each series of determination.

The following formula was used to calculate the activity of lipase.

$$\text{Activity} = \frac{A_{\text{Lipase}} \times 250 \times F}{A_{\text{Standard}} \times 10 \times V} \times F \ (U/L)$$

Where A is the absorbance at 440nm, F is the dilution factor and V, the sample volume.

Protein estimation: Protein determination was by the method of Lowry *et. al.,* (1951).

Localization of lipase in various fractions of the oil seed endosperm: Lipase was isolated from the three different varieties as earlier stated under enzyme isolation and assay. The centrifuged fractions were separated into the following fractions; lipid bodies (top layer), water soluble fraction (supernatant) and the pellets (particulate fractions). Lipase activity and protein content of each fraction were also determined. In a separate experiment, lipase was extracted at different pH from 3-day germinated white melon seeds using grinding buffers of different pH's (Mcvaine's buffer pH 4.0 – 6.0, phosphate buffer pH 7.0 – 8.0, Tris-HCl pH 8.0 – 9.0). Enzyme activity was assayed in the uncentrifuged homogenate (crude), isolated lipid bodies and the supernatants (water soluble fraction). The various supernatants were subjected to differential ammonium sulphate precipitation (0-90%).

Effect of sonnication; The lipid bodies were sonnicated for 5 sec in an ultrasonnicator and then treated with diethylether (1:2 v/v, respectively) to remove triacylglycerols. Both sonnicated and unsonnicated lipid bodies were examined under the microscope to find the effect of such treatment on the shape of lipid bodies. Enzyme activity and protein content were determined

Results and Discussion

White melon (*Cucumeropsis manii)* seeds had the highest lipase activity (Table 1). Although all the fractions had activity, very little activity was found in the pellets after centrifugation. Major activities were found in the lipid bodies and the water soluble fraction (supernatants). Generally, lipase activity was higher in the germinated seeds than in the ungerminated seeds. Huang, *et. al.* (1983), showed that lipase activity was found to be highest in the dry seeds of

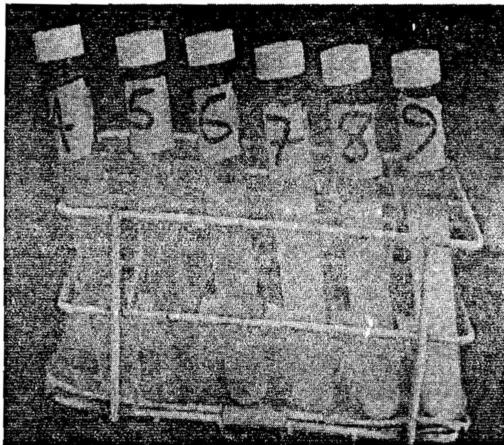

Fig 1: Effect of 10% NH_4 $(SO4)_2$ Saturation on the crude extract 4,5,6,7,8 and 9, are the different pH values.this shows the result of 10% NH_4 $(SO4)_2$ precipitation of the water soluble fraction prepared at different pH's.No precipitate was observed at pH 4 and 5, but from 6-9, lipid bodiesa were separated out on top of the solution which the solution becomes more cloudy as thepH increases with a big precipitate found at pH 8.0.

Fig. 2: Effect of 65% NH4 (SO_4) saturation on the crude extract 4,5,6,7,8 and 9, are the different pH valuesThis shows the result of . 65% NH_4 (SO_4) precipitation of the water soluble fraction from Fig 1 (the lipid bodies in Fig 1 were removed and the remaining solution was made up to 65% NH_4 (SO_4) saturation). The resulting soltion was clear and transparent with more lipid bodies separated out in increasing order as the pH increases from 4-9.

castor while virtually most of other oil seeds studied had none or little activity in the dry seed. In the dry ungerminated seeds, the highest activity was found in the water soluble fraction of the white melon seeds. Combining the lipid bodies and the supernatant augmented the activity of the enzyme. In the germinating seeds, highest activity was determined on the third day for white and yellow melon, and on the second day for pumpkin. Again, enzyme activity was shown to be highest in white melon seeds. Lipase activity was found to be higher in decoated seeds than in the undecoated. However, lipase activity appeared to be highest in the lipid bodies of the undecoated seeds while for the decoated seeds, the water soluble fraction (supernatant) had the highest lipase activity.

In summary, a mixture of lipid bodies and supernatant (Table 1) gave the highest lipase activity in all the oil seeds studied. Decoated germinated seeds had higher specific activity than undecoated germinated ones, with the highest activity observed in the decoated germinated white melon seeds on the 3rd day of germination. Also pumpkin and yellow melon had their peak lipase activities on the 2nd day of germination.

Homogenates of the white melon seeds were prepared at pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. After centrifugation, the supernatants were made up to 10% saturation. No separation of lipid bodies occurred at pH 4 and 5, though it was noticed at pH 6, 7, 8 and 9. This separation increases with increase in pH with lipase activity being highest at pH 8.0 (Fig. 2, Table 2). However when the supernatant was made up to 65% ammonium sulphate saturation, separation of the lipid bodies disappeared (Fig. 2, indicating that between 10% and 65% ammonium sulphate saturation, the lipid bodies redissolved. The enzyme activity in the clear water soluble fraction (supernatant) at 10% $(NH_4)_2$ SO_4 saturation (after centrifugation) was higher than that in the water soluble fraction (supernatant) at 45% $(NH_4)_2$ $S0_4$ saturation (Table 2), except at pH 4.0. In each case the activity of the enzyme was highest at pH 8.0. However, there was an increasing amount of lipid bodies separated out on top of the solution from pH 4 to pH 9. The highest amount of lipid bodies was noticed at pH 9.0 (Fig. 2). When the lipid bodies were tested for enzyme activities, it was interesting to observe that the specific activities at different pH's were higher at 45% $(NH_4)_2$ $S0_4$ saturation than at 10%. This suggests that more of the enzyme was separated out in form of lipid bodies at high

$(NH_4)_2$ SO_4 saturation especially at an alkaline pH of 8.0 (Table 3).

When the supernatants at pH 7, 8 and 9 (at 45% $(NH_4)_2$ $S0_4$ saturation) were brought to 90% saturation with $(NH_4)_2$ $S0_4$, and centrifuged, there was separation into two fractions, the top lipid bodies and the clear supernatant. The enzyme was precipitated from the supernatant by the $(NH_4)_2$ $S0_4$ (Table 4).

Table 4: 90% $NH_4)_2SO_4$ saturation*

pH	Specific activity (units/mg protein)	
	supernatant	lipid bodies
7.0	2.27±0.20	20.00±0.00
8.0	0.90±0.02	23.45±0.45
9.0	3.56±0.05	20.90±0.90

**Ammonium sulphate concentration was raised from 45% to 90%. Values are average of three determinations.*

Effect of Sonnication and diethylether on the shape of lipid bodies: Table 5 shows the effect of sonnication and treatment with diethylether on lipid bodies. The micrographs show lipid bodies that were only sonnicated (Fig 3a), those only washed with diethylether (Fig. 3b), and those sonnicated and further washed with diethylether (Fig.3c), and those neither sonnicated nor washed with diethylether (Fig. 3d).

Table 5: Effect of Ultrasonnication and/or diethyether on lipid bodies

Treatment	Specific activity (units/mg prot.)
None(control)	20.00±0.03
Sonnication only	21.45±0.23
Diethyether	22.00±0.50
Sonnication and diethyether	23.20±0.05

Values are average of three determinations.

Sonnication disrupted the membrane of the lipid bodies. Also when the lipid bodies were washed with diethylether, the triacylglycerols (TAG) were extracted from the membranes leaving the lipid bodies also disrupted. The fact that high activity was recorded after washing with diethylether suggests that lipase from white melon seed *(Cucumeropsis manii)* is a membrane protein. Moreover, lipid bodies are the

Fig. 3a: micrograph showing lipid bodies that were not sonicated and not treated with diethylether

Fig. 3b: micrograph showing sonicated lipid bodies that were not treated with diethylether

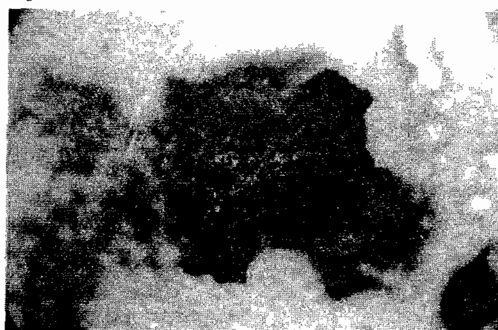

Fig. 3c: micrograph showing lipid bodies that were not sonicated but treated with diethylether.

Fig. 3d: micrograph showing lipid bodies that were sonicated and subsequently treated with diethylether.

source of lipase in oil seeds (Huang *et. al.*, 1983). Sonnication and treatment with diethylether disrupt the membrane and extract the TAG of the lipid bodies. [As TAG is, in a concentration dependent manner, an inhibitor of plant lipases, its removal by this process could augment the enzyme activity]. Also, repeated washing of the lipid bodies with buffers of different pH, as well as NaCl solution of increasing concentration could not solubilize the lipid bodies. This equally suggests that the enzyme is really a membrane protein. Research is currently going on in our laboratory on the extraction, purification and characterization of this membrane bound lipase.\

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