ISOLATION AND PURIFICATION OF LIPASE FROM COCOA BEANS (*Theobroma cacao.* L.) OF CLONE PBC 159

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ABSTRACT

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) was extracted and purified from acetone dry powder of cocoa (Theobroma cacao. L.) of clone PBC 159 extract. The Lipase from AcDP of cocoa beans was used for purification using 40-60 and 60-80% ammonium sulphate precipitation. The resulted indicated 44.73 and 60.51-fold purification with 26.74 and 33.31% recovery lipase activity (yield), respectively. The crude lipase enzyme from both precipitation were eluted, producing a single peak after applying through Sephacryl S-200 chromatography. The purified enzyme had a uniform specific activity throughout the final chromatography peak. The results from SDS-PAGE analysis showed that the molecular weight of the lipase enzyme was in between 45-66 kDa.

Keywords: *isolation, purification, lipase, cocoa beans*

INTRODUCTION

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are ester hydrolases or esterases since they hydrolyse the ester bonds of triacylglycerol molecules. In plants, this lipolytic enzyme plays an important role in fatty acid metabolism. Highly active lipases are found to catalyze the hydrolysis of reserved triacylglycerols. Triacylglycerols are usually localized in subcellular organelles of the higher plants called lipid bodies [1,2].

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 [3]. Many methods dealing with the purification of lipases had been reported and mostly the initial step of enzyme purification involves the precipitation of enzyme with ammonium sulphate salts or acetone fractionation. The subsequent steps used by many researchers are either gel exclusion chromatography, anion exchanger chromatography or hydrophobic interaction chromatography which gave satisfactory results. Mozaffar and Weete [4], reported that purified the extracellular lipase from *Phytium ultimum* using ammonium sulphate precipitation and by DEAE-Sepharose CL-6B and Sephacryl S-200 chromatography. The objective of this present study was to isolate and purify the lipase from cocoa beans (*Theobroma cacao.* L.) of clone PBC 159 .

EXPERIMENTAL SECTION

Materials

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

Instrumentation

Minipuls 3 Gilson Peristaltic Pump, Bio-Rad 2110 Fraction Collecter, Econo UV Detector, Glass Separation Colomn, Magnetic Stirrer, Shaker Water-bath, Electrophoreogram of SDS-PAGE.

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* [5]. 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 ^{o}C) for 8 hours in a soxhlet apparatus and reextracted for another 8 hours. Following that, all purine alkaloids were also removed by extraction with 250 mL chloroform for 8 hours. 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 ^{o}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 ^{o}C for 1 hours with frequent shaking at 20 min intervals. The suspension was then centrifuged at 10.000 x *g* for 15 min at 4 ^{o}C and the resulting pellet was re-extracted

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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 ^{o}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* [6]. 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 ^{o}C. The supernatant was dialysed against the same buffer at 5 ^{o}C for 2 days. The dialysate was centrifuged again at 10.000 x *g* for 30 min at 4 ^{o}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 [7]. The resulting crude extract from AcDP (300 mL) was fractional precipitated by 0-20 , 20-40 , 40-60 , 60-80 and 80-100% saturated of ammonium sulphate. The precipitate was separated by centrifugation at 10,000 rpm, 40 ^{o}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 ^{o}C. The dialysing medium was changed twice every 24 hours. The suspension was then lyophilised and stored at -20 ^{o}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 [8]. 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 1 g 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 ^{o}C before used for analysis.

Assay of Lipase Activity

The lipase enzyme activity was determined according to Kwon and Rhee method [9]. 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 ^{o}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.

Purification of Cocoa Lipase

Preparation of DEAE-cellulose Ion Exchanger

The DEAE cellulose resin was prepared according to Boyer method [10]. The DEAE-cellulose resin (100 gs) was subjected to pretreatment before being packed into a column with 1L of 0.5 M HCl stirred for 30 min. After stirring with 0.5 M HCl, the solution was allowed to settle for 30-40 min and after the bulk of HCl had been decanted, the DEAE-cellulose slurry was filtered through a layer of Whatman no: 1 using a Buchner funnel under gentle suction. The DEAE-cellulose was then washed several times with distilled water through the Buchner funnel until a neutral pH was attained. The DEAE-cellulose was washed and stirred for 30 min with one liter of 0.5 M NaOH, the solution was allowed to settle down 30-40 min. After the bulk of the NaOH was decanted off, the slurry was filtered through the Buchner funnel. The DEAE-cellulose was then washed with distilled water again through the Buchner funnel until the pH of the filtrate was in the region of neutral pH. Finally, the DEAE-cellulose was washed with one liter of 20 mM Tris buffer (pH 7.8) before being packed into a glass column. The size of the DEAE-cellulose column used

was 1.0 cm (diameter) x 25 cm (height). The DEAE-cellulose was properly packed into a column to a bed height of 20 cm.

Fractionation of Enzyme using DEAE-cellulose Ion

Exchanger Medium

The crude enzyme from 40-60 and 60-80% of ammonium sulphate precipitation was separately dialysed with 5 L of 20 mM of Tris buffer (pH 7.8) over night in the cold room (0-4 ^{o}C) and lyophilised for 24 hours. The solution of enzyme (5 mL) was deaerated and applied into a glass column at the top surface of gel column. The deaerated sample was washed with 200 mL of 20 mM Tris buffer (pH 7.8). The enzyme sample was then eluted with a 0.0–0.5 M NaCl (salt gradient) in 20 mM Tris buffer (pH 7.8) using a Minipuls 3 Gilson peristaltic pump at a flow rate 0.5 mL/min. The fraction of 5 mL each was collected using a Bio-Rad 2110 Fraction Collecter. The protein concentration of eluted fractions was continuously monitored by Econo UV Detector at 280 nm. Fractions with absorbance greater than 0.100 were pooled. After pooling, the fractions were dialysed with 20 mM of Tris buffer (pH 7.8), and then stored fraction at –20 ^{o}C for further use.

Preparation of Gel Filtration Medium

The gel of Sephacryl S-200 (25 g) was swelled in 500 mL of 20 mM sodium phosphate buffer (pH 6.8) containing 0.02% sodium azide as an antimicrobial agent for 3 days. The gel was then washed and decanted several time using elution buffer (20 mM sodium phosphate) to removing all floating particles before being packed into a glass column. The size of the Sephacryl S-200 gel used was 1.5 cm (diameter) x 50 cm (height). The gel was then deaerated and packed into a column to a bed height (35 cm). After packing, the gel was eluted with 1.0 mL 0.01% (w/v) bromophenol blue. Finally, the gel column was eluted again with deaerated 20 mM sodium phosphate buffer (pH 6.8).

Fractionation Enzyme using Sephacryl S-200 Gel
Filtration Medium

The fractions of DEAE-cellulose ion exchanger were dialysed with 5 L of 20 mM sodium phosphate buffer (pH 6.8) over night and lyophilised for 24 hours. The solution of enzyme (5 mL) was deaerated and applied into a glass column at the top surface of gel column. The sample of enzyme was then eluted with 20 mM sodium phosphate buffer (pH 6.8) by a Minipuls 3 Gilson peristaltic pump at flow rate of 0.5 mL/min. The fraction of 5 mL each was collected using a Bio-Rad 2110 Fraction Collecter. The protein concentration of eluted fractions was continuously monitored by Econo UV Detector at 280 nm. Fractions with absorbance greater than 0.100 were pooled. After pooling, the fractions were dialysed with 20 mM sodium phosphate buffer (pH 6.8), and then fractions were stored for further use.

Preparation of Sodium Dodecyl Sulphate – PAGE

The SDS-PAGE was prepared according to Laemmli method [11]. 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 ^{o}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 (isolation of lipase from the precipitation of crude extract or AcDP) 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* [2] 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 (Table 1). 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.

DEAE-cellulose Ion Exchanger Chromatography

The results showed that lipase from both 40-60 or 60-80% of fractions could not be eluted through the DEAE-cellulose column using 20 mM Tris buffer (pH 7.8). Therefore, it is assumed that the lipase from both fractions was still bound to the resin. This was indicated by the absence of lipase activity in the eluted fractions. Thereafter the lipases which were bound to the DEAE-cellulose column matrix would have to be eluted out by high salt concentration. The DEAE-cellulose column was eluted using 0.0–0.5 M sodium chloride solutions as a linear gradient. It was found that there were 3 protein peaks and one of the peaks belonged to a lipase enzyme after the fractions was assayed. The peak was pooled from 50-70 tube number of fractions from 40-60% saturation and 40-65 tube number of fractions from 60-80% saturation. The elution profiles of 40-60 and 60-80 % saturation obtained by DEAE-cellulose ion exchanger chromatography are shown in the Fig 1 and 2, respectively.

According to Antonian [3], the lipase fractionation can be successfully carried out using DEAE-Sephacel anion exchanger chromatography.

Sephacryl S-200 Gel Filtration Chromatography

The fractions 0f 40-60 and 60-80% saturation eluted by DEAE-cellulose ion exchanger, were dialysed using 20 mM sodium phosphate (pH 6.8) and then lyophilised for 24 hours. The elution profiles of fractions of 40-60 and 60-80 % saturation eluted through Sephacryl S-200 Gel Filtration Chromatography are showed in the Fig 3 and 4, respectively. It was found that was one major protein peak and it belonged to a lipase enzyme.

A summary of the various steps utilised to purify the lipase for 40-60 and 60-80% ammonium sulphate precipitation is given in Table 2 and 3. The purification procedure carried out was repeated several times and was found to be highly reproducible. The first step of the lipase purification consisted of ammonium sulphate precipitation. Both the dialysed precipitate and dialysed supernatant obtained after 40-60 and 60-80% ammonium sulphate precipitation, respectively, contained negligible quantities of lipase activity.

Table 1. Lipase specific activity of crude enzyme from AcDP and ammonium sulphate precipitated fractions

Fractions	Volume (mL)	Activity (U)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)
Crude extract From AcDP	300	62.68	18804.00	474.0	39.67	100.00
$(NH_4)_2SO_4$:						
0-20%	159	63.44	10086.96	250.3	40.30	53.64
20-40%	130	65.43	8505.90	120.9	70.35	45.23
40-60%	110	50.62	5568.20	65.1	85.53	29.61
60-80%	76	35.23	2677.48	29.9	89.55	14.24
80-100%	59	18.02	1063.18	21.0	50.63	5.65

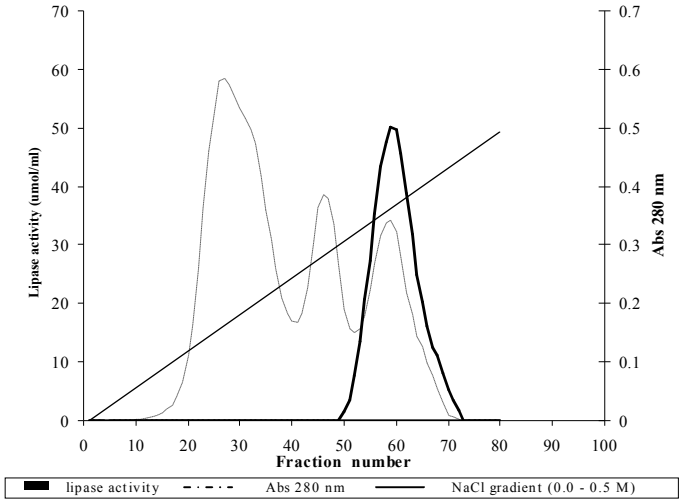

Fig 1. Elution profile of soluble lipase from 40-60% saturation of ammonium sulphate precipitation obtained by DEAE-cellulose chromatography. Note : Lipase activity was eluted from column using 0.0-0.5 M NaCl

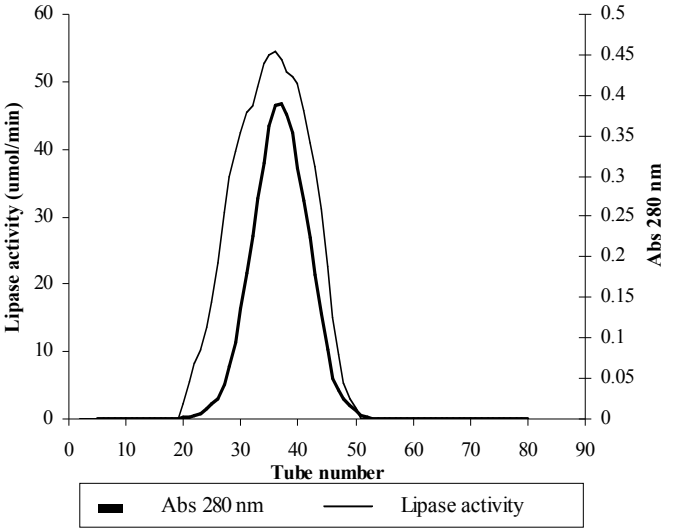

Fig 3. Elution profile of soluble lipase from 40-60% saturation of DEAE-cellulose elution was obtained by sephacryl S-200 gel filtration chromatography.

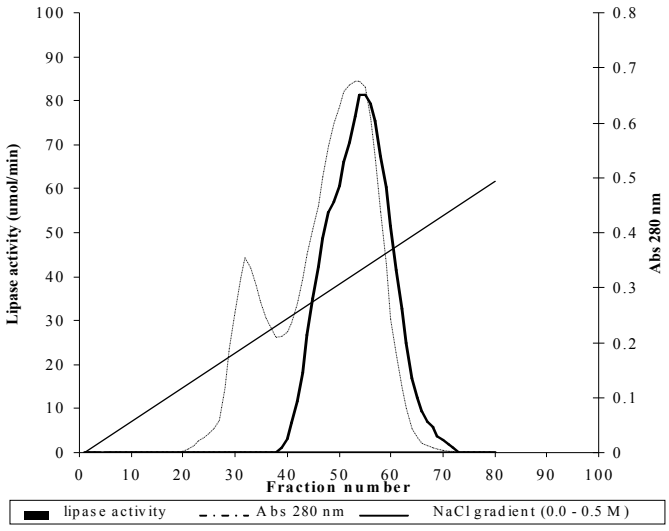

Fig 2. Elution profile of soluble lipase from 60-80% saturation of ammonium sulphate precipitation obtained by DEAE-cellulose chromatography. Note: Lipase activity was eluted from column using 0.0-0.5 M NaCl gradient.

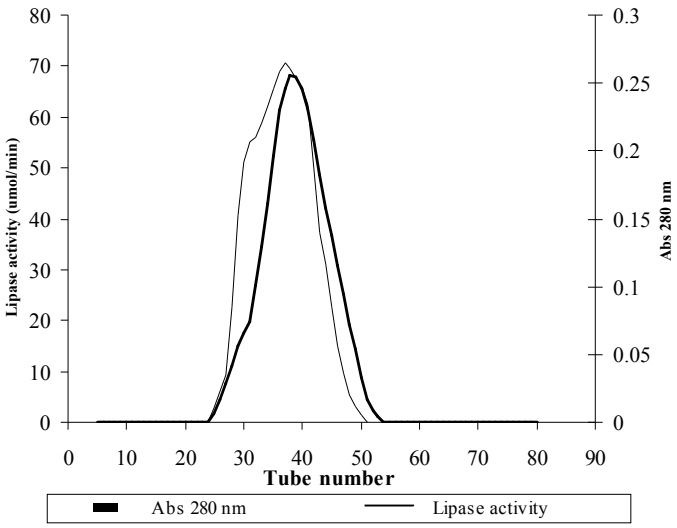

Fig 4. Elution profile of soluble lipase from 60-80% saturation of DEAE-cellulose elution was obtained by sephacryl S-200 gel filtration chromatography.

Table 2. Purification scheme of lipase from AcDP of cocoa bean (40-60% saturation of ammonium sulphate precipitation)

Purification steps	Volume (mL)	Activity (U)	Protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude AcDP	2350	15213.5	1200.75	12.67	1	100
$(NH_4)_2SO_4$(40-60%)	169	12872.7	145.45	88.50	6.99	84.61
DEAE-cellulose	58	7511.0	17.57	427.49	33.74	49.37
Sephacryl S 200	15	4068.0	7.18	566.57	44.72	26.74

AcDP: Acetone Dry Powder

Table 3. Purification scheme of lipase from AcDP of cocoa beans (60-80% sat. of ammonium sulphate precipitation)

Purification steps	Volume (mL)	Activity (U)	Protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Crude AcDP	2350	15213.5	1200.75	12.67	1	100
$(NH_4)_2SO_4$(60-80%)	100	13721.8	143.68	95.50	7.54	90.19
DEAE-cellulose	60	9511.0	18.03	527.51	41.63	62.52
Sephacryl S 200	30	5068.0	6.61	766.72	60.51	33.31

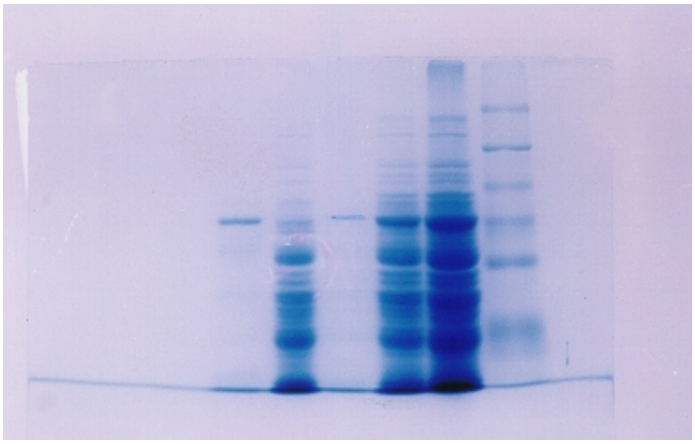

Plate 1. SDS-PAGE electrophoreograms of cocoa lipase. Note : The crude enzyme from AcDP and ammonium sulphate precipitation have been done to assay of lipase activity before applying through SDS-PAGE, Line 1: Standard molecular weight, Line 2: Crude enzyme from AcDP, Line 3: Ammonium sulphate precipitation (40-60%), Line 4: Lipase molecular weight from 40–60% saturation, Line 5: ammonium sulphate precipitation (60–80%), Line 6: Lipase molecular weight from 60-80% saturation.

The purity of the lipases in this experiment was examined by various criteria, such as, ion exchanger on DEAE-cellulose, gel filtration on Sephacryl S-200 and SDS-PAGE. The elution profile of the lipase from 40-60 and 60-80% of ammonium sulphate saturation on a DEAE-cellulose column is shown in Fig 1 and 2, respectively. The enzyme was found to be eluted as a single peak. The procedure adopted for purification from 40-60 and 60-80% of ammonium sulphate precipitation resulted in approximately 44.73 and 60.51-fold purification with 26.74 and 33.31% recovery of lipase activity, respectively. The enzyme was then further purified to homogeneity in the last step of purification, that was by gel-filtration chromatography on Sephacryl S-200 column. The elution patterns are as shown in Fig 3 and 4, respectively. All of the active fractions from 40-60 and 60-80% ammonium sulphate saturation that were obtained on Sephacryl S-200 column have shown approximately the same activity and a symmetrical profile was obtained. The electrophoreogram of SDS-PAGE of generally purified lipases fractions of 40-60 and 60-80% saturation had shown that the enzyme have molecular weight between 45-66 kDa (Plate 1). These finding is generally in agreement with those reported in the literature for molecular weight of lipase. The electrophoretic analyses revealed that lipase have molecular weight of acidic glycoproteins between 20 and 60 kDa although some forms aggregate in solution. Most purified lipases contain between 2 and 15% of carbohydrate, with the major glycoside residue being known as mannose [12].

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

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.

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