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Purification and Characterization of a Novel Pyrethroid Hydrolase from *Aspergillus niger* ZD11

WEI Q. LIANG,† ZHUO Y. WANG,† HE LI,† PEI C. WU, JI M. HU, NA LUO, LI X. CAO, AND YU H. LIU*

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

The pyrethroid pesticides residues on foods and environmental contamination are a public safety concern. Pretreatment with pyrethroid hydrolase has the potential to alleviate the conditions. For this purpose, a fungus capable of using pyrethroid pesticides as a sole carbon source was isolated from the soil and characterized as *Aspergillus niger* ZD11. A novel pyrethroid hydrolase from cell extract was purified 41.5-fold to apparent homogeneity with 12.6% overall recovery. It is a monomeric structure with a molecular mass of 56 kDa, a pI of 5.4, and the enzyme activity was optimal at 45 °C and pH 6.5. The activities were strongly inhibited by Hg^{2+}, Ag^{+}, and *ρ*-chloromercuribenzoate, whereas less pronounced effects (5−10% inhibition) were observed in the presence of the remaining divalent cations, the chelating agent EDTA and phenanthroline. The purified enzyme hydrolyzed various insecticides with similar carboxylester. *trans*-Permethrin is the preferred substrate.

KEYWORDS: Pyrethroid insecticides; pyrethroid hydrolase; purification; *Aspergillus niger* ZD11; degradation

INTRODUCTION

Synthetic pyrethroid insecticides have been widely used for controlling many agricultural and urban pests in agriculture, forestry, horticulture, animal and public health, and homes all around the world (*1*). They are likely to become more widely used as organophosphate insecticides including diazinon and chlorpyrifos are phased out due to the concerns regarding their safety (*2*). Extensive applications not only result in pest resistance to these insecticides, but also may lead to environmental issues and human exposure. A variety of personnel are exposed to pyrethroids during manufacture and application, diet, and drinking water. Although these compounds are widely considered safe for humans (*3*), numerous studies have shown that very high exposure to pyrethroids might cause potential problems to man (*4*). Such effects include suppressive effects on the immune system, endocrine disruption, lymph node and splenic damage, and carcinogenesis (*1*). In addition, most synthetic pyrethroids possess apparent toxicity to fish and other aquatic organisms, including aquatic invertebrates, often at concentrations less than 1 μg L^{-1} (*5*). Therefore, it is important to develop a rapid and efficient disposal process to eliminate or minimize contamination of surface water, groundwater, and agricultural products by pyrethroid insecticides.

The fate of pyrethroid pesticides in the environment is associated with both abiotic and biotic process, including evaporation, photooxidation, chemical oxidation, bioaccumulation, and microbial transformation (*6*). Microbial degradation has been deemed the most influential and significant cause of pesticides removal. Therefore, biodegradation is considered to be a reliable cost-effective technique for pesticides abatement and a major factor determining the fate of pyrethroid pesticides in the environment (*7*). Some pyrethroid carboxylesterases from pyrethroid-resistant insects have been purified and characterized (*8*). A few pyrethroid-degrading bacteria including *Bacillus cereus* SM3 (*9*), *Pseudomonas Fluorescens* (*10*), *Vibrio hollisae*, *Burkholderia picketti*, and *Erwinia carotovora* have been isolated from soils and rivers (*11*). The only pyrethroid-degrading enzyme from *Bacillus cereus* SM3 was purified and characterized. These studies have indicated that the first step in the microbial degradation and detoxification of pyrethroid compounds is the hydrosis of carboxylester linkage. There is, however, little information on pyrethroid pesticides biotransformation by fungi (*9*, *10*). Fungus is an important member of microorganisms that are critical to the biogeochemical cycle and are responsible for the bulk of the degradation of xenobiotics in the biosphere. To understand thoroughly the role of pyrethroid pesticides biodegradation by microbes, it is necessary to carry out studies on isolation of fungi, metabolic mechanism, and molecular biology, and evaluate objectively their role. Taking into consideration that many synthetic organic compounds in many cases, although biodegradable, may persist in nature for a long time, the populations and microorganisms bringing about their destruction are not large or active enough (*12*, *13*). A low biodegradation rate resulting from low microbial growth or insufficient quantities of organism is one of the common

* Author to whom correspondence should be addressed [telephone 86-20-84113712; fax 86-20-84036215; e-mail ls112@zsu.edu.cn].
† These authors contributed equally to this work.

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obstacles that must be overcome in developing bioremediation process for chemical pollutants; this limitation may be overcome by the following two ways. One way involves the introduction of appropriate plasmid-borne catabolic genes into well-established and competitive indigenous populations; an alternative approach is bioaugmentation by inoculation of a habitat with microorganisms that survive adverse condition (*14*−*16*). The ability of fungi to produce spores is an attractive feature for improving efficiency of remediation of contaminated environment; there is a great deal of interest in fungus with the broad-spectrum substrate specificity and high catalytic turnover rate for various pyrethroid pesticides, making it a promising candidate for managing exposure to pyrethroid pesticides. This paper describes the purification and characterization of a novel pyrethroid hydrolase from *Aspergillus niger* ZD11, previously isolated from the pesticides contaminated soil, in an attempt to better understand its specific role on pyrethroid pesticides degradation. To our knowledge, this is the first broad-spectrum pyrethroid hydrolase purified to homogeneity from fungi, which degrades various pesticides containing similar carboxylester linkage effectively, and further genetic studies may lead to the discovery of novel genes involved in the future.

MATERIALS AND METHODS

Microorganism Isolation and Culture Conditions. *Aspergillus niger* ZD11, which was used in all experiments, was isolated from the soils where pesticides were heavily used. The minimal medium (MM medium) consisted of 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% NaCl, 0.001% $MnSO_4$, and 0.005% $CaCl_2$ (pH 6.8). After being autoclaved, the pyrethroid pesticide was prepared by dissolving it into 10 times its volume of ethanol and added at the concentration (200 mg/L). The soil sample was inoculated into MM medium, and then pyrethroid-degrading fungi were enriched by repeating subcultivations. Appropriate dilutions of the enrichment culture were placed on MM agar plates and incubated at 30 °C for 3 days. The colonies were selected from the plates, and the positive colony was isolated and purified. The pyrethroid hydrolysis was further confirmed by pyrethroid-degrading experiments, quantification, and identification of pyrethroids, and transformation products were based on retention times and pear areas of pure standards through GC−MS (*4*, *17*). The pesticide assay was conducted using a Hewlett-Packard (Palo Alto, CA) 6890 gas chromatograph equipped with a 5973 mass spectrometer, and the MS chemstation with window system (1701AA series) software was used for analysis. A 30 m × 0.25 mm × 0.25 μm HP-5MS fuse-silica capillary column, coated with 5% biphenyl and 95% dimethylpolysiloxane (from HP Inc.), was employed. The column temperature program was set as follows: 90 °C (hold for 5 min) to 265 °C at 30 °C/min, 265 °C to 300 °C at 4 °C/min, hold for 4 min. The GC injector port was used in the "splitless" mode and held isothermally at 270 °C for injections for the duration of the run. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1} by using electronic pressure control. The GC/MS interface temperature was maintained at 280 °C. The MS was operated in the electron impact (EI) ionization mode with electron energy of 70 eV, and mass-to-charge ratio scan ranged from 50 to 550 amu to determine appropriate masses for selected ion monitoring. The MS ion source and mass filter (quad) temperatures were held at 230 and 150 °C, respectively. Extraction of the synthetic pyrethroids was performed using solid-phase microextraction (SPME) equipment. The solid-phase microextraction (SPME) manual fiber holder and poly(dimethylsiloxane) (PDMS) with 100 μm-thickness coated fibers were from Supelco Inc. (Bellefonte, PA). The SPME fibers were conditioned under helium for 1−2 h in hot GC injection port before extraction. The SPME sampling stand and heat/stir plate used in extraction were also from Supelco. The magnetic stirring bars (10 × 3 mm) were from Aldrich (St. Louis, MO). The sampling was performed by immersing a SPME fiber directly into the 3 mL sample solution or at 25 °C under magnetic stirring condition for an appropriate period for the absorption of analytes onto the fiber coating.

Chemicals and Reagents. Sephadex G-100, DEAE-Sepharose CL-6B, and DEAE-Sephacel were purchased from Amersham Pharmacia Biotech Co. (UK). Cypermethrin (98%), *trans*-permethrin and *cis*-permethrin (99%), fenvalerate (98%), malathion (98%), and deltamethrin (98%) were kindly provided by Zhong shan pesticide factory (Guang dong, 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.

Enzyme Purification. All of the experiments described below were carried out between 0 and 4 °C unless otherwise specified.

(a) Preparation of Crude Extract. For enzyme production, the medium was inoculated with *Aspergillus niger* ZD11 viable spores. The culture was incubated at 30 °C for 5 days in 500-mL Erlenmer flasks containing 200 mL of medium on a rotary shaker at 150 rpm, harvested by centrifugation at 10 000*g* for 20 min at 4 °C, washed twice with cold 50 mM Tris-HCl buffer (pH 6.8), and stored at −20 °C until used later.

Next, 12.6 g of washed mycelia was resuspended in 50 mM Tris-HCl buffer (pH 6.8) and disrupted in a vibration homogenizer (Vibrogen vi4, Edmund tubingen, German) with glass beads (0.1 mm in diameter). After standing at 4 °C overnight, the suspension was centrifuged (12 000*g* for 20 min at 4 °C) to remove the unbroken cells and cellular debris. The suspension was centrifuged at 20 000*g* for 30 min, the supernatant was subjected to ultracentrifugation at 100 000*g* for 1 h, and the resulting supernatant was used as an enzyme source for subsequent enzyme purification.

(b) Ammonium Sulfate Precipitation. The 100 000*g* supernatant was brought to 40% ammonium sulfate saturation and stirred for 30 min, the cloudy suspension was centrifuged at 20 000*g* for 30 min, and supernatant was brought to 90% ammonium sulfate saturation; after being stirred for 30 min, the pellet obtained by centrifugation at 20 000*g* for 30 min was dissolved in the smallest possible volume 50 mM Tris-HCl buffer (pH 6.8) and dialyzed 1000-fold against 50 mM Tris-HCl buffer (pH 6.8), and the supernatant was filtered through a 0.22 μm membrane (Millipore) and concentrated.

(c) Gel Filtration through Sephadex G-100. The concentrated enzyme solution was loaded onto Sephadex G-100 column (1.8 × 100 cm) preequilibrated with 50 mM Tris-HCl buffer (pH 6.8). The column was washed at a flow rate of 24 mL/h with 400 mL of the same buffer, and 5-mL fractions were collected. Proteins were eluted in fractions 15−76, whereas the enzyme activity was confined to fractions 26−34. The fractions with high specific activity were then pooled and concentrated for further purification.

(d) Ion-Exchange Chromatography. The concentrated enzyme was loaded onto a TSK-DEAE column (30 × 2.5 cm) that had been equilibrated with 50 mM Tris-HCl buffer (pH 6.8). The column was washed with 150 mL of the same buffer, and proteins were eluted with a linear gradient of NaCl from 0 to 1 M. Fractions (2.5 mL) were collected every 6 min and screened for enzyme activity; fractions that eluted with the running buffer and exhibited enzyme activity were pooled, dialyzed overnight, lyophilized, dissolved in 50 mM Tris-HCl buffer (pH 6.8), and placed on a DEAE-Sepharose CL-6B column (30 × 2 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 6.8). The column was eluted with a linear gradient of NaCl from 0 to 0.5 M at a flow rate of 24 mL/h, 2-mL fractions were collected and tested for enzyme activity, and active fractions that eluted with the NaCl gradient were pooled, dialyzed 1000-fold against 50 mM Tris-HCl buffer (pH 6.8) and lyophilized, and loaded on a Sephacryl S-200 column (90 × 1 cm). They were then equilibrated and washed with 50 mM Tris-HCl buffer (pH 6.8), and 1-mL fractions were collected. Active fractions were pooled for subsequent analysis.

Determination of Protein. Protein amounts were determined by the method of Bradford. Bovine serum albumin (Sigma) was used as standard for calibration (*18*).

Determination of 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 substrate was performed at 30 °C for 10 min in 50 mM sodium phosphate buffer (pH 6.5) containing 1% acetonitrile. The production of ρ-nitrophenol was monitored at 405 nm by Labsystems Dragon Wellscan MK3. One unit of enzyme activity was defined as the amount

Table 1. Summary of Purification of Pyrethroid Hydrolase from *Aspergillus niger* ZD11

purification step	total protein (mg)	total activity (units)[a]	Sp act (units mg^{-1})	purification (fold)	yield (%)
crude extract	151 ± 8	693 ± 35	4.6 ± 0.24	1	100
$(NH_4)_2SO_4$	37.6 ± 1.8	613 ± 31	16.3 ± 0.82	3.5	88
Sephadex G-100	10.5 ± 0.51	432 ± 22	41 ± 2	8.9	62.3
TSK-DEAE	2.48 ± 0.12	216 ± 11	87 ± 4.6	18.9	31
DEAE-sephrose CL-6B	1.23 ± 0.06	152 ± 7	123 ± 6	26.7	22
Sephacryl S-200	0.46 ± 0.02	87 ± 4	191 ± 9.6	41.5	12.6

[a] Enzyme activity was measured using ρ-nitrophenyl acetate as substrate.

of enzyme that produced 1 μmol of ρ-nitrophenol per minute from substrate under these conditions (*19*). Pesticide hydrolase activity assays were carried out according to the methods of Huang et al., Wheelock et al., and Stok et al. (*20*−*22*). All pesticides were prepared in ethanol (25 mM). The enzyme specificity was determined by adding 4 μL (25 mM in ethanol) of substrate solution to 2 mL enzyme solution for a final concentration of 25 μM and incubating for 5 min at 30 °C. After that, samples were extracted, and the remaining pesticides were quantified by GC−MS. In every measurement, the effect of nonenzymatic hydrolysis of substrates was taken into consideration and subtracted from the value measured when the enzyme was added.

Determination of Molecular Mass and pI. 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 (*23*). Proteins were stained with Coomassie brilliant blue G. The molecular mass of the native protein by gel filtration on a Superose 12HR 5/30 column, gamma globulin (160 000 Da), bovine serum albumin (67 000 Da), ovalbumin (43 000 Da), carbonic anhydrase (30 000 Da), was used as the reference protein. 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 Isoelecric Focusing Calibration (Pharmacia LKB) according to recommendations by the supplier.

Effect of Temperature and pH on Pyrethroid Hydrolase Activity. For determination of the optimum pH, the activity was determined by incubating purified enzyme (0.2 μg/mL) with *trans*-permethrin as a substrate at 30 °C for 5 min in 50 mM buffer at pH values between 4 and 10. The buffers (at a final concentration of 50 mM) used for the measurement are described below: citric acid−NaOH (pH 4.0−5.5); potassium phosphate (pH 5.0−7.0); Tris-HCl buffer (pH 6.5−9.0), glycine-NaOH buffer (pH 8.5−10.0). Overlapping pH values were used to verify that there were no buffer effects on substrate hydrolysis. For the pH stability determination, samples were incubated in 50 mM buffers from pH 4 to 10 at 30 °C for 2 h. The relative residual activity was assayed as described above. The optimum temperature was determined analogously with a constant pH of 6.5 and different temperatures. For determination of the thermostability, purified enzyme (0.2 μg/mL) was incubated in reaction buffer for 1 h at temperatures ranging from 20 to 70 °C, and retaining pyrethroid hydrolase activity was measured as before.

Effect of Various Chemicals on Pyrethroid Hydrolase Activity. The effects of various chemicals on the enzyme activity were investigated by preincubating the enzyme with chemicals in 50 mM Tris-HCl buffer (pH 6.8) for 30 min at 30 °C and then measuring the residual activities by using *trans*-permethrin as a substrate as described above.

Determination of Substrate Specificity and Kinetic Measurement. Substrate specificity against ρ-nitrophenyl esters was determined using ρ-nitrophenyl acetate ranging from 0.025 to 1.5 mM, ρ-nitrophenyl propionate ranging from 0.02 to 0.8 mM, ρ-nitrophenyl butyrate ranging from 0.01 to 0.5 mM, ρ-nitrophenyl valerate ranging from 0.005 to 0.5 mM, ρ-nitrophenyl caproate ranging from 0.015 to 0.8 mM, and ρ-nitrophenyl caprylate ranging from 0.1 to 3.5 mM as a substrate in 50 mM sodium phosphate buffer (pH 6.8) containing 1% acetonitrile at 30 °C. An additional 0.04% Triton X-100 was included in the reaction mixture in the case of ρ-nitrophenyl caprylate. Substrate specificity against different pesticides was analogously determined by measuring

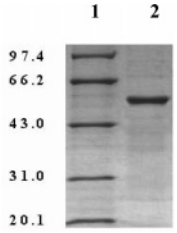

Figure 1. SDS-PAGE analysis of the purified pyrethroid hydrolase from *Aspergillus niger* ZD11 (lane 2) and protein markers (lane 1) stained with Coomassie blue. Markers from top to bottom are phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (20.1 kDa).

enzyme activity over a range of final concentrations from 0.01 to 9 μM according to different pesticides. All initial velocities were determined at five time points at which no more than 10% of the substrate had been consumed, and solution content never exceeded 1% of the total assay volume, so the decrease in substrate concentration remains linear with time over the period of measurement and the rate was almost constant throughout the assay. The *r*-square values ranged from 0.963 to 0.982 according to different substrates. Initial reaction velocities measured at various concentrations of substrates were fitted to the Lineweaver−Burk transformation of the Michaelis−Menten equation (*6*). Kinetic analyses by curve fitting were performed with the SigmaPlot software.

All of the experiments were carried out at least in triplicate, and the results were expressed as means of those data. Standard deviations were determined and reported.

RESULTS

Enzyme Production and Purification. *Aspergillus niger* ZD11 was aerobically cultivated at 30 °C in MM with *trans*-premethrin as the main carbon sources. Pyrethroid hydrolase activity was detected at the late log phase and reached the maximum level 5 days after the start of cultivation. However, when premethrin in the MM medium was replaced by an equal amount of glucose, cell extract showed no or only a trace level of the enzyme activity. These results suggested that the enzyme was induced by *trans*-premethrin in the medium. The enzyme activity was detected at the late log phase and reached the maximum level 5 days after the start of cultivation. Pyrethroid hydrolase was purified from the cell-free extract of *Aspergillus niger* ZD11 by ammonium sulfate precipitation, gel filtration on SephadexG-100, TSK-DEAE, DEAE-sephrose CL-6B, and Sephacryl 200 HR chromatography. The data on the purification are summarized in **Table 1**. The enzyme was purified 41.5-fold to a specific activity of 191 U/mg protein from the cell with a yield of 12.6%. The purified enzyme gave a single band in SDS-PAGE. This indicated that the purified sample was electrophoretically homogeneous under the dissociating conditions. The molecular mass of the purified enzyme was estimated by SDS-PAGE analysis and was approximately 56 kDa (**Figure 1**). The relative molecular mass of native enzyme estimated by gel filtration on a calibrated column of Sephacryl 200 HR was around 55 kDa. Hence, it is assumed that the native pyrethroid-degrading enzyme is a monomer. The pI value was estimated to be 5.4. The purified enzyme catalyzed the hydrolysis of permethrin to equimolar amounts of 3-phenoxybenzyl alcohol and 2,2-dimethyl-3-(2,2-dichlorovinyl)-cyclopropanecarboxylic acid (**Figure 2**); transformation products were identified and confirmed by GC−MS. The same hydrolysis was reported by permethrinase from *Bacillus cereus* SM3 (*9*).

Effects of pH and Temperature on Activity and Stability of the Purified Enzyme. The effect of pH and temperature on

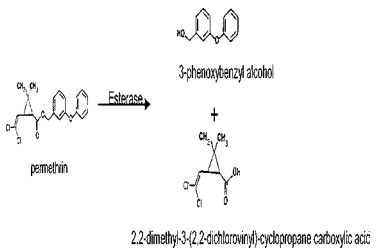

Figure 2. Hydrolysis of permethrin of pyrethroid hydrolase from *Aspergillus niger* ZD11.

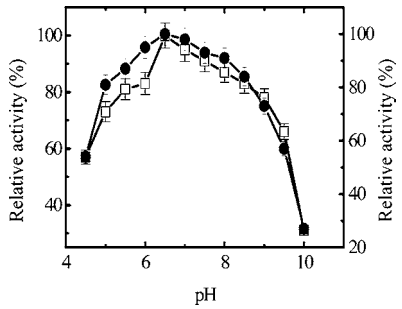

Figure 3. Effect of pH on activity (□) and stability (●) of pyrethroid hydrolase from *Aspergillus niger* ZD11.

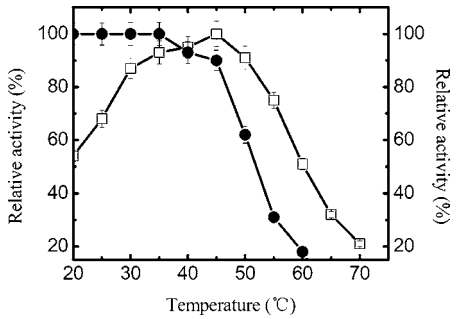

Figure 4. Effect of temperature on activity (□) and stability (●) of pyrethroid hydrolase from *Aspergillus niger* ZD11.

the catalytic activity was studied by using *trans*-premethrin as a substrate. The pH−activity profile of the enzyme was bell-shaped, with maximum values at pH 6.5 (**Figure 3**), and the enzyme was found to be stable in the pH range between 5.0 and 9.0. The optimum temperature was determined at the range of 20−70 °C. The optimal temperature for the enzyme was 45 °C (**Figure 4**). The enzyme was fairly stable up to 45 °C and had 62% of its activity at 50 °C. It was completely inactivated at 65 °C.

Effects of Various Compounds and Metal Ions on Enzyme Activity. The effects of various chemicals on the enzyme activity were investigated by addition of the tested compounds into the reaction mixture at the final concentration. The activity was then measured with *trans*-premethrin as substrate and expressed as a percentage of the activity obtained in the absence of the added compound (**Table 2**). The presence of Hg^{2+} and Ag^{+} caused a complete inhibition at 1.0 mM, while less pronounced effects (5−10% inhibition) were observed in the presence of the remaining divalent cations. The enzyme activity was strongly inhibited by 1 mM *ρ*-chloromercuribenzoate (PCMB), whereas the chelating agents EDTA and phenanthroline (10 mM) showed little effect on the enzyme activity.

Substrate Specificity. The substrate specificity toward *ρ*-nitrophenyl esters of various fatty acids was shown in **Table 3**. The purified enzyme showed the highest activity with *ρ*-nitrophenyl valerate (473 U/mg) among the *ρ*-nitrophenyl esters examined. Both K_m and k_{cat} values of purified enzyme decreased with increases in aliphatic chain length up to *ρ*-nitrophenyl valerate (C_5). 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 substrate. Short-chain *ρ*-nitrophenyl esters seemed to be preferred substrates, whereas *ρ*-nitrophenyl esters of long-chain fatty acids were poor substrates, and the purified enzyme had no activity against *ρ*-nitrophenyl laurate. Taking into consideration that lipases prefer substrates with relatively long aliphatic chains, these results showed the purified enzyme is an esterase and not a lipase.

A range of pesticides such as malathion, *cis*-permethrin, *trans*-permethrin, cypermethrin, fenvalerate, and deltamethrin were tested for substrate specificity of the purified enzyme. Although the purified enzyme hydrolyzed all pesticides tested at different hydrolysis rates, the hydrolysis of all pesticides was much lower than the hydrolysis of some *ρ*-nitrophenyl esters of fatty acids. The *trans*-permethrin was hydrolyzed most rapidly, while deltamethrin was the least readily attacked. *cis*-Permethrin was hydrolyzed at an approximately equal rate toward *trans*-permethrin. The K_m and k_{cat} values were calculated by fitting the data to the Michaelis−Menten equation (**Table 4**). The purified enzyme showed comparable affinity against structurally similar pesticides with K_m values ranging from 0.16 to 1.67 μM.

Table 2. Effect of the Various Substances on Relative Activity of Pyrethroid Hydrolase from *Aspergillus niger* ZD11

substances	relative activity (%)
none	100
1 mM $MgCl_2$	94 ± 4.2
1 mM $CuSO_4$	92 ± 4.1
1 mM $ZnSO_4$	93 ± 4.1
1 mM $MnCl_2$	92 ± 4.3
1 mM $AgNO_3$	8 ± 0.3
1 mM $HgCl_2$	6 ± 0.2
10 mM EDTA	96 ± 4.5
10 mM 1,10-phenanthroline	91 ± 4.1
1 mM PCMB	0

Table 3. 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	187 ± 9	211 ± 9	152 ± 6	0.72
ρ-nitrophenyl propionate	243 ± 12	152 ± 7	193 ± 9	1.27
ρ-nitrophenyl butyrate	386 ± 19	93 ± 4	306 ± 15	3.3
ρ-nitrophenyl valerate	473 ± 22	62 ± 3	375 ± 18	6.05
ρ-nitrophenyl caproate	331 ± 16	125 ± 6	263 ± 13	2.1
ρ-nitrophenyl caprylate	82 ± 4	651 ± 33	65 ± 3	0.1
ρ-nitrophenyl laurate	0	0	0	0

Table 4. 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	29.3 ± 1.5	0.18 ± 0.008	0.91 ± 0.05	5.05
trans-permethrin	31.1 ± 1.5	0.16 ± 0.006	0.97 ± 0.045	6.06
cypermethrin	8.4 ± 0.4	0.23 ± 0.012	0.26 ± 0.014	1.13
fenvalerate	3.1 ± 0.016	1.12 ± 0.06	0.096 ± 0.0043	0.086
deltamethrin	0.47 ± 0.024	1.67 ± 0.082	0.015 ± 0.0007	0.009
malathion	2.3 ± 0.11	1.31 ± 0.061	0.072 ± 0.0034	0.055

DISCUSSION

A few bacterial isolates possessing pyrethroid hydrolase activity have been isolated, whereas pyrethroid-degrading enzymatic preparations have not been reported to date on the fungus. This is the first report to our knowledge on the production, purification, and properties of a pyrethroid hydrolase from fungus. The production of pyrethroid hydrolase from *Aspergillus* niger ZD11 depended strongly on the carbon sources. When glucose was added in the minimal medium as carbon source, cell extract showed no or only a trace level of the enzyme activity. The pyrethroid was the best carbon source for the production of intracellular pyrethroid hydrolase. The specific activity of the purified enzyme under optimal conditions was 31.1 U mg^{-1} of protein on *trans*-permethrin, whereas the specific activities reported for pyrethroid hydrolase toward *trans*-permethrin were from 0.0146 to 0.597 U mg of $protein^{-1}$ (*9*, *21*, *25*). Therefore, the purified enzyme from *Aspergillus niger* ZD11 is among the most efficient pyrethroid hydrolase described so far. As a monomeric 53 kDa protein, the purified enzyme is also similar to some known pyrethroid hydrolase, whose molecular masses range from 58.6 to 61 kDa (*9*, *21*, *25*). The optimum pH of the purified enzyme was lower than that recorded from *Bacillus cereus* (pH 7.5) (*9*). The optimal temperature of 45 °C was higher than that recorded for *Bacillus cereus* (37 °C) (*9*).

The effects observed in the presence of potential inhibitors or activators of the purified enzyme activity were investigated. The purified enzyme was significantly affected by sulfhydryl oxidant metals (Hg^{2+}, Ag^{+}), while other metal ions did not have a remarkable effect on the activity. This suggested that thiol may be involved in the active catalytic site. Furthermore, activity was completely inhibited by thiol-modifying reagents such as PCMB, therefore, suggesting again that sulfhydryl groups may be involved in the catalytic center of the enzyme, substrate binding, and/or recognition (*26*). EDTA and 1,10-phenanthroline did not affect activity, indicating that divalent cations are not required for enzyme activity. This property is similar to that noted in *Bacillus cereus*, lacking any requirement for metal ions at the active site (*9*).

The purified enzyme not only hydrolyzed various *ρ*-nitrophenyl esters of short-medium chain fatty acids, but also degraded many pesticides with similar carboxylester such as cypermethrin, permethrin, fenvalerate, deltamethrin, and malathion, which is an organophosphorus pesticide, indicating that the purified enzyme is an esterase with broader specificity. However, this observation does not quite agree with data reported by Motoyama et al., Maloney et al., and Stok et al. (*9*, *21*, *25*). The pyrethroid-hydrolyzing carboxylesterase (BAC36707) from mouse liver microsomes and permethrinase from *Bacillus cereus* 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 the fenvalerate, and three other forms possessed approximately equal activity toward these two insecticides. Furthermore, the hydrolysis of *cis*-permethrin, *trans*-permethrin, cypermethrin, fenvalerate, and deltamethrin by the purified pyrethroid hydrolase was much higher than that of pyrethroid-hydrolyzing carboxylesterase (BAC36707) from mouse liver microsomes and carboxylesterase E3 from *Nephotettix cincticeps* Uhler (*21*, *25*). In a previous paper, there was a preference in both mammal and insect carboxylesterases for permethrin to cypermethrin, and *trans*-permethrin to *cis*-permethrin (*21*, *25*). Yet the carboxylesterase from *Nephotettix cincticeps* Uhler preferred *cis*-permethrin to *trans*-permethrin. In this study, pyrethroid hydrolase possessed approximately equal activity toward permethrin isomers. This feature is different from that of carboxylesterase E3 from *Nephotettix cincticeps* Uhler (*21*) and carboxylesterase (BAC36707) (*25*), indicating that the purified enzyme lacked stereoselectivity. The comparison of K_m and k_{cat} revealed that the pyrethroid hydrolase has about 10-fold higher affinity toward *trans*-permethrin than deltamethrin and can hydrolyze the former about 65-fold faster than the latter. The catalytic efficiencies (k_{cat}/K_m) are considered as a measurement of the enzyme specificity; among these, *trans*-permethrin is clearly the preferred substrate, due to either steric hindrance of hydrolysis or stabilization of the est bond. Replacement of the chlorovinyl group of the pyrethoids with bromovinyl also affected the rate of hydrolysis by the enzyme. Cypermethrin was more readily transformed than deltamethrin, in agreement with findings for the permethrinase from *Bacillus cereus* (*9*), pyrethroid-hydrolyzing carboxylesterase (BAC36707) from mouse liver microsomes, and the esterases associated with mammalian microsomal fractions *(27*), suggesting that the pyrethroid hydrolase has a specific preference for the properties of the halogen atoms.

In conclusion, the pyrethroid hydrolase described here differs from those previously reported in at least one of the following aspects: molecular mass, pI, pH, and temperature optima. These differences of the isofunctional enzyme suggest diversity in evolution and a spread of pyrethroid hydrolase gene among different microorganisms. Environments contaminated with pyrethroid are regarded as hazardous because the pesticides are considered to be an endocrine-disrupting chemical (*28*). Thus, a technique for rapid degradation of the compounds is required. In view of the resistance to many metals, leakage requirement for cofactors, relatively broad pH and temperature optimum, and higher activity against a range of pesticides, the purified enzyme could conceivably be developed to fulfill the practical requirements to enable its use in situ for detoxification of pyrethroids where they cause environmental contamination problems. Further study is helpful to establish more molecular knowledge on gene overexpression and regulatory mechanisms.

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