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CHARACTERIZATION AND PARTIAL PURIFICATION OF A LIPASE FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY

1. An extracellular lipase (glycerol ester hydrolase, EC 3.1.1.3) has been isolated from the culture medium of *Pseudomonas aeruginosa*. The bacterium also produces a similar, though lower, cell-bound activity.

2. The exo-enzyme has an optimal pH of 8.9, shows maximal activity at 40°, and attacks emulsified mono-, di- and triglycerides.

3. Thermal inactivation experiments indicate that hydrolysis of glycerides is due to a single, unspecific enzyme.

4. The enzyme is inhibited by protamine and sodium taurocholate but is not affected by NaCl, heparin, sulfhydryl group inhibitors or 10 μM eserine.

5. An approx. 35-fold purification has been achieved by filtration through Sephadex G-200.

6. Disk electrophoresis separates the purified enzyme into two protein fractions, only one of which has lipolytic activity.

7. The purified enzyme is capable of rapidly degrading the lipoprotein fractions of human serum.

INTRODUCTION

Lipolysis seems to be a widespread characteristic among microorganisms. Two recent studies carried out on almost 100 strains of bacteria, both Gram-positive and Gram-negative[1,2], indicated that all produced diffusible lipases in some degree. Another survey[3], conducted on 234 strains of Gram-negative bacteria isolated from seawater or marine sediments, showed that almost 90% of the organisms possessed lipolytic activity. However, few enzymes of this type have been fully characterized, and almost nothing is known about their metabolic or physiological significance.

In the course of a study on human lipoprotein lipase, an agar plate with an inclusion of coconut oil[4] was accidentally contaminated by a strain of *Pseudomonas*

aeruginosa. The area of contamination became evident by a clearing zone of the lipid material. The production of lipases by this species has been mentioned in a report on esterases by SIERRA[5] and in a paper by NASHIF AND NELSON[6].

EXPERIMENTAL

Cultures and production of enzyme

P. aeruginosa, Strain 10145, obtained from the American Type Culture Collection, was maintained on a liquid medium containing 2% Trypticase (BBL), 0.08% Ediol and 0.5% glucose, in 0.1 M phosphate buffer (pH 7.6).

Lipolytic enzyme was produced in a semisolid medium prepared by adding Ionagar No. 2 (Oxoid, England) to the above liquid medium to a final concentration of 0.5%. The semisolid medium was stored in 200-ml bottles each containing 45 ml. For use they were heated in a boiling-water bath, to melt the agar, then cooled to 50–55° and inoculated with 2 ml each of a 24-h liquid culture of *P. aeruginosa*. After being well mixed, the contents of each bottle were poured into a 15-cm-diameter petri dish and incubated at 28° for 24 h. At the end of this time the petri dishes were put in a freezer at −20° for a few hours and then left to thaw at room temperature, in order to favor exudation of the liquid containing the enzyme. This liquid was filtered through a nylon stocking to remove the agar, and the filtrate was centrifuged at 10 000 × *g* for 1 h at 4°. All further operations were performed in the cold. The supernate was stored at −20° till further use. A flocculent precipitate, which appeared when this fraction was thawed after storage, was separated by centrifuging for 30 min at 7200 × *g*. The clear supernate ("crude enzyme") contained 4–8 mg of protein/ml. The specific activity varied between 0.5 and 1.0 μequiv of free fatty acids liberated per h per mg protein, in the conditions of the standard assay.

The crude enzyme could be lyophilized with little loss of activity. The powder was redissolved in distilled water, immediately before use, to a concentration of 30–40 mg protein per ml ("lyophilized crude enzyme"). About 70% of this protein, as measured by the method of LOWRY *et al.*[7] with bovine serum albumin as standard, was dialyzable.

Standard enzyme assay

The reaction mixture consisted of 0.2 ml of a 5% Ediol emulsion, 0.2 ml of a 10% bovine serum albumin solution (Cohn Fraction V, Armour), 0.60 ml of 0.2 M sodium phosphate buffer (pH 7.9) and 1.0 ml of crude enzyme or 0.1 ml of lyophilized crude enzyme, in a final volume of 2.0 ml. It was incubated for 1 h at 37°, after which the free fatty acids were extracted and measured by the method of Dole, slightly modified according to TROUT *et al.*[8]. Blanks were prepared by heating the enzyme preparation for 10 min in a boiling-water bath before incubation. All assays were carried out in duplicate. Results are expressed as μequiv of free fatty acids liberated per ml of the reaction mixture per h. One unit of activity was defined as that amount which liberates 1.0 μequiv of free fatty acids in 1 h.

Other assays

When tributyrin was used as substrate, the butyric acid liberated was measured titrimetrically with 0.10 M NaOH, using phenolphthalein as indicator.

Fatty acids liberated from all other substrates were extracted and measured by the Dole method.

Electrophoresis on plates of agar gel with inclusion of the enzymic substrate

It has been observed by FINKELSTEIN *et al.*[4] that the properties of agar gel plates as a support medium for electrophoresis of serum proteins and lipids are not modified by inclusion in the gel of a lipidic substrate. This affords a very sensitive method for detection of lipases.

Agar–Ediol (100 ml) contained 1.2 g of Ionagar No. 2, 97.9 ml of sodium barbital buffer ($I = 0.05$), 1 ml of a 1:1000 merthiolate solution and 1.1 ml of a sonified 3.6% Ediol emulsion in water. For agar–tributyrin, Ediol was replaced by 1.7 ml of a 5% tributyrin emulsion in water.

Runs were performed at a voltage gradient of 5 V/cm for 3–4 h. Plates were then incubated at room temperature for 24–48 h and stained for lipids. The presence of lipolytic activity was denoted by a clearing zone where the substrate had been hydrolyzed; this zone did not take up the dye. Lipids present in sera were shown up by increased staining over the background.

Microimmunoelectrophoresis was performed according to GRABAR[9] and staining for lipids and proteins following URIEL[10].

Sephadex gel filtration

Lyophilized crude enzyme (3 ml) was applied to a 1.8 cm × 40 cm Sephadex G-200 column fitted with a syphon to reduce the hydrostatic pressure, and previously washed with 0.05 sodium phosphate buffer (pH 7.9). Elution was performed with the same buffer at a velocity of 0.3 ml/min. Fractions of 3 ml were collected. Blue dextran (mol. wt. 500 000) was used to determine the void volume of the gel.

Disk electrophoresis

Analytical electrophoretic runs on 7.0% acrylamide gel were performed according to the technique of DAVIS[11], using 0.8 mm × 60 mm tubes. Samples (200 μg of protein) were loaded in 40% (w/v) sucrose solution with a small quantity of bromophenol blue as marker.

Substrates

Ediol (SchenLabs Pharmaceuticals) and Lipomul (Upjohn) are emulsions containing 50% coconut oil and 66% corn oil by weight, respectively. Other glycerides were obtained commercially. Emulsions of water-insoluble substrates were prepared by homogenizing 250 mg of the appropriate glyceride with 10 mg of Tween 60 and 5 ml of water for 2 min in a Branson sonifier with an output of 30 W, immediately before use. Both Ediol and Lipomul were subjected to sonication in the same conditions, since the enzyme was very sensitive to the degree of dispersion of the substrate (use of sonified Ediol could more than double the activity obtained with an untreated emulsion). Tween 20 and Tween 60 were diluted with water to the desired concentration.

RESULTS

Enzyme content at different stages of growth

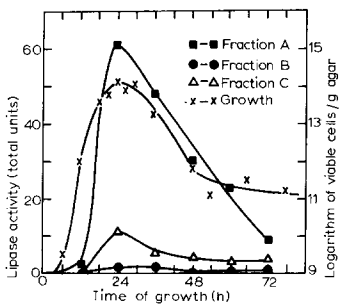
P. aeruginosa from a 24-h liquid culture was used to innoculate eighteen petri dishes containing a semi-solid medium. This technique was adopted because production of lipolytic activity was consistently higher in semi-solid than in liquid media, even when very shallow layers of the latter were used.

Every 6 or 12 h after inoculation three of the dishes were removed from incubation and an equal section of each agar disk was cut out. The three pieces were weighed together and gently homogenized in a glass Potter, with saline. An aliquot of the resulting suspension was used to estimate the number of viable cells per g agar, by effecting a colony count, using the pour plate technique[12]. The agar remaining in each petri dish was frozen, thawed and filtered as described in EXPERIMENTAL, and the liquid was pooled before centrifuging for 1 h at 10 000 × *g*. The supernate (Fraction A) was separated, and the pellet of bacteria was washed twice with 1 ml of saline. The washings were pooled (Fraction B) The pellet was resuspended in 0.5 ml saline (Fraction C). Suitable aliquots of each fraction were incubated in the conditions of the standard assay.

The results of a typical experiment are shown in Fig. 1. As can be seen, lipolytic activity appeared in the extracellular medium about 6 h after the onset of the logarithmic phase and increased steadily with the viable count. Both parameters reached a maximum after 24 h of growth. At this time lipolysis could also be measured in whole cells, though it was less than 20% of the total activity produced by the bacteria. This fraction was not removed by two successive washes with saline.

Both the extracellular and the cell-bound enzyme were unstable upon prolonged growth of the cells, as witnessed by the rapid decline in activity after 24 h.

On one occasion a slightly different pattern of enzymic activity was obtained. The maximum of extracellular activity appeared between 36 and 48 h, although the total amounts of enzyme produced by the bacteria and its distribution were similar to those of previous experiments. This shift in time was accompanied by an abnormally sharp decline in the viable count after 24 h of growth. When 24-h-old cells from



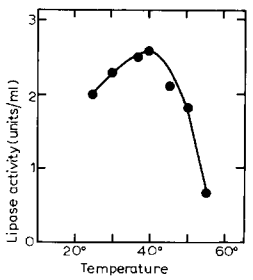


Fig. 1. Production of lipolytic activity as a function of bacterial growth. Fraction A, extracellular medium. Fraction B, pooled saline washings of whole cells. Fraction C, whole cells. Activity is expressed as total units contained in each fraction. Bacterial growth is expressed as the logarithm of the viable cells per g agar. Other experimental details are given in the text.

Fig. 2. Effect of temperature on the enzymic activity. Conditions as in the standard assay, with crude enzyme.

this experiment were ruptured by sonication there was a 4-fold increase in activity over the level which could be measured in whole organisms, whereas sonication of such cells from the experiment shown in Fig. 1 did not release any additional enzyme. Apparently, the bacteria had retained that portion of enzyme which should have been extruded, perhaps owing to a structural or metabolic anomally of this particular culture, which also affected the death rate. The enzyme was later released owing to autolysis of the cells.

All further studies were conducted on the enzyme contained in Fraction A. A flocculent precipitate which appeared when this fraction was stored in the cold was separated by centrifuging for 30 min at 7200 × *g* immediately before use.

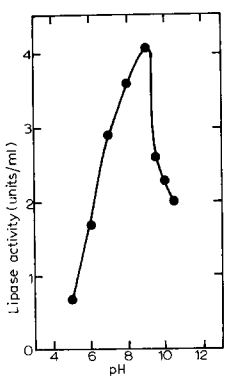
Localization of cell-bound enzyme

Cells were suspended in saline and disrupted by sonication for 5 min at 50 W. The extract was centrifuged at 1500 × *g* for 15 min, the pellet was resuspended in saline and the supernate was again centrifuged at 100 000 × *g* for 90 min. Almost 80% of the activity originally present in the sonic extract was recovered in the 1500 × *g* pellet.

Characterization of lipase

The rate of hydrolysis (free fatty acids production) of a coconut oil preparation containing a final glyceride concentration of 2.5 g/l was linear for the first 50 min and then declined slowly. The results of determining lipolysis at various temperatures are presented in Fig. 2. Measurable activity could be demonstrated between 25 and 55°, with an optimum at 40°.

When hydrolysis of Ediol by the *P. aeruginosa* enzyme was studied as a function of pH it was found that the optimum corresponded with that of the culture medium, 8.8–9.1, though activity could be detected over a much wider range (Fig. 3). Thermal inactivation at 50, 55 and 60° was studied (see Fig. 4). After 50 min heating at 50° about half the activity was lost, whereas at 60°, 3 min sufficed to produce the same inactivation.



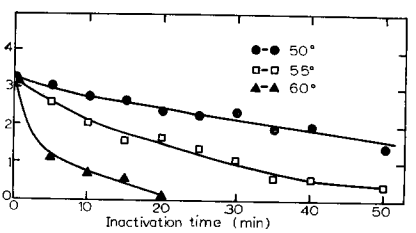
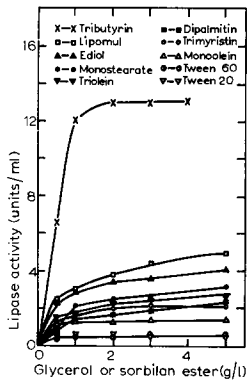


Fig. 3. Dependence of reaction rate on pH. The reaction mixture was that of the standard assay, with crude enzyme. The pH was adjusted to the values noted in the figure, by addition of 1 M HCl or 2 M NaOH. Incubation time was 20 min.

Fig. 4. Residual activity of crude enzyme after thermal inactivation. The enzyme preparation was heated in a water bath held at the appropriate temperature, for the periods of time noted in the figure, and then rapidly cooled in ice. The activity was then measured under standard conditions.



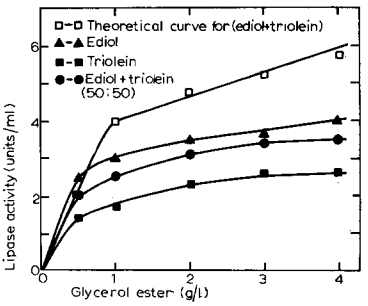


Fig. 5. Hydrolysis of glycerol and sorbitan esters by the lyophilized crude enzyme. The conditions were these of the standard assay. Substrates were prepared as described in EXPERIMENTAL.

Fig. 6. Activity of lyophilized crude enzyme on a mixture of 50% Ediol and 50% triolein (w/v). The total amount of glyceride present in the incubation mixture was equivalent to that of the standard assay in all cases.

Substrate specificity

As can be seen in Fig. 5, hydrolysis of tributyrin prodceeed at a much higher rate than that of the long-chain glycerol esters, though comparable activity was obtained with all of the latter, regardless of the nature of the fatty acid chains.

At the maximal concentration of Ediol used as substrate in our experiments, only 15 mg/100 ml of glycerol monostearate and 20 mg/100 ml of Tween 60 were present in the incubation mixture. The graphs obtained with these two compounds prove that, at such low concentrations, neither of them can contribute significantly to the production of free fatty acids from Ediol. Coconut oil must therefore be the true substrate.

Presence of one or more lipolytic activities in the enzyme preparation

The possibility that two or more enzymes were being assayed simultaneously was investigated by using a mixture composed of 50% Ediol and 50% triolein as substrate. As shown in Fig. 6, the experimental curve differs widely from that calculated for the sum of two independent activities, but the possibility could not be excluded that two enzymes were nevertheless present, each one acting preferentially on one of the substrates and partially inhibited by the presence of the other substrate.

Another experiment was performed in which the residual activity on a number of glycerides was measured after thermal inactivation of the enzymic preparation at 60°. The results shown in Fig. 7 point to the conclusion that only one enzyme is operative in the conditions of the assay, since activity is destroyed at virtually the same rate for all the substrates.

Additional evidence in support of this view is provided by electrophoretic runs performed on agar plates in which either Ediol or tributyrin had been included in the gel. Comparison of Samples 1 in Figs. 8A and 8B, shows that the pattern of migration and clarification of the glyceride emulsion was identical for both substrates. Furthermore, the same result was obtained when a purified enzyme preparation was used (Samples 2 in Figs. 8A and 8B).

TABLE I

EFFECT OF VARIOUS SUBSTANCES ON ENZYMIC ACTIVITY

The reaction mixture was that of the standard assay except for the addition of the compounds indicated. A lyophilized crude enzyme preparation was used.

Compound	*Concentration in the incubation mixture*	*Inhibition* (%)*
Protamine	100 μg/ml	25
	2000 μg/ml	50
Sodium taurocholate	1 mM	49
	5 mM	67
NaCl	1 M	0
Heparin	100 μg/ml	0
Eserine	1 mM	0
	5 mM	27
p-Hydroxymercuribenzoate	1 mM	0
Iodacetate	1 mM	0
Sodium pyrophosphate	1 mM	0
NaF	1 mM	0
	10 mM	30
EDTA	0.1 mM	25
	1 mM	60
Mercaptoethanol	1 mM	0

* Mean value of at least three separate experiments.

Effect of several substances on enzymic activity

Inhibition studies were carried out with the compounds shown in Table I. The enzyme from *P. aeruginosa* differs from lipoprotein lipase in that it is not affected by high concentrations of NaCl, nor by excess heparin[13,14], though it is inhibited 50% by protamine at 2 mg/ml. The strong inhibition produced by 0.05% sodium taurocholate distinguishes our enzyme from pancreatic lipase, which is stimulated by bile salts[15], and from lipoprotein lipase which is not affected by concentrations 10 times higher than those used in these experiments[16].

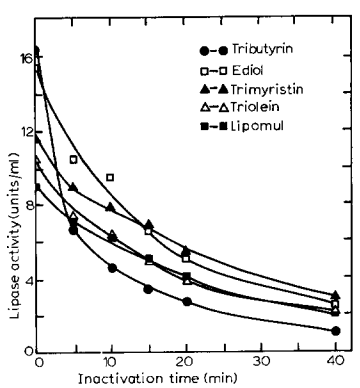


Fig. 7. Residual activity of lyophilized crude enzyme on different substrates, after inactivation at 60°. Conditions as described in the text.

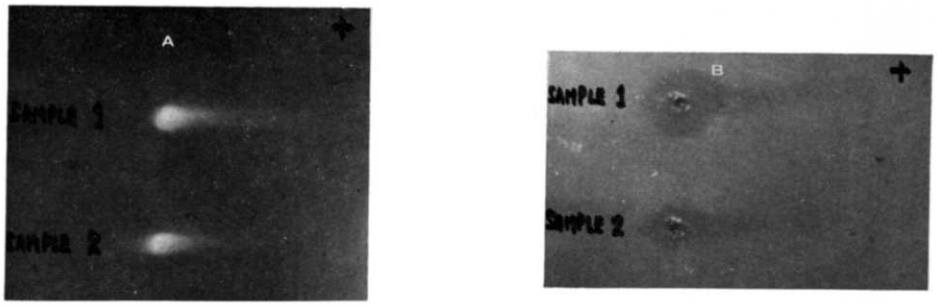


Fig. 8. Electrophoresis on agar gel with inclusion of the enzymic substrate. Plates were prepared, and incubated for 24 h at room temperature as described in EXPERIMENTAL. A. Inclusion of Ediol. Sample 1, lyophilized crude enzyme (10 μg of protein); Sample 2, Sephadex pool (0.33 μg of protein). The plate was stained for lipids. B. Inclusion of tributyrin. Samples 1 and 2 the same as in A. The plate was not stained.

The inhibitory effect of 1 mM EDTA could be almost totally reversed by addition of 5 mM $MgSO_4$. However, 2.5 mM Ca^{2+} inhibited 50% of the activity, although at lower concentrations (0.5 mM) it could substitute for albumin as an acceptor of free fatty acids. Eserine, a potent inhibitor of esterases at very low concentrations (10–0.1 μM), had no effect at concentrations below 5 mM, a concentration that inhibits other true lipases[14,17]. NaF, which has been reported to inhibit the esterases of mycobacteria[18] and a staphylococcal lipase[19], had little effect on the enzyme from this organism.

Two sulfhydryl group inhibitors, *p*-hydroxymercuribenzoate and iodoacetate, were without effect, in agreement with similar findings for the lipase of *Pseudomonas fragi*[20].

Purification of enzyme

As with other lipases, that from *P. aeruginosa* was easily adsorbed on calcium phosphate gel. However, the strong binding of the enzyme with the calcium salts rendered the method impracticable.

An alternative purification procedure was adopted. Lyophilized crude enzyme was filtered through a Sephadex G-200 column, as described in EXPERIMENTAL. A single peak with lipolytic activity was eluted almost immediately after the void volume. The active fractions were mixed (Sephadex pool). Recovery of the enzymic activity varied between 30 and 100% and purification between 15 and 60 times, in individual experiments, though the amount of protein recovered in the peak was practically the same every time. No improvement was observed when albumin (0.25%), NaCl (0.25 M), $MgSO_4$ (1 mM), or mercaptoethanol (10 mM), either singly or in various combinations, were added to the elution buffer. Recovery was considerably decreased by high concentrations of NaCl.

Stability of the enzyme

The crude enzyme was stable at room temperature for a few hours. It could be stored at −20° for over two years, and at 4° for at least a month, without loss of activity. The lyophilized crude enzyme was stable idefinitely. The enzyme eluted from Sephadex G-200 could be stored at −20° for over a month and repeatedly frozen and thawed without appreciable inactivation. However, more than 60% of the

Fig. 9. Acrylamide gel electrophoresis of the enzyme eluted from Sephadex G-200. After electrophoresis the gel was stained with 1% Amido black, destained electrophoretically, and stored in 7% acetic acid. Band I, fast-moving unidentified protein; Band II, slow-moving active enzyme.

activity was lost when it was stored overnight at 4°. It was found that addition of bovine serum albumin in a final concentration of 0.5% reduced inactivation of the Sephadex pool, during the process of lyophilization, from 50 to 25%. The dry powder (lyophilized pool) was stable for at least two months, regardless of whether albumin had been added or not.

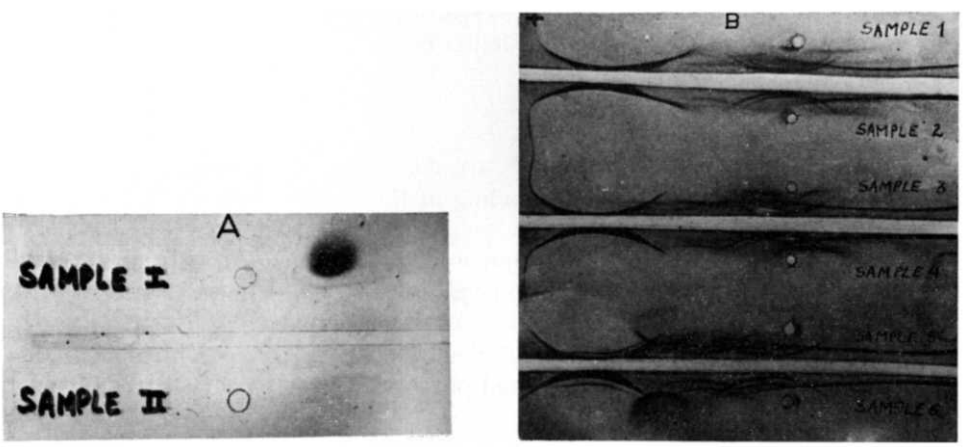


Fig. 10. Immunoelectrophoresis of normal human serum before and after incubation with lipolytic enzyme preparations. Agar plates were prepared and stained for lipids as described in EXPERIMENTAL. A. 0.1 ml serum (Sample I) and 0.1 ml serum *plus* 10 mg Sephadex pool (Sample II), were incubated for 1 h at 37°. Afterwards 3 μl of each incubation mixture were placed in the wells and subjected to electrophoresis for 2 h. Troughs were filled with 0.18 ml of rabbit anti-β-lipoprotein serum. B. Samples 1 and 2: 25 mg of acetone-precipitated enzyme preparation were dissolved in 0.5 ml of before-fat-intake plasma and after-fat-intake plasma (sample withdrawn 1 h after an intake of 250 ml of milk and 30 g of butter), respectively. Samples 3 and 4: the same as Samples 1 and 2, except that an alcohol-precipitated enzyme preparation was used. Samples 5 and 6: 0.5 ml of before-fat-intake plasma and 0.5 ml of after-fat-intake plasma, respectively. All the samples were incubated for 2 h at 37°, after which 5 μl of each was placed in the corresponding well and subjected to electrophoresis. Troughs were filled with human total anti-serum. This plate was also stained for proteins.

Acrylamide gel disk electrophoresis

During electrophoresis on acrylamide gel of a sample of lyophilized pool (without added albumin) two bands appeared (see Fig. 9). They were removed from the gel by continuing the electrophoresis and collecting fractions in a small compartment closed off by a dialysis membrane. An aliquot of each fraction was run by electrophoresis on an agar–Ediol plate. Measurable lipolytic activity was associated with the slow-moving fraction, which corresponds to Band II.

Recovery of protein from the acrylamide gel was 85%, but inactivation of the enzyme was too high to permit evaluation of the purification factor.

Effect on human serum lipoproteins

When immunoelectrophoresis was performed with a sample of normal human serum before and after incubation with different preparations of the lipolytic enzyme from *P. aeruginosa*, a profound effect was observed on the lipoprotein fractions. In Plate A, Fig. 10, the well-defined spot corresponding to β-lipoprotein (Sample I) practically disappeared after 1 h of incubation with Sephadex pool (Sample II). Two other enzyme preparations, obtained by fractionation of the crude enzyme with ammonium sulfate and precipitation with either alcohol or acetone, were incubated with plasma obtained from a subject before and after a fatty meal (see Plate B, Fig. 10). Samples 5 and 6 correspond to before-fat-intake plasma and after-fat-intake plasma, respectively. The precipitin arcs, parallel to those for albumin, correspond to α-lipoprotein, and the spots that have stained for lipid material correspond to β-lipoprotein. The latter was increased after a fatty meal. Samples 1 and 2 correspond to before-fat-intake plasma and after-fat-intake plasma incubated with the acetone precipitate, and Samples 3 and 4 to the same incubated with the alcohol precipitate. In both the disappearance of the α-lipoprotein and the large decrease in β-lipoprotein are clearly visible.

DISCUSSION

The enzyme described in this paper should be termed a lipase rather than an esterase, since it is active on water-insoluble fatty acid esters of glycerol. The very low activity on soluble substrates and the lack of inhibition by 10 μM eserine also support this criterion.

The enzyme's characteristics are similar to those of other microbial lipases, insofar as substrate requirements, pH and temperature optima, thermal stability and lack of specificity are concerned.

The majority of microbial lipases described in the literature have been found in the culture supernatant fluid, and the extracellular nature of the enzyme has been well documented in at least one instance, that of *P. fragi*[21]. However, few authors seem to have investigated the existence of cell-bound lipases, whether truly intracellular or surface-located[22].

In the present case it would seem that the major part of the total enzyme is extracellular, since it appears in the medium surrounding the cells during the logarithmic phase of growth. Although this is not conclusive evidence, because a certain amount of autolysis may occur even in young cultures, the striking paralellism between the level of enzymic activity and the colony count indicates that the presence of the

enzyme in the culture medium is not due to liberation from broken or damaged cells but rather to an active process of extrusion effected by viable organisms. About 20% of the enzyme is firmly bound to the cells: it cannot be removed by two successive washes with saline. Since this fraction is capable of metabolizing an emulsion of fat which is unlikely to be able to penetrate the cell, the enzyme should be located on or near the bacterial surface (*i.e.* outside the main permeability barrier of the cytoplasmic membrane). This hypothesis is supported by the fact that almost 80% of the activity of sonified cells was recovered in the 1500 × *g* pellet, which is mainly composed of cell-wall debris. It remains to be seen whether one or more distinct protein entities are responsible for these differently located activities.

It has been stated that the staphylococci alone, among the potentially pathogenic bacteria, are known to produce an exo-lipase[17,23]. It is obvious that other potentially pathogenic genera, notably the pseudomonads, are also active in producing diffusible lipases. Although the significance of this observation is not clear, since no relationship between lipolytic activity and toxigenicity has been established as yet, it is perhaps noteworthy that production of lipase by *P. aeruginosa* could be increased by repeated passage of bacterial cells through an animal host, a procedure which normally enhances the virulence of a strain.

An important characteristic of this enzyme, in view of the practical implications, is its ability to act upon human serum lipoproteins. Further studies on this aspect are in progress.

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