Low-Temperature Lipase from Psychrotrophic Pseudomonas sp. Strain KB700A

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We have previously reported that a psychrotrophic bacterium, Pseudomonas sp. strain KB700A, which displays sigmoidal growth even at −5°C, produced a lipase. A genomic DNA library of strain KB700A was introduced into Escherichia coli TG1, and screening on tributyrin-containing agar plates led to the isolation of the lipase gene. Sequence analysis revealed an open reading frame (KB-lip) consisting of 1,422 nucleotides that encoded a protein (KB-Lip) of 474 amino acids with a molecular mass of 49,924 Da. KB-Lip showed 90% identity with the lipase from Pseudomonas fluorescens and was found to be a member of Subfamily I.3 lipase. Gene expression and purification of the recombinant protein were performed. KB-Lip displayed high lipase activity in the presence of Ca2+. Addition of EDTA completely abolished lipase activity, indicating that KB-Lip was a Ca2+-dependent lipase. Addition of Mn2+ and Sr2+ also led to enhancement of lipase activity but to a much lower extent than that produced by Ca2+. The optimal pH of KB-Lip was 8 to 8.5. The addition of detergents enhanced the enzyme activity. When p-nitrophenyl esters and triglyceride substrates of various chain-lengths were examined, the lipase displayed highest activity towards C10 acyl groups. We also determined the positional specificity and found that the activity was 20-fold higher toward the 1(3) position than toward the 2 position. The optimal temperature for KB-Lip was 35°C, lower than that for any previously reported Subfamily I.3 lipase. The enzyme was also thermolabile compared to these lipases. Furthermore, KB-Lip displayed higher levels of activity at low temperatures than did other enzymes from Subfamily I.3, indicating that KB-Lip has evolved to function in cold environments, in accordance with the temperature range for growth of its psychrotrophic host, strain KB700A.

Lipases (glycerol ester hydrolases) are hydrolases acting on the carboxyl ester bonds present in acylglycerols to liberate fatty acids and glycerols. Lipases are versatile enzymes that are distributed throughout living organisms. A vast number of bacterial lipases with different enzymological properties and substrate specificities have been found (20). They have a wide range of potential applications in the hydrolysis, esterification, and transesterification of triglycerides and in the chiral selective synthesis of esters (17, 19). They also lack an N-terminal signal peptide and are secreted through an ATP-binding cassette (ABC) transporter system (1, 2, 12). At present, lipases from Subfamilies I.1 and I.2, lipases from Subfamily I.3 are considerably larger (Pseudomonas sp. strain MIS38, 65 kDa [3]; Pseudomonas fluorescens SIK W1, 50 kDa [10]; Serratia marcescens, 65 kDa [26]), do not have cysteine residues, and do not require any additional gene product for correct folding. They also lack an N-terminal signal peptide and are secreted through an ATP-binding cassette (ABC) transporter system (1, 2, 12). At present, in contrast to the abundant information on lipases from Subfamilies I.1 and I.2, lipases from Pseudomonas sp. strain MIS38 (3), P. fluorescens SIK W1 (25), and S. marcescens (26) are the only proteins that have been studied among members of Subfamily I.3.

We have isolated from a subterranean environment a psychrotrophic strain, KB700A, which showed sigmoidal growth even at −5°C (27). We have previously shown that strain KB700A produced an extracellular lipase. This study reports the identification of the lipase gene from the psychrotrophic Pseudomonas strain KB700A and gives detailed enzymatic characterization of its gene product.

MATERIALS AND METHODS

Bacterial strains and plasmids. Pseudomonas sp. strain KB700A was isolated from a water sample collected 700 to 1,800 m below the surface (27). Escherichia coli strain TG1 and plasmids pUC18 and pUC118 were used for subcloning of the gene fragments and DNA manipulation. E. coli strain BL21(DE3) (Novagen, Madison, Wis.) was used as a host cell along with the expression vector pET-25b (Novagen).

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General DNA manipulation. Restriction enzymes and DNA polymerase were purchased from Toyobo (Osaka, Japan) as well as Takara Shuzo (Kyoto, Japan). Each enzyme was used according to the recommendations of the manufacturer. DNA ligations were performed using DNA ligation kit (Toyobo). Genomic DNA and plasmid DNA were isolated using genomic and plasmid DNA isolation kits, respectively (Qiagen, Hilden, Germany). A DNA purification kit (Toyobo) was used to recover DNA fragments from agarose gels.

Construction of genomic library. *Pseudomonas* sp. strain KB700A genomic DNA was partially digested with *Sma*I; 3- to 9-kbp DNA fragments were excised from agarose gels (0.7%), and DNA was recovered. Genomic DNA fragments were ligated with pUC118, which had been previously digested with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase. The ligation products were introduced into *E. coli* TG1.

Sequencing and analysis of the lipase gene. DNA sequencing was performed using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, Calif.). Nucleotide and deduced amino acid sequence analyses, open reading frame search, multiple alignment, and molecular-mass and isoelectric-point calculations were performed using DNASIS software (Hitachi Software, Tokyo, Japan). Database homology search was performed with the Basic Local Alignment Search Tool (BLAST) program provided by DNA Data Bank of Japan (DDBJ).

Expression of the lipase gene in *E. coli*. The open reading frame of the putative lipase gene was amplified by PCR and inserted into pET-25b expression vector (Novagen). This recombinant plasmid, designated as pET-lip, was used for the expression of the lipase gene in *E. coli.* The strain BL21 (also called Top10) was grown for 16 h at 37°C in NZCYM medium (1% NZ amine, 0.5% yeast extract, 0.5% NaCl, 0.1% Casamino Acids, and 0.2% MgSO4·7H2O [pH 7.0]) containing ampicillin (50 μg/ml). The precul- ture was inoculated (1%) into fresh NZCYM medium containing ampicillin, and cultivation was continued until the optical density at 660 nm reached 0.4. The culture was then supplemented with 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated for another 4 h at 37°C for optimal expression of the lipase gene. Cells were harvested by centrifugation at 6,000 × *g* for 10 min and washed with 50 mM Tris-HCl buffer (pH 8.0). The cell pellet was resuspended in the same buffer, and the cells were then disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation (15,000 × *g* for 30 min). The recombinant lipase in the insol- uble form was denatured with 6 M urea and then refolded by fractional dialysis in 3, 1.5, and 0.5 M urea in 50 mM Tris-HCl (pH 8.0). The refolded protein was purified by HiTrap Q column (Amersham Pharmacia Biotech, Upsalla, Sweden). The purity of the protein was examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, Calif.) according to the manufacturer’s instructions. Bovine serum albumin was used as a standard.

Lipase assay. Lipase production by colonies on agar medium was detected by the tributyrin agar diffusion assay. Agar plates were incubated at 30°C for 30 min with magnetic stirring at 500 rpm. The enzyme reaction was stopped by the addition of 20 ml of ethanol. The amount of fatty acids released during the incubation was determined by titrating the mixture with 50 mM KOH to pH 10.0 using an APB-117 titrator (Kyoto Electronics, Kyoto, Japan). One unit of lipase activity was defined as the activity required to release 1 μmol of fatty acids per min under the above conditions.

Determination of temperature, pH, and detergent effects. The optimum temperature and pH for enzyme activity were determined photometrically with p-NPP as a substrate. The assay was performed by incubation of the reaction mixture at various temperatures and pHs. The effects of detergents on the lipase activity were analyzed by addition of 1% (wt/vol) detergent in Tri-HCl buffer (pH 8.0) to the enzyme solution described above. Activity measurements were carried out immediately and after 1 h of incubation at 25°C. As 5 μl of enzyme solution was added to the reaction mixture (1 ml), the final concentration of detergent in the reaction mixture was 0.005%. Analysis of the effects of detergents on the activity of KB-Lip was based on the measurements carried out immediately after addition of detergent, while analysis of the stability against detergents was based on the measurements taken after incubation for 1 h. Activity was measured by spectrophotometric assay with p-NPP as a substrate.

Thin-layer chromatography. Positional specificity of the lipase was examined by thin-layer chromatography of the reaction product obtained by using pure triolein as a substrate (29). Triolein was purified from a commercial product by passing it through a column of Wakogel C-200 (Wako Pure Chemical Industries, Osaka, Japan) with n-hexane–ethyl acetate (98.2: vol/vol) as the bed solvent. A reaction mixture composed of 0.5 g of triolein, 5 ml of 50 mM acetate buffer (pH 5.6), and 200 U of the enzyme was incubated at 30°C for 15 min with magnetic stirring at 500 rpm. After incubation, the reaction product was extracted with 20 ml of ethyl ether. Aliquots of the ether layer were applied to a silica gel 60 plate (Merck KgaA, Darmstadt, Germany) and developed with a 95:4:1 (vol/vol) mixture of chloroform, acetone, and acetic acid. The spots were visualized by spraying the plate with 50% (vol/vol) H2SO4 in methanol and then heating in an oven at 150°C until charring occurred. The contents of triolein, 1,3-diolein, 1,3(3)-diolein, monolein, and oleic acid were determined with a thin-layer chromatography and flame ionization detection analyzer (Iatron MK-5; Ia- tron, Tokyo, Japan) after developing with a mixture of benzene, chloroform, and acetic acid (50:20:0.7, vol/vol).

Nucleotide sequence accession number. The nucleotide sequence data of the KB-lip gene reported in this paper will appear in the DDBJ, EMBL, and GenBank DNA databases under the accession number AB063391.

RESULTS

Cloning of the lipase gene from KB700A. We have previously reported that the psychrotrophic bacterium *Pseudomonas* sp. strain KB700A produces a lipase (KB-Lip) (27). The lipase activity in the culture supernatant was detected only when strain KB700A was grown below 25°C and in moderately poor medium such as 0.2% Luria-Bertani medium. No activity could be detected when cells were grown at 30°C (data not shown).

As there was a possibility that KB-Lip was a lipase that could function at low temperatures, we attempted to clone its gene. A partially digested genomic DNA library was introduced into *E. coli* TG1 cells, and about 13,000 recombinant colonies were screened on tributyrin agar plates for lipase or esterase activity. Twelve colonies were found positive, forming halos on tributyrin agar plates for lipase or esterase activity.

Analysis of the nucleotide sequence. In the 3.5-kbp DNA fragment, we found an open reading frame (*KB-lip*) consisting of 1,422 nucleotides that encoded a protein (KB-Lip) of 474 amino acids with a molecular mass of 49,924 Da. A putative ribosome binding site (5′-GAGG-3′) was found 7 bp upstream of an initiation codon, ATG, and a putative transcription termination signal, poly(TC), was located 20 nucleotides downstream of the stop codon, TAA.
of the open reading frame of \textit{KB-lip} showed a G+C content of 61.8%.

\textbf{Amino acid sequence comparison.} The amino acid sequence of \textit{KB-Lip} displayed high similarity with lipases classified in Subfamily I.3. \textit{KB-Lip} was 90\% identical to the lipase from \textit{P. fluorescens} B52 (31), 88\% identical to \textit{Pseudomonas} sp. strain LS107d2 (21), and 87\% identical to \textit{P. fluorescens} no. 33 (24). Among enzymes whose properties have been studied, \textit{KB-Lip} displayed 88\% identity with the lipase from \textit{P. fluorescens} SIK W1 (10), 58\% identity with the lipase from \textit{Pseudomonas} sp. strain MIS38 (3), and 48\% identity with the lipase from \textit{S. marcescens} (26). The GXSXG motif, which includes the activiteserine residue (4), was found from residues 205 to 209. The amino acid sequence analysis revealed that the lipase from \textit{KB700A} lacked a typical N-terminal signal peptide for its secretion. This is as in the case of the lipase from \textit{P. fluorescens} B52, for which it has been shown that secretion is dependent on the C-terminal region of the protein (12). The C-terminal domain of \textit{KB-Lip} was highly similar to that of \textit{P. fluorescens} B52, suggesting similar mechanisms for secretion of \textit{KB-Lip}. In addition, an extreme C-terminal motif consisting of a negatively charged amino acid followed by four hydrophobic residues, proven necessary for the secretion of metalloprotease PrtG from \textit{Erwinia chrysanthemi} (15), was found near the C terminus of \textit{KB-Lip}. We also found multiple GXXGXD motifs, which are supposed to be involved in Ca$^{2+}$ binding in proteases and lipases (7, 16, 28).

\textbf{Production and purification of the lipase.} We expressed the lipase gene in \textit{E. coli} BL21(DE3) cells under the control of the T7 promoter. Although we expressed the gene under various conditions, the protein product was consistently produced in an insoluble form as inclusion bodies. Therefore, we denatured the protein in 6 M urea, and refolding conditions were examined. We found that fractional dialysis in 3, 1.5, and 0 M urea in 50 mM Tris-HCl (pH 8.0) led to efficient refolding of the enzyme. The solubilized, active protein was further purified by ion exchange chromatography. The homogeneity of the protein was demonstrated by SDS-polyacrylamide gel electrophoresis (Fig. 1).

\textbf{Effects of metal ions.} Using p-NPP as a substrate, purified \textit{KB-Lip} exhibited lipase activity without addition of any metal ions. However, when 5 mM EDTA was added in the reaction mixture, enzyme activity was completely abolished, indicating that \textit{KB-Lip} was dependent on divalent cations. Dialysis of \textit{KB-Lip} did not abolish its activity, suggesting that addition of chelating agents was necessary to remove the cations bound to the protein. We examined the effects of various metal ions and found that maximum lipase activity was found in the presence of 5 mM Ca$^{2+}$, indicating that \textit{KB-Lip} was a Ca$^{2+}$-dependent lipase (Table 1). Enzyme activity was slightly activated by Sr$^{2+}$ and Mn$^{2+}$ (1.2- to 1.5-fold). In contrast, the lipase activity was inhibited by other divalent cations, particularly Co$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$.

\textbf{Effects of pH and temperature on the enzyme activity.} With p-NPP as a substrate, \textit{KB-Lip} was most active at pHs between 8 and 8.5. We then examined the effects of temperature on the activity of \textit{KB-Lip} at pH 8.0. \textit{KB-Lip} showed maximum activity at 35°C (Fig. 2). The specific activity towards p-NPP at pH 8.0 and 35°C was 54 U/mg. Heat treatment at 60°C for 5 min resulted in a 70\% decrease in enzyme activity (data not shown).

\textbf{Effects of various detergents.} The effects of various detergents on \textit{KB-Lip} are summarized in Table 2. In general, we observed an increase in \textit{KB-Lip} activity with the addition of detergents. The presence of SDS or 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate (CHAPS) slightly increased the activity (10 to 23\%), and approximately twofold increases in activity were observed with Triton X-100, Tween 80, and Tween 20. However, we found that levels of activity of the enzyme after incubation with these detergents at 1% (wt/vol) for 1 h did not display significant differences from the activity observed without addition of detergent.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{Divalent cation (5 mM)} & \textbf{Relative activity (\%)} \\
\hline
None & 100 \\
EDTA & 0 \\
Ca$^{2+}$ & 560 \\
Cr$^{2+}$ & 30 \\
Co$^{2+}$ & 5 \\
Cu$^{2+}$ & 4 \\
Mg$^{2+}$ & 40 \\
Mn$^{2+}$ & 120 \\
Ni$^{2+}$ & 15 \\
Sr$^{2+}$ & 150 \\
Zn$^{2+}$ & 2 \\
\hline
\end{tabular}
\caption{Effects of various metal ions\footnote{Metal chlorides were used in the assay.} on \textit{KB-Lip} activity}
\end{table}
1,3-diolein was produced when lipase from the proportion of 1,2(2,3)-diolein to 1,3-diolein. Similarly, no positional specificity, we obtained the expected 2:1 ratio for using lipase from was considered negligible because of the short reaction time. The extent of hydrolysis was 10%. Spontaneous acyl migration chromatography (Fig. 3). The reactions were carried out until activity with triolein as a substrate was examined by thin-layer activity was measured at various temperatures at pH 8.0. Five milli- molar Ca$^{2+}$ was present in the reaction mixture.

**Substrate specificity.** The relative lipase activities towards various p-nitrophenyl esters were examined at 30°C at pH 8.0. Lipase showed the highest activity with p-nitrophenyl caprate (C$_{10}$ acyl group) among the p-nitrophenyl esters examined (Table 3). Medium-chain acyl group p-nitrophenyl esters seemed to be good substrates for KB-Lip, while p-nitrophenyl esters with acyl groups shorter than C$_{8}$ were poor substrates. Activity toward p-nitrophenyl palmitate in the latter category was also very low, lower than that of p-nitrophenyl caprate by a factor of 10. When activity toward triacylglycerol substrates was examined, the lipase also showed highest activity toward tricaprin (C$_{10}$ acyl group) (Table 4). However, a relatively high activity was observed for triglycerides containing C$_{18}$ acyl groups.

**Positional specificity.** The hydrolytic product of the lipase activity with triolein as a substrate was examined by thin-layer chromatography (Fig. 3). The reactions were carried out until the extent of hydrolysis was 10%. Spontaneous acyl migration was considered negligible because of the short reaction time. Using lipase from *Candida rugosa*, an enzyme reported to have no positional specificity, we obtained the expected 2:1 ratio for the proportion of 1,2(2,3)-diolein to 1,3-diolein. Similarly, no 1,3-diolein was produced when lipase from *Rhizopus delemar*, an enzyme specific to the 1,3-position, was used. When KB-Lip was examined, 1,2(2,3)-diolein was formed in large quantities from the initial stage of hydrolysis, while the formation of 1,3-diolein remained rather low. These results indicate that KB-Lip hydrolyzed 1- or 3-positioned ester bonds in preference to 2-positioned ester bonds in triolein. The amount of 1,3-diolein formed at 10% hydrolysis was 2.5% of that of 1,2(2,3)-diolein, suggesting that the enzyme cleaves the 1(3)-positioned ester bond 20 times as fast as the 2-positioned ester bond.

### TABLE 2. Effects of various detergents on KB-Lip activity

<table>
<thead>
<tr>
<th>Detergent</th>
<th>Relative activity (%)$^a$</th>
<th>Residual activity (%)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS</td>
<td>123</td>
<td>81</td>
</tr>
<tr>
<td>SDS</td>
<td>110</td>
<td>80</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>193</td>
<td>80</td>
</tr>
<tr>
<td>Tween 80</td>
<td>205</td>
<td>89</td>
</tr>
<tr>
<td>Tween 20</td>
<td>204</td>
<td>86</td>
</tr>
</tbody>
</table>

$^a$ In the presence of 0.005% detergent.  
$^b$ After 1 h of incubation with 1% detergent.

### TABLE 3. Comparison of KB-Lip activities on p-nitrophenyl esters

<table>
<thead>
<tr>
<th>Substrate</th>
<th>U/mg</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitrophenyl acetate (C$_{3}$)</td>
<td>32.6</td>
<td>6</td>
</tr>
<tr>
<td>p-Nitrophenyl propionate (C$_{4}$)</td>
<td>81.5</td>
<td>15</td>
</tr>
<tr>
<td>p-Nitrophenyl butyrate (C$_{5}$)</td>
<td>103</td>
<td>19</td>
</tr>
<tr>
<td>p-Nitrophenyl caprate (C$_{6}$)</td>
<td>337</td>
<td>62</td>
</tr>
<tr>
<td>p-Nitrophenyl caproate (C$_{10}$)</td>
<td>543</td>
<td>100</td>
</tr>
<tr>
<td>p-Nitrophenyl palmitate (C$_{16}$)</td>
<td>54.3</td>
<td>10</td>
</tr>
</tbody>
</table>

### DISCUSSION

*Pseudomonas* sp. strain KB700A was isolated from a water sample collected about 700 m below the water surface and was identified as a *Pseudomonas* sp. exhibiting maximum similarity at the 16S rRNA level with *Pseudomonas marginalis*. Strain KB700A was a psychrotroph, exhibiting sigmoi- dal growth even at −5°C (27). We found that the strain produced an extracellular lipase when grown in liquid medium at temperatures below 25°C, while no activity could be detected when cells were grown at 30°C either with or without tributyrin. On the other hand, clear zone formation was observed around the KB700A colonies when grown at 30°C on tributyrin agar plates, indicating that the gene could be expressed at this temperature. The absence of lipase activity in the culture supernatant at 30°C is likely to be due to the thermolability of the enzyme. In support of this, we have observed that KB-Lip was much more thermolabile than previously reported lipases.

The primary structure of KB-Lip indicated that it was a member of Subfamily I.3 of bacterial lipolytic enzymes. Sequence comparison suggested that secretion of KB-Lip in strain KB700A was dependent on its C-terminal region, as in the case of lipase from *P. fluorescens* SIK W1 (1) and *P. fluorescens* B52 (12). These proteins are transported via an ABC exporter system. In the 9-kbp DNA fragment which was isolated in the initial screening, we also found another open reading frame encoding a putative ABC transporter component (data not shown). The sequences with highest similarity in the database were the ATP transporter protein genes from *P. fluorescens* SIK W1 (1) and *P. fluorescens* no. 33 (23), both adjacent to the lipase genes. These facts suggest that KB-Lip is secreted from the cells via a mechanism similar to that of *P. fluorescens* SIK W1.

Activity of KB-Lip was dependent on divalent cations,

### TABLE 4. Comparison of KB-Lip activities on triacylglycerides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>U/mg</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacetin (C$_{2}$)</td>
<td>150</td>
<td>9</td>
</tr>
<tr>
<td>Tributyrin (C$_{4}$)</td>
<td>390</td>
<td>24</td>
</tr>
<tr>
<td>Tricaprin (C$_{8}$)</td>
<td>480</td>
<td>29</td>
</tr>
<tr>
<td>Tricaprylin (C$_{10}$)</td>
<td>330</td>
<td>20</td>
</tr>
<tr>
<td>Tricaprin (C$_{12}$)</td>
<td>2,410</td>
<td>145</td>
</tr>
<tr>
<td>Tributyrin (C$_{14}$)</td>
<td>1,660</td>
<td>100</td>
</tr>
<tr>
<td>Trilinolein (C$_{18}$)</td>
<td>1,830</td>
<td>110</td>
</tr>
</tbody>
</table>
targets of site-directed mutagenesis studies to elucidate rationally the mechanism of cold tolerance or thermostability. Another point to be stressed is that the activity levels of KB-Lip at low temperatures were higher than the activity of the enzyme from strain MIS38. At 30°C, with p-nitrophenyl caprate (p-NPC) as a substrate, we observed a specific activity of 543 U/mg. Under the same conditions and with its optimal substrate p-nitrophenyl caprate, MIS38 lipase displayed only 110 U/mg (3). Based on the results shown in Fig. 2 and Table 3, KB-Lip should display over 130 U/mg even at 20°C. The activity levels of KB-Lip are comparable to those of the lipase from cold-adapted Pseudomonas sp. strain B11-1 (a member of Family IV). With p-nitrophenyl butyrate (p-NPB) as a substrate, this lipase displayed activities of 164 U/mg at 25°C and approximately 121 U/mg at 20°C (9). The above observations suggest that KB-Lip has adapted, in terms of temperature dependency, to the growth range of its host. Strain KB700A displays growth between −5 and 35°C and optimal growth between 25 and 30°C, and no growth is observed at 40°C. Our results indicate that this adaptation is the result not only of an increase in thermostability at higher temperatures but also of an increase in activity at lower temperatures.

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