Cloning, Nucleotide Sequencing, and Expression in *Escherichia coli* of a Lipase and Its Activator Genes from *Pseudomonas* sp. KWI-56

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Received March 19, 1991

A lipase gene (*lip*) and its activator gene (*act*) on a 2.9 kb *Bgl*II-*Eco*RI fragment from *Pseudomonas* sp. KWI-56 were cloned in *Escherichia coli* using pUC19 as a vector plasmid. From the sequencing results, the open reading frames of the *lip* and the *act* were found to contain 1092 and 1032 nucleotides, respectively. The *act* existed downstream of the *lip* with the same orientation. When the *lip* was expressed in *E. coli* using the *lac* promoter on the pUC plasmid vector, the lipase activity of *E. coli* carrying both the *lip* and the *act* was 200-fold greater than that carrying only the *lip*. This result suggested the *act* was important in the expression of the *lip* in *E. coli*.

Lipase catalyzes the hydrolysis of triacylglycerol and is a well-known extracellular, inducible enzyme of the genus *Pseudomonas*. Recently, some structural genes of lipase from genus *Pseudomonas* have been cloned.^{1–4)} The nucleotides of the lipase genes from *P. fragi* were sequenced, and they were expressed in *Escherichia coli*.^{3,4)} However, few reports have appeared concerned with genetic studies on regulation, secretion, and expression of lipase activity.

During the course of cloning of the lipase gene (*lip*) from *P. sp.* KWI-56, an extracellular thermostable lipase producer,⁵⁾ we have found that the lipase activity was enhanced in *E. coli* by the function of the activator gene (*act*) existing downstream of the *lip*.⁶⁾ This paper describes the cloning and sequencing of the *lip* and the *act* from *P. sp.* KWI-56.

Materials and Methods

Bacterial strain and plasmids. *E. coli* HB101 and *E. coli* JM103 were used as host for the recombinant plasmids. *Pseudomonas* sp. KWI-56 (KWI-56)⁵⁾ was used as a DNA donor. The vectors used for the cloning experiments were M13mp18, M13mp19, pUC18, pUC19,⁷⁾ and pHSG299.⁸⁾

Media and cultivation. Nutrient broth and LB were used for the cultivation of KWI-56 and *E. coli*, respectively. Tributyrin-LB agar³⁾ was used to detect the lipase activity of transformants. When the transformants were cultivated, ampicillin (Ap) and kanamycin (Km) were added at a final concentration of 50 µg/ml each.

Recombinant DNA techniques. Chromosomal DNA was extracted from KWI-56 by the method of Smith.⁹⁾ Plasmid DNA was prepared by the rapid alkaline extraction method and was purified by Sepharose CL-4B column chromatography.¹⁰⁾ DNA was ligated using a DNA ligation kit (Takara Shuzo Co., Kyoto). Restriction enzymes, T4 polymerase, and a bacterial alkaline phosphatase were obtained from Toyobo Co., Osaka, and used under the recommended conditions. Artificial frameshift mutagenesis was done by an 8 mer *Eco*RI-linker (GGAATTCC) insertion after repair to the blunt end from cohesive end using mung bean nuclease (Toyobo Co., Osaka). The construction of deletion phages for the DNA sequencing was done using a deletion kit (Takara Shuzo Co., Kyoto). The DNA was sequenced by the dideoxy chain termination method.¹¹⁾ Deaza-dGTP and *Taq* polymerase (Promega Co. Wisconsin) were used for the sequencing reaction.

Enzyme assay. The measurement of the lipase activity was described in a previous paper.⁵⁾

Identification of amino acid composition, N-terminal amino acid sequence, and C-terminal amino acid. The purification of the lipase from KWI-56 was described in

a previous paper.⁵⁾ The purified lipase was hydrolyzed at 110°C for 24 hr with 5.7 N HCl in a sealed evacuated glass tube. Cysteine and cystine were measured as cysteic acid after performic acid oxidation.¹²⁾ The hydrolysates were analyzed with a Shimadzu LC-3A amino acid analyzer by the method of Ishida *et al.*¹³⁾ Tryptophan was measured by the method of Bencze *et al.*¹⁴⁾ The N-terminal amino acid sequence was identified by Edman degradation.¹⁵⁾ The C-terminal amino acid was identified using carboxypeptidase A (Seikagaku Kogyo Co., Tokyo).

Cell fractionation. The cell fractionation was done by the osmotic shock method.¹⁶⁾ The cells were washed with 30 mM Tris-HCl (pH 8.0) containing 0.86% NaCl, and were suspended in an osmotic solution (30 mM Tris-HCl pH 8.0, 20% sucrose, 1 mM EDTA) for 10 min at 25°C with shaking. After centrifugation at 7000 × *g* for 10 min, the cells were suspended in cold water and shaken for 10 min at 4°C. The suspension was centrifuged at 9000 × *g* for 10 min. The supernatant was used as a periplasmic fraction, and the pellet was suspended in 30 mM Tris-HCl (pH 7.5) and sonicated. The suspension was used as a cytoplasmic fraction.

Western blotting. Proteins were electroblotted onto a nitrocellulose paper from the gel after SDS-PAGE. The paper was washed with blocking solution (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 2% BSA) and incubated with the same buffer containing rabbit antiserum against the purified lipase from KWI-56. After washing, the paper was incubated with blocking solution containing the second antibody coupled to alkaline phosphatase. The colorimetric detection was done using 5-bromo-4-chloroindoxyl phosphate and nitro blue tetrazolium by the method of Blake.¹⁷⁾

Results

Cloning of the lip from KWI-56 chromosomal DNA

The chromosomal DNA from *Pseudomonas* sp. KWI-56 (KWI-56) was partially digested with *Sau3AI*, and was ligated to *Bam*HI-digested pUC19. Then, *E. coli* HB101 was transformed with the recombinant plasmids. Among approximately 15,000 transformants, 18 colonies forming a clear zone on the tributyrin-LB agar were isolated. Among those, only one transformant was found to produce the lipase at 0.03 U/ml. The other 17 transformants did not show any lipase activity, and seemed to contain esterase genes. The lipase-producing transformant harbored a

recombinant plasmid containing 11.5-kb of foreign DNA. It was designated pLP6.

Subcloning and sequencing of the lip

Subcloning experiments were done to locate the *lip* on the 11.5-kb DNA fragment. As shown in Fig. 1, *E. coli* HB101 strains harboring pLP63, pLP64 (containing a 2.9-kb *Bgl*II-*Eco*RI DNA fragment) and pLP65 (containing a 2.1-kb *Bgl*II-*Bam*HI DNA fragment) formed a clear zone on the tributyrin-LB agar, although the strains harboring pLP61 and pLP62 did not. These results show that the *lip* exists on the 2.1-kb *Bgl*II-*Bam*HI DNA fragment.

The cohesive ends of the 2.9-kb *Bgl*II-*Eco*RI fragment from pLP64 were converted to blunt ends using the Klenow fragment. The DNA fragment was joined to the *Hinc*II site on M13mp19. Several deletion plasmids were constructed, and the nucleotide sequences of both directions of the 2916-bp DNA fragment were identified as shown in Fig. 2. The DNA sequence indicated the presence of major four open reading frames (ORFs), positions 297 to 1470 (ORF1), 374 to 1213 (ORF2), 1476 to 2507 (ORF3), and 1565 to 2551 (ORF4), in the 2916-bp *Bgl*II-*Eco*RI DNA fragment.

The N-terminal amino acid sequence, Ala-Asp-Gly, of the purified lipase from KWI-56 found by Edman degradation is identical

Fig. 1. Subcloning of the DNA Coding for the *lip* from KWI-56.

The thick and thin lines indicate pUC19 vector DNA and KWI-56 chromosomal DNA, respectively. Arrows indicate the direction of transcription from the *Plac* on pUC vector DNA. The lipase activity of the transformant is detected by clear zone formation on the tributyrin-LB agar containing 50 µg/ml of Ap. The restriction enzymes are as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III.

to the predicted amino acid sequence of the ORF1. The C-terminal amino acid was found to be Val, which coincided with that predicted from the nucleotide sequence of the ORF1. In addition, the analyzed amino acid composition agreed with that (from Ala-1 to Val-320) deduced from the nucleotide sequence of this ORF (Table I). These results indicated the ORF1 contained the *lip*.

The ORF1 has six translational start codons, ATG, at positions 297, 378, 390, 429, 465, and 492. The presence of a typical SD (Shine-Dalgarno) sequence in *E. coli* or in *Pseudomonas*, AGGAG, 12 bases upstream of the ATG codon at position 378 indicates that the *lip* consists of 1092 bp starting at position 378. Thus, the N-terminal sequence from -44 to -1 seemed to be a signal sequence, and the mature lipase was predicted to be a 320 amino acid protein with a molecular weight of 33,196.

Table I. AMINO ACID COMPOSITIONS OBSERVED FROM THE PURIFIED LIPASE FROM KWI-56 AND CALCULATED FROM THE NUCLEOTIDE SEQUENCE

Amino Acid	No. of residues	
	Observed	Calculated
Asx	32.1	32
Thr	30.1	32
Ser	21.1	24
Glx	21.8	21
Pro	11.6	11
Gly	35.7	36
Ala	39.9	39
CysH	0.4	2
Val	28.7	30
Met	0.9	1
Ile	9.9	11
Leu	34.0	33
Tyr	14.4	15
Phe	6.8	7
His	6.6	7
Lys	8.1	7
Arg	8.7	9
Trp	5.3	3
Total	316.1	320

Calculated from the nucleotide sequence without the signal sequence. Observed by amino acid analysis and based on volume from M. W. 33,000.

Expression of the *lip* on *E. coli*

When *E. coli* JM103 (pLP64) was cultivated in LB containing Ap and 0.1 mM IPTG (isopropyl- β -D-thiogalactopyranoside), the lipase activity reached 22.5 U/ml as shown in Table II. To investigate the cellular localization of the lipase molecule, the cells were fractionated by the osmotic shock method. Approximately 95% of the lipase activity was in the cytoplasmic fraction, and 5% in the periplasmic fraction. Very low activity was detected in the supernatant. The molecular weight of the lipases from the cytoplasmic and periplasmic fractions were measured by western blotting. The lipase protein from the cytoplasm appeared in two molecular forms (33-kD and 38-kD), although the molecular mass of periplasmic lipase was 33-kD as a single band and consisted with that of mature lipase from KWI-56 as shown in Fig. 3.

The plasmid pLP640 (Fig. 4) had a *lac* promoter (*Plac*) in an opposite direction to pLP64. The lipase activity of JM103 (pLP640) lysate was 0.2 U/ml (Table II), and was only 1% of that of JM103 (pLP64). This fact

Table II. LIPASE ACTIVITY OF *E. coli* JM103 CARRYING pLP64 DERIVATIVES

Plasmids	Antibiotics in LB	Lipase Activity (U/ml)
none		0
pLP64	Ap	22.5
pLP65	Ap	0.13
pLP640	Ap	0.20
pLP641	Ap	0.10
pHSGL68	Km	0
pLP65 + pHSGL68	Ap, Km	16.5
pLP65 + pHSGL68B	Ap, Km	14.8
pLP65 + pHSGL68M	Ap, Km	0.10
pLP65 + pHSGL68K	Ap, Km	0.08
pLP65 + pHSGL68N	Ap, Km	0.08
pLP65 + pHSGL68E	Ap, Km	17.5

Overnight culture of *E. coli* JM103 carrying plasmids was transferred to fresh LB (1% inoculation) containing 0.1 mM IPTG and 50 μ g/ml of antibiotics (Ap and/or Km), and incubated at 37°C for 14 hr with shaking. Cells from 5 ml of the culture broth were washed and suspended in 5 ml of 30 mM Tris-HCl (pH 7.5). The suspension was treated by ultrasonic disintegration, and its lipase activity was measured.

suggested that the *lip* on pLP64 was mostly expressed by the *Plac* in the pUC vector DNA.

On the other hand, the lipase activity of JM103 (pLP65) reached only 0.13 U/ml in spite of the presence of the *lip*. JM103 carrying pLP64l, which had an artificial frame shift mutation by the insertion of an 8 mer

*Eco*RI-linker DNA (GGAATTCC) in the *Bam*HI restriction site (position 2099), also produced 0.10 U/ml of the lipase (Fig. 4 and Table II). In these cases, only very low activity was detected in the supernatant. These results suggested that the downstream region was important for high expression of the lipase

Fig. 2.

1441	ATGCGAACCG GCTGAAGCTG GCGGGGCTGT AATCGATGAC GTCACTGAA GGACGGCGCG
	A N R L K L A G V * M T S R E G R A P
	320 act
1501	CGCTGGGCGG GCGCGCGGTG GTCTATGGTG TGCTGGGGCT GGCGGGGATT GCGCGGTGG
	L A R R A V V Y G V V G L A A I A G V A
	10 20
1561	CGATCTGGAG CCGCGCGGGC TGGCATCGCG CAACGGGGCG TTCCGGGAG TCGCGGAGG
	M W S G A G W H R A T G A S G E S P E A
	30 40
1621	CGCTCGGTGC AGGGGGATCG GTTACCGCAC CGCGCGGGCG AGCGGTGCG GCAAGCACGG
	S V A G G S V T A P P Q A A V P A S T G
	50 60
1681	GCTTGGCGCC GTCACTCGCC GGCTCCAGCG CGCGCGGGTT GCGCTCGAT GCGCGGGCG
	L P P S L A G S S A P R L P L D A G G H
	70 80
1741	ATCTCGGAA GTGCGCGCA GTGCGGGATT TCCTCGACTA CTGCTCAAC GCGCAGAGCG
	L A K S R A V R D F F D Y C L T A Q S D
	90 100
1801	ACCTGAGCGC GCGCGGTCTC GACGCGTTCG TCATGCGCGA GATTGCGCA CAGCTCGAG
	L S A A G L D A F V M R E I A A Q L D G
	110 120
1861	GTACCGTTGC GCAAGCGAG GCGCTCGACG TGTCGCAACG GTATCGCGCG TATCTCGAG
	T V A Q A E A L D V W H R Y R A Y L D A
	130 140
1921	CACCTCGGAA ATTGCGGAT GCGCGCGCGG CGACAAGTC CGACCTGGC GGTTCGCAAC
	L A K L R D A G A A D K S D L G A L Q L
	150 160
1981	TGCGCTCGA CCAGCGCGCG TCGATCGGT ACCGCAAGCT CGGCGACTGG AGCCAGCGCT
	A L D Q R A S I A Y R T L G D W S Q P F
	170 180
2041	TCCTCGGTGC GGAGCAGTGG CGGCAGGCT ACGACCTGGC GCGACTGAAG ATCGCGCAGG
	F G A E Q W R Q R Y D L A R L K I A Q D
	190 200
2101	ATCCACGCT GACGGATCG CAGAAGCGG AACGGCTCGC GCGCTCGAA CAGCAGATCC
	P T L T D A Q K A E R L A A L E Q Q M P
	210 220
2161	CGSCGAAGA ACGCGCGCG CAGCAGCACA TCGACGCA GCGTGGCGG ATCGACCAGA
	A D E R A A Q Q H I D Q Q R A A I D Q I
	230 240
2221	TGCGCAATT GCAGAAGAGC GCGCGGACAC CGGATGCGAT GCGCGCAAA CTGACGAGA
	A Q L Q K S G A T P D A M R A Q L T Q T
	250 260
2281	CGCTGGGCC CGAAGCGGC GCGCGGTTCG CGCAGATGCA GCAGGAGAC GCATGTGGC
	L G P E A A A R V A Q M Q Q D D A S W Q
	270 280
2341	AGAGCGCTA CGCGACTAT GCGCGCAGC GCAAGCAGAT CGAATGGGC GCGCTGTGCG
	S R Y A D Y A A Q R T Q I E S A G L S P
	290 300
2401	CGCAGGATG CGAAGCGCAG ATCGCGCGCG TGCGGCAGCG CGTGTTCAGC CGGCGCGCGG
	Q D R D A Q I A A L R Q R V F T R P G E
	310 320
2461	AAGCGGTGG TGCGGCATCG CTGATGCGG GGGCGGGCAG CGCGCGGTAA CGCGCGCGCG
	A V R A A S L D R G A G S A R *
	330 340
2521	GCAGGCGATG ACGTTCGCTA TGCGCGCGCG GTGAGATGCC GCGCGAGGTG TTGCTCGATG
2581	GTATTGCGCA GCGTGTGAA CGCGCGCTCG ATGCTTCTGT GCGGAGCGCA TGCATAACCG
2641	AGCAGCAGGC CGCGCGCGCG GGTGTGAGG CTGCTGTAGT AGCTGGTGG CCGCGCGCAG
2701	ATCAGCGCG CATCGAAGCG GCTCTGGGTG ACGCGCGGAT CGTGCAGGC GTGGGCGAGG
2761	CGAGCAGCA GATGAGGCC TGCTCTGTCC CCATCACCG GCAGCGGCTC CGCGAAGCGC
2821	GCGCGGATCG CGTGTATCAT CAGTTCGCGG CGCTCGCGGT ACAGGTTGCG CATCGCTCTG
2881	ACGTGCGAGG TCAGGTGGCC GTCCATGAAT GAATTC

Fig. 2. Nucleotide Sequence of the 2.9kb *Bgl*II–*Eco*RI Fragment Containing the *lip* and the *act*.

The entire amino acid sequence deduced from the nucleotide sequence is shown below the coding region. The arrow indicates the cleavage site of the lipase with signal peptidase, which was estimated from the N-terminal amino acid sequence (underlined) from the purified lipase. SD indicates the SD sequence of the *lip*. The double underline indicates the common sequence of lipase, G-X-S-X-G. Open and shaded boxes indicate the possible translational start codons and the SD-like sequences of the *act*, respectively.

Fig. 3. Western Blot Analysis of the Lipase in Cell Fractions of *E. coli* HB101 (pLP64).

Lane 1, purified lipase from KWI-56; lane 2, periplasmic fraction of HB101 (pLP64); lane 3, cytoplasmic fraction of HB101 (pLP64); lane 4, cell lysate of HB101 (pUC19).

activity.

To find whether the downstream region has a trans-acting function, complementation tests were carried out. A plasmid, pHSG68, was constructed by ligating the 1.9-kb *Pst*I–*Eco*RI fragment from pLP64 to *Eco*RI and *Pst*I digested pHSG299 which is a Km resistant pUC type vector (Fig. 4). When JM103 carrying both pLP65 and pHSG68 was cultivated in LB containing 0.1 mM IPTG, Ap, and Km, the lipase activity of the lysate reached 16.5 U/ml (Table II). JM103 (pHSG68) did not produce the lipase. These results suggested that an activator gene (*act*) was located downstream of the *lip*, and the gene product enhanced the lipase activity about 200-fold as a trans-element.

Nucleotide sequence of the *act*

To determine the localization of the *act*, derivatives from pHSG68 were constructed using the restriction cleavage sites, *Bst*EII, *Mlu*I, *Kpn*I, *Not*I, and *Eco*T22I at position 1640, 1822, 1860, 2295, and 2630 (Fig. 4). To

Fig. 4. Restriction Maps of pLP64 and Its Derivatives.

The thick and thin lines indicate vector DNA and KWI-56 chromosomal DNA, respectively. An open and a shaded box indicate the ORFs of the *lip* and the *act*, respectively. Arrows indicate the direction of transcription from the *Plac* on PUC vector. A triangle under the line indicates the linker insertion site for the artificial frameshift mutation. Restriction enzymes are as follows: B, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; E, *Eco*RI; Et, *Eco*T22I; K, *Kpn*I; M, *Mlu*I; N, *Not*I; P, *Pst*I.

construct plasmids pHSG68B, pHSG68M, pHSG68K, pHSG68N, and pHSG68E, pHSG68 was digested by *Pst*I–*Bst*EII, *Pst*I–*Mlu*I, *Pst*I–*Kpn*I, *Not*I–*Eco*RI, and *Eco*T22I–*Eco*RI, respectively. The resulting fragments were treated with T4 polymerase and self-ligated. The lipase activity of *E. coli* JM103 carrying both pLP65 and the derivative was measured. pHSG68B and pHSG68E increased the lipase activity (14.8–17.5 U/ml) in JM103, but pHSG68M, pHSG68K, and pHSG68N did not (0.08–0.1 U/ml) as shown in Table II. These results showed the start and stop codons of the *act* must be located upstream of the *Mlu*I site and downstream of the *Not*I site, indicating that either the ORF3 or the ORF4 contained *act*. In pHSG68B, it was expected that the translation was started from its own start codon and/or the *lacZ* start codon on the vector. The ORF3 had two

translational start codons, GTG, at position 1665 and 1761 between the *Bst*II site and the *Mlu*I site, but the ORF4 did not. The ORF3 was in the same translational reading frame as the vector's *lacZ* gene, and resulted in the expression of an β -galactosidase fusion protein. Thus, these results indicated that the *act* was presented in the ORF3 which encoded a polypeptide of 344 amino acid residues with a molecular weight of 36,544. No homologies between this protein and other proteins in the NBRF-PIR and the SWISS-PROT data bases could be found using the method of Lipman and Pearson.¹⁸⁾ This ORF existed at 7bp downstream of the *lip* with the same orientation, and had eight possible translational start codons at positions 1476, 1518, 1533, 1557, 1563, 1626, 1665, and 1761. The SD-like sequences, GAGGCG and AGGCAG, were in front of GTG codons at positions 1626 and 1665, respectively.

Discussion

Kugimiya *et al.*³⁾ and Aoyama *et al.*⁴⁾ reported the cloning of lipase genes from *P. fragi* IFO-3458 and IFO-12049. The nucleotide sequences of these genes were identical with each other except for 3 nucleotides. On a taxonomical study, KWI-56 was a close relative of *P. cepacia*,⁵⁾ and was different from *P. fragi* according to rRNA homologies.^{19,20)} In spite of the difference in the taxonomic classification, there is 30% overall homology between the two lipases from KWI-56 and IFO-12049. This result suggests that the structure of lipases from genus *Pseudomonas* might be similar.

When comparing the lipases from *Pseudomonas* to that of *Staphylococcus*,^{21,22)} five regions of amino acid sequence were conserved, although the overall homology was very low. In addition, the orders of the five conserved regions on the primary structures of these lipases were also identical (Fig. 5). Therefore the conserved regions may be essential for the expression of the enzyme activity. In fact, the 2nd conserved region includes the common sequence of lipases, Gly-X-Ser-X-Gly, and the

serine in this peptide is considered as an active serine residue (Ser-87). Furthermore, one of the histidine and aspartic acid residues in the conserved regions might form a catalytic center with the Ser-87, Ser-His-Asp triad, as was already shown in the lipases from *Rhizomucor miehei* and human pancreas by X-ray crystallography.^{23,24)}

No homology except for the common sequence of Gly-X-Ser-X-Gly was found between KWI-56 and other lipases from most of the eukaryotic sources such as pancreas,^{25,26)} liver^{27,28)} and eukaryotic microorganisms.^{29,30)} However, the sequence of His-X-Asp-X-Ile in the 5th region as shown in Fig. 5 existed in the human gastric³¹⁾ and the rat lingual³²⁾ lipases as well.

In *E. coli*, 95% of lipase activity was located in the cytoplasm, and the lipase appeared in two molecular forms (33-kD and 38-kD). On the other hand, it was confirmed that the lipase also existed in the periplasm, and its molecular mass was 33-kD as a single molecule (Fig. 3). These results suggest that the cloned lipase is produced as a 38-kD proenzyme, and cleaved by signal peptidase to be secreted into the periplasmic space as a 33-kD mature enzyme. The 33-kD lipase may be a membrane-bound structure in *E. coli* because of its hydrophobicity, and the minor part is detected in the periplasm.

The *act* was found downstream of the *lip*, and the *lip* and *act* might be expressed as a polycistronic mRNA in *E. coli* (pLP64) using *Plac*, because consensus sequence for the *E. coli* promoter was not found on upstream of the *act*. Though the *act* has eight possible translational start codons, it is not clear which codon is used in *E. coli*. Further work is needed to clarify this question by identifying the N-terminal sequence and molecular weight of the purified activator.

The activation mechanism by the activator is not understood. It is supposed that the hypothetical mechanisms include the following: (1) activation at the level of transcription; (2) stabilization of the lipase mRNA; (3) activation at the level of translation; (4)

Fig. 5. Partial Homology of the Amino Acid Sequences among the Lipases from KWI-56, *P. fragi* IFO-12049,⁴⁾ *S. hyicus*²¹⁾ and *S. aureus*.²²⁾

A: Position of the conserved regions (thick lines, 1–5) on the lipase primary structures as a mature protein (KWI-56) and pre-proteins (others).

B: Homologous amino acid residues in the conserved regions (1–5) are boxed. The aspartic acid, histidine, and serine residues that are identical in all sequences are marked by circles. The numbers beside the sequences indicate the amino acid numbers of mature protein (KWI-56) and each pre-protein (others) from their N-terminus.

processing or folding of the lipase precursor; and (5) activation by co-factor. Generally, the regulatory mechanism of expression of a gene functions at the level of transcription. In this case, the role of the transcriptional activator is a positive factor which increases the amount of mRNA by binding to operator or repressor. However, this activation system must not function in the case of the *lip*, because the *lip* was controlled by the *Plac* on pUC vector and

the promoter from KWI-56 does not play an important role in the lipase expression in *E. coli*.

On the other hand, the activation of lipase by a co-factor is known for the lipases from *Saccharomycopsis lipolytica*,³³⁾ human pancreas,³⁴⁾ and castor beans.³⁵⁾ If a co-factor is essential for the expression of the lipase activity from KWI-56, a drastic decrease of the activity may be found in some purification step, but

it was not found in our previous purification experiment. The purified enzyme, which showed a single band on SDS-PAGE, had lipase activity, and its molecular weight, 33,000, consists of that from the nucleotide sequence, so there is no possibility that a co-factor combines with the lipase for the expression of the lipase activity.⁵⁾ Accordingly, it seems that the *act* encodes neither a co-factor nor a co-factor synthesizing enzyme.

Further investigations are in progress to clarify the activation mechanism mentioned above.

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