Localization and Characterization of Lipolytic Enzymes Produced by *Lysobacter enzymogenes*

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Lipolysis by *Lysobacter enzymogenes* is due to the production of two major extracellular esterases. These were investigated using a turbidimetric assay with Tween 20 as the substrate. One esterase is cell-associated and found in the particulate fraction of cell-free extracts, the other esterase is secreted into the culture medium. In batch culture with 0.8% yeast extract as the medium the particulate esterase was produced mainly during the exponential growth phase and its yield was increased about twofold by the addition of olive oil and other substrates. The supernatant esterase was produced mainly after the exponential growth phase and was induced six- to tenfold by olive oil. PAGE, combined with activity staining using Tween 20 as the substrate, indicated that the two enzymes have different electrophoretic mobilities. Intact cells expressed 87% of the particulate cell-associated esterase activity. The enzyme was released from cells or from the particulate fraction by incubation with 0.2% Zwittergent 3-14. Tween 20 (2%, v/v) or 1 mM-EDTA released little of the enzyme from whole cells. Separation of the inner and outer membranes of *L. enzymogenes* showed that the particulate esterase was localized in the outer membrane. Both esterases were very active with Tween 20 as the substrate, but they hydrolysed other compounds at very different rates. The relative activities with Tween 20, *p*-nitrophenyl palmitate, tributyrin and olive oil were 100, 3, 11 and 0 for the particulate esterase and 100, 73, 0 and 28 for the secreted esterase. In addition to these two extracellular esterases, *L. enzymogenes* contains a hydrolase in the cytoplasm which is most active with tributyrin.

INTRODUCTION

The genus *Lysobacter* contains Gram-negative gliding bacteria which are noted for the production of many extracellular enzymes and for their role in biodegradation (Christensen & Cook, 1978; Reichenbach & Dworkin, 1981; Rosenberg & Varon, 1984). All species of the genus, except two strains of *Lysobacter brunescens*, were found to hydrolyse Tween when tested on solid media containing this detergent (Christensen & Cook, 1978). A number of the extracellular enzymes produced by *Lysobacter* species have been characterized (Jackson & Matsueda, 1970; Whitaker, 1970; Hedges & Wolfe, 1974; von Tigerstrom, 1980, 1981, 1984; von Tigerstrom & Stelmashuk, 1986, 1987*b*), but the enzymes responsible for the lipase activity have not been investigated.

Preliminary experiments indicated that *L. enzymogenes* produces two extracellular Tween-degrading hydrolases: one associated with the cells, the other secreted into the culture medium. Although the secreted enzyme can use olive oil as a substrate and might be called a lipase (Jensen, 1983), both enzymes prefer water-soluble substrates and for this reason we call the enzymes esterases rather than lipases (Sugiura, 1984).

This paper describes the production of the two esterases by *L. enzymogenes* under different culture conditions, the major differences between the two enzymes and the localization of the cell-associated esterase.

Abbreviations: pNPP, *p*-nitrophenyl palmitate; KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonate).

METHODS

Chemicals. The substrates used for the enzyme assays were obtained from Sigma except the β -lactamase substrate (PADAC) which was from Calbiochem. Phosphorylase *b* and cytochrome *c* also were from Sigma and the other protein standards for the SDS-PAGE were from Pierce. Catalase from bovine liver was obtained from Calbiochem and *Escherichia coli* C₄F₁ phosphatase was prepared in this laboratory (von Tigerstrom, 1983).

Organisms and culture conditions. *Lysobacter enzymogenes* UASM 495 (ATCC 2987) and the other *Lysobacter* species were obtained from Dr F. D. Cook, University of Alberta. Except where indicated otherwise, liquid cultures were grown at 22 °C in 0.8% (w/v) yeast extract, 0.1% (v/v) olive oil as described earlier (von Tigerstrom, 1980). The olive oil was not emulsified. It was added to the medium before autoclaving and became dispersed during the shaking of the culture. The minimal medium contained 15 mM-potassium phosphate, 7.6 mM-(NH₄)₂SO₄, 0.5 mM-MgCl₂, 0.2% sodium aspartate, 0.5% glucose, 0.5 µg FeSO₄ ml⁻¹ and trace metals, pH 7.2. The cultures were harvested, usually in the stationary growth phase, by centrifugation at 16300 *g* for 15 min at 4 °C to obtain the cells and the culture supernatant. This removed 99.99% of the viable cells from the culture supernatant. The cell-free extract was prepared by suspending the cells in 10 mM-Tris/HCl, 50 mM-NaCl, 1.5M-MgCl₂, pH 7.4, and disrupting the cells with a French pressure cell at 6.9 kPa. Intact cells were removed by centrifugation at 3020 *g* for 15 min.

Enzyme assays. Esterase activities were determined routinely by a turbidimetric assay using Tween 20 as the substrate (von Tigerstrom & Stelmashuk, 1989). The assay solution contained 20 mM-Tris/HCl, 1.8% (v/v) Tween 20, 3 mM-CaCl₂, pH 8.5. The increase in the optical density at 500 nm (OD₅₀₀) was determined at 37 °C after addition of the enzyme source. A volume of 0.3 ml of enzyme source, containing up to 0.15 units of activity, was added to 3.7 ml of assay solution. Samples of 0.9 ml were removed at timed intervals to determine the OD₅₀₀. The rate of increase of OD₅₀₀ was linear up to OD₅₀₀ 1.2 and the rate of increase was essentially zero without added enzyme source. The spectrophotometric esterase assay with *p*-nitrophenyl palmitate (pNPP) (Winkler & Stuckman, 1979) and the titrimetric assay with olive oil and tributyrin have been described previously (von Tigerstrom & Stelmashuk, 1989).

Cell-free extracts and cells incubated with 0.2% (w/v) Zwittergent 3-14 for 30 min at 37 °C were used as sources of the cell-associated esterase and the culture supernatants as the source of the supernatant esterase. These were diluted with 20 mM-Tris/HCl, pH 8.0, if necessary. The activity of the cell-associated esterase was twofold higher at 37 °C than at 22 °C and the supernatant esterase had the same activity at both temperatures. The cell-associated enzyme was stable for at least 2 h at 22 °C and 37 °C and the secreted enzyme was stable for at least 2 h at 22 °C and lost only 10% of its activity in 2 h at 37 °C. The presence of Zwittergent 3-14 had little or no effect on the stabilities.

Catalase (Chance & Maehly, 1955), alkaline phosphatase (von Tigerstrom, 1984), NADH oxidase (Osborne *et al.*, 1972) and glucose-6-phosphate dehydrogenase (DeMoss, 1955) were assayed as described in the references. β -Lactamase was assayed by following the absorbance at 570 nm of an assay solution containing 50 mM-potassium phosphate, 4 µg PADAC ml⁻¹, pH 7.0. Enzyme activities are expressed in units. In the catalase assay, 1 unit = a change of 1 A₂₃₀ unit min⁻¹. For all other enzymes, 1 unit = 1 µmol product formed min⁻¹. It was established by comparison with a titrimetric assay that a change of 1.0 OD₅₀₀ unit in the turbidimetric assay with Tween 20 is equivalent to the release of 0.91 µmol fatty acid (ml of reaction mixture)⁻¹.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out on slabs of 10% (w/v) polyacrylamide using the method of Maizel (1971), as described earlier (von Tigerstrom & Stelmashuk, 1985). The protein standards were prepared under the usual denaturing conditions. The esterase preparations were dialysed against 10 mM-Tris/HCl, pH 8.0, and, in order to preserve the enzymic activities, they were made to 1% (w/v) SDS just before application and were not heated. Staining for protein was done by the method of Fairbanks *et al.* (1971). To locate the esterase activity in gels, the gels were blotted briefly to remove excess moisture and overlaid with 1% (w/v) agarose, 50 mM-Tris/HCl, 1.8% (v/v) Tween 20, 3 mM-CaCl₂, pH 8.5, on 'Gel Bond Film' (Marine Colloids Division, FMC Corporation). They were then incubated at 37 °C for about 30 min, or until bands of precipitation developed in the overlay. The overlay was photographed against a dark background or the positions of the bands were marked on the 'Gel Bond Film' in order to obtain a permanent record of the activity stain.

Separation of cell wall components. This was done by sucrose gradient centrifugation using the method of Hancock & Nakaido (1978). The details of the procedure used for *L. enzymogenes* have been reported (von Tigerstrom & Stelmashuk, 1985).

Preparation of shock fluid. Cells grown to early stationary phase were subjected to the MgCl₂-shocking procedure of Cheng *et al.* (1970) as described in detail for *L. enzymogenes* (von Tigerstrom & Stelmashuk, 1985). A cell-free extract was prepared from the shocked cells as described above and this was centrifuged for 60 min to obtain the particulate fraction (48000 *g* pellet) and the cytoplasmic fraction (48000 *g* supernatant).

Glycerol gradient centrifugation. To obtain an estimate of the *M_r* of the native *L. enzymogenes* esterases, the enzymes were treated with 0.2% (w/v) Zwittergent 3-14 in 10 mM-Tris/HCl, pH 8.0, and centrifuged through a glycerol gradient as described by von Tigerstrom & Stelmashuk (1986), except that the gradient also contained 0.05% (w/v) Zwittergent 3-14. Catalase (*M_r* 230000), *Escherichia coli* alkaline phosphatase (*M_r* 84000), ovalbumin (*M_r* 45000) and cytochrome *c* (*M_r* 12500) were also used as the standards.

Analytical methods. Protein concentrations were determined by the Lowry method. Controls were included to correct for interference due to buffer components and detergents. The absorbance at 280 nm (A_{280}) was used to estimate the protein in the fractions of the sucrose and glycerol gradients. In the sucrose gradients approximately 50% of the A_{280} values was due to turbidity. Cytochrome *c* was detected by determining the A_{280} and the A_{530} . Concentrations of 2-keto-3-deoxyoctonate (KDO) were estimated according to the method of Law & Slepecky (1961).

RESULTS AND DISCUSSION

Esterase production

The esterase production by different species of *Lysobacter* was determined. We found that *L. antibioticus*, *L. enzymogenes* and *L. gummosus* produced similar levels of cell-associated esterase [from 0.4 to 0.7 units (ml of culture)⁻¹] in 0.8% (w/v) tryptone broth when grown to OD₆₀₀ values of 2.0, 2.7 and 2.9, respectively. *L. brunescens* produced only about 0.1 unit ml⁻¹ when grown to OD₆₀₀ 1.7 in the same medium supplemented with 2 mM-MgCl₂ to prevent cell lysis. The esterase activities in the culture supernatants were generally low, about 0.01 units ml⁻¹.

L. enzymogenes, which has been used in a number of enzyme studies (Whitaker, 1970; von Tigerstrom, 1980, 1981, 1983, 1984; von Tigerstrom & Stelmaschuk, 1985, 1986, 1987*a*), was chosen for further experiments. We had noted that the composition of the medium had a great effect on the production of extracellular enzymes by *L. enzymogenes* (von Tigerstrom, 1980, 1983); therefore, the organism was grown in different media in order to try and increase the esterase production. The activities of the cell-associated esterase were 0.7 units ml⁻¹ for cells grown in tryptone or yeast extract broth, 0.6 units ml⁻¹ for cells grown in soy peptone broth and 0.4 units ml⁻¹ for cells grown in peptone broth or minimal medium. The supernatant esterase activities were similar (0.1 to 0.2 units ml⁻¹) in all the media except for tryptone broth (usually only 0.01 units, ml⁻¹).

Yeast extract broth was chosen for studies of the enzyme production because *L. enzymogenes* grew well in this medium and produced relatively high levels of both esterases. The effects of various supplements to the growth medium on the esterase yield are shown in Table 1. The additions had little or no effect on cell growth, but all increased the yield of the cell-associated esterase. Their effects on the activity of the supernatant esterase were more specific. Mineral oil decreased the supernatant esterase activity, but no effect was seen with tributyrin. However, Tween 20 and olive oil increased the activities, two- and sixfold, respectively. It is interesting that olive oil had a great effect on the yield of the supernatant esterase, but not on the cell-associated esterase. As will be shown below, olive oil is a substrate for the supernatant enzyme only. Therefore, 0.8% yeast extract, 0.1% olive oil was used routinely as the medium for the production of the supernatant esterase and 0.8% yeast extract, with or without olive oil, for the production of the cell-associated esterase. Mineral oil was included as a control to show that the increased activity is specific for substances that might be substrates for the enzyme. The result

Table 1. *Effect of supplements to the growth medium on the production of cell-associated and supernatant esterases by L. enzymogenes*

The organism was grown in 0.8% yeast extract with the additions (0.1% v/v) indicated. The cultures were harvested in the early stationary phase. The cell-associated esterase activities were determined in the cells after treatment with Zwittergen 3-14 and the supernatant esterase was determined in the culture supernatant.

Addition	Esterase activity (units ml ⁻¹)	
	Cells	Supernatant
None	0.63	0.11
Tween 20	1.1	0.23
Tributyrin	1.1	0.12
Olive oil	1.2	0.61
Mineral oil	1.0	0.051

also indicated that the increased activity in the supernatant when olive oil is added is not due to release of the enzyme from the cells in the presence of a hydrophobic substance, since it does not occur with mineral oil or tributyrin.

Further evidence that olive oil increased the production of the supernatant enzyme, rather than enhanced the release of the esterase from the cell surface, was obtained in experiments involving inhibition of protein synthesis. When olive oil was added to a culture growing in 0.8% yeast extract medium in the early stationary phase, the esterase activity in the supernatant increased twofold over the next 6 h as compared to the control without olive oil. This increase was prevented by the addition of 50 µg chloramphenicol ml⁻¹ and was, therefore, due to the synthesis of the enzyme. In addition, incubation of the cell-associated or supernatant enzyme with olive oil did not increase the enzyme activities, indicating that the olive oil did not activate the enzymes.

The growth of the organism and the production of the two enzymes in 0.8% yeast extract broth and 0.8% yeast extract, 0.1% olive oil are shown in Fig. 1. The growth was followed by determining the increase in the cell-associated protein, since OD₆₀₀ determinations, especially during the first 5 h, were unreliable in the culture containing olive oil due to the interference by the emulsion. This growth corresponded to an increase in the OD₆₀₀ of *L. enzymogenes* from 0.23 to a final OD₆₀₀ of 5.3. After a short lag, the cultures grew exponentially for about 10 h with a generation time of 140 min.

The rate of production of the cell-associated esterase was exponential during the active growth period. Little or no enzyme was produced during the stationary phase and the yield of this esterase was approximately doubled in the presence of olive oil. Thus, the cell-associated esterase seems to be produced constitutively and its production is unaffected by catabolite repression which would be greatest during exponential growth. The production of the supernatant esterase is induced by olive oil. Since it is produced after the exponential growth phase it seems likely that the enzyme is under catabolite repression. Although catabolite repression is not understood in detail in *Lysobacter* and many other organisms, this interpretation is consistent with current ideas on the control of extracellular enzyme biosynthesis (Priest, 1984).

The results in Fig. 1 and Table 1 indicate that *L. enzymogenes* produces at least two different esterases. They do not rule out a possible precursor-product relationship between the two enzymes. However, as will be shown below, differences in electrophoretic mobilities and substrate specificities of the two enzymes makes this an unlikely possibility.

PAGE of esterase preparations

The *L. enzymogenes* esterases differ with respect to their cellular locations, times of production in batch culture and, probably, in their biosynthetic control mechanisms, as just described. In addition, after PAGE of the crude esterase preparations in the presence of SDS each enzyme had one clearly defined band of activity (Fig. 2) and a mixture of the two resulted in the two separate bands. After prolonged incubation of the activity stain, a faint second band (with the faster mobility of the supernatant enzyme) could be observed with the cell extract. Thus, the cell extract may contain a trace of the active supernatant esterase, but the supernatant appears to be free of the cell-associated enzyme.

The supernatant esterase has a faster electrophoretic mobility and, therefore, probably a lower M_r than the cell-associated enzyme. The electrophoretic mobilities could not be used to calculate the M_r values for the enzymes since the esterase preparations were not heated in the presence of SDS before PAGE was carried out. The M_r markers were included to indicate the approximate sizes of the enzymes.

Glycerol gradient centrifugation also was carried out on the partially purified esterases. The cell-associated enzyme was found just before the ovalbumin peak and the secreted enzyme just after the ovalbumin peak. Therefore the M_r values of the esterases are quite similar: 45 ± 5 kDa for the cell-associated esterase and 38 ± 5 kDa for the supernatant esterase.

Fig. 1

Fig. 1. Growth and production of esterases by *L. enzymogenes*. The organism was grown in 0.8% yeast extract, 0.1% olive oil (●) and 0.8% yeast extract (○) and growth was determined by the increase in amount of cell-associated protein. The cell-associated esterase activities in the culture with (■) and without (□) olive oil and the supernatant esterase activities in the culture with (▲) and without (△) olive oil were determined. This experiment is representative of two carried out with the same media. It was also carried out with 0.8% tryptone but much lower activities were obtained.

Fig. 2

Fig. 2. PAGE. The organism was grown in 0.8% yeast extract, 0.1% olive oil and the cell-free extract and the culture supernatant were prepared as described in Methods and dialysed against 10 mM-Tris/HCl pH 8.0. The electrophoresis and staining of esterase activity in the gel are described in Methods. The overlay was in contact with the gel for 1 h at 37 °C and the photograph was taken after 5 h incubation at 22 °C of the overlay alone. Lane 1 contained 20 µl cell-free extract (0.03 esterase units), lane 2 contained 75 µl supernatant (0.03 esterase units) and lane 3 a mixture of the two preparations. Protein staining (not shown) resulted in multiple bands for each preparation but none seemed to correspond to the activity bands. The experiment was carried out three times and was very reproducible.

Localization of the cell-associated esterase

We noted that the cell-associated esterase could be determined reliably both in cell extracts and in cells treated with Zwittergent 3-14 for 30 min to obtain maximum activity. Whole, untreated cells expressed $87 \pm 1.7\%$ of the maximum activity (mean $\pm$ SD, $n = 3$). Since whole cells express much of the esterase activity, the enzyme is probably located at the cell surface,

Table 2. Release of cell-associated esterase from *L. enzymogenes* cells

The additions were made to a cell suspension of $OD_{600} = 4.8$. The cell suspensions were incubated for 30 min at 37 °C before centrifuging a sample of each at 17400 *g* for 10 min. The cell suspensions and the resulting supernatant solutions were assayed for esterase activities. Centrifugation immediately after the additions released 0% to 2% of the activities. Although the preincubation with Tween 20 reduces the activity, none of the compounds added interfered with the assay.

Addition	Esterase activity (units ml ⁻¹)		
	Total	Released	Percentage of activity released
None	0.65	~0	~0
Zwittergent 3-14 (to 0.2%)	0.75	0.70	93
Tween 20 (to 2%)	0.37	0.012	3
EDTA (to 1 mM)	0.67	0.028	4
Tween 20 + EDTA	0.27	0.10	37

readily accessible to the substrate. An alternative explanation would be that the Tween 20 used as the substrate could have solubilized the enzyme during the assay. This was investigated and the results are shown in Table 2. The table compares the release of the esterase from whole cells exposed to Tween 20, Zwittergent 3-14 or EDTA. Only 3% of the activity was released after 30 min incubation with Tween 20, whereas considerably more was released with a combination of Tween 20 and EDTA, or with Zwittergent 3-14. Therefore, the results indicate that the enzyme is quite tightly bound, and active, when associated with the cell surface.

When cell-free extracts of *L. enzymogenes* were centrifuged at 48000 *g*, the cell-associated esterase was mainly present in the particulate (48000 *g* pellet) fraction. This step, and subsequent solubilization from the particulate fraction with Zwittergent 3-14, have been used to partially purify the enzyme (von Tigerstrom & Stelmaschuk, 1989). The particulate nature of the enzyme and the solubilization with detergent indicated that the esterase might be membrane-associated.

The outer membrane and the cytoplasmic membrane of *L. enzymogenes* were separated by sucrose gradient centrifugation (Hancock & Nikaido, 1978; von Tigerstrom & Stelmaschuk, 1985) to determine the location of the particulate cell-associated esterase. The results are shown in Fig. 3. The NADH oxidase and the cell-associated phosphatase (Osborne *et al.*, 1972; von Tigerstrom & Stelmaschuk, 1985) were used as marker enzymes for the cytoplasmic membrane and outer membrane, respectively. In addition, KDO concentrations were determined in pooled fractions. Fractions 3–5, fractions 6–8 and fractions 13–16 contained 1.3, 0.9, and 0.2 µg KDP ml⁻¹, respectively. The results indicate that the enzyme is associated with the outer membrane of the organism.

Substrate specificities

The esterases have different substrate specificities, as indicated in Table 3. Cell-free extract was centrifuged to obtain the 48000 *g* insoluble (particulate) fraction and the 48000 *g* soluble fraction. The particulate cell-associated enzyme was most active with Tween 20. It degraded tributyrin at a much lower rate and had little or no activity with pNPP or olive oil. The enzyme in the culture supernatant also was most active with Tween 20. In contrast to the particulate cell-associated enzyme, it degraded pNPP and olive oil relatively rapidly and tributyrin at a very low rate or not at all. The analysis of the soluble portion of the cell-free extract indicated that *L. enzymogenes* contained another lipolytic activity which was most active with tributyrin.

In an attempt to determine the location of the 'tributyrin hydrolase', we subjected whole cells to the MgCl₂-shocking procedure and obtained periplasmic, particulate and cytoplasmic fractions as described in Methods. Results from two experiments indicated that the cytoplasmic fraction contained 79 ± 3% of the 'tributyrin hydrolase' and, as expected, 93 ± 2% of the

Fig. 3. Localization of the cell-associated esterase by sucrose density gradient centrifugation. Cell-free extract (5.7 ml per tube) prepared from a cell suspension with an OD_{600} of 25 was used as described in Methods. The activity of the esterase (●), NADH oxidase (■), outer-membrane-associated phosphatase (○), and the A_{280} (□) were determined. The experiment was done twice in duplicate and found to be very reproducible.

Table 3. *Distribution of the esterases and their activities with different substrates*

The organism was grown to early stationary phase. The esterase activities were determined in the pellet (particulate) and supernatant (soluble) fraction of cell-free extract, obtained by centrifugation at 48000 g for 60 min, and in the culture supernatant. The numbers in parentheses are relative values. Activities obtained with Tween 20 were arbitrarily assigned a value of 100.

Substrate	Esterase activity [units ml culture ⁻¹]		
	Cell extract		Culture supernatant
	Particulate	Soluble	
Tween 20	0.93 (100)	0.28 (100)	0.74 (100)
pNPP	0.027 (3)	0.025 (9)	0.54 (73)
Tributyrin	0.10 (11)	2.28 (814)	~0 (~0)
Olive oil	~0 (~0)	~0 (~0)	0.21 (28)

marker enzyme, glucose-6-phosphate dehydrogenase. The periplasmic fraction contained $63 \pm 6\%$ of the cellular β -lactamase but only $4 \pm 4\%$ of the 'tributyrin hydrolase'. Therefore, the 'tributyrin hydrolase' appears to be cytoplasmic and probably contributes little to the extracellular esterase activity of *L. enzymogenes*. We have not attempted to further characterize this enzyme.

Purification of the esterases

Purification of the esterases has been difficult, especially in the case of the secreted enzyme. The secreted esterase was concentrated tenfold from 2 litres of the culture supernatant by ammonium sulphate precipitation at 75% saturation. This also concentrated gummy material, probably polysaccharide, resulting in a very viscous solution and little purification. The viscosity could be decreased somewhat by vigorous homogenization in a Waring blender. Enzyme instability was a major obstacle to further purification. For example, after dialysis against 2 mM-Tris/HCl, pH 8.0, and ion-exchange chromatography with DEAE-Sephacel, more than 90% of the activity was lost.

The cell-associated esterase is more stable. Using cells from 2 litres of culture, the enzyme was purified 19-fold with a 23% yield. Cell-free extract was prepared and centrifuged at 48000 g for 60 min to obtain crude cell envelopes containing 70% of the esterase activity. The cell envelopes were extracted with 2% Triton X-100 to remove some particulate proteins and then the esterase was solubilized with 0.2% Zwittergent in 20 mM-Tris/HCl, pH 8.0. It was purified by chromatography on a DEAE-Sephacel column in the presence of 0.1% Zwittergent 3-14 and by gel filtration using Sephacryl S-200. Electrophoresis of the final preparation, carried out as described for Fig. 2, indicated that many hydrophobic proteins had co-purified with the enzyme. A number of other purification methods were explored unsuccessfully. Thus, further work to try and obtain highly purified esterases in microgram quantities does not seem to be justified at this time.

Other bacterial lipolytic enzymes have been described in the literature. Sugiura (1984) reviewed the subject and reported the purification of excreted lipases from *Pseudomonas fluorescens* and *Chromobacterium viscosum*. Extracellular lipases of two other pseudomonads, *P. nitroreducens* and *P. fragi* were studied by Watanabe *et al.* (1977). The effect of polysaccharides on exolipase production was determined in *Serratia marcescens* (Winkler & Stuckmann, 1979) and the influence of cultural conditions on the production of lipolytic enzymes in a number of microbes was reported by Sztajer & Maliszewska (1988).

The lipolytic enzymes of *Pseudomonas aeruginosa* seem to be the most interesting with respect to the *Lysobacter* enzymes. In addition to a phospholipase (Berka & Vasil, 1982), this organism also produces two esterases. As is the case in *L. enzymogenes*, one esterase is located in the outer membrane (Ohkawa *et al.*, 1979) and the other is secreted (Stuer *et al.*, 1986). The times of production of the *P. aeruginosa* enzymes during the cell growth cycle seem to be similar to those observed with *L. enzymogenes* and we noted recently that the two *P. aeruginosa* enzymes also are very active with Tween 20 as the substrate (von Tigerstrom & Stelmashuk, 1989).

The *Lysobacter* esterases probably have a nutritional function. They are two more enzymes in a large arsenal of extracellular hydrolases, such as proteases (Jackson & Matsueda, 1970; Whitaker, 1970), DNA- and RNA-degrading enzymes (von Tigerstrom, 1980, 1981), phosphatases (von Tigerstrom, 1984, von Tigerstrom & Stelmashuk, 1986), β -glucanase and chitinase (Hedges & Wolfe, 1974) and amylases (von Tigerstrom & Stelmashuk, 1987b), that these organisms produce to degrade generally high- M_r substances in their environment.

Of the many extracellular hydrolases produced by *Lysobacter*, some are produced in 'duplicate', one being cell-associated, the other being secreted into the medium. This was shown to be true for the two phosphatases of *L. enzymogenes* (von Tigerstrom, 1984) and now appears to be true also for the esterases. In both cases the cell-associated enzymes are produced constitutively, whereas the secreted enzymes are produced after the exponential growth phase. One might speculate that it is more efficient and economical for these saprophytes to have hydrolytic enzymes located at the cell surface so the cell can afford their constitutive production. Secreted enzymes may be synthesized as a 'last resort' once readily available substrates are depleted.

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