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Density-Gradient and Chromatographic Fraction of Leptospiral Lipase

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Fractionation of leptospiral lipase by CsCl density gradients and G-200 Sephadex chromatography yielded five active protein peaks. Two were obtained from the density gradients and three from G-200 Sephadex columns. Esterase activity of these fractions was demonstrated by electrophoretic examination. Several protein bands were visible when disc electrophoresis was performed on the respective fractions. Lipolytic and esterolytic activities were both present, and the overlapping of these activities was discussed.

Kemenes and Lovrekovich (9) first studied the "fat splitting" ability of various leptospiral cultures. They found that strains of *Leptospira icterohaemorrhagiae*, *L. canicola*, and *L. pomona* had greater extracellular lipase activity than 31 other strains of virulent and avirulent serotypes of *Leptospira*. Parnas et al. (13) showed that there was variability in lipase production within the same serotype. Bertok and Kemenes (2) were first to indicate that whole-culture lipase assays had maximal activity with tributyrin substrate. They also found that lipase was elaborated in the log phase of growth and reached the highest concentration at the peak of the growth curve. Although no purification was attempted, Bertok and Kemenes also showed that the lipase was filterable.

The first detailed attempt at characterization of the leptospiral lipase was carried out in this laboratory (14). Ethyl alcohol and acetone extraction and freeze-drying produced a brown powder, stable in dry and liquid states, which had maximal activity at *p*H 8 and 30 C; it was not inhibited by metals, proteolytic enzymes, or −SH blocking agents, and possessed a Michaelis constant of 4.76×10^{-2}. It was this product that was subjected to density-gradient ultracentrifugation, chromatographic separation, and electrophoretic analysis for a more detailed biochemical investigation.

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MATERIALS AND METHODS

Cultures. Thirty-seven strains of leptospires were assayed for lipolytic activity. Inocula were grown in albumin-Tween 80 medium (5, 6) for 5 days, washed, and then inoculated into 100-ml volumes of this media; they were incubated at 30 C for 7 days.

After incubation, the cells were removed by centrifugation, washed, and discarded. The washings were added to the supernatant fluids and filtered to insure cell-free preparations. After dialysis, the preparations were freeze-dried and stored at −50 C.

Serotype *L. pomona* LC-12 was the source of the leptospiral lipase. The growth medium and conditions of culture and methods of isolation were as previously described (14).

Lipase activity. Lipase activity was assayed by titrating the hydrolyzed fatty acids liberated by the incubation of the enzyme with various triglycerides. All the titrations were conducted with a Fisher Automatic Titrimeter, as reported in a previous paper (14). Activity was expressed as micromoles of fatty acid neutralized per minute per milligram of protein at 30 C.

Substrates. The substrates used in these experiments included triacetin, tributyrin, tricaproin, tricaprylin, trilaurin, trimyristin, tripalmitin, tristearin, triarachidin, trioleien, trilinolein, trilinolenin, caprylic methyl ester, capric methyl ester, lauric methyl ester, myristic methyl ester, and palmitic methyl ester, all at a final concentration of 0.166 M.

Density-gradient centrifugation. Cesium chloride was used to make the gradients. A 4-ml amount of a solution of 1.32 g of cesium chloride per ml (Harshaw Chemical Co., Cleveland, Ohio) was placed in a mixing chamber together with tris(hydroxymethyl)-aminomethane (Tris) buffer (*p*H 8.0; 0.2 M) as the diluting fluid. Three density-gradient tubes were prepared that contained cesium chloride with a density

range of 1.32 to 1.15 g/ml. The density gradients were overlayed with 1 ml of original leptospiral lipase preparation containing 60 mg of material. The three tubes were then placed into an SW39L swinging bucket rotor, in a Spinco model L centrifuge.

The samples were centrifuged for 8 hr at 125,000 × *g*. The centrifuge was allowed to stop without braking, and the tubes were removed and photographed. The density-gradient tubes were frozen (11) and then cut in an aluminum cutting jig molded to the shape of the centrifuge tube (1). It was possible, in this manner, to cut the centrifuge tube into eight samples by means of a jeweler's saw.

Microliter pipettes containing gradient samples were weighed on an analytical balance to determine their density to three decimal places, corrected to 4 C.

Density-gradient fractions were scanned at 280 nm in a Beckman DU spectrophotometer (Beckman Instruments Co., Fullerton, Calif.) to determine the existence of invisible protein bands. In addition, the fractions were assayed for protein content and for lipase and esterase activities.

For further concentrations, the respective CsCl fractions were pooled and dialyzed at 2 C against deionized water until the fractions were free of CsCl. The pooled fractions were precipitated with three volumes of 95% ethyl alcohol precooled to −50 C and centrifuged at 2,500 × *g* for 30 min at 2 C. The supernatant fluid was discarded and the precipitates were then freeze-dried and yielded brown powders.

Electrophoresis. Separation of protein fractions of the lipase was carried out in acrylamide gel, according to the method of Davis (3). Each sample containing 125 μg of protein was subjected to electrophoresis with a constant current of 5 ma/tube until the tracking dye reached a point 5 mm from the base of the gel. The gels were then stained for 2 hr at room temperature with 0.5% amido black in 7.5% acetic acid. Destaining was accomplished at a constant current of 15 ma/tube until the blue protein bands were visible in a clear gel.

Horizontal starch-gels were prepared by the use of hydrolyzed starch (Connaught Medical Research Laboratory, Toronto, Ontario, Canada) in 0.03 M borate buffer (*p*H 7.6). Starch molds 200 by 20 by 6 mm were procured (15) and, after solidification, the gels were wrapped in plastic and stored overnight at 4 C.

Samples from each isolated fraction containing 5% protein were applied by saturating Whatman no. 1 filter paper and placing this in a slit cut 8 cm from the cathodic end of the gel. The gels were placed on buffer trays containing 0.3 M borate buffer (*p*H 7.6), and electrophoresis was carried out at 4 C for 6 hr with a constant voltage of 7.5 v/cm. The gels were then sliced longitudinally and the exposed, cut surface was subjected to enzyme staining.

Enzyme localization. To identify esterase activity, α-naphthyl acetate and Fast Red TRN (Dajac Laboratories, Borden Chemical Co., Philadelphia, Pa.), and α-naphthyl butyrate and Fast Blue RR (Sigma Chemical Co., St. Louis, Mo.) were used according to the procedures of Markert and Hunter (12). The gels were incubated until bands became evident and then washed for 5 min in running tap water before being drawn and photographed.

Chromatography. Since the lipase preparations showed no detectable separation with the lower dextrans, G-200 Sephadex was used for the column chromatographic separation. The method of Flodin (8) was followed to obtain a satisfactory Sephadex column.

Samples were applied by draining the buffer from the column until the Sephadex surface was exposed, and 400 mg of lipase that contained 75% protein in 10 ml of 0.05 M Tris (*p*H 8.0) was added to the top of the gel. After the sample was drawn into the column, more buffer was added and the column was started. The brownish color of the sample allowed visual checks on its migration; when the band had moved 60 cm, the column was connected to an auto-

TABLE 1. *Lipase activity of extracellular preparations from Leptospira*

Serotype	Strain	Activity ($\times 10^{-2}$)[a]
L. alexi		4.6
L. andaman		0
L. atlantae	LT-81	0
L. australis	Ballico	0
L. autumnalis	Akiyama A	0
	Rachmat	3.6
L. ballum	S-102	0
	M-127	0
L. bataviae	Van Tienen	1.5
L. biflexa	Waz	2.1
	LT-430	7.3
	CDC	1.1
	Patoc I	0.6
	Sao Paulo	7.4
L. canicola	Hond Utrecht	0.6
	Moulton	1.2
	Reubusch	4.6
	Swafford	1.6
L. grippotyphosa	Moscow V	0
L. hebdomadis	Hebdomadis	3.0
L. hyos	LT-79	0
	Mitis Johnson	0
L. icterohaemorrhagiae	AB	0.7
	RGA	0
	LC-199	0.7
L. mini	LT-117	0
L. pomona	LC-34	1.3
	Johnson	1.2
	4822	2.9
	LC-12	1.4
	Wichard	1.8
	MLS	1.2
	S-91	2.2
	Pomona	1.0
L. pyrogenes	Salinem	2.6
L. sejroe	Mallersdorf	0
L. semarang		5.8

[a] Values are expressed as micromoles of fatty acid neutralized per minute per milligram of protein at 30 C. Tributyrin substrate was 0.166 M.

matic fraction collector and 3-ml fractions were collected at a rate of 15 ml/hr. A total of 1,800 ml of buffer was used in this collection. Tubes were analyzed for protein at 280 nm. Respective peaks were pooled, dialyzed overnight against deionized water, and freeze-dried; after checking the fractions for lipase activity, they were stored at −50 C.

Protein determination. The protein content of each of the various leptospiral fractions was determined by the method of Lowry et al. (10). A control uninoculated sample of leptospiral medium prepared in a similar manner was also tested for protein.

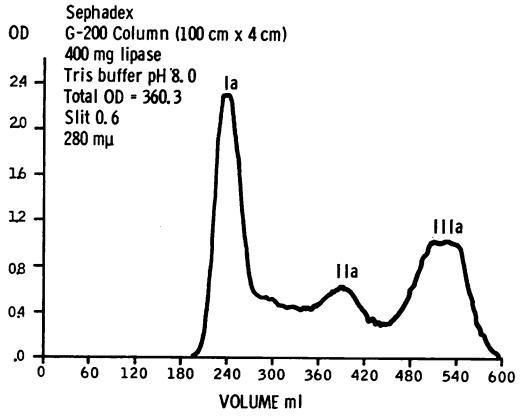

FIG. 1. *Elution pattern of Sephadex G-200 column from original lipase of Patel et al. (14).*

TABLE 2. *Protein content of various lipase preparations from Leptospira*

Preparation	Protein
Uninoculated media	95
Crude lipase	86[a]
Sephadex fraction Ia	62.5
Sephadex fraction IIa	56
Sephadex fraction IIIa	62.5
CsCl fraction I	70
CsCl fraction II	50

[a] Data obtained from Patel et al. (14).

TABLE 3. *Lipolytic activity of lipase fractions from Leptospira*

Preparation	Activity ($\times 10^{-2}$)[a]
Crude lipase	2.9[b]
Sephadex fraction Ia	2.4
Sephadex fraction IIa	1.0
Sephadex fraction IIIa	0.5
CsCl fraction I	2.0
CsCl fraction II	2.1

[a] Values are expressed as micromoles of fatty acid neutralized per minute per milligram of protein at 30 C. Tributyrin substrate was 0.166 M.

[b] Data obtained from Patel et al. (14).

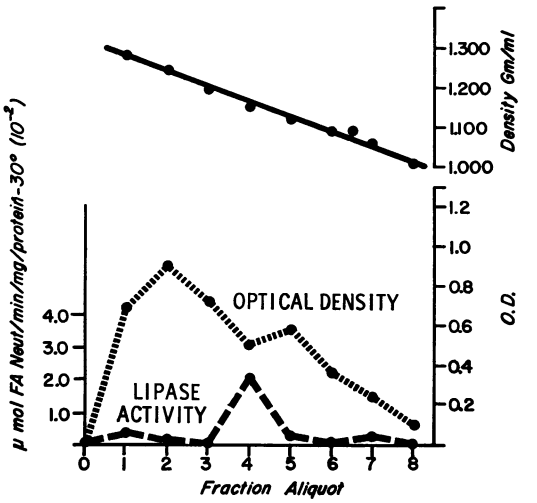

FIG. 2. *Density distribution of fraction I leptospiral lipase.*

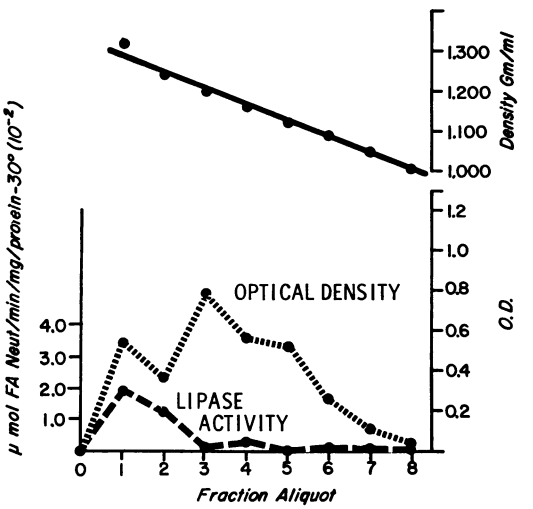

FIG. 3. *Density distribution of fraction II leptospiral lipase.*

RESULTS

The lipolytic activities of the 37 strains of leptospires are recorded in Table 1. Twelve strains showed no lipase activity upon the tributyrin substrate, and 25 strains showed variable activity with this triglyceride.

The crude lipase preparation, originally extracted by the method of Patel et al. (14), yielded three protein fractions by Sephadex chromatography; they were designated Ia, IIa, and IIIa (Fig. 1). These fractions had various protein contents and lipolytic activities (Tables 2 and 3).

The existence of two distinct bands was resolved in the crude leptospiral lipase after centrifugation

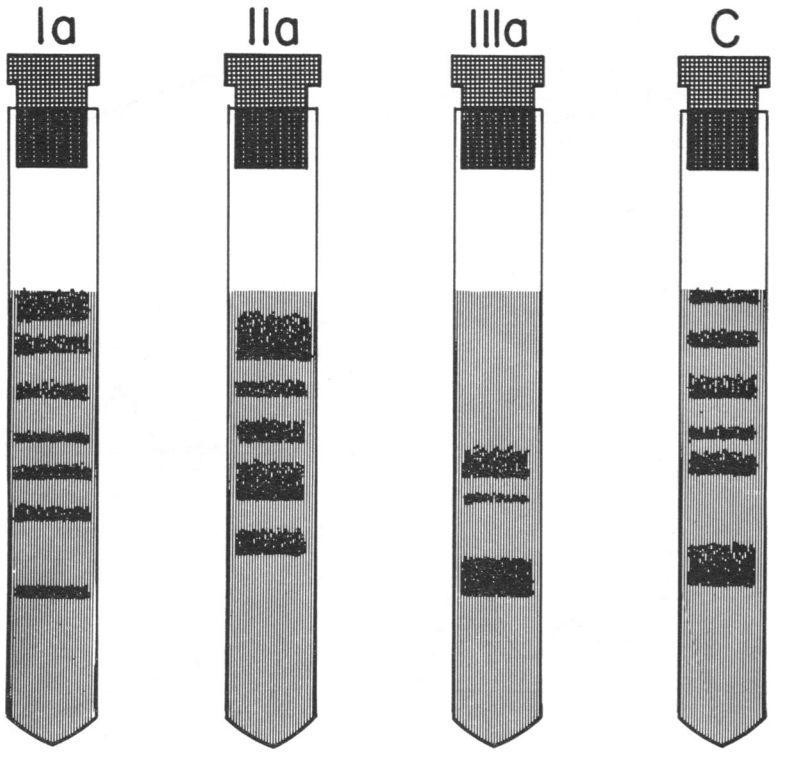

FIG. 4. *Diagram of disc electrophoresis of Sephadex fractions Ia, IIa, and IIIa and original lipase C.*

for 8 hr at 125,000 × *g*). An homogeneous, brown upper band and a white, particulate lower band were arbitrarily designated fractions I and II, respectively.

Buoyant density determinations of fractions I and II revealed optimal enzyme activity at 1.140 and 1.260 g/ml, respectively. Additional spectrophotometric analysis of the separate fractions showed the presence of two protein peaks in each fraction. In fraction I, an active peak at a buoyant density of 1.120 g/ml and an inactive peak at 1.230 g/ml were demonstrated (Fig. 2). In fraction II, an inactive peak at a density of 1.20 g/ml and an active peak at 1.260 g/ml were demonstrated (Fig. 3).

Electrophoresis. The disc electrophoresis of the crude preparation and Sephadex fractions showed numerous protein bands (Fig. 4). Analysis of fraction I revealed three prominent bands and two diffuse bands, whereas fraction II had one prominent band and four or more diffuse bands (Fig. 5).

Esterase activity was found in the leptospiral preparation by the use of both triacetate and tributyrate as substrates. The density gradient fractions showed no enzyme activity with triacetate as substrate. However, all the Sephadex and the density gradient fractions had esterase activity against the butyrate. The control media (uninoculated) also had two bands of esterase activity which migrated similarly to some of the lipase fractions (Fig. 6).

Substrates. Maximal activity against tributyrin was obtained with crude lipase and fractions I and II. Increased activity was observed with the unsaturated triglyceride trilinolein by use of the crude preparation and fraction II. Fraction II also had increased activity with palmitic methyl ester. In general, all of the lipase fractions showed decreased activity with increased saturated acyl side chains. For the methyl ester, the rate of hydrolysis was low for all lipase preparations.

Optimal *p*H, temperature, and stability of the density-gradient fractions were similar to those found by Patel et al. (14) with leptospiral lipase.

DISCUSSION

Many lipolytic enzymes also have esterase activity and are complex mixtures with multiple substrate specificities. Most fatty acid esterases attack short-chain esters as opposed to lipases, which hydrolyze long-chain esters. In view of the observed overlapping activity, clearer terminology has come about in redefining esterases and lipases.

Enzymes which hydrolyze soluble substrates are termed esterases and those which hydrolyze insoluble substrates are termed lipases. Another possible method of differentiating these enzymes makes use of the fact that diisopropylfluorophosphate inhibits esterases and not lipases (4).

Lipolytic activity in the genus *Leptospira* has been reported by a number of investigators (2, 7, 13, 14). These reports indicate that lipase production was variable throughout the genus. The results presented here agree with those of Parnas et al. (13) in that approximately two-thirds of the cultures tested produced lipase.

Esterase activity was present in the crude lipase, density-gradient, and Sephadex fractions, confirming the overlapping specificities that arise in this group of enzymes.

Tributyrin has been noted to be the most acceptable substrate for leptospiral lipase; it was also observed that other substrates can function in this capacity. The hydrolysis of palmitic methyl ester suggested esterase activity, and this was demonstrated in the control medium, crude lipase, and all isolated fractions. These general findings have been reported for other lipase investigations (4). The identification of esterase in the fractions indicates that attempts to classify the lipase as a tributyrinase requires careful examination of the enzyme preparations for additional proteins with enzymatic activity. Since leptospiral lipase exhibits greatest activity against a tributyrin substrate, some authors have attempted to classify such enzymes as trybutyrinases (2, 7). However, with the positive identification of an enzyme which shows affinity for a synthetic ester, it seems doubtful that such a classification of leptospiral or, for that matter, microbial lipases could be valid. This is particularly true in instances where esterase activity was not reported and where whole bacterial cultures, crude media extracts, or precipitates were used to demonstrate tributyrinase activity.

In view of the presence of a number of protein bands from the chromatographic and gradient analysis, it was assumed that these bands had different enzymatic properties. The subsequent investigation of the separated fractions substantiated this hypothesis.

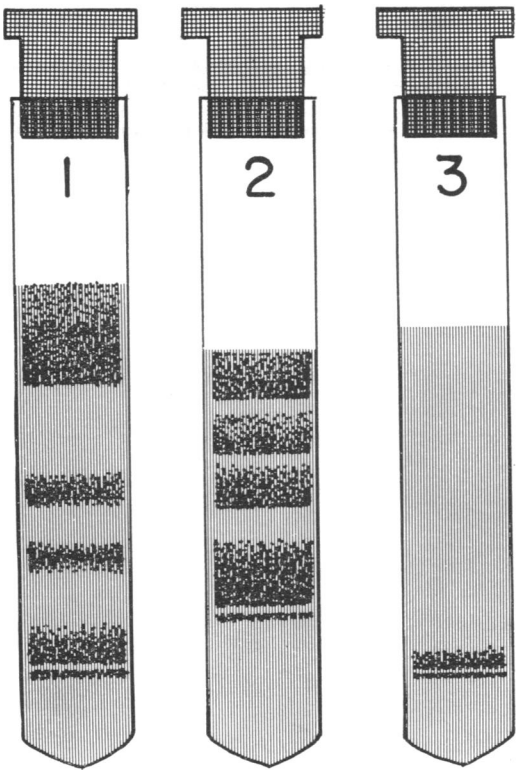

FIG. 5. *Disc electrophoresis of fraction II (1), fraction I (2), and microbial lipase [Mann (3)].*

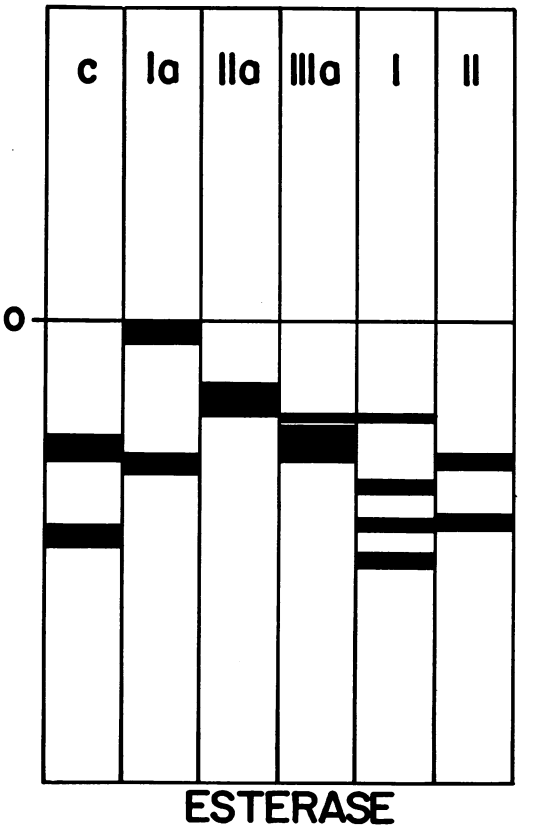

FIG. 6. *Diagram of esterase patterns from lipase fractions. Symbols: c, control media; Ia, IIa, and IIIa, sephadex fractions; I and II, CsCl gradients.*

Fraction Ia had a greater lipolytic activity than did the other fractions, and some extraneous protein was removed, as indicated by its total protein content. This is not seen in the other Sephadex fractions since there is a decrease in both protein content and lipolytic activity when compared to the lipase of Patel et al. (14). The density gradient fractions had very similar activity, but fraction II had 20% less protein. This may constitute removal of some esterase activity (Fig. 6) but not purification of the true lipolytic portion of the preparation.

It is evident from the esterase patterns that the media had activity and that this was not removed from all fractions. Aside from the control media, there were similarities and differences in the electrophoretic mobility. These differences include areas of activity (fractions Ia, IIa, and I) that are not common to the other fractions tested. The question of leptospiral isozymes is brought out at this point and can only be resolved by further investigation of these preparations.

Additional work is also necessary to definitely determine whether the enzymic activity is truly lypolytic or esterolytic, or whether both activities will be retained by a pure enzyme preparation.

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