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Nonionic detergent-induced activation of an esterase from *Bacillus megaterium* 20-1

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Abstract

An esterase-producing *Bacillus megaterium* strain (20-1) was isolated from a soil sample collected in South Korea. The cloned gene showed that the esterase 20-1 composed of 310 amino acids corresponding to a molecular mass (M_r) of 34,638. Based on the M_r and the protein sequence, the esterase 20-1 belonged to the H lipase/esterase group. The optimum temperature and pH of the purified His-tagged enzyme were 20–35 °C and 8.0, respectively. The esterase 20-1 showed a 'nonionic detergent-induced activation' phenomenon, which was a detergent type- and concentration-dependent process. In comparison with the native enzyme, the Tween 80-treated enzyme had relatively a similar k_{cat} value of 274 s^{-1} but a very low K_m value of 0.037 mM for PNPC (C_6), therefore, it showed a 14-fold increase in k_{cat}/K_m value.

Keywords: Esterase; *Bacillus megaterium*; *Alicyclobacillus acidocaldarius*; Detergent-induced activation

1. Introduction

Lipases and esterases are hydrolases (E.C. 3.1.1.1) acting on the carboxyl ester bonds of acylglycerols and/or some water-soluble esters. They are ubiquitous enzymes that have been purified and studied from different sources, including animals, plants, and microorganisms. These proteins are divided into three large groups based on their sequence identity [1]: the C group, which includes cholinesterases and fungal lipases, the L group, which includes lipoprotein lipases and bacterial lipases, and the H group, named after the hormone-sensitive lipases (HSL) discovered by Vaughan et al. [2], which comprises of proteins showing sequence similarity with HSL. Among bacterial enzymes, *Alicyclobacillus acidocaldarius* esterase (EST2) [3], *Archaeoglobus fulgidus* esterase (AFEST) [4], and brefeldin A esterase [5] from *Bacillus subtilis* were recently turned out to belong to the H group.

Although both lipase and esterase hydrolyze ester bonds, they show some fundamental difference in kinetics, based upon the properties of the substrates they hydrolyze [6]. Esterase (E.C. 3.1.1.1) catalyzes the

Abbreviations: AFEST, *Archaeoglobus fulgidus* esterase; EST2, *Alicyclobacillus acidocaldarius* esterase; HSL, hormone-sensitive lipase; PNPC, *p*-nitrophenyl caproate; T80, Tween 80; TBN, tributyrin

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cleavage of ester bonds of short chain length (less than C_8). The hydrolytic mechanism of most known esterases resembles that of Michaelis–Menten saturation kinetics. They contain a catalytic triad that consists of Ser, His, and Asp with the Ser located in the consensus sequence G–X–S–X–G at the active site [7].

Both lipase and esterase have a number of unique characteristics: substrate specificity, regio-specificity, and chiral selectivity [8,9]. Therefore, they are important for the biotechnological applications in medicine, synthetic chemistry (e.g. synthesis of chiral compounds) and food processing [10,11].

An esterase-producing *Bacillus megaterium* (20-1) was isolated from soil sample and the corresponding esterase gene was cloned, which was turned out to be somewhat similar with EST2 enzyme [3]. The biochemical properties were characterized in detail and compared with EST2 in this paper.

2. Materials and methods

2.1. Isolation of esterase-producing bacteria

Soil samples collected near Daejon, South Korea were suspended in sterile water and spread onto TBN-LB plate, esterase-screening plate prepared as follows: a tributyrin (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 mixed with 450 ml of a LB agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar). A strain showing the most excellent ability to form halo on the screening plate was selected after 24 h incubation at 37 °C and further isolated.

2.2. Cloning and expression of the esterase 20-1 gene

Genomic DNA from the isolated strain (*B. megaterium 20-1*) was prepared, completely digested with *Hin*dIII, ligated into pUC19 vector, and used to transform *E. coli* XL1-Blue. A colony forming a clear halo on TBN-LB plate containing ampicillin (50 μg/ml) was selected. Recombinant plasmid (pL20-1) was purified from the transformant and the insert DNA sequence was analyzed with the Big Dye DNA Sequencing Kit and ABI PRISM sequence analyzer (3.3) (Perkin-Elmer, USA).

To express the esterase 20-1, we used an *E. coli* expression vector, pET22b. First, two oligomeric primers (primers 1 and 2) and *B. megaterium* genomic DNA (in pL20-1) were used to generate PCR product carrying a *Nco*I restriction site at its 5′ end (immediately upstream of the initiation codon ATG of the esterase 20-1 gene) and a *Bam*HI site at its 3′ end. The nucleotide sequences of the two primers are as follows:

- primer 1: 5′-T TCC ATG GCT ATG CCG TTA GAT CCG CAT-3′;
- primer 2: 5′-T TGG ATC CGG AAT AGA ATC AAA TAC TTG-3′.

After digestion with *Nco*I and *Bam*HI, the PCR product was ligated with pET22b vector. *E. coli* XL1-Blue was transformed with the ligation mixture and the resulting recombinant plasmid, pMLE, was obtained from a transformed *E. coli* cell. *E. coli* BL21(DE3) was transformed by electroporation with the pMLE plasmid.

2.3. Purification of esterase 20-1

Transformed *E. coli* was cultivated in LB medium at 30 °C. After IPTG (1 mM) induction and ultrasonic cell lysis, total soluble proteins were recovered from the cell extract. They were attached to the Ni-NTA column and, after washing with 20 mM Imidazole, 300 mM NaCl, and 50 mM NaH_2PO_4 buffer (pH 7.0), the bound esterase 20-1 was eluted with 200 mM Imidazole, 300 mM NaCl, and 50 mM NaH_2PO_4 buffer (pH 7.0). It was dialyzed against 50 mM Tris–HCl buffer (pH 8.0) and used to characterize its biochemical properties.

2.4. Esterase assay

Activity was measured with *p*-nitrophenyl caproate (PNPC) or other PNPEs [12]. The reaction mixture consisted of 0.01 ml of 10 mM substrate in acetonitrile, 0.04 ml of ethanol, and 0.95 ml of 50 mM Tris–HCl buffer (pH 8.0) containing an appropriate amount (10 μl) of the enzyme. In the assay of revealing the biochemical properties of the purified enzyme, 0.05 mg/ml of BSA was added to enzyme solution as a stabilizer. The enzyme reaction was performed

for 3 min at 35 °C, unless otherwise specified. The amount of *p*-nitrophenol liberated during the reaction was measured by its absorbance at 405 nm. One esterase unit is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol/min.

To detect the active esterase band on the polyacrylamide gel, zymographic analysis was done as follows. Sodium dodecyl sulfate (SDS)-PAGE (12%) was performed using slab gels and renaturation of the enzyme was done by soaking the gel in 50 mM Tris–HCl buffer (pH 8.0) for 1 h. Activity assay of the renatured enzymes was achieved by attaching the gel to TBN agarose plate and incubating at 37 °C.

2.5. Temperature and pH effects on esterase 20-1

The optimum temperature of the esterase 20-1 was measured by assaying their hydrolytic activities of PNPC at various temperatures (10–50 °C). To examine the thermostability of the esterase 20-1, the enzyme was incubated at various temperatures (10–60 °C) for 30 min in the presence of 0.05 mg/ml of BSA and

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                                                        AAGCTTATATAAACAATAATCATCGGTGTGCCTCCTTTGTACG   -181
TATTTATATTTATATAGGTGAGAATAAAATATATACGAATATCATCCTCTTTATTTAATTGTTATACCCATTTTTAATCAAAACAAACAA    -91
AAGCCTGCCGAGAAGACAATATATTGGATTTTTTCTGAAATTTCATTCATCATTACATATAGAGTTACCTTGAAAGGAGAGAGAAGAAGA     -1
ATGCCGTTAGATCCGCATATTCAAATATTTCTAAATCAATATAATGAAATGCCCCGTCCTTCTTTAGAGGACGTTACACCCCCACAGCTG     90
 M  P  L  D  P  H  I  Q  I  F  L  N  Q  Y  N  E  M  P  R  P  S  L  E  D  V  T  P  P  Q  L    30

AGGGAAATGGAAAAGATGTCCTTAACTCCTTCTAAAGAAGCAGTTAAAAAAGTATATAATGAAGAAATTGAATTAAATGAACGTACGCTC    180
 R  E  M  E  K  M  S  L  T  P  S  K  E  A  V  K  K  V  Y  N  E  E  I  E  L  N  E  R  T  L    60

ACTCTACGAGTGTATGAACCTGAAGGAACAGGGCCATTTCCCGCTCTTGTTTATTATCACGGAGGAGGATGGGTATTAGGCAGCCTGGAT    270
 T  L  R  V  Y  E  P  E  G  T  G  P  F  P  A  L  V  Y  Y  H  G  G  G  W  V  L  G  S  L  D    90

ACTCATGACTCCATATGCAGGTCGTATGCAAATGAAACAAACTGTATTGTAGTGTCTGTTGATTACCGTCTTGCTCCTGAGAGTAAATTT    360
 T  H  D  S  I  C  R  S  Y  A  N  E  T  N  C  I  V  V  S  V  D  Y  R  L  A  P  E  S  K  F   120

CCCGCTGCAGTAAACGATGCCTATGACGCCTTGGATTGGATTTCAGCTCATGCGTCTCAATTAAATATCGATTCAAACAAAATTGCCGTC    450
 P  A  A  V  N  D  A  Y  D  A  L  D  W  I  S  A  H  A  S  Q  L  N  I  D  S  N  K  I  A  V   150

GGTGGCGACAGTGCCGGTGGTAACCTTGCTGCGGTTGTAAGCATTTTAGCAAAACAAAGACAAGGTCCATCCATTGTTCATCAGCTGCTT    540
 G  G  D  S  A  G  G  N  L  A  A  V  V  S  I  L  A  K  Q  R  Q  G  P  S  I  V  H  Q  L  L   180
          †
ATTTATCCGTCTGTAGGATTTAAAAATCAACACCCTGCCTCTATGAAAGAAAACGCCGAAGGATATCTTCTTTCAAAAGATCTCATGGAT    630
 I  Y  P  S  V  G  F  K  N  Q  H  P  A  S  M  K  E  N  A  E  G  Y  L  L  S  K  D  L  M  D   210

TGGTTTCGCCTTCAGTACTTAAATAATAAAGAAGAAGAACAGCACCCCTATAACGCTCCAGTATTACTAGAAGATTTATCGAGTCTACCG    720
 W  F  R  L  Q  Y  L  N  N  K  E  E  E  Q  H  P  Y  N  A  P  V  L  L  E  D  L  S  S  L  P   240

AGCGCTACCATTATTACAGCACAATATGATCCTTTAAGAGATAGCGGAAAAGACTACGCGGACGCATTAAAAAATCACGGTGTCCCCGTC    810
 S  A  T  I  I  T  A  Q  Y  D  P  L  R  D  S  G  K  D  Y  A  D  A  L  K  N  H  G  V  P  V   270
                            †
ACCTATGAAAATTATGAAACAATGATTCACGGGTTTTTAGGGTTTCATGAATTCGTCCCACTCGCTCAGCAGGCGATCAATAAAAGCGCA    900
 T  Y  E  N  Y  E  T  M  I  H  G  F  L  G  F  H  E  F  V  P  L  A  Q  Q  A  I  N  K  S  A   300
                            †
GCTCAACTGCGTCAAGTATTTGATTCTATTTAAGGCGCAAAGATATATAGTGTCATAAAATATTTAATTAAAAAAATACTAAAAATATAG    990
 A  Q  L  R  Q  V  F  D  S  I  ***                                                           310

TAAAAGTTAAAAAGTTCTTTCCTTAATTGGATTGGGCTTTTTTACTTTTCAATTTTCTCGTATAATTCATCCATTTTAACATTCAAAACG   1080
TCAGCTAACTTAAGTACTTTATCCATGCTAGAACAAACTGAAAAAGAAGAAAAGAAATTCTTGAGGTTATGGAAAGTACGATAATGGATT   1170
ATAGAGAAGAAGATATGGGCAATATTAGCGATTTTAACGGAAATTTATTGGAAAATAAAAAAGAAATATAATTGAAAAAATAACAAAAAT   1260
AACCTATTTCATGGTATTATTTACTCGTTGAAGGGCTGAGTACTGTGACAGAGCAATTGTAGGGGTTAAGCTT                   1333
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the esterase 20-1 gene isolated from *B. megaterium* 20-1. The numbering of nucleotides starts at the 5′ end of the esterase gene and the putative active site residues (†) are indicated. *Hin*dIII sites are underlined. The sequence has been submitted to GenBank under accession number AF514856.

then the residual activity was measured at 35 °C and pH 8.0.

The optimum pH of the esterase 20-1 was measured by assaying at various pHs (pH 4–10). And to find out the pH stability of the enzyme, it was incubated at various pHs (pH 4–10) for 30 min in the presence of 0.05 mg/ml BSA and the residual esterase activities were measured at 35 °C and pH 8.0. GTA buffer (100 mM 3,3-dimethylglutaric acid, 100 mM Tris, 100 mM 2-amino-2-methyl-1,3-propanediol), an universal buffer, was used in this experiments.

2.6. Detergent effects on the esterase 20-1

The purified esterase 20-1 was mixed with various detergents (1%, v/v or w/v) containing 0.05 mg/ml BSA and incubated for 30 min. With 10 μl volumes of pretreated mixtures, the esterase activity was measured at 35 °C and pH 8.0 as described above.

3. Results and discussion

A bacterial strain (20-1) isolated from soil sample collected near Daejon, South Korea, showed a strong lipolytic activity on TBN-LB plates. The strain was Gram-positive, rod-shaped, endospore-forming, aerobic, and catalase-positive. API 50CHB test showed that it is very similar with *B. megaterium* (data not shown). In addition, the determined 16S rRNA sequence showed 99.4% identity with that of *B. megaterium*. So, the isolated strain 20-1 was designated as *B. megaterium* 20-1.

At present, *B. megaterium* has been known to produce three different kinds of lipolytic enzymes; two extracellular enzymes (65 and 19 kDa sized) and one cell-bound enzyme (58 kDa sized) [13]. The gene of 19 kDa-sized enzyme has been recently cloned and classified into subfamily I.4 of bacterial lipases [11,13]. And the *p*I and molecular weight of the 58 kDa-sized enzyme was known to be similar to PnbA from *B. subtilis* [14], EstAI from *Bacillus* sp. BP-7 [13], or EstA from *Paenibacillus* sp. BP-23 [15].

The molecular weight of the corresponding enzyme was determined to be about 38 kDa by zymographic analysis. Since its molecular size is quite different from the previously known *B. megaterium* esterases, it seems to be a novel enzyme. So, gene cloning and gene expression were done to characterize the enzyme.

The chromosomal DNA library of *B. megaterium* 20-1 was used to transform *E. coli* XL1-Blue. An *E. coli* transformant formed a clear halo on TBN-LB plate after 24 h-incubation. The recombinant plasmid (pL20-1) isolated from this transformant had a 1.6 kb-sized insert DNA. The nucleotide sequence showed one major open reading frame of 933 bp, which encoded a polypeptide of 310 amino acid sequences (Fig. 1) and corresponds to a molecular mass

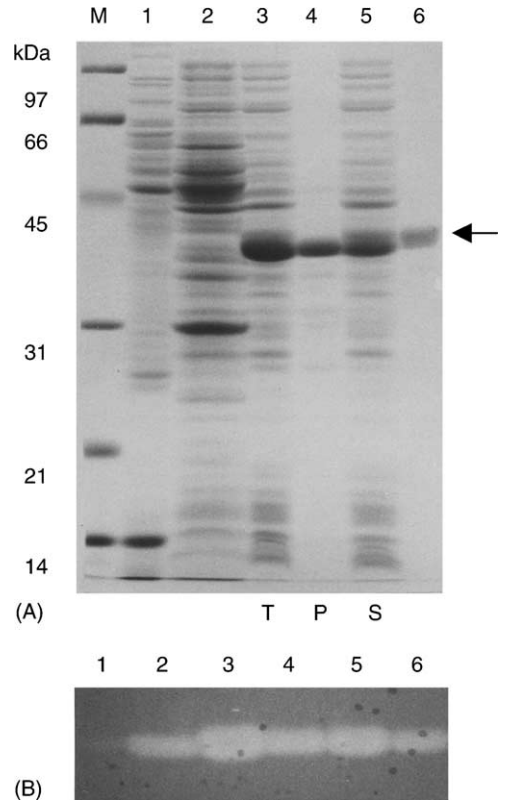

Fig. 2. SDS-PAGE of the expressed esterase 20-1. (A) Lane M is standard protein markers. Lane 1 is cell extract of *B. megaterium* 20-1. Lane 2 is cell extract of *E. coli* XL1-Blue/pL20-1. Lane 3–5 are the cell fractions of *E. coli* BL21(DE3)/pMLE cultivated at 37 °C for 4 h after IPTG was added (T: total cell extract; P and S: insoluble and soluble proteins after ultrasonic cell lysis, respectively). Lane 6 is the purified esterase 20-1 by Ni-NTA column chromatography. (B) After SDS-PAGE was performed, zymographic analysis was done with TBN-agarose gel. Lane 1–6 correspond to those in A panel, respectively. Arrow indicates the esterase 20-1.

of 34,638. The predicted amino acid sequence was compared with protein sequences in the PDB data bank using BLAST program. The most similar enzymes were those from *Alicyclobacillus acidocaldarius*, *Archaeoglobus fulgidus*, and *Bacillus halodurans*, which had 55.8, 37.6, and 33.8% identities, respectively. The X-ray crystal structure of *A. acidocaldarius* esterase was recently revealed [16]. By comparison with that structure, three amino acid residues (Ser154, Asp250, and His280) were identified as active sites in the esterase 20-1.

In *B. megaterium* 20-1 strain, the presence of cell-bound esterase was observed on TBN-LB plate, but the enzyme could not be purified from the cell extract because of its low level of production. Therefore, we tried to express the enzyme in *E. coli* BL21(DE3) strain with an expression vector, pET22b. Esterase 20-1 gene was inserted into the vector and a recombinant vector, pMLE, was constructed. The recombinant protein was designed to contain N-terminal 24-extra amino acids and C-terminal 22-extra amino acid containing six His residues.

Esterase activity in *E. coli* cell extract was measured to be 3600 U/l and the His-tagged enzyme was purified easily by Ni-NTA column chromatography (Fig. 2). The final specific activity was estimated to be 124 U/mg when PNPC (C_6) was used as a substrate.

The optimum temperature of esterase 20-1 for the hydrolysis of PNPC was measured to be 25–35 °C. The enzyme was relatively stable up to 45 °C, then a drastic decrease in stability occurred above 45 °C (Fig. 3).

The optimum pH of the esterase was measured to be 8.0 and it was stable at a pH range of 6.0–9.0 (Fig. 3).

Therefore, although it has exactly the same optimum pH with those of EST2 and AFEST enzymes, it is unexpectedly quite different from the two enzymes in that the two enzymes are highly thermostable enzymes having the optimum temperature of 70 °C [17].

The effects of various detergents on the esterase 20-1 were shown in Fig. 4A. When the esterase 20-1 was mixed and incubated with 1% (w/v) sodium dodecyl sulfate, an anionic detergent, it was completely inactivated. The same concentration of sodium deoxycholate (DOC) and sodium taurocholate (TC) showed no noticeable effects on the enzyme activity. But Triton X-100, a neutral detergent, activated the

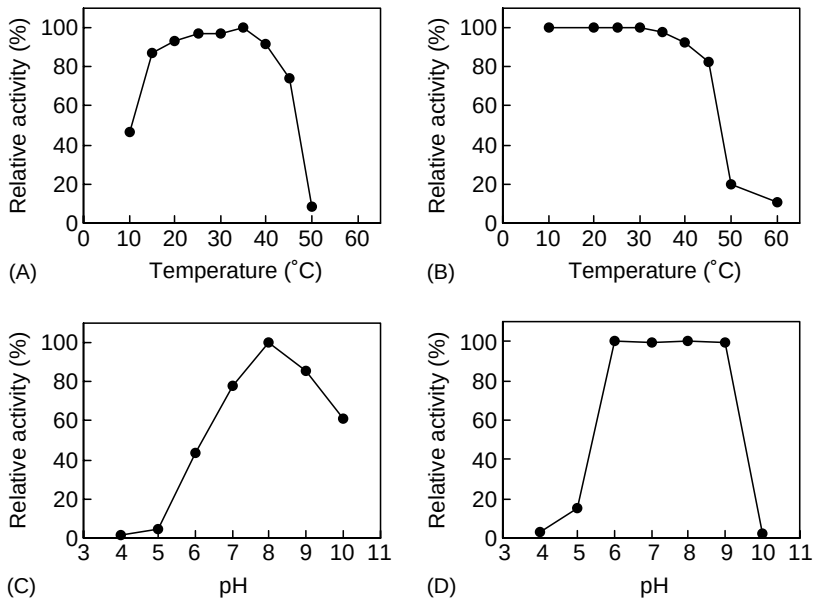

Fig. 3. Effects of temperature and pH on the esterase 20-1. (A) The esterase activity was measured at various temperatures (10–50 °C). (B) Enzyme was pre-incubated at various temperatures for 30 min at pH 8.0 and the residual activity was measured at 35 °C, pH 8.0. (C) The esterase activity was measured at various pHs (4.0–10.0) at 35 °C. (D) Enzyme was pre-incubated at various pH buffers for 1 h at 30 °C and the residual activity was measured at 35 °C, pH 8.0.

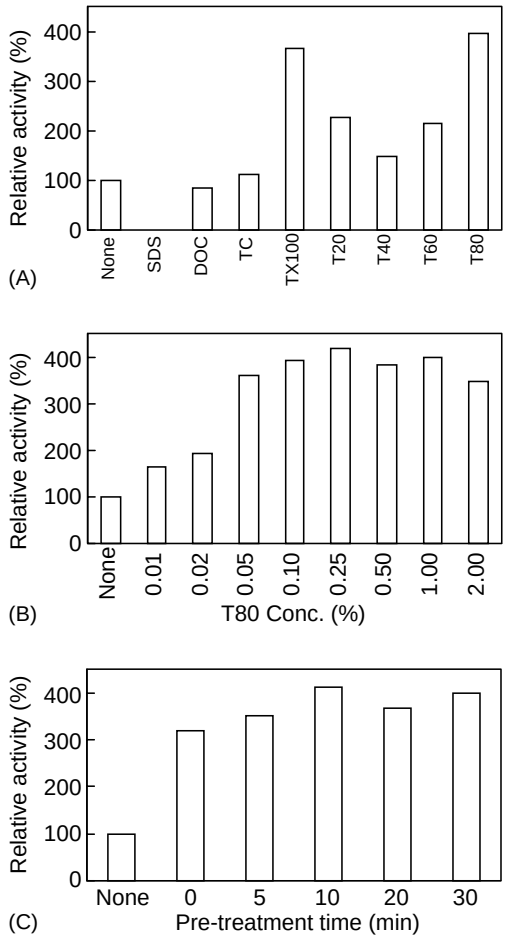

Fig. 4. Effects of Tween 80 on the esterase 20-1. (A) The esterase was mixed with various detergents (1%, w/v or v/v) and incubated at 25 °C for 30 min. After then, the esterase activities of each mixture were measured. (B) The esterase was mixed with various concentration of T80 and after 30 min incubation, the esterase activities were measured. (C) Time-dependent activation of the esterase by T80 (0.1%) was measured. Pre-treatment time means the time at which enzyme are mixed with T80 before reaction starts.

enzyme activity as much as fourfold. In addition, all Tween detergents strongly activated the esterase 20-1 up to two- to fourfold. Tween detergents have polyoxyethylene (POE) sorbitan as a common moiety and one varying acyl chain; Tween 20, 40, 60, and 80 have lauryl, myristyl, palmityl, and oleyl groups as their acyl chain, respectively. In particular, as T80 activated the enzyme most strongly, we further studied the T80 effects on the enzyme in detail.

To determine whether or not the enhancement of the esterase activity was due to the direct interaction of T80 molecule with the esterase 20-1 enzyme, The T80 concentration dependency to the enzyme activation was, at first, tested (Fig. 4B). The enzyme was activated with as low concentration as 0.01% T80. At higher concentration over 0.05%, T80 fully activated the esterase 20-1 (fourfold).

Secondly, time dependency of the enzyme activation was checked. After 0.1% T80 was pre-mixed and incubated with the esterase molecule for various times (0–30 min), enzyme activities were measured. The 0 min means T80 was mixed just before the reaction started (in fact, it took about 5 s to mix and measure the OD). Fig. 4C shows that T80-induced enzyme activation occurred within a few seconds.

When T80 (0.001%, final concentration) was directly added to the reaction mixture, no increase in enzyme activity was observed (data not shown). This result showed that T80 interacted directly with the esterase enzyme and excluded the possibility that the enhancement of the esterase activity was due to the T80-induced changes in the physicochemical state of PNPC substrate.

Table 1 displays the kinetic constants of the esterase 20-1 for the hydrolysis of PNP esters with acyl chain length of 2–6 at 35 °C. Reaction velocity increased with increasing chain length, reaching a maximum with PNP-caproate. Affinity constants showed a decrease as chain length increased. For the best substrate, a k_{cat} of 352 s^{-1} and K_m of 0.67 mM were estimated. T80-treated enzyme showed similar kinetic constants with native enzyme for C_2 and C_3 substrates, but it had quite different kinetic constants for C_4 and C_6 substrates. In comparison with native enzyme, it had a similar k_{cat} value of 274 s^{-1} but had a remarkably decreased K_m value of 0.037 mM for $PNPC_6$. Therefore, its k_{cat}/K_m value for PNP-caproate is 14-fold higher than that of native enzyme. These results demonstrated that T80 treatment changed the enzyme to have a high affinity to the $PNPC_6$ molecule.

As it is cell-bound enzyme, its interaction with some lipid molecule including T80 has been expected even though the detailed binding mode was not understood at all. Therefore, further biochemical and biophysical analysis are needed to understand the nature of the interaction, for it seems that the nonionic

Table 1
Kinetic parameters for the native and the modified esterases

Acyl chain length	Acetate (C_2)			Propionate (C_3)			Butyrate (C_4)			Caproate (C_6)		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)
20-1	1.43	108	75.5	1.57	322	205	0.14	46.7	334	0.67	352	525
T80-treated 20-1	1.15	89.7	78.0	1.39	529	381	0.45	255	567	0.037	274	7410

detergent-treated esterase can be exploited efficiently in the fatty acid production.

Acknowledgements

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