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The extracellular nature of the lipase of *Pseudomonas fragi*

The lipase (glycerol ester hydrolase, EC 3.1.1.3) of *Pseudomonas fragi* exhibits a substrate specificity[1] similar to that reported by DESNUELLE AND SAVARY[2] for pancreatic lipase and is of interest in that it could be used as a tool for determination of triglyceride structure. This interest has stimulated further investigations into the nature and characteristics of the enzyme. During these investigations it became apparent that although the lipase had been assumed to be an extracellular enzyme[3], there were no definitive data to support the assumption. POLLOCK[4] points out that the mere presence of an enzyme in the culture medium is not sufficient proof of the extracellular nature of the enzyme; it must be established that the enzyme has not been released into the medium by cell autolysis. This objective can be achieved by demonstrating that (1) the enzyme appears early in the growth of the culture when cells are not prone to lysis; (2) the enzyme is not found inside the cells; and (3) when the enzyme appears in the culture medium, an intracellular marker does not simultaneously appear. These three criteria were satisfied in this investigation.

The first criterion was satisfied by determining at various time intervals the lipase activity and turbidity of cultures of *P. fragi* incubated statically at 20° in a synthetic medium[5]. Fig. 1 shows that lipase activity was present in the medium as early as 16 h, paralleled the increase in turbidity, and reached a maximum at 32 h.

To demonstrate that the lipase is not inside the cells, suspensions of washed cells were ruptured in a French pressure cell after one and two days incubation. The data in Table I show that no significant lipase activity was detected in the cell extract

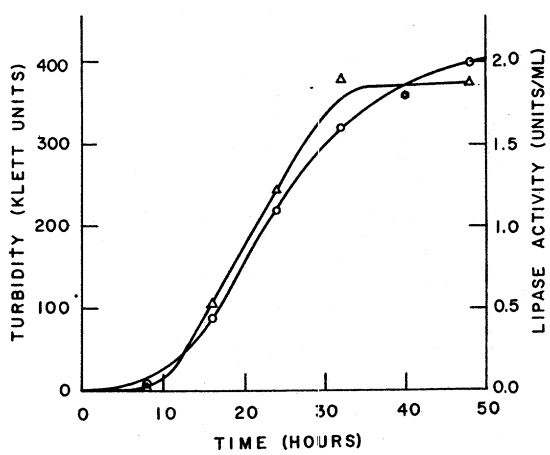

Fig. 1. Lipase activity in culture supernatant as related to growth of *P. fragi*. The organism, medium, and lipase assay used were as described by ALFORD AND PIERCE[5] except that $(NH_4)_2SO_4$ was omitted from the medium. Turbidity, as measured with the Klett colorimeter (no. 66 filter), and the lipase activity were determined on parallel cultures of 20 ml of medium per 125 ml Erlenmeyer flasks incubated statically at 20° for the times indicated. Unit of lipase activity is defined as that amount of enzyme which liberates 1 microequivalent of acid per min under the assay conditions. △——△, lipase activity; ○——○, turbidity.

TABLE I

DISTRIBUTION OF LIPASE ACTIVITY IN CULTURE OF *P. fragi*

	1st day assay		*2nd day assay*	
	Lipase activity (units)*	*Protein*** (mg)*	*Lipase activity (units)*	*Protein (mg)*
Culture supernatant*	3.6	0.3	2.6	0.2
Cell extract**	0.2	16.0	0.1	28.8
Cell debris**	0.1	7.6	0.2	23.6

* Culture supernatant was obtained by centrifugation of the culture at 0° at 13 200 × *g* for 10 min. Unit of lipase activity, growth medium, and growth conditions as in legend in Fig. 1.
** Cell extract from a 250-ml culture was prepared by suspending twice-washed cells in 10 ml of 0.25 M phosphate buffer (pH 6.0), rupturing in a chilled French pressure cell (American Instrument Company) at about 20 000 lb/in², and centrifuging at 0° at 34 500 × *g* for 30 min. The sediment, taken up in 10 ml of 0.25 M phosphate buffer (pH 7.0), was designated as cell debris.
*** Protein was determined by the method of LOWRY *et al.*[6] with human serum albumin as standard.

or debris at any time during the period of active growth and enzyme synthesis. Had the lipase activity been intracellular or cell-bound, it should certainly have been detected at two days when the assays of the cell extract and of the cell debris contained over 100 times more protein than that of the culture supernatant. When subjected to similar treatment in the French press, the culture supernatant lost only 25% of its lipase activity.

The degree of lysis occurring in the culture was determined by following the release into the culture medium of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49), an intracellular enzyme. This enzyme was chosen as a marker because it was found in appreciable quantity in the cell extracts of *P. fragi*. Further, control experiments had ascertained that the enzyme could be detected if as little as 1% of the culture had lysed and that the enzyme was

TABLE II

GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY AND LIPASE ACTIVITY IN CULTURE SUPERNATANT OF *P. fragi*

Cultural conditions, lipase assay, and medium as described in legend of Fig. 1.

Incubation time (h)	*G6PD activity* (units/ml)*	*Lipase activity (units/ml)*	*% lysis***
24	<0.02	1.07	<1.0
48	0.02	1.85	1.0
72	0.13	2.20	6.5

* Glucose-6-phosphate dehydrogenase activity (G6PD) was determined by measuring reduced nicotinamide adenine dinucleotide phosphate[7] with a Beckman Model DB spectrophotometer at 340 mμ. A unit of activity is defined as the amount of enzyme which causes a change of 0.001 absorbance units/min.
** Per cent lysis was estimated by the following equation:

$$\% \text{ Lysis} = \frac{[\text{G6PD}]_c \times 100}{[\text{G6PD}]_e \times \text{total protein}}$$

in which:
$[\text{G6PD}]_c$ is units of G6PD per ml of culture supernatant;
$[\text{G6PD}]_e$ is units of G6PD per mg of protein in cell extract;
total protein is protein in culture (supernatant plus cellular protein) in mg/ml.

stable for at least three days in a growing culture. Table II clearly shows that 50% of the total lipase activity appeared within 24 h, at which time there had been less than 1% lysis. Moreover, the six- to seven-fold increase in lysis which occurred between 48 and 72 h was accompanied by only a 1.2-fold increase in lipase activity.

Although the extracellular nature of this enzyme cannot be unequivocally established without the demonstration of its liberation from individual cells, it has been determined to a very high degree of probability.

We wish to acknowledge the technical assistance of Mrs. ELSIE E. STEINLE of this laboratory. This work is part of a dissertation to be submitted by the senior author to the Department of Microbiology and Tropical Medicine, Georgetown University School of Medicine, Washington, D.C. (U.S.A) in partial fulfillment of the requirements for the Ph.D. degree.

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Received June 4th, 1965