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Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2

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Extracellular lipase was purified from a Tween 80-limited continuous culture of *Pseudomonas aeruginosa* EF2 by ultrafiltration of the culture supernatant followed by anion-exchange and gel-filtration FPLC. The lipase was composed of a single subunit (M_r 29000, pI 4·9), which was capable of a variable degree of aggregation, and which exhibited both lipase activity, measured with the insoluble substrate olive oil (predominantly triolein), and esterase activity, measured with the soluble substrates *p*-nitrophenyl acetate and Tween 80. Lipase activity was approximately eight times higher than either type of esterase activity (k_{cat} approximately 3000 s^{-1} for the hydrolysis of olive oil). The enzyme showed a marked regiospecificity for the 1,3-oleyl residues of radiolabelled triolein, was relatively stable at moderate temperatures (exhibiting a biphasic loss of activity with an initial $t_{\frac{1}{2}}$ of 17·5 min at 60 °C) and was very stable to freezing and thawing. Lipase activity was only weakly inhibited by the serine-active reagent 3,4-dichloroisocoumarin, and was not inhibited by the chelating agent EDTA (1 mM). The N-terminal amino acid sequence of the *Ps. aeruginosa* EF2 lipase showed a marked similarity to those of several other bacterial lipases.

Introduction

Recent interest in the potential use of microbial lipases in biotechnology (see Macrae, 1983; Macrae & Hammond, 1985; Harwood, 1989) has stimulated work on the purification and characterization of several of these enzymes from bacteria, principally from mesophilic and psychrotrophic species of *Pseudomonas* (Dring & Fox, 1983; Fox & Stepaniak, 1983; Stuer *et al.*, 1986; Yamamoto & Fujiwara, 1988). Most of these lipases are particularly thermolabile at moderate temperatures and/or are inhibited by chelating agents: both properties may limit the potential applications of these enzymes for detergent formulations.

Pseudomonas aeruginosa strain EF2 is a newly-isolated, thermotolerant bacterium which produces an extracellular lipase. The physiological regulation of lipase expression by this organism, and the optimization of lipase production during growth in continuous culture, have recently been reported (accompanying paper: Gilbert *et al.*, 1991).

This paper describes the purification of the *Ps. aeruginosa* EF2 lipase together with some of its biochemical and physicochemical properties.

Abbreviations: DCI, 3,4-dichloroisocoumarin; FPLC, fast protein liquid chromatography.

Methods

Organism and culture conditions. Pseudomonas aeruginosa EF2 was grown in continuous culture under Tween 80 (polyoxyethylene sorbitan monooleate) limitation (dilution rate, D 0·05 h^{-1}, pH 6·5, 37 °C) as described previously (Gilbert *et al.*, 1991).

Preparation of culture supernatants. Cultures were harvested by centrifugation in an MSE high-speed centrifuge at 10000 *g* for 15 min, and the supernatant was carefully removed and stored at −20 °C until required.

Purification of lipase. The culture supernatant (697 ml) was reduced in volume to approximately 10 ml by passage under nitrogen at 4 °C through a 202 Ultrafiltration unit (Amicon) containing a YM10 (M_r 10000) cut-off filter. Excess salts were removed by passage through a PD10 desalting column (Pharmacia) to give a final volume of 14 ml. The concentrated, desalted supernatant was passed through an acrodisc filter (0·2 μm pore size; Gelman), then loaded on to an FPLC 10/10 Mono-Q anion-exchange column (Pharmacia) equilibrated with 20 mM-Tris/HCl buffer, pH 7·5, and eluted with the same buffer containing a linear gradient of NaCl (0–1 M over 40 min) at a flow rate of 3 ml min^{-1}. The seven fractions containing the highest lipase activities were pooled, then concentrated by passage under nitrogen at 4 °C through an 8010 Ultrafiltration unit (Amicon) containing a PM10 (M_r 10000) cut-off filter and centrifuged at full speed for 10 min in a

Microcentaur centrifuge (MSE). The supernatant was divided into two 0·75 ml portions, each of which was loaded on to an FPLC Superose 6 gel-filtration column (Pharmacia) and eluted at a flow rate of 0·25 ml min^{-1} with 20 mM-Tris/HCl buffer, pH 7·5, containing 100 mM-NaCl. The fractions containing the highest lipase activities were analysed by SDS-PAGE, and those which contained pure lipase were pooled and stored at −20 °C.

Enzyme assays. Lipase activity was assayed titrimetrically at pH 9·0 with a standard olive oil emulsion as substrate (Sigma), and esterase activity was assayed spectrophotometrically at pH 7·0 with *p*-nitrophenyl acetate as substrate, both at 37 °C, as described previously (Gilbert *et al.*, 1991). Esterase activity was also measured titrimetrically at pH 9·0 and 37 °C, as described for lipase, except that the olive oil emulsion was replaced by 20% (v/v) Tween 80 as substrate. In some experiments lipase and esterase (Tween 80) activities were measured at a series of pH values over the range 7·0 to 10·5, and temperatures over the range 20 to 70 °C. Lipase activity was expressed as lipase units (LU) $(mg\ cells)^{-1}$ or LU $(mg\ protein)^{-1}$ (1 LU was defined as the release of 1 μmol titratable fatty acid per min). Esterase activities were expressed as μmol min^{-1} $(mg\ cells)^{-1}$ or μmol min^{-1} $(mg\ protein)^{-1}$.

Inhibition of lipase and esterase activity by DCI. The effect of the serine-active reagent 3,4-dichloroisocoumarin (DCI) on the activity of the purified lipase was determined by incubating the enzyme (approximately 20 μg ml^{-1}) at 30 °C in 20 mM-Tris/HCl buffer, pH 7·5, containing up to 100 μM-DCI (added from a 10 mM stock solution in methanol). Small samples of the mixture removed over a 3·5 h period and assayed for lipase and esterase (Tween 80) activity. Control samples were assayed for the effect of methanol alone.

Determination of regiospecificity. This was carried out at 37 °C using a modification of the method of Okumura *et al.* (1976). For qualitative analysis the reaction mix (final volume 2·5 ml) contained 2·15 ml 20 mM-Tris/HCl buffer, pH 7·5, containing 250 mM-NaCl and 50 mM-$CaCl_2$, 0·25 ml triolein (99% pure) or diolein (85% 1,3-diolein, 15%, 1,2-diolein), and 0·1 ml purified lipase. Identical mixes were placed in several 10 ml glass scintillation vials and agitated at 37 °C. Each reaction was started by the addition of enzyme to a final concentration of approximately 20 μg ml^{-1}, and terminated at intervals up to 7 h by three successive extractions into 2·5 ml dichloromethane. The solvent was then removed by rotary evaporation, and the residue was resuspended in 2·5 ml dichloromethane. Samples (1 μl) were applied to a 0·25 mm thick silica gel 60 F-254 chromatography plate, and were resolved in a 70:30:1 (by vol.) mix of ligroin (mineral spirits)/diethyl ether/acetic acid. The spots were visualized by spraying the plate with 20% (v/v) H_2SO_4 in ethanol and then heating in an oven at 100 °C until charring occurred, and were identified by comparing their R_F values with those of known standards. For quantitative analysis the procedure used was as described above except that the substrate consisted of 0·25 ml ^{14}C-labelled triolein (glycerol tri[1-^{14}C]oleate; 12 mCi $mmol^{-1}$, 0·44 GBq $mmol^{-1}$) and 2 μl samples were applied to the chromatography plate. After separation, the spots were scraped off the plate and placed in plastic vials containing 4 ml Optiphase T scintillation fluid prior to measuring the radioactivity by scintillation counting.

Non-dissociating (native)-PAGE. Discontinuous native-PAGE was carried out at high pH (stacking gel pH 8·3, running gel pH 9·5) using 7·5% (w/v) polyacrylamide slab gels (Hames, 1981) which were stained for protein using Kenacid blue R. Staining for lipase activity was carried out by incubating the gel at 37 °C for 24 h in contact with a 1% (w/v) agarose overlay attached to 1850-101 Gel Bond Film (LKB). The overlay contained either (*a*) a 5% (v/v) olive oil emulsion (Sigma) containing 0·01% (w/v) Victoria blue B, or (*b*) 2% (v/v) Tween 80 plus 3 mM-$CaCl_2$ in 50 mM-Tris/HCl buffer, pH 8·5, and lipase activity was detected by the appearance of, respectively, a blue zone on a white background (due to the release of oleic acid and its interaction with the pH indicator) or of a white zone on a clear background (due to the precipitation of calcium oleate).

Other methods. Discontinuous SDS-PAGE and determination of protein using the Bradford method was carried out as described previously (Silman *et al.*, 1989; Gilbert *et al.*, 1991). The gels were stained for protein with Kenacid blue R, then destained and, where appropriate, scanned at 633 nm using an LKB laser densitometer linked to a recording integrator. Determination of the N-terminal amino acid sequence and the pI value of the purified lipase was carried out using previously described procedures (Silman *et al.*, 1991).

Chemicals. [^{14}C]Triolein (glycerol tri[1-^{14}C]oleate; 60 mCi $mmol^{-1}$, 2·22 GBq $mmol^{-1}$) was purchased from Amersham. All other chemicals were obtained from Sigma or BDH and were of the finest grade available.

Results

Purification and characterization of lipase

Ps. aeruginosa EF2 was grown in Tween 80-limited continuous culture under near-optimum conditions for expression of maximum lipase activity (D 0·05 h^{-1}, pH 6·5, 37 °C), and the lipase was purified from the culture supernatant by ultrafiltration followed by anion-exchange (Mono-Q) and gel-filtration (Superose) FPLC (Table 1, Fig. 1). SDS-PAGE followed by scanning densitometry showed that the enzyme was 99·5% pure. The calculated purification factor of 31 indicated that the lipase concentration in the culture supernatant was approximately 2 μg ml^{-1} (equivalent to approximately

Table 1. *Purification of extracellular lipase from Ps. aeruginosa* EF2

Lipase was purified from the culture supernatant of a Tween 80-limited continuous culture of *Ps. aeruginosa* EF2 (D 0·05 h^{-1}, pH 6·5, 37 °C). The supernatant was concentrated by ultrafiltration and the lipase was subsequently purified using anion-exchange (Mono-Q) and gel-filtration (Superose) FPLC as described in Methods.

Fraction	Protein concentration (mg ml^{-1})	Total protein (mg)	Specific activity (LU mg^{-1})	Purification	Total activity (LU)	Yield (%)
Supernatant	0·07	48·8	213	1·0	10406	100
Concentrated supernatant	1·43	20·0	468	2·2	9377	90
Mono-Q	1·75	5·4	1226	5·7	6650	64
Superose	0·02	0·28	6606	31·0	1853	18

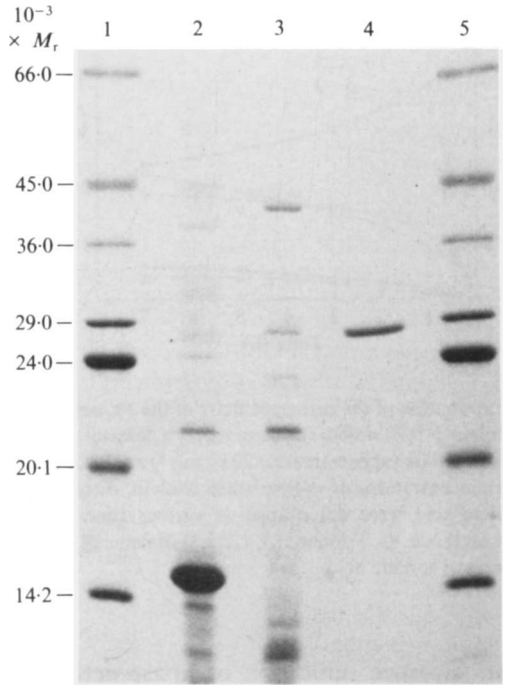

Fig. 1. SDS-PAGE showing the purification of lipase from a culture supernatant of *Ps. aeruginosa* EF2 grown under Tween 80 limitation. Proteins were separated using SDS-PAGE and stained with Kenacid blue R as described in Methods. Tracks: 1, M_r standards; 2, concentrated culture supernatant; 3, pooled Mono-Q fractions; 4; pooled Superose fractions; 5, M_r standards.

3% of the total protein). However, subsequent purifications using identical FPLC procedures showed that the purification factor was extremely variable and could be as low as 5 (equivalent to approximately 20% of the protein in the culture supernatant). This variation presumably reflected the difference, albeit slight, in the extent of cell lysis in the various cultures.

SDS-PAGE showed that the lipase was composed of a single type of subunit with an M_r of approximately 29000. Gel-filtration FPLC on a Superose column, precalibrated with various M_r standards, indicated that the enzyme was capable of undergoing variable but extensive aggregation, which was subsequently confirmed by the observation that the enzyme smeared badly when subjected to native-PAGE. In spite of this, isoelectric focusing of the lipase on non-dissociating polyacrylamide gels led to sufficiently clear focusing of the enzyme to indicate an isoelectric point (pI) of 4·9, a value which was commensurate with the ability of the enzyme to bind to the anion-exchange resin (Mono-Q) during FPLC.

The purified enzyme exhibited both lipase activity, measured with olive oil (predominantly triolein) as substrate, and esterase activity, measured with *p*-nitrophenyl acetate and Tween 80 as substrates, confirming an earlier suggestion based on the physiological studies that both activities were expressed by a single enzyme (Gilbert *et al.*, 1991). Lipase activity was significantly enhanced by the presence of NaCl (optimum concentration 250 mM) and/or $CaCl_2$ (optimum concentration 50 mM). The lipase activity of the purified enzyme from *Ps. aeruginosa* EF2 was 6606 LU mg^{-1} (assayed at pH 9·0, 37 °C) which was equivalent, assuming a minimum native M_r of 29000, to a k_{cat} of 3193 s^{-1}. The lipase activity was approximately eight times the esterase activities obtained with *p*-nitrophenyl acetate and Tween 80, indicating that the enzyme is much more active on an olive oil emulsion than on the two soluble substrates tested, and that it should therefore be regarded as a true lipase which also exhibits some esterase activity. This was confirmed by the observation that the ratios of these three activities remained essentially constant during the entire purification procedure [average ratios lipase/esterase (*p*-nitrophenyl acetate)/esterase (Tween 80), 8·2:0·9:1·0], which also indicated that this enzyme was probably the only lipase/esterase present in the original culture supernatant.

Measurements of the effect of temperature and pH on the lipase and esterase (Tween 80) activities of the purified enzyme showed that maximal activities were observed at 50 °C, and at pH 8·5 and 9·0, respectively.

The enzyme exhibited a biphasic loss of lipase activity during exposure to high temperatures, with an initial $t_{\frac{1}{2}}$ of 17·5 min at 60 °C (Fig. 2*a*). The extent of this initial inactivation phase varied with temperature, increasing from a 48% loss of activity at 55 °C to a complete loss of activity at 80 °C. The effect of temperature was analysed in more detail by measuring the time taken (D, h; see Fox & Stepaniak, 1983) for the enzyme to lose 90% of its activity during exposure to various temperatures (measured by extrapolation, as required, of the initial inactivation phase), and then replotting the log of the D values against temperature to determine the increase in temperature which was required to decrease D by an order of magnitude (defined as Z, °C; see Fox & Stepaniak, 1983) (Fig. 2*b*). A Z value of 9·75 °C was obtained by this procedure.

The lipase was very stable to freezing and thawing, losing only 16% of its activity after six freeze–thaw cycles.

Regiospecificity of lipase

The regiospecificity of the *Ps. aeruginosa* lipase was determined by measuring the release of ^{14}C-labelled dioleins, monooleins and oleic acid from [^{14}C]triolein labelled in the C-1 position of each of the fatty acyl

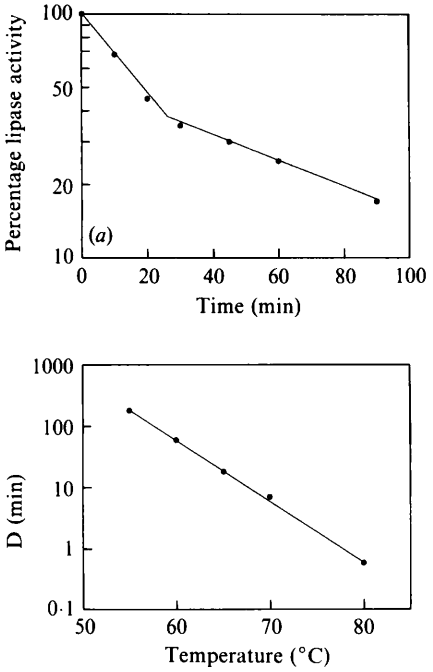

Fig. 2. Determination of the thermostability of the *Ps. aeruginosa* EF2 lipase. Purified lipase (approximately 20 μg ml^{-1} in 20 mM-Tris/HCl buffer, pH 7·5) was incubated at various temperatures over the range 55 to 80 °C. Samples were taken at regular intervals and assayed for lipase activity, and the D value (the time taken for the enzyme to lose 90% of its original activity measured by extrapolation of the initial rapid phase of inactivation) was calculated for each temperature. (*a*) Percentage lipase activity versus time of incubation at 60 °C, (*b*) D versus temperature (slope = Z).

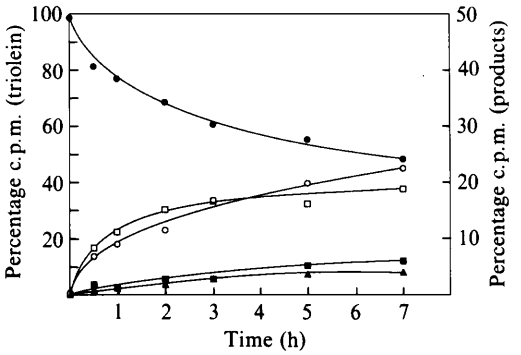

Fig. 3. Determination of the regiospecificity of the *Ps. aeruginosa* EF2 lipase on triolein. [^{14}C]Triolein (glycerol tri[1-^{14}C]oleate) was incubated with purified lipase (approximately 20 μg ml^{-1}) at 37 °C, pH 8·5, for 7 h and the concentrations of radiolabelled triolein, dioleins, monooleins and oleic acid were determined at various time intervals as described in Methods. ●, Triolein; □, 1,2(2,3)-diolein; ■, 1,3-diolein; ▲, monooleins; ○, oleic acid.

residues (Fig. 3). Slightly over half of the radioactivity in the triolein was released during the 7 h period of the experiment, the majority of which appeared in 1,2(2,3)-diolein (19%) and oleic acid (23%), with much smaller amounts in 1,3-diolein (6%) and monoolein (4%). No detectable hydrolysis of triolein occurred in the absence of added lipase. It was concluded from these results that the lipase predominantly hydrolysed the peripheral (1,3) ester bonds. This was confirmed by showing that lipase discriminated between a mixture of unlabelled dioleins (85% 1,3-diolein, 15% 1,2(2,3)-diolein), hydrolysing the 1,3-diolein much faster than the 1,2(2,3)-diolein.

Sensitivity to inhibitors

The divalent metal-chelating agent EDTA (1 mM) caused no significant inhibition of lipase or esterase (Tween 80) activity following preincubation with the enzyme for 30 min at 30 °C, indicating that the enzyme is probably not a metalloprotein.

Preincubation of the enzyme with the serine-protease inhibitor DCI (100 μM) at 30 °C elicited a weak, time-dependent, biphasic inhibition of lipase activity. The initial, fast rate of inactivation (25% inhibition after 5 min incubation) was still extremely slow compared with the effect of DCI on serine proteases (Harper *et al.*, 1985), and the subsequent slow phase rarely led to more than 50% inhibition even after 3 h incubation. DCI also had a similar effect on esterase (Tween 80) activity. It was not possible from these results to draw unambiguous conclusions with respect to the presence or absence of an essential serine residue in this enzyme.

N-terminal amino acid sequence

N-terminal amino acid analysis of the *Ps. aeruginosa* EF2 lipase (Fig. 4) showed that the enzyme exhibited considerable similarity with the N-terminal sequences of the lipases from *Ps. aeruginosa* TE3285 (originally *Ps. fluorescens*), *Ps. pseudoalcaligenes*, *Ps.* sp. ATCC 21808, *Ps. cepacia* and *Ps. fragi*, as determined by direct analysis of the purified enzymes and/or from the DNA sequences of the cloned genes (Kugimiya *et al.*, 1986; Aoyama *et al.*, 1988; Nakanishi *et al.*, 1989; Andreoli *et al.*, 1989; Jørgensen *et al.*, 1991; Kordel & Schmid, 1991; Nishioka *et al.*, 1991; T. Nishioka, personal communication). In spite of the failure to identify unambiguously the N-terminal amino acid in the *Ps. aeruginosa* EF2 lipase, 11 of the next 12 amino acids were identical with those in the *Ps aeruginosa* TE3285 enzyme, and at least five were identical with those in the other *Pseudomonas* enzymes. Several of the DNA sequences contained a region coding for a lengthy signal sequence, an observation which was commensurate with the extracellular nature of these lipases. All of the enzymes exhibited the consensus

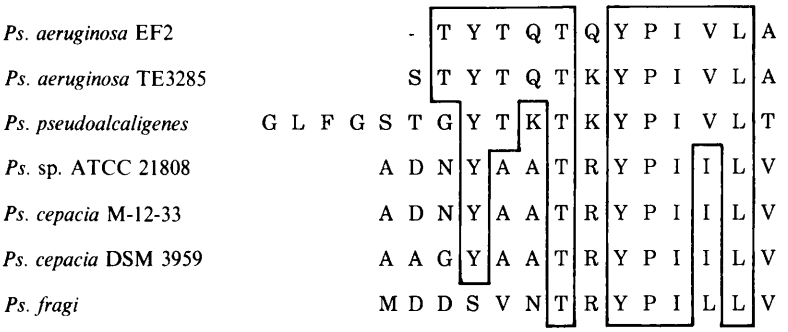

Fig. 4. Comparison of the N-terminal amino acid sequence of *Ps. aeruginosa* EF2 lipase with that of other bacterial lipases. The purification and N-terminal amino acid sequencing of the *Ps. aeruginosa* lipase were carried out as described in Methods. The N-terminal amino acid sequences of the other lipases were determined either by analysis of the purified enzyme or from the DNA sequence of the cloned lipase gene. *Ps aeruginosa* TE3285 (originally called *Ps. fluorescens*; Nishioka *et al.*, 1991; T. Nishioka, personal communication), *Ps. pseudoalcaligenes* (Andreoli *et al.*, 1989), *Ps.* sp. ATCC 21808 (Kordel & Schmid, 1991), *Ps. cepacia* (Nakanishi *et al.*, 1989; Jørgensen *et al.*, 1991) and *Ps. fragi* (Kugimiya *et al.*, 1986; Aoyama *et al.*, 1988).

sequence −TXYPIXL− close to the N-terminus, where the first variable residue was a basic amino acid (glutamine, arginine or lysine) and the second variable residue was a hydrophobic amino acid (isoleucine, leucine or valine).

Discussion

The essentially constant activity ratio for the hydrolysis of olive oil, *p*-nitrophenyl acetate and Tween 80 which was observed during the purification of the lipase from *Ps. aeruginosa* EF2, together with the approximately eightfold higher activity with the olive oil emulsion than with the two soluble substrates, confirmed that the enzyme is a true lipase which also exhibits some esterase activity (i.e. it preferentially hydrolyses ester bonds at fat–water interfaces). It is therefore appropriate to compare the properties of the purified lipase from *Ps. aeruginosa* EF2 with those of other bacterial lipases, mainly, but not exclusively, from *Pseudomonas* spp. which have also been investigated (Dring & Fox, 1983; Bozoğlu *et al.*, 1984; Kugimiya *et al.*, 1986; Stuer *et al.*, 1986; Lee & Iandolo, 1986; Yamamoto & Fujiwara, 1988; Aoyama *et al.*, 1988; van Oort *et al.*, 1989).

The *Ps. aeruginosa* EF2 lipase, like all of these other bacterial lipases, is composed of a single type of subunit which can apparently undergo a variable degree of aggregation. The subunit M_r (29000) is very similar to that of several other *Pseudomonas* lipases (29000 to 35000), but substantially different from those reported for the *Ps. fragi* AFO 3458 (14600), *Ps. fluorescens* MC 50 (55000), *Staphylococcus aureus* (70000) and *Staphylococcus hyicus* (86000) enzymes (Bozoğlu *et al.*, 1984; Kugimiya *et al.*, 1986; Lee & Iandolo, 1986; van Oort *et al.*, 1989).

The observation that lipase activity was significantly enhanced in the presence of $CaCl_2$ and NaCl probably reflects the ability of these salts to react with free fatty acids adhering to the oil droplets, to diminish interfacial charge effects and/or to increase droplet surface area (see Brockerhoff & Jensen, 1974). In terms of lipase activity, the specific activity (6606 LU mg^{-1}) and the calculated k_{cat} (3193 s^{-1}) of the purified enzyme from *Ps. aeruginosa* EF2 are two- to six-fold higher than those reported for the lipases from *Ps. fluorescens*, *Pseudomonas* sp., *Pseudomonas* sp. ATCC 21808 and *S. hyicus* (Dring & Fox, 1983; Yamamoto & Fujiwara, 1988; van Oort *et al.*, 1989; Kordel & Schmid, 1991), but lower than for the lipase from *Ps. aeruginosa* PAC1R (Stuer *et al.*, 1986), all measured under similar, but not identical, conditions.

The optimum pH for lipase and esterase activity (pH 8·5–9·0) is within the range (pH 7·0–9·5) exhibited by other *Pseudomonas* lipases, whereas the optimum temperature (50 °C) is significantly higher than that of the lipases from *Ps. aeruginosa* PAC1R, *Pseudomonas* sp., and a psychrotrophic strain of *Ps. fluorescens* (< 40 °C) and lower than the lipase from *Ps. fragi* (65–80 °C). Importantly, the thermostability of the *Ps. aeruginosa* EF2 lipase at 60 °C ($t_{\frac{1}{2}}$ 17·5 min) is significantly greater than that of the other *Pseudomonas* lipases which have been examined in this way ($t_{\frac{1}{2}}$ 1–10 min) (Dring & Fox, 1983; Fox & Stepaniak, 1983; Yamamoto & Fujiwara, 1988).

The insensitivity of the purified lipase to EDTA probably indicated that, in contrast to other *Pseudomonas* lipases (Dring & Fox, 1983; Bozoğlu *et al.*, 1984; Yamamoto & Fujiwara, 1988), the lipase activity is not dependent on the presence of a divalent metal activator.

It is possible that both the relatively high thermostability and the resistance to inhibition by EDTA reflect the growth conditions under which *Ps. aeruginosa* EF2 was isolated, i.e. at a relatively high temperature (50 °C) in an olive oil minimal medium supplemented with the chelating agent nitrilotriacetic acid (Gilbert *et al.*, 1991).

The marked specificity of the *Ps. aeruginosa* EF2 lipase for the 1,3-oleyl residues of triolein is very similar to that previously observed for several fungal lipases, and

contrasts with the lack of specificity exhibited by other fungal lipases and by the lipase from *S. hyicus* (Okumura *et al.*, 1976; van Oort *et al.*, 1989).

The N-terminal amino acid sequence of the *Ps. aeruginosa* EF2 lipase is strikingly similar to that of the other *Pseudomonas* lipases which have recently been sequenced, either by direct analysis of the purified enzymes or from the DNA sequences of the lipase genes (Kugimiya *et al.*, 1986; Aoyama *et al.*, 1988; Andreoli *et al.*, 1989; Nakanishi *et al.*, 1989; Jørgensen *et al.*, 1991; Kordel & Schmid, 1991; Nishioka *et al.*, 1991), indicating that all of the enzymes are probably closely related to each other. As they include the *Ps. fragi* IFO 3458 and *Ps. fluorescens* MC50 lipases, which have been reported to have subunit M_r values of 14600 and 55000, respectively (Bozoğlu *et al.*, 1984; Kugimiya *et al.*, 1986), it must be concluded that these two enzymes contain a non N-terminal deletion and addition, respectively. It should be noted that none of the *Pseudomonas* lipases show any N-terminal sequence similarity to the lipases from *S. aureus* and *S. hyicus* (Lee & Iandolo, 1986; van Oort *et al.*, 1989).

Several of the *Pseudomonas* lipases have been shown to contain the consensus sequence −GXSXG− at their putative active site (Kugimiya *et al.*, 1986; Andreoli *et al.*, 1989; Nakanishi *et al.*, 1989; Jäger *et al.*, 1991; Jørgensen *et al.*, 1991) and/or to be inhibited by the serine-active reagent diethyl *p*-nitrophenyl phosphate (Jäger *et al.*, 1991; Kordel & Schmid, 1991), indicating that a serine residue is essential for lipase activity. The relatively slow and incomplete inhibition of the *Ps. aeruginosa* EF2 lipase by the serine-active reagent DCI (compared with the effect of the latter on various serine proteases; Harper *et al.*, 1985) was therefore rather unexpected, and suggests either that the enzyme does not contain a serine residue at its active site or, more likely, that serine residue is present but is relatively inaccessible to DCI.

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