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MOLECULAR CLONING AND NUCLEOTIDE SEQUENCE OF THE LIPASE GENE FROM PSEUDOMONAS FRAGI

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The gene coding for the lipase of Pseudomonas fragi was cloned into Escherichia coli JM83 by inserting Sau3A-generated DNA fragments into the BamHI site of pUC9. The plasmid isolated, pKK0, was restriction mapped and the position of the lipase gene on the 2.0 kb insert was pinpointed by subcloning. DNA sequencing revealed that the open reading frame comprises 405 nucleotides and gives a preprotein of 135 amino acids with a predicted Mr of 14643. By comparing the putative lipase amino acid sequence with porcine pancreatic, rat lingual and Staphylococcus hyicus lipases the amino acid sequence around the reactive serine was found to be common among the types of lipase which have been reported.

Lipase (E.C.3.1.1.3) catalyzes the hydrolysis of fats to give free fatty acid, partial glycerides and glycerol, and in reverse the formation of glycerides from glycerol and free fatty acid under certain conditions (1). Furthermore, it can be used as a catalyst for the interesterification of oils and fats (2,3). Use of some lipases having 1·3 positional specificity gives products which have valuable properties for the oil and fat industry (4,5).

Lipases have been found in many species of animal, plant and microorganisms, and some have been partially purified. However, perhaps due to the instability of this enzyme, there have been only a few reports of the primary structure, and the relation between the structure and the function of the enzyme is unknown.

In the present paper, the molecular cloning of the lipase gene from Pseudomonas fragi which is known to have 1·3 positional specificity (6,7) and its amino acid sequence predicted from nucleotide sequencing are described. It is suggested that the amino acid sequence around the active center of the enzyme is conserved.

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Materials and Methods

Bacterial strains, plasmids and medium

Pseudomonas fragi IFO 3458 was obtained from the Institute for Fermentation, Osaka, and used as a cloning host organism. The vector for cloning experiments was plasmid pUC9 harboring ampicillin resistance (8). Escherichia coli JM83 was employed as the host for recombinant plasmids (9). L-broth (10g peptone, 5g yeast extract and 5g NaCl per liter of distilled water, pH 7.2) was the basal medium for E.coli strains and for the P.fragi strain which were grown at 37°C and 25°C, respectively.

Recombinant DNA techniques

DNA was extracted from P.fragi by the modified method of Marmur as described by Coleman et al (10). Plasmid DNA was prepared as described by Klein et al (11). Restriction digests and ligation reactions were performed according to the manufacturers recommendation. All enzymes were purchased from Takara Shuzo Co.,Kyoto.

Cloning and screening procedures

The tributyrine diffusion agar method is more convenient and more sensitive for the detection of lipase activity than other existing assay techniques (12,13,14). This method was used to clone the lipase gene from P. fragi. 0.5% (V/V) of tributyrine were added to 25 ml of L-broth agar which contained ampicillin (50 μg/ml) in a test tube. After vigorous vortexing, the solution was immediately poured into a Petri dish. Tributyrine was diffused homogeneously in the agar. E.coli JM83 transformed with a DNA library of P.fragi carried by pUC9 was grown on a tributyrine agar plate for 24 hrs at 37°C, and the colony forming a clear zone was isolated.

DNA sequencing analysis

For nucleotide sequencing, fragments of DNA were subcloned into pUC9 or pUC8 and transformed into JM83 for sequence determination by the dideoxy procedure (15).

Result and Discussion

Cloning of the P.fragi lipase gene

P.fragi produces an extracellular lipase (7 ,16). In order to isolate the coding gene, chromosomal DNA prepared from the Pseudomonas host was partially digested with Sau3A and fragments of 2 to 6 kb in length were isolated by means of sucrose gradient centrifugation. The sized DNA was ligated with alkaline phosphatase-treated, BamHI-cut pUC9 and transformed into E.coli JM83. Then, about 3000 recombinant colonies were repeatedly screened on tributyrine agar plates, and a single halo-forming colony was detected. In Fig.1a the lipase production of E.coli JM83 containing pUC9 (negative control), E.coli JM83 harboring plasmid (pKK0) and P.fragi host (lipase gene donor strain) are shown.

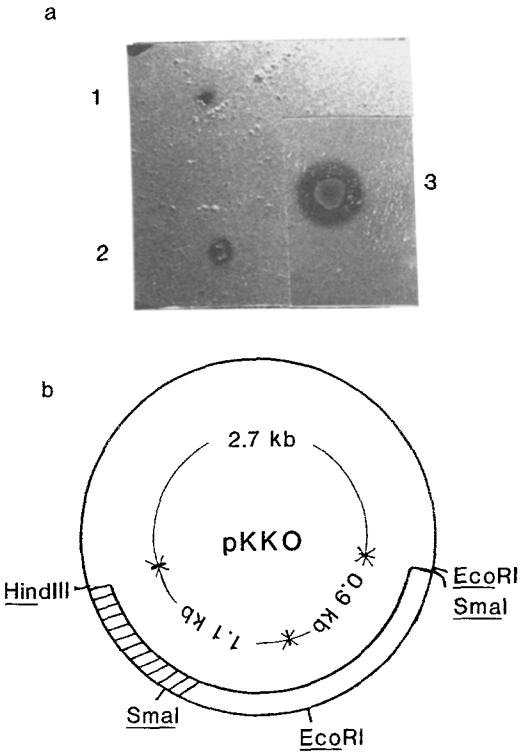

Fig.1a. Lipase production by various strains on the tributyrine agar plate (see Materials and Methods). After 24 hrs incubation at 37℃ and 25 ℃ for E.coli and for Pseudomonas strains, respectively, visible clear zones due to lipase activity are formed around the colonies. (1)E.coli JM 83 containing pUC9 (negative control) . (2) E.coli JM 83 harboring pKKO. (3) P.fragi (lipase gene donor strain) .
b ; Restriction map of pKKO. pKKO consists of the 2.7 kb of plasmid vector pUC9 (thin line) and the 2.0 kb of P.fragi DNA fragment (double line) which was cloned in the BamHI site of pUC9. The lipase gene is located within the 1.1 kb HindIII-EcoRI DNA fragment (dashed region).

Classical restriction mapping of the recombinant plasmid, pKKO, revealed the presence of a 2.0 kb insert of Pseudomonas DNA (Fig.1b).

Subcloning of the lipase gene in E.coli

DNA was prepared from pKKO and cleaved with the restriction endonucleases HindIII and EcoRI. These fragments were then subcloned into EcoRI and EcoRI-HindIII sites of pUC9, respectively, and transformed into E.coli JM83. A plasmid carrying the 0.9 kb EcoRI DNA fragment did not make a clear zone on the tributyrine agar plate. However, a plasmid with the 1.1 kb DNA fragment between HindIII site and EcoRI site made a clear zone at the same level as pKKO (data not shown). Furthermore, when the HindIII-EcoRI 1.1 kb DNA fragment was

subcloned into pUC8 which had a reversed multicloning site of pUC9, no clear zone was formed (data not shown). Since the promoter region of lac.Z gene derived from pUC9 is located immediately upstream from the HindIII site (17), these data seem to suggest that the lipase gene inserted nearby downstream from the HindIII site of pKK0 is expressed in E.coli by means of the promoter of lac. Z gene.

Nucleotide sequence of the lipase gene

The nucleotide sequence was determined for the 1.1 kb DNA fragment between the HindIII site and the EcoRI site of pKK0. The nucleotide sequence of the lipase gene is shown in Fig.2a. It was observed that the open reading frame comprises 405 bp and gives a preprotein of 135 amino acids with a predicted Mr of 14643 in the position suggested by the subcloning analysis. Upstream from ATG of the lipase gene there is a 5'-GAGA-3' sequence which might function as a Shine-Dalgarno (SD) sequence (18). The methionine precedes a sequence of hydrophobic amino acids, and these may be the core of a signal sequence for secretion (19).

Comparison of the lipase amino acid sequence

The sequence of P.fragi lipase was compared to those of porcine pancreatic (20), rat lingual (21) and Staphylococcus hyicus (22) lipases. The sequence of P.fragi lipase is remarkably shorter than others and bears little amino acid sequence homology over all. However, there are similarities between P.fragi lipase and rat lingual lipase at 2 segments of five amino acids. One is the sequence -His-Gly-Leu-Phe-Gly- found in the sequence of N-terminal region (Fig. 2b). It is interesting that the sequence around the N-terminal region of the rat lingual lipase is present in the same position as that of P.fragi lipase, but the significance of this mutual sequence is unclear. Another similarity is in the region of the active site described below.

Many hydrolytic enzymes are "serine" enzymes ; they contain a reactive serine residue that attacks the substrate with its alkoxy oxygen as a nucleophile (23). In a porcine pancreatic lipase, proteolytic digestion of the lipase labeled with diethyl p-nitrophenyl phosphate yielded a peptide of the

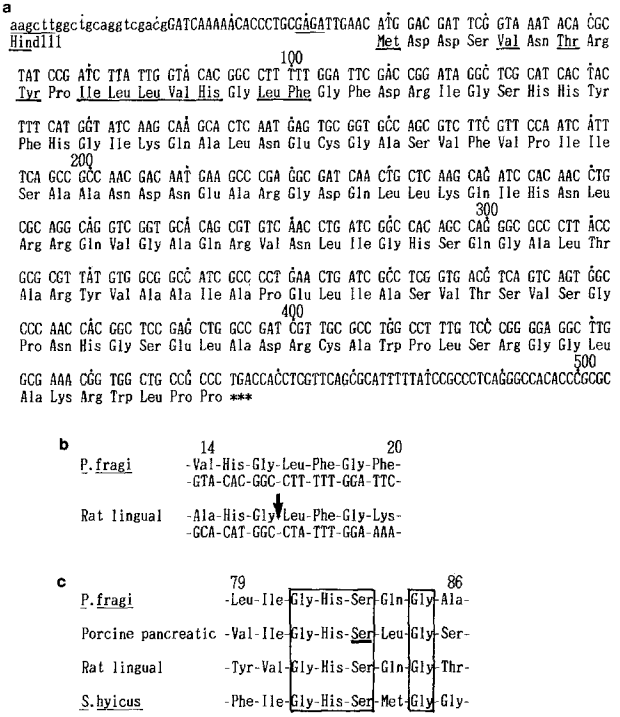

Fig.2a. Nucleotide sequence encoding the lipase and the predicted amino acid sequence. The nucleotide sequence is presented from HindIII site (nucleotide 1). The amino acid sequence is shown beneath the nucleotide sequence. Hydrophobic amino acids in the putative signal peptide (amino acids 1 to 18) are underlined. A probable SD sequence is shown by a solid line below the nucleotide sequence. Asterisks indicate a stop codon. Small letters show the nucleotide derived from the plasmid, pUC9 vector.
b ; DNA sequence and predicted amino acid sequence of P.fragi lipase (described in this report) were compared to N-terminal regione of rat lingual lipase (21). The vertical line associated with an arrow indicates position in releasing its signal peptide of rat lingual lipase. Numerals above the amino acids indicate the order counted from the translation start site (Met) as 1 with regard to the lipase from P.fragi.
c ; Comparison of the amino acid sequences of P.fragi (described in this paper) with porcine pancreatic (20), rat lingual (21) and Staphylococcus hyicus (22) lipases around the putative reactive serine. The reactive serine residue of the porcine pancreatic lipase is underlined. Rectangles show the identical amino acids among all of these sequences.

formula -Gly-His-Ser(P)-Leu-Gly- in which the phosphate was indeed bound to a serine residue (24). This result suggested that the lipase can be considered as a serine enzyme. We found an amino acid sequence homologous to that formula around the reactive serine in an equivalent position of P.fragi, rat lingual and S.hyicus, as shown in Fig.2c. Thus, it is postulated that the lipase is a "serine" enzyme, and that the region including the reactive serine is common to many types of lipases.

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