Purification and Properties of Lipase from the Anaerobe *Propionibacterium acidi-propionici*

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A lipase secreted by the anaerobe *Propionibacterium acidi-propionici* was purified 52-fold with 27% recovery by employing a three-step purification protocol. The enzyme has a small molecular mass (Mr = 6000–8000) as determined by gel filtration and ultracentrifugation. It hydrolyzed palm oil, coconut oil, castor oil, olive oil, groundnut oil and tributyrin. Enzyme activity was inhibited by Ni^{2+}, Ba^{2+}, Mg^{2+}, Cu^{2+}, ethylenediaminetetraacetic acid, iodoacetamide, N-acetylimidazole and nonidet P-40 but stimulated by Ca^{2+}, Co^{2+}, K^{+}, Fe^{2+}, sodium dodecyl sulfate and N-bromosuccinamide. The enzyme showed substrate inhibition for both tributyrin and *p*-nitrophenyl acetate.

KEY WORDS: Lipase, *Propionibacterium acidi-propionici.*

Several sources of organic waste material are being studied in our laboratory for production of methane gas by the anaerobic digestion process. The tomato processing industry generates a considerable amount of waste comprised of skin, pulp and seed (1). Besides other components, tomato processing waste characteristically contains significant levels of lipid (~24% wt/wt). We have isolated a number of anaerobic bacterial cultures that produce extracellular hydrolases from the digester and found one of them, *Propionobacterium acidi-propionici*, excreting a lipase of an unusually small molecular mass. Since there is practically no information on the lipases produced by anaerobic microorganisms, we carried out some detailed studies on the lipase produced by *P. acidi-propionici.* These studies were also aimed at understanding the role of lipase in the overall anaerobic degradation process of tomato processing waste (TPW).

MATERIALS AND METHODS

Chemicals. The molecular weight markers for gel filtration, iodoacetamide, N-acetyl imidazole, Sephadex gels, and *para*-nitrophenyl acetate were procured from Sigma Chemical Company, St. Louis, MO.

Organism and growth conditions. Propionibacterium acidi-propionici was isolated from TPW slurry undergoing anaerobic digestion in a 1-L digester. The culture was identified by using the key according to Holdeman *et al.* (2). For production of extracellular lipase, the organism was grown anaerobically at 30°C for 36 h in Henderson medium (3) (yeast extract, 6.0 g; tryptone, 7.0 g; K_2HPO_4, 0.45 g; KH_2PO_4, 0.45 g; $NaHCO_3$, 6.0 g; cysteine HCl, 0.5 g; glycerol, 3.0 g/L; resazurin (0.1% wt/vol), 1 mL per liter). The supernatant after pelleting the cells was taken for purification of the enzyme. All operations were carried out at 4°C.

Enzyme assay. Lipase was assayed by the slightly modified procedure of Lee and Lee (4) with either tributyrin or *p*-nitrophenyl acetate as the substrate. The reaction mixture consisted of 5 mL of tributyrin emulsion and 5 mL of 50 mM phosphate buffer pH 7.0 with known aliquots of enzyme preparation. Tributyrin emulsion was made by taking 10 mL of tributyrin, 90 mL of distilled water, 0.5 g gum arabic and 0.2 g sodium benzoate and blending them in a homogenizer. The reaction mixture was incubated for 1 h at 30°C, and the reaction was stopped by keeping the reaction mixture at 90°C for 10 min. Each assay had a control in which heat inactivated enzyme was added prior to incubation of the reaction mixture. The liberated free fatty acids were titrated with 0.05M NaOH. One unit of enzyme is defined as the amount that causes the generation of one micromole of butyric acid or one micromole of *p*-nitrophenol per minute.

Protein was estimated by the method of Lowry *et al.* (5) with bovine serum albumin as standard. During purification of the enzyme, protein was monitored by absorbance at 280 nm. Discontinuous polyacylamide gel electrophoresis was performed on slab gels for both nondenaturing and sodium dodecyl sulfate (SDS)-denaturing (20% wt/vol acrylamide) gel according to the method of Laemmli *et al.* (6).

The molecular weight of the native enzyme was determined by gel permeation chromatography in ephadex G-100 with β-lactalbumin (18,400), lysozyme (14,300) and cytochrome C (11,700) as molecular weight markers.

RESULTS

Purification of lipase. The results of a typical purification experiment is given in Table 1. Enzyme was concentrated by salting out with ammonium sulphate at 70% saturation, leading to 91.6% enzyme recovery and fourfold increase in specific activity. The second step involved molecular sieving in Sephadex G-50 (Fig. 1), which yielded a 33-fold purified lipase with 88% recovery. The elution profile of enzyme in Sephadex G-50 suggested that the lipase under study was a small molecule. Hence, it was rechromatographed in Sephadex G-25. To do this the pooled active fractions from G-50 chromatography were precipitated by ammonium sulphate at 80% saturation after increasing the protein content by addition of bovine serum albumin (BSA). This was necessitated because of the low concentration of protein in the pooled fractions after Sephadex G-50 chromatography. Rechromatography of the enzyme preparation thus obtained on Sephadex G-25 (Fig. 2) revealed two apparent lipase fractions, one of which eluted in the void volume, along with a large quantity of the contaminating protein, BSA. This first lipase fraction has presumably resulted from the interaction of BSA with a part of the lipase. It is known that BSA can interact hydrophobically with a large number of ligands (7). Despite some loss of the lipase content in this manner, it was possible to get 27% recovery of the enzyme in the second fraction, which was found to have been purified further in relation to the preceding step. The second lipase fraction was pooled, concentrated and again rechromatographed on Sephadex G-25, showing an elution profile symmetrical in itself and coinciding with the single protein peak.

Properties of lipase. The enzyme was found to be

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TABLE 1

Purification of Lipase from *Propionibacterium acidi-propionici*

Treatment/step	Volume (mL)	Protein (mg/mL)	Enzyme (units/mL)	Total units	Specific activity	Purification fold	% Recovery
Culture supernatant	2750	0.24	0.021	57.70	0.09	1.00	100.00
Ammonium sulphate precipitation (70%)	4.4	35	12.020	52.87	0.34	3.77	91.60
Sephadex G-50	26	0.65	1.958	50.90	3.01	33.40	88.00
Sephadex G-25	11	0.30	1.416	15.58	4.72	52.40	27.00

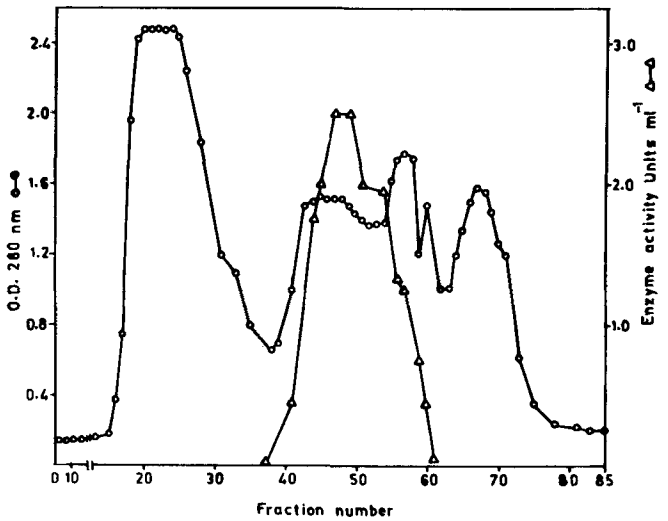

FIG. 1. Elution profile of lipase in Sephadex G-50 chromatography. The enzyme solution from 70% ammonium sulphate precipitation corresponding to 154 mg of protein was loaded on a column (60 × 2 cm); 2.0-mL fractions were collected at a flow rate of 12 mL h^{-1}.

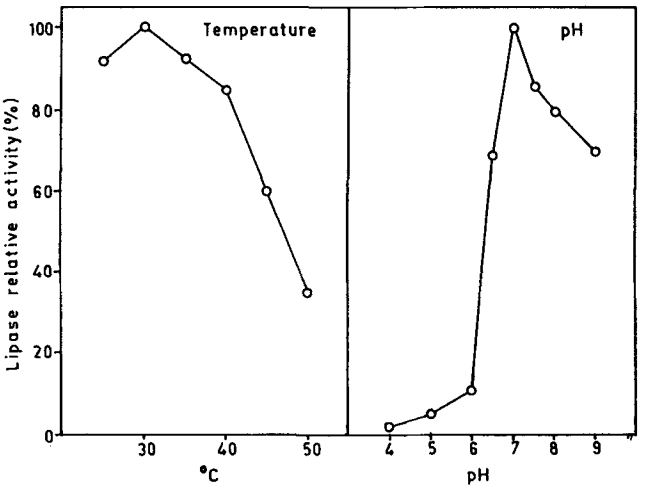

FIG. 3. Influence of pH and temperature on relative activity of lipase of *Propionibacterium acidi-propionici*. The effect of temperature on lipase activity was studied by incubating the reaction mixture at different temperatures for 1 h; the effect of pH was studied by carrying out the reaction at different pH values (pH 4–5 citrate-phosphate, pH 6–8 phosphate, pH 9 Tris-HCl) for 1 h.

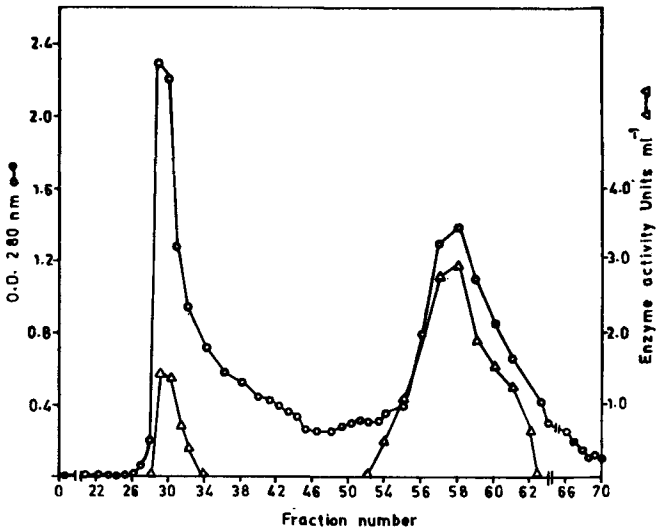

FIG. 2. Elution profile of lipase in Sephadex G-25 chromatography. The active eluate from Sephadex G-50 was concentrated by ammonium sulphate precipitation and loaded on Sephadex G-25 column (70 cm × 1.4 cm); 1.5-mL fractions were collected at a flow rate of 6 mL h^{-1}.

optimally active at pH 7.0 and at 30°C (Fig. 3). The homogeneity of the enzyme preparation could not be further substantiated after G-25 chromatography either by polyacrylamide gel electrophoresis (PAGE) or by ultracentrifugation. In PAGE, the protein could not be stained with silver or Coomassie stains despite loading high levels (480 μg) of protein (determined by the procedure of Lowry *et al.* [5]). By gel filtration, the relative molecular mass of the enzyme was indicated as 8,000 daltons (Fig. 4). The purified enzyme was capable of hydrolyzing the oils of groundnut, olive, castor, palm and coconut (Table 2), of which the palm oil was found to be hydrolyzed with maximum efficiency.

The effect of different concentrations of tributyrin as well as *p*-nitrophenyl acetate was examined and revealed substrate inhibition due to both compounds. The K_m for the former substrate was calculated as 4.16 mM tributyrin with a corresponding V_{max} of 12.35 μM butyric acid per min per mg protein (Fig. 5). For *p*-nitrophenyl acetate, the calculated K_m was 0.045 mM and V_{max} was 2.5 μM of *p*-nitrophenol per min per mg protein (Fig. 6).

The effect of various metal ions and functional group reagents on lipase activity is indicated in Table 3. Ni^{2+}, Mg^{2+} and Ba^{2+} caused significant inhibition of the enzyme while Cu^{2+} caused only slight inhibition, and Hg^{2+} at the level tested (1×10^{-3}M) did not cause any inhibition. The polar detergent SDS stimulated enzyme activity while the nonpolar nonidet P-40 was inhibitory. The indole reagent N-bromosuccinamide was found to stimulate enzyme activity.

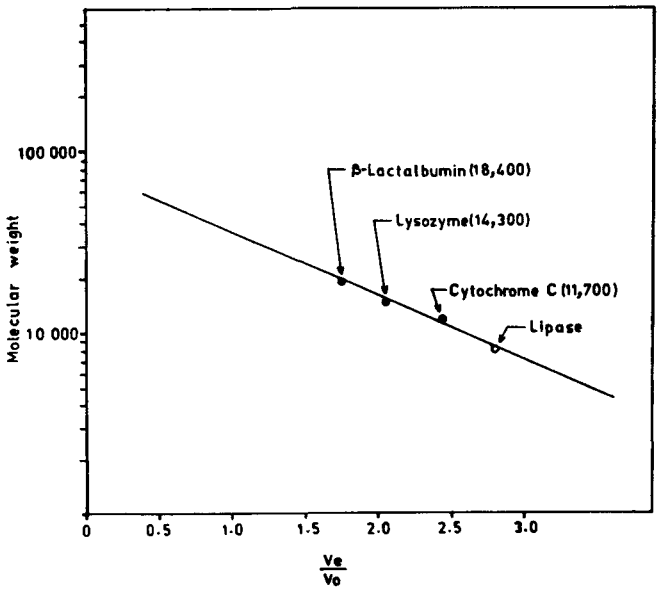

FIG. 4. Molecular weight determination by gel filtration (Sephadex G-100).

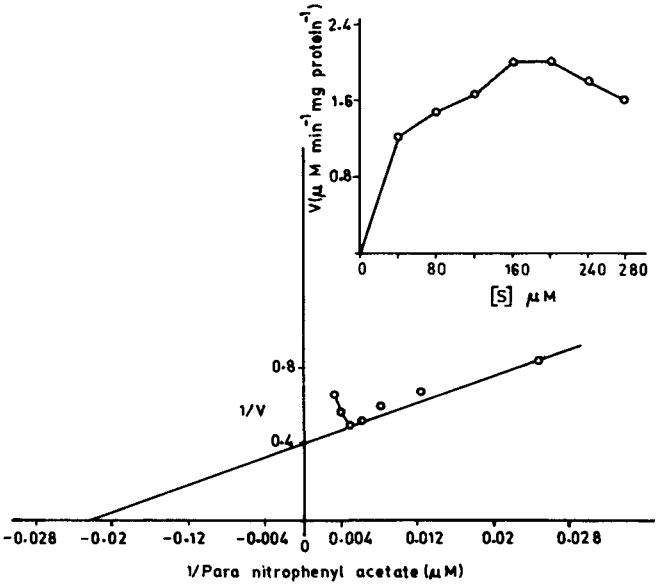

FIG. 6. Influence of *p*-nitrophenyl acetate concentration on initial velocity of lipase (Lineweaver-Burk plot; insert: Michaelis-Menton plot).

TABLE 2

Lipase Activity with Different Oils as Substrate Taking Tributyrin as Standard[a]

Oil	Lipase relative activity (%)
Tributyrin	100.00
Groundnut oil	88.23
Olive oil	91.18
Castor oil	98.50
Palm oil	136.40
Coconut oil	117.65

[a]Substrate emulsion contained 100 mg of oil prepared in 1% gum acacia in 20 mM phosphate buffer pH 7.0 and assayed at 30°C. The specific activity of the enzyme was 8.26 μmoles min^{-1} mg $protein^{-1}$ with respect to tributyrin.

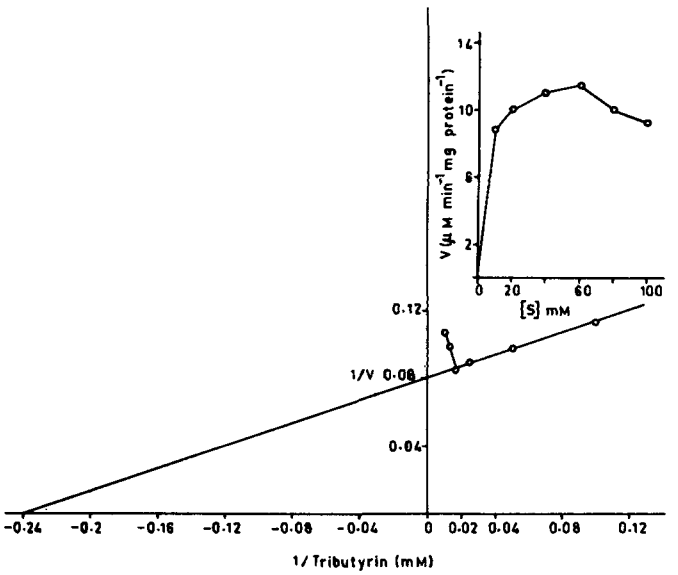

FIG. 5. Influence of tributyrin concentration on initial velocity of lipase (Lineweaver-Burk plot; insert: Michaelis-Menton plot).

TABLE 3

Effect of Activators and Inhibitors on Lipase Activity[a]

Effectors	Lipase relative activity (%)
Control	100.00
Calcium chloride	106.50
Cobalt chloride	106.20
Potassium chloride	110.70
Cupric chloride	93.93
Barium chloride	83.14
Nickel chloride	60.046
Magnesium chloride	67.667
Manganese chloride	101.62
Mercuric chloride	100.00
Ferrous sulphate	107.85
Ethylenediaminetetraacetic acid	75.29
Nonidet P-40[b]	84.68
Sodium dodecyl sulfate[b]	113.93
Iodoacetamide	95.30
N-bromosuccinamide	121.30
N-acetyl imidazole	89.60
Parachloromercuribenzoic acid	110.89

[a]The assay was carried out at 30°C as described in Methods with tributyrin as substrate. The specific activity of the enzyme was 8.26 μmole min^{-1} mg $protein^{-1}$.
[b]0.4%, Other compounds, 1×10^{-3} M.

DISCUSSION

This is perhaps the first detailed study on purified lipase excreted by a lipolytic microorganism involved in the anaerobic digestion of organic matter. However, some information on the lipases produced by *Anaerovibrio lipolytica*, a rumen organism and *Propionibacterium acnes* from the human skin is available. TPW was found to contain nearly 25% lipid. Therefore, lipases can be expected to play an important role in the overall anaerobic digestion process in this particular organic waste.

An unusual feature of the lipase under study was its

small size, with a molecular mass of 8,000 as determined by gel filtration. The molecular mass of lipase produced by *P. acnes* was reported to be 46,770 (8). Another unexplained feature of the lipase studied in the present work was the difficulty to stain it in electrophoresis gels with conventional staining reagents (Coomassie blue, silver stains). Because the protein could be measured with phenol reagent, it was suspected that it has an unusually high content of tyrosine and tryptophan and perhaps of cysteine, cystine and histidine as well (9). It became difficult, therefore, to determine the homogeneity of the enzyme preparation by using the electrophoresis technique, mainly because the protein could not be stained, despite the fact that a fairly high amount of protein (as determined by phenol reagent) was loaded on the gel, which had a high crosslinking (20% polyacrylamide) to resolve small-sized proteins. However, based on the elution profile of the highly purified enzyme in Sephadex G-25, it appeared that the preparation was homogeneous. Yet another unusual feature was the ineffectiveness of Hg^{2+} (at 1×10^{-3}M) to inhibit the enzyme and stimulation of enzyme activity by the sulfhydryl reagent, *para*-chloro-mercuribenzoate. This could mean that the masking or inactivation of cysteine residues in the enzyme protein in some way could cause enhancement of enzyme activity. This may have been due to reduction in polarity and an increase in hydrophobicity of the enzyme protein. During the attempts to purify the enzyme, we found it incapable of binding to ion-exchange resins at various pH values, which suggests an already existing overall hydrophobicity in the protein, which is to be expected of enzymes that act on hydrophobic substrates such as lipids. Furthermore, we also found the present lipase a poor elicitor of antibody response in rabbits.

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