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Extracellular lipase from *Pseudomonas aeruginosa* is an amphiphilic protein

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Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) secreted by *Pseudomonas aeruginosa* PAC1R was purified from cell-free growth medium by preparative isoelectric focusing. After blotting the N-terminal amino acid sequence and the amino acid composition were determined and compared to *P. fragi* and *P. cepacia* lipases yielding significant homology between all three species. Additionally, a consensus sequence K-Y-P-i-v-l-V-H-G was identified residing at the N-terminus of *Pseudomonas* lipases and in the central part of *Staphylococcus* lipases. Treatment of lipase with the serine-specific inhibitor diethyl *p*-nitrophenyl phosphate caused a rapid and complete inhibition of enzyme activity indicating the presence of a serine at the catalytic site as expected from lipase consensus sequences. Upon charge-shift electrophoresis the electrophoretic mobility of purified lipase was shifted either anodally or cathodally in the presence of sodium deoxycholate and cetyltrimethylammoniumbromide, respectively. This result demonstrates that extracellular lipase of *P. aeruginosa* exhibits an amphiphilic character like intrinsic membrane proteins.

Introduction

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) hydrolyzes triacylglycerols acting at an oil-water interface. A considerable interest in lipases stems from their potential for industrial use which is broadened by the fact that lipases remain active in organic solvents [1]. Bacterial lipases have been characterized from gram-positive and gram-negative species [2] with special emphasis on lipases from the genus *Pseudomonas* [3]. Lipase from *P. fluorescens* was biochemically characterized and crystallized [4]. The structural genes of lipases from *P. fragi*, *P. cepacia* and *P. aeruginosa* have been cloned and sequenced [5–7, S. Wohlfarth, personal communication). We have purified the lipase from *P. aeruginosa* to electrophoretic homogeneity by isoelectric focusing [8]. The molecular weight was found to be 29 kDa and the isoelectric point 5.8, the enzyme showed a broad substrate specificity and no positional preference [9]. Further biochemical characterization revealed that *P. aeruginosa* lipase was associated with lipopolysaccharide (LPS) when isolated from cell-free growth medium, leading us to assume that the lipase should exhibit an amphiphilic character [10].

The present study was undertaken to further characterize the lipase from *P. aeruginosa* with respect to its N-terminal amino acid sequence and amino acid composition. Inhibitor studies should reveal whether it belonged to the group of serine-hydrolases and charge shift electrophoresis was used to characterize the enzymatically active purified lipase protein.

Materials and Methods

Bacterial strain and culture conditions

P. aeruginosa PAC1R [11] was used and the culture conditions were as described [8] with the following exception: medium contained 8 g nutrient broth (Difco Laboratories), 4 g NaCl, 10 mM $MgCl_2$ and 1 mM $CaCl_2$ per liter. Growth medium was sterile-filtered and concentrated by ultrafiltration as described previously [8].

Isoelectric focusing

A gel trough of $22 \times 14 \times 0.2$ cm was formed by mounting plastic spacers onto a glass plate. At a distance of 1.5 cm parallel to the anode a sample applicator was placed onto the glass plate which consisted of

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two metal strips in a fixed distance of 1.7 cm. During the focusing run the gel was cooled to 4°C. The focusing gel was prepared by solubilizing 800 mg of the zwitterionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in 37.3 ml of 1 mM Tris hydrochloride buffer (pH 8.0) containing 0.5 mM EDTA. After stirring for 1 h at room temperature, 1.6 g of Sephadex-IEF were added, and the gel slurry left overnight at 4°C. Then 2.67 ml Ampholyte 5–7 were added and the slurry poured into the gel trough on both sides of the sample applicator. Lipase containing concentrated growth medium (usually 3–4 ml) was solubilized in a total vol. of 6.53 ml 20 mM Tris hydrochloride buffer (pH 8.0), 2 mM EDTA and 300 mg CHAPS. After magnetic stirring for 1 h at 4°C, 0.47 ml of Ampholine 5–7 and 0.28 g of Sephadex-IEF were slowly added and the slurry poured into the sample applicator zone. The sample applicator was removed and the whole gel was lightly sprinkled with dry Sephadex-IEF to ensure a smooth and even gel surface. The anode strip was saturated with 0.1 M H_3PO_4 and the cathode strip with 0.1 M NaOH. Isoelectric focusing usually lasted for 3500 to 4500 Vh with current setting at 20 mA and power at 20 W. Lipase containing fractions were detected by placing 11 pieces of filter paper for a few seconds on top of the gel in a distance of 1 cm from each other. These filter papers were than put into test tubes containing lipase substrate and assayed for lipase activity. For recovering focused lipase protein a fractionation grid was pressed into the gel at the position of highest enzyme activity and usually four different gel fractions (wet volume: 1.5 ml/fraction) were removed. After addition of 3 ml elution buffer (10 mM Tris hydrochloride (pH 8.0), 20 mM NaCl, 1% (w/v) (CHAPS) per fraction and thoroughly mixing the Sephadex-IEF was separated by filtration and lipase activity per fraction was determined with *p*-nitrophenylpalmitate (p-NPP) as a substrate. The pH-gradient was determined potentiometrically after suspending appropriate aliquots of the focusing gel in an aqueous solution of 10 mM KCl.

Charge-shift electrophoresis

The method was a modification of the procedure described by Helenius and Simons [12]. Gels were prepared from 40 ml of 50 mM glycine buffer (pH 9.0) containing 100 mM NaCl, 0.4 g of agarose, 0.5% (v/v) Triton X-100, and alternatively 0.25% (w/v) sodium deoxycholate (DOC) or 0.05% (w/v) cetyltrimethylammonium bromide (CTAB). The solutions were cast onto Gel Bond film and the gel stored in a moistened chamber for 15 h at 4°C.

IEF-purified lipase was concentrated in centrifugal microconcentrators (Centricon 10, Amicon, Witten, Germany), washed several times with endotoxin-free water (Sigma, Munich, Germany) and finally with 2 ml of a solution of 0.1% (v/v) Triton X-100. Residual detergent was removed by chromatography on a column of Bio-Beads SM4 (Bio-Rad, Munich, Germany) as described [13]. Lipase containing fractions were pooled, concentrated as described above and freeze-dried. Samples containing 1 μg lipase protein were solubilized in 50 mM Tris hydrochloride buffer (pH 9.0) containing 10 mM EDTA, 100 mM NaCl, 2% (v/v) Triton X-100, and alternatively 2% (w/v), DOC and CTAB. Reference proteins were ovalbumin (Sigma, Munich, Germany), cytochrome *c* (Serva, Heidelberg, Germany), and ADP,ATP-carrier (an intrinsic membrane protein from beef heart mitochondria, kindly provided by M. Klingenberg, University of Munich, Germany). Running buffer was 50 mM glycine (pH 9.0), 100 mM NaCl, 0.5% (v/v) Triton X-100 and 0.25% (w/v) DOC or 0.05% (w/v) CTAB, respectively. The gels were run for 3.5 h at a constant voltage of 100 V with current setting at 150 mA and power at 15 W. Proteins were detected by silver staining [14].

Determination of N-terminal amino acid sequence

IEF-purified lipase (4 μg) was subjected to SDS-polyacrylamide gel electrophoresis in a 12% gel [15]. Markers were rainbow molecular weight marker (Amersham, Oakville, Ontario, Canada) and carbonic anhydrase (M_r 29000, Sigma, St. Louis, MI, USA). After electrophoresis the separated proteins were transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA, USA) by the method of Towbin et al. [16] with blotting buffer containing 0.1% (w/v) SDS. Electroblotting was carried out in a Bio-Rad transblot apparatus for 18 h at 10 mA. The blotting membrane was stained with Ponceau S [17], the lipase-containing band (29 kDa) cut out, destained in distilled water (10 min), 50% (v/v) methanol (5 s), and again distilled water (30 min) and air-dried. The N-terminal amino acid sequence was determined using a Beckman 890-C spinning cup sequencer.

Amino acid composition analysis

Preparation of IEF-purified lipase was as described above with the following exceptions: electrophoresis and blotting were carried out in a Bio-Rad Mini Protean II system. Blotting was for 5 min at 300 mA with 10 mM borate buffer (pH 9.2) containing 20% (v/v) methanol and 0.1% (w/v) SDS. The blotting membrane was stained with Coomassie brilliant blue R-250, destained and the lipase-containing band subjected to amino acid analysis. The sample was hydrolyzed in an atmosphere developed from 6 M HCl at 150°C for 1 h. Derivatization of the free amino acids with phenylisothiocyanate was carried out as described [18]. The phenylthiocarbamoyl derivatives were separated by reversed-phase HPLC using the 130A separation system (Applied Biosystems, Weiterstadt, Germany). The data

TABLE I

Amino acid composition of lipases from three Pseudomonas species

Amino acid	Amino acid composition (mol%)		
	P. cepacia [a]	*P. aeruginosa* [b]	*P. fragi* [a]
G	11.3	16.1 (±1.5)	9.4
A	12.5	9.2 (±0.7)	11.2
V	9.1	7.8 (±0.6)	6.1
L	10.9	9.8 (±1.0)	11.2
I	3.1	4.1 (±0.6)	4.7
S	7.8	11.2 (±1.8)	8.3
T	9.4	7.1 (±0.4)	4.3
C	0.6	n.d.	0.4
M	0.3	1.9 (±0.4)	1.4
D/N	10.0	6.3 (±3.5)	10.8
E/Q	6.6	4.6 (±1.5)	6.9
R	2.8	5.0 (±1.4)	7.6
K	2.2	4.5 (±1.1)	1.1
H	1.9	1.9 (±0.7)	4.7
F	2.2	4.0 (±0.4)	4.3
Y	4.7	3.8 (±0.1)	2.5
W	0.9	n.d.	0.7
P	3.8	3.6 (±0.2)	4.3

[a] Data obtained from the nucleotide sequences [5,6].

[b] Data are mean values from triplicate determinations, standard deviations are given in parentheses.

given in Table I were not corrected for destruction or slow liberation during hydrolysis.

Inhibition studies

Diethyl *p*-nitrophenyl phosphate (DNPP) was dissolved in a mixture of 2-propanol (0.5–20 mM in 300 μl), acetate buffer (pH 6.0, 100 mM, 1.5 ml) and CHAPS (4% (w/v), 0.75 ml). This DNPP-solution was mixed with IEF-purified lipase and destilled water (final vol.: 3 ml) and incubated at 20°C. Aliquots (100 μl) were withdrawn and lipase activity was followed as a function of time and DNPP-concentration.

Assay for lipase activity

Lipase activity was determined photometrically with p-NPP as a substrate with 1 nkat representing about 1.32 ng of pure lipase protein [8].

Computer analysis

Analysis of the N-terminal amino acid sequence data was performed on an IBM-compatible PC using the computer program PROSIS (Pharmacia-LKB, Uppsala, Sweden). The N-terminal amino acid sequence of *P. aeruginosa* lipase was used to search the NBRF-protein data bank for homologous sequences. Equivalence groups were (i) acidic amino acids N, D, B (Asx), E, Q and Z (Glx); (ii) basic amino acids H, R and K; (iii) aliphatic amino acids M, L, I and V; and (iv) aromatic amino acids F, Y and W.

Results

Purification by IEF

A typical isoelectric focusing experiment in a Sephadex-IEF matrix revealed a sharp peak of lipase activity corresponding to an isoelectric point of 5.7 (Fig. 1). When starting with about 100 μg of lipase protein as estimated from specific activity the total yield was about 20%. This means that the use of Sephadex-IEF instead of agarose which we used earlier [8] increased the total recovery of lipase protein by a factor of ten.

Charge-shift electrophoresis

The behaviour of *P. aeruginosa* lipase upon charge-shift electrophoresis was studied in the presence of Triton X-100 as the nonionic, sodium deoxycholate as the anionic or cetyltrimethylammonium bromide as the cationic detergent. Among the reference proteins cytochrome *c* migrated to the cathode (2 cm) and ovalbumin to the anode (2.2 cm), irrespective of the detergent used. As shown in Fig. 2A lipase shifted its mobility both anodally (1.5 cm) and cathodally (1.0 cm) depending on the detergent used. The same behaviour was observed with the intrinsic membrane protein ADP, ATP-carrier (1.5 cm anodally, 1 cm cathodally). Fig. 2B shows a schematic summary of the results of the charge-shift electrophoresis experiments. It is obvious that lipase from *P. aeruginosa* exhibited a marked charge-shift similar to the one observed for the intrinsic membrane protein ADP,ATP-carrier.

N-terminal amino acid sequence

The N-terminal amino acid sequence of *P. aeruginosa* lipase is shown in Fig. 3. The sequence was determined for the first 40 residues, amino acids at position numbers 14, 28, 33, 36 and 37 could not be

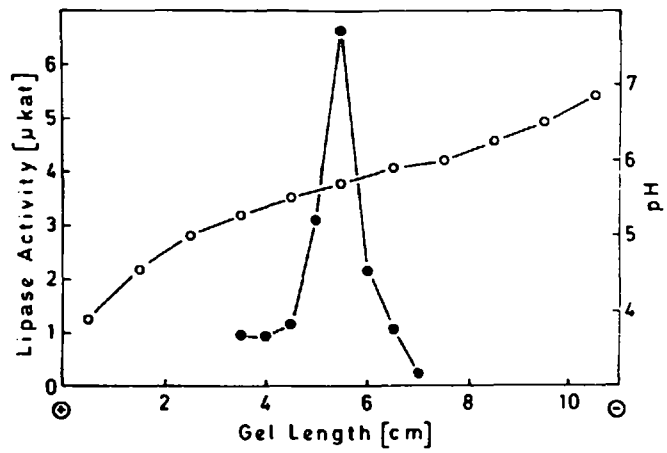

Fig. 1. IEF of lipase in a Sephadex-gel containing CHAPS (2%, w/v). Lipase activity (●) was determined with p-NPP as the substrate and pH values (○) potentiometrically. Positions of the cathode and the anode are indicated.

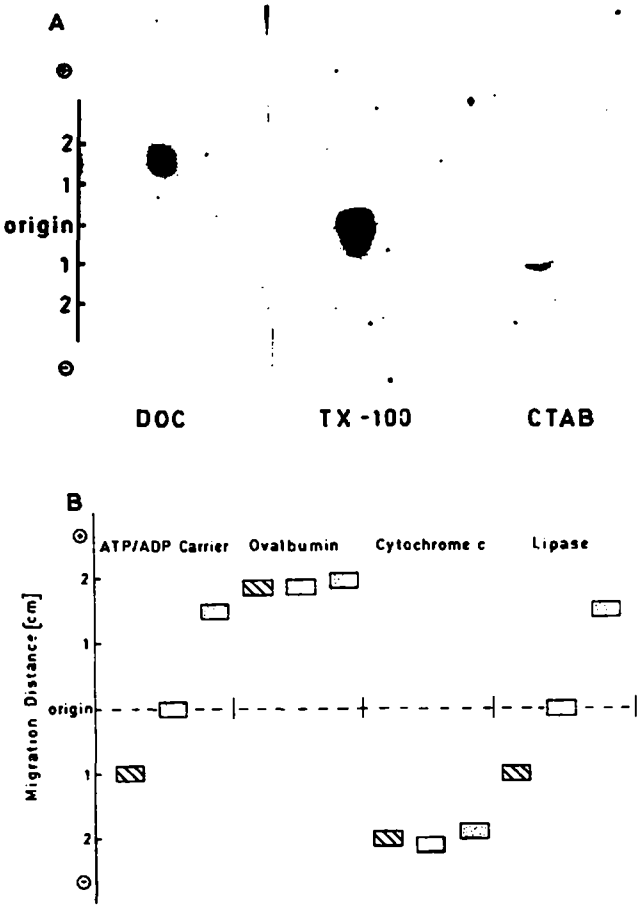

Fig. 2. Charge-shift electrophoresis of lipase and reference proteins ovalbumin, cytochrome *c* and ADP,ATP-carrier protein. Agarose gels were run in the presence of 0.5% (v/v) Triton X-100 (TX-100, □), and additionally with 0.25% (w/v) sodium deoxycholate (DOC, ▤) or 0.05% (w/v) cetyltrimethylammonium bromide (CTAB, ▨). (A) Silver-stained gels containing IEF-purified lipase (1 μg/gel) and the indicated detergents. (B) Schematic presentation of the combined results.

identified definitively. Comparison of the sequence with those from *P. fragi* and *P. cepacia* lipases showed a high degree of homology. The matching percentage was 62% for *P. fragi* lipase (total window: 36, alignment window: 29, matching: 18 residues with 15 identical and 3 equivalent) and 56% for *P. cepacia* lipase (total window: 36, alignment window: 32, matching: 18 residues with 15 identical and 3 equivalent).

Interestingly, a comparison of the *P. aeruginosa* sequence with amino acid sequences of two lipases from gram-positive *Staphylococci* showed a comparable degree of homology with the homologous regions residing in the middle of the sequences. When using the 10 amino acid consensus sequence of *P. aeruginosa* lipase for comparison we found 6 identical and 1 equivalent residue for *S. aureus* lipase (position numbers 303 to 312) and 5 identical and 1 equivalent residue for *S. hyicus* lipase (position numbers 261 to 270).

Amino acid composition

The amino acid analysis of *P. aeruginosa* lipase yielded an overall composition similar to the lipase of *P. fragi* and *P. cepacia* (Table I), although the content of glycine and serine was somewhat higher in *P. aeruginosa* lipase. Based on the measured content of valine, methione, isoleucine, leucine, alanine, phenylalanine, tyrosine and proline the calculated relative hydrophobicity was 44% which is again in the same range as the values for *P. fragi* lipase (46%) and *P. cepacia* lipase (47%). The ADP/ATP carrier which was used as a reference protein in charge-shift electrophoresis experiments has a relative hydrophobicity of 49% as calculated from the primary structure [21].

Inhibition of lipase activity

A solution of IEF-purified lipase emulsified in CHAPS (5 nkat/experiment) was mixed with a solution of DNPP in acetate buffer (pH 6.0), incubated at room temperature and aliquots were assayed for lipase activity with p-NPP as the substrate. The inactivation was studied as a function of time (Fig. 4A) and DNPP-concentration (Fig. 4B). The concentration of DNPP causing a 50% inactivation of the original lipase activity was 5 mM (Fig. 4B) which represents a molar excess

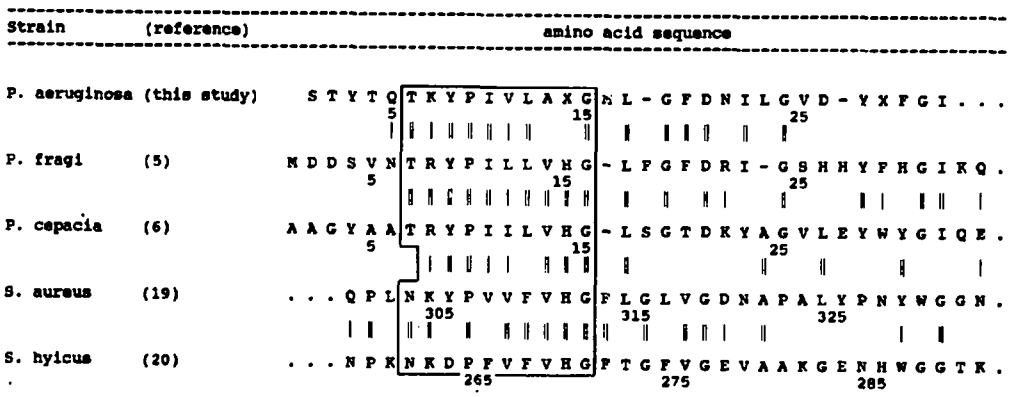

Fig. 3. Comparison of N-terminal amino acid sequences of lipases from three *Pseudomonas* species and internal amino acid sequences of lipases from two *Staphylococcus* species. (X) means no identification during sequencing; (–) is a gap introduced by the computer program; equivalent amino acids are connected by single lines and identical amino acids by double lines.

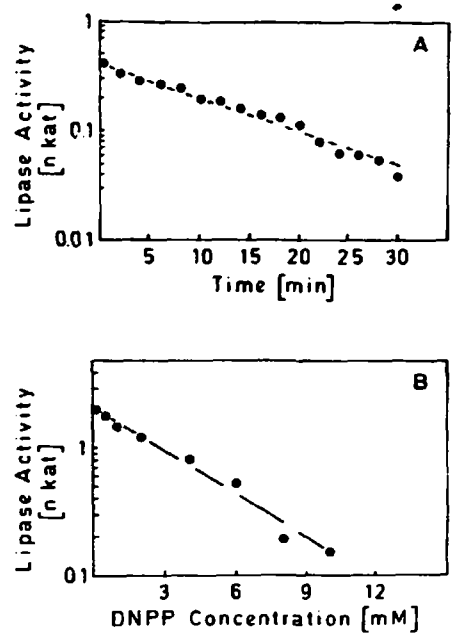

Fig. 4. Inactivation of lipase by DNPP in the presence of CHAPS (1% w/v). Lipase activities were determined photometrically and plotted semilogarithmically as a function of (A) incubation time in the presence of 2 mM DNPP and (B) DNPP-concentration at 10 min incubation time.

of approx. 7000-fold. Comparable results were obtained with a titrimetric assay and trioleoyl glycerol as the substrate (data not shown). On the other hand, treatment of lipase with the serine hydrolase inhibitor phenylmethanesulphonyl fluoride (PMSF) under identical experimental conditions did not yield in inactivation of lipase activity.

Discussion

Extracellular lipase from *P. aeruginosa* has been purified by preparative isoelectric focusing in a Sephadex gel matrix. The p*I* was determined to be 5.7 corresponding to the one determined by analytical IEF in in an agarose gel matrix [8]. The preparative approach was chosen mainly because it allowed to use large sample vols. of up to 40 ml. However, initial focusing experiments did not yield in a sufficient resolution; instead a broad distribution of lipase activity was observed ranging from the cathode to the anode. Therefore, a newly designed sample application device allowing a locally restricted sample application was placed near the anode to ensure that lipase and LPS migrated into opposite directions thereby avoiding a reaggregation of both components during the focusing run. Although the sample volume had to be reduced to about 5 ml the focusing effect significantly improved yielding a sharp peak of lipase activity (Fig. 1).

Lipase from *P. aeruginosa* was present in the growth medium in association with LPS forming lipase-LPS micelles [10]. This observation together with biochemical evidence [8] led us to conclude that the surface of the lipase protein consisted of at least some hydrophobic domains allowing interaction with the lipid A part of the LPS [9]. Charge-shift electrophoresis provides an appropriate experimental method to test such a prediction [12]. It had already been used to characterize a hormone-sensitive lipase from rat adipose tissue [22]. *P. aeruginosa* lipase showed a marked shift both towards the anode and the cathode depending on the charge of the detergent used (Fig. 2). This behaviour is consistent with the assumption that the enzyme is amphiphilic although it does not prove its location as an intrinsic membrane protein. By Western blotting using a rabbit anti-lipase antiserum raised against purified lipase protein we found that during early stationary growth phase about 10% of the enzyme molecules remained cell-bound whilst about 90% were released into the growth medium. So far, we have no evidence for a secretion mechanism involving cleavage of membrane-bound lipase leading to the release of one protein fragment and leaving another fragment anchored to the membrane. (H.B. Koch and K.-E. Jaeger, unpublished observations).

Analysis of the total amino acid composition of lipase did not reveal a surplus amount of hydrophobic residues. The same result was found for lipases of *P. fragi* and *P. cepacia* (Table I). Such a finding, however, does not necessarily exclude the possibility of hydrophobic domains being exposed to the environment [23]. Another interesting observation was the similarity in overall amino acid composition between all three *Pseudomonas* lipases despite a certain degree of uncertainty caused by experimental determination of the amino acid composition. The assumption that all three *Pseudomonas* lipase proteins show a high degree of similarity at least at the level of primary structure gained further support from comparison of their amino-terminal amino acid sequences (Fig. 3). A consensus box consisting of ten amino acid residues could be identified based on the assumption that amino acid No. 14 in *P. aeruginosa* lipase should be a His residue which has been confirmed by determination of the nucleotide sequence of the lipase gene (S. Wohlfarth, personal communication). The same consensus box including a His-9 has been found at the amino-terminus of lipases from *P. glumae* [24] and *P. alcaligenes* [25]. Just recently, Nishioka et al. [26] and Ihara et al. [27] published sequences of lipases from unknown species of the genus *Pseudomonas* with an amino-terminus which is identical to the one we have determined. We therefore suggest that those strains were *P. aeruginosa*. It has been speculated that the conserved aminoterminal region may somehow be involved in the active site of lipases [20]. Our results further substantiate this assumption which we would extend to propose that an amino-terminal ten amino acid consensus box should

be present in all lipases of the species *Pseudomonas*. Interestingly, such a region of homology has also been found in lipases from the gram-positive bacteria *S. aureus* and *S. hyicus* [19,20] where they reside in the middle of the sequences. In case of *S. hyicus* lipase the amino-terminal part of the protein could be proteolytically cleaved to yield a 46 kDa lipase with a 3-fold increased specific activity [20]. This processed lipase then contained a His residue at position No. 24 of the sequence which is comparable to the His positions found in *Pseudomonas* lipases.

It is tempting to speculate whether His-9 of the consensus box is part of the catalytic triad consisting of Asp-His-Ser that has been found in eukaryotic lipases from *Rhizomucor miehei* [28] and human pancreas [29]. Evidence for the presence of an active site Ser was obtained from studies with the Ser-specific inhibitor DNPP. Lipase from porcine pancreas was rapidly and almost completely inhibited by emulsions of DNPP [30] as was *P. aeruginosa* lipase (Fig. 4). The concentration of the inhibitor and the molar ratio of inhibitor to enzyme were comparable in both cases. Recently, it has been shown that a 100-fold molar excess of DNPP emulsified in sodium deoxycholate was needed to label the catalytic site Ser residues of gastric and pancreatic lipases [31]. We therefore conclude that DNPP inactivation of *P. aeruginosa* lipase was due to reaction with an essential Ser residue present in the well known lipase consensus sequence Gly-x_1-Ser-x_2-Gly [2] where x_1 is His in prokaryotic lipases.

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Note added in proof:

Recently, E.J. Gilbert et al. (J. Gen. Microbiol. (1991) 137, 2223–2229) have published a comparison of N-terminal amino acid sequences of seven *Pseudomonas* lipases including an N-terminal consensus sequence (-TXYPIXL-). However, their consensus sequence differs from the one described in this paper (see Fig. 3) in that it does not contain His-9. Furthermore, these authors did not observe the occurrence of the consensus sequence in lipases of *Staphylococci*

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