Purification and Properties of a Glycerol Ester Hydrolase (Lipase) from *Propionibacterium shermanii*

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Received for publication 12 March 1970

An intracellular glycerol ester hydrolase (lipase) from *Propionibacterium shermanii* was recovered from cell-free extracts and purified by ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography on diethylaminoethyl-cellulose. Maximum enzyme activity was observed at pH 7.2 and 47°C when an emulsion of tributyrin was used as substrate. The enzyme was stable between pH 5.5 and 8. Heating the enzyme solution at 45°C for 10 min resulted in a 75% decrease in activity. Maximum rate of hydrolysis of triglycerides was observed on tripalmitin, followed in order by tributyrin, tricaprin, and tricaprylin. The lipase was strongly inhibited by mercury and arsenicals, but specific sulfhydryl reagents had little or no inhibiting effect on the enzyme activity. The enzyme also showed some esterase activity, but the hydrolysis of substrates in solution was small as compared to the hydrolysis of substrates in emulsion.

The formation of monocarboxylic acids from carbohydrates by the propionic acid bacteria has been extensively studied, and the pathway is known to contain a number of enzymes, many of which have been purified and characterized (2). The formation of monocarboxylic acids from triglycerides by propionic acid bacteria, however, has not been investigated. In another report (Oterholm, Ordal, and Witter, J. Dairy Sci., in press), we presented evidence that *Propionibacterium shermanii* possesses a glycerol ester hydrolase (EC 3.1.1.3) which forms monocarboxylic acids from triglycerides as the substrate. The present work is concerned with the purification and characterization of this enzyme in an effort to obtain further information of microbial lipases in general and of the glycerol ester hydrolyzing properties of the propionic acid bacteria in particular.

MATERIALS AND METHODS

**Organism and cultural conditions.** The strain of *P. shermanii* used in this work was obtained from Chr. Hansen’s Laboratory, Inc., Milwaukee, Wis. The organism was routinely grown in 5-liter batches as stationary culture at 30°C for 38 to 40 hr in a medium containing 5 g of yeast extract (Difco), 20 g of Trypticase, and 5 g of glucose per liter (20). Stock cultures and cultures for daily use were prepared as previously described (20), with the above broth as the basal medium.

**Preparation of cell-free extract.** After harvesting by centrifugation, the cells were washed three times in 0.01 M ammonium chloride buffer and resuspended in 200 ml of the same buffer. Cell-free extract was then prepared as described by Oterholm, Ordal, and Witter (20). The protein content of the cell-free extract was determined by the method of Lowry et al. (16) with crystalline serum albumin as the standard.

**Lipase assay.** The glycerol ester hydrolase (lipase) activity of the cell-free extract was determined by measuring the initial rate of hydrolysis by continuous titration of the liberated acids with 0.1 N CO2-free KOH by using an automatic recording pH-stat (E. H. Sargent & Co., Chicago, Ill.). Controls containing boiled enzyme were similarly titrated. An emulsion of tributyrin in 10% gum arabic (5) was routinely used as the substrate. The assays were conducted at 35°C and at pH 7.2. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of one nano-equivalent of acid per minute. The simple triglycerides, esters of fatty acids and aromatic esters, used as substrates were all reagent grade commercial preparations. Emulsions of the various substrates were prepared by ultrasonic treatment by the procedure described for the assay of lipase from the lactic acid bacteria (20).

**Purification procedure.** Pulverized ammonium sulfate (Mallinckrodt Chemical Works, St. Louis, Mo.) was slowly added to the crude cell-free extract with constant stirring. During this step, a pH of 6.8 was maintained. Material which precipitated between
0.30 and 0.50 saturation after standing overnight at 4°C contained most of the glycerol ester hydrolase activity. The precipitate was collected by centrifugation and resuspended in distilled water. The salts remaining in the enzyme-protein solution after ammonium sulfate fractionation were removed by gel filtration by using Sephadex G-25 as described by Flodin (8). The packed column (2.5 by 3.5 cm) was equilibrated with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.2) before desalting.

Further purification of the enzyme was obtained by ion-exchange chromatography. Type 40 diethylaminoethyl (DEAE)-cellulose (Schleicher and Schuell Co., Keene, N.H.) was prepared by the procedure of Peterson and Sober (21). The DEAE-cellulose column (1.5 by 2.5 cm) was equilibrated with 0.01 M Tris-hydrochloride buffer (pH 7.2) by slowly percolating large volumes of the buffer through the packed material. A sample of the desalted-enzyme preparation was applied to the column, and the protein was eluted by a linear gradient of NaCl obtained by using 270 ml of 0.6 M NaCl in 0.01 M Tris-hydrochloride buffer (pH 7.2) in the reservoir and 270 ml of the same buffer in the mixing chamber. The column was connected to an automatic fraction collector, and fractions of 5 ml were collected and analyzed for protein and glycerol ester hydrolase activity. The flow rate was controlled at 0.5 ml/min by a Beckman Solution Metering Pump (model 746). The active fractions were pooled and dialyzed against 50 volumes of 0.01 M Tris-hydrochloride buffer (pH 7.2) for 30 hr with one change of buffer. The enzyme preparation was concentrated by using coarse beads of Sephadex G-25. A weighed and precooled amount of Sephadex was added to the protein solution under stirring and allowed to swell for 25 to 30 min. The swollen gel grains were then removed by centrifugation with modified centrifuge tubes (Occomy Associates, Chicago, Ill.). This concentrated enzyme preparation was stored at 0°C and used in all of the subsequent work on the properties of the enzyme.

Effect of pH. The effect of hydrogen ion concentration on enzyme activity was determined by measuring the initial velocity of the reaction at various pH values simply by changing the setting of the pH-stat. The effect of pH on enzyme stability was studied by exposing the enzyme to different pH values for 5 min at 35°C and then measuring the remaining activity after readjusting the solution to pH 7.2 (6).

Effect of temperature. The effect of temperature on both enzyme activity and enzyme stability was determined. The influence of temperature on lipase activity was studied over the range of 20 to 50°C by measuring the reaction rate at pH 7.2. The various temperatures were obtained by changing the setting of the pH-stat. Data were collected at 5-degree increments, and the activation energy was calculated from the linear portion of an Arrhenius plot of log k versus 1/T (where k is the initial velocity and T the absolute temperature). The effect of temperature on the stability of the enzyme was studied over a range of 35 to 47.5°C at pH 7.2 as follows. A 0.15-ml amount of the enzyme solution was added to 9.85 ml of distilled water which had been previously equilibrated at the appropriate test temperature. The diluted enzyme solution was kept at the temperature being tested for 10 min. At the end of this incubation period, the solution was brought to the assay temperature of 35°C by addition of 2 ml of an ice-cold tributyrin emulsion, and the activity was determined by continuous titration of the liberated acids.

Michaelis constant. The relation between substrate concentration and enzyme activity was obtained by determining the reaction rate on various concentrations of tributyrin emulsion at pH 7.2 and 35°C. The Michaelis constant, K_M, for the enzyme was determined by the double-reciprocal plot method of Lineweaver and Burk (15).

RESULTS AND DISCUSSION

The scheme of treatment used for the purification of glycerol ester hydrolase from Propionibacterium shermanii is given in Table 1. The specific activity of the purified enzyme was 141-fold over that of the starting cell-free extract, and the overall yield was about 44%.

The lipase protein was eluted from the DEAE-cellulose column at a concentration gradient ranging from 0.32 to 0.37 M NaCl and was contained in the very last portion of the eluted proteins. An attempt to purify further the enzyme by gel filtration on Sephadex G-75 was not successful, because the lipase activity was found in the major protein peak which came off the column with the void volume. This indicated, however, that the lipase protein was a relatively large molecule since the molecular weight exclusion limit of Sephadex G-75 is approximately 50,000.

The rate of hydrolysis of the tributyrin emulsion obeys zero-order kinetics as shown in Fig. 1.

| Treatment | Total protein mg | Total activity units | Spec. activity* | Yield % |
|-----------|-----------------|---------------------|----------------|--------|
| Cell-free extract | 2,167 | 420,000 | 193 | 100 |
| (NH₄)₂SO₄ | 798 | 331,000 | 415 | 78.8 |
| Sephadex G25 | 684 | 299,200 | 437 | 71.2 |
| DEAE-cellulose | 6.85 | 187,500 | 27,300 | 44.6 |

* Reaction mixture contained 58 mmoles of tributyrin sonically dispersed in 10% aqueous gum arabic, water, and enzyme in a total volume of 6 ml. Rate of hydrolysis was determined at pH 7.2 and at 35°C by continuous titration of the liberated acids with a pH-stat.

* Expressed as units per milligram of protein.

* One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of one nano-equivalent of acid per minute.
This linear relationship continued for about 20 min, after which a slow but steady decrease in the rate of hydrolysis was noted. This supported the findings of Desnuelle (4) that hydrolysis slows down relatively early and that assay systems based on long incubation times may lead to inaccurate results.

A linear relationship was also evident when enzyme activity was measured as a function of protein concentration (Fig. 2). The same direct proportionality was also found when crude extract was used as the source of enzyme.

The effect of the hydrogen ion concentration on the activity of the enzyme (Fig. 3) shows that the lipase had a rather broad pH optimum with maximum activity in the region of pH 7.2. This was in agreement with the general findings that most bacterial lipases have their optimum activity at a neutral or slightly alkaline pH (1, 13, 17, 23, 24). The fairly rapid decline in the reaction velocity on either side of pH 6 and 8 may be due to a decreased saturation of the enzyme with substrate because of a decreased stability of the enzyme, or a combination of the two. These effects can be distinguished experimentally by exposing the enzyme to a range of pH values and then testing the activity after readjusting the pH to a standard value. The results of such an experiment are shown in Fig. 4. Apparently, the fall on the alkaline side of pH 8 is primarily due to destruction of the enzyme, whereas the fall on the acid side of pH 6 is partly due to a decreasing affinity of the lipase for its substrate and partly due to an irreversible destruction of the enzyme protein.

The majority of microbial lipases have been reported to be most active within a temperature...
The lipase of *P. shermanii* was an exception to this as it had an apparent optimum activity at 47 C (Fig. 5). At temperatures as high as about 45 C, the main effect of heat was an increase in the reaction rate. Above 45 C, the thermal inactivation of the enzyme increased, and a rapid decrease in the activity of the enzyme was experienced.

The rather marked difference between the optimal temperature of the lipase of *P. shermanii* and most other lipases was probably due to differences in the assay conditions used. Many workers have based their temperature-activity studies on the relatively long incubation periods necessitated by most lipase assay systems, rather than on the initial velocity as reported here. Since thermal destruction of an enzyme is progressive, the shape of the curve and the apparent optimum temperature depend on time and would cause the optimum temperature to fall as the time interval was increased.

The activation energy for the lipase-catalyzed reaction was obtained from the linear portion of an Arrhenius plot of the log of the reaction rate against the reciprocal of the absolute temperature and was found to be 8,100 cal/mole. The corresponding temperature coefficient, Q10, was 1.6. Activation energies of 7,000 and 8,500 cal/mole given by Sizer and Josephson (26) and Schwartz (24), respectively, for the action of pancreatic lipase on tributyrin are not much different from the value obtained for the lipase of *P. shermanii* on the same substrate. A somewhat higher value, 11,800 cal/mole, was reported by Shah and Wilson (25) for *Staphylococcus aureus* on trilin as the substrate, whereas Kvamme, Clagett, and Treumann (14) found the energy of activation of wheat germ lipase to be 8,000 cal/mole. The activation energies which hitherto have been reported for bacterial lipases appear to be of the same order of magnitude as that of *P. shermanii*.

The procedure used for studying the thermal inactivation of the lipase (see above) should eliminate the inactivation resulting from the time necessary to heat or cool the enzyme solution to the given temperatures. The inactivation of *P. shermanii* lipase by exposure to various temperatures is given in Table 2. After 10 min at 35 C in the absence of substrate, there was some loss in activity. This loss increased to greater than 80% after 10 min at 47.5 C. Apparently, the lipase of *P. shermanii* does not belong to the group of markedly thermostable bacterial lipases found in *Pseudomonas fragi* (17), *Achromobacter lipolyticum* (18), *Pseudomonas mucidolens* (22), and others. The lipase of *P. shermanii*, however, was much more stable in the presence of its substrate than it was in aqueous solution. The enzyme lost only 12% of its activity in 10 min at 47.5 in the presence of an emulsion of tributyrin as compared to a loss of 82% in aqueous solution at the same temperature. Exactly the opposite effect has been reported for a "tributyrinase" from wheat germ (23), whereas the thermostability of the lipase of *P. fragi* appears to be increased in the presence of fat or protein in the medium (17).

For the action of pancreatic lipase, Desnuelle (4) has shown that the interfacial area is more
TABLE 2. Thermal stability of the lipasea

| Temp C | Activity units | Relative activity % |
|--------|----------------|---------------------|
| 35.0   | 278            | 85.8                |
| 37.5   | 267            | 82.4                |
| 40.0   | 189            | 58.3                |
| 42.5   | 145            | 44.7                |
| 45.0   | 83             | 25.6                |
| 47.5   | 58             | 17.9                |

a An 8.95-ml amount of distilled water was equilibrated at the respective temperatures followed by the addition of 0.15 ml of the enzyme solution. After 10 min of incubation at the appropriate temperatures, the enzyme solution was brought to the assay temperature, 35 C, by the addition of 2 ml of an ice-cold tributyrin emulsion, and the remaining activity was determined by continuous titration of the liberated acids at pH 7.2.

b No decrease in the reaction rate was observed when the enzyme was incubated for 10 min at 35 C and at pH 7.2 in the presence of substrate, and the activity under these conditions was taken as 100%.

Table 3. Ratio of esterase activity to lipase activity during purification

| Purification procedure | Specific activity | Ratio of esterase activity to lipase activity |
|------------------------|-------------------|---------------------------------------------|
| Crude extract . . . .  | 193               | 0.048                                       |
| (NH₄)₂SO₄ fractionation | 415               | 0.038                                       |
| Desalting by Sephadex G-25 | 437              | 0.045                                       |
| DEAE-cellulose chromatography . . . | 27,300 | 0.048                                       |

a Assay conditions were those described in footnote a of Table 1.

b Esterase assay was performed in 0.2 M aqueous solution of triacetin. Rate of hydrolysis was determined at pH 7.2 and at 35 C by continuous titration of the liberated acids with a pH-stat.

d Enzyme was prepared at tributyrin emulsion under the conditions described in footnote a of Table 1 and was taken as 100%.

and lipase activity is presented in Fig. 6. From the inserted double reciprocal plot of reaction rate versus tributyrin concentration, the Kₘ value was calculated to be 2 × 10⁻⁴ M. This value is somewhat larger than the Kₘ of 6 × 10⁻⁴ M found for pancreatic lipase (27).

As indicated in another report (Oterholm, Ordal, and Witter, J. Dairy Sci., in press), the crude extract of P. shermanii exhibited small amounts of esterase activity when assayed on a typical esterase substrate such as triacetin in solution. The various preparations obtained during purification were also found to contain a weak esterase activity. However, the ratio of esterase

![Graph](https://example.com/graph.png)

**Fig. 6. Effect of tributyrin concentration on the reaction rate of lipase.** Except for the changes in the concentration of tributyrin, the assay conditions were those described under Fig. 1. Kₘ (2.0 × 10⁻⁴ M) was calculated from these data.

important than the substrate concentration per se. Therefore, when evaluating the effect of substrate concentration on enzyme activity, all different concentrations were made from the same 10% tributyrin emulsion. When the substrate concentration in the reaction mixture is increased, the interfacial area should increase proportionally. No attempt was made, however, to define the Michaelis constant in terms of interfacial area.

The relation between substrate concentration
P-Hydroxymercuri-
inemulsion
to
drolysis
acetinin
whereas
rate
footnote
sharp
activity
activity
activity
ase
TABLE
VOL.
CaC12
100
Na2HAsO4
Tributyrin
Tripropionin.
TABLE
NaCl
100.0
2.86 \times 10^{-2} 
8.30 \times 10^{-2} 
1.67 \times 10^{-4} 
1.67 \times 10^{-4} 
8.3 \times 10^{-2} 
4.44 \times 10^{-4} 
4.44 \times 10^{-4} 
5.0 \times 10^{-2} 
2.7 \times 10^{-2} 
% 
96 
79 
0 
0 
52 
100 
85 
55 
68 

a Assay conditions were those described in footnote a of Table 1.

b Activity on tripropionin was taken as 100%.

c Data on pancreatic lipase (29).

TABLE 6. Effect of enzyme inhibitors on the activity of the lipase

| Compound | Concen | Activity |
|----------|--------|----------|
| None     |        | 100      |
| NaF      |        |          |
| NaH2AsO4 |        |          |
| HgCl2    |        |          |
| CaCl2    |        |          |
| Iodoacetate |      |          |
| p-Hydroxymercuribenzoate | |          |
| Ethylenediaminetetraacetate | |          |
| Sodium taurocholate | |          |

a Assay conditions were those described in footnote a of Table 1 after addition of the enzyme to the reaction mixture containing the inhibitor.

activity to lipase activity was almost constant throughout purification, indicating that the esterase activity was due to an activity of the lipase itself (Table 3).

To evaluate further the dual esterase and lipase activity of the glycerol ester hydrolase, the enzyme activity toward substrates in solution as well as in emulsion was determined. The rates of hydrolysis given in Table 4 are all relative to the rate of the enzyme activity on an emulsion of tributyrin. The results show that no activity was found at the lowest concentration of triacetin, whereas 0.5 saturation gave the same slight activity found in the cell-free extract and partially purified preparations. Moreover, a relatively sharp increase in the reaction rate was evident when triacetin in solution was changed to triacetin in emulsion. The activity on methylbutyrate was the same as that observed during purification, whereas no activity was found on such typical esterase substrates as \(\alpha\)-naphthyl acetate, Tween 60, and Tween 80. These results were evidence that the enzyme from \(P.\) \textit{shermanii} preferentially hydrolyzed substrates in emulsion and did not act, or acted very slowly, on substrates in solution. It was therefore concluded that the glycerol-ester-hydrolyzing enzyme from \(P.\) \textit{shermanii} was a lipase and that the small esterase activity towards substrates in solution was due to an activity of the lipase itself. Previously presented kinetic studies were further evidence for this conclusion. Similar dual substrate properties have been reported for many other microbial lipases, including the crystalline lipase of \textit{Aspergillus niger} (12) and the purified lipase of \textit{Rhizopus delemar} (9) and \(P.\) \textit{fragi} (17).

Wills (29) was one of the first investigators to observe that mammalian lipases preferentially hydrolyze triglycerides of short chain fatty acids, notably tripropionin and tributyrin. With the apparent exception of the lipase from \(P.\) \textit{fragi} (17), microbial lipases also preferentially hydrolyze triglycerides of short-chain fatty acids.

The substrate specificity of the lipase of \(P.\) \textit{shermanii} (Table 5) corresponded to other microbial lipases (7, 11, 23, 25) by showing a maximum rate of hydrolysis toward tripropionin and a decreasing rate with increasing fatty acid chain length.

The effect of selected inhibitors on the activity of the lipase of \(P.\) \textit{shermanii} is given in Table 6. In common with many hydrolytic enzymes (3, 28), this lipase was strongly inhibited by mercuric ions and arsenate. Inhibition by heavy metals and arsenate suggested the possible participation of sulfhydryl groups in the active sites of enzyme activity. This suggestion was negated, however, by the lack of or very little inhibition caused by the more specific sulfhydryl inhibitors of \(p\)-hydroxymercuribenzoate and iodoacetate. Since the rate at which lipases hydrolyze triglycerides is dependent upon the interfacial area between the emulsified substrate and soluble enzyme, agents which reduce this area may act as inhibitors even though they do not combine with the enzyme itself. Inhibition of lipolytic activity by the surface-active agents ethylenediaminetetraacetate and sodium taurocholate may act in this manner. The sensitivity of this lipase to calcium ions differs from some other microbial lipases which have been shown to be activated by equivalent concentrations (12, 27, 30), but generally the pattern of inhibition of \(P.\) \textit{shermanii} lipase is similar to that of other microbial lipases.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Training Grant FD 00-004.
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