Spoilage of Coconut Oil Purification and Properties of a Fungal Lipase that Attacks Coconut Oil

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ABSTRACT

A lipase was isolated from a strain of *Aspergillus flavus* which attacked coconut kernel and oil with the liberation of free fatty acids. The enzyme was purified 109-fold by ammonium sulphate precipitation, diethyl aminoethyl-cellulose and Sephadex G-200 chromatography. The optimum pH of the enzyme reaction was 6.2. The action of the enzyme on pure triglycerides was studied. The triglycerides of the shorter chain fatty acids were more rapidly hydrolyzed, while hydrolysis of tristearin was not detected under the conditions of assay. K_m for trilaurin and trimyristin were 9.09×10^{-4} and 1.42×10^{-3} M, respectively. Para-chloromercuricbenzoate was an inhibitor. Thin layer chromatography and gas liquid chromatography of the esterified products of enzymatic hydrolysis of coconut oil showed the presence of oleate, palmitate, myristate, laurate, caprate, caproate and caprylate but not stearate, although stearate was present in coconut oil.

INTRODUCTION

Lipolytic enzymes are found in many tissues, seeds and microorganisms, and have been discussed by Jensen (1). Lipases from aspergilli growing on peanut oil and castor oil have been studied by Ramakrishnan and Banerjee (2). Crystallization and properties of lipase of a strain of *Aspergillus niger* have been described by Fukumoto et al. (3).

Coconut oil which is commercially extracted from copra, i.e., sun-dried coconut endosperm using screw presses, is a mixture of a number of mixed triglycerides of lauric, myristic, palmitic, capric, caproic, caprylic, stearic and oleic acid (4,5). Coconut oil extracted from well dried and properly stored copra has low free fatty acid content. However, if the oil has been extracted from under-dried, badly stored copra, the incidence of free fatty acids is very high. While studying the development of free fatty acids in coconut oil, we isolated a strain of *Aspergillus flavus* which actively attacks coconut oil, liberating free fatty acids. The strain which we have isolated grows on copra, on fresh coconut kernel and on coconut oil containing traces of coconut kernel.

In this paper the isolation of the lipase, its purification and properties, and the products of hydrolysis are discussed.

MATERIALS

Cooks Joy is a refined coconut oil manufactured and marketed by British Ceylon Corp., Colombo. Sephadex G-200 and diethyl aminoethyl (DEAE) cellulose were purchased from Pharmacia, Uppsala, Sweden. Methyl esters of fatty acids used as standards in thin layer chromatography and gas liquid chromatography analysis were obtained from Polyscience Corp., Evanston, Ill. Fine grade Silica Gel G (60 μ) was purchased from C. Desaga GmbH, Heidelberg, West Germany. Synthetic triglycerides were received from M. Nakajima, Kyoto University.

METHODS

Enzyme Assay

Lipase activity was monitored by measuring the free fatty acids released using coconut oil as the substrate. The reaction mixture normally consisted of 10 ml coconut oil, 24 ml 0.01 M potassium phosphate buffer pH 6.5 and 1 ml enzyme solution in a 100 ml Erlenmeyer flask. The incubation was at 26 C for 10 min on a shaker at 120 strokes per minute. In some instances gum arabic was added as a stabilizer of the emulsion and the incubation mixture was mixed well just before incubation. There was no enhancement in enzyme activity in the presence of an emulsifier as long as the reaction mixture was incubated with shaking. After incubation the free fatty acids were extracted thrice with 30 ml chloroform. The combined extract was titrated with 0.02 N alcoholic potassium hydroxide using phenolphthalein as an indicator. The difference between the milliequivalents of alkali required for titration of the test solution and the amount for the blank was recorded as milliequivalent of free fatty acids liberated during the incubation. One unit of lipase is then one milliequivalent fatty acid released in 10 min. In the case of purified enzyme, 1 ml of a suitably diluted enzyme solution was added to the incubation mixture.

Protein Estimation

Protein estimation was done according to the method of

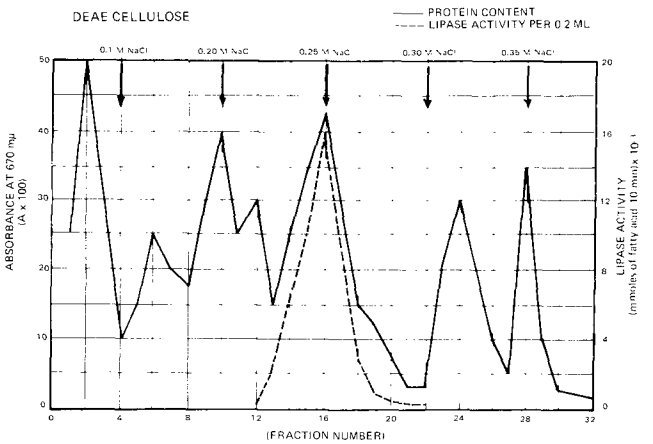

FIG. 1. Diethyl aminoethyl-cellulose chromatography. (C.I.S.I.R. SK. No. 326-1.)

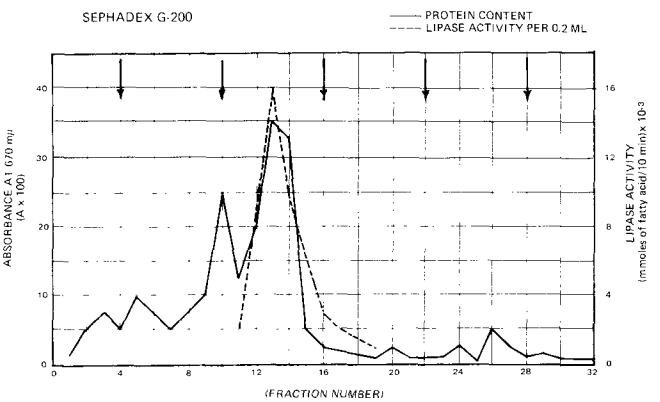

FIG. 2. Sephadex G-200 fractionation. (C.I.S.I.R. SK. No. 326-2.)

TABLE I

Purification of Lipase from a Strain of *Aspergillus flavus*

Fractions	Volume, ml	Total activity, units	Protein, mg/ml	Specific activity, units/mg protein	Yield, %	Purification
Crude extract	46	120	16	0.16	100	1
Ammonium sulphate precipitate	25	80	0.9	3.5	67	22
Diethyl aminoethyl-cellulose	18	10.8	0.08	7.5	9	47
Sephadex G-200	12	8.4	0.04	17.5	7	109

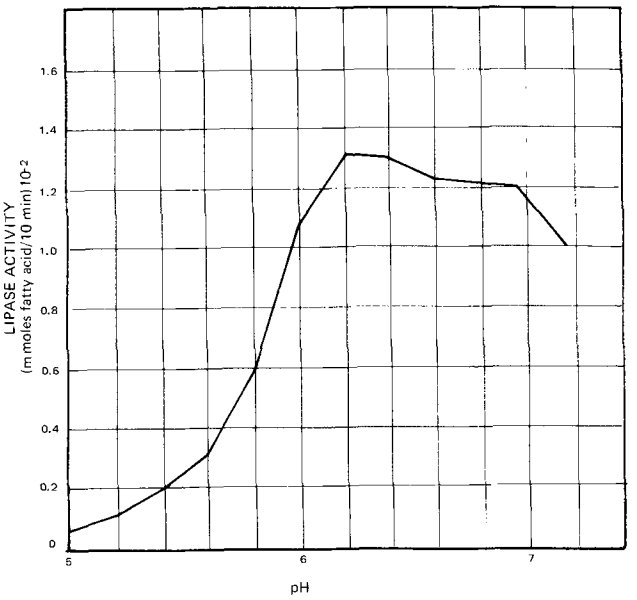

FIG. 3. Effect of pH on the reaction of purified lipase. (C.I.S.I.R. SK. No. 326-3.)

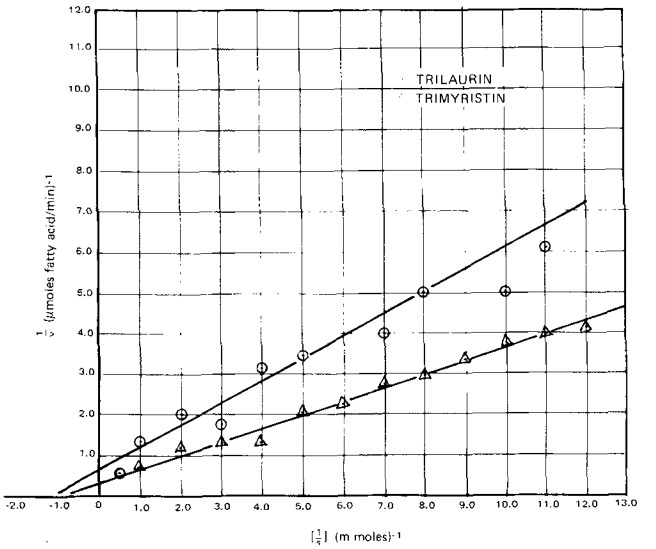

FIG. 4. Lineweaver-Burk plot using trilaurin and trimyristin as the substrates. (C.I.S.I.R. SK. No. 326-9.)

Lowry et al. (6). Casein was used to prepare the standard protein solutions.

TLC and GLC of Fatty Acids

Urea adducts of the liberated fatty acids were prepared according to the method of Moreno and Roncero (7), and the free fatty acids were methylated according to the method of Stoffel et al. (8). TLC of the methyl esters was carried out according to the method of Malins and Mangold (9). The esters were also analyzed in a Varian Gas Liquid Chromatograph equipped with a thermal conductivity detector. The column (5 ft x 1/8 in.) was packed with 20% diethylene glycol succinate (DEGS) on 60/80 mesh chromosorb W. All samples were applied in 2 μl quantities. The operating parameters of the instrument were as follows: injector temperature, 200 C; detector temperature, 240 C; column temperature, 190 C; carrier gas, helium; flow rate, 60 ml/min; recorder range, 1 mV.

TABLE II

Effect of Lipase on Triglycerides

Substrate	Milliequivalents of fatty acid produced/10 min/incubation mixture x 10^{-1}
Trilaurin	14.0
Trimyristin	9.0
Tripalmitin	4.0
Triolein	1.6
Tristearin	0.0

RESULTS AND DISCUSSION

Isolation of an *Aspergillus flavus* Strain and Extraction of the Lipase

Isolation of the fungus: Freshly grated coconut kernel

TABLE III

Effect of Inhibitors on the Activity of Purified Lipase

Inhibitor	Molar concentration x 10^{-3}	Milliequivalents of fatty acid liberated x 10^{-2}	Inhibition, %
No inhibitor		14.0	0
NaF	100	14.0	0
$FeCl_3$	10	11.0	22.8
	50	10.0	30
Iodoacetamide	1	13.0	7.1
	100	12.4	11.4
p-Chloromercuric benzoate	1	7.0	50
	2	5.0	65.5
	10	4.7	66.4
EDTA	1	13.4	4.3
	100	13.0	7.1

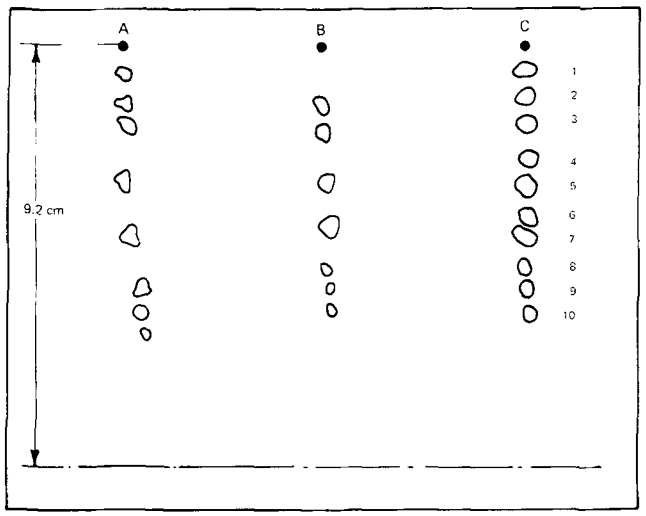

FIG. 5. Thin layer chromatography of the methyl esters of fatty acids. A. Methyl esters of fatty acids of coconut oil obtained by saponification; B. methyl esters of fatty acids of coconut oil released by lipase; C. standard methyl esters. 1. Methyl stearate; 2. methyl oleate; 3. methyl palmitate; 4. methyl linoleate; 5. methyl myristate; 6. methyl linolenate; 7. methyl laurate; 8. methyl caprate; 9. methyl caprylate; 10. methyl caproate. (C.I.S.I.R. SK. No. 326-6.)

was kept exposed to the atmosphere for 7 days. At the end of this period, the kernel had a very offensive odor and was brownish in color. A small portion of this kernel was incubated at 28 C in a flask containing 20 ml coconut oil and 20 ml 1% glucose. After 4 days of incubation several species of bacteria and fungi were growing on the surface. Among these a fungus was found that liberated considerable amounts of fatty acids from coconut oil. Pure cultures of this fungus were obtained and grown on Czapek's agar medium. The color of the colonies varied from greenish yellow to a persistent deep green in the older heads. The conidiophores appeared colorless and variable in length (400-1000 μ). The sterigmata were mostly in two series. From these and other morphological data this fungus was identified as a strain of *Aspergillus flavus*.

Extraction of crude enzyme: The fungus was grown on the surface of an incubation mixture consisting of 20 ml 0.05 M potassium phosphate buffer pH 7.0, containing 1% glucose (w/v), and 0.1% nutrient broth and 20 ml coconut oil. After 5 to 7 days of incubation at 28 C, the fungi were harvested by centrifugation (10 min at 2000 g). No lipase activity was detected in the culture supernatant. The mycelia were ground in a chilled mortar with about the same weight of chilled washed sand and just sufficient ice to keep the mixture moist. The mixture was centrifuged at 10,000 g for 10 min. The supernatant that contained the enzyme was recentrifuged for further clarification.

Purification of the Enzyme

Ammonium sulphate precipitation: The supernatant was saturated with ammonium sulphate, left for 1 hr at 8 C and centrifuged for 10 min at 10,000 g. The sediment was suspended in a minimal amount of 0.05 M potassium phosphate buffer, pH 8.0, dialyzed for 1 hr against 400 ml distilled water and then for 1 hr against 400 ml 0.01 M potassium phosphate buffer pH 8.0.

DEAE-cellulose fractionation: A DEAE-cellulose column (10 x 2.5 cm) was packed and equilibrated with 0.01 M potassium phosphate buffer, pH 8.0. Twenty-five milliliters of the dialyzed solution was applied to it. The column was washed with 20 ml 0.1 M potassium phosphate buffer, pH 8.0, and then sequentially with equal volumes of the same buffer, containing 0.1 M, 0.2 M, 0.25 M and 0.35 M NaCl. Five milliliter fractions were collected using an automatic fraction collector. The elution patterns of proteins and lipase activity are shown in Figure 1.

Sephadex G-200 fractionation: Three very active fractions obtained by DEAE-cellulose chromatography were pooled and dialyzed for 1 hr against 400 ml 0.01 M potassium phosphate, buffer pH 8.0. The dialysate, ca. 15

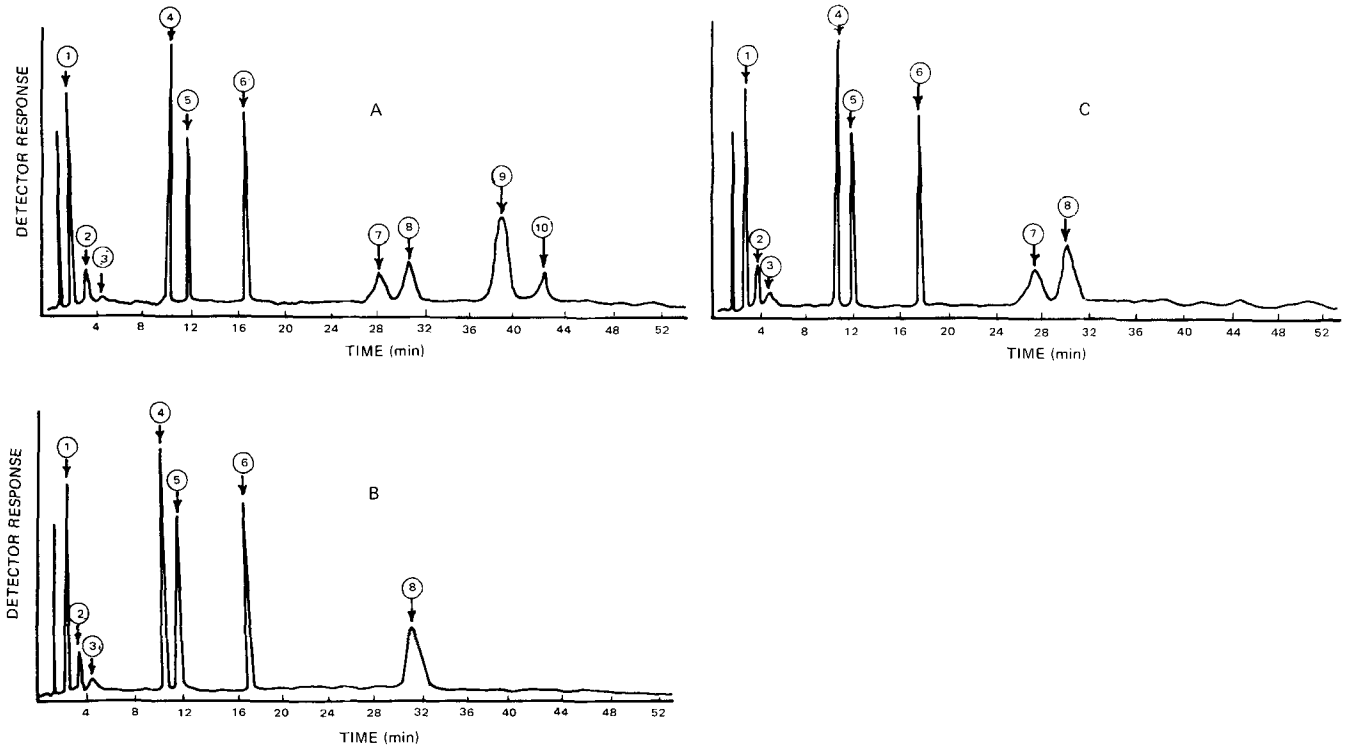

FIG. 6. A. Gas liquid chromatography (GLC) of standard methyl esters of fatty acids; B. GLC of methyl esters of fatty acids of coconut oil released by lipase; C. GLC of methyl esters of fatty acids of coconut oil obtained by saponification. 1. Methyl caproate; 2. methyl caprylate; 3. methyl caprate; 4. methyl laurate; 5. methyl myristate; 6. methyl palmitate; 7. methyl stearate; 8. methyl oleate; 9. methyl linoleate; 10. methyl linolenate. (C.I.S.I.R. SK. No. 326-7.)

ml, was applied to a Sephadex G-200 column (40 x 3.5 cm) that had previously been equilibrated with the same buffer. The column was eluted with the same buffer and 5 ml fractions were collected. Figure 2 shows the elution patterns of proteins and the lipase activity.

Table I is a summary of the various stages of purification, the corresponding yields and the degree of purification.

Properties of the Enzyme

The enzyme preparation after chromatography on Sephadex G-200 was characterized as follows.

Effect of pH: The reaction mixture consisted of 20 ml coconut oil, 24 ml 0.2 M potassium phosphate-citrate buffer of varying pH and 1 ml of the diluted, purified enzyme preparation. After 10 min incubation the fatty acids were extracted and titrated as described. The effect of pH on enzyme activity is shown in Figure 3. The optimum pH was ca. 6.2.

Selective action of lipase on pure triglycerides: The rate of hydrolysis of trilaurin, trimyristin, tripalmitin, triolein and tristearin is shown in Table II. Each flask contained 50 μmol of the substrate, 10 ml of 0.02 M potassium phosphate buffer, pH 6.5, 1.0 ml of 1% gum arabic, 1.0 ml enzyme preparation (1.04 mg protein) and water to 20 ml. After 10 min incubation on a shaker at 28 C, the fatty acids released were assayed as described. Hydrolysis was fastest with trilaurin followed by trimyristin, tripalmitin and triolein. Tristearin was not acted on by the enzyme.

The K_m for trilaurin and trimyristin were determined by varying the substrate concentration from 0.091 to 2 millimol per incubation mixture. Each flask contained the appropriate amount of substrate, 15 ml of 0.02 M potassium phosphate buffer pH 6.5, 1.0 ml of 1% gum arabic, 1.0 ml enzyme preparation (1.04 mg protein) and water to 25 ml. After 10 min incubation on a shaker at 28 C, the fatty acids released were assayed as described. The K_m for trilaurin and trimyristin are 9.09×10^{-4} and 1.42×10^{-3} M, respectively. Figure 4 gives the Lineweaver Burk plots using trilaurin and trimyristin as substrates.

Effect of various ions and inhibitors: Effects of some inhibitors were investigated at concentrations described in Table III. Our results, compared with those obtained by Lu and Liska (10), using a lipase preparation from *Pseudomonas fragi,* showed some interesting similarities and differences. We obtained only moderate inhibition (23%) with Fe^{+3}, whereas Lu and Liska observed 82% inhibition of their enzyme. On the other hand, their lipase closely resembled our lipase, in the extent of inhibition by NaF, EDTA and parachloromercuric benzoate.

The persistence of enzyme activity in the presence of parachloromercuric benzoate in concentrations as high as 1×10^{-2} M makes it probable that SH groups are not directly involved in lipase activity. The diminished activity may be due to conformational changes in the enzyme molecule in the presence of the inhibitors.

Analysis of the fatty acids released: Experiments were carried out to establish whether there were any qualitative differences between enzyme hydrolysis and alkaline hydrolysis of coconut oil. The methyl esters of the fatty acid mixture obtained in each case were subjected to TLC and GLC. As shown in Figures 5 and 6, stearic acid was not released by the lipase under the conditions of assay.

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