Purification and Characterization of Lipase (EC-3.1.1.3) from the Seeds of *Cucumeropsis manni* (White Melon)

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**Abstract**

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3.), has attracted a lot of attention in recent years because of its diverse biotechnological applications. Lipase occurs widely in nature, but plant lipase, is significant because of its unique substrate selectivity/specificity. Lipase was isolated from the endosperm of 4-day germinated seeds of *Cucumeropsis manni* using extraction buffer containing 1.95% (w/v) Tween 80. The crude enzyme was purified through a four step purification procedures of combined ammonium sulphate ((NH₄)₂SO₄) and cold acetone precipitated proteins, followed by dialysis and repeated gel filtration on sephadex G-200 column chromatography. This yielded enzyme with two activity peaks of lipase indicating the presence of two forms of lipase identified as acidic and alkaline lipases based on their respective pH optimum at 5.9 and 7.5. The acid lipase was found to be stable between pH 4.5 and 6.0 while the alkaline lipase was stable between 6.5 and 8.0 pH ranges. The two lipase fractions showed optimum activities at 37°C and were found to be stable at 45°C after incubation for 1 hr. Ca²⁺ was observed to be a very good activator and Pb²⁺ a potent inhibitor of the two forms of lipases. The acid lipase was purified to 11.0 fold while the alkaline lipase showed 5.4 purification fold. The acid lipase showed a Vmax of 166.7 Unit and Km of 15.67 g L⁻¹ while the alkaline lipase showed Vmax of 142.8 Unit and Km of 13.28 g L⁻¹.

**Keywords:** lipases, *Cucumeropsis manni*, purification, assay, properties

**Introduction**

Lipases (triacylglycerol acylhydrolase EC.3.1.1.3.) are enzymes which hydrolyze triacylglycerol at oil-water interface (Jensen, 1982), to release free fatty acids and glycerol (Abolemonaem et al., 2011). Lipases are present in animals, plants and microorganisms (Ejedegba et al., 2007). Many biotechnological applications for lipases have been described in food, detergent, pharmaceutical, oil and fat industries (Barros et al., 2010). Lipases have attracted much interest in recent years (Hellyer et al., 1999) owing to their unique substrate specificity consequently; plant lipases may represent interesting substitutes for microbial and animal lipases in biotransformation reactions (Palocci et al., 2003). Plant lipases contrary to those of microorganisms and animals are not well known (Bhari, 2000). Opute (1975) has demonstrated high level of lipase activity in *Cucumeropsis edulis* while Eze et al. (2005) have reported high level of lipase activity and its localization in *Cucumeropsis manni* seeds. The stored triacylglycerol of oil rich seeds such as *Cucumeropsis manni* (Badifu and Ogunsua, 1991) which represents a very good source of lipase, are hydrolyzed during germination of such seeds by the action of endogenous lipases (Ivan et al., 1995). *Cucumeropsis manni*, commonly known as white melon seed is a member of the Cucubitaceae family. The plant is a species of melon native to tropical West-Africa where its cultivation is usually associated to banana plant, corn and cassava (Fomekong et al., 2008). It is consumed largely as thickener of traditional soup called egusi soup in Nigeria(where it is found in abundance and grows very well), Republic of Benin.
and pistachio soup in Coted'Ivore (Koffi et al., 2008; Hanno and Susanne, 2010). Despite its agronomic and cultural (traditional medicine) importance, the plant lacks attention from research and development and as such is categorized as orphan crop (Loukou et al., 2003). The limit of proper knowledge of other possible utilization of the seed apart from consumption as food and in traditional medicine is a major deterrent to its wider production, which should result to increased income for the local farmers and conversion to value added products. Finding its use as a source of industrial material would encourage its production and therefore improve the local economy. In our quest to finding a cheap source of lipases we therefore report the isolation, purification and characterization of lipases from the endosperm of germinating seeds of *Cucumeropsis manni*.

**Materials and Methods**

**Materials**

*Cucumeropsis manni* seeds were bought from a local market in Nsukka, Enugu State, Nigeria.

**Reagents**

BSA was obtained from BDH England, Gum Arabic was a product of Qualikems India, other reagents and solvents were of analytical grade and freshly prepared unless where otherwise stated.

**Seed Germination**

The seeds of *Cucumeropsis manni* were germinated following the method of Eze and Chilaka (2010).

**Extraction and Isolation of Lipase**

Lipase was isolated from the endosperm of *Cucumeropsis manii* seeds following the method of Huang et al. (1983), with slight modifications as in Eze et al. (2005). The seeds were dehulled, washed in distilled water and homogenized with pestle and mortar in a cold 150 mM Tris-HCl grinding buffer pH 7.5 containing 0.4M sucrose, 0.6mM EDTA, 2.0mM Beta-Mercaptoethanol, and 1.95% (w/v) Tween 80. The homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged at 5000xg for 30 min at 4°C. The top layer called lipid bodies (fat pad) was removed using spatula, the supernatant (water soluble portion) and the pellet were collected into separate containers. The supernatant was designated crude lipase. The pellet was washed in buffer and lipase activity was assayed for in the 3 fractions.

**Lipase Assay**

This was done according to the method of Ducombe (1963) using olive oil as substrate. The free fatty acids released were converted to copper soap and quantified calorimetrically using sodiumdiethyldithiocarbamate as colour reagent. The absorbance was measured at 440nm against an appropriate blank. The concentration of fatty acid released was read off from an oleic acid standard curve.

**Purification of Lipase**

Lipase was extracted from 4-day germinated seeds of *Cucumeropsis manii* as described above. One half of the crude extract was precipitated with ammonium sulphate at 70% saturation. After 32 h the fatty upper part was carefully removed and the precipitate collected by centrifugation at 5000xg for 20 min. The second one half of the crude was saturated with cold acetone at 50% (v/v) and allowed to stand for 24 h in an ice bath. The precipitate formed was collected by centrifugation at 5000xg for 20 min. This was merged with that of ammonium sulphate and re-dissolved in Tris-HCl buffer pH 7.5. This was dialyzed against 0.05M Tris-HCl buffer pH 7.5 for 24 h. The dialyzed lipase was then loaded onto a sephadex G–200 column (2.5×62 cm) pre-equilibrated with Tris-HCl buffer pH 7.5. The enzyme was eluted with the same buffer and collected into separate test tubes.

**Estimation of Protein**

Protein was determined in the eluted fractions by measuring the absorbance at 280nm. Protein from other fractions was determined using the method of Lowry et al. (1951).

**Effect of Temperature and pH on Activity and Stability of Enzyme**

For the determination of optimum temperature for lipolytic activity, lipase was assayed at temperatures ranging between 30-80°C in a temperature controlled water bath using the
Ducombe (1963) standard method of assay. For temperature stability, enzyme solutions were incubated at 30-80°C for 30 min and then cooled at 0°C for another 30 min (Eze and Chilaka, 2010), the enzyme residual activity was determined as earlier described. Lipase activity was also assayed for in the pH range of 3.0-9.0 using the following buffer system; sodium acetate (pH 3.0-4.5); Sodium phosphate (pH 5.0-6.5), and Tris-HCl (7.0-9.0). pH stability was determined by incubating the enzyme solutions in various buffers of different pH range between 3.0-9.0 (as in above) for 1 h at room temperature and thereafter, the enzyme residual activity was monitored as earlier described.

Effect of Metal Ion on Enzyme Activity

This was carried out using the method of Islam et al. (2009). The enzyme was incubated with equal volume of various compounds containing various metal ions. The activity of lipase was measured after 30 min of incubation at 30°C.

Results and Discussion

Purification of Lipase

A 4-step purification process for Cucumeropsis manni lipase was used by combination of proteins from 70% ammonium sulphate and 50% cold acetone precipitation. This was followed by dialysis and repeated gel filtration on sephadex G-200. Two lipase activity peaks were obtained from gel filtration. The fraction from peak A and peak B were designated lipase A and lipase B respectively with purification fold of 5.4 and 11.0 and an enzyme activity yield of 0.7 for lipase A and 1.76 for lipase B. Table 1 shows the purification table for the lipase. Figure 1 shows the chromatogram for the gel filtration column chromatography on sephadex G-200 and Figure 2; the chromatogram of the re-chromatographed proteins on the same gel.

Effect of pH and Temperature on the Activity and Stability of the Enzyme

The pH profiles for the lipolytic activity of the two lipase fractions for olive oil hydrolysis showed that lipase A had its optimum activity at pH 7.5 while lipase B showed optimum activity at pH 5.9 (Figure 3). This suggests that there are two different enzymes present in Cucumeropsis manni seeds, an alkaline lipase and an acid lipase. Though, Hills and Murphy (1988) observed that some buffers cause substantial inhibition of lipase activity, but in the present study, the same assay buffers were used to assay both enzymes, it is therefore clear that a difference between the two lipases exists. The pH stabilities were found to be between pH 6.5 and 8.0 for the alkaline lipase and pH 3.5-6.0 for the acid lipase (Figure 4). Our result is in agreement with that of Muto and Beevers (1974) who isolated both alkaline and acidic lipase from castor bean (pH 5.0 and 9.0). Huang (1982) also recorded a pH optimum of 9.0 with glycine-HCl and 6.5 with imidazole-HCl buffers for soybean lipase. The advantage of the presence of the acid and alkaline lipase in a seed may help the seedlings to adapt to both acidic and alkaline environment and will also enable the use of lipase from such seeds for a wider range of industrial activity with limiting effect of pH.
Table 1  Purification table.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Volume (mL)</th>
<th>Protein (mg mL⁻¹)</th>
<th>Total Protein (μg)</th>
<th>Enzyme Activity (μm min⁻¹)</th>
<th>Total Activity (μm min⁻¹/mg prot)</th>
<th>Specific Activity (%)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>400</td>
<td>88.8</td>
<td>35,520</td>
<td>67.37</td>
<td>26,948</td>
<td>0.76</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Combined protein from (NH₄)₂SO₄ and Acetone precipitation</td>
<td>30</td>
<td>81.5</td>
<td>2,445</td>
<td>71.67</td>
<td>2150.1</td>
<td>0.79</td>
<td>8.0</td>
<td>1.04</td>
</tr>
<tr>
<td>Dialysis</td>
<td>37</td>
<td>67.4</td>
<td>2493.8</td>
<td>131.1</td>
<td>4850.7</td>
<td>2.0</td>
<td>18.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Chromatography on sephadex G-200 Lipase A</td>
<td>7.2</td>
<td>6.4</td>
<td>46.15</td>
<td>33.40</td>
<td>240.5</td>
<td>5.2</td>
<td>0.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Chromatography on sephadex G-200 Lipase B</td>
<td>9.4</td>
<td>6.3</td>
<td>59.22</td>
<td>50.60</td>
<td>475.6</td>
<td>8.03</td>
<td>1.76</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Temperature optimum of 37°C was obtained for the two lipases (Figure 5). The two lipases were also found to be stable at temperature up to 45°C above which there was a steady decline (Figure 6). However, the enzymes were fairly active at higher temperatures.

Effect of Metal Ion on Enzyme Activity
In this study, Ca²⁺ and Zn²⁺ increased the enzymes activity by about 50% and 10% respectively while Na²⁺ and Mg²⁺ showed slight inhibition, Lipase inhibition was more (85% and 50% respectively) in the presence of Pb²⁺ and Al³⁺. Calcium ion has been previously shown to have stimulatory effect on lipolytic enzymes. EDTA, a chelating agent caused about 14% inhibition on the activity of the enzyme (Figure 7). Inhibitory effects of EDTA on lipase activity have been reported by Eze et al. (2005) on lipase from maize seeds, Lin and Huang (1983), on lipase from castor seeds. A decrease in lipase activity in the presence of EDTA may be as a result of interference with enzyme absorption on the substrate water interface.

Effect of Substrate Concentration
The effect of substrate concentration investigated on lipases A and B using olive oil showed that there was an increase in enzyme
activity with increase in substrate concentration until saturation point of about 0.06mg ml\(^{-1}\). The decline after this concentration may be due to the effect of enzyme substrate concentration ratio or enzyme inhibited by the excess of substrate concentration or change of physiochemical characteristics (Ejedegba et al., 2007). Ejedegba et al. (2007) observed saturation point of about 8mM with lipases isolated from coconut seed.

Figures 8 and 9 show the Lineweaver-Burk plot for the acid and alkaline lipases from *Cucumeropsis manii*. Results show maximum velocity and Michaelis-Menten’s constant (V\(_{\text{max}}\) and K\(_{\text{m}}\)) for acid lipase (lipase B) of 166.7 Unit min\(^{-1}\) and 15.67g L\(^{-1}\) while that for the alkaline lipase A (lipase A) show V\(_{\text{max}}\) of 142.8 Units min\(^{-1}\) and K\(_{\text{m}}\) of 13.28g L\(^{-1}\) (with olive oil) respectively. Note should be made of the observation by Hills and Murphy (1988) that lipases act on insoluble substrates at the interface between the aqueous and non-aqueous phases, so it is not possible to make simple kinetic interpretations of substrate-concentration effects in the classical manner of soluble substrates.

**Conclusions**

On the bases of the above observations, it could be inferred that *Cucumeropsis manii* seeds are cheap sources of lipase and that this exists in two forms: acid and alkaline lipases (optimum pH 5.9 and 7.5 respectively) with a single optimum temperature at 37°C and are stable at temperature bellow 50°C. Ca\(^{2+}\) activated the enzymes while Pb\(^{2+}\) inhibited them. These properties tend to suggest that *Cucumeropsis manii* lipase could prove usefulness in industrial processes such as detergent industry and more research in this direction will enable the conversion of the seeds into more value added products. Whether the two forms are isoenzymes or not will be the focus of our next paper

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References


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