Nucleotide sequence of a novel arylesterase gene from *Vibrio mimicus* and characterization of the enzyme expressed in *Escherichia coli*

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A gene coding for an arylesterase of *Vibrio mimicus* was cloned. Sequence determination reveals that the esterase gene has an open reading frame of 600 nucleotides which encodes a protein of M_r 22300. The deduced amino acid sequence contains a pentapeptide GDSLS (residues 27–31), which was also found in the phospholipid–cholesterol acyltransferase from *Aeromonas hydrophila*. Substitution of Ser-29 by alanine or cysteine in the cloned gene abolished the esterase activity in the tributyrin plate assay. On the other hand, the activity was not lost when Ser-31 was changed to alanine. The cloned gene was expressed in *Escherichia coli*, and the protein purified by a four-step procedure. The purified protein migrated on SDS/PAGE as a single band with an apparent M_r of 22100. This enzyme favoured the hydrolysis of several arylesters and was classified as an arylesterase (EC 3.1.1.2). N-Terminal analysis showed that Ser-20 was the first amino acid of the mature secreted protein, suggesting that the N-terminal 19 hydrophobic amino acids served as a signal peptide.

INTRODUCTION

Esterases (ester hydrolase) (EC 3.1.1) have become increasingly important in biotechnology [1]. They are widely distributed in nature and catalyse the hydrolysis of ester bonds. The characteristic properties such as substrate specificity, regioselectivity and enantioselectivity among various esterases allow wide applications of these enzymes. For example, arylesterases (EC 3.1.1.2) show a preferential substrate specificity for aromatic esters. In mammalian systems, arylesterases play an important role in the detoxification of organophosphorus compounds [2], and diagnosis of liver cirrhosis is made possible by measuring the decrease in serum arylesterase content [3]. In contrast, the physiological functions of microbial arylesterases remain largely unknown. As arylesterases are extracellular enzymes, it is possible that bacteria may use them as tools to obtain energy and essential nutrients from the environment or to detoxify xenobiotics. Bacterial arylesterases may also be used for industrial and environmental purposes, especially considering the fact that they have broad substrate specificities. Little is known about their genetics and biochemistry. An arylesterase gene from *Pseudomonas fluorescens* have been cloned and its gene product characterized [4]. The *Ps. fluorescens* enzyme is a typical arylesterase and does not contain an active-site serine. An esterase-producing strain of *Vibrio mimicus* has been isolated from a Taiwan shrimp aquaculture pond (H.-C. Chen, personal communication). This strain produces only one extracellular esterase which favours hydrolysis of aromatic esters over other substrates (J.-F. Shaw, R.-C. Chang, K.-H. Chuang, Y.-T. Yen, Y.-J. Wang and F.-F. Wang, unpublished work). In the present paper, we report the cloning and nucleotide sequence of this arylesterase gene and the expression, purification and characterization of the gene product. Our results suggest that it is a novel arylesterase which contains a putative active-site serine in a hydrophobic domain.

MATERIALS AND METHODS

Bacterial strains and plasmids

V. mimicus NTOU 66 was used as the DNA source for cloning of the esterase gene. The vector for the cloning experiment was phagemid pGEM3Zf(+) (Promega). *E. coli* JM83 and JM101 were the hosts for the recombinant plasmids (neither strain showed any extracellular esterase activity).

Media and growth conditions

E. coli and *V. mimicus* were grown in L-broth (10 g of peptone, 5 g of yeast extract and 10 g of NaCl per litre of water) at 37 °C and 25 °C respectively. When needed, ampicillin was added at a concentration of 50 μg/ml.

Recombinant DNA procedures

Plasmid DNA was prepared by the procedure of Klein et al. [5], and restriction digestions and ligation reactions were performed according to the manufacturer's recommendations (Boehringer-Mannheim). Exonuclease III unidirectional deletion was carried out as described [6].

Construction of the *V. mimicus* genomic library

Chromosomal DNA from *V. mimicus* NTOU 66 was prepared by the method of Coleman et al. [7]. The DNA was partially digested with *Sau*3A, and the DNA fragments ranging in size from 2 to 6 kb were isolated after electrophoresis in agarose gel using a QIAEX gel extraction kit (Institute of Molecular Biological Diagnostic GmbH). The DNA fragments were ligated to the *Bam*HI site of pGEM3Zf(+), and the ligated DNA was transformed into *E. coli* JM83. Ampicillin-resistant colonies were selected on tributyrin/agar plates [8] at 37 °C after 24 h incubation.

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The sequence data reported in this paper have been submitted to the EMBL/Genbank/DDBJ Nucleotide Sequence Databases under the accession number X71116.

DNA sequence analysis

The DNA sequence was determined by a modified dideoxynucleic acid sequencing method [9] using the Sequenase version 2.0 kit (United States Biochemical).

Esterase purification

E. coli JM101 harbouring the plasmid pL662dHE was inoculated into a 500 ml culture of L-broth with 50 μg/ml ampicillin. Cells were grown at 37 °C for 48 h in 2-litre baffled flasks, and the culture media were aerated by rotary shaking at approximately 200 rev./min. Culture media were harvested after removal of cell debris by centrifugation at 6000 *g* for 30 min. The enzyme was precipitated by 40–70 %-satd. $(NH_4)_2SO_4$ fractionation. The crude enzyme was loaded on to a hydrophobic interaction column (Bio-Rad) pre-equilibrated with 25 mM disodium hydrogen phosphate/sodium dihydrogen phosphate buffer (pH 6.8) containing 2.4 M $(NH_4)_2SO_4$ (buffer A). After the column had been washed with buffer A, the bound proteins were gradually eluted with a 25 mM disodium hydrogen phosphate/sodium dihydrogen phosphate buffer, pH 6.8 (buffer B). The fractions with esterase activity were pooled and concentrated by Amicon YM2 membranes (1000 M_r cut-off point). The concentrated fractions were loaded on to an ion-exchange column (Mono Q; Bio-Rad) pre-equilibrated with buffer B. After the column had been washed with buffer B, the bound proteins were gradually eluted with a gradient of 0–1 M NaCl in buffer B. The activity-containing fractions were pooled and concentrated once more.

Protein concentration measurement, SDS/PAGE and gel-staining methods

Protein concentration was determined by a Bradford dye-binding procedure with BSA as standard [11]. Continuous SDS/PAGE was carried out using 5–20 % (w/v) gradient polyacrylamide slab gels [12]. Samples were preboiled for 5 min in loading buffer. Low-M_r markers were purchased from Pharmacia. The gels were silver-stained [13] for proteins and activity-stained [14] for esterase.

pH-stat method

The pH-stat method [15] was carried out at pH 8.0 with a Radiometer pH-stat titration system (PHM 61 standard pH-meter, TTT 80 titrator and ABU 80 autoburette; Copenhagen, Denmark).

The esterase activity of the enzyme was assayed by using *p*-nitrophenyl esters of fatty acids as substrates. Detailed procedures are described in [10].

N-Terminal sequence determination

Purified enzyme was dialysed, freeze-dried and N-terminal amino acid analysis was performed (by the Southern Instrument Center, National Cheng-Kung University, Tainan, Taiwan) using an Applied Biosystems 477A gas–liquid–solid-phase protein sequenator equipped with an Applied Biosystems 610A data-analysis system.

Site-directed mutagenesis

Site-directed mutagenesis of the cloned esterase gene was performed by the strategy of overlap extension using PCR as described by Higuchi et al. [16]. The first round of PCR was carried out with the primer combinations of 5′-flanking primer and mutant primer-2 or 3′-flanking primer and mutant primer-1. The reaction products from the two PCRs were purified from agarose gel to remove template and primer DNAs. They were mixed at a molar ratio of 1:1 and used as DNA templates for the second-round PCR. The second PCR was performed using 3′- and 5′-flanking oligonucleotides as primer pairs. The oligonucleotide primers used were: 5′-flanking primer: 5′-CATCTTTAAGGATCCTTAATTGTTCTTG-3′; 3′-flanking primer: 5′-TTCGAGCTCAAAATTAGAG-3′; mutant primer: for Ser-29 to Ala-29 mutation: primer-1: 5′-GTTCTTGTTGATGCCTTGAGTGCGGGCT-3′; primer-2: 5′-AGCCCGCACTCAAGGCATCACCAAGAAC-3′; for Ser-29 to Cys-29 mutation: primer-1: 5′-GTTCTTGGTGATTGCTTGAGTGCGGGCT-3′; primer-2: 5′-AGCCCGCACTCAAGCAATCACCAAGAAC-3′; for Ser-31 to Ala-31 mutation: primer-1: 5′-GGTGATAGCTTGGCTGCGGGCTATCAA-3′; primer-2: 5′-TTGATAGCCCGCAGCCAAGCTATCACC-3′.

Chemicals

All chemicals were purchased from commercial sources and were of reagent grade.

RESULTS AND DISCUSSION

Cloning of the *V. mimicus* esterase gene

V. mimicus NTOU 66 was shown to have extracellular esterase activities (H.-C. Chen, personal communication). To initiate the isolation of the esterase gene, a genomic library was constructed in vector pGEM3Zf(+), transformed into *E. coli*, and grown in agar plates containing tributyrin as substrate. The plates were prepared by pouring a mixture containing 1.5 % (w/v) agar, 1 % (v/v) tributyrin and ampicillin (50 μg/ml) in L-broth medium into a Petri dish. Among 6000 ampicillin-resistant clones grown on tributyrin/agar plates, two clear halo-forming clones were identified. One of these, designated pL662, was chosen for further analysis.

Restriction mapping of plasmid pL662 revealed the presence of a 2.6 kb DNA insert (results not shown). The esterase expression, as indicated by the size of the halos on tributyrin/agar plates, was independent of the orientation of the 2.6 kb insert DNA in pGEM3Zf(+) (results not shown). Moreover, the activity remained relatively invariant with or without the presence of isopropyl β-D-thiogalactoside, an inducer for the *lac* promoter located upstream from the multiple cloning sites. These data suggest that the expression of the cloned *V. mimicus* DNA in *E. coli* may be under the control of its own promoter.

Plasmid pL662 was subjected to a series of deletion analyses, as shown in Figure 1. One of the clones, pL662dHE, carrying a 0.77 kb *V. mimicus* DNA fragment, was shown to express higher esterase activity in *E. coli* than the parental pL662 clone.

Nucleotide and deduced amino acid sequences of the cloned esterase gene

The nucleotide sequence (Figure 2) of the 0.77 kb *V. mimicus* DNA fragment in pL662dHE was determined. This sequence contains a single open reading frame at positions 162 to 761; a protein of 200 amino acid residues and a M_r of 22264 may be deduced from the coding region. The isoelectric point of this fragment was calculated to be 4.9. The N-terminal region of the deduced sequence contains a segment of 19 consecutive hydro-

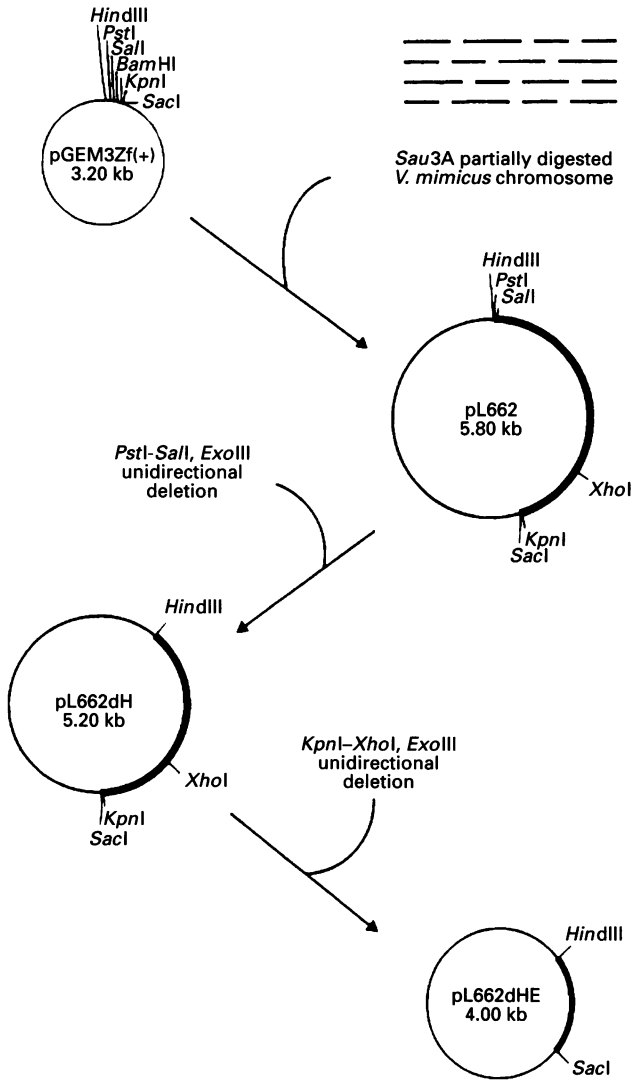

Figure 1 Construction of plasmid pL662dHE

Thick lines, DNA segments originating from the chromosomal DNA of *V. mimicus*; thin line, DNA segment originating from pGEM3Zf(+).

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5'-AAGCTTTGCCTGCACCAGATGTGCCGACAATTGCGATGGTCTCGCCCTTTCTGATGTCAA  60
  HindIII
CATTTACATCTTTAAGGATTGTTAATTGTTCTTGATTAGTAGAGACTTGTTTAGAAACCA 120
AATTCGCTTGAACAATAGGTGCTTTGAGTACAGGTGTTTGCATG ATT CGT TTA CTT  176
                                SD      MET Ile Arg Leu Leu    5

TCC TTA GTT CTC TTT TTT TGT CTT TCC GCC GCA TCG CAA GCA AGC    221
Ser Leu Val Leu Phe Phe Cys Leu Ser Ala Ala Ser Gln Ala Ser    20
                    Signal peptide
GAA AAG CTT CTT GTT CTT GGT GAT AGC TTG AGT GCG GGC TAT CAA    266
Glu Lys Leu Leu Val Leu Gly Asp Ser Leu Ser Ala Gly Tyr Gln    35
                Putative active site
ATG CCG ATT GAG AAG AGT TGG CCT AGC TTA CTC CCT GAC GCG CTG    311
Met Pro Ile Glu Lys Ser Trp Pro Ser Leu Leu Pro Asp Ala Leu    50

CTC GAA CAC GGG CAA GAT GTA ACA GTT ATT AAT GGC AGC ATC TCC    356
Leu Glu His Gly Gln Asp Val Thr Val Ile Asn Gly Ser Ile Ser    65

GGA GAC ACT ACT GGC AAC GGT TTG GCT CGC CTC CCG CAA CTG CTT    401
Gly Asp Thr Thr Gly Asn Gly Leu Ala Arg Leu Pro Gln Leu Leu    80

GAC CAA CAT ACA CCC GAC TTA GTT CTT ATC GAG TTG GGC GCC AAT    446
Asp Gln His Thr Pro Asp Leu Val Leu Ile Glu Leu Gly Ala Asn    95

GAT GGG TTG CGT GGC TTC CCT CCA AAA GTT ATT ACG TCG AAT CTG    491
Asp Gly Leu Arg Gly Phe Pro Pro Lys Val Ile Thr Ser Asn Leu   110

TCG AAA ATG ATT AGC CTG ATC AAA GAT TCT GGT GCC AAT GTG GTC    536
Ser Lys Met Ile Ser Leu Ile Lys Asp Ser Gly Ala Asn Val Val   125

ATG ATG CAA ATT CGT GTC CCA CCT AAC TAC GGC AAG CGC TAC AGC    581
Met Met Gln Ile Arg Val Pro Pro Asn Tyr Gly Lys Arg Tyr Ser   140

GAC ATG TTT TAC GAC ATC TAT CCC AAA TTG GCT GAG CAT CAA CAA    626
Asp Met Phe Tyr Asp Ile Tyr Pro Lys Leu Ala Glu His Gln Gln   155

GTT CAG TTA ATG CCG TTC TTC TTA GAG CAT GTG ATC ACC AAA CCA    671
Val Gln Leu Met Pro Phe Phe Leu Glu His Val Ile Thr Lys Pro   170

GAG TGG ATG ATG GAT GAT GGC TTA CAT CCG AAA CCT GAA GCT CAA    716
Glu Trp Met Met Asp Asp Gly Leu His Pro Lys Pro Glu Ala Gln   185

CCT TGG ATT GCG GAA TTC GTT GCT CAA GAA TTG GTT AAA CAT CTC    761
Pro Trp Ile Ala Glu Phe Val Ala Gln Glu Leu Val Lys His Leu   200

TAA TTTTGAGCTC-3'
 *      SacI
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Figure 2 Nucleotide and deduced amino acid sequences of the cloned *V. mimicus* esterase

The deduced amino acid sequence is shown beneath the nucleotide sequence. A potential Shine–Dalgarno (SD) sequence and the putative signal peptide sequence are indicated. The deduced amino acid sequence similar to several esterases and an acyltransferase (see Table 1 for details) is boxed. The stop codon is indicated by an asterisk. The *Hin*dIII site and the *Sac*I site in the multiple cloning region of pGEM3Zf(+) are indicated. This gene is cloned in the opposite direction to the vector.

phobic amino acids which may serve as a signal peptide [17]. Upstream from the translation start site at base 152 is the sequence 5′-AGGTGT-3′ which may function as a Shine–Dalgarno sequence [18].

Sequence comparison of the cloned esterase with other known esterases failed to reveal any significant similarity. Structural data from the X-ray crystallography of several lipolytic enzymes indicate the presence of a catalytic triad of either Ser, His, Asp or Ser, His, Glu [19–21]. Similar constellations of these residues were found in many serine proteases and are known as the charge-transfer relay system important for catalysis [22]. A comparison of the amino acid sequences in a variety of lipases and esterases indicates that most of the lipases share an active-site serine-containing consensus motif GXSXG which is often preceded by four hydrophobic amino acids [23]. Although the cloned *V. mimicus* gene did not contain the GXSXG sequence, a search for the conserved nine-amino-acid segment reveals the presence of a similar but not identical sequence -LLVLGDSLS- (residues 23–31). It is of interest to note that the same pentapeptide GDSLS was also found in phospholipid–cholesterol acyltransferase from *Aeromonas hydrophila*. Table 1 summarizes a comparison of various hydrolytic enzymes that contain the four preceding hydrophobic amino acids and a GXSXX consensus motif. The hydropathy indices [32] of the nine-amino-acid segment were also calculated, and range from 2.7 to 13.9 in this class of hydrolases. The presence of the active serine in a hydrophobic pocket is presumably to facilitate the binding of hydrophobic substrates. This contrasts with the hydrophilic nature of amino acid sequences surrounding the active serine in serine proteases which show hydropathy indices ranging from −2.7 to −10.8 [33–35]. The relatively high hydropathy index (13.9) for the putative active site of *V. mimicus* esterase may reflect the substrate preference of the enzyme for aromatic esters.

Role of Ser-29 in the cloned esterase

In order to evaluate the importance of the serine residue in the pentapeptide GXSXS, we constructed mutated genes by overlap-extension strategies using PCR as described in the Materials and methods section. Substitution of the middle serine (Ser-29) by alanine or cysteine abolished the ability of the transformant to form a halo in the tributyrin plate assay, indicating loss of enzymic expression in the Ser-29-mutated esterases. In contrast,

Table 1 A comparison of a nine-amino acid conserved sequence among esterolytic enzymes

Abbreviations: *Vm*A, *V. mimicus* arylesterase; *Ah*A, *A. hydrophila* phospholipid–cholesterol acyltransferase; *Pf*A, *Ps. fluorescens* arylesterase; *Pf*C, *Ps. fluorescens* carboxylesterase; *Dp*E, *Drosophila pseudoobscura* esterase; *Pr*L, *Pseudomonas fragi* lipase; *Sa*L, *Staphylococcus aureus* lipase; *Sh*L, *Staphylococcus hyicus* lipase; *Rm*L, *Rhizomucor miehei* lipase; *Pf*L, *Ps. fluorescens* lipase; rHL, rat hepatic lipase.

Species and enzyme	Amino acid sequence and position	Hydropathy indices	Reference
*Vm*A	23 31 LLVLGDSLS	13.9	This study
*Ah*A	10 18 IVMFGDSLS	11.7	24
*Pf*A*	–	–	4
*Pf*C	108 116 IFLAGFSQG	10.6	25
*Dp*E	205 213 VLLVGHSAG	13.0	26
*Pr*L	77 85 VNLIGHSQG	2.7	8
*Sa*L	406 414 VHLVGHSMG	6.1	27
*Sh*L	363 371 VHLVGHSMG	5.4	28
*Rm*L	138 146 VAVTGHSLG	8.5	29
*Pf*L	200 208 VLVSGHSLG	10.4	30
rHL	76 84 VHLIGYSLG	10.2	31

* Consensus sequence was not found in *Pf*A.

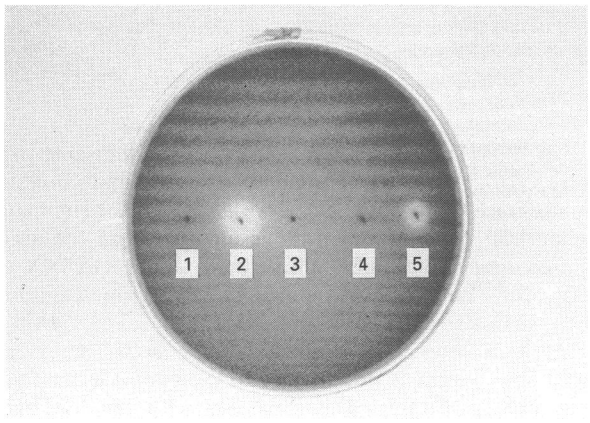

Figure 3 Tributyrin plate assay of the Ser-29 and Ser-31 mutated esterases

Site-directed mutagenesis of the cloned lipase was performed as described in the Materials and methods section. The mutated clones were transformed in *E. coli* and grown in agar plates containing the esterase substrate, tributyrin. The halo-forming clones indicate expression of esterase activity. 1, pGEM3Zf(+) vector control; 2, wild-type clone; 3, S29A mutant; 4, S29C mutant; 5, S31A mutant.

the conversion of the C-terminal serine in the pentapeptide (Ser-31) into alanine did not affect expression of the esterase (Figure 3). These results suggest that the middle serine (Ser-29) of the pentapeptide may very well serve as the active-site serine of the cloned esterase.

Table 2 Purification profile of *V. mimicus* esterase

One unit of enzyme activity is defined as the formation of 1 μmol of *p*-nitrophenol from *p*-nitrophenyl butyrate/min [10].

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Supernatant	2424.60	1163.80	2.09	100.0
$(NH_4)_2SO_4$ fractions (40–70%)	542.10	247.00	2.20	22.4
Hydrophobic interaction column	153.73	67.04	2.03	6.3
Mono Q column	112.61	4.96	22.70	4.6

An arylesterase gene from *Ps. fluorescens* has been cloned and its gene product characterized [4]. This enzyme is a typical arylesterase which shows a preferential substrate specificity for aromatic esters. The enzyme was not inhibited by di-isopropyl fluorophosphate, a serine hydrolase inhibitor. Sequence analysis failed to reveal the presence of a GXSXX consensus motif in the protein. These findings indicate that the *Pseudomonas* arylesterase may not have an active-site serine [4,36], which is in clear contrast with our findings for the *V. mimicus* enzyme.

The mechanism of arylesterase action has been proposed to include the formation of a thioester intermediate [37]. For the *V. mimicus* esterase, only one cysteine was found and it is located in the putative signal peptide of the protein, which would exclude the possibility that the enzyme is a cysteine esterase. Furthermore, results from the present study strongly imply that the enzyme is a serine esterase. The lack of cysteine residue in the mature *Vibrio* esterase suggests that the protein is a relatively flexible molecule, the tertiary structure of which depends on weaker non-covalent interactions. The conformational flexibility may allow the arylesterase to pass more freely through the cell wall. This is consistent with the observation that many extracellular bacterial proteins contain a low level of cysteine [38].

Purification of the *V. mimicus* esterase from an *E. coli* clone

The *E. coli* JM101 clone harbouring plasmid pL662dHE showed the highest extracellular esterase activities and was used as a source to purify this enzyme. The specific activity of the purified enzyme increased to 22.70 units/mg, representing an overall purification of 10.1-fold (Table 2). The purified enzyme was homogeneous as judged by the presence of a single protein band with an apparent M_r of 22100 on SDS/PAGE (Figure 4). This M_r is very close to that predicted from the DNA sequence and is the same as that of arylesterase from *V. mimicus* NTOU 66 (results not shown). It appears that a similarly processed arylesterase was produced in the *E. coli* clone.

Characterization of the cloned esterase

The *V. mimicus* esterase purified from *E. coli* harbouring pL662dHE was used for several biochemical analyses as follows. First, a pH–activity profile for the enzyme was investigated using tributyrin as substrate, and the optimal pH was found to be 7–9 (Table 3). Secondly, enzyme activities at various temperatures were analysed with *p*-nitrophenyl butyrate as substrate, and the optimal temperature was found to be 50 °C (Table 4). Thirdly,

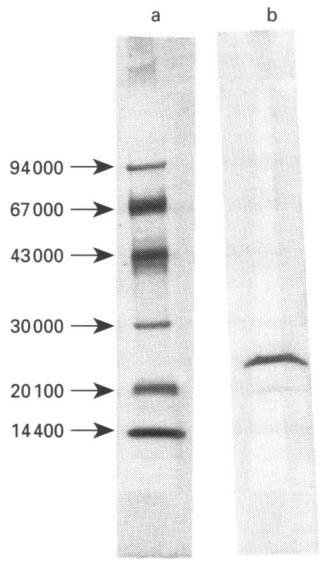

Figure 4 SDS/PAGE of the purified esterase

Proteins were separated by SDS/PAGE with a 5–20% gradient gel. The proteins were visualized by silver staining. Lane a, M_r markers; lane b, purified esterase.

Table 3 Effect of pH on the *V. mimicus* esterase

The activity was assayed at 37 °C using the pH-stat method. The substrate for the assay was tributyrin (4.08%, v/v). The relative activity at a given pH was calculated by dividing the absolute reaction rate by that at pH 8.0.

pH	Relative activity (%)
5.0	15
6.0	50
7.0	97
8.0	100
9.0	95

Table 4 Effect of temperature on the activity of *V. mimicus* esterase

Esterase activity was monitored by the formation of *p*-nitrophenol from *p*-nitrophenyl butyrate [15]. The relative activity at a given temperature was calculated by dividing the absolute rate by that at 50 °C.

Temperature (%)	Relative activity (%)
30	58
40	72
45	76
50	100
55	50
60	48
70	21

the ability of the enzyme to hydrolyse esters of fatty acids of various chain length was studied; esters of octanoate, decanoate and laurate were clearly favoured (Table 5). Finally, a broad

Table 5 Relative activities of the purified *V. mimicus* esterase

Esterase activity was monitored by observing the formation of *p*-nitrophenol from *p*-nitrophenyl esters [15]. The relative activity for a given substrate was calculated by dividing the absolute activity by that of *p*-nitrophenyl decanoate (1836 μmol of *p*-nitrophenol formed/ml per min).

Substrates	Relative activity (%)
p-Nitrophenyl acetate	14
p-Nitrophenyl butyrate	18
p-Nitrophenyl hexanoate	40
p-Nitrophenyl octanoate	75
p-Nitrophenyl decanoate	100
p-Nitrophenyl dodecanoate	77
p-Nitrophenyl myristate	30
p-Nitrophenyl palmitate	20
p-Nitrophenyl stearate	3

Table 6 Hydrolysis of various esters by the *V. mimicus* esterase

The rate of hydrolysis was assayed by the pH-stat method. The average reaction rate for each substrate was obtained from three independent experiments. The relative activity for a given substrate was calculated by dividing the average rate by that of *p*-nitrophenyl butyrate (5460 μmol of *p*-nitrophenol formed/ml per min) multiplied by 100.

Substrates (50 mM)	Relative activity (%)
p-Nitrophenyl acetate	83.25
p-Nitrophenyl butyrate	100.00
p-Nitrophenyl decanoate	13.47
p-Nitrophenyl laurate	9.33
p-Nitrophenyl palmitate	0.11
p-Nitrophenyl stearate	0.00
α-Naphthyl acetate	30.63
α-Naphthyl butyrate	14.66
α-Naphthyl laurate	1.08
α-Naphthyl palmitate	0.47
Triacetin	41.63
Tributyrin	15.91
Tricaprin	0.00
Triolein	0.00
Olive oil	0.00
Menthyl acetate	0.00
Phenyl acetate	36.65
Benzyl acetate	13.17
Cholesteryl *n*-butyrate	0.00
Cholesteryl *n*-decylate	0.00

substrate survey for the enzyme was carried out (Table 6), and it was found to preferentially hydrolyse several aryl esters. On the basis of this substrate specificity, the enzyme is classified as an arylesterase. These biochemical properties are similar to those of partially purified extracellular arylesterase from *V. mimicus* (J.-F. Shaw, R.-C. Chang, K.-H. Chuang, Y. T. Yen, Y.-J. Wang and F.-F. Wang, unpublished work). This suggests that the cloned gene encoded the extracellular esterase of *V. mimicus*.

N-terminal determination

We determined the N-terminal amino acid residues up to the 10th cycle, and this showed that the first amino acid was serine and the entire sequence determined was SEKLLVLGDS- which corresponds to codons 20–29 in the open reading frame. These

results strongly suggest that the first 19 amino acids of the deduced sequence are indeed a signal sequence.

V. mimicus NTOU 66 isolated from a Taiwan freshwater pond was kindly provided by Professor Hsing-Chen Chen, Department of Marine Food Science, National Taiwan Ocean University, Keelung, Taiwan, Republic of China. We thank Dr. Chien Chen for his valuable suggestions during the preparation of this manuscript. This research was supported by a grant from the National Science Council, Republic of China (NSC 82-0418-B-001-112-BA) to J.F.S.

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Received 2 June 1993/21 September 1993; accepted 12 October 1993