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CHARACTERISTICS OF THE EXTRACELLULAR LIPASES FROM *CORYNEBACTERIUM ACNES* AND *STAPHYLOCOCCUS EPIDERMIDIS**

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ABSTRACT

The lipases (glycerol-ester hydrolases, 3.1.1.3) from two cutaneous bacteria *Corynebacterium acnes* (*Propionibacterium acnes*, Group II) and *Staphylococcus epidermidis* have been partially purified and characterized to permit the screening of possible enzyme inhibitors. Ultimately one of these inhibitors may be useful in the topical treatment of acne vulgaris. Although these enzymes are similar in function, they are distinctive in their characteristics.

The enzymes were partially purified from liquid culture broth by either ultrafiltration or precipitation followed by gel filtration on Sephadex G-100 and chromatography with a cellulose ion exchanger. These enzyme preparations demonstrated their greatest catalytic activity against emulsions of tributyrin > triolein > tricaprylin > trilaurin > tristearin. The patterns of triglyceride hydrolysis by the two lipases, however, was different. The enzyme from *S. epidermidis* behaved similarly to pancreatic lipase and cleaved the α positions of the triglycerides more rapidly than the β position, producing monoglyceride intermediates. The *C. acnes* lipase cleaved both positions with equal facility, producing only glycerol and free fatty acids.

C. acnes lipase was stable in the acidic pH range between 5–8; while the *S. epidermidis* lipase was inactivated in that range and stable between pH 7–10. *C. acnes* lipase was generally more sensitive to enzyme inhibitors and was very effectively inhibited by diisopropylfluorophosphate (DFP) and some of its analogs. *S. epidermidis* lipase was not inhibited by DFP although some of the DFP analogs were effective inhibitors. The lipase activity present within comedones removed from acne patients was inhibited 70% by DFP (0.1 mM), suggesting that the major lipase in comedones originates from *C. acnes*.

The etiology of acne vulgaris is complex, involving androgenic stimulation of the sebaceous glands at puberty, hydrolysis of triglycerides in sebum by bacteria, and a hyperkeratotic reaction to the fatty acids by the sebaceous follicles [1]. The bacterial enzymes which hydrolyze triglycerides have been isolated and inhibitors of the enzymes have been screened. Our ultimate goal is the evaluation of several of these enzyme inhibitors in the topical treatment of acne vulgaris. If a topical enzyme inhibitor significantly lowers the fatty acids in sebum, the role of the fatty acids in the pathophysiology of acne vulgaris can be realistically assessed. If a topical enzyme inhibitor significantly lowers the fatty acids in sebum, the role of the fatty acids in the pathophysiology of acne vulgaris can be realistically assessed.

When this study was begun in 1970 [2], it had been reported that the enzymes could be recovered from liquid culture broth [3,4] but the optimal methods of purification were not known. Another study described a purification scheme quite similar to the one presented in this report, but bacterial inhibitors were not studied in detail [5].

MATERIALS AND METHODS

Materials

Bacterial culture broths were purchased from Difco Corporation. Tributyrin, tricaprylin, trilaurin, triolein, and tristearin, in addition to [1-^{14}C]trioctanoin and [1-^{14}C]triolein (specific activities 84 mCi/mMole) were obtained from Applied Science Labs. Diisopropylfluorophosphate was purchased from Sigma Chemical Co. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, while Reeve Angel Co. supplied the preswollen microgranular CM-cellulose (Whatman CM-52) and DEAE cellulose (Whatman DE-52). Pancreatic lipase was purchased from Worthington Biochemical Corp. For the polyacrylamide electrophoresis, Canalco supplied ammonium persulfate and their two premixed solutions, RDS-A and RDS-C. Sodium dodecyl sulfate obtained from Fisher Scientific Co., was recrystallized from ethanol. Coomassie brilliant blue was purchased from Colab Laboratories, Inc. The analogs of DFP were reference standards donated by the United States Pesticides Repository, Perrine Primate Research Branch, Environ-

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Abbreviations: PNPA, *p*-nitrophenyl acetate; DFP, diisopropylfluorophosphate; EDTA, ethylenediamine tetraacetic acid; PCMB, p-chloromercuribenzoate; SDS, sodium dodecyl sulfate; PVP-I_2, povidone-I_2.

mental Protection Agency. All other chemicals were of reagent grade.

Growth of Microorganisms

Corynebacterium acnes (*Propionibacterium acnes*, Groups I and II) and *Staphylococcus epidermidis* were isolated from facial follicles of 15 males and females. These organisms were maintained as stock cultures on brain-heart infusion agar and the most active lipase producers (as assayed against tributyrin) were used as initial sources of enzyme. The four isolates of *C. acnes* studies in detail were all Group II or phage-resistant organisms [6]; the five isolates of *S. epidermidis* were classified as *Staphylococcus*, type II, using the method of Baird-Parker [7]. For batch purification, 3-day subcultures from the agar plates were inoculated into 2 liters of Eugon broth or brain–heart infusion broth enriched with 10 mM potassium phosphate buffer (pH 7.5).

Initially, the effects of enzyme yield of additional enrichment of the broth with tributyrin (33 mM), glycerol (108 mM), and increasing concentrations of glucose (27.5, 55, 110, 165, and 275 mM) were studied. To insure anaerobiasis, the broth was gassed with 95% nitrogen:5% carbon dioxide for 15 min before inoculation. Cultures were maintained in a shaking metabolic incubator at 35°, 37°, or 39° for periods up to 10 days and the bacteria separated from the broth by centrifugation in a Sorvall® RC 2-B at 22,500 × *g* for 60 min.

Purification Procedure

Four separate methods were evaluated for the initial purification of the enzymes from broth:

1. $(NH_4)_2SO_4$ precipitation using the nomograph of Dijeso [8]. Enough solid $(NH_4)_2SO_4$ was added to produce 60% saturation at 4°. After stirring for 2 hr, the solution was centrifuged and the supernatant discarded. The precipitate was dissolved in water (100 ml) and dialyzed against running tap water overnight. Following lyophilization, the material was stored at −20°.

2. Alcohol precipitation following the procedure outlined by Kaufman [9]. A beaker containing 1 liter of broth was placed in a large basin filled with a mixture of acetone–water–dry ice at −10°. As the temperature of the broth reached −4°, 95% ethanol (cooled to −30°) was added in discrete drops until 1 liter was added. After continual stirring for 60 min the solution was centrifuged at −10° for 30 min at 5000 × *g*. The precipitate was resuspended in 100 ml of water and lyophilized.

3. Ultrafiltration utilizing the Diaflo® 402 chamber, equipped with the XM-50 filter (Amicon Corp.). Stirring was controlled to avoid foaming and positive pressure was obtained with a nitrogen reservoir at 60 psi. Filtration was conducted at 4° until the volume was reduced to 10 ml. This volume was directly added to the Sephadex G-100 column as the second step of purification.

4. Ultrafiltration with cellulose acetate hollow fibers (Bio-Rad® ultrafilter). Running tap water was used as the suction source for 6 hr at 4°. The reduced volume (10 ml) was directly added to the Sephadex G-100 column.

The retentate or precipitate, was percolated onto a Sephadex G-100 column (3.5 × 35 cm) and eluted with distilled water, pH 6, in the case of *C. acnes* and with 0.005 M phosphate buffer, pH 7.5, for *S. epidermidis*. Following lyophilization of the fractions containing enzymatic activity, the preparations were reconstituted to 5 ml and further purification was obtained by ion-exchange chromatography. For the lipase of *C. acnes*, chromatography of the enzyme preparation through a CM-cellulose column (2.0 × 20 cm) was conducted at pH 6.0. The column was initially equilibrated and eluted with 5 mM ammonium formate (150 ml), followed by 50 mM ammonium formate (75 ml), and in the final step, enzymatic activity was released from the column with 500 mM ammonium formate (75 ml). The lipase from *S. epidermidis* was eluted from DEAE-cellulose with phosphate buffer, pH 7.5, between a concentration gradient of 0.05 to 0.5 M. Both preparations were desalted by ultrafiltration in the Diaflo® 402 chamber, lyophilized and stored at −20°.

Measurement of Catalytic Activity

The enzyme preparations were routinely assayed for lipase activity with an olive oil emulsion in the Metrohm® pH stat following the method of Shalita and Wheatley [10]. Fatty acid release was monitored by potentiometric titration with 10 mM NaOH. The specific activity of the enzyme preparation was expressed in units/mg protein; one unit being equivalent to one nmole of NaOH consumed per min. Protein concentration was determined by the method of Lowry et al [11] using bovine serum albumin as standard.

To evaluate the relationships between different lipid substrates and enzymatic activity, purified tributyrin, tricapylin, trilaurin, triolein, and tristearin were used. Upon sonication,‡ these triglycerides (10 mM) formed stable emulsions in the presence of sodium cholate (1.25% w/v). The velocity of cleavage of these substrates was compared at concentrations between 0.125 and 5.0 mM.

[1-^{14}C]trioctanoin (0.06 μmol) and [1-^{14}C]triolein (0.06 mmol) were utilized to evaluate preferential hydrolysis of the positions on the triglyceride. An ethanolic solution of these lipids was pipetted into the reaction vessel and evaporated to dryness with nitrogen gas. Krebs–Ringer phosphate buffer (pH 7.5) and the enzyme solution were added and sonicated‡ to disperse the radioactive compound. Incubations in a metabolic shaker at 37° were terminated after 15, 30, or 60 min by the addition of chloroform–methanol (2:1). Following a Folch extraction of the lipids [12], the residue was spotted on silica gel plates for chromatography using the method of Freeman and West [13]. Radioactive triglycerides, diglycerides, monoglycerides, and fatty acids were monitored on the Vanguard autoscanner [14]. For comparison, simultaneous experiments were conducted with pancreatic lipase.

A water-soluble substrate, *p*-nitrophenyl acetate, was also examined. The velocity of hydrolysis at pH 7.8 was measured with the Cary 15 spectrophotometer at 405 nm. The rate of hydrolysis was calculated using the molar extinction coefficient of 1.84×10^4 for *p*-nitrophenolate [15].

Cell-bound lipase activity against the olive oil emulsion was determined following 3 saline washes (10,000 × *g* × 30 min) and sonication‡ of the bacterial pellet.

pH and Temperature Dependence

The effect of hydrogen-ion concentration on enzyme stability was studied by incubating the enzyme preparations in distilled water in a pH range from 3 to 11 for 30 min at 23° and, thereafter, measuring the residual hydrolytic activity at pH 7.8 against the olive oil emulsion in the Metrohm® pH stat. The effect of pH on enzyme activity was determined by measuring initial velocities at pH values between 5.5 and 8.5 in the pH stat using the olive oil as substrate.

‡ Sonifier Cell Disruptor, Model W 185 E, Heat Systems, Ultrasonics, Inc.; using three bursts at 150 watts for 1 min each with constant cooling in an acetone–dry ice bath.

The effect of temperature on enzyme stability of these lipases was studied between 23° and 85°. 0.1 ml of enzyme solution was diluted to 1 ml in either distilled water, pH 5.2, or in phosphate buffer (0.005 M), pH 7.6, and heated to the desired temperature for 15 min. Immediately thereafter it was plunged into an ice bath for 30 min before assaying the residual activity at 23° (pH 7.8) against the olive oil emulsion. The influence of temperature on lipase activity was examined between 3° and 55°. Measurements were obtained by changing the temperature setting on the pH stat and cooling or heating the jacketed reaction vessel containing the substrate and enzyme preparation with a Lauda-Thermostat*.

Enzyme Inhibitors

Potential inhibitors were added to the enzyme preparation at pH 7.6 and preincubated for 120 min at 23°. Activity was monitored in the presence of these inhibitors on the pH stat using the olive oil emulsion at pH 7.8. Compounds studied included cupric acetate, zinc chloride, EDTA, PCMB, tetracycline HCl, erythromycin, pentamidine, stilbamidine, PVP-I_2, H_2O_2, succinic acid peroxide, benzoyl peroxide, DFP, and several analogs of DFP.

Polyacrylamide Electrophoresis

The purity of the enzyme preparation was analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis following the Weber and Osborn [16] modifications of the technique of Shapiro et al [17]. The proteins were denatured by overnight incubation in 1% 2-mercaptoethanol and 1% SDS at 37° as were the following reference materials: bovine serum albumin, salivary amylase, carbonic anhydrase, chymotrypsinogen-A, and lysozyme. Fifty- to 100-μg samples were added to each tube of 5% polyacrylamide–0.1% SDS gels along with 1 drop of glycerol and tracking dye (0.05% bromphenol blue). Phosphate buffer (0.1 M), pH 7.1, containing 0.1% SDS was layered on top of each sample and also used to fill the two compartments of the Canalco model 12 electrophoresis apparatus. A current gradient of 8 ma/tube was passed at 23° until the tracking dye had migrated the length of the tube (approximately 3 hr). Gels were routinely fixed and stained with 0.1% Coomassie blue in 10% acetic acid for 2 hr and then destained with 10% acetic acid. To determine molecular weights of the resulting protein bands the mobility of these bands was compared with the mobilities of the reference proteins of known molecular weights using a semilogarithmic plot.

Studies on Comedones

Comedones were extracted from four individuals, sonicated‡ in the presence of Krebs–Ringer phosphate buffer (pH 7.5) and each sample was divided into two equal aliquots to be incubated separately with [1-^{14}C]trioctanoin, 0.06 μmol, at 37° for 1 hr. One aliquot was incubated in the presence of DFP (0.1 mM), while the other aliquot served as the control. Following extraction of the lipids with a Folch wash [12] and evaporation of the solvent, the lipid residue was spotted on a thin-layer silica plate and chromatographed by the method of Freeman and West [13]. Analysis of the hydrolytic products was conducted on the Vanguard autoscanner and compared to lipid standards [14].

RESULTS

Rapid bacterial growth occurred within the first 48 hr of inoculation into liquid broth; thereafter, the total cell mass showed no significant change. Lipase activity demonstrated a continuous increase throughout the usual 6- to 8-day incubation period, leveling off thereafter. The effects of the enrichment of the basic broth on enzyme yields are summarized in Table I and Figure 1. The use of BHI broth, supplemented with phosphate buffer and glucose, and incubated at 35° provided optimal enzyme yields from *C. acnes*. Additions of tributyrin or glycerol had no additional effect. Of the four *C. acnes* strains screened in these initial studies, strain III demonstrated the most cellular and extracellular activity (Table II). This strain was utilized routinely for the subsequent studies.

TABLE I

Enrichment of the liquid broth

Incubations	Units/liter ($\times 10^{-3}$)	
	C. acnes	*S. epidermidis*
Eugon broth, 35°	590	2166
BHI broth, 35°	1580	249
Temperature		
35°	461	1197
37°	135	958
39°	74	*
Glycerol, 108 mM		
with	585	*
without	610	*
Tributyrin, 33 mM		
with	485	*
without	461	*

* Not done.

Enzyme units are the total extracellular activity in 1 liter of broth following 6 days of anaerobic growth. Concentrations of glucose (110 mM) and phosphate (10 mM) were the same in all experiments.

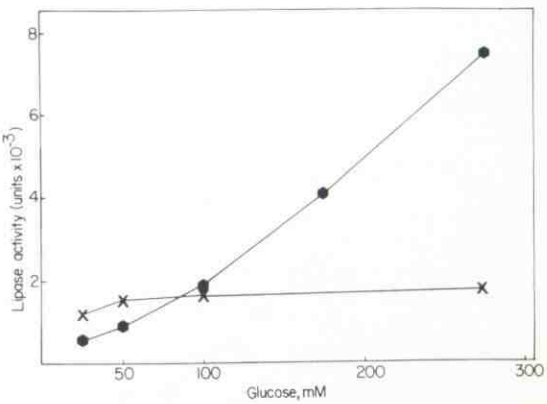

FIG. 1: Effects of supplemental glucose on enzyme yield from liquid broth. Lipase activity in a liter of culture broth was measured against on olive oil emulsion after 6 days of anaerobic growth. (●——●, *C. acnes*; ×——×, *S. epidermidis*).

The *S. epidermidis* strain selected for further study had remarkable extracellular lipase activity, but no detectable cell-bound enzyme. With this bacteria, lipase production was best in Eugon broth when incubated at 35°; additional glucose had no potentiating effects (Fig. 1). We have noted no change in lipase activity in these organisms following repeated subculturing.

The four alternate steps employed in the initial purification of the enzymes are summarized in Table III. For the lipase of *C. acnes*, precipitation of the enzyme with ethanol was the most satisfactory; with the lipase of *S. epidermidis*, concentration with the Bio-Rad® hollow fibers appeared more useful. The *C. acnes* lipase is completely excluded from Sephadex G-100 or G-200 and was significantly purified from low-molecular-weight material (Fig. 2). With ion-exchange chromatography this lipase is recovered at moderate ionic strength, yielding a substantially further purification of the enzyme (Table IV). The *S. epidermidis* enzyme behaved similarly, but was not completely excluded from Sephadex G-100.

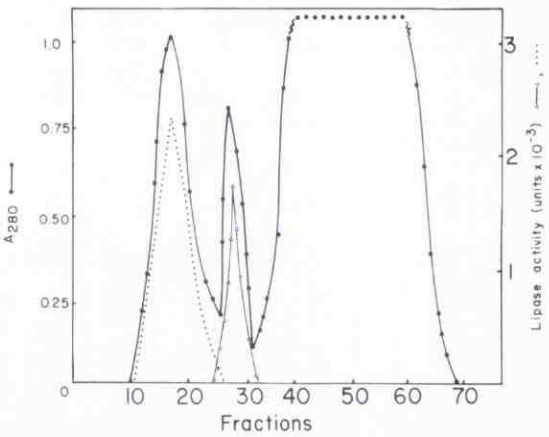

FIG. 2: The enzyme elution pattern from Sephadex G-100. The lipase activity from *C. acnes* (·····) was eluted from a 3.5 x 35 cm column with distilled water, pH 6. With the lipase of *S. epidermidis* (Δ——Δ), the activity was eluted with 0.005 M phosphate buffer at pH 7.5. Column flow was adjusted to 30 ml/hr.

TABLE II

Enzyme yield from bacterial isolates

Isolates	Enzyme units/liter (× 10^{-3})	
	Cell-bound activity	Extracellular activity
C. acnes		
I	2.3	68
II	4.4	60
III	1092.0	326
IV	0	107
S. epidermidis	0	2166

Enzyme units are expressed as total activity in 1 liter of broth (extracellular) or in the corresponding bacterial pellet following sonication (cell-bound) after 6 days of anaerobic growth at 35°C. BHI culture broth was used for *C. acnes*, while Eugon broth was used for *S. epidermidis*.

TABLE III

Comparison of precipitation to ultrafiltration as the initial step in enzyme purification

Initial step	Specific activity units/mg (× 10^{-3})	
	C. acnes	*S. epidermidis*
Diaflo® chamber	45	41
Bio-Rad® hollow fibers	91	240
Ethanol precipitation	595	140
$(NH_4)_2SO_4$ precipitation	48	*

* Not done.

The specific activity of the enzymes isolated from 1 liter of broth following ultrafiltration with either the Diaflo® or Bio-Rad® system was compared to that obtained following precipitation of the enzymes with alcohol or ammonium sulfate.

TABLE IV

Purification scheme for the extracellular lipases

Fractions	Total protein mg	Specific activity units/mg	Fold purification	Yield %
C. acnes				
Culture supernatant	16800	26	—	—
Alcohol precipitation	128	2560	98	75
Gel filtration	38	5870	226	51
CM-cellulose	8	12480	480	23
S. epidermidis				
Culture supernatant	13860	43	—	—
Ultrafiltration	340	140	33	80
Gel filtration	447	913	212	72
DEAE-cellulose	14	11405	327	33

The catalytic activity of both lipases against triglycerides at 23° was linear for at least 30 min. Of the purified substrates, tributyrin demonstrated the greatest velocity while the other substrates were cleaved at a slower rate (Fig. 3). Unlike pancreatic and *S. epidermidis* lipases, the lipase from *C. acnes* cleaved PNPA at a significant rate, although less rapid than the rate observed for tributyrin, yielding an apparent V_{max} of 4.25 units/mg of protein.

Chromatographic analysis of the cleavage of [1-^{14}C]trioctanoin and [1-^{14}C]triolein by the *C. acnes* lipase after 15, 30, or 60 min revealed no monoglyceride intermediate such as that found following cleavage with *S. epidermidis* lipase or pancreatic lipase (Fig. 4). This suggests that the former bacterial enzyme has no selectivity towards acyl sites on the triglyceride and does not release a monoglyceride intermediate.

pH studies revealed that the *C. acnes* enzyme was stable in the acidic range, but very labile at a pH greater than 8 (Fig. 5). Catalytic activity was also pH-dependent, with insignificant hydrolysis below pH 5.5. Optimal activity was between pH 7.6 and 8.0. The lipase of *S. epidermidis* was remarkably different since it was stable in the range between 7–10, yet the optimal pH for hydrolysis was also 7.8. These studies indicated that the *C. acnes* enzyme should be stored at pH 5, while the *S. epidermidis* preparation would be more stable under storage at pH 8. At these respective pH values both enzymes have been stable for at least 6 months at −20°.

The lipase from *C. acnes* was relatively stable to heat at pH 5.5; requiring 15 min at 75° to decrease the catalytic activity greater than 50% (Fig. 6). At

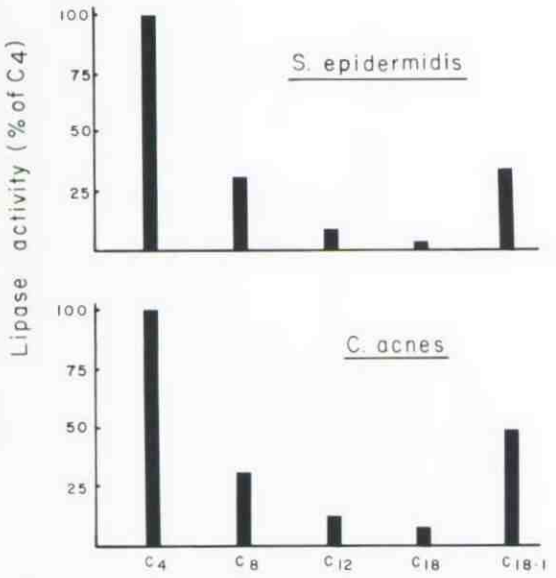

FIG. 3: Enzyme hydrolysis of triglycerides. Purified triglycerides were assayed against the lipase of *C. acnes* and *S. epidermidis* in the pH stat. Results are expressed as percentage of maximal activity, using tributyrin (C_4) as the reference lipid.

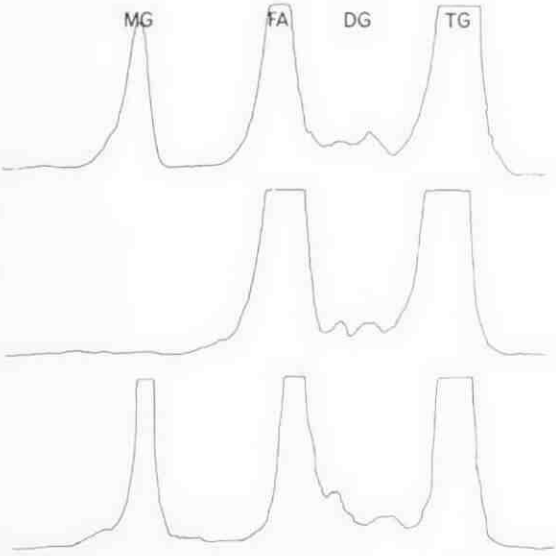

FIG. 4: The radiochromatogram of products of [1-^{14}C] trioctanoin hydrolysis by the lipase of *S. epidermidis* (top panel), *C. acnes* (middle panel), and hog pancreas (bottom panel). MG, monoglycerides; FA, fatty acids; DG, diglycerides; TG, triglycerides.

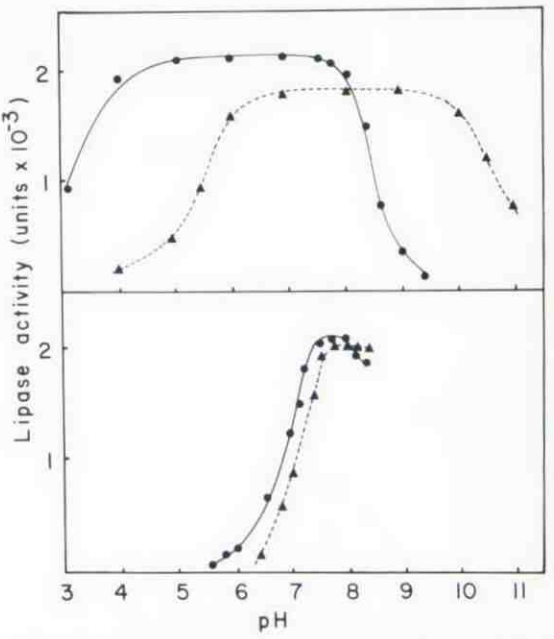

FIG. 5: pH stability and activity studies. pH stability (top panel) was determined after exposing the lipases of *C. acnes* (●——●) or *S. epidermidis* (▲-----▲) to the indicated hydrogen-ion concentration for 30 min. The effect of pH on activity (bottom panel) was determined by measuring initial enzyme velocities between pH 5.5 and 8.5.

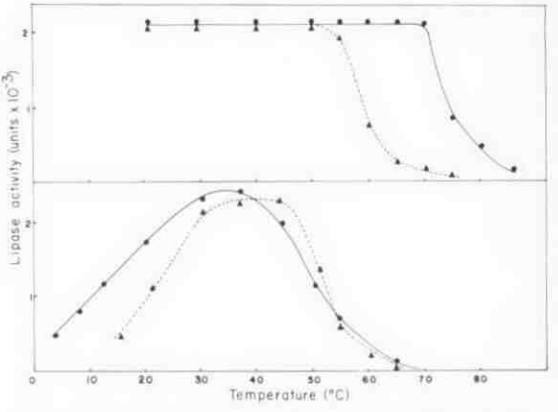

FIG. 6: Temperature stability and activity studies. The stability to heat (top panel) was examined between 20° and 85° at pH 5.2 for the lipase of *C. acnes* and pH 7.6 for the lipase of *S. epidermidis*. The effect of heat or enzyme activity (bottom panel) was examined between 3° and 55° using a jacketed reaction vessel on the pH stat. (*C. acnes*, ●——●; *S. epidermidis*, Δ——Δ).

pH 7.6 this enzyme was more labile, losing more than 50% of activity after 15 min at 55°. Enzyme activity was depressed after cooling the reaction vessel to 3°; whereas, maximal hydrolysis occurred at 37°, only to decrease again at 45° and higher.

Heat denaturation studies could not be conducted on the lipase from *S. epidermidis* at pH 5.5 because of instability at the pH; at pH 7.6 the enzyme was slightly more stable than the *C. acnes* lipase, requiring 15 min at 60° before there was a significant drop in hydrolytic activity. The catalytic activity of the *S. epidermidis* lipase was also influenced by temperature (Fig. 6).

In general, enzyme inhibitors were more effective on the lipase of *C. acnes* (Table V). Inhibition was possible with two heavy metals, but EDTA and PCMB were ineffective. Other inhibitors included tetracycline, pentamidine, stilbamidine, and PVP-I_2. DFP was the most potent inhibitor of the *C. acnes* lipase, being effective at 10^{-6} M. Several of the DFP analogs were effective inhibitors of both enzymes.

Analysis of the *S. epidermidis* preparation by SDS-polyacrylamide electrophoresis revealed one

TABLE V

Inhibition studies

Compound	mM	Inhibition (%)	
		C. acnes	*S. epidermidis*
$Cu(CH_3COO)_2$	1.0	87%	0%
$ZnCl_2$	1.0	27	5
EDTA	1.0	0	0
PCMB	1.0	0	0
Tetracycline HCl	1.0	100	67
	0.1	41	17
Erythromycin	1.0	0	0
Pentamidine	1.0	75	0
Stilbamidine	1.0	65	46
PVP-I_2	*	100	100
H_2O_2	1.0	27	13
Succinic acid peroxide	1.0	0	16
Benzoyl peroxide	<1.0†	0	13
DFP	1.0	100	25
	0.1	95	0
	0.01	53	0
	0.001	34	0
Analogs of DFP			
Carbaryl®	1.0	78	0
Trichlorophon®	1.0	91	80
Dichlorvos®	1.0	100	82
Malathion®	1.0	0	0
Ronnel®	1.0	0	0

* Tested as a 1% solution

† Very insoluble in water

Potential inhibitors were preincubated with the enzymes at pH 7.6 for 120 min at 23°. Residual activity was monitored with the pH stat, using the olive oil emulsion.

FIG. 7: The SDS-polyacrylamide electrophoresis pattern of the lipase of *C. acnes* (left panel) and *S. epidermidis* (right panel).

TABLE VI

The hydrolysis of [1-^{14}C]trioctanoin by comedones

Subject	Controls		DFP, 1 mM		% Inhibition
	TG	FFA	TG	FFA	
I.	39%	61%	79%	21%	66%
II.	60	40	98	2	96
III.	65	35	82	18	52
IV.	77	23	92	8	66
Avg.	60%	40%	88%	12%	70%

Following the incubation of comedones with [1-^{14}C] trioctanoin in the presence and absence of DFP, the lipids were extracted and chromatographed. The percent of [^{14}C] label remaining in the triglyceride fractions (TG) was compared to the percent that appeared in the free fatty acid fraction (FFA).

protein band with an approximate molecular weight of 26,000. Three dominate protein bands existed in the *C. acnes* preparation, with respective molecular weights of >100,000, 54,000, 43,000 (Fig. 7).

The hydrolysis of [1-^{14}C]trioctanoin by the lipase present in comedones was impressive (Table VI). In the control studies 40% of the radiolabelled lipid was converted to free fatty acids during 1 hr of incubation. The addition of DFP (1 mM) decreased this hydrolysis by 70%.

DISCUSSION

The lipases of *C. acnes* and *S. epidermidis* have been partially purified and characterized. Although similar in function, the two enzymes are quite unique in their catalytic activity. Both hydrolyze triglycerides (tributyrin > triolein > tricaprylin > trilaurin > tristearin) but the enzymes use different mechanisms in the process. The *S. epidermidis* lipase is similar to pancreatic lipase in producing a monoglyceride intermediate, while the *C. acnes* lipase produces free fatty acids and glycerol. Only the *C. acnes* lipase will attack the water-soluble substrate, *p*-nitrophenyl acetate. The inhibition of the *C. acnes* lipase by low concentrations of DFP suggests that the amino acid serine is present at the catalytic site of this enzyme, similar to other hydrolases such as chymotrypsin, elastase, and cholinesterase [18–21]. Additional studies in this laboratory with [^{32}P]DFP have demonstrated this reactive serine [22]. Serine may also be present in the active site of *S. epidermidis* but it will remain impossible to prove this hypothesis until one of the inhibitory analogs of DFP is radiolabeled to provide a tag to identify the reactive amino acid. The ultimate goal of this study remains to be fulfilled as the clinical effectiveness of the analogs of DFP are only currently being evaluated in the topical treatment of acne.

Altering the culture conditions had considerable influence on enzyme yield. For example, lipase production for *C. acnes* was better in BHI broth, enriched with high concentrations of glucose and incubated at 35°. Eugon broth was more ideal for *S. epidermidis* and additional glucose had no significant effect. Other growth effectors, such as pH, are still being studied. Variations among bacterial isolates were also apparent as the enzyme distribution could be predominately cell-bound or extracellular depending on the strain studied. For these present studies we chose the two strains with the greatest extracellular activity. Further studies are needed to ascertain the significance of cell-bound vs. soluble enzyme activity.

Differences were also apparent in the purification of the two enzyme preparations. During the initial steps ethanol provided a clean precipitate of the *C. acnes* lipase, but not the *S. epidermidis* lipase. Ultrafiltration was much more satisfactory for the latter. The Bio-Rad® chamber was superior to the Diaflo® as the former yielded a higher specific activity and it was virtually impossible to filter the preparation to dryness.

Elution of the enzyme activity at or near the void volume of Sephadex G-100 is typical of many native lipases and this behavior has been attributed to large-molecular-weight aggregates of protein and lipid [23–25]. Evidence for this lipid–protein or protein–protein aggregate was suggested by the polyacrylamide electrophoresis where the predominate dissociated protein bands are present in low-molecular-weight regions of the gel. By utilizing [^{32}P]DFP [22] it has been possible to radiolabel our lipase preparation from *C. acnes* and demonstrate that the protein band with a molecular weight of 54,000 is tagged. Studies are currently planned to identify the possible lipid components within these lipase preparations.

The results of the pH stability studies dictated the choice of cellulose ion exchangers for purification as chromatography of the *C. acnes* preparation was limited to the pH range between 4 and 7, while the *S. epidermidis* preparation required a range between 7 and 9. Predictably, DEAE-cellulose would not release the *C. acnes* lipase at pH 6.0, while CM-cellulose would not bind the lipase from *S. epidermidis* at pH 8.0.

The lack of substrate specificity was quite apparent with the *C. acnes* lipase. This enzyme cleaves the α and β positions of triglycerides with equal facility. In addition, di- and monoglyceride substrates are attacked [22], as well as the water-soluble substrate *p*-nitrophenyl acetate. Pancreatic lipase and *S. epidermidis* lipase preferred the α position of the triglyceride and neither enzyme cleaved *p*-nitrophenyl acetate.

Compared to DFP and its analogs, other inhibitors were relatively ineffective. A more extensive study of the analogs of DFP may prove exciting for the eventual topical treatment of acne vulgaris. In our initial studies several leads are apparent; for example, the substitution of thiophosphate for oxyphosphate diminishes activity. The relatively nontoxic carbamates may be useful. The most potent organophosphates are frequently substituted with methyl groups. One would consider elongating these methyl groups to 8 or 10 carbons to model the inhibitor more like a triglyceride and less like acetylcholine. This may lead to selective toxicity so an analog of DFP may eventually be useful in the topical treatment of acne vulgaris.

It is apparent from these studies that the purification and characterization of one lipase, such as *C. acnes* lipase, does not predict the character and optimal growth conditions for another lipase, such as the *S. epidermidis* preparation. For example, until conducting pH stability studies we continually lost the lipase from *S. epidermidis* since the culture broth has a final pH of 5.0. After adjusting the pH of this broth to 8.0 immediately after the incubation period the enzyme yield improved.

Reinkel and Shen commented on these pH effects in 1969 [4].

It is interesting to speculate that the "acid mantle" of the facial skin is controlled by these lipases. The pH of sebum and comedones is usually 5.5 which is, coincidentally, the acid termination point for catalysis by these lipases. Sebum is probably around physiologic pH at the time of sebaceous cell rupture. Cleavage of triglyceride, or the release of free fatty acids, must be rapid in the sebaceous follicle as hydrolysis is nearly complete once the sebum exits onto the surface of the skin [26]. As the pH drops, lipase activity would decrease, eventually stopping when the pH reaches 5.5; thus producing the "acid mantle."

For anatomic reasons it has usually been assumed that the lipase from *S. epidermidis* was not so important in the pathogenesis of acne since this bacteria dwells predominately on the surface of skin and not in the follicular depths. Our data appear to support this assumption since DFP inhibits only the *C. acnes* lipase and not the lipase from *S. epidermidis* and the addition of this organophosphate to comedones effectively inhibited triglyceride hydrolysis. Additionally, the pattern of triglyceride hydrolysis by comedones was identical to the pattern of hydrolysis produced by the lipase of *C. acnes* as no monoglyceride intermediate could be identified. However, other follicular hydrolases such as the *Pityrosporum ovale* lipase and possibly tissue lipases may be important, but these were not studied in this paper.

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