PARTIAL CHARACTERIZATION OF LIPASE FROM COCOA BEANS (*Theobroma cacao.* L.) OF CLONE PBC 159

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ABSTRACT

A study was carried out to characterize the cocoa lipase from cocoa beans (Theobroma cacao, L.*) of clone PBC 159. The optimum temperature of cocoa lipase was 30-40 °C and the pH optimum was 7.0-8.0. The moleculer weight of the lipase enzyme was in between 45-66 kDa. The results indicate that K_m value for cocoa bean lipase was 2.63 mM, when trimyristin was used as a substrate. The incubation of cocoa bean lipase with triolein and tributyrin (as substrate) yielded K_m of 11.24 and 35.71 mM, respectively. The V_{max} value obtained from the incubation of the lipase with a wide range of substrates, including tributyrin, trimyristin and triolein, are expressed as µmole acid/min/mg protein for cocoa lipase. V_{max} values decreased with the increase in the triacylglycerol chain-length, with V_{max} values of 27.78, 13.16 and 11.63 µmole acid/min/mg protein when incubated with tributyrin, trimyristin and triolein, respectively. Inhibition of lipase occurred in the presence of diisopropyl flourophosphate, N-bromosuccinimide and 5,5-dithiobis-(-2-nitrobenzoic acid).*

Keywords: *characterization, lipase, cocoa beans*

INTRODUCTION

Lipases are ester hydrolases or esterases since they hydrolyse the ester bonds of triacylglycerol molecules. The lipases are more active with insoluble fatty acid esters and hydrolyse the ester bonds present only at the water-oil interface, whereas carboxylic ester hydrolases that are specific for the soluble esters are simply termed esterases [1]. According to Jensen *et al*. [2], lipases or acylglycerol hydrolase are enzymes which catalyze the hydrolysis of long chain aliphatic acids from acylglycerol at the oil/water interface. The systematic name is acylglycerol acylhydrolase. The interface is usually provided by emulsion globules or lipoprotein particles, the latter are primarily chylomicrons and very low density lipoprotein. The element providing the interface has been termed the super substrate. One of the most important enzymes found in cocoa beans is lipase. This lipolytic enzyme plays an important role in fatty acid metabolism [3]. The lipases from different sources have been studied extensively and their properties have been characterized. These enzymes have been partially purified or purified to homogeneity. Purified lipases subsequently have been characterized for molecular size, metal binding capabilities, glycoside and phosphorous contents, and substrate specificities [4].

According to Jensen [5], most lipases are active in wide range of temperature from 20-60 °C but the usual range was 30-45 °C. The lipase hydrolases a variety of fatty acid ester and has an optimum pH of about 7. The enzyme retained its full activity at 20 to 55 °C [6]. Abigor *et al.* [7] reported that the oil palm mesokarp lipase has an optimal activity upon its substrates at 30 ^{o}C. According to Hassan [8] the lipase showed that the optimum temperature for oil palm mesocarp lipase activity range between 20.0 to 32.5 °C.

According to Jensen [5], the pH optimum of most lipases is in between 7 and 9. Muto and Beevers [9] reported that two lipases were found in extracts from castor bean endosperm. One, with optimal activity at pH 5.0 (acidic lipase), was present in dry seeds. The seconds, with an alkaline pH optimum (alkaline lipase), was particularly active at pH 9.0. The acidic lipase of castor bean is present in the dormant seed showed the pH optimum of enzyme was 4.2 and it was rather heat stable [10,11]

According to Schuepp *et al.* [1] the substrate specifity determination was performed with triacetin, tributyrin, trimyristin and triolein, where exo- and endolipase demonstrated higher affinity for trimyristin, with *Km* values of 5.11 and 3.24 mM, respectively. Inhibition of the exo- and endolipase occured in the presence of a range of chemicals, including sodium deoxycholate, ferrous and ferric chloride, diisopropyl flourophosphate, *N*-bromosuccinimide and 5,5-dithiobis-(-2-nitro-benzoic acid). The purpose of the study is to characterize the cocoa lipase from cocoa beans (*Theobroma cacao*, L.) of clone PBC 159.

MATERIALS AND METHODS

Material

Mature cocoa (*Theobroma cacao,* L.) pods of clone PBC 159 were used. They were obtained from the Golden Hope Plantations Berhad, Perak Darul Ridzuan, Malaysia. The pods were harvested around

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the age of 160 days (400 pods per clone), and then within a day transported to the Department of Biochemistry and Microbiology, Putra Malaysia University, Serdang, Selangor Darul Ehsan, Malaysia.

Instrumentation

Thermolyne Maxi Mix II Vortex, Haake SWB 20 Shaker Water-bath. UV/VIS Spectrophotometer Pye-Unicam Model 8625.

Procedure

Isolation of Cocoa Lipase

Preparation of Acetone Dry Powder (AcDP). Acetone Dry Powder (AcDP) of cocoa beans was prepared according to method of Kirchcoff *et al.* [12]. The pulp and cotyledone was lyophilized using a freeze dryer machine (Edward, UK) and crushed with a cold mortar and pestle before they were defatted with 250 mL petroleum ether (40-60 °C) for 8 hours in a soxhlet apparatus and reextracted for another 8 h. Following that, all purine alkaloids were also removed by extraction with 250 mL chloroform for 8 h. The resultant defatted and purine-free alkaloid cotyledons were then sieved (size, 300 µM mesh) in order to obtain a uniform particle size cotyledone powder. In order to remove polyphenols, 50 g of the powder were treated with cold aqueous acetone (kept at -20 °C for overnight) containing 5 mM sodium ascorbate and 0.1% thioglycolic acid. The mixture was shaken vigorously for 30 sec and then incubated at 20 min intervals. The suspension was then centrifuged at 10.000 x *g* for 15 min at 4 °C and the resulting pellet was re-extracted twice with 80% cold acetone, and four times with 70% cold aqueous acetone. The residual water was removed by dehydration with 100 mL of 100% acetone. The resultant acetone dry powder was stored at -20 °C before being used for analysis.

Extraction of Crude Enzyme from AcDP. Crude enzyme from Acetone Dry Powder (AcDP) was extracted according to method of Seow *et al.* [13]. The acetone dry powder (1 g) was resuspended in 100 mL of chilled 20 mM sodium phosphate buffer (pH 7.5) in ratio one to hundred (1:100) and then extracted using a cold mortar. After extracting, the suspension was centrifuged at 10,000 x *g* for 30 min at 4 °C. The supernatant was dialysed against the same buffer at 5 °C for 2 days. The dialysate was centrifuged again at 10.000 x *g* for 30 min at 4 °C and the supernatant was used as the enzyme solution for the determination of the lipase enzyme activity.

Precipitation of Enzyme using Ammonium Sulphate. The enzyme was precipitated using ammonium sulphate as described by Green and Hughes [14]. The resulting crude extract from AcDP (300 mL) was fractional precipitated by 0-20, 20-40, 40-60, 60-80 and 80-100% ammonium sulphate. The precipitate was separated by centrifugation at 10,000 rpm, 40 °C for 10 min and resuspended in a minimal volume of 20 mM sodium phosphate buffer (pH 6.8), before dialysed against 20 mM sodium phosphate buffer (pH 6.8) at 40 °C. The dialysing medium was changed twice every 24 hours. The suspension was then lyophilised and stored at -20 °C before use.

Determination of Specific Lipase Activity

Assay of Soluble Protein Content. The soluble protein content was determined according to Lowry *et al.* method [15]. The copper content was prepared by adding 1.0 mL of 0.5% copper sulphate pentahydrate and 1% sodium potassium tartate to 50 mL of 0.1 M sodium hydroxide, containing 2% sodium carbonate. The protein solution (0.5 mL) was added to 5.0 mL of the alkaline copper reagent and the solution mixture was allowed to stand for 10 min. Then, 0.5 mL of the Folin-Ciocalteus phenol reagent (which had been diluted 1:1 with distilled water) was rapidly added and mixed. This solution was allowed to stand for 30 min, and the absorbance was measured at 580 nM. A standard curve was prepared using Bovine Serum Albumin at concentration 50-200 µg/mL.

Substrate Emulsion Preparation. The substrate emulsion was prepared by mixing one gram of Arabic gum with 80 mL of distilled water. The mixture was then added with 1.21 g (0.10 M) of Tris, 0.077 g (5mM) of dithiothreitol, 1.0 mL of olive oil and before it was mixed to emulsify using a Thermolyne Maxi Mix II Vortex. The mixture was adjusted to pH 6.5-7.0 with distilled water. The substrate emulsion was kept in a refrigerator at 5 °C before used for analysis.

Assay of Lipase Activity. The lipase enzyme activity was determined according to Kwon and Rhee method [16]. Approximately 1.0 mL of crude enzyme from AcDP was added with 3.0 mL of substrate emulsion. The solution was then homogenized using a Thermolyne Maxi Mix II vortex and was incubated at 30 °C for 2 hours and placed in a Haake SWB 20 shaking water bath at 50 rpm. After adding 4.0 mL of isooctane, the sample was homogenized using a vortex mixer and then heated in boiling water for 5 min. During sample heating, the top of sample tube was covered with a layer of aluminium foil. The suspension was then centrifuged at 5.000 *g* for 5 min. Two mL of upper layer (isooctane layer) was taken and mixed with 1.0 mL of 5% cupric acetate-pyridine. The mixture was homogenized using a vortex mixer before the absorbance was read using a Pye-Unicam model 8625 UV/VIS spectrophotometer at the wavelength 715 nM. The preferred IUPAC-IUB units (U) was used to calculate a unit of lipase activity such as µmole free fatty acids released/min. Enzyme specific activity expressed in µmole free fatty acids released/min/mg

protein was seldom used. It would be helpful if all who investigate lipases, reporting activity as U or preferably as enzyme specific activity.

Characterization of Cocoa Lipase. Lipase specific activity was also assayed using three substrates: tributyrin, trimyristin and triolein. The acetate buffer (pH 4.0–6.0) and phosphate buffer (pH 5.0–9.0) were used to determine the effect of pH and temperature on lipase specific activity. The inhibitory effect of selected range of inhibitors on the enzyme activity of cocoa lipase was investigated using diisopropyl flourophosphate, *N*-bromosuccinimide and 5,5-dithiobis-(-2-nitro-benzoic acid).

Detection of Molecular Weight. The SDS-PAGE was prepared according to Laemmli method [17]. The gel cassettes was assembled using clean glass plates as instructed by the manufacturer. The resolving gel was prepared as follows: in a disposable plastic tube, the appropriate volume of solution containing the desired concentration of acrylamide was prepared, using the values given above (solutions for preparing resolving gels – 10%, 30 mL). The components were mixed in the order shown. Polymerization began as soon as the TEMED was added. Without delaying, the mixture was swirled rapidly and proceed to the next step. The acrylamide solution was poured into the gap between the glass plates. Sufficient space for the stacking gel was left. The acrylamide solution was overlayed carefully with distilled water using a pasteur pipette. The gel was placed at room temperature. After polymerization was completed (30 min), the overlay was poured off and the top of gel was washed several times with deionised water to remove any unpolymerized acrylamide and then was drained as much fluid as possible from the top of the gel, and was then removed any remaining water with the edge of a paper towel. The stacking gels were prepared as follows: in a disposable plastic tube, the appropriate volume of solution containing the desired concentration of acrylamide was prepared using the values given above (solutions for preparing stacking gels – 4%,10 mL). The components were mixed up in the order shown. Polymerization began as soon as the TEMED was added. Without delaying, the mixture was swirled rapidly and proceeded to the next step. The stacking gel solution was poured directly onto the surface of the polymerized resolving gel. A clean comb was then immediately inserted into the stacking gel solution. More stacking gel solution was added to fill up the spaces of the comb completely. The gel was placed in a vertical position at room temperature. While the stacking gel was polymerised, the sample was prepared by heating them to 100 °C for 5 min in loading buffer (1:1) to denature the proteins. After polymerization was completed, the wells were removed carefully. The wells were washed immediately with deionized water in order to remove any unpolymerized acrylamide. The gel was mounted in the electrophoresis apparatus. The tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. These were best done with a bent hypodermic needle attached to a syringe. The volume loaded was dependent on the protein content of individual samples but was usually in the range 5–15 µL The electrophoresis apparatus was attached to an electric power supply. A voltage of 30 V/cm was applied to the gel. After the dye front was moved into the resolving gel, the voltage was increased to 60 V/cm and run the gel until the bromophenol blue reached the bottom of the resolving gel. Then the power supply was turned off. The glass plates were removed from the electrophoresis apparatus and placed them on a paper towel. Using a spatula, the plates were dried apart. The orientation of the gel was marked by cutting the corner. The gel was then fixed and stained with Commassie Brilliant Blue dye.

RESULT AND DISCUSSION

Isolation of Cocoa Lipase

The isolation of crude enzyme from Acetone Dry Powder (AcDP) with ammonium sulphate precipitation showed an optimum lipase activity compared to the original crude extract. Schuepp *et al* [1] reported that the lipases can be successfully isolated using ammonium sulphate precipitation method. Crude enzyme from AcDP was gradually precipitated by 0-20, 20-40, 40-60, 60-80, and 80-100% of saturations by the addition of ammonium sulphate.

The two highest levels of lipase specific activity recorded were at 85.53 µmole/min/mg protein with 29.61% recovery and 89.85 µmole/min/mg protein with 14.24% recovery in 40-60 and 60-80% of saturation respectively while the crude enzyme obtained from AcDP and 0-20% saturation has the least lipase specific activity. This is due to the relatively higher protein content in the crude enzyme of AcDP and 0-20% of saturation, which is about 3 times compared with that of 40-60% of saturation. Higher sample volume was obtained from AcDP and 0-20 % fraction. The 40-60 and 60-80% fractions were then chosen for further studies due to their higher lipase.

Temperature and pH Study

According to Jensen [5], most lipases are active in a wide range of temperature from 20–65 °C but the usual range was 30–45 °C. The lipase has an optimum pH of 7.0. The enzyme retained its full activity at 20 to 55 °C. The partially purified lipase from *Theobroma cacao* was incubated at a wide range of temperatures (10-60 °C) and enzyme activity was measured spectrometrically using olive oil as substrate. Abigor *et al.* [7] reported that the oil palm mesocarp lipase has

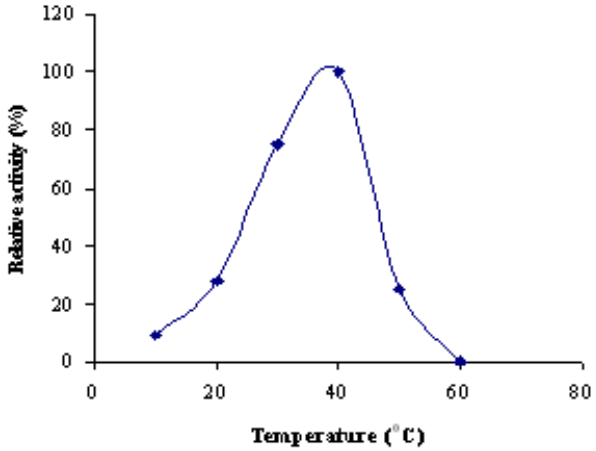

Figure 1. Effect of temperature on lipase specific Activity of cocoa beans

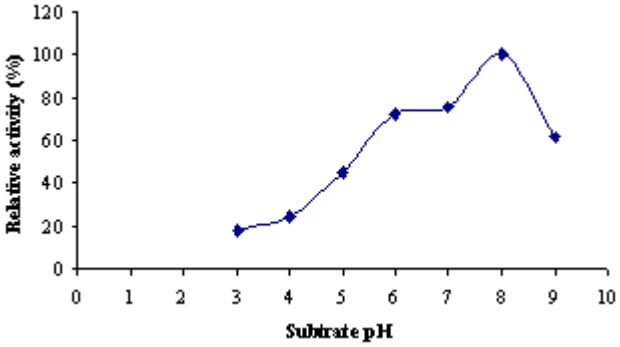

Figure 2. Effect of pH on lipase specific activity of cocoa beans

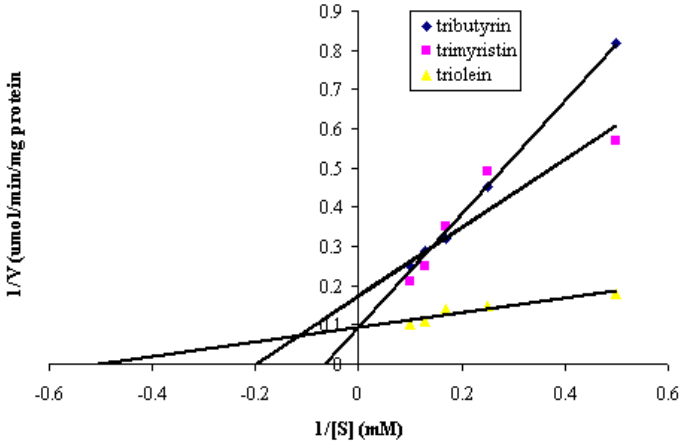

Figure 3. Lineaweaver-Burk double reciprocal plots for the cocoa lipase using tributyrin, trimyristin and triolein as a substrate

an optimal activity of 30 °C. While Hassan [8] reported that it was in the range of 20–32.5 °C. Figure 1 shows that the optimum temperature for partially purified relative cocoa lipase activity is between 30–40 °C. Above this temperature range, the relative cocoa lipase activity dropped sharply at higher temperatures (40–60 °C) until 0% relative lipase activity was obtained.

According to Jensen [5], the pH optimum of most lipases is in between 7 and 9. The results of the pH study which used olive oil as substrate for lipase assay is shown in Figure 2. The pH optimum of cocoa beans lipase activity was 7.0-8.0.

Substrate Specificity

Lineweaver-Burk double-reciprocal plots of the activity of cocoa bean lipase incubated with a wide range of substrate (tributyrin, trimyristin and triolein) are shown in Figure 3. The results indicate that K_m value for cocoa bean lipase was 2.63 mM, when trimyristin was used as a substrate. The incubation of cocoa bean lipase with triolein and tributyrin (as substrate) yielded K_m of 11.24 and 35.71 mM, respectively. Schuepp *et al.* [1] reported that K_m value of 5.11, 9.66 and 21.52 mM for the lipases from *P. fragi,* using trimyristin, triolein, tributyrin as a substrate, respectively. The incubation of cocoa lipase with a substrate, triacetin resulted in a K_m value of 7.10 mM.

The V_{max} value obtained from the incubation of the enzyme with a wide range of substrates, including tributyrin, trimyristin and triolein, are expressed as µmole acid/min/mg protein for cocoa lipase. V_{max} values decreased with the increase in the triacylglycerol chain-length, with V_{max} values of 27.78, 13.16 and 11.63 µmole acid/min/mg protein when incubated with tributyrin, trimyristin and triolein, respectively. These findings are in agreement with the previous reports which indicated that the lipase enzyme from *P. fragi* was most active with triacylglycerols of intermediate carbon-chain lengths and trilaurin was most rapidly hydrolysed, followed by tripalmitin, whereas longer chain lengths were hydrolysed more slowly [1].

Effect Of Inhibitors On Cocoa Beans Lipase Activity

The use of diisopropyl flourophosphate (DFP) at low concentration (0.2 µM) resulted in 90% inhibition of cocoa beans lipase activity and complete inhibition occured at 0.25 µM. According to Schuep *et al.* [1] the incubation of exolipase with 0.1 and 0.2 µM diisopropyl flourophosphate (DFP) resulted in 50 and 100% inhibition of cocoa beans lipase activity, respectively. DFP is reported to inactivate serine-esterases, including lipases and proteases by reacting with the serine residue at the active site [18]. *N*-Bromosuccinimide (NBS) was also an effective inhibitor for cocoa beans lipase. The incubation of cocoa lipase with *N*-bromosuccinimide (NBS) at 0.6 µM resulted in more than 50% loss of lipase activity, whereas total inhibition occured at 1.0 µM (Table 1). According to Schuepp *et al.* [1] the inhibition of exolipase by 0.5 µM of *N*-bromosuccinimide (NBS) resulted in a 50% loss of the original enzyme activity, whereas complete inhibit-

Table 1. Effects of inhibitors on lipase specific activity of cocoa lipase

Inhibitors used:	Inhibitor concentration (uM)	Residual Activity (%)
Diisopropyl flourophosphate (DFP)	0.05	92
	0.10	70
	0.15	36
	0.20	10
	0.25	0
N-Bromosuccinimide (NBS)	0.20	80
	0.40	65
	0.60	40
	0.80	25
	1.00	0
5,5-Dithiobis-(-2-nitrobenzoic acid)	0.05	70
	0.10	45
	0.15	32
	0.20	15
	0.25	0

ion of lipase activity occured at 2.0 µM. *N*-bromosuccinimide (NBS) has been used for the oxidation of tryptophan and tyrosine residues in proteins. Tryptophan is a highly hydrophobic amino acid and this characteristic may play an important role in the formation of the enzyme-substrate complex and in the maintenance of protein conformation [19].

The inhibition of cocoa beans lipase activity by 0.2 µM of 5,5-Dithiobis-(-2-nitrobenzoic acid) resulted in a 15% of residual activity of cocoa lipase, whereas complete inhibition occured at 0.25 µM. According to Schuepp *et al.* [1], the incubation of exolipase with 0.075 µM of 5,5-Dithiobis-(-2-nitrobenzoic acid) resulted in the inhibition of cocoa beans lipase activity by 75%. However, lower concentration of this inhibitor reagent did not show any inhibitory effect (Table 1). Complete inhibition of the enzyme activity could be achieved by the addition of 2.5 µM 5,5-Dithiobis-(-2-nitrobenzoic acid) [18].

Moleculer Weight of Lipase

Lipase was succesfully isolated from acetone dry powder of cocoa beans by solid ammonium sulphate precipitation in 20 mM sodium phosphate buffer. Five fractions, that were lipolytically active against olive oil were isolated, using 0-20%, 20-40%, 40-60%, 60-80% and 80-100% saturations of solid ammonium sulphate. Purified lipase extract were obtained after applying through DEAE cellulose and Sephacryl S-200 chromatography and have been done to assay of lipase activity before applying through SDS-PAGE. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis of the cocoa lipase showed the presence of a major band with a molecular weight of range in 45-66 kDa.

CONCLUSION

The results of temperature study on the characterize of cocoa lipase showed that the optimum temperature of partial purified cocoa lipase is between 30–40 °C, The pH study with olive oil as a substrate for lipase assay and it is shown a pH optimum of lipase activity between pH 7.0-8.0. K_m value for cocoa beans lipase was 2.63 mM, when trimyristin was used as a substrate. The incubation of cocoa bean lipase with triolein and tributyrin and yielded K_m of 11.24 and 35.71 mM for cocoa lipase, respectively, as the substrate and V_{max} values decrease with the increase in the triacylglycerol chain-length when incubated with tributyrin, trimyristin and triolein shown, with V_{max} values of 27.78, 13.16 and 11.63 µmol acid per min per mg protein, respectively. Inhibition of lipase occurred in the presence of diisopropyl flourophosphate, N-bromosuccinimide and 5,5-dithiobis-(-2-nitrobenzoic acid). Purified lipase extract were obtained after applying through DEAE cellulose and Sephacryl S-200 chromatography and have been done to assay of lipase activity before applying through SDS-PAGE. Sodium dodecyl sulphate – polyacrylamide gel electrophoresis of the cocoa lipase showed the presence of a major band with a molecular weight of range in 45-66 kDa. saturations of solid ammonium sulphate.

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