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Enantioselectivity of a New Cold-adapted lipase from *Photobacterium lipolyticum* That is Closely Related to Filamentous Fungal Lipases

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Psychrophilic lipases have attracted attention for their increasing use in the organic synthesis of chiral intermediates due to their low optimum temperature and high activity at very low temperatures, which are favorable properties for the production of relatively frail compounds. In addition, these enzymes have an advantage under low water conditions due to their inherent greater flexibility.

A new lipolytic *Photobacterium* strain M37 was recently isolated and identified, and named *P. lipolyticum* sp. nov. due to its lipolytic activity. The corresponding lipolytic enzyme is also expected to be a psychrophilic enzyme, as the *Photobacterium* strain was originally isolated from a marine habitat where the surrounding temperature was as low as 1~3℃, representing a new enzyme due to the absence of any other reports on a lipolytic enzyme from a *Photobacterium* species. After the genome sequencing of *Photobacterium profundum* SS9, about 10 genes were assigned as putative lipase/esterase genes based on their amino acid sequences, however, none of their actual activities have yet been proven experimentally.

Cloning of lipolytic protein gene from *Photobacterium lipolyticum* sp. nov.

Although *P. lipolyticum* sp. nov produced a large halo on a tricaprylin agar plate, the amount of the extracellular enzyme was not enough to purify and characterize its biochemical properties. Accordingly, shotgun cloning of the corresponding gene was performed. A DNA library of *P. lipolyticum* M37 was created with the *Sau*3A1 enzyme and used to transform *E. coli* XL1-Blue. The *E. coli* transformant then made a clear zone on a TCN-LB plate after 24 hours of incubation. The recombinant plasmid isolated from the cells was found to contain a 1.6 kb-sized insert DNA with one major open reading frame of 1,023 bp encoding a polypeptide chain of 340 amino acids corresponding to an M_r of 38,026.

The predicted amino acid sequence was compared with other protein sequences deposited in the SWISSPROT databank using the BLAST program. The most similar enzymes were those from various filamentous fungi, including *Thermomyces lanuginosa* (O59952, 12.6%), *Penicillium camemberti* (P61870, 5.7%), *Rhizomucor miehei* (P19515, 16.0%), and *Rhozopus niveus* (P61872, 11.9%). Interestingly, no sequence similarity was found with any of the bacterial lipases/esterases or putative lipases from the *Photobacterium profundum* SS9 genome. A primary sequence alignment of the M37 protein with the four above mentioned fungal lipases revealed that many regions were conserved over the entire sequences. A

comparison of the X-ray crystal structures previously elucidated for the four fungal liapses with the primary structure for the M37 protein showed three amino acid residues (Ser174, Asp236, and His312) constituting the active site and RG residues (Arg90 and Gly91) making an oxyanion hole sequence. The M37 protein was found to be more closely related with the abovementioned filamentous fungal lipases rather than other bacterial enzymes.

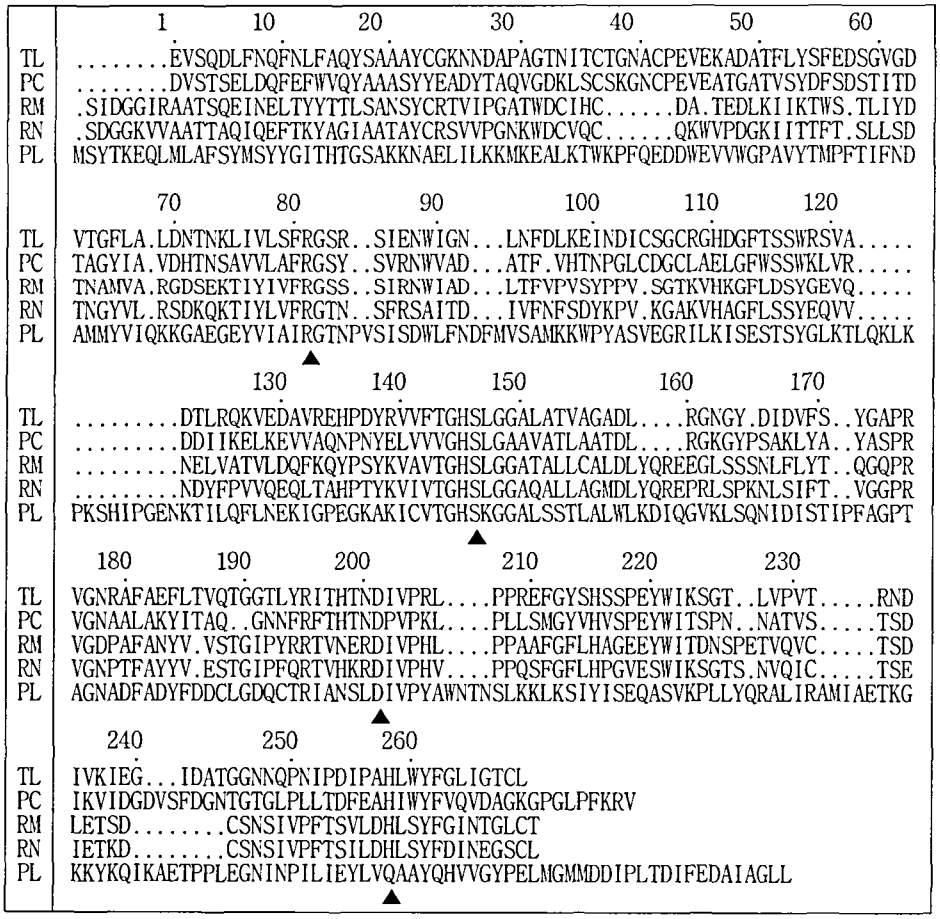


Fig. 1 Sequence alignment of lipase M37 with four filamentous fungi lipases.
Triangles represent residues involved in catalytic activity.

Expression of M37 lipase

The M37 protein gene was inserted into an expression vector, pET22b, and a recombinant plasmid constructed. *E. coli* BL21 (DE3) cells were then transformed with the plasmid and induced to express a recombinant protein using IPTG at two different growth temperatures. When cultivated and induced at 37℃, the resulting protein was exclusively insoluble, however, at a lower culture temperature of 18℃, the resulting protein was both soluble and insoluble. The soluble protein exhibited hydrolytic activity toward an olive oil emulsion, suggesting that the M37 protein was a typical lipase enzyme. Since the recombinant enzyme included a His-tag in its C-terminal part, it bound tightly to an Ni-NTA column and was then eluted using a 250 mM imidazole-buffer and observed homogeneously on an SDS-PAGE gel.

Biochemical characterization of M37 lipase

Since the M37 lipase was originally produced from a psychrophilic *Photobacteium* strain, it was anticipated to have a rather low optimum temperature and cold-adapted property. As expected, the M37 lipase had a relatively low optimum temperature (25℃) for the hydrolysis of olive oil, and the activation energy was as low as 2.07 kcal/mol within a temperature range of 5~25℃, indicating that the M37 lipase was a typical cold-adapted enzyme. For comparison, the same properties were measured for the *Rhizomucor miehei* lipase, which exhibited an optimum temperature of 50℃ and activation energy of 12.1 kcal/mol within a temperature range of 5~50℃, revealing that even though the M37 lipase and *R. miehei* lipase shared a sequence similarity, only the former was cold-adapted.

The thermal stability was measured using two different methods. The M37 lipase remained fairly stable up to 20℃, then lost almost all its activity above 35℃. A structure-unfolding kinetic experiment also showed a similar result, as the M37 lipase started to unfold above 20℃ with a T_m value of 30℃. Therefore, these two experiments showed that the M37 lipase was a typical psychrophilic enzyme.

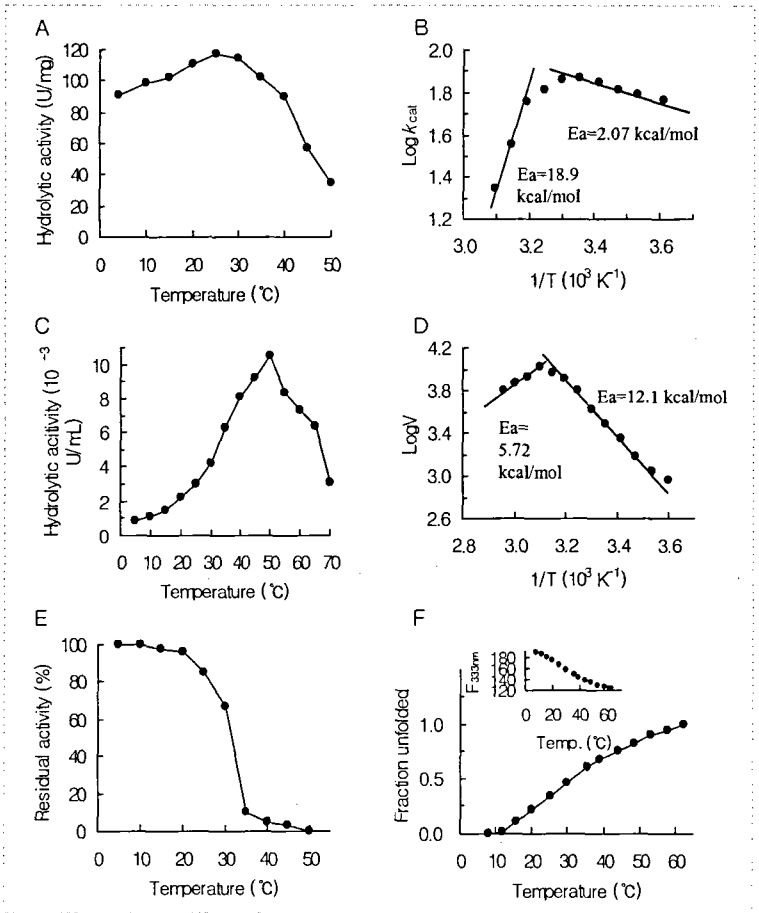
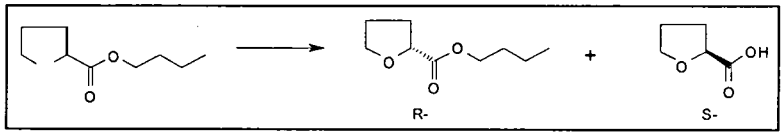


Fig. 2 Effect of temperature on hydrolytic activityof lipase M37.
Hydrolytic activity of lipase M37 (A) and R. mieheilipase (C) measured at different temperatures using olive oil-gum arabic emulsion. Activation energy and inactivation energy calculated for lipase M37 (B) and *R. miehei* lipase (D). Residual activity of lipase M37 measured at 25℃ after heat-treatment at different temperatures for 30 min (E). Unfolding kinetics of lipase M37 measured while increasing temperature (F).

The M37 lipase also had a narrow chain-length specificity, showing a relatively high hydrolytic activity toward tributyrin (C_4) and triolein ($C_{18:1}$), yet very low activity toward most of the other triglycerides tested. As such, this narrow specificity implies that the M37 lipase could be used as a biocatalyst in chemical synthesis reactions, rather than as an enzyme additive in washing detergents.

Enantioselective hydrolysis of THFA ester

Varous chiral compounds were used as intermediate in pharmaceutical industry. Tetrahydrofuroic-2-acid (THFA) is on of important chiral compounds used for the production of Terazosin, which is an alpha-adrenergic blockers used to treat a hypertensive patients. The M37 lipase could hydrolyze THFA-butyl compound and in the reaction process, it showed some degree of chiral selectivity. After 2-hr reaction, conversion rate was above 50% and reached up to 75% within 7-hr. The e.e.s value increased continuously and reached up to 32% after 7-hr. As such the M37 lipase hydrolyzed rapidly (S)-THFA butyl ester, while (R)-form ester more slowly. This meant that in the reaction mixture the ratio of remaining (R)-form ester to (S)-form ester increased gradually with time course and that the enriched (R)-form THFA ester could by extracted with organic solvent and used for the production of Terazosin.



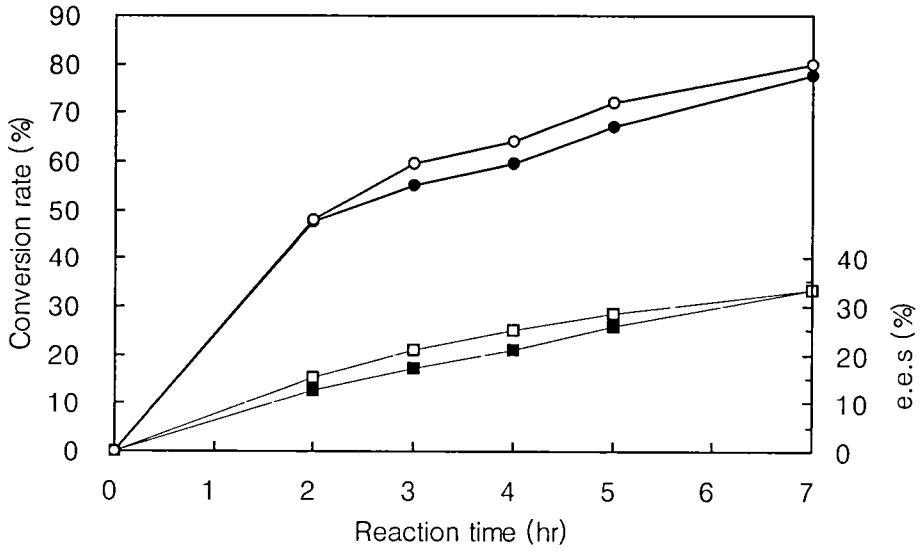


Fig. 3 Time course changes of conversion rate and enantiomeric excess of THFA-butyl ester.
(A) The chiral selective resolution of THFA-butyl was shown. (B) The open circle and closed circle mean conversion rate at 30℃ and 20℃, respectively. The open square and closed one mean enantiomeric excess value at 30℃ and 20℃, respectively. The e.e.s was calculated from the ratio (%) of [(R-form) - (S-form)]/[(R-form) + (S-form)].

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