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Expression and Characterization of a New Esterase Cloned Directly from *Agrobacterium tumefaciens* Genome

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Abstract A new functional lipolytic enzyme (AT4) has recently been found from *Agrobacterium tumefaciens* C58 Cereon using a genome-wide approach. The enzyme has some sequence similarity to *E. coli* acetyl hydrolase, *Emericella nidulans* lipase, *Moraxella* sp. lipase, *Acinetobacter lwoffii* esterase, and *Streptomyces hygroscopicus* acetyl hydrolase. However, the sequence similarities are very low (less than 25%), suggesting that it is a new lipase/esterase enzyme. In the present study, intact cell of the *A. tumefaciens* strain was shown to have lipolytic activity on a tributyrin-LB plate. The AT4 gene was then expressed at a high level in *E. coli* BL21 (DE3) cells and the enzyme was purified simply by Ni-NTA column chromatography. The purified enzyme showed hydrolytic activity toward *p*-nitrophenyl caproate, but not toward olive oil, suggesting that the AT4 enzyme was a typical esterase rather than lipase. AT4 esterase had a maximum hydrolytic activity at 45°C and pH 8.0, when *p*-nitrophenyl caproate was used as a substrate. It was relatively stable up to 40°C and at pH 5.0–9.0. Calcium ion and EDTA did not affect the activity and thermal stability of the enzyme. As for substrate specificity, AT4 enzyme could rapidly hydrolyze acetyl and butyl groups from *p*-nitrophenyl esters and 1-naphthyl esters. In addition, it also released acetyl residues from acetylated glucose and xylose substrates. Therefore, this new esterase enzyme might be used as a biocatalyst in acetylation and deacetylation reactions performed in the fine chemical industry.

Key words: *Agrobacterium tumefaciens*, esterase, substrate specificity

Lipase/esterases (EC 3.1.1.-) catalyzing the hydrolysis of ester bonds are diverse enzymes that were isolated and characterized from various animals, plants, and microorganisms [4, 10, 11, 19, 20]. Generally, each enzyme has its own unique substrate specificity and reaction property. They are used in a number of valuable biotransformation reactions such as resolution of racemic mixtures, synthetic reactions, blocking and unblocking of catalytic groups in peptide chemistry, and modification of sugars [5, 12, 17]. Since the scope of organic synthesis performed by these enzymes is enormous, the supply of suitable enzymes is very important. Recently, a genome-wide screening method has been developed for retrieving novel enzymes from a microbial genome database [8, 18]. According to the method, many putative enzymes could directly be tested for their lipase/esterase signature sequence and enzyme activity. Two putative lipase/esterase genes from *Agrobacterium tumefaciens* C58 Cereon have been uncovered by such an approach to have lipolytic activity [8]. However, there has yet been no report on any lipolytic enzyme isolated from this bacterial species. In the present study, we attempted to characterize its biochemical properties including substrate specificity.

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Purification of Esterase AT4

E. coli BL21 (DE3) cells were transformed with a recombinant plasmid containing the AT4 gene. The *E. coli* cells were cultured at 30°C in LB medium containing ampicillin (100 μg/ml) until the optical density at 600 nm reached to 0.5. After 1 mM isopropylthiogalactoside was added, cells were cultured for 5 h at 30°C, harvested, and ruptured by ultrasonication. After centrifugation (10,000 ×*g*, 20 min), proteins in the soluble and insoluble fractions were analyzed by SDS-PAGE (12% acrylamide gel). The following enzyme purification procedure was employed: the soluble fraction was loaded onto a Ni-NTA column and, after washing the column with 100 mM imidazole, 300 mM NaCl, and 50 mM NaH_2PO_4 buffer (pH 7.0), the bound AT4 enzyme was eluted with the same buffer containing 300 mM imidazole. The eluted fractions were analyzed by SDS-PAGE (12% acrylamide gel). The purified enzyme was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) and used to characterize its biochemical properties.

Esterase Assay

Esterase activity was measured colorimetrically with *p*-nitrophenyl caproate ($PNPC_6$) [7]. The reaction mixture contained 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 (10 µl) of the enzyme. The enzyme reaction was performed for 3–5 min at 35°C, unless otherwise specified. The amount of *p*-nitrophenol released during the reaction was measured by its absorbance at 405 nm. One esterase unit was defined as the amount of enzyme to release 1 µmol of *p*-nitrophenol per min. Esterase activity of the intact *A. tumefaciens* cell was detected on a TBN-LB plate prepared as follows: A tributyrin (TBN) emulsion was made by emulsifying 5 ml of TBN in 45 ml of 200 mM NaCl, 10 mM $CaCl_2$, and 5% (w/v) gum arabic solution. The TBN emulsion (50 ml) was mixed with 450 ml of LB agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) and used to make the TBN-LB plate [6].

The optimum temperature of esterase AT4 was measured by assaying its hydrolytic activities on $PNPC_6$ at various temperatures (20–55°C). To examine the thermostability of esterase AT4, the enzyme was pre-incubated at various temperatures (20–55°C) for 30 min in the presence of 0.05 mg of bovine serum albumin (BSA)/ml, and the residual activity was then measured at 35°C and pH 8.0. The optimum pH of the AT4 esterase was measured by assaying at various pHs (pH 4.0–9.5). To determine the pH stability of the enzyme, it was pre-incubated at various pHs (pH 4.0–9.5) for 30 min in the presence of 0.05 mg BSA/ml and the residual esterase activities were measured at 35°C and pH 8.0. Various buffers (100 mM sodium acetate for pH 4.0–6.0, 100 mM potassium phosphate for 6.0–7.5, 100 mM Tris-HCl for 7.5–9.5) were used.

Hydrolytic reactions were carried out with increasing concentrations of *p*-nitrophenylacetate (pNPA) (0–1.0 mM) and *p*-nitrophenylbutyrate (pNPB) (0–0.7 mM). The reaction mixtures consisted of 0.01 ml each of substrate (100× conc.) in acetonitrile, 0.04 ml of ethanol, and 0.95 ml of 100 mM potassium phosphate buffer (pH 7.0) containing an appropriate amount (~10 µl) of the enzyme. The reaction was performed for 3–5 min at 25°C, and the increase of absorbance at 405 nm was measured spectrophotometrically [7]. Enzyme reactions were performed with increasing concentrations of 1-naphthyl acetate (0–1.0 mM) and 1-naphthyl butyrate (0–1.0 mM). The reaction mixture consisted of 0.06 ml each of substrate (10× conc.) in ethanol, 0.54 ml of 50 mM Tris-HCl buffer (pH 8.0) containing an appropriate amount (~5 µl) of the enzyme. The reaction was performed for 10 min at 35°C, then 0.3 ml solution containing 0.01% Fast Red TR salt, 10% Tween 20, and 1 M sodium acetate buffer (pH 4.3) was added. Absorbance at 430 nm was then measured spectrophotometrically [14]. Enzyme reactions were also carried out with increasing concentrations of 7-amino cephalosporanic acid (0–2.5 mM), glucose pentaacetate (0–2.5 mM), and xylose tetraacetate (0–2.5 mM). The reaction mixture consisted of 0.5 ml each of substrate (2× conc.) in methanol and 0.5 ml of the enzyme [2]. The enzyme reaction was performed for 5 min at 35°C and stopped by heating at 70°C for 10 min. The amount of acetic acid in the reaction mixtures was analyzed by following the protocol described in the acetate analysis kit (catalog No. 10148261035) (R-Biopharm AG, Germany).

Sequence Analysis and Expression of AT4 Enzyme

Two putative lipolytic enzymes (AT1 and AT4) were recently found in the *Agrobacterium tumefaciens* genome sequence [8]. This observation was of great interest, because there had been no previous report of any esterase enzyme from this bacterial species. In the present study, the AT4 enzyme was characterized, since it had a higher lipolytic activity than the AT1 enzyme. The protein sequence of the AT4 enzyme was compared with other protein sequences in the Swissprot databank using the Blast Search program. The enzymes with the most homology included *E. coli* acetyl hydrolase (P23872, 24.8%) [13], *Emericella nidulans* lipase (Q00675, 24.5%) [1], *Moraxella* sp. lipase (P24484, 23.2%) [3], *Acinetobacter lwoffii* esterase (P18773, 19.5%) [16], and *Streptomyces hygroscopicus* acetyl hydrolase (Q01109, 18.4%) [15]. All these enzymes belong to the esterase/lipase group and the two enzymes were named as acetyl hydrolases based on their substrate specificity. Although the sequence similarities were very low (less than 25%), they had many conserved regions over the entire length of the proteins, including N-terminal HG region and the 'GDSXG' sequence (Fig. 1). In the present study, we showed for the first time that the intact cell of *A. tumefaciens* strain had lipolytic activity on a tributyrin-agar plate (data not shown),

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AT4     ----MISDPQVLAFIRKTEASYPAQANTASAAENRAYYDA-----MCADFRAPRPENVIVV----DRSIS   57
P23872  MKPENKLPVLDLISAEMKTVVNTLQPDLPPWPATGTIAEQRQYYTLERRFWNAGAPEMATRAYMVPTKYG   70
Q00675  ------------------------MQGWKTIVGKLMSRYDF-----PLPDL-----SVQAE----DKILG   32
P18773  MKFGTVWKYYFTESLLKATIRTPSQLNLAPN-ALRPVLDQ-----LCRLFPQN--PTVQIR----PIRLA   58

AT4     GVACRLYG---LASPVCVLYLHGGGLVVGGLDSHDDVCVEIADATGLQIISVDYRLAPEHRWPAQINDVE  104
P23872  QVETRLFCP-QPDSPATLFYLHGGGFILGNLDTHDRIMRLLASYSQCTVIGIDYTLSPEARFPQAIEEIV  119
Q00675  GVPTRIYTPPDVADPPLALYFHAGGWVMGSIDEEDGFVRTLCKLARTRIFSVGYRLAPEFRFPMALDDCL   82
P18773  GVRGEEIKA-QASATQLIFHIHGGAFFLGSLNTHRALMTDLASRTQMQVIHVDYPLAPEHPYPEAIDAIF  107

AT4     AVWRAVDQP----------VVVVGDSAGG--LLAAALCLSQK--GKRQP----LGQVLIYPG-LGGD---  172
P23872  AACCYFHQQAEDYQINMSRIGFAGDSAGAMLALASALWLRDK-----QIDCGKVAGVLLWYG---LYGLR  201
Q00675  TVARSVLETY-----PVQSICFIGASAGGNMAFSTALTLVSDGLGDRVQGVVALAPVTVHPDSVSADNRD  167
P18773  DVYQALLVQG----IKPKDIIISGDSCGANLALALSLRLK-Q-----QPELMPSGLILMSPY---EDLTL  184

AT4     -GDAASYRENAEAPLLRASDVLTYRDFVFGGQPATDPIARPLKAPDLS-DLAPAFVVTADVDPLRDDGWA  240
P23872  DSVTRRLLGGVWDGLTQQDLQMYEEAYLSNDADRESPYYCLFNN-DLTREVPPCRIAGAEFDPLLDDSRL  255
Q00675  RGEYTSYEENDRLTINTGSAMRSF--FDCYGAPPDDPRLSCLLHPGLG-KLNKVYMAVGDADTLRDDVRL  219
P18773  TSESLRFNQKHDALLSIEALQAGIKHYLTDDIQPGDPRVSPLFD-DLD-GLPPTLVQVGSKEILLDDSKR  237

AT4     YAERLAAAGVPVTLRNEAQLPHGFLRARIMSDRARRSFQAVIAAISRFASNEVPLANFPHEP  282
P23872  LYQTLAAHQQPCEFKLYPGTLH----AFLHYSRMMKTADEALRDGAQFFTAQL  304
Q00675  MRDALVALEVPVKCDEYPGYPH----FSWLFPSPALREHQA-L-----FFGNLLSGICWVCE  286
P18773  FREKAEQADVKVHFKLYTGMWN----NFQMFNAWFPEAKQALADIAEFATSLDLD  288
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Fig. 1. Sequence alignment of AT4 protein with other enzymes.
Protein sequences of AT4, *E. coli* acetyl hydrolase (P23872), *Emericella nidulans* lipase (Q00675), and *Acinetobacter lwoffii* esterase (P18773) were aligned. Conserved amino acids are boxed, and the N-terminal HG region and catalytic sites are marked (*).

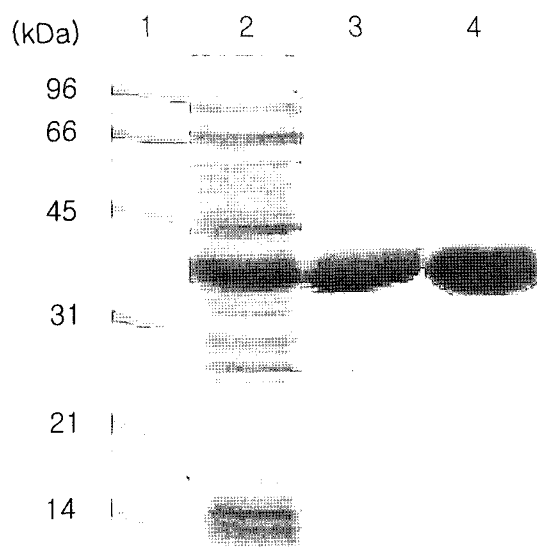

Fig. 2. SDS-PAGE of AT4 esterase.
AT4 esterase was analyzed by SDS-PAGE. Lane 1 is protein size markers. Lanes 2 and 3 are proteins in the soluble and insoluble fractions of transformed *E. coli* cells. Lane 4 is the AT4 enzyme purified via Ni-NTA column.

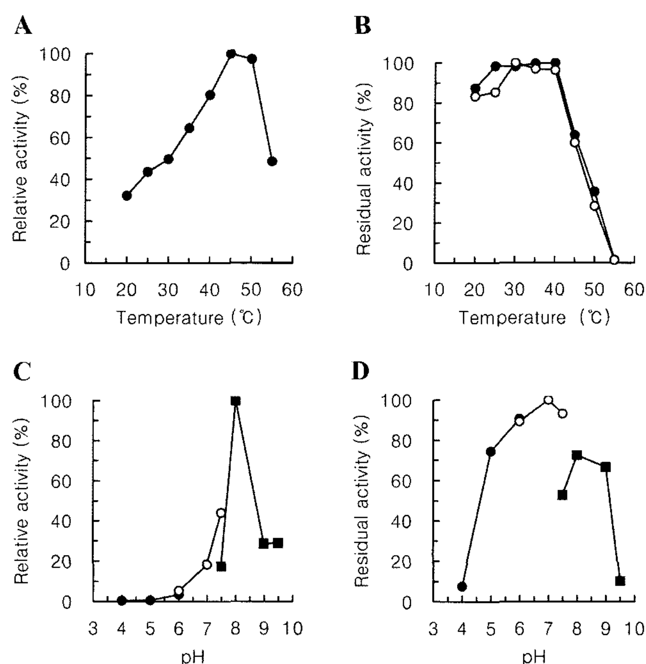

Fig. 3. Effects of temperature and pH on esterase AT4.
A. Hydrolytic activity toward $PNPC_6$ was measured at various temperatures. **B**. The enzyme was incubated at various temperatures for 30 min in the presence of 5 mM calcium ion (○) or 5 mM EDTA (●). Then, the residual activities were measured at 35°C. **C**. Hydrolytic activity was measured at various pHs. **D**. The enzyme was incubated at various pHs for 30 min and the residual activity was measured at pH 8.0.

and this esterase activity might have been due to the AT1 and AT4 enzymes.

To overexpress this novel AT4 enzyme in *E. coli* cells, the recombinant plasmid pET-AT4 was constructed. This plasmid contained a 906 bp-sized coding region, which was ligated with pET-22b vector to make a recombinant protein, composed of 302 amino acids plus an additional 13 C-terminal amino acids containing 6 histidine residues. This recombinant protein was expressed in *E. coli* cells. When the cells were cultured at 30°C and induced to produce the recombinant proteins, AT4 protein was expressed in both soluble and insoluble forms (Fig. 2), of which only the former had the lipolytic activity. The fraction of soluble to insoluble proteins did not increase, even though the culture temperature was lowered to 20°C (data not shown). The expressed enzyme was tightly bound to a Ni-NTA column, which might have been due to its His-tag located at the C-terminal. Then, it was eluted from the column with a buffer containing 300 mM imidazole and analyzed on SDS-PAGE gel (Fig. 2).

Biochemical Characterization of AT4 Esterase

The purified enzyme showed hydrolytic activity toward *p*-nitrophenyl caproate, but not toward olive oil, suggesting that the AT4 enzyme is a typical esterase rather than lipase. AT4 esterase had an optimum temperature of 45°C when $pNPC_6$ was used as substrate (Fig. 3A), which implied that it is a typical mesophilic enzyme. It was stable up to 40°C when assayed after pretreatment at each temperature for 30 min. Its thermostability was exactly the same in the presence or absence of calcium ion (Fig. 3B). This meant that calcium ion had no effect on its activity or stability, and also suggested that the protein had no calcium binding site [9]. In addition, it had an optimum pH of 8.0 and was stable in pH range of 5.0–9.0 (Figs. 3C and 3D). Most

Table 1. Kinetics parameters of AT4 esterase toward various substrates.

Substrate	K_m (mM)	V_{max} (μmol min^{-1} ml^{-1})[a]	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} mM^{-1})
*p*NPA	0.761	20.1	11.8	15.5
*p*NPB	0.860	18.1	10.6	12.3
1-NA	1.01	20.2	11.6	11.5
1-NB	1.10	32.6	18.7	17.0
7-ACA	3.02	20.2	11.8	3.90
Glucose pentaacetate	2.01	7.22	4.22	2.10
Xylose tetraacetate	2.16	14.0	12.2	5.64

[a]Protein concentration used in this experiments was 0.029 μmol/ml.

metal ions had no effect on the enzyme except cobalt, zinc, and cadmium ions, which showed strong inhibitory effects. Tween detergents had no inhibitory or activating effects on the enzyme. However, Triton X-100 strongly inhibited and SDS completely inhibited the enzyme activity (data not shown).

Hydrolytic activity was measured with various substrates (Table 1). The enzyme had a K_m value of 0.761 mM and k_{cat} value of 11.8 sec^{-1} with *p*-nitrophenyl acetate. These kinetic parameters were similar to the values obtained with *p*-nitrophenyl butyrate. With 1-naphthyl esters, the butyl group was released more rapidly than the acetyl group from the substrates, and the resulting k_{cat}/K_m value for 1-naphthyl acetate was three times higher than that for 1-naphthyl butyrate. In addition, AT4 esterase could release acetyl residues from 7-amino cephalosporanic acid (7-ACA) and acetylated glucose and xylose molecules with comparable rates. As such, AT4 esterase was able to hydrolyze short-chain acidic groups in several synthetic substrates as well as sugar molecules. Such substrate specificity suggests that this novel esterase enzyme might potentially be used as a biocatalyst in acetylation and deacetylation reactions performed frequently in the fine chemical industry. In summary, we demonstrated in this paper that the novel AT4 gene cloned directly from the *A. tumefaciens* genome was expressed well in the *E. coli* system, and that, on the basis of substrate specificity, the expressed protein was a typical esterase.

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