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Partial Purification and Some Biochemical Properties of Lipase from Germinating Oil Palm Seeds

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Abstract: Lipases hydrolyze lipids to yield fatty acids and glycerol which are used as raw materials in food, cosmetics and pharmaceutical industries. This study was conducted to characterize lipase from germinating oil palm seeds. The lipase was extracted, purified and the biochemical properties were studied. Lipase assay was carried out using *p*- ntrophenyl palmitate as the substrate. The lipase was subjected to 80% ammonium sulphate precipitation, purified on ion exchange chromatography column using DEAE Sephadex A50 resin and gel filteration on Sephadex G-100. Relative molecular weight of the enzyme was determined on a calibrated gel filtration column using Sephadex G-100 while the subunit molecular weight was estimated on 10% Sodium Dodecyl Sulphate Polycarylamide gel electrophoresis. Kinetic parameters (K_m and V_{max}), effect of temperature, pH, and sensitivity to metal ions as well as substrate specificity of the lipase were studied. The lipase had a specific activity of 27.78 μmol/min/mg, a purification fold of 30.32%. The relative molecular weight of the enzyme was estimated to be 40kDa, while the K_m and V_{max} values were 2.5mM and 32.6 μmol/min/ml respectively. $ZnCl_2$, KCl, EDTA and $MnCl_2$, inhibited the enzyme activity, the highest inhibition of 8.53% was observed with EDTA while its activity was enhanced by $CaCl_2$. Optimal pH was 7.0 and the temperature for maximal lipase activity was 45^{o}C. The enzyme hydrolyzed coconut oil at about twice the rate of palm kernel oil and palm oil, and at a higher rate than olive oil and raphia oil. The study concludes that germinating oil palm is a good source of lipases which can be of use industrially.

KEYWORDS: Partial purification, Biochemical properties, Lipases, Germinating oil palm seeds

1.0 Introduction

Lipases are a group of enzymes that catalyzes the hydrolysis (cleavage) and synthesis of various forms of lipids (Svendson, 2000). They belong to a subclass of the esterases and have been reported to be active at the oil water interface (Ibrahim, 1996). Oil seed lipases have great potential for commercial exploitation as industrial enzymes. The demand for lipolytic enzymes has been increased due to its potential use in the various manufacturing processes of industrial goods such as food and pharmaceuticals (Boland *et al*., 1991), in the production of biodiesel and industrial detergent (Freire and Castilho, 2008) as well as in the analysis of triacylglycerols raw materials in food, cosmetics and pharmaceutical industries (Forgia *et al*., 1995). Fat splitting, with lipase as a catalyst, is advantageous compared to conventional process due to low energy consumption, low cost, high product quality, ease of purification and safer process.

Lipases are widespread in nature and have been found in animals, plants and microorganisms (Ohnishi *et al*, 1994). In fact, germinating seeds have been shown to have an exceptionally high lipolytic activity probably in order to meet its energy requirements. Lipases are considered to be rate limiting/controlling during germination enzyme and hence often subjected to change(s) in activity during

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germination (Brockerhoff and Jensen, 1974; Ejedegba *et al*., 2007).

Oil seeds have been explored and most widely studied with respect to lipase extraction and characterization, (Sadeghipour and Bhatla, 2003; Enujiugha *et al.*, 2004; Sagiroglu and Arabaci, 2005). Therefore palm seed (kernel) which is imbedded in the fruit of the oil palm tree (*Eleais guineensis*) is readily available and is a source of lipase enzyme. In this study we report the isolation, purification and characterization of lipase from germinated oil palm seeds with the view that it could be used to hydrolyze vegetable oils for production of fatty acid and glycerol.

2.0 Materials and Methods

2.1 Materials

Germinating oil palm seeds (2 weeks old) were obtained from Nigerian Institute for Oil Palm Research (NIFOR) in Benin city, Nigeria. Raphia oil was extracted by Soxhlet using n-Hexane as solvent and olive oil was purchased from a supermarket in Benin, Edo State, Nigeria. All chemicals, reagents, resins, standard protein and solvents used in the experiment were of analytical grades and were obtained from Sigma chemical Company, St Louis, Mo., USA, Pharmacia Fine Chemical, Uppsala, Sweden and BDH Chemicals Limited, Poole, England.

2.2 Enzyme preparations

Entire germinating and seed tissues were used for enzyme preparations. The enzyme isolation was carried out according to the modified method of Lin and Haung (1983) as described briefly. The seed tissues were washed with distilled water and homogenized for 10 minutes in 0.15M Tris buffer. The homogenate was filtered through one layer of cheese cloth and centrifuged for 30 minutes at 10,000g using High Speed Refrigerated Centrifuge. It yielded a fat layer, a supernatant liquid and a pellet. Small portion of the supernatant was used for the assay of lipase activity and protein concentration determination, while the remaining supernatant was fractionally precipitated using 70% ammonium sulphate.

2.3 Assay of enzyme activity

Lipase activity was determined by the colorimetric method of Lotrakul and Dharmsthiti, (1997), in which p-nitrophenyl palmitate (p-NPP) was used as substrate. The reaction contained 180µl of solution A (0.062g of *p*-NPP in 10ml of 2-propanol, sonicated for 2 min before use), 1620µl of solution B(0.4% Triton X-100 and 0.1 % gum Arabic in 50 mM Tris-HCl, pH 8.0) and 200µl of properly diluted enzyme sample. The product was detected at 410 nm wavelength after incubation for 15 minutes at 37^{o}C. One unit of lipase activity (U) was defined as the amount of enzyme required to release 1µmol of p- nitrophenol (p-NP) per/ml/minute under standard assay condition.

2.4 Determination of protein concentration

Protein concentration of the samples was determined according to Lowry's method (1951) using BSA (1mg/ml) as the standard protein.

2.5 Enzyme purification

The crude extract from the centrifugation step was fractionally precipitated to 80% ammonium sulphate saturation (129 g/L) according to the method of Doonan (1996). After being left undisturbed for 1 hour in an ice bath, the suspension was centrifuged at 10,000 x *g* for 15 minutes at 4^{o}C, the supernatant was discarded, precipitate collected and dialyzed against Tris buffer for 4 hours to remove traces of ammonium sulphate.

Ion exchange chromatography was performed using DEAE Sephadex A-50 as the resin. The resin was packed into a 1.5 cm x 40 cm column. 5ml of the dialyzed extract was carefully layered on the column bed surface that has been previously equilibrated with about 3 litres of Tris buffer, pH 7.5, containing 1mM EDTA and was allowed to drain into the bed. The column was washed with Tris buffer to remove unbound protein, followed by elution with linear gradient of 1M NaCl in Tris buffer. Elution was performed at a flow rate of 30ml/hr and fraction of 5ml each was collected. Protein content was determined spectrophotometrically at 280nm. The fractions were also assayed for lipase

activity and those having lipase activity were pooled and stored in the refrigerator for further purification.

The enzyme fraction was further purified on gel filtration chromatography using Sephadex G-200 as the resin. 5ml of the DEAE-Sephadex sample was layered on the column (1.5 × 40 cm). Elution was performed at a flow rate of 10 ml/hour and fractions of 5 ml each were collected. Protein content was again determined spectrophotometrically at 280 nm. The fractions were also assayed for lipase activity. The fractions having lipase activity were pooled.

2.6 Determination of Molecular Weight

2.6.1 Apparent molecular weight

The native molecular weight of lipase from geminating oil palm seeds was determined under non- denaturation condition by gel filtration on a column of sephadex G-100 (1.5 x 100 cm) using the following standard protein markers: Gamma globulin (150,000 Da), creatine phosphokinase (81,000 Da), bovine serum albumin (68,000 Da), ovalbumin (45,000 Da), Alpha chymotrypsinogen (25,700 Da). Void volume (V_o) was determined with Blue Dextran-2000. Apparent molecular weight of the enzyme was estimated from the calibrated plot of Logarithm of Molecular weights of standard proteins against their elution volume

2.6.2 Subunit molecular weight

The subunit molecular weight of purified lipase was estimated on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoreses (SDS-PAGE) according to the method of Laemmli (1970) About 0.1 ml (5mg/ml) of purified protein was loaded on to the gel. The gel was stained with 0.1 % Coomassie Brilliant Blue R-250 and de-stained in de-staining solution

2.7 Determination of kinetic parameters

The Michelis-Menten constant (K_m) and the maximum velocity (V_{max}) were determined by determining the enzyme activity of the purified lipase at various concentrations of substrates. The kinetic parameters (K_m and V_{max}) were calculated by plotting 1/V against 1/[S] in Lineweaver Burk plot.

2.7.1 Effect of temperature on enzyme activity

A thermo-stated, water-jacketed reaction chamber was employed in determining the temperature dependence effects on lipase activity. The optimum temperature was determined by assaying the relative lipase activity at different temperatures of 30, 40, 50, 60, and 70^{o}C after 1 hour incubation at pH 7.5 as standard conditions. The values of lipase activities were plotted against temperature.

2.7.2 Determination of the thermostability of the lipase

Thermal stability was determined by incubating the enzyme at different temperatures ranging from 30^{o}C to 70^{o}C for various duration of time (30, 60, 90, 120, and 150 minutes) at pH 7.5. Residual lipase activity was determined and expressed as a percentage of the relative lipase activity. All tests were conducted in triplicate.

2.7.3 Effect of pH on enzyme activity

The effect of pH on the activity of purified enzyme was studied by determining the enzyme activity at various pH ranges of 3.5 to 10.0 for 1 hour at room temperature using p- nitrophenyl palmitate as the substrate. The lipase activity was assayed under standard conditions.

2.7.4 Enzyme inhibition

The following salts at the concentration of 5mM - 20mM were tested for their possible inhibitory effects on the activity of the purified lipase enzyme: zinc chloride, manganese chloride, calcium chloride, potassium chloride and EDTA at room temperature for 1hour and the lipase activities were determined.

2.7.5 Effects of substrates (hydrolysis reactions)

Palm oil, raphia oil, olive oil, palm kernel oil and coconut oil were used as lipase substrates.

The degree of lipolysis was determined by the assay method described previously.

3.0 Results

The elution profile of the crude extract from ion – exchange chromatography column of DEAE Sephadex A-50 is presented in Figure 1. The enzyme was not bound to the DEAE-Sephadex A-50 as it came out in the flow through fractions. No lipase activity was recovered in the fractions obtained by gradient elution. The crude protein was adsorbed on the DEAE- Sephadex A-50 column and this was eluted with a linear NaCl gradient (Figure 1), single peak was obtained. The active fractions from the peak was pooled together and used for further purification by gel filtration chromatography. Single peak was also obtained; active fractions were pooled and used for the assay of the enzyme activity. A summary of the purification procedures is presented in Table 1. The partially purified enzyme from the germinating oil palm seed tissues had a specific activity of 27.78 µmol/min/mg, a purification fold of 30.32 and a percentage yield of 8.53.

The apparent molecular weights of the protein as determined by gel filtration on sephadex G-100 under non-denaturing condition was estimated to be 40 kDa. The SDS-polyacrylamide gel electrophoresis produced a single band as seen in Figure 2. The subunit molecular weight was estimated to be 40 kDa.

The Michelis constant (K_m) was determined from the Lineweaver Burk plot (Figure 3) by dividing the slope of the line with the intercept. The partially purified enzyme was found to have K_m of 2.5 mM and V_{max} of 32.6µmol//min/ml.

The purified lipase was stable at temperature ranges of 40^{o}C -50^{o}C, with maximum activity at 50^{o}C, followed by rapid decrease of activity at higher temperatures (Figure 4). Results of pH showed that the enzyme was most active at pH 7.0, retaining about 95% of its relative activity. Below or above pH 7, activity of lipase decreased (Figure 5).

The enzyme exhibited thermal stability at 50^{o}C for 60 minutes, retaining about 95 % of its activity. After heating for 90 minutes, the highest relative activity of the enzyme was achieved as 100 % at 50^{o}C (Figure 6).

Enzyme stability was also determined using various inhibitors at 5mM -20mM concentrations of $ZnCl_2$, $MnCl_2$, $CaCl_2$, EDTA and KCl. Results showed that $CaCl_2$ enhanced the enzyme activity with maximum activity at 5 mM concentration. All other salts inhibit the activity of the enzyme as the concentration increases, with maximum inhibitory effect shown by EDTA at 20 mM concentration (Table 2).

Hydrolytic reaction of partially purified lipase with some known vegetable oils is summarized in Table 3. The lipase showed maximum hydrolytic rate with coconut oil, followed by comparable rates of hydrolysis between olive oil and raphia oil. Palm kernel oil and palm oil had lowest hydrolytic rate.

4.0 Discussion

The precipitation of enzymes was carried out by ammonium sulphate since this salt is highly soluble in water, cheap and had no deleterious effect on structure of protein. Results revealed that purification using Sephadex G-200 and DEAE Sephadex A-50 resins led to increase of 8.53 % yield and a purification fold of 30.32. This result is similar to the findings of Abigor *et al* (2003) who reported 28-fold increase in relative activity of partial purification of lipase from *Jatropha curcas L.*

An apparent molecular weight of 40 kDa for germinated oil palm obtained on gel filtration is similar to the molecular weight of *Aspergillus niger* lipase of 35 kDa on Sephadex G-100 gel filtration chromatography and SDS-PAGE (Sugihara *et al*., 1988) and that of monomeric lipase from *P. fluorescens* with molecular weights of 45 kDa. The purified lipase was monomeric and had a subunit molecular weight of 40kDa (germinated seeds) on SDS-PAGE. This result is similar to reports by Sharma *et al*. (2002) and Sadeghipour and Bhatla, (2003) on smaller lipases with molecular weights of 37kDa and 40-50 kDa respectively.

The kinetic parameters (K_m and V_{max}) of lipase obtained by Lineweaver Burk plot when *p*-nitrophenyl palmitate was used as substrates was 2.5mM and 32.6 µmol/min/ml respectively. The K_m obtained is similar to that of cell bound

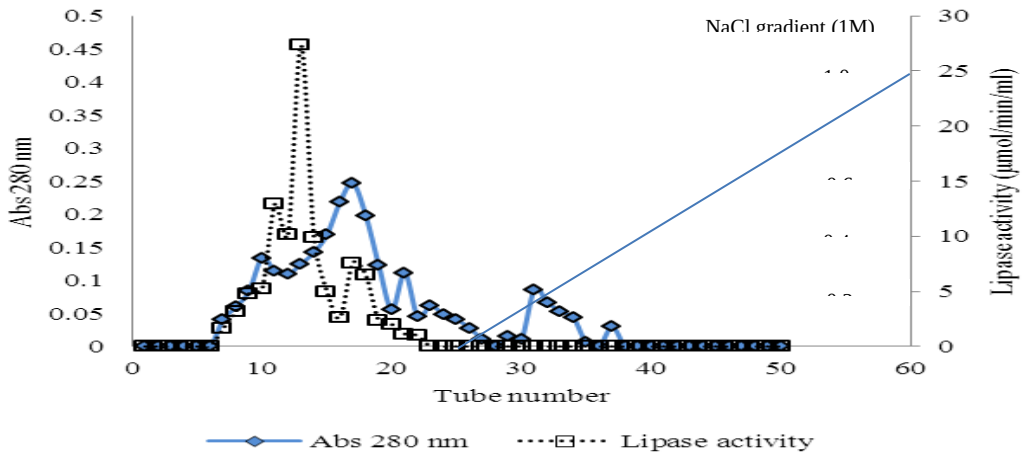

Figure 1: Elution profile of Lipase from germinated oil palm seeds on DEAE Sephadex A-50 5ml of the dialyzed extract was carefully layered on the DEAE Sephadex A-50 column bed (1.5 x 40) surface that has been previously equilibrated with about 3 litres of Tris buffer, pH 7.5, containing 1mM EDTA and was allowed to drain into the bed. The column was washed with Tris buffer to remove unbound protein, followed by elution with linear gradient of 1M NaCl in Tris buffer. Elution was performed at a flow rate of 30ml/hr and fraction of 5ml each was collected. Protein was monitored spectrophotometrically at 280nm. The fractions were also assayed for lipase activity. The fractions 7 – 21 were pooled for further purification.

Table 1: Summary of purification of lipase from germinated oil palm seeds

	Purification Parameters							
Step	**Vol.**	**LPA**	**LPC**	**TLA**	**TLP**	**SP**	**Yield**	**Fold**
Crude extract	100.	28.54	31.15	2854.00	3115.00	0.91	100	1.00
$(NH_4)_2SO_4$ ppt	10.60	38.4.0	12.48	407.04	132.28	3.07	14.26	3.35
Ion Exchange	5.10	44.00	4.07	224.40	20.75	10.81	7.86	11.79
Gel	3.70	65.85	2.37	243.64	8.76	27.78	8.53	30.32

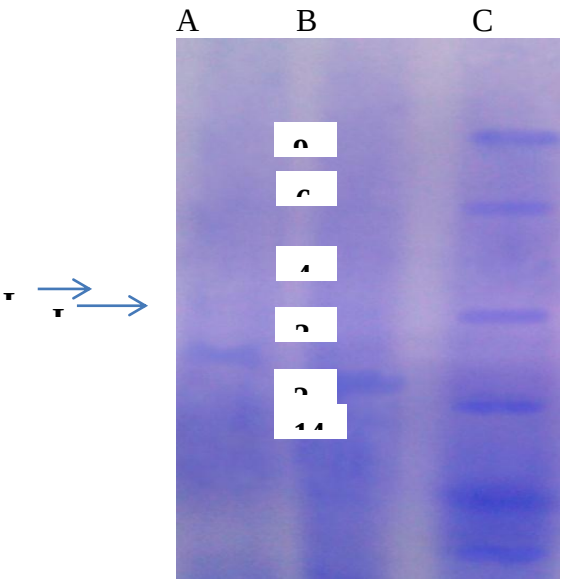

Figure 2: Polyacrylamide gel electrophoresis of lipase from germinating oil palm seeds. SDS-PAGE was performed on 12% gel using SDS phosphate buffer system at pH 7.2. Protein markers used were phosphorylate B (97,000Da), Bovine albumin (66,000Da), chicken ovalbulmin (45,000Da), soyabeans trypsin inhibitor (30,000Da), bovine lactoglobulin (20,000Da), and egg white lysozyme (14,000Da).
A= Gel for Gel-filtration; B= Gel for SDS-PAGE; C= Gel for standard protein markers.

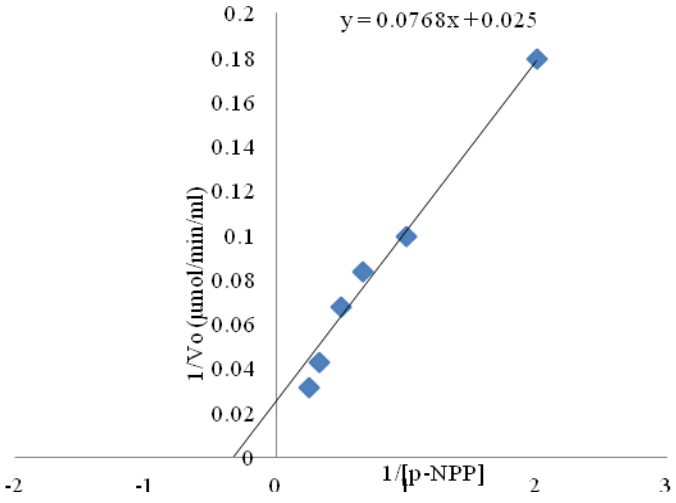

Figure 4: Linewaever-Burk plot of the lipase

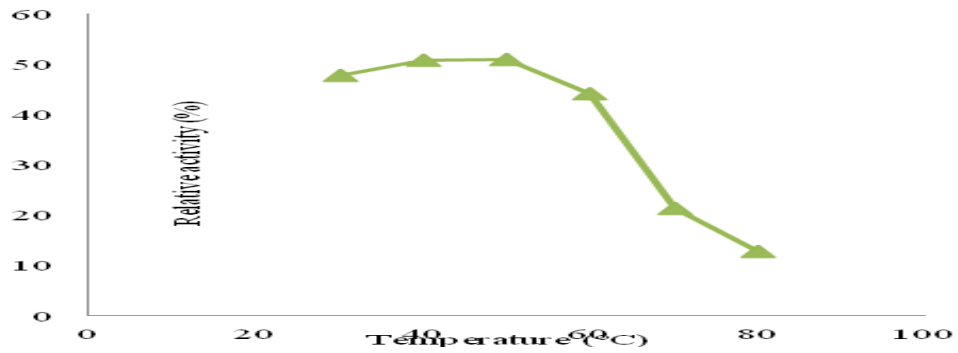

Fig.5. Effect of temperature on lipase activity from germinating oil palm seeds

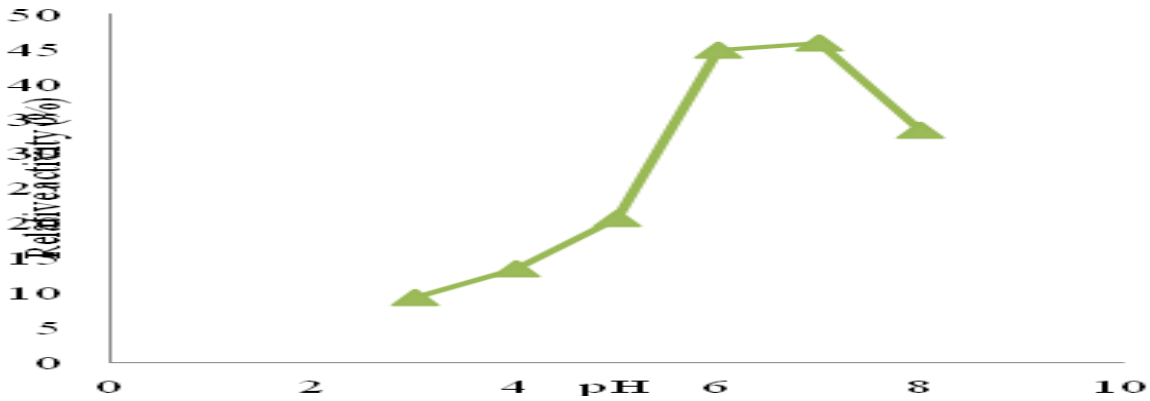

Fig.6. Effect of pH on lipase from germinating oil palm seeds

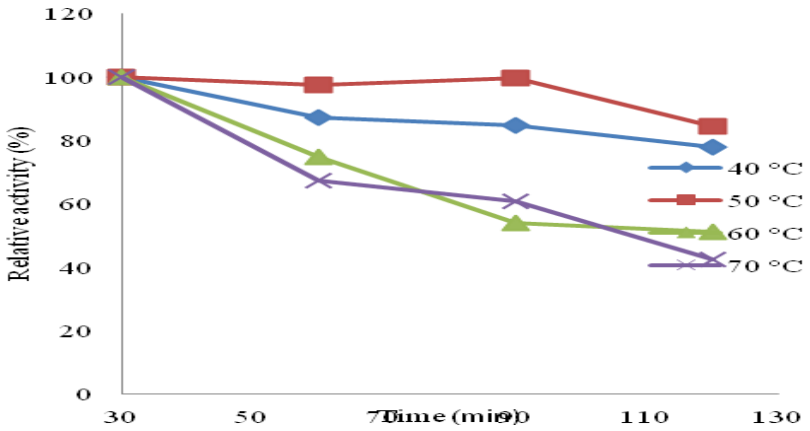

Figure 6: Thermostability of lipase from germinating oil palm seeds

Table 2: Effect of salts on lipase from germinated oil palm seeds

	5 mM	10 mM	15 Mm	20 mM
Metal ion	**%Relative Activity**	**%Relative Activity**	**%Relative Activity**	**%Relative Activity**
$ZnCl_2$	29.95	23.63	22.20	22.86
$MnCl_2$	60.91	49.57	41.66	39.61
$CaCl_2$	122.64	109.37	106.62	105.59
EDTA	20.45	12.85	12.17	8.78
KCl	47.14	66.63	52.53	48.90
Control	100	100	100	100

Table 3: Hydrolytic activity of lipase from germinated seeds of oil palm

Substrate	**Lipase Activity (µmol/min/ml)**
Coconut oil	71.57
Palm kernel oil	35.08
Palm oil	29.93
Olive oil	42.95
Raphia palm	41.84

lipase of *Geotrichum candidum* whose K_m was reported to be 2.46mM when *p*-nitrophenyl palmitate was used as the substrate (Brabcová *et al*., 2010). For a *P. cepacia* lipase, Pencreac'h and Baratti (1996) reported V_{max} values of 30 mmol/min/ml, respectively, when pNPP was used as substrate. The K_m reported in this study probably suggests that the enzyme has high affinity for the substrate and could catalyze the hydrolysis reaction for production of glycerol and fatty acid.

One of the factors that affect the activity of enzymes is temperature and is often used to characterize enzyme. In this study, the optimum temperature of lipase was 50ºC. Activity was rapidly lost at temperatures greater than 50ºC. This is similar to the findings of Abigor *et al* (2003) on lipase from germinating seeds of *Jatropha curcas L*.

According to Sharon *et al* (1998), the changes in pH affect the protein structure and the enzyme activity. The maximum activity of both lipase from germinating oil palm was observed to be at pH 7 which dropped onwards and is similar to the results of lipase from germinating *Jatropha curcas L* seed whose pH optima was near neutrality (Abigor *et al*., 2003). *Pentaclethra* lipase also had optimum pH near neutrality (Enujiugha *et al*., 2004) while partial purified cocoa lipase had an optimum pH of 7.0 **(**Ratna, 2008).

The thermal stability experiment showed that the lipase was sensitive to thermal activation; this may be as a result of thermal distortion of its structure.

Metal ions have been reported to stimulate lipase-catalyzed hydrolysis of oil by removing the fatty acids from the oil-water interface and allowing lipase to act freely on oil molecules (Ohnishi *et al*., 1994). From this study on the effects of metallic salts on lipase activity of germinated oil palm seeds, all the metal salts used inhibited the lipase activity at differing levels of inhibition. The highest level of

inhibition was observed with $Zn^{2+}$and the least inhibition was observed with $Mn^{2+.}$ Lipase activity of germinating oil palm seeds was enhanced by $CaCl_{2,}$ this could be that Ca^{2+} forms a prosthetic group with the lipase enzyme and is needed as a cofactor. Monnet *et al* (2012) reported that calcium ion was found to enhance lipase activity, while $Mn^{2+,}$ Na^{+}, K^{+}, $Fe^{3+,}$ Fe^{2+} and Zn^{2+} strongly inhibited the same. Sharon *et al*. (1998) reported a lipase of *P. aeruginosa* KKA-5 that retained its activity in the presence of Ca^{2+} and Mg^{2+} but was slightly inhibited by Mn^{2+}, Cd^{2+}, and Cu^{2+}. Kambourova *et al* (2003) suggested that the positive effect of Ca^{2+} is due to formation of insoluble ion-salts of fatty acids during hydrolysis, thus avoiding the product inhibition. Rahman *et al*., (2005) stated that metal ions will bind to the enzyme and change the enzyme's conformation to achieve better stability and hence greater activity. EDTA was also observed to inhibit the activity of the enzyme and this is in agreement with the report of Monnet *et al* (2012) that lipase from dormant ripe and unripe *Terminalia catappa Linn* were strongly inhibited by EDTA, thus suggesting that the lipases are metalloenzymes. According to Enujiugha (2004) the inhibition could be attributed to its chelating process which may disrupt the formation of the enzyme substrate complex. This invariably affects the formation of the end product (Enujiugha *et al*., 2004).

The result of the hydrolytic activity shows that the enzyme hydrolyses coconut oil at a higher rate than other oils used. Palm oil and palm kernel oil were hydrolyzed at comparable rates; also olive oil and raphia oil were hydrolyzed at comparable rates. The result showed that the lipase hydrolyze medium chain triacylglycerol at a faster rate than long chain triacylglycerol. This is in contrast to the findings of Abigor *et al* (2003) who reported that lipase from germinating *J. curcas* L. seeds hydrolyzed long-chain TAG than medium chain triacylglycerol.

In conclusion, this work established the presence of lipase from germinating oil palm seeds, it also revealed that this enzyme from had some physico-chemical properties that are similar to what have been obtained from other plant and microbial sources. Some of these properties could make the enzyme to find extensive applications in chemical, pharmaceutical, food, and industrial processes.

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