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A Direct Approach for Finding Functional Lipolytic Enzymes from the *Paenibacillus polymyxa* Genome

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Abstract A direct approach was used to retrieve active lipases from *Paenibacillus polymyxa* genome databases. Twelve putative lipase genes were tested using a typical lipase sequence rule built on the basis of a consensus sequence of a catalytic triad and oxyanion hole. Among them, six genes satisfied the sequence rule and had similarity (about 25%) with known bacterial lipases. To obtain the six lipase proteins, lipase genes were expressed in *E. coli* cells and lipolytic activities were measured by using tributyrin plate and *p*-nitrophenyl caproate. One of them, contig 160-26, was expressed as a soluble and active form in *E. coli* cell. After purifying on Ni-NTA column, its detailed biochemical properties were characterized. It had a maximum hydrolytic activity at 30°C and pH 7–8, and was stable up to 40°C and in the range of pH 5–8. It most rapidly hydrolyzed $pNPC_6$ among various PNP-esters. The other contigs were expressed more or less as soluble forms, although no lipolytic activities were detected. As they have many conserved regions with lipase 160-26 as well as other bacterial lipases throughout their sequence, they are suggested as true lipase genes.

Key words: Lipase, *Paenibacillus polymyxa*, genome database

Lipases are triacylglycerol acylhydrolases (EC 3.1.1.3) that catalyze both hydrolysis and synthesis of long-chain triacylglycerols [7]. They exhibit various substrate specificity, including chain-length selectivity, position selectivity, and stereoselectivity. As such, these enzymes are important elements in the production of fatty acids, interesterification of fats, and synthesis of useful esters and peptides in the food, pharmaceutical, and fine chemical industries [8, 20, 25].

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Many diverse lipase proteins have been purified and characterized from animal, plant, and microorganisms, and their genes have been cloned and the nucleotide sequences analyzed. From those sequence analyses, it has been known that most lipase proteins have the following characteristic features in their primary sequence [3, 7]. First, they contain a catalytic triad sequence, consisting of Ser, Asp, and His, wherein the active site serine residue is normally located in a highly conserved pentapeptide sequence (Gly-X-Ser-X-Gly). Second, the HG (or RG in some fungal lipases) sequence, which constitutes an oxyanion hole in the three-dimensional protein structure, is positioned about 70 to 100 amino acids ahead of the active site serine residue. Third, the signal sequence (SS, 35 amino acids) or SS inclusive of a Pro part (400 amino acid) is located 10 to 40 amino acids ahead of the HG sequence.

Recently, whole bacterial genomes from many different species of *Bacteria* and *Archaea* have been analyzed, and the genome information has been accumulated and released in public databases (TIGR, http://www.tigr.org). Based on protein sequence analyses and comparison, some genes have been annotated for their putative function [23]. In particular, as a result of such microbial genome research, the number of putative lipase genes from many microbial strains studied is rapidly increasing.

Paenibacillis polymyxa, belonging to endospore-forming low-G+C Gram-positive bacilli, produces antimicrobial compounds and secretes diverse hydrolyzing enzymes [2, 15, 26, 27]. Some strains in the species can enhance the growth of plants and antagonize harmful soil microorganisms that inflict plants through its ability to synthesize plant hormones and to produce peptide antibiotics. *P. polymyxa* E681 is a plant-growth-promoting rhizobacterial strain capable of suppressing plant diseases, which was isolated from the rhizosphere of winter barley grown in South Jeolla Province,

Korea. Recently, the *P. polymyxa* E681 genome sequence was analyzed completely [14, 21].

In this work, we analyzed some putative lipase sequences retrieved from the *P. polymyxa* genome for the presence of the typical lipase signature sequence and measured their real enzyme activities in the cell after gene cloning and protein expression.

MATERIALS AND METHODS

Materials

P. polymyxa E681 (=KCTC 8801P) was kindly provided by Prof. Chang Seuk Park at Gyeongsang National University. The chromosomal DNA was prepared, using a genomic DNA extraction kit (Intron Biotechnology, Korea). The tributyrin (TBN), tricaprylin (TCN), and *p*-nitrophenyl esters were purchased from Sigma, and the other enzymes and chemicals from commercial companies.

Retrieval of Putative Lipase from *P. polymyxa* Genome

ORFs in the draft genome sequence of *Paenibacillus polymyxa* E681 were predicted and, using the BLASTP program [1], the nr database (nr=all non-redundant GenBank CDS translations+RefSeq Proteins+PDB+SwissProt+PIR +PRF) was searched for homologs of the deduced amino acid sequences. ORFs showing highest similarities to lipases or esterases were further examined for the presence of esterase/lipase-specific motifs with InterProScan [18].

PCR Cloning of Typical Lipase Genes

Based on the sequence rule described above, 6 typical lipase genes were selected from 12 putative lipase genes. To obtain the lipase genes, each set of forward and reverse primers were synthesized. The forward primers contained an *Nde*I (or *Nco*I) site plus coding sequence (18mer), while the reverse primer contained a *Hin*dIII (or *Xho*I) site plus coding sequence (18mer, stop codon was omitted). The *P. polymyxa* chromosomal DNA prepared was used as template DNA. The PCR reaction was performed according to routine procedure. The thermal cycles of denaturation (95°C, 1 min), annealing (45°C, 1 min), and polymerization (72°C, 1.5 min) were performed 25 times. Thereafter, the PCR products were digested with *Nde*I and *Hin*dIII, ligated with a pET22b (Novagen, Madison, WI, U.S.A.) vector, and transformed into *E. coli* XL1-Blue. The resulting recombinant plasmids were then purified and used to transform *E. coli* BL21 (DE3) cells.

The transformed *E. coli* cells were cultured on a lipase-screening TBN-LB plate prepared as follows [12, 14]: A TBN emulsion was made by emulsifying 5 ml of TBN in 45 ml of a 200 mM NaCl, 10 mM $CaCl_2$, and 5% (w/v) gum arabic solution for 2 min in a Waring blender. This TBN emulsion (50 ml) was then mixed with 450 ml of LB agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) and used to make TBN-LB plates.

Lipase Expression

The recombinant *E. coli* BL21 (DE3) cells were cultured at 30°C in an LB medium containing ampicillin (100 μg/ml). Lipase expression was induced by adding IPTG (0.5 mM) and lowering culture temperature to 18°C. After 4 h of cultivation, the *E. coli* cells were collected and lysed by ultrasonication, then the water-soluble fractions were recovered by centrifugation and used as the cell extract.

Lipase Activity Assay

The lipase activity was measured with $PNPC_6$(*p*-nitrophenyl caproate), as previously described [11, 17, 19], where the reaction mixture consisted of 0.01 ml of 10 mM $PNPC_6$ in acetonitrile, 0.04 ml of ethanol, and 0.95 ml of 50 mM Tris-HCl buffer (pH 8.0) containing an appropriate amount of the cell extract. The enzyme reaction was carried out for 3 min at 35°C, and the amount of *p*-nitrophenol (PNP) liberated during the reaction was measured by its OD at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of PNP per min at 35°C.

Purification of Lipase 160-26

Cell extract of *E. coli* cells harboring the lipase 160-26 gene was dialyzed with a binding buffer (50 mM Tris, pH 8, 300 mM NaCl, 5 mM imidazole). The enzyme suspension was loaded onto a Ni-NTA column (Qiagen, Valencia, CA, U.S.A.), and unbound protein was washed out with 50 mM Imidazole. The His-tagged lipase 160-26 bound to Ni-NTA resin was then eluted with 300 mM imidazole solution.

To check the purity of lipase 160-26, SDS-PAGE (12%) was performed by using slab gels, as described by Laemmli [9]. The proteins on the gels were stained with Coomassie Brilliant Blue R-250.

Characterization of Lipase 160-26

The optimum temperature of the lipase 160-26 was determined by assaying its hydrolytic activity on $PNPC_6$ at various temperatures (5-55°C). To examine the thermostability, the enzyme was incubated at various temperatures (5-60°C) for 30 min in a 50 mM Tris-HCl buffer (pH 8.0) containing 0.05 mg/ml of BSA, and the residual activity was then determined at 35°C and pH 8.0 [16].

The optimum pH of the lipase 160-26 was determined by assaying at various pHs (pH 6-10). GTA buffer (100 mM 3,3-dimethylglutaric acid, 100 mM Tris, 100 mM 2-amino-2-methyl-1,3-propanediol), a universal buffer, was used in these experiments. To determine the pH stability of the enzyme, it was preincubated at various pHs (pH 4-10) for 30 min in the presence of 0.05 $mg \cdot ml^{-1}$ BSA and the

Table 1. List of putative lipolytic enzymes of *P. polymyxa*.

Contig	Signiture sequence[a]		Protein size (aa)	Best hit protein[b]
	HG	GXSXG		
4-3	+ (71)	+ (145)	307	*Morzxella* sp. lipase (P24484)
160-26	+ (81)	+ (182)	368	*Acinetobacter iwoffii* esterase (P18773)
236-10	+ (175)	+ (249)	403	*Acinetobacter iwoffii* esterase (P18773)
236-11	+ (92)	+ (166)	323	*Moraxella* sp. lipase (P24484)
263-5	+ (105)	+ (179)	344	*Moraxella* sp. lipase (P24484)
345-1	+ (48)	+ (122)	279	*Moraxella* sp. lipase (P24484)
159-6	+ (37)	+ (115)	335	Yeast hypothetical protein (P28321)
150-7	-	-	212	*Bacillus subtilis* hypothetical protein (P42969)
158-18	-	-	418	*Bacillus subtilis* hypothetical protein (P42304)
172-12	-	-	386	*Bacillus subtilis* hypothetical protein (P42969)
303-6	-	-	373	*Bacillus subtilis* hypothetical protein (P42969)
438-17	-	-	513	*Butyrivibrio fibrisolvens* xylanase (P26223)

[a]Plus (+) and minus (-) mean the presence and absence of signature sequence, respectively. The numbers in parentheses are the location of the signature sequence in the proteins.

[b]The numbers in parentheses indicate GenBank-id.

residual esterase activity was then determined at 35°C and pH 8.0. In this case, GTA buffer was also used.

RESULTS AND DISCUSSION

Retrieval of Lipase Genes from *P. polymyxa* Genome

Nucleotide sequence analysis of all contigs (4,200 genes) from the *P. polymyxa* genome sequence showed that as many as twelve genes possibly code for lipase proteins (Table 1). The number of putative lipase genes was too many in comparison with those from other bacterial genomes reported until now, which contained usually less than five putative lipase genes.

As described above, all lipases have typical signature sequences. Investigation of the above twelve contigs showed that only seven contigs coded proteins with the signature sequences (Table 1). The remaining five contigs had neither the HG region nor the GXSXG sequence. In addition, when BLAST search was done, only six proteins matched with other bacterial lipases/esterases. The six proteins were, of course, those with the lipase signature sequences. Therefore, the six contigs were considered to be true lipase/esterase genes.

The best-hit bacterial proteins include *Moraxella* sp. lipase [6], *Acinetobacter iwoffii* esterase [24], and *Escherichia coli* acetyl esterase [22] (Table 1). The percent identities between the six contigs and the recruited bacterial lipases were, however, very low (less than 25%) (Table 2). Therefore, these six putative lipases seemed to be novel lipases/esterase, at least in their primary structure. In addition, sequence comparison showed that these six proteins were also very different from one other. Except for contig 236-10 and contig 236-11, which have a high percent identity (64.4%), the others had very low percent identities (12–36%) (Table 2).

These low similarities amongst them as well as towards other bacterial lipase provided a motive to investigate whether

Table 2. Percent identity among six contigs and three bacterial lipases.

Contig	No.[a]	1	2	3	4	5	6	7	8	9
1.	236-10	100[b]	64.4	28.7	16.9	23.7	13.3	18.1	19.4	23.4
2.	236-11		100	28.0	18.0	25.8	11.8	24.5	18.5	21.8
3.	4-3			100	26.1	30.1	19.9	20.2	19.5	19.8
4.	263-5				100	36.2	14.2	19.2	14.1	18.5
5.	345-1					100	16.5	23.3	20.8	20.8
6.	160-26						100	14.4	15.4	16.5
7.	P24484							100	17.9	19.1
8.	P23872								100	24.4
9.	P18773									100

[a]P24484, P23872, and P18773 are GenBank-ids of *Moraxella* sp. lipase, *E. coli* acetyl esterase, and *Acinetobacter iwoffii* esterase, respectively.

[b]Percent identity was calculated using DNAStar program.

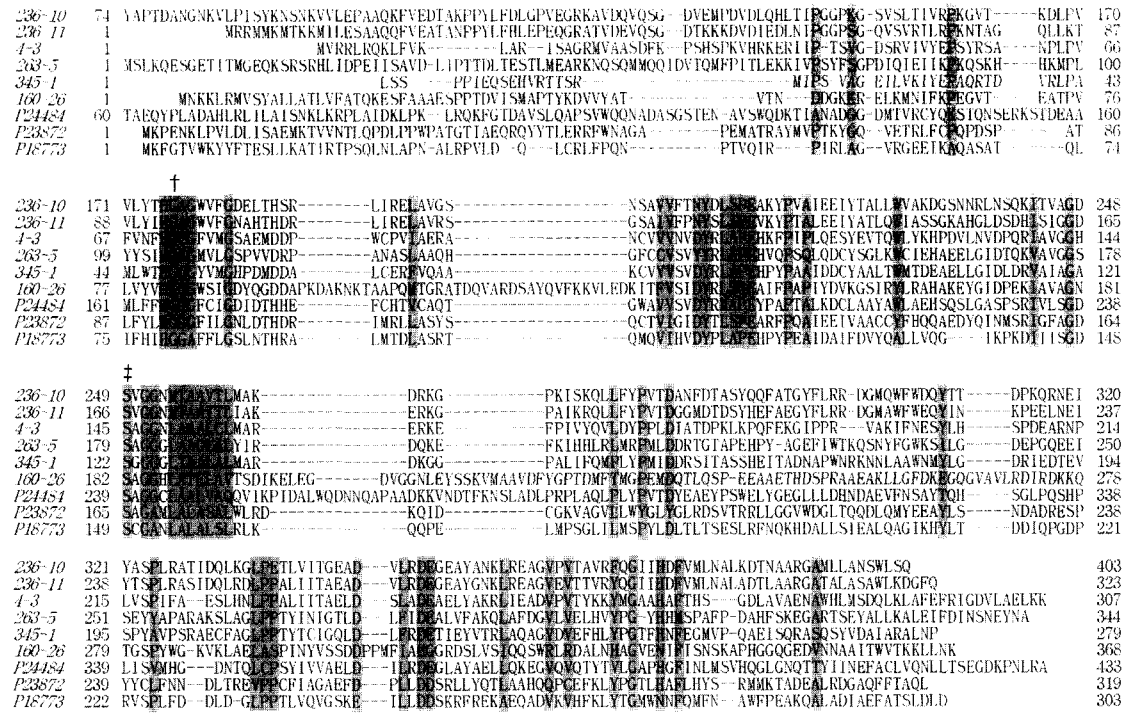

Fig. 1. Multiple sequence alignment of 6 contigs and 3 bacterial lipase/esterases.
The numbers indicate the sequence of amino acids in the proteins. The shaded regions indicate the conserved regions among the 9 enzymes. The symbols, † and ‡, indicate the oxyanion regions and active site serine residues, respectively.

all the six contigs really code for true lipase proteins in the cell. One positive clue came from the result of multiple sequence alignment. The Clustal W method (in DNAStar suite of programs) showed that all six putative lipases and three bacterial lipases could be aligned well in spite of their low sequence similarity (Fig. 1). That is, they had many conserved regions throughout their sequence, strongly implying that all six putative lipases may be true lipase genes.

PCR Cloning of Putative Genes

To obtain the six lipase genes, each set of forward and reverse primers were synthesized and PCR reactions performed as described in Materials and Methods. Among the six PCR reactions, only five reaction mixtures amplified DNA with the expected sizes. The PCR products were ligated into a pET22 vector and the resulting recombinant plasmids were used to transform *E. coli* BL21 (DE3). Three recombinant *E. coli* cells harboring the lipase genes (contig 4-3, 160-26, 345-1) exhibited clear zones around colonies on a TBN plate (Table 3). Meanwhile, the quantitative $PNPC_6$ assay of *E. coli* cell extracts revealed the functional expression of only one lipase gene (contig 160-26) in the *E. coli* cell. That is, *E. coli* cells transformed with two lipase genes (contigs 4-3, 345-1) had an esterase activity too weak to be detected by colorimetric assay. *E. coli* cells with the other genes (contigs 236-1, 263-5) showed no activity at all, even on TBN plate.

Protein profiles of the cell extracts of the five recombinant *E. coli* cells were analyzed by SDS-PAGE (Fig. 2). For four contigs (160-26, 236-11, 263-5, 345-1), target protein bands with expected sizes were detected in both soluble and insoluble fractions on the gel. This meant that in the cases of three contigs (236-11, 263-5, 345-1), a detectable amount of protein was expressed in soluble form in *E. coli* cells. However, no hydrolytic activity toward $PNPC_6$ was measured in the cell extract. The three expressed proteins had very low esterase activity, if any, and the genes seemed

Table 3. Measurement of lipolytic activity of the cloned proteins.

Contig	Calculated Mr[a] (kDa)	Expression[b] (soubility)	Hydrolytic activity toward	
			TBN[c]	$PNPC_6$[d]
4-3	34.4	n.d.	+	-
160-26	40.0	S, P	+	+ (25 U/L)
236-11	35.7	S, P	-	-
345-1	30.5	S, P	+	-

[a]Molecular weight was calculated from the deduced amino acid sequence using the DNAStar program.

[b]S, soluble protein; P, insoluble protein; n.d. not detected.

[c]Plus (+) and minus (-) mean the presence and absence of clear zones around colonies of the transformed cells, respectively.

[d]Relative hydrolytic activity of cell extract of the transformed *E. coli* expressed as enzyme unit per liter of culture volume.

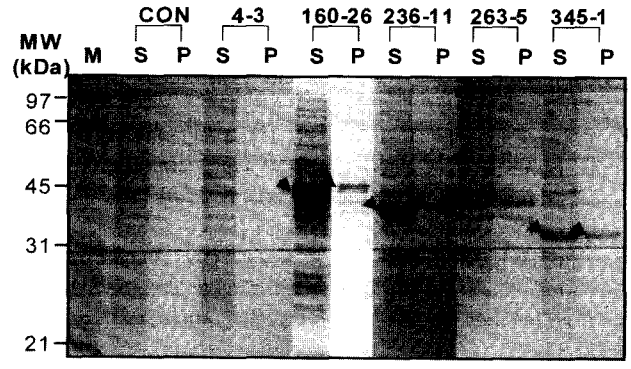

Fig. 2. SDS-PAGE of cell extracts from five recombinant *E. coli* cells.
Arrows indicate expected lipase proteins. CON means protein bands of *E. coli* BL21(DE3) transformed with pET22 vector. S, soluble fraction; P, insoluble fraction.

to be vestige genes in *P. polymyxa*. In the case of contig 4-3, the target protein band was not seen on the gel.

Characterization of *P. polymyxa* Lipase 160-26

Contig 160-26 was the only lipase gene expressed at a high level in *E. coli* BL21(DE3). Following induction of the culture with IPTG, the incubation temperature was lowered to 18°C. This temperature shift increased the expression level of the protein. His-tagged lipase 160-26 in the cell extract was easily purified by Ni-NTA column chromatography. Purified lipase had a specific activity of 0.99 U/mg toward $PNPC_6$, a relatively low value in comparison to other lipases.

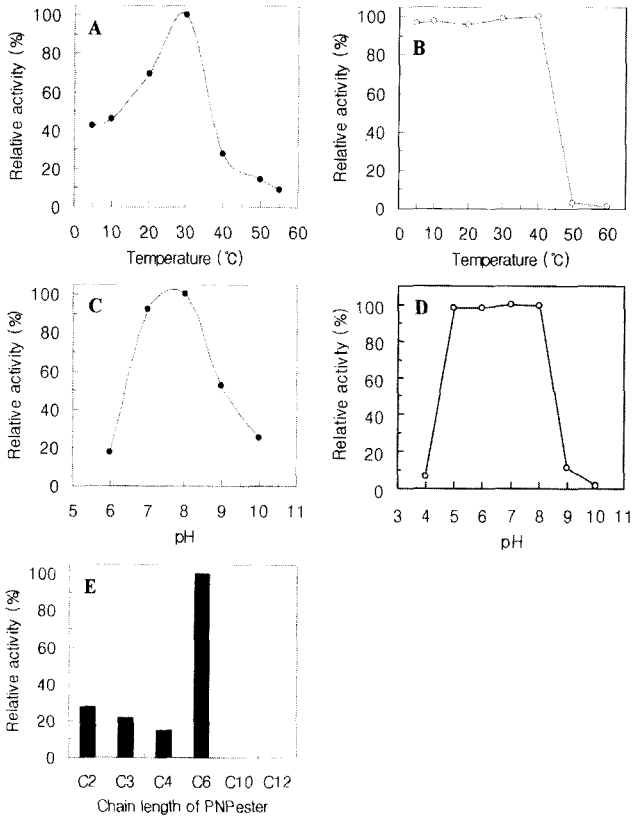

Fig. 3. Biochemical properties of lipase 160-26.
Hydrolytic activity at various temperatures (A) and pHs (C), enzyme stability at various temperatures (B) and pHs (D), and chain length specificity (E) were all measured.

Simple biochemical properties of the purified enzyme were characterized for comparison with other bacterial lipases. When $PNPC_6$ was used as substrate, the enzyme had a maximum hydrolytic activity at 30°C (Fig. 3A). The above enzyme was thermostable up to 40°C, while its stability decreased rapidly above 40°C (Fig. 3B). The enzyme had a maximum hydrolytic activity at pH 7-8 (Fig. 3C) and the stability was maintained within the pH range of 5-8 (Fig. 3D). Chain length specificity for PNP-acetate (C2), -propionate (C3), -butyrate (C4), -caproate (C6), -caprate (C10), and -laurate (C12) was also measured, and the enzyme was found to hydrolyze PNP-caproate most rapidly (Fig. 3E). PNP-acetate, -propionate, and -butyrate were hydrolyzed relatively slowly, but PNP-caprate and -laurate were not hydrolyzed at all. Taken together, although the protein sequence of lipase 160-26 was a little unique, the basic biochemical properties were very similar to other bacterial lipases.

Many lipases are being used in diverse bioconversion reactions as biocatalysts [7, 8, 20, 25]. To choose a suitable lipase, it is needed to collect in advance diverse lipases as an enzyme pool. Consequently, lipase 160-26 can readily be used as a member in the enzyme pool.

Cell extract of *P. polymyxa* has a weak but measurable hydrolytic activity (4.2 mU/ml) toward $PNPC_6$ (unpublished data). Hence, our present results show that this activity may come from the protein 160-26, however, this conclusion needs to be further confirmed by experiments such as knocking out of the gene.

In conclusion, *P. polymyxa* genome contained total six putative lipase/esterase genes. One of them, 160-26, was expressed well in *E. coli*, but the rest of the genes were not expressed well in this work. However, since they have many conserved regions with lipase 160-26 as well as other bacterial lipases throughout their sequence, they appear to be true lipase genes. Recently, various directed enzyme evolution techniques have been developed to improve enzyme activity as well as other biochemical properties [4, 5, 10]. Since the six *P. polymyxa* genes have quite novel amino acid sequences, they could possibly be used as gene sources to make useful industrial enzymes.

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