Isolation and Characterization of a Staphylococcal Lipase

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A number of coagulase-negative staphylococci isolated from human skin were found to produce lipase. Lipolytic activity appeared in the growth medium during the stationary phase of growth but did not appear as a result of autolysis of the cells. Maximal lipase synthesis was obtained when the medium was adjusted to pH 7.5 before inoculation. The purified enzyme hydrolyzed tributyryl and tridecanoin most actively, and a relatively high rate of hydrolysis of triolein was also noted. The optimal activity of the purified lipase was at pH 7.5. The characteristics of the concentrated crude enzyme and purified lipase were compared.

The ability of staphylococci to hydrolyze triglycerides and the relatively wide distribution of this ability in various staphylococcal types have been known for some time (2–4). However, systematic studies of these lipases relating to their isolation and characterization are relatively rare.

The pathological significance of lipases from staphylococci is unknown; however, it has been suggested that they influence the development of acne (9) and the presence of oleic acid released by the hydrolysis of serum lipids by staphylococcal lipases could be pathologically important (8). It has also been reported (11) that the egg yolk opacity reaction shown by some staphylococci, which is of potential aid in the detection of pathogenic strains, is due to lipolytic activity. With few exceptions, there are almost no reports of attempts to purify and characterize these lipases and especially those lipases synthesized by the coagulase-negative staphylococci.

The present studies constitute an attempt to obtain a relatively pure lipase from a coagulase-negative Staphylococcus isolated from the human skin and to characterize this enzyme.

MATERIALS AND METHODS

Isolation and growth of microorganisms. The Staphylococcus strain used in these studies was isolated from scalp washings obtained by placing 5 ml of 0.1% peptone-water in a glass cylinder (25 mm in diameter) firmly held in place on the scalp. This area was scrubbed for 30 sec with a glass rod flattened at the end, and 1 ml of the scrubbing fluid was plated on Brain Heart Infusion Agar (BHI Agar, Difco) and incubated at 37 C. Colonies were picked and subsequently maintained on BHI Agar slants.

Lipase (EC 3.1.1.3) production was routinely obtained in BHI broth adjusted to pH 7.5. Incubation was at 37 C for 40 hr.

Lipase assay. The relative incidence of lipase-producing staphylococci was determined by plating 1-ml samples of the above-described scalp washings in BHI Agar containing 0.5% tributyryl and 5.0% NaCl (emulsified ultrasonically before autoclaving). Colonies of staphylococci surrounded by clear zones were considered active producers of lipase.

Potentiometric assay. Lipase activity was determined by measuring initial reaction velocities with a Titiraph-Titrator (Radiometer, Copenhagen, Denmark) utilizing the pH-stat function of this instrument. Substrate consisted of 100 amoles of the indicated triglyceride in 0.003 M histidine buffer (pH 8.0) and 0.002 M CaCl2. This reaction system was emulsified with an ultrasonic probe (Branson Sonifier, Branson Instruments, Great Neck, N.Y.) for 15 sec at the maximal power setting. The activity rates were determined as the number of microequivalents of KOH required to maintain the pH of the reaction mixture at pH 8.0 per minute. Reaction rates were routinely followed for 5 min.

Coagulate test. The coagulate test was performed by using the microslide technique and confirmed by the tube method. In both tests, a coagulate-positive strain of S. aureus was employed as a positive control.

To perform the microslide presumptive technique, lipase-positive colonies were picked from the agar surface and mixed into a drop of distilled water on a microscope slide. A small drop of coagulate plasma was then added to the bacterial suspension and the resulting mixture was observed for clumping.

The tube test consisted of dissolving an ampoule of coagulate plasma (Difco) in an equal volume of distilled water. Two drops of a heavy suspension of the Staphylococcus grown in BHI broth was then added to 0.5 ml of the plasma solution in a small test tube, incubated at 37 C, and examined for clotting at 4 and 12 hr.
Table 1. Concentration and purification of lipolytic activity

| Determination         | Volume (ml) | Protein (mg/ml) | Activitya (μEq per min per ml) | Total activity (μEq/min) | Specific activity (μEq per min per mg of protein) |
|-----------------------|-------------|-----------------|---------------------------------|--------------------------|-----------------------------------------------|
| Spent medium          | 5,000       | 1.04            | 0.23                            | 1,150                    | 0.2                                           |
| Vacuum dialysis conc. | 200         | 1.2             | 2.6                             | 780                      | 2.2                                           |
| Sephadex G-100        | 20          | 0.63            | 2.0                             | 40                       | 3.2                                           |
| Lyphogel conc.        | 10          | 1.3             | 4.0                             | 40                       | 6.4                                           |

a All activities were measured with a tributyrin substrate (100 μmoles) at pH 8.0.

Protein assay. Protein content of enzyme solutions was determined by the method of Lowry et al. (5). Ovalbumin was the standard.

Enzyme concentration and isolation. The soluble enzyme was separated from whole cells by centrifugation at 18,400 × g in a centrifuge (model LCA-1, Lourdes Instrument Co., Brooklyn, N.Y.) fitted with a continuous feed head. The cell-free supernatant (Table 1) was then concentrated in multiple lengths (30 cm each) of dialysis tubing (size 8, Union Carbide Corp., Chicago, Ill.) suspended in a vacuum chamber for 24 to 36 hr. This procedure reduced the volume of the spent medium from 5 liters to 300 ml. Portions (5 ml) of the concentrate containing the enzyme were then placed on a Sephadex G-100 column (50 by 2.5 cm) and eluted with 0.02 M tris(hydroxymethyl)amino methane (Tris) buffer (pH 7.5) at 4°C. The eluted peak (as determined by absorbance at 280 nm) possessing lipase activity (Fig. 1) was then concentrated to one-half its original volume with Lyphogel beads (Gelman Instrument Co., Ann Arbor, Mich.), and the resulting enzyme solution was then characterized.

Electrophoresis. Electrophoresis was carried out on a portion of the eluted, concentrated material by using cellulose polyacetate strips as the supporting medium in a sodium barbital-barbituric acid-Tris buffer (Gelman Instrument Co., Ann Arbor, Mich., pH 7.8). A current of 2 ma per strip was applied for 45 min, followed by staining with Ponceau S or Amido schwartz dyes.

Materials. The triglycerides employed in these studies were obtained from Eastman Organic Chemicals, Rochester, N.Y. The methyl esters were gifts of R. G. Folzenlogen of this laboratory. The sample of p-chloromercuribenzoate (PCMB) was obtained from General Biochemicals, Chagrin Falls, Ohio.

RESULTS AND DISCUSSION

Lipase production. The scalps of three subjects were examined for the relative incidence of organisms capable of hydrolyzing tributyrin. The results of these experiments show that on two of the subjects lipase-positive strains exceeded lipase-negative strains. Both tributyrinase-positive and -negative strains were coagulase-negative. All of the isolated strains fermented glucose anaerobically and were found to correspond to Baird-Parker subgroup 2 (1). A number of colonies showing no hydrolysis zones were subcultured and assayed for lipase activity potentiometrically to assure that the colonies were lipase-negative. In these instances, no detectable levels of lipolysis were found.

A gradual increase occurred in lipase activity throughout logarithmic and early maximum stationary growth phases (Fig. 2). Maximum lipase...
activity did not appear in the growth medium until well after the maximum stationary phase of growth was reached. Cell suspensions harvested during logarithmic and early stationary growth were lysed ultrasonically (60% breakage confirmed microscopically) and found to contain no detectable lipolytic activity. Hence the increase in activity appearing at 20 to 24 hr was probably not due to cell autolysis. The synthesis of lipase during the late stationary phase is somewhat at variance with the sequence of synthesis of other staphylococcal extracellular products. Enterotoxin, for example, is synthesized during late logarithmic and early maximum stationary phase (6). The lipase could be intracellular and released during autolysis. However, our results with lysed cells would discount this possibility.

The relative purity of the concentrated, eluted enzyme was demonstrated by additional gel filtration and a single peak which possessed lipase activity. Further evidence of increased purity was obtained by electrophoretic techniques which demonstrated the loss of two of the three stained bands after gel filtration, selection of the single lipase peak, and its subsequent concentration. A similar, unstained cellulose polyacetate strip, when placed on agar containing 0.05% tributyrin (emulsified ultrasonically), produced clearing at a position corresponding to the stained band.

pH Effects. Figure 3 shows that pH 7.5 appears to be the optimal, initial pH level for enzyme synthesis as determined by analysis of crude enzyme concentrates obtained from medium adjusted to the indicated pH levels before growth. The optimum pH level for activity of the purified enzyme also is pH 7.5 (Fig. 3). There appears to be little activity below pH 6.0 which agrees with the work of Renshaw and San Clemente (10), who, however, noted an optimal pH of approximately 8 when triolein was used as a substrate.

Temperature optimum. The optimal temperature for activity of the purified lipase was obtained with a jacketed reaction vessel through which water at various temperatures was circulated. The optimal temperature appeared to be approximately 32 C (Fig. 4), but considerable activity also appeared at 25 C. All subsequent lipase assays were therefore performed at room temperature (23 to 25 C).

Substrate specificity. The lipase concentrate and purified lipase used in these studies hydrolyzed pure triglycerides and olive oil but did not hydrolyze the methyl esters of butyric, octanoic, myristic, or oleic acids. Maximum lipolysis (Fig. 5) was obtained with triglycerides possessing

![Graph showing the effect of initial pH on the synthesis of crude enzyme concentrate and on the activity of the purified enzyme.](image1)

![Graph showing the effect of temperature on purified lipase activity. Tributyrin substrate, pH 8.0.](image2)

![Graph showing the effect of triglyceride fatty acid chain length on lipase activity. Unlined columns represent lipolytic activity of the crude concentrated enzyme. Cross-hatched columns refer to the lipolytic activity of the purified enzyme. Triglyceride designated C18= represents triolein.](image3)
chain lengths of 4 and 10 carbon atoms. Triolein was also readily hydrolyzed by both the enzyme concentrate and the purified lipase. Virtually no hydrolysis could be noted for saturated acyl groups of greater than 10 carbon atoms. The inability to hydrolyze methyl esters was somewhat surprising in view of the activity shown for many of the triglyceride esters. Sterol esters were similarly not hydrolyzed.

**Stability.** The enzyme preparations used in these studies were stable for as long as 4 weeks if maintained in the frozen condition. When stored at 4 °C, activity of the crude concentrate diminished at a very slow rate, reaching a 25% reduction after refrigeration for 5 days. Under similar conditions, the purified enzyme underwent a 40% loss in activity. At room temperature, however, the purified enzyme lost approximately 20% of its activity after 4 hr.

Studies on the temperature stability of the concentrated and purified enzyme showed that heating at 70 °C (pH 8.0) for 4.0 min resulted in 100% inactivation of tributyrylase activity, whereas 3.0 min was required for 100% inhibition at 80 °C. The purified enzyme was somewhat less stable than the crude enzyme.

**Metal ion effects and inhibitors.** The activity of the purified enzyme was assayed in the presence of increasing concentrations of Ca²⁺. With tributyrin as a substrate, appreciable activity (Fig. 6) was obtained at very low Ca²⁺ levels (10⁻⁴M) and in the absence of this cation. A slight increase in lipase activity was noted at Ca²⁺ levels between 10⁻⁴ and 10⁻³ M. Increasing cation concentrations beyond this level inhibited the activity. The hydrolysis of triolein was considerably more dependent on Ca²⁺ concentration than was the hydrolysis of tributyrin, and measurable activity with this substrate did not occur until the Ca²⁺ level reached 10⁻³ M. Maximal trioleinase activity was obtained at a Ca²⁺ concentration of 7.5 × 10⁻³ M. The observation that the trioleinase activity was dependent on cation concentration whereas tributyrylase activity appeared to be unaffected by metal ions might be expected, since the probable function of such ions is to remove inhibitory free fatty acids from the lipid-water interface by the formation of insoluble soaps. Such a function is critical if the free fatty acid liberated by enzyme action is insoluble as is the case with oleic acid. The soluble product of the lipase action on tributyrin, however, would not be expected to accumulate at the interface and thus soap formation is not critical. The other divalent cation tested, Mg²⁺, produced similar results.

The addition of NaCl to the reaction mixture produced a measurable decline in the hydrolysis of tributyrin. This effect is quite different from that obtained with pancreatic lipase which shows a stimulatory effect with the addition of NaCl (7). The influence of NaCl on other microbial lipases has not been reported in the literature; however, complete inhibition of tributyrylase activity probably does not occur at concentrations as high as 1 M since appreciable clearing of tributyrin emulsions occurs in agar containing 5.0% NaCl.

The sulfhydryl group inhibitor PCMB does not produce appreciable inhibition of tributyrylase activity at concentrations at which it might be expected to be an inhibitor at the active site. High PCMB levels (5 × 10⁻³ M) produced a 63% inhibition of tributyrylase activity, which could be due to the nonspecific effect of Hg²⁺. From these data, it is probable that functional sulfhydryl groups are not essential for activity of this enzyme.

The soluble lipase obtained in these studies from coagulase-negative staphyloccoci isolated from the human skin seems to be a relatively active enzyme. It does not appear to be greatly different from lipases isolated from coagulase-positive organisms which have been reported in the literature. However, some differences can be seen, most notably the capacity to hydrolyze triacylglyceride substrates and the time of lipase synthesis during growth. Other characteristics, such as stability, pH for optimal synthesis, and activity, appear to be relatively similar to reports in the literature.

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