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Full Length Research Paper

Isolation and preliminary characterization of conophor nut (*Tetracarpidium conophorum*) lipase

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Lipase in oilseeds helps to hydrolyze the ester bonds of storage triacylglycerols. The crude lipase from the conophor nut (*Tetracarpidium conophorum*) was isolated and assayed via quantification of the free fatty acids liberated by the hydrolysis of the oilseed triacylglycerols. Optimum pH and temperature for the enzyme activity of the conophor nut lipase was pH 8.0 and at 30°C with substantial lipolysis at 80°C, underscoring the thermostability of the enzyme. The effects of different ions on the activity of the isolated lipase were examined. NaCl and EDTA inhibited activity by various degrees, while Ca^{2+} and Hg^{+} enhanced the enzyme activity. The results of the present study show that the lipase from conophor nut can favourably be exploited to complement existing lipase sources.

Key words: Conophor nut, lipase activity, temperature, pH.

INTRODUCTION

Lipases (triacylglycerol acyl hydrolases EC 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of triacylglycerols and a large variety of esters. Oilseed lipases have been discovered to have great commercial prospects as industrial enzymes (Enujiugha et al., 2004). They represent cheap sources of industrial lipases with remarkable potentials for biocatalytic hydrolysis. However, lipase activity is generally absent in ungerminated seeds, and progressively increases during germination. According to Hills et al. (1990) during the growth of oilseed plants, lipases are produced in large amounts to hydrolyze triacylglycerol to fatty acids and glycerol, which supports for the growth of young plants. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved.

The Conophor plant (*T. conophorum* Mull. (Arg) Euphorbiaceae), commonly called the African walnut, is a perennial climbing shrub found in the moist forest zones of Sub-Saharan Africa. It is cultivated principally for the nuts which are cooked and consumed as snacks, along with boiled corn (Enujiugha, 2003). Conophor nut contains 49.2% dry wt. of oil (Enujiugha and Ayodele-Oni, 2003), which is liquid and golden yellow in color, with taste and odor resembling those of linseed oil. The residue after oil expression contains over 50% protein. Gas chromatographic analysis of the seed oil has shown a high level (>66% dry wt.) of the sn-3 fatty acid, linolenic acid (Ogunsua and Adebona, 1983), which is considered essential to the well being, growth and development of children (Innis, 1991). The purpose of this work was to isolate and partially characterize crude lipase from conophor nut and determine its substrate specificity. This is expected to give insight into its potentials for industrial utilization.

MATERIALS AND METHODS

Sources and preparation of materials

The raw conophor nut used in the study was obtained from local farmers at Akure, Ondo state, Nigeria. The nuts were carefully cracked, sliced to about 1-2 cm thickness with a kitchen knife and milled into powder using Waring blender and kept at 4°C prior to analysis. The four oil substrates used for the analysis namely, soybean, cottonseed, groundnut and palm kernel were obtained from the King's market at Akure. All the chemicals and reagents used in the study are of analytical grade and procured from E. Merck AG (Germany).

Crude enzyme preparation

The acetone powder of the conophor nut lipase was prepared according to the method of Hassanien and Mukherjee (1986) with some modifications. 30 g of the seed cotyledon was ground with 30 ml of cold acetone using a Waring blender. The acetone extract

Table 1. Effect of different ions on the activity of lipase from conophor nut on different substrates.

Salt/ion	Soybean oil Activity (%FFA)[b]	Groundnut oil Activity (%FFA)[b]	Palm kernel oil Activity (%FFA)[b]	Cottonseed oil Activity (%FFA)[b]
NaCl	0.07±0.002	0.08±0.008	0.05±0.001	0.05±0.042
$CaCl_2$	0.33±0.020	0.28±0.035	0.27±0.003	0.26±0.013
$HgCl_2$	0.27±0.004	0.36±0.020	0.26±0.005	0.24±0.004
EDTA	0.25±0.001	0.23±0.010	0.13±0.001	0.20±0.001
Control	0.27±0.005	0.25±0.003	0.22±0.010	0.20±0.004

[a]Reaction conditions: enzyme-substrate ratio 1:5; 1 ml of 0.01M of salt solutions; phosphate buffer (pH 7) used as control. [b]Values represent means of triplicate determinations (mean ± SME).

was filtered through a cheese cloth (Enujiugha et al., 2004) and washed four times with 20 ml each of cold acetone. The residue was air -dried at room temperature (26°±1°C) to yield the acetone powder which was kept at 4°C.

Assay of enzyme activity

The assay of the lipase activity was carried out using the titrimetric method of Khor et al. (1986) as modified by Enujiugha et al. (2004). 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. Each incubation process was terminated with the addition of 25 ml acetone-ethanol (1:1; v/v) to facilitate the extraction of the free fatty acids liberated. The liberated free fatty acids were quantified by direct titration with 0.01 M NaOH using phenolphthalein as indicator. The lipase activity was expressed as the percentage of free fatty acids liberated after 1 h incubation at 30°C (Wetter, 1957).

Effect of substrate and ions on lipase activity

Four different substrates (soybean oil, cottonseed oil, groundnut oil, and palm kernel oil) were substitutively used at 5 g in the assay mixture with subsequent incubation at room temperature for 1 h at 60% relative humidity (RH). The oils were dried at 50^{o}C for 4 h in an air oven before being used in the assay. Lipase activity was mea-sured for each substrate so as to determine the fatty acids speci-ficity of the lipase from conophor nut.

Approximately 0.01 M solutions of sodium chloride (NaCl), calcium chloride (CaCl2), mercury chloride (HgCl2) and ethylene diamine tetra-acetic acid (EDTA) were prepared. The control solution was prepared using 0.01 M phosphate buffer at pH 7. Using the method of Mukundan et al. (1985), 1 ml of each of the solutions was added to separate assay mixtures and then incubated for 1 h with continuous stirring. After which the lipase activity was quantified as described earlier.

Effect of pH and temperature on enzyme activity

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 hexane. The mixture was incubated at 30°C for 1 h with continuous stirring, and the activity determined for each pH. A thermostatic, water-jacketed reaction chamber with shaker was employed to determine the temperature dependence of lipase activity. The assay mixtures were incubated at different temperatures (30 to 80°C) for 1 h and the activities measured.

RESULTS AND DISCUSSION

Rate of lipolysis of different substrates

The present study has highlighted the potential for exploitation of industrial lipase from a common and inexpensive plant source. The results of the lipolysis of soybean, cottonseed, groundnut and palm kernel oils by the lipase in conophor nut are presented in Table 1. In systems without added ions, lipolysis was more pronounced in soybean oil, and groundnut oil (comprising <C20 fatty acids). In a previous work, Enujiugha et al. (2004) reported a pronounced lipolysis in palm kernel and coconut oil with short–chain FFA. It has also been observed by Huang and Moreau (1978) that oilseed lipases are more active on triacylglycerols containing short-chain fatty acids. The above results are in conformity with the observation of Mukundan et al. (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. The amount of oils available at the interface determines the activity of the lipases. Hexane was added to the assay mixture according to the method of Khor et al. (1986) so as to increase the interfacial area of the activity of the enzyme. Mukherjee (1990) observed that the actual site of lipolysis is at the interface. Also, Enujiugha et al. (2004) reported that the enzymatic activity of a lipase is related to the interfacial area of the water-insoluble substrate.

Effects of ions on lipolysis

The effects of ions on the activities of lipase from conophor nut were found to be variable (Table 1). Sodium chloride was observed to significantly reduce the activity in all the substrates which it was exposed to, although Mukundan et al. (1985) observed that the chloride ion did not cause any inhibitory effect but the metal ions did. This means that the Na^{+} could therefore be main causative effect for the decline in activity. The Ca^{2+} and Hg^{2+} enhanced the activity considerably. The observed calcium effect is in agreement with the observation of

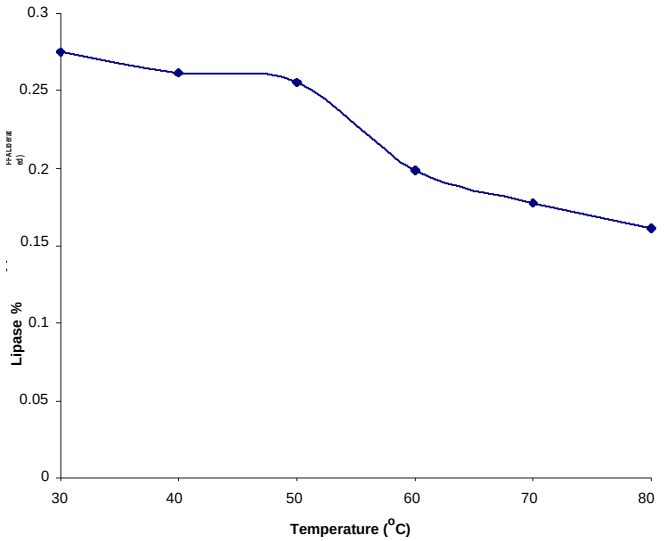

Figure 1. Effects of temperature on conophor nut lipase activity using soybean oil in hexane (enzyme-substrate ratio1:5; pH 8.0; 1 h incubation period).

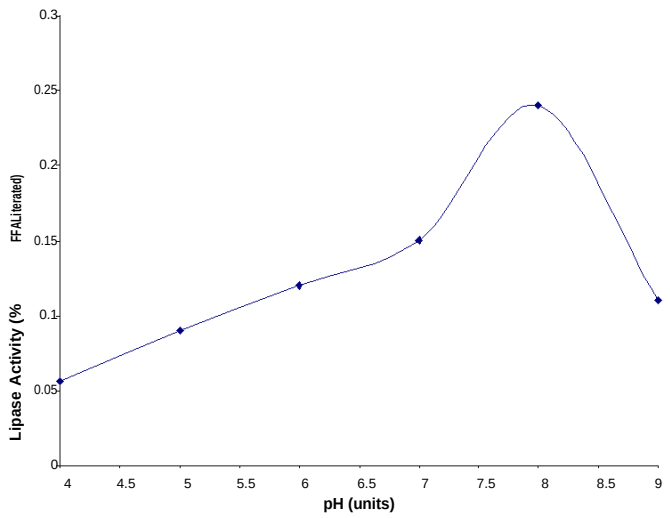

Figure 2. Effect of pH on the activity of conophor nut lipase at 30^0C using soybean oil in hexane (enzyme-substrate ratio1:5; 1 h incubation period).

Enujiugha et al. (2004) that calcium ion in the reaction mixture brought about 64% increases in activity. Abigor et al. (2002) and Haas et al. (1992) also observed an increase in lipase activity by calcium ion inclusion. The inhibition of activity by EDTA was more pronounced in the palm kernel oil. The inhibition of the activity could be attributed to its chelating process of the system and thereby disrupting the formation of the enzyme substrate complex. This invariably affects the formation of the end product (Enujiugha et al., 2004) . A slight difference was observed in the effect of Hg^{2+} on the enzyme activity. The Hg^{2+} slightly favored lipolysis in the oils, which is in con-trast with the observation of Sanders and Pattee (1975) that 10^{-4} M solution of Hg^{2+} brought about complete inhi-bition of lipase activity in peanut. The inhibition of lipase activity by mercury chloride is an indication of the pre-sence of sulfhydryl group in the enzyme molecule (Mukundan et al., 1985). Although the Hg^{2+} did not inhibit lipolysis in this study, there was indication that it could inhibit lipolysis in cottonseed oil as there was a gradual drop in activity relative to other substrates.

Effects of temperature and pH on lipase activity

Figures 1 and 2 show the respective effects of tempera-ture and pH on the activity of the lipase from conophor nut (*T. conophorum*). Abigor et al. (1985) observed that purified oil palm lipase has been shown to have optimal activity at 30°C, above which there was a steady decline. It was observed in the present study that there was gra-dual decline in the activity of the lipase from conophor nut with successive increases in temperature, from 30 to 80^0C. However, the enzyme was fairly active at higher temperatures. The basis for sustained activity at relatively higher temperatures is still unclear, but it might be linked to the fact that the lipases are highly hydrophobic. Enujiugha et al. (2004) reported that there was a decline in activity above 30°C, though there was a substantial lipolysis, even at 80°C, indicating a fairly high thermo-sta-bility of the enzyme. The present study agrees with this observation using lipase obtained from *T. conophorum*.

A comparison of activity profile of the lipase at different pH is shown on Figure 2. The lipase was found to have highest lipolysis between pH 7.0 and 8.0. Past studies indicated different pH optima for lipases from different plant sources. The pH optimum for *Pentaclethra* lipase is near neutrality (Enujiugha et al., 2004). Ory et al. (1962) found out that some other lipases are optimally active at acid pH (e.g. pH 5.0 for ungerminated castor bean li-pase); while oil palm mesocarp lipase has pH 4.2 (Abigor et al., 1985). The results of the present study revealed a peak at pH 8.0, followed by a slight drop at pH 9.0. The advantage of alkaline pH range lies in the absence of cor-rosion problems associated with acidic environments in industrial processes.

Conclusion

An alkaline lipase (optimum pH 8.0) with a fair thermo-activity was isolated from the cotyledons of raw conophor nut (*T. conophorum*) and the substrate specificity exa-mined. The conophor nut lipase could prove useful in industrial biocatalytic hydrolysis. It could also be inferred from the present preliminary characterization that the conophor nut lipase could prove useful in processes that require lower cooling costs and minimal corrosion pro-blems. Lipolysis was more pronounced in soybean and groundnut oils. Ca^{2+} and Hg^{2+} enhanced the enzyme acti-vity, while Na^{+} and EDTA caused various degrees of inhi-

bition. The results show that the conophor nut lipase could be exploited in industrial processes.

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