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Cloning of the *Pseudomonas glumae* Lipase Gene and Determination of the Active Site Residues

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The *lipA* gene encoding the extracellular lipase produced by *Pseudomonas glumae* PG1 was cloned and characterized. A sequence analysis revealed an open reading frame of 358 codons encoding the mature lipase (319 amino acids) preceded by a rather long signal sequence of 39 amino acids. As a first step in structure-function analysis, we determined the Ser-Asp-His triad which makes up the catalytic site of this lipase. On the basis of primary sequence homology with other known *Pseudomonas* lipases, a number of putative active site residues located in conserved areas were found. To determine the residues actually involved in catalysis, we constructed a number of substitution mutants for conserved Ser, Asp, and His residues. These mutant lipases were produced by using *P. glumae* PG3, from which the wild-type lipase gene was deleted by gene replacement. By following this approach, we showed that Ser-87, Asp-241, and His-285 make up the catalytic triad of the *P. glumae* lipase. This knowledge, together with information on the catalytic mechanism and on the three-dimensional structure, should facilitate the selection of specific modifications for tailoring this lipase for specific industrial applications.

Lipases (triacylglycerol hydrolase; EC 3.1.1.3), particularly those produced by microorganisms, have received increasing attention over the past few years (11). Since these enzymes are relatively stable and are capable of catalyzing a variety of reactions, they are potentially of importance for diverse industrial applications (15).

In recent years information on the structural and mechanistic properties of lipases has become available. The nucleotide and amino acid sequences of several microbial lipases have been published, including the lipases of *Pseudomonas fragi* (1), *Pseudomonas cepacia* (17), *Pseudomonas* sp. (21), *Staphylococcus hyicus* (12), *Staphylococcus aureus* (19), *Geotrichum candidum* (24, 25), *Candida cylindracea* (18), and *Rhizomucor miehei* (2). Recently, the crystal structures of human pancreatic lipase (HPL) (31), *R. miehei* lipase (RML) (3), and *G. candidum* lipase (23) have been published. So far, however, no three-dimensional structure of a bacterial lipase has been elucidated. The notion that lipases have a catalytic triad consisting of Ser-Asp-His was confirmed by the HPL and RML structures, whereas for the *G. candidum* lipase the catalytic triad was found to be Ser-Glu-His. In all three cases the side chains of the active site amino acids form a configuration which is stereochemically very similar to that of serine proteases. In contrast to the proteases, the lipases share the common feature that the active site is buried in the protein. In the case of the HPL and RML the active site is covered by a short amphipathic helix or "lid" whereas the active site of *G. candidum* lipase seems to be covered by two nearly parallel amphipathic helices. Brzozowski et al. (4) have shown that in the case of RML the lid moves away upon interaction with the substrate. It has been proposed that this conformational change results in activation of these enzymes at an oil-water interface.

To obtain a lipase for use in detergent formulations, an extensive screening of microbial lipases was performed by assaying them in a model wash system. The lipase produced by the gram-negative bacterium *Pseudomonas glumae* PG1 performed best in these studies (27, 28). In order to optimize this lipase for detergent application, we initiated a research project that included analysis of its catalytic properties (7), determination of the three-dimensional structure (5), and determination of structure-function relationships.

In this paper we describe the isolation and characterization of the structural gene for the extracellular lipase of *P. glumae* PG1. As a first step in the analysis of structure-function relationships we determined the amino acid residues which constitute the catalytic triad.

MATERIALS AND METHODS

Bacterial strains and growth conditions. We used a nonfluorescent *Pseudomonas* strain which is classified as a member of the *Pseudomonas solanacearum* rRNA complex (i.e., rRNA group II) and is closely related to *P. glumae* (on the basis of determinations by K. Kersters, University of Ghent, Ghent, Belgium); below this strain is designated *P. glumae* PG1. *P. glumae* was grown at 30°C in PG medium, which contained (per liter) 6 g of $(NH_4)_2SO_4$, 3.5 g of KH_2PO_4, 3.5 g of K_2HPO_4, 0.02 g of $CaCl_2$, 1 g of $MgSO_4 \cdot 7H_2O$, and 2 g of yeast extract (Difco) (pH 6.5), or on minimal medium E agar (14) (15 g of agar/liter). Glucose (5 g/liter), olive oil (Sigma) (10 ml/liter), or oleic acid (Sigma) (10 ml/liter) was added as a carbon source. In addition, we used BYPO medium, which contained (per liter) 10 g of Trypticase peptone (BBL), 3 g of yeast extract, 5 g of meat extract (Oxoid), 5 g of NaCl, 7 g of KH_2PO_4, 15 g of agar, and 50 ml of an olive oil emulsion consisting of 100 ml of olive oil per liter and 100 g of gum arabic (Merck) per liter. The formation of clear zones surrounding bacterial colonies grown on BYPO medium plates was used as a qualitative measure of lipase production. *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth or on Luria-Bertani agar (22). If necessary, appropriate antibiotics were added to the media

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at the following concentrations: ampicillin, 100 μg/ml for *E. coli*; tetracycline, 25 μg/ml for *E. coli* and 50 μg/ml for *P. glumae*; and kanamycin, 25 μg/ml for *E. coli* and 100 μg/ml for *P. glumae*.

Lipase production and characterization. The *P. glumae* lipases were produced in laboratory scale, fed-batch fermentation cultures (approximately 8 liters) containing olive oil or oleic acid as the sole carbon source. To recover the lipase from the culture medium, cells were removed by centrifugation in a Sorvall superspeed centrifuge equipped with a type GSA rotor at 10,000 rpm for 30 min. After ultrafiltration with a Nephross Andante type HF dialyzer (Organon Teknika), impurities were removed by isopropanol (50%, vol/vol) precipitation and Amberlite type XAD8 (Sigma) hydrophobic interaction chromatography. The enzyme was bound to the Amberlite in 2 M NaCl–20 mM Tris-HCl (pH 8.5) and after washing with 20 mM Tris-HCl (pH 8.5) was eluted with 10 mM Tris-HCl (pH 8.5)–50% (vol/vol) ethanol. Further purification was achieved by anion-exchange chromatography on DEAE-Trisacryl; samples were applied to the column in 20 mM Tris-HCl (pH 8.5) and were eluted with an NaCl gradient (concentrations from 0 to 100 mM).

Lipase activity was routinely assayed in pH-stat equipment (model PHM84 research pH meter, model ABU80 autoburette [Radiometer, Copenhagen, Denmark], Apple II computer for data handling and pH-stat control) at pH 9.0 and 30°C. An olive oil emulsion (3%, stabilized with 3% gum arabic) was used as the substrate, and 0.05 N NaOH (Titrisol [Merck]) was used for titration; 1 lipase unit corresponded to 1 μmol of free fatty acid released per min. Lipase K_m values were determined by using the activity assay described above, in which the substrate concentration was varied. Over the substrate range studied no lag phase was observed. The data were fitted directly to a hyperbola by using nonlinear regression. It should be mentioned that apparent K_m values were obtained, as surface concentration rather than absolute substrate concentration determines the activity of this enzyme.

The N-terminal amino acid sequence of purified lipase was determined by using an Applied Biosystems gas phase protein sequencer. The sequence obtained was 1-ADTYAA TRYPVILVHGLAGTDK-22.

DNA manipulation. Restriction enzymes and other DNA-modifying enzymes were obtained from Amersham International or Biolabs and were used according to the manufacturer's instructions. The nucleotide sequence was determined by the Sanger dideoxy chain termination method (22), using a Sequenase kit, 7-deaza-dGTP (United States Biochemical Corp.), and universal M13 primers, as well as internal primers. Chromosomal DNA was isolated essentially as described by Marmur (20). Transformation of *E. coli* with recombinant DNA molecules, plasmid isolation, and purification of DNA fragments were done as described by Sambrook et al. (22). Plasmid DNA was introduced into *P. glumae* by performing biparental matings with *E. coli* S17-1 (26) containing the relevant plasmid. Matings were performed on Luria-Bertani agar for 16 h at 30°C. Transconjugants were selected on minimal medium E plates supplemented with 0.5% glucose and the appropriate antibiotic(s).

Gene replacement. To facilitate the replacement of the lipase gene by a selectable drug resistance marker, the approximately 1,050-bp *Cla*I-*Pst*I fragment of pUR6001 (Fig. 1A), which contained almost all of the lipase structural gene, was replaced by the 1.4-kb *Eco*RI-*Ava*I fragment of pBR322 (22), which contained the tetracycline resistance gene. After partial digestion of the resulting plasmid, pUR6103, with *Bam*HI, a 2.5-kb fragment (comprising the tetracycline resistance gene flanked by the lipase 5′ and 3′ border sequences) was isolated and introduced into the unique *Bam*HI site of suicide plasmid pRZ102 (16), yielding pUR6107. By biparental mating with *E. coli* S17-1(pUR6107), this plasmid was introduced into *P. glumae* PG1; transconjugants were selected on minimal medium E plates containing glucose and tetracycline. A Southern blot analysis of the chromosomal DNA was performed to confirm that the lipase gene was replaced by the tetracycline resistance gene.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (22). Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were prepared either in the presence (reduced) or in the absence (oxidized) of β-mercaptoethanol. Isoelectric focusing was performed by using a phastSystem apparatus (Pharmacia).

Computer analysis. To analyze DNA and protein sequences, the computer programs of UWGCG (8) were used along with the EMBL nucleotide sequence data bank (release 29) and the SWISS-PROT protein sequence data bank (release 20).

RESULTS AND DISCUSSION

Isolation and characterization of the *P. glumae* PG1 lipase gene. A genomic library of *P. glumae* PG1 was constructed in cosmid vector c2RB and was maintained in *E. coli* 1046, as described by Sambrook et al. (22). On the basis of the N-terminal amino acid sequence of the strain PG1 lipase, mixed probe oligonucleotides were prepared and used to screen the genomic library (22). In this way, several positive cosmid clones were obtained. To subclone the lipase gene, one of these cosmid clones, pUR6000, was digested with different restriction enzymes and ligated with linearized pEMBL9 (22). The subclones obtained were screened with the mixed probes, and in this way we obtained pUR6001, which contained a 2.2-kb *Bam*HI chromosomal DNA fragment (Fig. 1A). Figure 1B shows the nucleotide sequence of an approximately 1.2-kb fragment. An analysis of this sequence revealed the presence of an open reading frame from ATG at position 89 to TGA at position 1165, preceded by a Shine-Dalgarno sequence (GGAGA). The putative protein that is encoded is 358 amino acids long and contains the amino acid sequence of the purified lipase, as determined by Edman degradation (Fig. 1B), confirming the isolation of the *lipA* gene. The codon usage corresponds to the codon preference table of *Pseudomonas aeruginosa* (30), and the high G+C content (approximately 70 mol%) is typical for a *Pseudomonas* gene.

***lipA* gene product.** The *lipA* gene codes for mature lipase and a preceding remarkably long signal sequence of 39 amino acids. Compared with the general architecture of signal sequences (10), the lipase signal sequence has an exceptionally long c region. The mature lipase consists of 319 amino acids and has a calculated molecular mass of 33.1 kDa. The two cysteine residues at positions 191 and 279 are connected by a disulfide bond, as the apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels is increased by reduction with β-mercaptoethanol. The calculated isoelectric point for the mature lipase is 6.0, which contrasts with the measured value of 7.2, indicating that some of the negatively charged residues may be obscured by cationic counterions (e.g., calcium) (7). High

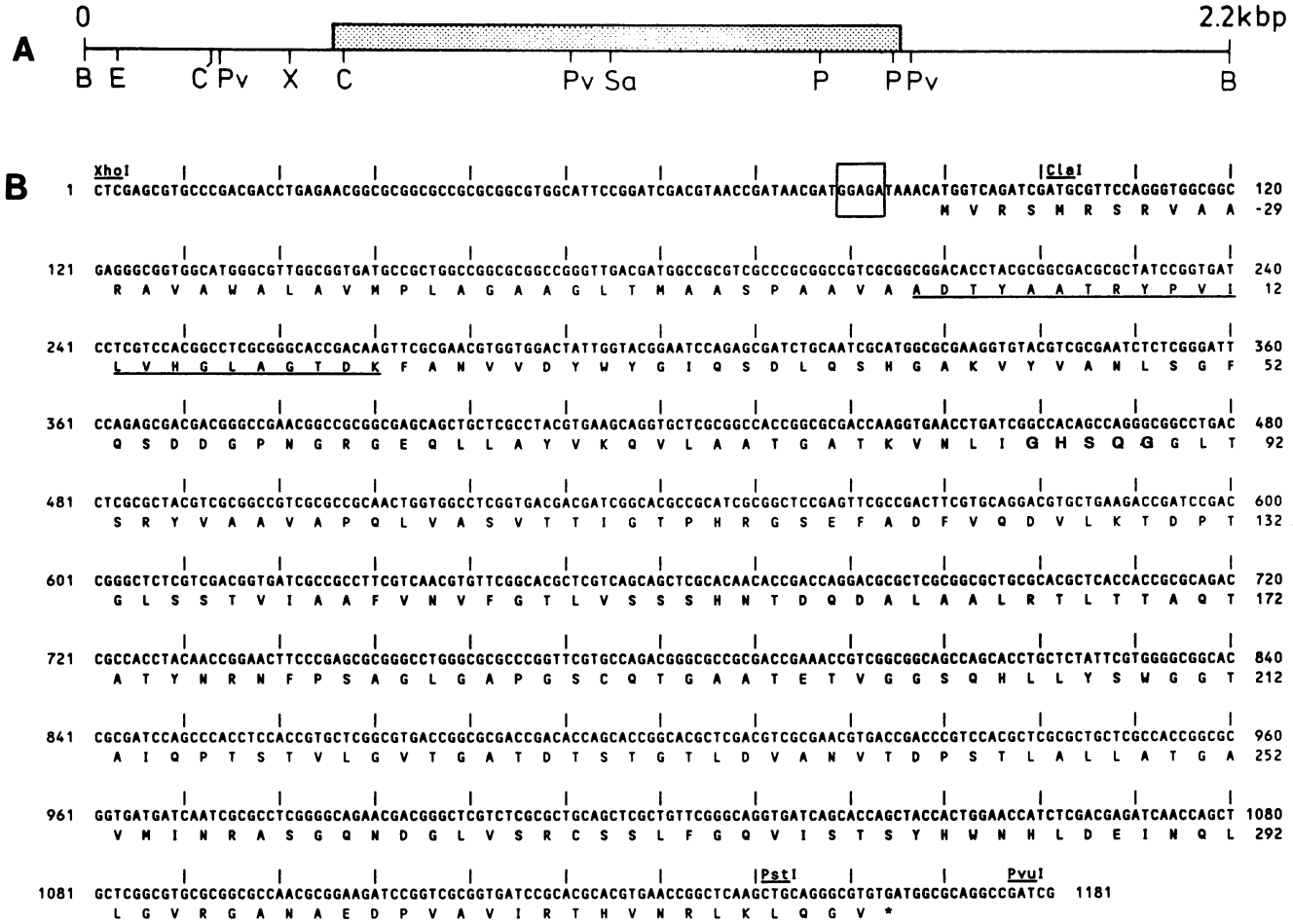

FIG. 1. (A) Restriction map of the 2.2-kb chromosomal DNA fragment of *P. glumae* present in pUR6001, including the lipase gene (stippled box). Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; Sa, *Sal*I; X, *Xho*I. (B) Nucleotide sequence of *lipA* gene and deduced amino acid sequence of the lipase of *P. glumae* PG1. The putative Shine-Dalgarno sequence is enclosed in a box. The N-terminal amino acid sequence, as determined by Edman degradation of mature lipase, is underlined.

levels of homology were found with the lipases of *P. cepacia* (89%), *Pseudomonas* sp. (62%), and *P. fragi* (60%).

Construction of a lipase-negative strain. To produce engineered lipases, we used the homologous host *P. glumae*. To ensure that the mutant lipase which we produced was not contaminated with wild-type lipase, the wild-type lipase gene was inactivated by replacing the chromosomal *lipA* gene by a tetracycline resistance gene (see Materials and Methods). One of the transconjugants having the Lip^- Tc^r Km^s phenotype was designated *P. glumae* PG3. After we reintroduced the wild-type lipase gene into strain PG3, the resulting organism had the Lip^+ phenotype, indicating that this strain can be used for the production of mutant lipases.

Identification of the active site residues. Recent crystallographic data for several eukaryotic lipases (3, 23, 31) revealed that these enzymes belong to the class of serine hydrolases. Other members of this class of enzymes are serine proteases and esterases. The active sites of these hydrolases are composed of a serine, a histidine, and a carboxylic amino acid (aspartate or glutamate residues). While the architecture of this active site is retained in most instances, little structural homology is found. This is true for proteases (e.g., trypsin and subtilisin families) and for lipases and esterases. One common feature is apparent; among lipases and esterases the order of active site residues in the amino acid sequence is Ser-Asp(Glu)-His, while the order is His-Asp-Ser in trypsins and Asp-His-Ser in subtilisins. Additional amino acid sequence homology among the enzymes is low, except for closely related enzymes like the subtilisins produced by bacilli and the *Pseudomonas* lipases mentioned above. The sequence homology within this family of lipases and the sequence homology with more distantly related lipases were used to identify conserved regions surrounding Ser, His, and Asp residues. To assess the potential function of the selected amino acids as part of the catalytic triad, site-directed mutagenesis was used to replace the residues with other amino acids.

(i) **Active site serine.** Assignment of the active site Ser residue was straightforward, as the fully conserved Ser-87 in the sequence Gly-His-Ser-Gln-Gly conforms to the Gly-X-Ser-X-Gly sequence pattern found in many hydrolases. Replacing Ser-87 by alanine (lipase PGL21) yielded inactive enzyme (Table 1), confirming the functional importance of this residue; our results were in line with the results of identical studies carried out previously with rat hepatic lipase (6) and lipoprotein lipase (9).

TABLE 1. K_m and V_{max} values of mutant lipases, compared with wild-type lipase values

Lipase	Mutation	Activity[a]	K_m (mM)	V_{max} (lipase units/mg)
PGL1	Wild type	+	0.7	4,000
PGL21	S87A	−	ND[b]	ND
PGL12	H15A	+	1.2	1,300
PGL62	H285A	−	ND	ND
PGL30	D263E	+	1.0	1,800
PGL60	D121E	+	0.6	1,000
PGL67	D241E	+	0.6	300

[a] Clearing zone formation on BYPO medium plates was used as an initial measure for the production of active lipase (+) or inactive lipase (−).
[b] ND, not determined.

(ii) Active site histidine. Of the eight histidine residues present in lipase PGL1, only His-15, His-86, and His-285 are found in regions that are conserved in the family of *Pseudomonas* lipases. His-86 is situated next to the active site Ser-87 and is probably not a member of the catalytic triad because of structural constraints. To discriminate between His-15 and His-285, we replaced both residues by alanine, resulting in lipases PGL12 and PGL62, respectively. Changing His-15 to alanine resulted in a mutant lipase having a K_m that was almost double the wild-type lipase K_m and a decreased maximum initial velocity (V_{max}) (Table 1), whereas the mutation His-285-Ala (H285A) resulted in an inactive lipase, indicating that His-285 is part of the active site triad. As in the *Pseudomonas* lipases, the active site histidines in HPL (His-263) and in RML (His-257) are located in the sequence (Asn or Asp)-His-Leu (Fig. 2B), supporting the hypothesis that His-285 is an active site residue in *P. glumae* lipase. The decreased activity of the His-15-Ala mutant, the highly conserved sequence of hydrophobic residues preceding His-15, and the homology with pancreatic, gastric, and *Staphylococcus* lipases (29) point to an important functional role for the region between amino acids 7 and 17. Interestingly, it has been suggested that in HPL the backbone nitrogen of Phe-77 is one of the two donors to the oxyanion (31). Considering the high level of homology with the *Pseudomonas* enzyme (Fig. 2A), we tentatively assign this role to the backbone nitrogen of Leu-17.

(iii) Active site aspartate. The most difficult task was the assignment of the active site carboxylate residue, because structurally this residue is not as highly constrained as the other two residues in the catalytic triad. No conserved sequences surrounding glutamate residues were discovered among the *Pseudomonas* lipases, indicating that an aspartate should be a member of the catalytic triad. When we compared the family of *Pseudomonas* lipases, only five aspartate residues were located in more or less conserved regions. In lipase PGL1 three of these (residues 121, 241, and 263) (Fig. 2) are the most likely candidates if the sequential order Ser-Asp-His indicated above is considered. To identify the active site aspartate residue, the three possible candidates, aspartate 121, aspartate 241, and aspartate 263 were first replaced by a glutamate residue. In these cases the functional carboxylate group is retained, but small conformational effects may occur because of the introduction of an extra methylene group. Mutants Asp-121-Glu (lipase PGL60), Asp-263-Glu (lipase PGL30), and Asp-241-Glu (lipase PGL67) were all found to be catalytically active. However, while the V_{max} values of lipases PGL60 and PGL30 are of the same order as the V_{max} of the wild-type lipase, the V_{max} observed for mutant Asp-241-Glu (lipase PGL67) (Table 1) is more than 1 order of magnitude lower than the V_{max} of the wild-type lipase.

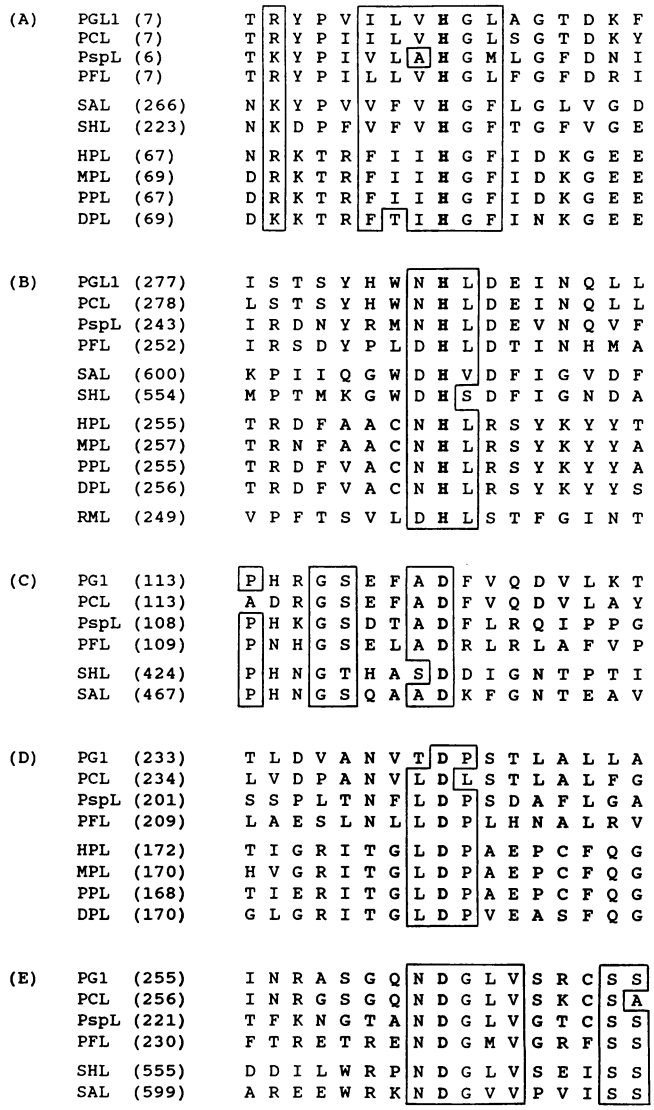

FIG. 2. Regions surrounding putative active site residues of lipase PGL1 compared with corresponding regions in other lipases: regions surrounding histidine 15 (A), histidine 285 (B), aspartate 121 (C), aspartate 241 (D), and aspartate 263 (E). Abbreviations: PCL, *P. cepacia* lipase (17); PspL, *Pseudomonas* sp. (21); PFL, *P. fragi* (1); SAL, *S. aureus* (12); SHL, *S. hyicus* (19); HPL, human pancreatic lipase (31); MPL, mouse pancreatic lipase (13); PPL, pig pancreatic lipase (13); DPL, dog pancreatic lipase (13); and RML, *R. miehei* lipase (3). Similar amino acids conserved in most of the lipases are enclosed in boxes.

To obtain further evidence, three more mutants were made by replacing Asp-121, Asp-241, and Asp-263 by alanine, resulting in lipases PGL85, PGL87, and PGL89, respectively. In these mutants the carboxylate group is removed, which obviously has a more drastic effect on lipase activity. For lipase PGL85 a V_{max} comparable to that of lipase PGL1 (wild type) was found, which proved that Asp-121 is not the active site aspartate. With respect to the

lipase PGL89 mutation, the V_{max} was determined to be 900 lipase units/mg, indicating that Asp-263 is not involved in the catalytic triad. When the strain producing the lipase with mutation Asp-241-Ala was grown, no lipase activity was detected, indicating that Asp-241 is the catalytic site residue. Furthermore, this finding is supported by secondary structure predictions, which suggest that Asp-241 is located on the C-terminal side of a β-strand, which is similar to the position of the active site Asp in the crystal structures of RML (3) and HPL (31).

A remarkable observation was that mutations Asp-241-Ala and Asp-263-Ala resulted in a considerable decrease in the amount of lipase produced, whereas the production levels of the other mutants were comparable to the production level of the wild type.

Conclusion. On the basis of the primary sequence homology observed in a group of four *Pseudomonas* lipases, several conserved regions containing putative active site amino acids (serine, histidine, or aspartate) were found. These conserved regions may be important for structural and/or functional reasons. In order to determine the residues that actually make up the catalytic triad of *P. glumae* PG1 lipase, we replaced all of the potential active site residues and produced mutant enzymes in lipase-negative *P. glumae* PG3. From the results obtained we conclude that Ser-87, His-285, and Asp-241 make up the catalytic triad of lipase PGL1 and that the corresponding amino acids in the homologous *Pseudomonas* lipases share the same role.

This result, together with increasing knowledge concerning the catalytic mechanism (7) and the known three-dimensional structures of other lipases, should help workers construct a model of lipase PGL1 structure. This model should guide us to select specific modifications for protein engineering to develop a superior lipase for industrial application.

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