

Cloning, sequencing and expression of the lipase gene from *Pseudomonas fragi* IFO-12049 in *E. coli*

Shigeyuki Aoyama, Naoyuki Yoshida and Satoshi Inouye⁺

Research Center, Chisso Petrochemical Corporation, Goi kaigan, Ichihara, Chiba 290 and ⁺Basic Research Laboratories, Chisso Corporation, Kamariya 2, Kanazawa-ku, Yokohama 236, Japan

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The lipase gene from *Pseudomonas fragi* IFO-12049 was isolated using the expression library and the primary structure of lipase deduced from the nucleotide sequence was determined. It is composed of 277 amino acid residues and a protein of M_r 29 966, which was close to the value of the lipase expressed in *E. coli*.

Lipase; DNA cloning; Sequencing; Expression; (*Pseudomonas fragi*)

1. INTRODUCTION

Lipase (EC 3.1.1.3) is a lipolytic enzyme that hydrolyzes the ester bond of the triglycerol and is widely distributed in various animals, plants and microorganisms [1–6]. These lipases have a variety of properties in substrate specificity, activator requirements and so on. Especially lipases from microorganisms are an important enzyme for practical purposes and used in the dairy and other food processes [7,8]. Further it is also known that lipases catalyze the ester synthesis by the reversal reaction of the hydrolysis and was useful for the stereo-specific synthesis [9,10].

Recently several reports for the molecular cloning of the lipase gene have been published and revealed the primary structure of their lipases [11–16]. The determination of the primary struc-

ture of lipases is necessary to understand the molecular mechanism of the catalytic reaction and the relationships between the function and the structure of lipase.

Here we report the cloning, complete nucleotide sequence, chromosomal localization and expression of the lipase gene from *Pseudomonas fragi* IFO-12049 in *E. coli*.

2. MATERIALS AND METHODS

2.1. Materials

Restriction endonucleases were from Nippon Gene (Toyama, Japan) or Toyobo Co. (Osaka, Japan) and used according to manufacturer's recommendations. T_4 polynucleotide kinase, Klenow fragments of polymerase I from *E. coli* and bacterial alkaline phosphatase were products of Takara Shuzo (Kyoto, Japan). [α -³²P]dCTP (– 600 Ci/mmol) was from New England Biolabs (MA, USA). Tributyrin was from Tokyo Kasei (Tokyo, Japan). All reagents were of analytical grade unless otherwise stated.

2.2. Bacterial strains, plasmids and medium

P. fragi IFO-12049 was from the Institute for Fermentation, Osaka and used as a DNA donor for cloning. *E. coli* JM83 and pUC9 [17] were used as a host strain and a cloning vector, respectively. L-Broth medium (10 g bactotryptone, 5 g yeast extract, both from Difco, Detroit, USA and 5 g NaCl per liter) was used for both *E. coli* and *P. fragi* strains, which were cultured at 37°C and 30°C, respectively.

Correspondence address: S. Aoyama, Research Center, Chisso Petrochemical Corporation, Goi Kaigan, Ichihara, Chiba 290, Japan

Abbreviations: bp, base pair; PAGE, polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl

The nucleotide sequence presented here have been submitted to the EMBL/GenBank database under the accession number Y00975

2.3. Plasmid construction, DNA preparations and transformation

Standard recombinant DNA methods were carried out essentially as in [18]. Chromosomal DNA from *P. fragi* IFO-12049 was purified as in [19] and the preparation of plasmid was performed by the alkali method [18]. DNA fragments were isolated from 0.7% agarose gel by binding to DEAE-81 paper (Whatman, USA) and eluting with 1.0 M NaCl. *E. coli* was transformed as in [20].

2.4. DNA sequencing

For sequence determination, fragments of DNA were subcloned into pUC9 and were confirmed by a modified dideoxynucleic acid sequencing method [12] using either an M13 dideoxy sequencing kit (Takara Shuzo, Kyoto, Japan) or an M13 deaza-sequencing kit (Nippon Gene, Toyama, Japan).

2.5. Cloning and screening procedures

The tributyrin diffusion agar method was carried out essentially as in [11] except the concentration of tributyrin was 1% (v/v). The transformant harboring the plasmid containing the lipase gene formed a clear zone.

2.6. SDS-PAGE

SDS-PAGE was run essentially as in [22]. The sample dissolved in Laemmli's sample buffer was heated at 95°C for 5 min and cooled before being applied to a gel. The gel was fixed and stained with 0.1% (w/v) Coomassie brilliant blue R-250 (Eastman Kodak). The standard marker was obtained from Pharmacia fine chemicals (Uppsala, Sweden).

3. RESULTS

3.1. Cloning of the lipase gene from *P. fragi* IFO-12049

P. fragi IFO-12049 DNA was partially digested with *Sau*3A, and 2–6-kbp fragments were collected from electrophoresed agarose gel using DEAE-81 paper. The partial *Sau*3A fragments were inserted into the *Bam*HI site of pUC9 and the ligated DNA was transformed into *E. coli* JM83 on the L-plate containing 1% tributyrin and ampicillin (50 µg/ml). Among 1500 transformants, one colony formed a clear zone due to lipase activity (fig.1b). A recombinant plasmid containing a 3.3-kb segment of the insert DNA was recovered from the transformant and designated pFL-1. Restriction enzyme analysis results in the map of pFL-1 as in fig.1a.

3.2. Subcloning, sequencing and chromosomal localization

Subcloning experiments were performed to identify the minimum region of the insert DNA necessary for the production of lipase. The

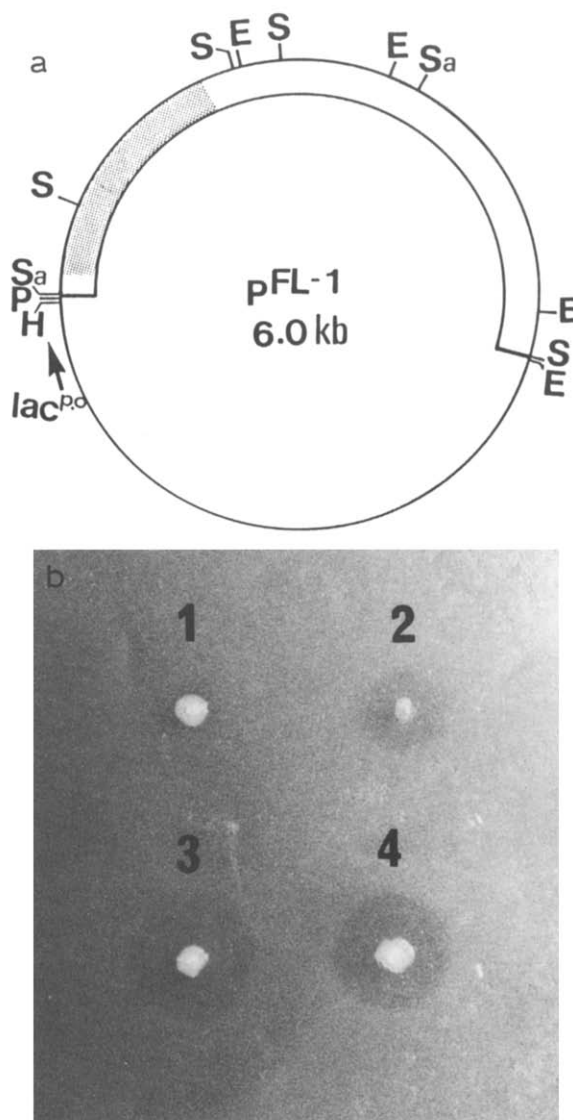


Fig.1. (a) Physical map of plasmid pFL-1. The thick and thin lines represent the *P. fragi* DNA and vector pUC9, respectively. The shaded region in the thick line shows the region coding the lipase gene. The arrow indicates the direction of transcription of the *lac* promoter from pUC9 and its size corresponds to size of the gene. Restriction enzymes: E, *Eco*RI; P, *Pst*I; H, *Hind*III; Sa, *Sal*I; S, *Sma*I. (b) Detection of the lipase activity on the 1% tributyrin agar plate. After 30 h incubation at 30°C, the clear zone was formed due to lipase activity. Lanes: 1, JM83/pUC9; 2, *P. fragi* IFO-12049; 3, JM83/pFL-1; 4, JM83/pFL-2.

plasmid pFL-2 was constructed by the self-ligation of the *Eco*RI site of pFL-1. As shown in fig.2, these results indicated that the 0.2-kb region from

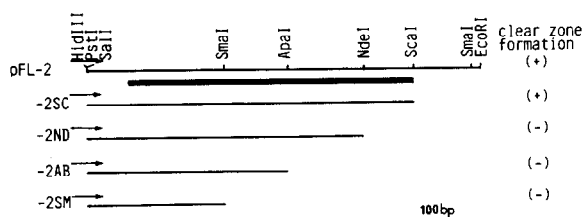


Fig.2. Subcloning of the DNA coding *P. fragi* lipase from pFL-1. Arrows indicate the direction of transcription from the *lac* promoter on the pUC9 vector. The lipolytic phenotype was determined by the halo formation on 1% tributyrin/L-broth plate. The thick underlining indicates the *RasI* fragment, used as a probe for Southern hybridization. Numbers are in 100 bp.

the *EcoRI* site at the end of the insert of pFL-2 was essential for lipase activity.

As in fig.3, the nucleotide sequence of the insert region of pFL-2 was determined and was found to contain a single open reading frame. The primary structure of the lipase deduced from the nucleotide sequence shows that lipase is composed of 277 amino acid residues. The molecular mass calculated from the expected amino acid sequence is 29966 Da.

Southern blot hybridization of *PstI* digestion of *P. fragi* DNA with a 0.9-kb *RsaI* fragment of pFL-2 as a probe (thick underlining in fig.2) show-

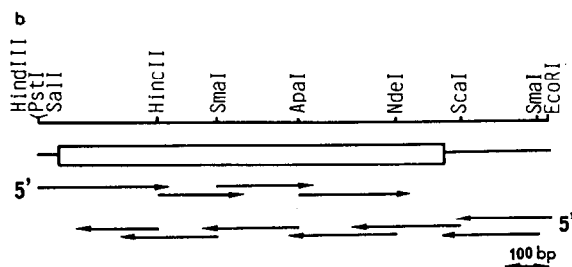
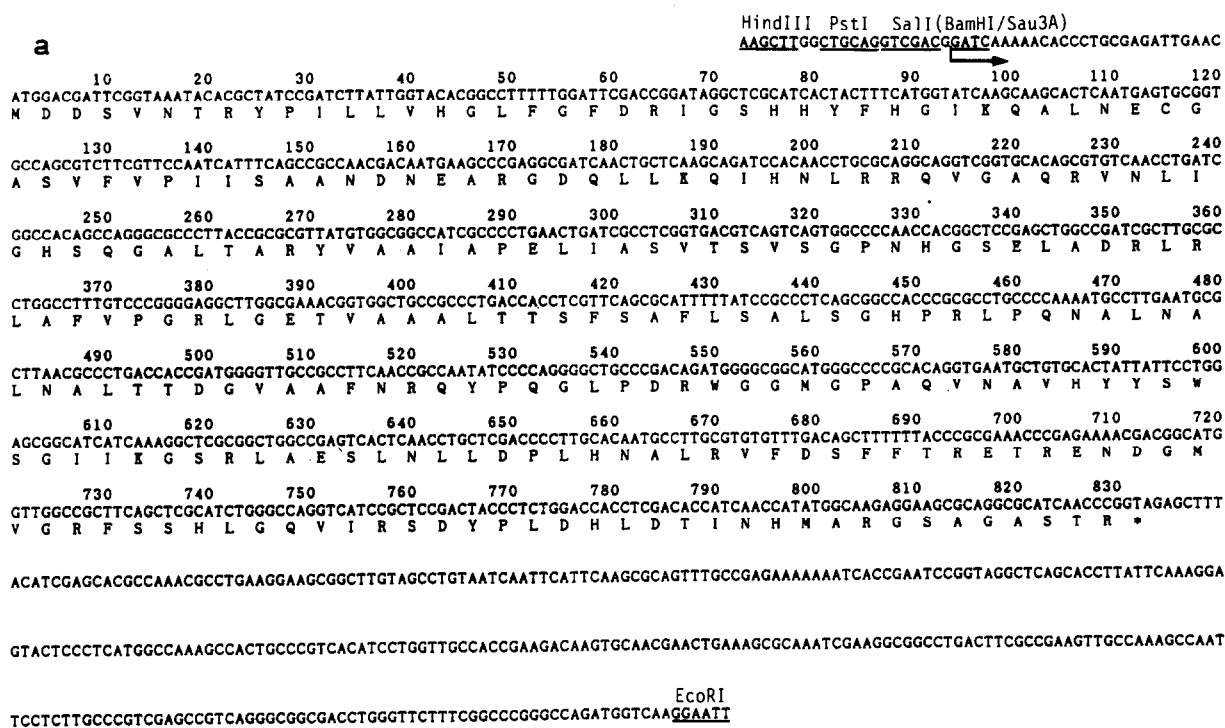


Fig.3. (a) Nucleotide sequence encoding the lipase and the predicted amino acid sequence. The horizontal arrow shows the region derived from *P. fragi* DNA. (b) Sequence strategy of pFL-2. The open box indicates the region coding the lipase gene. Numbers are in 100 bp.

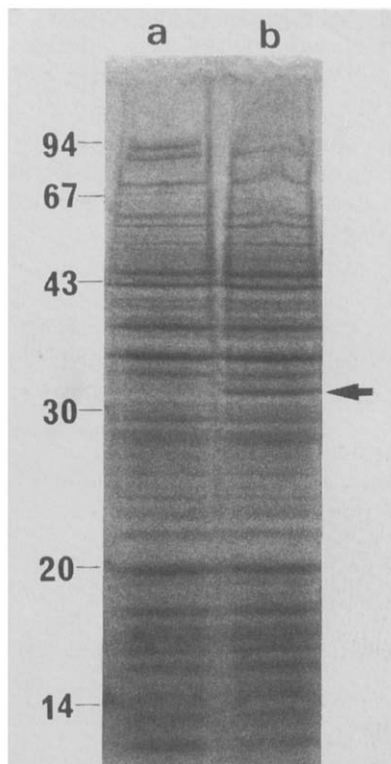


Fig.4. SDS-PAGE analysis of the expressed lipase gene from *P. fragi* in *E. coli*. 30 μ l of the cultured cells was applied on a 15% SDS-gel for 16 h at 37°C. Lanes: a, JM83/pUC9; b, JM83/pFL-2. The arrowhead shows the position of the expressed lipase protein. The molecular mass markers (in thousands) are indicated on the left.

ed a 4.3-kb single positive band (not shown). These results suggested that the cloned lipase gene was a single copy gene in *P. fragi* IFO-12049.

3.3. Expression of the lipase gene from *P. fragi* in *E. coli*

The lipase gene from *P. fragi* was expressed in *E. coli* using pFL-2 with the control of the *lac* promoter of pUC9. The level of production of the lipase in *E. coli* was detected by electrophoresis on SDS-polyacrylamide gel (15%). The molecular mass of the expressed lipase was estimated to be about 32 kDa as shown in fig.4.

4. DISCUSSION

A partial *Sau*3A fragment of *P. fragi* IFO-12049 DNA cloned in *E. coli* was shown to direct the pro-

duction of lipase whose activity could be detected in situ on plates using tributyrin (fig.1b). A 3.3-kbp *Sau*3A fragment containing the active gene was cloned into pUC9 under the control of *lac* promoter. From the nucleotide sequence, the predicted molecular mass of the lipase is 29966 Da and this value agrees with that of the lipase expressed in *E. coli* as estimated by SDS-PAGE (fig.4).

Recently Kugimiya et al. [11] reported the cloning of the lipase gene from *P. fragi* IFO-3458. The restriction map and the molecular mass of pFL-1 obtained from *P. fragi* IFO-12049 were different from those of *P. fragi* IFO-3458. However, the nucleotide sequence analysis suggested that the two *P. fragi* lipase genes will have diverged from the same origin, because the nucleotide sequence was completely identical except at positions 354, 444 and 451, shown in fig.3.

The purification of the lipases from *Pseudomonas* sp. has been performed and the molecular mass was determined. These results demonstrate that their molecular masses are about 29–33 kDa [4–6]. The experimental results from the subcloning, nucleotide sequencing and expression using pFL-2 agreed with the molecular masses of the lipases from *Pseudomonas* sp.

The amino-terminal region of the lipase deduced from the nucleotide sequence has a signal peptide-like sequence [11,14], but the cleavage of this region in *E. coli* was not determined. The tributyrin halo, which is caused by hydrolysis with *E. coli*-harboring pFL-2, points to the extracellular production of lipase in *E. coli* (fig.1b). However, it is not yet clear whether the apparent extracellular production is due to the lysis of cells or the secretion through the inner/outer membrane in *E. coli*. So it is necessary to purify and characterize the lipase expressed in *E. coli*. The expression of *P. fragi* lipase in *E. coli* will enable the production of large amounts of the recombinant lipase, which can be used for enzymological and structural studies, and for the commercial purposes.

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