JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Molecular Cloning, Purification, and Biochemical Characterization of a Novel Pyrethroid-Hydrolyzing Esterase from *Klebsiella* sp. Strain ZD112

PEI C. WU,† YU H. LIU,*,† ZHUO Y. WANG,† XIAO Y. ZHANG,† HE LI, WEI Q. LIANG, NA LUO, JI M. HU, JIA Q. LU, TIAN G. LUAN, AND LI X. CAO

State Key Laboratory of Biocontrol, School of Life Science, Zhongshan University, Guangzhou 510275, People's Republic of China

The gene encoding pyrethroid-hydrolyzing esterase (EstP) from *Klebsiella* sp. strain ZD112 was cloned into *Escherichia coli* and sequenced. A sequence analysis of the DNA responsible for the *estP* gene revealed an open reading frame of 1914 bp encoding for a protein of 637 amino acid residues. No similarities were found by a database homology search using the nucleotide and deduced amino acid sequences of the esterases and lipases. EstP was heterologously expressed in *E. coli* and purified. The molecular mass of the native enzyme was approximately 73 kDa as determined by gel filtration. The results of sodium dodecyl sulfate−polyacrylamide gel electrophoresis and the deduced amino acid sequence of EstP indicated molecular masses of 73 and 73.5 kDa, respectively, suggesting that EstP is a monomer. The purified EstP not only degraded many pyrethroid pesticides and the organophosphorus insecticide malathion, but also hydrolyzed *ρ*-nitrophenyl esters of various fatty acids, indicating that EstP is an esterase with broad substrates. The K_m for *trans*- and *cis*-permethrin and k_{cat}/K_m values indicate that EstP hydrolyzes both these substrates with higher efficiency than the carboxylesterases from resistant insects and mammals. The catalytic activity of EstP was strongly inhibited by Hg^{2+}, Ag^{+}, and *ρ*-chloromercuribenzoate, whereas a less pronounced effect (3−8% inhibition) was observed in the presence of divalent cations, the chelating agent EDTA, and phenanthroline.

KEYWORDS: Pyrethroid insecticides; pyrethroid-hydrolyzing esterase; molecular characterization; kinetic properties; *Klebsiella* sp. strain ZD112

INTRODUCTION

Pyrethroid insecticides have been developed as alternatives to the recalcitrant organochlorine insecticides to control insects in crop production and around households for over four decades (*1*). Although the pyrethroid insecticides are much less toxic to mammals than are many other ones, they are not free from adverse effects (*2*, *3*). These adverse health effects of pyrethroids have been reported including suppression of the immune system after exposure, lymph node and splenic damage, carcinogenesis, and hormonal activity (*4*). The historical concern about the use of pyrethroid insecticides is their apparent toxicity to fish and aquatic invertebrates, often at concentrations of less than 1 *μ*g/L (*5*). In addition, epidemiological accounts, clinical reports, and other laboratory studies indicate that pyrethroids possess estrogenic and antiprogestagenic activities as well as other pesticides and have thus been classified as endocrine disruptors (*6*).

As a result of their widespread and repeated use, a great deal of unused and obsolete pesticide is threatening the environment and public health. Therefore, there has been a serious need to develop remediation processes to eliminate or minimize contamination in food and the environment. Microbial degradation has been deemed the most influential and significant cause of pesticide removal and a major factor determining the fate of pesticides in the environment. As a result, biological remediation is generally considered to be the safest, least disruptive, and most cost-effective technique for pesticide abatement. Carboxylesterases are a family of enzymes that are important in hydrolysis of numerous xenobiotic and endogenous ester-containing compounds; they play an important role in the detoxification of carbamates and pyrethroids by the hydrolysis of ester bonds (*7*). Some pyrethroid carboxylesterases hydrolyzing permethrin into 3-phenoxybenzyl alcohol and 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid from pyrethroid-resistant insects and mammal organs have been purified and characterized (*7*), but those enzymes often show a narrow substrate specificity and low activity (*8*, *9*). Given that pesticidal residues are comprised of a mixture of the pesticides, those properties restricted usefulness in bioremediation. However, the organophosphate and carbamate insecticide-degrading enzymes from bacteria usually have advantages in terms of

* To whom correspondence should be addressed. Phone: 86-20-84113712. Fax: 86-20-84036215. E-mail: ls112@zsu.edu.cn.
† These authors contributed equally to this work.

10.1021/jf052691u CCC: $33.50
Published on Web 01/04/2006

broader substrate specificities and superior kinetics (*10*−*12*); a similar feature may be observed in enzymes hydrolyzing pyrethroid insecticides from bacteria. Although some pyrethroid-degrading bacteria including *Bacillus cereus* SM3 (*13*), *Pseudomonas fluorescens*, *Vibrio hollisae*, *Burkholderia picketti*, and *Erwinia carotovora* have been isolated (*14*), and a few pyrethroid-degrading enzymes from *B. cereus* SM3 and *Aspergillus niger* (*15*) have been purified and characterized, no further research on the nucleic acid level was reported (*13*). In the present study, we screened *Klebsiella* sp. strain ZD112 and found a novel pyrethroid-hydrolyzing esterase (EstP) with higher activity and broader substrate specificities, as well as cloning, sequencing, and recombinant expression of its gene. To our knowledge, this is the first report so far on information about the pyrethroid-hydrolyzing esterase gene from microorganisms. Further study is helpful to obtain an excellent detoxifying enzyme for use as a bioremediation agent.

MATERIALS AND METHODS

Chemicals and Reagents. Cypermethrin (98%), *trans*-permethrin and *cis*-permethrin (99%), envalerate (98%), malathion (98%), and deltamethrin (98%) were kindly provided by the Zhongshan Pesticide Factory (Guangdong, China). All *ρ*-nitrophenyl esters were purchased from Sigma. All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless otherwise stated.

Isolation and Culture Conditions. The minimal medium with permethrin (MM medium) contained the following constituents, g/L (20 mM potassium phosphate buffer [pH 7.0]): NaCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2 \cdot H_2O$, 0.05; $(NH_4)_2SO_4$, 0.5; $FeSO_4$, 0.02; $ZnSO_4$, 0.002. After autoclaving, the permethrin was prepared by dissolving it into 10 times its volume of ethanol and added at that concentration (200 mg/L). The sewage from pyrethroid pesticide manufacture was inoculated into MM medium, and then pyrethroid-degrading bacteria were enriched by repeating five subcultivations. Appropriate dilutions of the enrichment culture were placed on MM agar plates and incubated at 37 °C for 3 days. The fastest colonies were selected from the plates, and the positive colony was isolated and purified. The pyrethroid hydrolysis was confirmed through GC−MS according to the method previously reported by Liang et al. (*15*).

DNA Manipulation. Routine DNA manipulation was carried out as described by Sambrook et al. (*16*). Restriction enzymes and DNA polymerase were purchased from Takara (Dalian, China). Each enzyme was used according to the recommendations of the manufacturer. DNA ligations were performed using T4 DNA ligase (Fermentas). Genomic DNA and plasmid DNA were isolated using genomic and plasmid DNA isolation kits, respectively (QIAamp DNA Mini Kit, EZNA Plasmid Miniprep Kit I). A DNA purification kit (EZNA Gel Extraction Kit) was used to recover DNA fragments from agarose gels.

Construction of Genomic Libraries and Screening for the Pyrethroid-Hydrolyzing Esterase Gene. To prepare genomic DNA fragments of approximately 3−10 kb, genomic DNA from *Klebsiella* sp. strain ZD112 was subjected to partial digestion with *Sau*3AI, fractions containing DNA fragments of the desired size were pooled, and the resultant DNA fragments were ligated into pUC18, which had been previously digested with *Bam*HI and dephosphorylated. *Escherichia coli* DH5α was transformed with the library and plated onto Luria−Bertani (LB) agar plates containing 100 *μ*g/mL ampicillin, 100 *μ*M 5-bromo-4-chloro-3-indolyl *β*-D-galactopyranoside (X-Gal), and 0.1 mM isopropyl *β*-D-thiogalactopyranoside (IPTG). The white transformants with foreign fragment DNA were chosen as candidate clones. The white transformants were subsequently replicated onto LB agar plates containing 100 *μ*g of ampicillin/mL and 100 *μ*M 5-bromo-4-chloro-3-indolyl caprylate (X-caprylate); blue colonies were selected because the hydrolysis of X-caprylate by the esterase produced a blue precipitate. The blue colonies were further tested for their ability to hydrolyze permethrin through GC−MS analyses. The transformants with pyrethroid-hydrolyzing esterase were obtained and confirmed. *E. coli* DH5α (pestP1) and *E. coli* DH5α (pestP2) were isolated and sequenced. The fragment size of the two plasmids was 5.8 kb. pestP1 and pestP2 were further digested with various endonucleases. The size of the insert of the plasmid pestP1 was reduced by using the *Hin*dIII restriction site. The resulting DNA fragment of 3.2 kb from plasmid pestP1 was ligated into a *Hin*dIII-digested, dephosphorylated pUC18 plasmid. The ligated DNA was introduced into *E. coli* DH5α; the resulting clone *E. coli* DH5α (pestP3) was tested for its ability to hydrolyze permethrin through GC−MS analyses.

Sequencing and Analysis of the Pyrethroid-Hydrolyzing Esterase Gene. Sequencing reactions were performed using a BigDye Sequencing Kit according to the instructions of the manufacturer. The sequencing products were analyzed on an ABI 377 DNA sequencer. Nucleotide and deduced amino acid sequence analyses, an open reading frame search, and multiple alignment, molecular mass, and isoelectric point calculations were performed using DNASIS software. A database homology search was performed with the BLAST program provided by NCBI.

Southern Blot Analysis. Total DNA from *Klebsiella* sp. strain ZD112 digested with a restriction enzyme was separated with 1% agarose gel electrophoresis, transferred onto a Hybond-N nylon membrane, and probed with the labeled pyrethroid-hydrolyzing esterase gene (*estP*) fragment. All hybridization procedures were performed as described by Sambrook et al. (*16*). The DNA probe was labeled using a random-primed labeling kit, following the manufacturer's recommendations.

Gene Expression and Purification of Pyrethroid-Hydrolyzing Esterase. The open reading frame of the putative *estP* was amplified by PCR and inserted into the pET29a expression vector. This recombinant plasmid, designated as pET−*estP*, was used for the expression of the pyrethroid-hydrolyzing esterase gene in *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) carrying pET−*estP* was grown in LB medium containing ampicillin (100 *μ*g/mL) at 37 °C by induction with 0.1 mM IPTG when the optical density at 660 nm reached 0.4. After induction for 12 h, 9.4 g of cell pellets were harvested and disrupted by sonification. Cell debris was removed by centrifugation (10000*g* for 20 min at 4 °C). The supernatant was the crude extract of the enzyme. Solid ammonium sulfate was added to the supernatant to 30% saturation with constant stirring. After being stirred for 20 min, the mixture was centrifuged at 15000*g* for 20 min. The precipitate was discarded. Additional solid ammonium sulfate was added to the supernatant to 80% saturation with constant stirring. The precipitate was collected by centrifugation at 15000*g* for 20 min, dissolved in the smallest possible volume of 50 mM potassium phosphate buffer (pH 7), dialyzed 100-fold against the same buffer, and concentrated by ultrafiltration through a YM-30 membrane (Millipore). The concentrated enzyme solution was loaded onto a 500 mL Phenyl Sepharose column that had been preequilibrated with 50 mM potassium phosphate buffer (pH 7) containing a 25% saturation of ammonium sulfate. Subsequently, the column was washed with 100 mL of the equilibration buffer; then proteins were eluted with a decreasing gradient of ammonium sulfate. Fractions containing the enzyme activity were pooled, concentrated, dialyzed against 50 mM potassium phosphate buffer (pH 7), and loaded onto a DEAE-Sepharose CL-6B ion-exchange column preequilibrated with 50 mM potassium phosphate buffer (pH 7). The column was washed at a flow rate of 20 mL h^{-1} with 500 mL of the same buffer, and proteins were eluted with a linear gradient of NaCl from 0 to 1.0 M. The active fractions were collected and concentrated by ultrafiltration through a YM-30 membrane (Millipore). The concentrated enzyme solution was applied to a Sephacryl 200 HR column equilibrated with 50 mM potassium phosphate buffer (pH 7). Proteins were eluted at a flow rate of 0.3 mL min^{-1}, and fractions of 3 mL were collected. The protein concentration was determined by the method of Bradford; bovine serum albumin (Sigma) was used as the standard for calibration (*17*).

Determination of the Molecular Mass and Isoelectric Point. The molecular mass of the denatured protein was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). An SDS−12.5% polyacrylamide gel was prepared by the method of Laemmli (*18*). Proteins were stained with Coomassie Brilliant Blue G. The molecular mass of the enzyme subunit was estimated using a protein marker as the standard. The molecular mass of the native protein by gel filtration on a Superose 12HR 5/30 column, *γ*-globulin (160000 Da), bovine serum albumin (67000 Da), ovalbumin (43000 Da), and carbonic anhydrase (30000 Da), was used as the reference protein

molecular mass. The isoelectric point (pI) was estimated by PAGE with 6.25% ampholine (pH 3.5−10) in a gel rod (0.5 × 10 cm) using a kit for isoelectric focusing calibration according to recommendations by the supplier.

Enzyme Activity. The esterase activity against ρ-nitrophenyl esters was determined by measuring the amount of ρ-nitrophenol released by esterase-catalyzed hydrolysis. The hydrolysis of the substrate was performed at 30 °C for 10 min in 50 mM sodium phosphate buffer (pH 7.0) containing 1% acetonitrile. The production of ρ-nitrophenol was monitored at 348 or 405 nm according to different pH values by a Labsystems Dragon Wellscan MK3. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of ρ-nitrophenol/min from the substrate under these conditions (*19*). The pyrethroid-hydrolyzing esterase activities against pesticides and the pesticide assay were performed as described by Liang et al. (*15*).

The optimum pH of the pyrethroid-hydrolyzing esterase was measured using ρ-nitrophenyl acetate as a substrate (1 mM) at 30 °C. The buffers (at a final concentration of 50 mM) used for the measurement are described as follows: citric acid−NaOH (pH 3.5−5.5), potassium phosphate (pH 5.0−7.0), Tris−HCl buffer (pH 6.5 to 9.0), glycine−NaOH buffer (pH 8.5−10.0). Overlapping pH values were used to verify that there were no buffer effects on substrate hydrolysis. The optimum temperature was determined analogously by measuring the esterase activity at pH 7.0 in the temperature range of 20−70 °C. The pH stability was tested after incubation of the purified enzyme (0.23 μg/mL) for 2 h at 30 °C in the above different buffers. The temperature stability was measured after incubation of the purified enzyme (0.23 μg/mL) in 50 mM sodium phosphate buffer (pH 7.0) for 1 h at different temperatures (*20*, *21*). The effects of various chemicals ($CaCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $MnCl_2$, $AgNO_3$, $HgCl_2$, EDTA, phenanthroline, ρ-chloromercuribenzoate) on the pyrethroid-hydrolyzing esterase activity were investigated by addition of the tested compounds into the reaction mixture, which was preincubated for 30 min at 30 °C. The activity was then measured as described above and expressed as a percentage of the activity obtained in the absence of the added compound. Taking into consideration the poor solubility of most metal ions in phosphate buffer, we use 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)−NaOH buffer (pH 7.0) in place of 50 mM sodium phosphate buffer.

Determination of Kinetic Parameters. Kinetic parameters against ρ-nitrophenyl esters were determined by measuring the enzyme activity using ρ-nitrophenyl acetate ranging from 0.02 to 1.5 mM, ρ-nitrophenyl propionate ranging from 0.01 to 0.8 mM, ρ-nitrophenyl butyrate ranging from 0.008 to 0.5 mM, ρ-nitrophenyl caproate ranging from 0.003 to 0.2 mM, ρ-nitrophenyl caprylate ranging from 0.005 to 0.4 mM, and ρ-nitrophenyl laurate ranging from 0.03 to 2 mM as a substrates in 50 mM sodium phosphate buffer (pH 7.0) containing 1% acetonitrile at 30 °C. Kinetic parameters against different pesticides were analogously determined by measuring the enzyme activity over a range of final concentrations from 0.005 to 7 μM dependent on the different pesticides. All initial velocities were determined at five time points, at which which no more than 10% of the substrate had been consumed, and the solution content never exceeded 1% of the total assay volume, so the decrease in substrate concentration remained linear with time over the period of measurement and the rate was almost constant throughout the assay; r^2 values ranged from 0.976 to 0.988 according to the different substrates. Initial reaction velocities measured at various concentrations of substrates were fitted to the Lineweaver−Burk transformation of the Michaelis−Menten equation (*22*). Kinetic analyses by curve fitting were performed with SigmaPlot software (*15*).

Nucleotide Sequence Accession Numbers. The nucleotide sequence data reported here have been submitted to the Nucleotide Sequence Database under accession numbers AY995176 (*estP*) and AY989899 (16S ribosomal DNA [rDNA]).

RESULTS

Isolation and Identification. The enrichment procedures generated a pure culture designated ZD112 that hydrolyzed many pyrethroids quickly. The strain was identified on the basis of classification schemes published in *Bergey's Manual of Systematic Bacteriology* and the 16S rRNA gene sequence (*23*, *24*). The strain ZD112 is Gram stain and catalase negative and oxidase, urease, and nitrate reduction positive, G ± C content 55.4 mol %, straight rod-forming shape with a capsule and dimensions of 0.9−1.2 μm width and 2.1−2.5 μm length. The 16S rRNA gene sequence of strain ZD112 was 99% similar to that of the 16S rRNA gene of *Klebsiella* sp. strain zmmo (GenBank accession no. U31075), 98% similar to that of the 16S rRNA gene of *Klebsiella pneumoniae* (GenBank accession no. X87261), and 98% similar to that of the 16S rRNA gene of *K. pneumoniae* ssp. *ozaenae* (GenBank accession no. AF228919). According to these properties described above, the isolate was designated *Klebsiella* sp. strain ZD112.

Cloning and Sequence Analysis of the Pyrethroid-Hydrolyzing Esterase Gene. Genomic DNA was digested with *Sau*3AI and inserted into pUC18. *E. coli* DH5α transformants were plated onto LB agar plates containing 100 μg of ampicillin/mL, 100 μM X-Gal, and 0.1 mM IPTG. The 38000 white transformants with foreign fragment DNA were chosen as candidate clones. These candidate white transformants were subsequently replicated onto LB agar plates containing 100 μg of ampicillin/mL and 100 μM X-caprylate; 67 blue colonies were selected and further tested for the hydrolysis of pyrethroid. The ability of the two positive blue colonies, *E. coli* DH5α (pestP1) and *E. coli* DH5α (pestP2), to hydrolyze pyrethroid was confirmed by GC−MS analysis. The two plasmids were isolated, sequenced, and found to contain identical inserts, indicating that both plasmids harbor the same fragment of chromosomal DNA inserted in the same orientation, most likely the same pyrethroid-hydrolyzing esterase gene. Taking into consideration that the pyrethroid-hydrolyzing esterase activity of *E. coli* DH5α (pestP1) was higher than that of *E. coli* DH5α (pestP2), *E. coli* DH5α (pestP1) was used for further analysis. The size of the cloned fragment in plasmid pestP1 was reduced when the fragment was digested with various restriction enzymes, and the deletion derivatives were screened according to the pyrethroid-hydrolyzing esterase activity, resulting in the subclone *E. coli* DH5α (pestP3). The sequence analysis indicated that a 3.2 kb *Hin*dIII fragment was found to exhibit pyrethroid-hydrolyzing esterase activity. The positive clone (pestP3) clearly showed that *estP* is present in the *Klebsiella* sp. strain ZD112 genome by Southern blot analysis. In the 3.2 kb DNA fragment, we found an open reading frame (ZD-Est) consisting of 1914 nucleotides, as shown in **Figure 1**, starting with an ATG initiation codon at position 115 and ending with a TGA termination codon at position 2027. A putative ribosome-binding site (GAAGGA) is located 21 nucleotides upstream from the initiation codon; possible −10 (TTAAA) and −35 (TTGAAG) promoter sequences separated by 20 nucleotides were detected upstream of the structural gene (*estP*) (*25*). The four tandem repeats of an A-rich box (AAAGAAA) existed between the −35 and −10 regions. The *estP* gene encoded a protein of 637 amino acids with a predicted molecular mass of 73000 Da. There is no signal peptide amino acid sequence in mature protein from the putative start codon to the N-terminal. The G + C content in the coding region is 41%.

Amino Acid Sequence Alignment. Similarities to the amino acid sequence of pyrethroid-hydrolyzing esterase from *Klebsiella* sp. strain ZD112 were sought among the sequences in the GenBank database by using the BLAST server. No putative conserved domains have been detected, but the search revealed significant similarities of EstP to several hypothetical proteins. The alignment of EstP (amino acids 1−637) with the *Salmonella enterica* hypothetical protein SC171 derived from AAS76447.1 (amino acids 222−860) showed 88% identical and 94% similar amino acids. The alignment of EstP (amino acids 1−633) with *Azoarcu*s sp. EbN1 hypothetical protein ebA4602 derived from

```
                                                                    GTCGCATCAATTCTTAGCAATGCACAAACCATCCTCAAAAAGCCCAAAGTTGAAGTG

                                                                                                                        -35

                                                                    AAAGAAATTCAAGCGTTAAAGCCAGCAAAAGAAGGACGTCCTGTTCCACGTACATTT

                                                                                        -10                 RBS

1       ATGGAGATTTGTACCAAAGGTTCGCGTAAGCATTTAACGAGCCGCGCATCTGAGCCG

1        M   E   I   C   T   K   G   S   R   K   H   L   T   S   R   A   S   E   P

58      AGTTACAACGTGCCAGAAAACCAGTATGTGCTGTATGTGGTTTCGAGCACGCTGAGC

20       S   Y   N   V   P   E   N   Q   Y   V   L   Y   V   V   S   S   T   L   S

        ..........................................................................................

1776    TACACACTTTACCGTGTATACCCACTGTGGTACATGCAATACACGATTAATTAAGAA

593      T   H   F   T   V   Y   T   H   C   G   T   C   N   T   R   L   I   K   N

1833    TGGCGAGTATTGGACCTATTTGTCTTTGATGCCTATGTCTTCAATTAACATAAAGTGT

612      G   E   Y   W   T   Y   L   S   L   M   P   M   S   S   I   N   I   K   C

1891    CCAAACTGCGAGTCTCCCGTATGACGGGTGACCTGCTCCCCGTATTTTCACACAGAC

631      P   N   C   E   S   P   V   *

        TGCTGTTAGTTACTTCCGCCTTTGGGAGTTTTCGGATCTGCGGTGGAAAAATGAGAAA

        GCAGGATATGCACAGCATATCCATATGCCGCGCATCTGCATAGGGCAGACATACGCATAT
```

Figure 1. Partial nucleotide sequence and deduced amino acid sequence of the *estP* gene from *Klebsiella* sp. strain ZD112. The proposed ribosomal binding site (RBS) and –10 and –35 regions of the putative promoter are underlined. The stop codon is marked by an asterisk.

YP159633.1 (amino acids 222−863) showed 36% identical and 56% similar amino acids.

Overexpression and Purification of Recombinant EstP. We produced recombinant EstP from *Klebsiella* sp. strain ZD112 in *E. coli* BL21 (DE3). The recombinant EstP was purified from the crude extract to homogeneity by successive ammonium sulfate precipitation and Phenyl Sepharose, DEAE-Sepharose CL-6B, and Sephacryl 200 HR chromatography. The results of the overall purification procedure are summarized in **Table 1**. Up to the final purification step, the enzyme was purified 66.6-fold, with a recovery yield of 16.3%. The homogeneity of the protein was confirmed by SDS−PAGE (**Figure 2**). The molecular mass of the purified protein was 73000 Da, in good agreement with the molecular mass deduced from the nucleotide sequence (73500 Da). The relative molecular mass of the native enzyme estimated by gel filtration on a calibrated column of Sephacryl 200 HR was 73000 Da. Hence, it is assumed that the purified enzyme (EstP) is a monomer. The pI value was estimated to be 8.6. The N-terminal amino acid sequence of the native protein, MEICTKGSRKHLTSRASE, was identical to the sequence deduced from the nucleotide sequence, confirming that the purified enzyme was the protein product of EstP.

Table 1. Summary of Purification of Recombinant EstP from *E. coli* BL21 (DE3)

purification step	total protein amt (mg)	total activity[a] (U)	specific activity (U/mg)	purification (fold)	yield (%)
crude extract	238 ± 11	879 ± 42	3.7 ± 0.17	1	100
$(NH_4)_2SO_4$	49 ± 2.3	554 ± 26	11.3 ± 0.53	3.05	63
Phenyl Sepharose	9 ± 0.4	281 ± 12	31.5 ± 1.62	8.5	32
DEAE-Sepharose CL-6B	2.9 ± 0.15	207 ± 10.3	71.4 ± 3.6	19.3	23.5
Sephacryl 200 HR	0.58 ± 0.03	143 ± 7.4	246.6 ± 11.8	66.6	16.3

[a] The enzyme activity was measured using *ρ*-nitrophenyl acetate (1 mM) as the substrate.

Effect of the pH and Temperature on the Catalytic Activity and Stability. With *ρ*-nitrophenyl acetate as a substrate, the pH ranged from 3.5 to 10.0 and the temperature ranged from 20 to 70 °C. Despite the absorption of *ρ*-nitrophenol varying when the pH is altered because of changes in the equilibrium between *ρ*-nitrophenol and *ρ*-nitrophenoxide, *ρ*-nitrophenol and *ρ*-nitrophenoxide had a pH-independent isosbestic wavelength at 348 nm. Therefore, the release of *ρ*-nitrophenol was monitored at 348 nm. The pH−activity profile of the enzyme was bell-

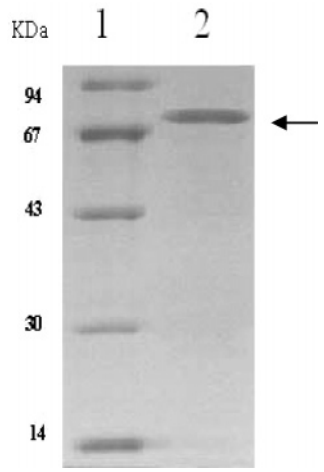

Figure 2. SDS–PAGE analysis of the purified EstP (lane 2) and protein markers (lane 1) stained with Coomassie Blue. Markers from the top to the bottom are phosphorylase b (94000 Da), bovine serum albumin (67000 Da), ovalbumin (43000 Da), carbonic anhydrase (30000 Da), and α-lactalbumin (14000 Da).

Table 2. Kinetic Constants for Hydrolysis of Various ρ-Nitrophenyl Esters

substrate	specific activity (U/mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
ρ-nitrophenyl acetate	91 ± 4.2	261 ± 13	81 ± 4	0.31
ρ-nitrophenyl propionate	269 ± 13	139 ± 7	238 ± 12	1.71
ρ-nitrophenyl butyrate	355 ± 17	92 ± 4	314 ± 15	3.41
ρ-nitrophenyl caproate	573 ± 28	37 ± 1.6	507 ± 23	13.7
ρ-nitrophenyl caprylate	407 ± 18	68 ± 3.3	360 ± 17	5.3
ρ-nitrophenyl laurate	67 ± 3	339 ± 16	59 ± 4	0.17
ρ-nitrophenyl myristate	0	0	0	0
ρ-nitrophenyl palmitate	0	0	0	0

shaped, with maximum values at pH 7.0. The enzyme was found to be stable in the pH range of 5.5−9.0. The optimal temperature for the enzyme was 40 °C. The enzyme was fairly stable up to 45 °C and had 54% of its activity at 50 °C. It was completely inactivated at 65 °C.

Effects of Reagents and Metal Ions on the Enzyme Activity. The presence of Hg^{2+} and Ag^{+} caused a complete inhibition at 0.5 mM, while a less pronounced effect (3−8% inhibition) was observed in the presence of the divalent cations (0.5 mM). The enzyme activity was strongly inhibited by 0.1 mM ρ-chloromercuribenzoate, whereas the chelating agent EDTA and phenanthroline (1 mM) showed little effect on the enzyme activity.

Substrate Specificity. The substrate specificity toward ρ-nitrophenyl esters of various fatty acids is shown in **Table 2**. EstP showed the highest activity with ρ-nitrophenyl caproate (573 U/mg) among the ρ-nitrophenyl esters examined. Both K_m and k_{cat} values of purified EstP decreased with increases in the aliphatic chain length up to C_6. The comparison of catalytic efficiency values (K_{cat}/k_m) for various substrates indicated that these values were dependent on the aliphatic chain length of the substrate. Medium-chain ρ-nitrophenyl esters seemed to be the preferred substrates, whereas ρ-nitrophenyl esters of longer chain fatty acids were poor substrates; EstP had no activity against ρ-nitrophenyl myristate and ρ-nitrophenyl palmitate. The activity against ρ-nitrophenyl esters of shorter chain fatty acids was lower than that of ρ-nitrophenyl caproate. Taking into consideration that lipases prefer substrates with relatively long aliphatic chains, these results showed the purified enzyme (EstP) is an esterase and not a lipase.

Table 3. Kinetic Constants for Hydrolysis of Various Pesticides

substrate	specific activity (U/mg)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
cis-permethrin	35.6 ± 1.7	0.16 ± 0.007	1.05 ± 0.051	6.56
trans-permethrin	38.8 ± 1.9	0.11 ± 0.005	1.14 ± 0.055	10.36
cypermethrin	9.8 ± 0.48	0.21 ± 0.01	0.288 ± 0.012	1.37
fenvalerate	3.5 ± 0.18	0.91 ± 0.043	0.103 ± 0.005	0.113
deltamethrin	0.53 ± 0.025	1.23 ± 0.056	0.016 ± 0.0007	0.013
malathion	2.4 ± 0.1	0.87 ± 0.041	0.07 ± 0.033	0.81

A range of pesticides such as malathion, pyrethroid, *cis*-permethrin, *trans*-permethrin, cypermethrin, fenvalerate, and deltamethrin were tested for substrate specificity of the recombinant EstP. EstP hydrolyzed the pesticides tested at different hydrolysis rates; *trans*-permethrin was hydrolyzed most rapidly, while deltamethrin was the least readily attacked, and *cis*-permethrin was hydrolyzed at a rate approximately equal to that of *trans*-permethrin. The K_m and k_{cat} values were calculated by fitting the data to the Michaelis−Menten equation (**Table 3**). The purified enzyme showed different K_m values ranging from 0.11 to 1.23 μM against a range of tested pesticides.

DISCUSSION

EstP from *Klebsiella* sp. strain ZD112 not only efficiently hydrolyzed ρ-nitrophenyl esters of medium- to short-chain fatty acids, but also degraded all pesticides tested, indicating that EstP is an esterase with broad substrates. Although there have been some reports on pyrethroid hydrolase from pyrethroid-resistant insects, mammal organ, *A. niger*, and *B. cereus* (*9*, *15*, *26*, *27*), some nucleotide sequences of the pyrethroid-hydrolyzing enzyme genes are available (accession nos. NM-133960, NM-144930, AY487948, Q964Q7, and Q27698). However, the pyrethroid pesticide degradative gene from the microorganisms has not as yet been reported. This is the first one to be determined for the pyrethroid-hydrolyzing enzyme gene from microorganisms.

Database searches revealed the similarity of pyrethroid-hydrolyzing esterase from *Klebsiella* sp. strain ZD112 to several putative proteins, but the sequences of EstP showed no similarities to any esterase or lipase sequence deposited in the databases. It is interesting that EstP reported herein showed that no conserved domains or binding sites were found in the sequence of pyrethroid-hydrolyzing esterase from *Klebsiella* sp. strain ZD112, considering that EstP possessed esterase activity. In general, the three-dimensional structures of many esterases and lipases show the characteristic α/β-hydrolase fold—a definite order of α-helices and β-sheets (*11*). The catalytic triad is composed of Ser-Asp-His (Glu instead of Asp for some lipases) (*28*), and usually also a conserved pentapeptide (Gly-X-Ser-X-Gly) is found around the active site serine. More recently, some esterases contained a Gly-X-X-Leu motif (*29*, *30*). Since pyrethroid-hydrolyzing esterase from *Klebsiella* sp. strain ZD112 has no sequence similarity to the esterases and lipases and also does not contain the conserved sequence motif G-X-Ser-X-G and Gly-X-X-Leu, it may be assumed that this enzyme may have evolved independently, as in the case of a novel carbaryl hydrolase from *Rhizobium* sp. strain AC100 (*31*). This carbamate pesticide hydrolase not only degraded many carbamate pesticides but also hydrolyzed 1-naphthyl acetate and 4-nitrophenyl acetate, indicating that the carbaryl hydrolase is an esterase, but no homology was found by a database homology search using nucleotide and deduced amino acid sequences of the esterases and lipases. The phloretin hydrolase from *Eubacterium ramulus* possesses properties similar to those of the two pesticide-hydrolyzing enzymes described above (*32*).

To isolate the gene responsible for pyrethroid-hydrolyzing esterase, taking into consideration the complexity in the course of directly cloning the pyrethroid-hydrolyzing esterase gene, in this study, we first selected 67 blue transformants with esterase activity in LB agar plates containing 100 μg of ampicillin/mL and 100 μM X-caprylate. The ability of those blue transformants with esterase activity to hydrolyze permethrin was demonstrated by assaying permethrin reduction and 3-phenoxybenzyl alcohol formation; only two transformants with esterase activity can hydrolyz permethrin. These results showed that not all esterases degrade pyrethroids; therefore, pyrethroid-hydrolyzing esterase from *Klebsiella* sp. strain ZD112 possessed broad substrates. The gene for EstP from *Klebsiella* sp. strain ZD112 was cloned from genomic DNA and expressed in *E. coli* BL21 (DE3), and its product was purified to apparent homogeneity and characterized. As a monomeric 73000 Da protein, the molecular mass of EstP is larger than those of the purified permethrinase (61000 Da) from bacteria, pyrethroid hydrolase (56000 Da) from *A. niger* ZD11, pyrethroid-hydrolyzing carboxylesterase (60000 Da) from mouse liver microsomes, and carboxylesterase E3 (58600 Da) from *Nephotettix cincticeps* Uhler. The pH optimum of EstP (pH 7.0) was lower than that recorded for *B. cereus* (pH 7.5) and higher than that reported for *A. niger* ZD11 (pH 6.5). The optimal temperature of 40 °C is similar to that recorded for *B. cereus* (37 °C) and lower that reported for *A. niger* ZD11 (45 °C) (*13*, *15*).

The substrate specificity was studied by the purified recombinant enzyme. The striking features for ρ-nitrophenyl caproate and ρ-nitrophenyl caprylate were quite different from the specificities of other microbial esterases (*33*), which are mainly specific for ρ-nitrophenyl esters of short-chain fatty acids. Only the enzymes from *Bacillus stearothermophilus* (*34*), *Sulfolobus acidocaldarius* (*35*), *Bacillus licheniformis* (*36*), and *Lactobacillus casei* CL96 (*37*) showed specificity patterns similar to that of EstP, since the purified recombinant enzyme hydrolyzed cypermethrin, permethrin, fenvalerate, deltamethrin, and malathion. Therefore, pyrethroid-hydrolyzing esterase seems to be capable of hydrolyzing a relatively wide range of compounds with similar chemical linkages (**Figure 3**), suggesting that it possesses broad substrates; the feature is the same as that of pyrethroid hydrolase from *A. niger* ZD11 (*15*). However, this observation does not quite agree with data reported by Motoyama et al. and Stok et al. (*9*, *38*). Pyrethroid-hydrolyzing carboxylesterase (BAC36707) from mouse liver microsomes did not hydrolyze malathion. On the other hand, Motoyama et al. found that one of five forms of carboxylesterases degraded malathion twice as fast as fenvalerate, and three other forms possessed approximately equal activity toward these two insecticides. In a previous paper, there was a preference in both mammal and insect carboxylesterases for permethrin over cypermethrin, and *trans*-permethrin over *cis*-permethrin (*8*, *9*), while carboxylesterase from *N. cincticeps* Uhler preferred *cis*-permethrin over *trans*-permethrin. However, pyrethroid-hydrolyzing esterase possessed approximately equal activity toward the permethrin isomers. Therefore, it lacked stereoselectivity; this feature is different from that of carboxylesterase E3 from *N. cincticeps* Uhler (*8*) and carboxylesterase BAC36707 (*9*). In addition, the apparent K_m values obtained for the purified enzyme (EstP) were lower than those for BAC36707 from mouse liver microsomes, carboxylesterase E3 from *N. cincticeps* Uhler, and pyrethroid hydrolase from *A. niger* ZD11 when the same substrates were used, indicating a higher affinity for these substrates in the case of EstP. The comparison of K_m and k_{cat} revealed that pyrethroid-hydrolyzing esterase has about 11-fold higher affinity toward *trans*-permethrin than deltamethrin and can hydrolyze the former about 71-fold faster than the latter.

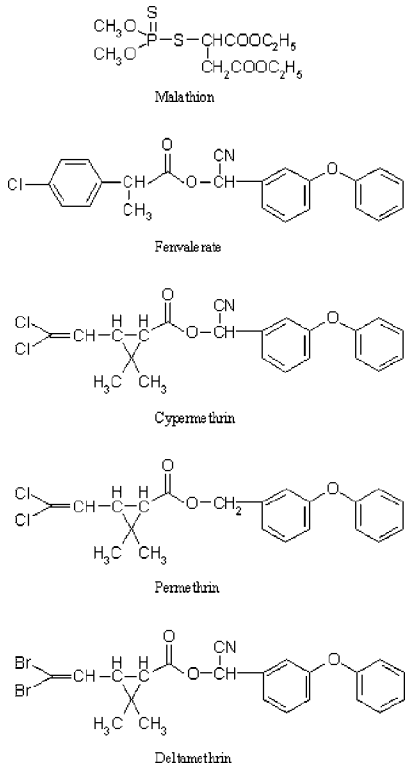

Figure 3. Structures of all pesticides tested.

The catalytic efficiencies (k_{cat}/K_m) are considered as a measurement of the enzyme's specificity; among these substrates *trans*-permethrin is clearly the preferred substrate.

In a word, the pyrethroid-hydrolyzing esterase gene from *Klebsiella* sp. strain ZD112 was cloned and sequenced, and the recombinant EstP was purified and characterized. To date, this is the first information available on the pyrethroid-hydrolyzing esterase gene from microorganisms. Taking into consideration that pesticidal residues resulting from agricultural production are complex mixtures, enzymatic bioremediation requiring the development of specific enzymes for each compound or isomer is unrealistic; therefore, the broader substrate specificities and higher activity of pyrethroid-hydrolyzing esterase are necessary to fulfill the practical requirements of bioremediation to enable its use in situ for detoxification of pyrethroids where they cause environmental contamination problems. The cloning and overexpression of the pyrethroid-hydrolyzing esterase gene can supply enzyme preparation at a low cost for environmental protection.

ACKNOWLEDGMENT

We are grateful to the National Natural Science Foundation of China (Grant 30570055), the Natural Science Foundation of Guangdong Province (Grant 031618), and the Science and Technology Plan Project of Guangdong Province (Grants 2003C32402 and 2005B10401010) for their financial support.

LITERATURE CITED

(1) Liu, W. P.; Gan, J. J.; Lee, S.; Werner, I. Isomer selectivity in aquatic toxicity and biodegradation of cypermethrin. *J. Agric. Food Chem.* **2004**, *52*, 6233−6238.

(2) Fakata, K. L.;Swanson, S. A.; Vorce, R. L.; Stemmer, P. M. Pyrethroid insecticides as phosphatase inhibitors. *Biochem. Pharmacol.* **1998**, *55*, 2017−2022.

(3) Miyamoto, J.; Kaneto, H.; Tsuji, R.; Okuno, Y. Pyrethroids, nerve posions: how their risks to human health should be assessed. *Toxicol. Lett.* **1995**, *82*, 933−940.
(4) Park, E. K.; Kim, J. H.; Gee, S. j.; Watanabe, T.; Ahn, K. C.; Hammock, B. D. Determination of pyrethroid residues in agricultural products by an enzyme-linked immunosorbent assay. *J. Agric. Food Chem.* **2004**, *52*, 5572−5576.
(5) Lutnicka, H.; Bogacka, T.; Wolska, L. Degradation of pyrethroids in an aquatic ecosystem model. *Water Res.* **1999**, *33*, 3441−3446.
(6) Garey, J.; Wolff, M. S. Estrogenic and antiprogestagenic activities of pyrethroid insecticides *Biochem. Biophys. Res. Commun.* **1998**, *251*, 855−859.
(7) Sogorb, M. A.; Vilanova, E. Enzymes involved in the detoxification of organophosphorus, carbamate and pyrethroid insecticides through hydrolysis. *Toxicol. Lett.* **2002**, *128*, 215−228.
(8) Chiang, S. W.; Sun, C. N. Purification and characterization of carboxylesterases of a rice green leafhopper *Nephotettix cincticeps* uhler. *Pestic. Biochem. Physiol.* **1996**, *54*, 181−189.
(9) Stok, J. E.; Huang, H. Z.; Jones, P. D.; Wheelock, C. E.; Morisseau, C.; Hammock, B. D. Identification, expression, and purification of a pyrethroid-hydrolyzing carboxylesterase from mouse liver microsomes. *J. Biol. Chem.* **2004**, *279*, 29863−29869.
(10) Horne, I.; Sutherland, T. D.; Harcourt, R. L.; Russell, R. J.; Oakeshott, J. G. Identification of an opd (organophosphate degradation) gene in an *Agrobacterium* isolate. *Appl. Environ. Microbiol.* **2002**, *68*, 3371−3376.
(11) Oakeshott, C.; Claudianos, C.; Russell, R. J.; Robin, G. C. Carboxyl/ cholinesterases: a case study of the evolution of a successful multigene family. *Bioessays* **1999**, *21*, 1031−1042.
(12) Sutherland, T. D.; Horne, I.; Weir, K. M.; Coppin, C. W.; Williams, M. R.; Selleck, M.; Rhssel, R. J.; Oakeshott, J. G. Enzymatic bioremediation: from enzyme discovery to applications. *Clin. Exp. Pharmacol. Physiol.* **2004**, *31*, 817−821.
(13) Maloney, S. E.; Maule, A.; Smith, A. R. W. Purification and preliminary characterization of permethrinase from a pyrethroid-transforming strain of *Bacillus cereus*. *Appl. Environ. Microbiol.* **1993**, *59*, 2007−2013.
(14) Lee, S. J.; Gan, J.; Kim, J. S.; Kabashima, J. N.; Crowley, D. E. Microbial transformation of pyrethroid insecticides in aqueous and sediment phases. *Environ. Toxicol. Chem.* **2004**, *23*, 1−6.
(15) Liang, W. Q.; Wang, Z. Y.; Li, H.; Wu, P. C.; Hu, J. M.; Luo, N.; Cao, L. X.; Liu, Y. H. Purification and characterization of a novel pyrethroid hydrolase from *Aspergillus niger* ZD11. *J. Agric. Food Chem*. **2005**, *53*, 7415−7420.
(16) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: a laboratory manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(17) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(18) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (London)* **1970**, *227*, 680−685.
(19) Khalameyzer, V.; Fischer, I.; Bornscheuer, U. T.; Altenbuchner, J. Screening, nucleotide sequence, and biochemical characterization of an esterase from *Pseudomonas fluorescens* with high activity towards lactones. *Appl. Environ. Microbiol.* **1999**, *65*, 477−482.
(20) Rhee, J. K.; Ahn, D. G.; Kim, Y. G.; Oh, J. W. New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Appl. Environ. Microbiol.* **2005**, *71*, 817−825.
(21) Wang, X. K.; Geng, X.; Egashira, Y.; Sanada, H. Purification and characterization of a feruloyl esterase from the intestinal bacterium *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.* **2004**, *70*, 2367−2372.
(22) Bzymek, K. P.; Holz, R. C. The catalytic role of glutamate 151 in the leucine aminopeptidase from Aeromonas proteolytica. *J. Biol. Chem.* **2005**, *279*, 31018−31025.
(23) Krieg, N. R., Holt, J. G., Eds. *Bergey's Manual of Systematic Bacteriology*; The Williams and Wilkins Co.: Baltimore, MD, 1984; Vol. 1.
(24) Lane, D. J. 16S/23S rRNA sequencing. In *Nucleic acid technique in bacterial systematics*; Stackebrandt, E., Goodfellow, M., Eds.; John Wiley & Sons: New York, 1991; pp 115−175.
(25) Abriouel, H.; Herrmann, A.; Starke, J.; Yousif, N. M. K.; Wijaya, A.; Tauscher, W.; Holzapfel, B. W.; Franz, M. A. P. Cloning and heterologous expression of hematin-dependent catalase produced by *Lactobacillus plantarum* CNRZ 1228. *Appl. Environ. Microbiol.* **2004**, *70*, 603−60.
(26) Feyereisen, R. J.; Koener, J. F.; Farnsworth, D. E. Isolation one sequence of cDNA encoding a cytochrome P450 from an insecticide-resistant strain of the house fly, *Musca domestica. Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 1465−1472.
(27) Kasai, S.; Shono, T. Molecular cloning and nucleotide sequence of a cytochrome P450 cDNA from a pyrethroid-resistant mosquito *Culex quinquefasciatus. Insect Mol. Biol.* **1998**, *7*, 185−191.
(28) Bresler, M. M.; Rosser, S. J.; Basran, A.; Bruce, N. C. Gene cloning and nucleotide sequencing and properties of a cocaine esterase from *Rhodococcus* sp. strain MB1. *Appl. Environ. Microbiol.* **2000**, *66*, 904−908.
(29) Bornscheuer, U. T. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS. Microbiol. Rev.* **2002**, *26*, 73−81.
(30) Wei, Y.; Schottel, J. L.; Derewenda, U.; Swenson, L.; Patkar, S.; Derewenda, Z. S. A novel variant of the catalytic triad in the Streptomyces scabies esterase. *Nat. Struct. Biol*. **1995**, *2*, 218−223.
(31) Hasshimoto, M.; Fukui, M.; Hayano, K.; Hayatsu, M. Nucleotide sequence and genetic structure of a novel carbaryl hydrolase gene (*cehA*) from *Rhizobium* sp. strain AC100. *Appl. Environ. Microbiol.* **2002**, *68*, 1220−1227.
(32) Schoefer, L.; Braune, A.; Blaut, M. Cloning and expression of a phloretin hydrolase gene rom *Eubacterium ramulus* and characterization of the recombintant enzyme. *Appl. Environ. Microbiol.* **2004**, *70*, 6131−6137.
(33) Riou, C.; Salmon, J. M.; Vallier, M. J.; Gunata, Z. Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryza. Appl. Environ. Microbiol.* **1998**, *64*, 3607−3614.
(34) Kim, H. K.; Park, S. Y.; Lee, J. K.; Oh, T. k. Gene cloning and characterization of thermostable lipase from *Bacillus. Stearothermophilus* L1. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 66−71.
(35) Sobek, H.; Gorisch, H. Further kinetic and molecular characterization of an extremely heat stable carboxylesterase from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. *Biochem. J.* **1989**, *261*, 993−998.
(36) Alvarez, M. E.; Augier, M. V.; Baratti, J. Characterization of a thermostable esterase activity from the moderate thermophile *Bacillus licheniformis*. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1865−1870.
(37) Choi, Y. J.; Miguez, C. B.; Lee, B. H. Characterization and heterologous gene expression of a novel esterase from *Lactobacillus casei* CL96. *Appl. Environ. Microbiol.* **2004**, *70*, 3213−3221.
(38) Motoyama, L. R. K.; Lin, P. T.; Dauterman, W. C. Dual role of esterases in insecticide resistance in the green rice leafhopper. *Pestic. Biochem. Physiol.* **1984**, *21*, 39−47.

Received for review October 30, 2005. Revised manuscript received December 3, 2005. Accepted December 5, 2005.

JF052691U