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Lipase activity in dormant seeds of the African oil bean (*Pentaclethra macrophylla* Benth)

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Abstract

Acetone powders were obtained from freshly harvested dormant seeds of African oil bean (*Pentaclethra macrophylla* Benth) and the activity as well as substrate specificity of the isolated crude lipase (glycerol ester hydrolase E.C. 3.1.1.3) were examined. The enzyme was more active on lauric oils (containing short-chain fatty acids), especially coconut oil. Hexane at 1:2 v/w solvent:substrate ratio was optimal in enhancing lipolysis of the oils. Optimum substrate concentration was 5 g in 2.5 ml of solvent, above which enzyme inhibition resulted. Lipolysis increased with enzyme concentration (non-linearly), with optimum rate at between 0.5 and 0.75 g and 60 min incubation. Optimum temperature for the activity of the oil bean lipase was 30 °C, although substantial lipolysis was still evident at 80 °C indicating the fairly high thermostability of the enzyme. pH optimum for enzyme activity was near neutrality. The effects of different ions on the activity of the isolated lipase were also investigated. The presence of Ca^{2+} increased activity by 64% while sodium chloride and mercuric chloride inhibited activity by 36% and 28.5%, respectively. On addition of EDTA, an inhibition in activity of 28% was observed. The results of the present study show that the oil bean seed will deteriorate with storage.

Keywords: *Pentaclethra macrophylla*; Dormant seeds; Lipase activity

1. Introduction

Lipases (glycerol ester hydrolases E.C. 3.1.1.3) in oilseeds help to hydrolyze the ester bonds of storage triacylglycerols. Acetone powders (acetone-insoluble residues obtained by extraction of the oilseed homogenates with chilled acetone) have been found effective in oil hydrolysis with preferential cleavage of fatty acids esterified at the sn-1,3 positions of the triacylglycerols (Hassanien & Mukherjee, 1986). It has been reported that, with few exceptions, lipase activity is absent in ungerminated (or dormant) seeds and increases rapidly in post-germination (Huang, 1984). In those cases where the enzyme is active in the dormant seeds, as in castor bean seed (Huang, 1984), peanut (Sanders & Pattee, 1975) and Barbados nut (Abigor, Uadia, Foglia, Haas, Scott & Savary, 2002), it is apparently inactive in vivo but highly active in vitro. In other words, as long as the seed is in the intact state, the enzyme will remain inactive, but any slight change in the seed or storage conditions will probably initiate activity.

Oilseed lipases have great potential for commercial exploitation as industrial enzymes, especially those oilseeds that are presently considered under-utilized, among which is the African oil bean seed. The African oil bean (*Pentaclethra macrophylla* Benth) produces a less-common oilseed whose use has been restricted to a nutritious snack and fermented soup condiment in some parts of sub-Sahara Africa (Enujiugha, 2000; Enujiugha & Olagundoye, 2001). The oil bean seed contains a substantial quantity of oil which could be exploited commercially (Enujiugha & Ayodele-Oni, 2003). An examination of the lipolytic enzymes contained in the membranes of the seed vacuole is important since improper storage and post-harvest handling may initiate

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lipolytic activity, thereby causing depreciation in the commercial value of the seed oil.

Already, in a previous report, the dormant seeds of African oil bean have been shown to contain active thermostable amylases (Enujiugha, Amadi, & Sanni, 2002). In the present study, the activity and some properties of the oil bean seed lipase are examined.

2. Materials and methods

2.1. Materials

Fresh seeds of the African oil bean were hand-picked from farms around NIFOR in Benin City, immediately after dehiscence of dry pods. The seeds were kept at 4 °C prior to analysis to avoid early lipolysis. Palm oil, palm kernel oil (PKO), coconut oil and raphia (*Raphia hookeri*) oil were obtained from the Nigeria Experiment Mill, NIFOR. The oils were refined before analysis, using the method of Jensen, Marks, Sampugna, Quinn, and Carpenter (1966). All the chemicals, reagents and solvents used in the experiments were of analytical grade and were products of E. Merck AG, Darmstadt, Federal Republic of Germany.

2.2. Enzyme preparation

All operations for the isolation of the enzyme were carried out at 4 °C (Muto & Beevers, 1974). Acetone powder of the oil bean seed lipase was prepared according to the method of Hassanien and Mukherjee (1986), with some modifications. The seeds were carefully cracked, to avoid bruising the endosperm, and the shells removed. 25 g of the seed cotyledons were ground with 30 ml of cold acetone using a Waring Blender. The acetone extract was filtered through a cheese cloth (Abigor et al., 2002) and washed four times, with 20 ml each time, of cold acetone (4 °C). The residue was air-dried at room temperature (26 ± 1 °C) to yield the acetone powder, which was kept at 4 °C until ready for assay.

2.3. Assay of enzyme activity

Lipase (glycerol ester hydrolase E.C. 3.1.1.3) activity was assayed using a modification of the titrimetric method of Khor, Tan, and Chua (1986). The assay mixture contained 5 g of substrate, 2.5 ml of hexane to solubilize the oil, and 1 g of the crude enzyme. The mixture was incubated at 30 °C for a period of 1 h with continuous stirring, using a magnetic stirrer. At the end of the incubation, 25 ml of acetone–ethanol (1:1 v/v) were added to stop the reaction and to extract the free fatty acids (FFAs) liberated. The FFAs in the mixture were then estimated by direct titration with 0.01 M NaOH using phenolphthalein as indicator. Lipase activity was expressed as the percent FFAs liberated after a 1 h incubation (Wetter, 1957). Corrections were made for endogenous fatty acid production (assay mixture without substrate) and non-enzymatic fatty acid production (assay mixture without enzyme preparation).

2.4. Effects of pH, temperature and time

Phosphate buffer (5 ml) at different pH (4–9) was added to 5 g of substrate, 1 g of enzyme preparation and 2.5 ml of hexane. The mixture was incubated at 30 °C for 1 h with continuous stirring, and the activity determined for each pH. A thermostatted, water-jacketed reaction chamber with shaker was employed for determining the temperature dependence of lipase activity. The assay mixtures were incubated at different temperatures (30–80 °C) for 1 h and the activity measured. The effect of time on lipase activity was determined by varying the time of incubation (15, 30, 45, 60 min) of the assay mixture at the same temperature (30 °C).

2.5. Effects of ions

To de termine the effects of ions, the method of Mukundan, Gopakumar, and Nair (1985) was adapted. For the investigation, 0.01 M solutions of sodium chloride (NaCl), calcium chloride ($CaCl_2$), mercuric chloride ($HgCl_2$) and ethylene diamine tetra-acetic acid (EDTA) were prepared. 1 ml of each of these solutions was added to separate assay mixtures and incubated for 1 h with continuous stirring. Lipase activity was determined as described above.

2.6. Effect of type and concentration of substrate on lipase activity

Different substrates (palm, palm kernel, raphia and coconut oils) were each used substitutively, at 5 g in the assay mixture, with subsequent incubation at 30 °C for 1 h. Lipase activity was measured for each substrate substitution, as described in this paper. The dependence of rate of lipolysis on substrate concentration was determined by assaying lipase activity with varying concentrations (1, 2, 3, 4, 5 g) of coconut oil (substrate) in 2.5 ml of solvent.

2.7. Effect of enzyme concentration on lipolysis

Different quantities of the oil bean lipase (0.25, 0.5, 0.75, 1 g of the acetone powder) were used in assay mixture with 5 g of substrate in 2.5 ml of hexane. Enzyme assay was carried out as described for lipase activity. For the control, assay mixture was incubated without added acetone powder.

3. Results and discussion

The results obtained in the present study show lipase activity in ungerminated (dormant) seeds of the African oil bean (*P. macrophylla* Benth). The common observation from past studies is that ungerminated (dormant) seeds have little or no lipase activity (Huang & Moreau, 1978; Wetter, 1957). Most investigations on plant lipases have been carried out on oleagenous seeds in which activity is generally found to become prominent upon germination (Hassanien & Mukherjee, 1986). However, lipase activity has been observed in ungerminated seeds of castor bean (Ory, St. Angelo, & Altschul, 1962) and *Jatropha curcas* (Abigor et al., 2002). In this study, African oil bean seed has shown lipase activity which is not manifested in vivo in sound seeds. However, it is expected that, if the seed is bruised, lipolytic activity will immediately be initiated.

The results of the lipolysis of palm, palm kernel, raphia and coconut oils by the lipase isolated from the dormant seeds of *P. macrophylla* are presented in Table 1. Lipolysis was more pronounced in palm kernel and coconut oils with short-chain FFAs; these are lauric oils that are predominantly made up of C_{12} fatty acids. Results of the present study are in agreement with previous observations that, generally (but with few exceptions)

Table 1
Lipolysis of different oils by the lipase isolated from dormant oil bean seed

Substrate	Activity (% FFA)[a]
Palm oil	0.2 ± 0.1
PKO	1.6 ± 0.2
Coconut oil	2.5 ± 0.1
Raphia oil	0.4 ± 0.1

[a] Figures represent means of triplicate determinations (mean $\pm$ SEM).

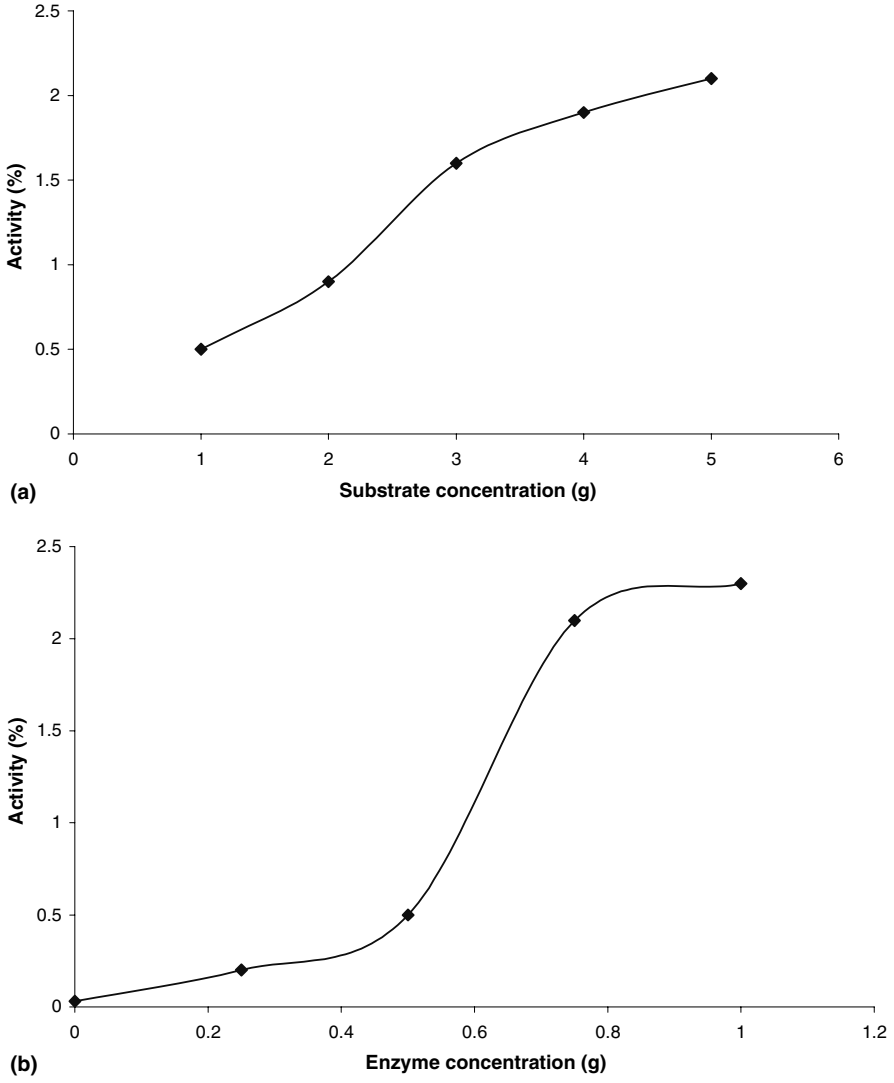

Fig. 1. Effect of different concentrations of (a) substrate and (b) enzyme on the activity of the lipase from dormant African oil bean seed.

oilseed lipases are more active on triacylglycerols containing short-chain fatty acids (Huang, 1984). Some mycelial lipases were also found to follow this trend (Tahoun & Ali, 1986). The results agree with the observation of Mukundan, Gopakumar and Nair (1985) that the short-chain fatty acids, owing to their comparatively higher water solubility, will have a smaller inhibitory effect in the lipid phase of the triglyceride emulsion, while the long-chain fatty acids, due to their lipophilic nature, may cause more inhibition in the lipid phase.

In this study the oils (substrates) were solubilized in hexane according to the method of Khor et al. (1986) so as to increase the interfacial area for the activity of the enzyme. It has been observed that the actual site of lipolysis is at the interface (Mukherjee, 1990). The enzymatic activity of a lipase is related to the interfacial area of the water-insoluble substrate. The optimal ratio found for hexane to oil is 0.5 ml of hexane to 1 g of substrate. Increasing or decreasing the hexane to oil ratio decreased the lipolysis reaction.

Fig. 1(a) and (b) shows the effect of different substrate and enzyme concentrations on the activity of the oil bean seed lipase. A linear logarithmic relationship between substrate concentration and rate of lipolysis was observed at concentrations up to 4 g. At higher concentrations, there was a drop in the rate of lipolysis,

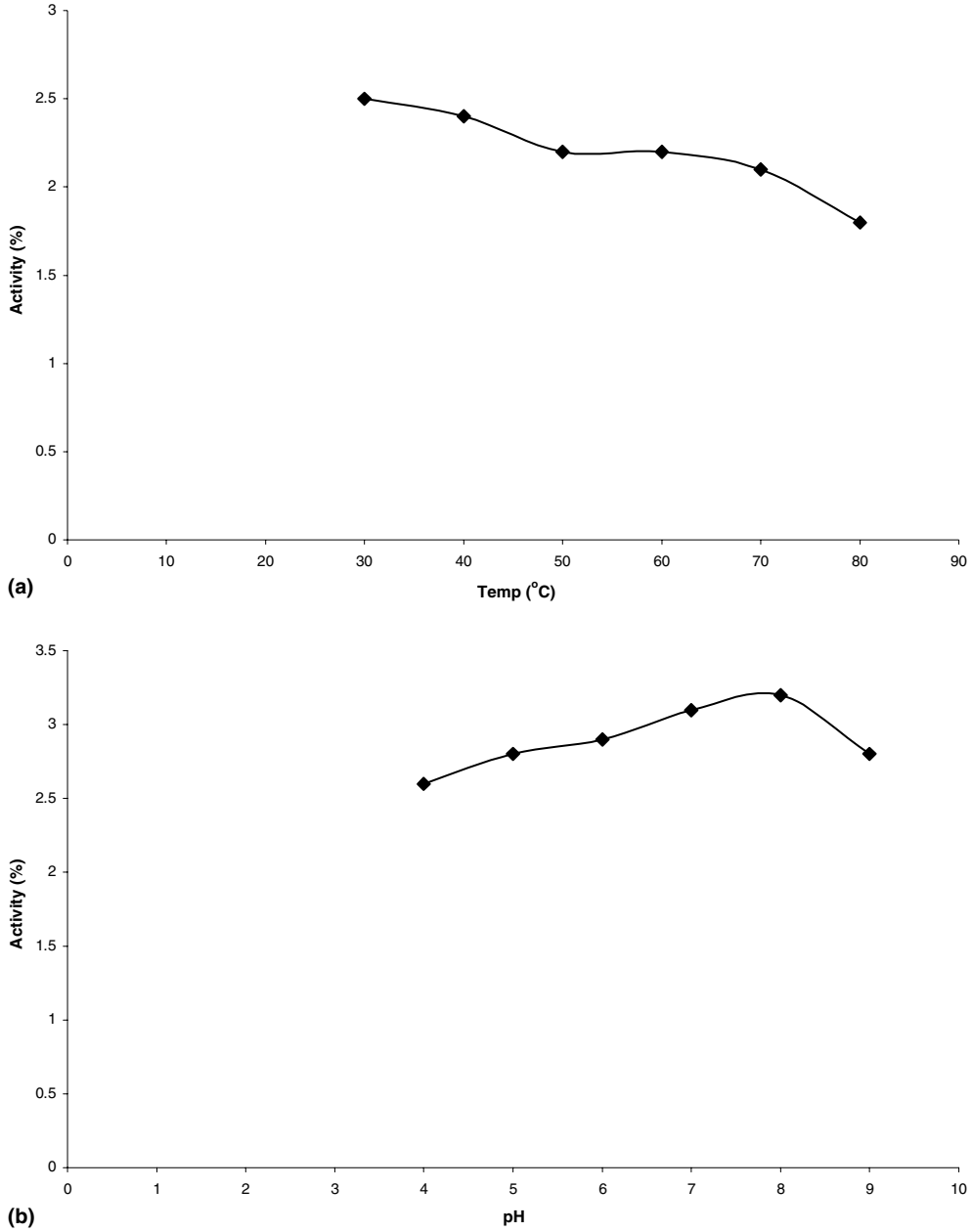

Fig. 2. Effect of (a) different temperatures and (b) pH on the oil bean lipase.

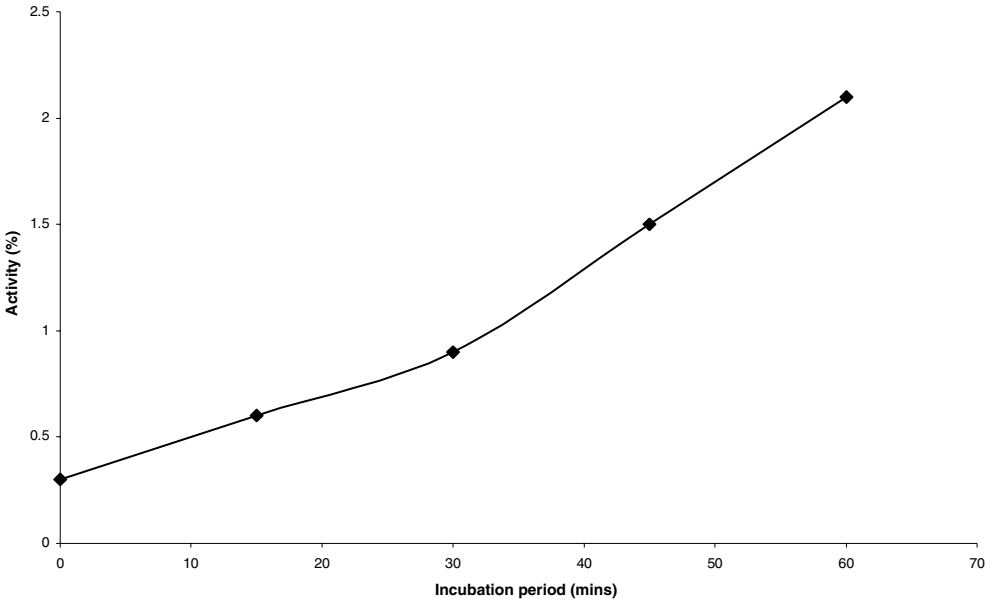

Fig. 3. Effect of incubation time on the lipase activity.

although there was no apparent decline in the activity of the enzyme. At this stage, increasing the concentration of the substrate would result in inhibition of the enzyme. The results of varying enzyme concentration with a constant substrate concentration, when plotted, were non-linear but incremental (Fig. 1(b)). This is in contrast to the findings of Hassanien and Mukherjee (1986) and Sanders and Pattee (1975), that the relationship is linear and passes through the origin for peanut, rape, mustard and lupine lipase preparations. In this study, at enzyme concentrations up to 0.5 g, there was a steady but slow fatty acid liberation. Between 0.5 and 0.75 g enzyme concentrations, a very high rate of lipolysis was observed. Beyond this, activity increased steadily, but rate of lipolysis was reduced. The results show that lipolysis of 5 g of oil could be optimally achieved at a 0.75 g enzyme concentration.

Figs. 2(a) and (b) show the respective effects of temperature and pH on the activity of the lipase from ungerminated *P. macrophylla* seed. The optimum temperature for the activity of the seed lipase was 30 °C, above which there was a steady decline. Purified oil palm lipase has been shown to have optimal activity at 30 °C, above which there was also a steady decline (Abigor, Opute, Opoku, & Osagie, 1985). In the present study, although activity declined above 30 °C, substantial lipolysis was still evident, even at 80 °C, indicating a fairly high thermostability of the enzyme.

The pH optimum for *Pentaclethra* lipase is near neutrality (Fig. 2(b)), which is similar to the pH 7.5 for lipase from *J. curcas* L. (Abigor et al., 2002) and pH 8.5 for peanut alkaline lipase (Sanders & Pattee, 1975). However, some other lipases have been found to be optimally active at acid pH, for example, pH 5.0 for ungerminated castor bean lipase (Ory et al., 1962) and pH 4.2 for oil palm mesocarp lipase (Abigor, Opute, Opoku & Osagie, 1985).

Effect of period of incubation on lipolysis is shown on Fig. 3. A linear logarithmic relationship was observed between reaction time and rate of lipolysis. The optimal activity was found to be at 60 min of incubation. Abigor et al. (1985) observed a linear relationship at shorter incubation periods for oil palm lipase. Some other researchers, working with lipase from different sources, also found that the rate of hydrolysis or lipolysis is linear on a logarithmic scale (Khor, Tan & Chua, 1986; Linfield, Olbrien, Serota, & Baraskas, 1984). With peanut alkaline lipase, a linear rate of hydrolysis was exhibited for over 60 min (Sanders & Pattee, 1975). This trend could be attributed to zero order reaction, indicating that the substrate is not limiting and the products of hydrolysis exhibit no inhibitory action.

Table 2 shows the effects of ions on the activity of lipase from dormant African oil bean seed. Calcium ion in the reaction mixture brought about 64% increase in

Table 2
Effects of different ions on the activity of lipase from dormant oil bean seeds[a]

Salt/ion	Activity (% FFA)[b]
NaCl	1.28 ± 0.25
$CaCl_2$	4.00 ± 0.50
$HgCl_2$	1.80 ± 0.09
EDTA	1.70 ± 0.31

[a] Figures represent means of triplicate determinations (mean ± SEM).

[b] Coconut oil/enzyme mixture without added salt served as control (activity $2.5 \pm 0.1\%$).

activity. Abigor et al. (2002) and Haas, Cichowicz, and Bailey (1992) also observed an enhancement of lipase activity by calcium ion inclusion. Sodium chloride, mercuric chloride and EDTA inhibited the activity of the *Pentaclethra* seed lipase by 36%, 28.5% and 28%, respectively. From the work of Mukundan et al. (1985), it is obvious that the chloride ion did not cause any inhibitory effect but the metal ions (Na^+, Hg^{2+}) did.

The inhibition of lipase activity by mercuric chloride is an indication of the presence of sulfhydryl groups in the enzyme molecule. Previous studies show that 10^{-4} M solutions of $HgCl_2$ brought about complete inhibition of lipase activity in peanut (Sanders & Pattee, 1975) and castor bean (Ory et al., 1962). Sodium and mercury were also found to inhibit oil palm lipase (Abigor et al., 1985). EDTA inhibition of the enzyme activity could be attributed to its chelating ability. It will naturally perform what is known as the 'chelation process' of the system and thereby disrupt the formation of the enzyme-substrate complex. This invariably affects the formation of the end-product.

4. Conclusion

An active lipase (acetone powder) has been isolated from dormant (ungerminated) seeds of the African oil bean (*P. macrophylla* Benth). The oil bean lipase was effective on triacylglycerols with short-chain fatty acids (especially lauric oils). The optimum conditions for lipolysis were found to be 30 °C and 60 min incubation time, with a pH optimum near neutrality. Calcium ion enhanced the enzyme activity, while sodium, mercury and EDTA inhibited lipolysis significantly ($P < 0.05$). The *Pentaclethra* seed lipase could be exploited commercially in industrial processes, especially with the current focus on oilseed lipases as convenient replacements for microbial lipases in biotechnological applications.

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