Purification, Characterization, and Molecular Cloning of Lactonizing Lipase from *Pseudomonas* Species*

(Received for publication, May 14, 1991)

Fumio Ihara, Yukari Kageyama, Mitsuyo Hirata, Takuya Nihira, and Yasuhiro Yamada‡

From the Department of Biotechnology, Faculty of Engineering, Osaka University, 2-1 Yamada-oka, Suita-shi, Osaka 565, Japan

An extracellular lipase catalyzing the synthesis of macrocyclic lactones in anhydrous organic solvents was purified to homogeneity from *Pseudomonas* nov. sp. 109, and characterized. The lipase showed a pI of 5.3 on isoelectric focusing and a M,

of 29,000 ± 1,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. With respect to substrate specificity, optimum chain length for acyl moiety varied depending on the type of reaction catalyzed: C18 in monomer lactone formation, C11 or shorter in dimer lactone formation, and C8 in ester hydrolysis. The amino-terminal 19 amino acid residues of the purified lipase were determined as Ser-Thr-Tyr-Thr-Gln-Thr-Lys-Tyr-Pro-Ile-Val-Leu-Ala-His-Gly-Met-Leu-Gly-Phe, and the gene encoding the lipase was identified by hybridization to a synthetic 20-nucleotide probe, cloned, and sequenced. Nucleotide sequence analysis predicted a 311-amino acid open reading frame, a putative ribosome-binding site, and a 26-amino acid sequence at the amino terminus of the sequence that is not found in the mature protein. This 26-amino acid sequence has many of the characteristics common to known signal peptides. The lipase gene encoded a sequence of Val-Asn-Val-Leu-Ile-Gly-His-Ser-His-Gly-Gly-Gly-Leu-Ile-Gly-His-Ser-His-Gly-Gly-Phe, which is very well conserved among lipases, and showed 38-40% overall homology to the amino acid sequences of lipases from *Pseudomonas fragi* and *Pseudomonas cepacia*, but showed little homology to those of other lipases, suggesting that some structural features are required for catalyzing macrocyclic lactone synthesis in organic solvents and are restricted to lipases of the *Pseudo-

monas* origin.

Lactone, an intramolecular ester, has generally been found in natural compounds. Especially, 14-membered or larger membered lactones (macro cyclic lactones) constituted key structures of many useful compounds, such as macrolide antibiotics (1). Although 5- or 6-membered lactones can be formed automatically and exclusively from corresponding hydroxy acids (2), the formation of macrocyclic lactones, especially the monomeric lactones, is not straightforward: the formation of monomer lactone should compete with the formation of linear oligomers and also with that of multimeric lactones, such as dimeric lactones (diolides). Therefore, several chemical methods have been developed to facilitate an intramolecular esterification, mainly by activating acyl carbonyl groups weakly with special reagents, such as 2,2'-dipyridyl disulfide (3), 2-chloro-1-methylpyridinium iodide (4), and carboxylic 2,4,6-trichlorobenzoic anhydride (5). However, the necessity to use very complicated, often very expensive, reagents or drastic conditions constitutes the main disadvantages of these chemical syntheses.

Recently, we have established that crude preparation of an extracellular lipase ("lipase P") produced by *Pseudomonas* nov. sp. 109 catalyzed in anhydrous organic solvents an intramolecular transesterification of methyl 16-hydroxyhexadecanoate leading to the efficient formation of cyclohexadecanol-ide (Fig. 1, n = 15) (6). Furthermore, when racemic mixtures of (ω-1)hydroxy acid methyl ester was used as substrate, the lactonizing lipase utilized only R-isomer, indicating the highly stereospecific nature of the lactonization. However, the lactonizing activity was not common to lipases. Our preliminary screening revealed that some lipases of *Pseudomonas* origin and porcine pancreas lipase possessed the lactonizing activity (6), which suggest that special structural features are necessary to catalyze the synthesis of macrocyclic lactones.

To initiate the detailed three-dimensional analysis of the lactonizing lipase, large amounts of highly purified enzyme would be necessary. To that goal, we described herein the purification of the lactonizing lipase from *Pseudomonas* nov. sp. 109, and cloning of the gene encoding the lactonizing lipase (lipL). Sequence analysis revealed the high homology to amino acid sequences of lipases from *Pseudomonas cepacia* (7) and *Pseudomonas fragi* (8, 9), which in turn predicted that those lipases may also have lactonizing activity.

MATERIALS AND METHODS

*Bacteria, Bacteriophage, and Plasmid*

*Pseudomonas* nov. sp. 109, the lipase P producer, was obtained from Nagase Biochemicals Ltd. and has been deposited to the Fermentation Research Institute, Agency of Industrial, Science and Technology, Tsukuba (FERM-P No. 3025). *Escherichia coli* JM105 was used as a host for recombinant plasmids, bacteriophage M13mp18 and M13mp19 for nucleotide sequencing, and plasmid pUC19 as a vector for cloning (10).

**Purification of Lactonizing Lipase**

Crude powder of lipase P was obtained from Nagase Biochemicals Ltd. (Osaka). All the purification procedures were done at 4 °C unless otherwise specified.

(1) **Preparation of Crude Extract**—Crude lipase P (5 g) was suspended in 30 ml of 0.05 M potassium phosphate buffer (pH 6.5) containing 1 mM MgCl₂, 5 mM 2-mercaptoethanol, and 20% glycerol. After standing for 1 h at 4 °C with mild stirring, the solution was...
Lactonizing Lipase from Pseudomonas Species

dialyzed for 8 h against 3 liters of the same buffer with two changes, and centrifuged for 10 min at 8,000 × g.

(ii) Isoelectric Focusing—Ampholine (pH 3.5–10, Pharmacia LKB Biotechnology Inc.) was added to the supernatant from step i to a final concentration of 2%, and the mixture was fractionated on Rotofor (Bio-Rad) for 4 h with 12 W constant power. Fractions 8–10 (containing 4.5–5.3) were mixed.

(iii) Gel Filtration on Sepharose CL-6B—Sample from step ii was applied to a Sepharose CL-6B column (2.8 × 50 cm, Pharmacia LKB Biotechnology Inc.) pre-equilibrated with 0.05 mM potassium phosphate buffer (pH 6.5) containing 1 mM MgCl₂ and 5 mM 2-mercaptoethanol, and developed with the same buffer. Fractions containing the lipase activity (fractions 41–46, 30 ml) were pooled and concentrated to 5.8 ml by ultrafiltration. Fractions containing 6.7 to 6.8 ml of the elution volume were pooled.

Chemicals

-p-Nitrophenyl acetate was obtained from Tokyo Kasei Co. p-Nitrophenyl butyrate, p-nitrophenyl hexanoate, p-nitrophenyl octanoate, p-nitrophenyl dodecanoate, p-nitrophenyl tetradecanoate, p-nitrophenyl hexadecanoate, p-nitrophenyl octadecanoate, and triaurin were obtained from Nacalai Tesque Ltd. Tricaprylin, tricaprin, tripalmitin, and tristearin were obtained from Wako Pure Chemicals Ltd. Tricaprin and trimiristin were obtained from Fluka Chemie AG and Sigma, respectively. p-Nitrophenyl decanoate was synthesized from decanol chloride and p-nitrophenol. Several chain lengths of ω-hydroxy acid methyl esters were synthesized as described previously (6).

SDS-PAGE

SDS-PAGE was done with a ready-made 4–20% linear gradient gel (Daichi Pure Chemicals Co. Ltd.) with a minigel apparatus (Daichi Pure Chemicals Co. Ltd.) according to Laemmli (11), and stained with Coo massie Brilliant Blue G-250. Silver staining was done with Ag stain (Daichi Pure Chemicals Co. Ltd.) according to the manufacturer’s protocol. Activity staining was done with α-naphthyl lactate and fast blue RR salt according to Gabriel (12).

Assay of Esterase and Lactonizing Activity

Esterase activity was routinely assayed by measuring the amount of p-nitrophenol formed from p-nitrophenyl acetate. Reaction mixtures (3.0 ml) contained 0.01 M potassium phosphate (pH 6.5), 2.5 mM p-nitrophenyl acetate, 5% CH₃CN, and enzyme. Reactions were initiated by addition of p-nitrophenyl acetate (50 mM, 0.15 ml) dissolved in dry CH₃CN to a solution of other components which had been preincubated at 37 °C for 2 min. During the reaction, absorbance at 400 nm originating from p-nitrophenol (ε₄₀nm = 14,200) was monitored. For determination of substrate specificity, p-nitrophenyl esters of various chain length were emulsified completely by sonication in the presence of 0.5% Triton X-100 in 0.05 mM potassium phosphate (pH 6.5). After preincubication at 37 °C for 3 min, reactions were initiated by addition of an equal volume of enzyme solution (1.5 ml) in the same buffer, followed by a further incubation at 37 °C for 10 min. Reactions were stopped by addition of acetic acid (3 ml). The mixtures were clarified by centrifugation, and the absorbance at 400 nm was measured. One unit of esterase activity was defined as the amount of enzyme which liberates 1 μmol of p-nitrophenol or fatty acid/min.

Lactonizing activity was assayed routinely by measuring the amount of cyclohexadecanolide formed from methyl 16-hydroxyhexadecanoate. After dialysis of enzyme sample against 0.05 M triethanolamine-HCl (pH 6.5) containing 1 mM MgCl₂, the dialyzed sample was lyophilized to remove water in a 50-ml round-bottom evaporation flask. The lactonization reaction was initiated by suspending the lyophilized sample with 1 mM methyl 16-hydroxyhexadecanoate in dry hexane. During the reaction with vigorous stirring at 40 °C, aliquots were withdrawn intermittently, mixed with an equal volume of octacosane (0.2 g/liter in dry hexane) as protein concentration was determined by the procedure of Lowry et al. (13) using crystalline bovine serum albumin as standard.

Amino Acid Sequence Analysis

A sample of purified protein (HPLC fraction) was passed through a TSK G2000SWXL column (0.78 × 30 cm) equilibrated with 0.01 M ammonium acetate (pH 6.5) containing 0.1% Naogen (Daichi Kogyo Seiyaku, Kyoto), protein fractions were pooled, and lyophilized to remove ammonium acetate. A sample of HDNA was then analyzed using an on-line phenylthiohydantoin-derivative analyzer 120A (Applied Biosystems). This analysis was carried out with the assistance of Dr. Hiroshi Kataoka (University of Tokyo, Dept. of Agriculture).

DNA Manipulations

Chromosomal DNA of Pseudomonas sp. 109 was obtained from cells grown in LB medium at 30 °C for 16 h by the modified method of Marmur as described by Coleman et al. (14). Plasmid DNAs were prepared by alkaline extraction (15). Restriction, ligation, end filling, and exonuclease treatment were done with enzymes from Takara Shuzo (Kyoto) or Toyobo Co. Ltd. (Osaka) under conditions recommended by the supplier. E. coli JM105 was transformed by the CaCl₂ method (16).

Southern Blot Analysis

High molecular weight genomic DNA was digested with PsI, SstI, or SmaI, and the digested DNAs were fractionated electrophoretically on a 1.0% agarose gel. The DNA was transferred to a nylon membrane (Hybond-N, Amersham Corp.), and hybridized with oligonucleotide probe end-labeled with γ-³²P]ATP (>3000 Ci/mmol, ICN Biomedicals Inc.) and T4 polynucleotide kinase. Hybridization was done in 5 × SSPE containing 0.5% SDS, 5 × Denhardt's solution, and 0.1 mg/ml of salmon sperm DNA for 20 h at 45 °C and washed twice with 0.1 × SSC containing 0.1% SDS for 10 min each at 45 °C (15, 17).

DNA Sequencing and Sequence Analysis

Nucleotide sequences were determined by the dideoxynucleotide chain termination method (18) using [α-³²P]dCTP (>3000 Ci/mmol, ICN Biomedicals Inc.) and M13 phage single stranded DNAs as template with Sequenase (United States Biochemical Corp.). The sequence was determined from both strands, and most nucleotides were sequenced several times in different overlapping clones.

Homology comparisons with EMBL, GenBank, NBRF, and SWISS-PROT sequences (release April 1991) were performed on a personal computer with GENETYX software package (Software Development Co. Ltd., Tokyo).

RESULTS AND DISCUSSION

Purification of Lactonizing Lipase—We have previously shown that crude powder of lipase from Pseudomonas sp. 109 (lipase P) catalyzed, in anhydrous organic solvents, intramolecular transesterification of methyl 16-hydroxyhexadecanoate leading to cyclohexadecanolide (n = 15, Fig. 1). However, when purified lipase constituting the major lipase activity in the crude powder was used instead, no lactonization was observed (data not shown). Therefore, Pseudomonas sp. 109 should produce at least 2 lipases, and the lactonizing lipase would be a minor component. Actually, our previous screening with commercial lipases demonstrated that only lipases from Pseudomonas species and porcine pancreas lipase (Sigma type II) possessed the lactonizing activity (6). Again,
Lactonizing Lipase from Pseudomonas Species

**TABLE I**

| Purification step        | Total volume | Protein | Lactonizing activity | Specific activity | Purification | Yield |
|--------------------------|--------------|---------|----------------------|-------------------|--------------|-------|
| Crude                    | 36.5         | 1717.7  | 108.6                | 0.063             | 1            | 100   |
| Isoelectric focusing     | 3.6          | 107.5   | 47.6                 | 0.422             | 7            | 44    |
| Sepharose CL-6B          | 24.6         | 11.0    | 93.9                 | 8.56              | 136          | 87    |
| HPLC                     | 430.5        | 5.5     | 172.2                | 31.30             | 497          | 158   |

**TABLE II**

| Substrate HO-          | Catalytic rate of lactone formation |
|------------------------|------------------------------------|
| (CH₂)n-COOCH₃ Monomer | % nmol/min/mg                      |
|                        | Diolide                             |
| n = 10                 | ND                                 |
| n = 11                 | 0                                  |
| n = 12                 | 12                                 |
| n = 13                 | 20                                 |
| n = 14                 | 20                                 |
| n = 15                 | ND                                 |
| n = 16                 | 7                                  |
| n = 17                 | 0.2                                |
| n = 18                 | 0.1                                |
| n = 20                 | 0                                  |

Lactonization activity of the lipase toward various ω-hydroxy acid methyl esters

Lactonization was performed with crude lipase P (200 mg) at a substrate concentration of 1 mM in dry hexane (20 ml) at 40 °C. The amount of corresponding monomer lactone and diolide were determined by gas chromatography as described (6). Reaction progress was followed intermittently, and catalytic rate was calculated from the linear part of each reaction.

**FIG. 1.** Lactonization reaction catalyzed by lipase from *Pseudomonas* nov. sp. 109 in anhydrous organic solvents using methyl esters of ω-hydroxy acids. I, substrate, n = 10–20; II, monomer lactone; III, dimer lactone (diolide).

**FIG. 2.** Purity and molecular weight determination of the lactonizing lipase by SDS-PAGE. A, samples were run on a 4–20% linear gradient gel, and stained with Coomassie Brilliant Blue G-250. Lane a, 0.5 μg of crude lipase P after dialysis and centrifugation; lane b, 2 μg of HPLC fraction; lane M, marker proteins (phosphorylase b, M₀ = 94,000; bovine serum albumin, M₀ = 67,000; ovalbumin, M₀ = 43,000; carboxy anhydrase, M₀ = 30,000; soybean trypsin inhibitor, M₀ = 20,000). B, log(M₀) versus relative mobility plot; M₀ position of purified lipase.

for porcine pancreas lipase, further purified enzyme (Sigma type VI) did not show any lactonizing activity, indicating that contaminating minor enzyme present in the rather crude preparation was responsible for the lactonization (data not shown).

To characterize in more detail the lactonizing lipase, the lipase was purified from the crude powder of lipase P. Fraction was monitored both by esterase activity toward p-nitrophenyl acetate and formation of cyclohexadecanolide from methyl 16-hydroxyhexadecanoate. On isoelectric focusing of the crude lipase P, two esterase peaks were observed at pH values of 5.3 and 7.0, respectively, and the lactonizing activity coincided with the former peak. The lactonizing lipase did not adsorb to CM-Sephadex C-25 at pH 7, naturally from its PI value of 5.3, and activity recovery was less than 50% with DEAE-Sepharose, thus eliminating the use of ion exchange chromatography for further purification. Furthermore, on hydrophobic chromatography with C₄, C₈, or phenyl resin, the lipase was strongly adsorbed and no activity was recovered even by 50% (v/v) ethylene glycol, indicating the very hydrophobic nature of the lactonizing lipase. However, gel filtration afforded a good separation and activity yield. After open column gel filtration using Sepharose CL-6B and gel filtration HPLC, nearly 500-fold purification was attained on the basis of specific lactonizing activity (Table I). However, during purification the yield in lactonizing activity exceeded 100%. This is due to the presence of polysaccharide or Ampholine, which makes it difficult to remove water before the assay of lactonizing activity. In lactonization, the anhydrous condition is necessary to afford intramolecular transesterification. The presence of water facilitated hydrolysis, rather than transesterification, of substrate, thus resulting in underestimation of the lactonizing activity. Therefore, actual purification should be around 300-fold. Electrophoresis of 0.5 μg of protein showed that the HPLC fraction is greater than 95% pure (Fig. 2) and migrated with an apparent molecular weight of 29,000 ± 1,000. Silver staining and activity staining also revealed a single band (data not shown). The mobility of samples run in gels in the absence of reducing agents did not change. However, apparent molecular weight estimated by gel filtration differed significantly (M₀ = 290,000 on Sephadex G-200 with 10 mM potassium phosphate, pH 7, and M₀ = 133,000 on TSK gel G2000SWXl, with 50 mM potassium phosphate, pH 7, containing 10% (v/v) CH₃CN), which indicated that the lipase existed as several aggregates and the aggregation state changed by the hydrophobicity of the eluent.

**Substrate Specificity of the Lactonizing Lipase—Chain**
TABLE III
Esterase activity of lactonizing lipase toward several p-nitrophenyl esters and triglycerides

Esterase activities were measured with purified lipase. Hydrolysis of p-nitrophenyl esters were measured at a fixed concentration (2.5 mM) of esters having an indicated chain length emulsified completely by sonication in the presence of 0.5% Triton X-100. Hydrolyses of triglycerides were measured at a fixed concentration (25 mM) of triglycerides emulsified as above. For triglycerides, after 60 min reaction at 37 °C with vigorous stirring, reaction was stopped by the addition of a 10-fold volume of ether/ethanol (1:1), and the amount of free fatty acid was determined by titration with 0.01 N KOH. Triton X-100 is added to ascertain micellar formation for all the substrates. In the absence of Triton X-100, the hydrolysis of tricaprin correlated well with the degree of micellar formation measured by iodine method (19), indicating that the lactonizing lipase preferentially hydrolyzes the micellar form of substrates.

| Substrate | p-Nitrophenyl ester hydrolyses, | Triglyceride hydrolyses, |
|-----------|---------------------------------|-------------------------|
| n = 0     | µmol/min/mg                      | µmol/min/mg              |
| n = 2     | 22.5                             | 1.6                     |
| n = 4     | 52.9                             | 3.0                     |
| n = 6     | 178.9                            | 3.1                     |
| n = 8     | 43.5                             | 3.2                     |
| n = 10    | 40.1                             | 1.5                     |
| n = 12    | 24.4                             | 0.41                    |
| n = 14    | 21.7                             | 0.24                    |
| n = 16    | 2.0                              | 0.33                    |

A, of PUY45 and sequencing strategy for cloned lipL gene (B). A, genomic DNA (5 µg/lane) digested completely by PstI (lanes 2 and 5), SαI (lanes 3 and 6), and SmαI (lanes 4 and 7) together with size markers (200 ng, AhinIII digests) were electrophoresed on 1.0% agarose gel and stained with ethidium bromide (lanes 1-4). After transfer to nylon membrane (Hybond N), the blot was hybridized with 32P-end labeled oligonucleotide probes (lanes 3-7) as described under "Materials and Methods," and exposed to x-ray film (Kodak X-Omat, −80 °C, 4 h). B, the strategy for sequencing is indicated with arrows representing the directions and length of the sequencing runs. A composite map of the overlapping clones with noncoding (white) and coding (striped) sequences is shown at the top of the figure with the initiation (ATG) and stop (TAG) codons together with restriction endonuclease sites. E, EcoRI; S, SalI; P, PstI. The scale is shown in kilobase pairs.

Lactonizing Lipase from Pseudomonas Species

Fig. 3. Southern blot analyses of genomic DNA of Pseudomonas nov. sp. 109 (A) and restriction endonuclease map of PUY45 and sequencing strategy for cloned lipL gene (B).
monomer lactone and diolide were catalyzed by different lipases.

**NH$_2$-terminal Amino Acid Sequence of the Lactonizing Lipase**—To isolate the lipase gene by hybridization to oligonucleotide probe, the NH$_2$-terminal region of the purified lipase was sequenced by automated Edman degradation. Only 1 amino acid was detected at each step of this process, confirming the homogeneity of the purified protein. By this procedure, the sequence of STYTQTKYPIVLAHGMLGF was synthesized.

**Isolation and Sequencing of a Gene Encoding the Lactonizing Lipase**—The probe was hybridized to restriction fragments generated from genomic DNA of Pseudomonas nov. sp. 109 (Fig. 3A), and 3.8-, 0.5-, and 6.0-kb bands were detected for digests by PstI, SalI, and Smal, respectively. After enrichment of the 6.0-kb Smal fragment by preparative agarose gel electrophoresis, the fragment was inserted into the Smal site of pUC19, and the ligated DNA was transformed into E. coli JM105 on the L-plate containing 0.5% tributyrin (8) and ampicillin (50 μg/ml). Among 300 transformants, two colonies formed a clear zone due to lipase activity. Recombinant plasmids recovered from the two colonies were found identical by restriction analyses. To further localize the lipase gene (lipL) in the 6.0-kb insert, various plasmids containing deletions at either end of the 6.0-kb fragment were generated by exonuclease III and mung bean nuclease, and a plasmid containing the 2.2-kb insert (pUY45) was found sufficient for lipase activity. Up to 3.3 kb could be removed from the left end of the 6.0-kb fragment and up to 0.5 kb could be removed from the right end without influencing activity (Fig. 3B).

The 2.2-kb insert encoded a 311-amino acid polypeptide.

---

**Concentrating Amino Acid Sequence**

**NH$_2$-terminal Amino Acid Sequence**

- V-Y-W-F-G-I-P-S-A-L-R-R-U-G-A-Q-V-Y-V-T-E-V-S-Q
- A-S-T-Y-T-Q-T-K-Y-P-I-V-L-A-H-G-M-L-G-F-D-N-I-L-G-F
- K-[Y-N-L-I-G-H-S-Q-G-A]-
- Y-N-L-I-G-H-S-Q-G-A-
- Y-N-L-I-G-H-S-H-G-G-
- V-M-I-F-G-E-S-A-G-A-
- V-H-F-I-G-H-S-M-G-G-
- V-H-V-I-G-H-S-L-G-A-
- V-H-L-V-G-H-S-M-G-

**Consensus Sequence**

- L-I-G-H-S-M-G-G-
- V-N-L-I-G-H-S-Q-G-A-
- Y-N-L-I-G-H-S-Q-G-A-
- Y-N-L-I-G-H-S-H-G-G-

**Consensus Function**

A: Hydrophobic; G: Charged amino acid; ?: Not detected.

**Comparison of lipase consensus sequences among various lipases (A) and overall amino acid sequences of lactonizing lipase from Pseudomonas species (B, C, D).** The amino acid sequences around the consensus region and the functional features were shown. The function of each amino acid is: Hyd, hydrophobic; Cha, charged amino acid; ?, not detected. B, sequence alignment was done according to Lipman and Pearson (31). Identical amino acids were represented by an asterisk (*), and similar amino acids were by dots (.), which were grouped as follows: Pro, Gly; Ser, Thr; Lys, Arg; Glu, Gln, Asn, Asp; Phe, Trp, His, Tyr; Ala, Ile, Val, Leu, Met, Cys (32).

**Fig. 5.** Comparison of lipase consensus sequences among various lipases (A) and overall amino acid sequences of lactonizing lipase from Pseudomonas species (B, C, D) with those of lipases from P. fragi and P. cepacia (B). A, the amino acid sequences around the consensus region and the functional features were shown. The function of each amino acid is: Hyd, hydrophobic; Cha, charged amino acid; ?, not detected. B, sequence alignment was done according to Lipman and Pearson (31). Identical amino acids were represented by an asterisk (*), and similar amino acids were by dots (.), which were grouped as follows: Pro, Gly; Ser, Thr; Lys, Arg; Glu, Gln, Asn, Asp; Phe, Trp, His, Tyr; Ala, Ile, Val, Leu, Met, Cys (32).

**Fig. 4.** Nucleotide sequence of lipL encoding the lactonizing lipase from Pseudomonas nov. sp. 109. The deduced amino acid sequence is denoted under the nucleotide sequence in the standard one-letter code. Amino acids are numbered starting with the first in-frame methionine as 1. Amino-terminal end of mature lactonizing lipase. The translation termination codon is designated by asterisks (**). The amino acid sequences obtained by sequencing of the amino-terminal end are underlined. The putative ribosome-binding site is indicated by a broken line. The lipase consensus sequence is boxed.
purified lipase was essentially identical to that deduced from the nucleotide sequence (data not shown), thus confirming that the cloned sequence was encoding the lactonizing lipase from *Pseudomonas* nov. sp. 109. The deduced amino acid sequence contains a sequence, -Val-Asn-Leu-Ile-Gly-His-Ser-His-Gly-Gly-, which match almost exclusively to the consensus sequence of lipase (Fig. 5A). Comparison with known sequences of lipases, such as from *P. fragi* (8, 9), *P. cepacia* (7), *Staphylococcus aureus* (21), *Staphylococcus hyicus* (22), *Geotrichum candidum* (23), *Candida cylindracea* (24), porcine pancreas (25), rat lingua (26), canine pancreas (27), human liver (28), and human pancreas (29), revealed that the lactonizing lipase have 38-40% homologies on the amino acid level with lipases of pseudomonads, but no overall homologies with amino acid sequences of other lipases. When the alignment of functionary similar amino acid residues are considered, similarities of lactonizing lipase toward lipases of pseudomonads increased (50% for lipase of *P. fragi* and 59% for *P. cepacia*, Fig. 5B). These high similarities on overall sequence together with the similarity in molecular weight weight suggest that those lipases from pseudomonads may have lactonizing activity, which lack in other lipases. For eucaryotic lipases, lipase activity has been reported to be inhibited by SH-blocking agents and the positions of Cys residues are well conserved (27, 30). However, for procaryotic lipases, Cys residues are lacking or low in its content, and seems not to participate in the catalytic activity. The lactonizing lipase contains only 2 Cys residues and its esterase activity was not affected by treatment of iodoacetic acid or 5,5'-dithiobis(2-nitrobenzoic acid) (no inactivation after incubation at 5 mM reagent at 37 °C for 1 h). Thus, similar to other procaryotic lipases, Cys residues in lactonizing lipase are not involved in its catalytic function.

No homologies were found when the nucleotide and predicted amino acid sequences were compared with the EMBL and GenBank nucleotide and National Biomedical Research Foundation and SWISSPROT protein sequences, except for the lipases mentioned above and one hypothetical protein from *Vibrio cholerae* (SWISSPROT, accession number P18488). Although the latter only shares the NH2-terminal part of the hypothetical protein, homology with lactonizing lipase is very high (68%), and, therefore, may represent a lipase-like enzyme of *Vibrio* origin.

The availability of genomic DNA clones for lactonizing lipase will provide tools for studying the mechanism involved in regulating the synthesis of the enzyme, and also make it possible to provide large quantities of highly purified enzyme, which is a prerequisite to initiate detailed investigations on the three-dimensional structural features which allow macrocyclic lactone formation.

Acknowledgments—We express our thanks to Nagase Biochemicals Ltd. for providing *Pseudomonas* nov. sp. 109, and also to Dr. H. Katsoka of the Department of Agricultural Chemistry, the University of Tokyo, for performing amino acid sequence analysis.

REFERENCES

1. Keller-Schierlein, W. (1973) *Fortschr. Chem. Org. Naturstoff.* 30, 313-340
2. Sisido, M. (1974) *Biophysics* 14, 135-146
3. Corey, E. J., and Nicolaou, K. C. (1974) *J. Am. Chem. Soc.* 96, 5614-5616
4. Mukaiyama, T., Narasaki, K., and Kikuchi, K. (1977) *Chem. Lett.* 441-444
5. Inanaga, J., Hirata, K., Saeki, H., Katsuki, T., and Yamaguchi, M. (1979) *Bull. Chem. Soc. Jpn.* 52, 1889-1903
6. Makita, A., Nihira, T., and Yamada, Y. (1987) *Tetrahedron Lett.* 28, 805-808
7. Jørgensen, S., Skov, K. W., and Diderichsen, B. (1991) *J. Bacteriol.* 173, 559-567
8. Kugimiya, W., Otani, Y., Hashimoto, Y., and Takagi, Y. (1986) *Biochem. Biophys. Res. Commun.* 141, 186-190
9. Aoyama, S., Yoshida, N., and Inouye, S. (1988) *FEBS Lett.* 242, 36-40
10. Perron, C., Jeffrey, V., and Missing, J. (1985) *Gene* (Amst.) 33, 103-119
11. Laemmli, U. K. (1970) *Nature* 227, 680-685
12. Gabriel, O. (1972) *Methods Enzymol.* 22, 578-604
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275
14. Coleman, K., Dougan, G., and Arbuthnott, J. P. (1983) *J. Bacteriol.* 153, 909-915
15. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Mandel, M., and Higa, A. (1970) *J. Mol. Biol.* 53, 164-1627
17. Southern, E. M. (1975) *J. Mol. Biol.* 98, 503-517
18. Sanger, F., Niclen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463-5467
19. Entessanglos, B., and Desnuelle, P. (1968) *Biochim. Biophys. Acta* 159, 285-295
20. Briggs, M. S., and Giersch, L. M. (1986) *Adv. Protein Chem.* 38, 192-190
21. Lee, C. Y., and Iandolo, J. J. (1986) *J. Bacteriol.* 166, 385-391
22. Götz, F., Popp, F., Korn, E., and Schleifer, K. H. (1985) *Nucleic Acids Res.* 13, 5985-5990
23. Shimada, T., Sugihara, A., Tomina, Y., Iizumi, T., and Tsunawasa, S. (1989) *J. Biochem. (Tokyo)* 106, 383-388
24. Kawaguchi, Y., Honda, H., Molimura, T. J., and Iwasaki, S. (1989) *Nature* 341, 164-168
25. DeCaro, J., Boudouard, M., Bonicel, J., Guidoni, A., Desnuelle, P., and Roversy, M. (1981) *Biochem. Biophys. Acta* 671, 129-138
26. Docherty, A. J. P., Bodmer, M. W., Angal, S., Verger, R., Riviere, C., Lowe, P. A., Lyons, A., Emtage, J. S., and Harris, T. J. R. (1985) *Nucleic Acids Res.* 13, 1891-1903
27. Mickel, F. S., Weidenback, F., Swarovsky, B., LaForge, K. S., and Scheele, C. G. (1988) *J. Biol. Chem.* 263, 12885-12901
28. Winkler, F. K., D’Arcy, A., and Hunziker, W. (1990) *Nature* 343, 771-774
29. Datta, S., Luo, C.-C., Li, W.-H., VanTuinen, P., Ledbetter, D. H., Brown, M. A., Chen, S.-H., Liu, S., and Chan, L. (1988) *J. Biol. Chem.* 263, 1107-1110
30. Shimada, T., Sugihara, A., Iizumi, T., and Tomina, Y. (1990) *J. Biochem. (Tokyo)* 107, 703-707
31. Lipman, D. J., and Pearson, W. R. (1985) *Science* 227, 1435-1441
32. Argos, P. (1987) *J. Mol. Biol.* 193, 385-396