JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

# A Novel L-Amino Acid Oxidase from *Trichoderma harzianum* ETS 323 Associated with Antagonism of *Rhizoctonia solani*

Chia-Ann Yang,[†] Chi-Hua Cheng,[‡] Chaur-Tsuen Lo,[∥] Shu-Ying Liu,[⊥] Jeng-Woei Lee,[*,§] and Kou-Cheng Peng[‡,*]

[†]Institute of Medical Science, Tzu Chi University, Hualien 97004, Taiwan, R.O.C.

[‡]Institute of Biotechnology, National Dong Hwa University, Hualien, 97401, Taiwan, R.O.C.

[§]Institute of Life Science, Tzu Chi University, Hualien 97004, Taiwan, R.O.C

[∥]Department of Biotechnology, National Formosa University, Yunlin 63208, Taiwan, R.O.C.

[⊥]Department of Molecular Biotechnology, Da-Yeh University, Changhua 51591, Taiwan, R.O.C.

Supporting Information

**ABSTRACT:** *Trichoderma* spp. are used as biocontrol agents against phytopathogens such as *Rhizoctonia solani*, but their biocontrol mechanisms are poorly understood. A novel L-amino oxidase (Th-LAAO) was identified from the extracellular proteins of *Trichoderma harzianum* ETS 323. Here, we show a FAD-binding glycoprotein with the best substrate specificity constant for L-phenylalanine. Although the amino acid sequence of Th-LAAO revealed limited homology (16−24%) to other LAAO members, a highly conserved FAD-binding motif was identified in the N-terminus. Th-LAAO was shown to be a homodimeric protein, but the monomeric form was predominant when grown in the presence of deactivated *Rhizoctonia solani*. Furthermore, in vitro assays demonstrated that Th-LAAO had an antagonistic effect against *Rhizoctonia solani* and a stimulatory one on hyphal density and sporulation in *T. harzianum* ETS 323. These findings further our understanding of *T. harzianum* as a biocontrol agent and provide insight into the biological function of L-amino acid oxidase.

**KEYWORDS:** L-amino acid oxidase, flavoprotein, *Trichoderma harzianum*, *Rhizoctonia solani*, biocontrol

## INTRODUCTION

*Rhizoctonia solani*, a soil-borne plant pathogen with worldwide distribution causes damping-off and root rot in a wide range of crops such as cabbage, cauliflower, potato, cucumber, and tobacco, resulting in serious agricultural losses.[1] The management approaches currently used to control *R. solani* include chemical pesticides and cultivation practices. However, these types of controls are restricted due to economic and ecological reasons. Alternative techniques, such as biological control, have been studied over the past few decades. Several *Trichoderma* spp. have been used as biocontrol agents against root rot pathogens, including *R. solani*.[1] *Trichoderma*, a filamentous fungus that is widely distributed in the soil, has been investigated for its biocontrol mechanisms during the past years. *Trichoderma* spp. contact *R. solani* hyphae via coiling and penetrate the host cell wall by secreting lytic enzymes.[2,3] Several glucanases and chitinases have been shown to be directly involved in the mycoparasitic interaction between *Trichoderma* species and *R. solani* [3,4]

Our previous study[5] identified several cell wall-degrading enzymes (i.e., Chitinase, cellulase, xylanase, $\beta$-1,3-glucanase, $\beta$-1,6-glucanase, and mannanase) and one putative L-amino acid oxidase from the extracellular proteins of *T. harzianum* ETS 323 grown in the presence of deactivated *R. solani* hyphae. L-amino acid oxidase (LAAO, E.C.1.4.3.2) is a dimeric flavoprotein that catalyzes the oxidative deamination of an L-amino acid substrate into a corresponding α-oxoacid with the production of $H_2O_2$ and ammonia.[6] It is well-known that these enzymes are widely distributed in various organisms.[7,8] Since Skarnes[9] first reported antibacterial activity for venom LAAO, LAAOs have become an attractive enzyme for biochemical studies and medical applications because of their effect on microbial organisms and diverse cell types, including cancer cells and platelets,[10,11] but its biological roles remain unclear.

While considerable attention has been focused on the glucanases and chitinases involved in the antagonism of *Trichoderma* spp. against *R. solani*, studies on the possible function of *T. harzianum* LAAO in this antagonism have not emerged. Therefore, the aims of this study were to identify the putative LAAO from *T. harzianum* ETS 323 and to ascertain the effect of this enzyme on the antagonism against *R. solani*.

## MATERIALS AND METHODS

All chemicals were purchased from J.T. Baker (Phillipsburg, NJ) and Sigma (St. Louis, MO) unless otherwise noted.

**Preparation of Fungal Materials.** *R. solani* hyphae were cultivated and deactivated as previously described.[5] *T. harzianum* ETS 323 was grown on PDA (Difco Laboratories) plates at 25 °C for 7 days. Following the addition of 2 mL of sterile water to each plate, the conidia were scraped off from each one. Cornidia ($10^6$/mL) were inoculated into 250 mL minimal medium (1.4 g $L^{-1}$ of $[NH_4]_2SO_4$, 0.2 g $L^{-1}$ of $KH_2PO_4$, 6.9 g $L^{-1}$ of $NaH_2PO_4 \cdot H_2O$, 0.3 g $L^{-1}$ of $MgSO_4 \cdot 7H_2O$, 1.0 g $L^{-1}$ of

Received: December 1, 2010
Accepted: March 24, 2011
Revised: February 9, 2011
Published: April 01, 2011

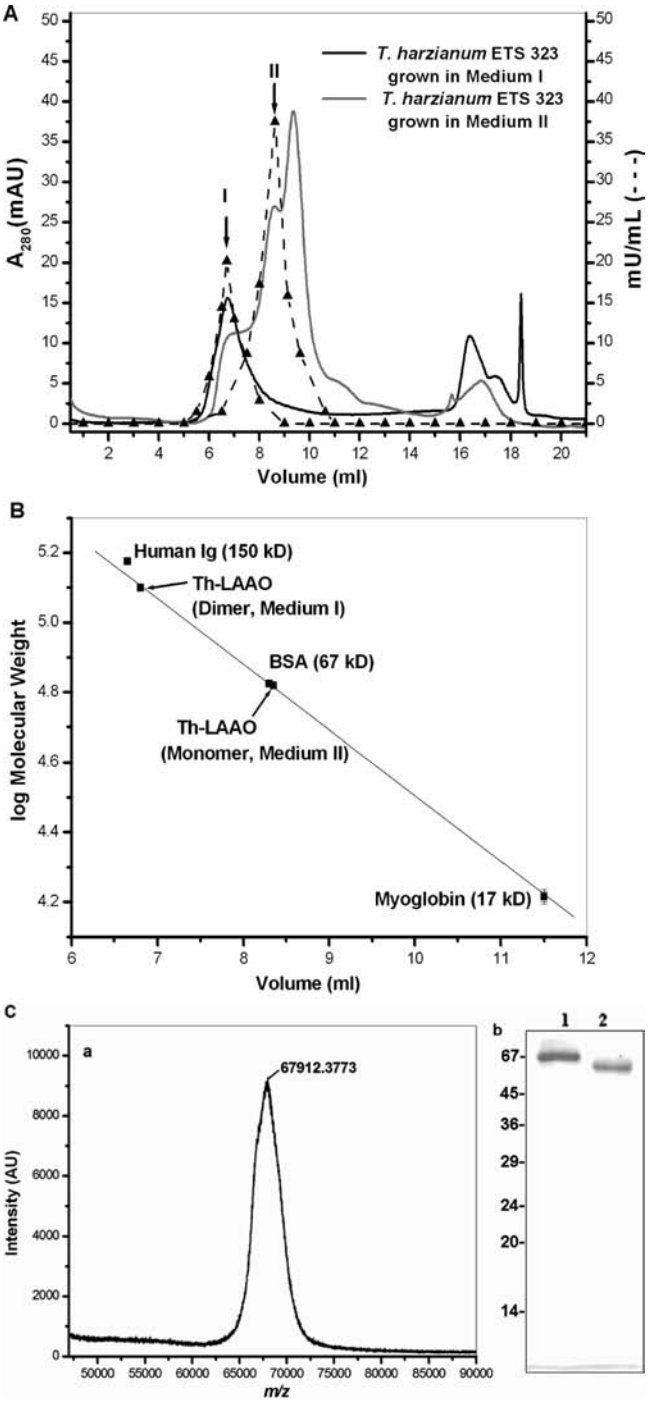


**Figure 1.** Purification of Th-LAAO by gel filtration chromatography (A). Elution volumes of the molecular mass standards and Th-LAAO (B). MALDI-TOF-MS of Th-LAAO and analysis of Th-LAAO deglycosylation by 10% SDS-PAGE (C). Th-LAAO-containing fractions obtained by a ConA-Sepharose column chromatography were applied to a gel filtration column. Arrows I and II indicate the Th-LAAO-containing fractions from medium I and II, respectively. The active fraction of LAAO is denoted by a dotted line. mU = milliunit. Panel b in Figure 1 (C) shows native and deglycosylated Th-LAAO. Th-LAAO: LAAO from *T. harzianum* ETS 323.

peptone, and 0.3 g $L^{-1}$ of urea). Each minimal medium was supplemented with either 10 g $L^{-1}$ of glucose (medium I) or 10 g $L^{-1}$ of glucose and 10 g $L^{-1}$ of deactivated *R. solani* hyphae (medium II) and further cultivated at 22 °C with shaking for 5 days.

**Assay for Antagonism Between *T. harzianum* ETS 323 and *R. solani*.** A thin layer of PDA was spread on a clean and sterile glass microscope coverslip placed in the middle of a sterile culture plate. The opposite sides of the medium-treated coverslip were seeded with two 4 mm diameter plugs of *T. harzianum* ETS 323 and *R. solani* hyphae cut from the growing edges of 5-day-old culture plates and then further incubated for a period of 6 days at 25 °C. The glass coverslip was stained with lactophenol cotton blue (Fluka Chemie, Buchs, Switzerland) to aid in the visualization of the hyphae under a light microscope (IX70; Olympus, Tokyo, Japan) equipped with an attached camera (SC35; Olympus, Tokyo, Japan). The hyphal interactions between the opposing colonies were photographed every 24 h.

**Purification of the Putative LAAO.** The resulting solution of extracellular protein extracts from medium I and II were concentrated using the Amicon Ultra Centrifugal Filter device with a 5-kDa molecular weight cutoff membrane (Ultra-4; Millipore, Billerica, MA). The Th-LAAO purification was carried out by ÄKTAprime plus 100 fast protein liquid chromatography (FPLC; Amersham Pharmacia Biotech, Piscataway, NJ) equipped with the columns mentioned hereafter in the paragraph. Protein-containing extracts were loaded onto a 5 mL ConA-Sepharose column (GE Healthcare Bio-Science, Piscataway, NJ). Afterward, the column was washed with a buffer (pH 7.4) containing 1 mM $CaCl_2$, 1 mM $MnCl_2$, 0.5 M NaCl, and 0.02 M Tris-HCl and then eluted with a linear gradient of methyl-α-D-manno-pyranoside (0−0.5 M) in 0.02 M Tris-HCl buffer (pH 7.4), containing 0.5 M NaCl at a flow rate of 1 mL $min^{-1}$. Glycoprotein fractions were pooled and concentrated with ultrafiltration and then subjected to FPLC with a Superdex HR 75 10/30 column (Amersham Pharmacia Biotech, Piscataway, NJ). The column was eluted with 20 mM sodium phosphate buffer (pH 7.4), at a flow rate of 0.2 mL $min^{-1}$. Protein molecular weight markers were used for calibration: myoglobin (17 kDa), bovine serum albumin (67 kDa), and human Ig (150 kDa). All chromatographic steps were monitored at 280 nm and performed at 25 °C. The purified Th-LAAO was lyophilized and identified with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

**Protein Glycosylation Analysis.** For glycosylation analysis, 2 μg of purified Th-LAAO was deglycosylated by incubation with 20 × $10^{-3}$ U glycolpeptidase F (Sigma-Aldrich St. Louis, MO) in 50 μL of 0.02 M sodium phosphate buffer (pH 7.6) at 37 °C for 17 h and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**SDS-PAGE.** The prepared extracellular proteins from media I and II were loaded onto 10% SDS/PAGE[12] at 25 mA for 2.5 h.

**MALDI-TOF-MS Analysis.** The molecular weights of Th-LAAO and the tryptic peptide mixture were analyzed using a 4800 plus MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA) equipped with a nitrogen laser, a delay extraction, and a reflector. The sample was prepared using a dried droplet method, with alpha-cyano-4-hyroxycinnamic acid as the matrix. The instrument was calibrated with des-Arg[1]-bradykinin ($MH^+$ 905.05), angiotensin ($MH^+$ 1296.6853), and neurotensin ($MH^+$ 1672.9175). The full-scan spectrum of typtic peptides in the MS mode was in an $m/z$ range of 1000−4000. All experiments were performed at least three times.

**Flavoprotein Analysis.** For verifying the presence of FAD in the purified Th-LAAO, a 200-μg aliquot of Th-LAAO was boiled in 1 mL for 20 min and filtered with an Amicon Ultra Centrifugal Filter device with a molecular mass cut off of 3 kDa (Ultra-4; Millipore, Billerica, MA). Afterward, the filtrate containing the chromophore (yellow color) was collected and subjected to electrospray ionization mass spectrometry (ESI-MS) analysis. The MS measurements of the chromophore and FAD-$Na_2$ standard (Sigma-Aldrich St. Louis, MO) were performed using a Thermo Finnigan LCQ Duo LC/MS/MS (Vernon Hills, Illinois) in the positive-ion mode under the following conditions: Samples were introduced by flow injection with a mobile phase of 25% methanol and 0.1% trifluoroacetic acid in water (v/v) (spray voltage: 4 kV; capillary temperature: 200 °C).

**Table 1. Protein Identification of the LAAO from *T. harzianum* ETS 323 by MALDI-TOF-MS**

| MALDI-TOF-derived amino acid sequence | $MH^+$ matched | $z$ |
|---|---|---|
| $_{1}$MVGAGVSGLYIAMILDDLKIPNLTYDIFESSSRTGGRLYTHHFTDAK $^{a}{}_{47}$ | 5175.8916 | 2 |
| $_{48}$ HDYYDIGAMR $_{57}$ | 1240.3527 | 1 |
| $_{58}$ YPDIPSMKR $_{66}$ | 1106.3019 | 1 |
| $_{67}$ TFNL FKRTGMPLIK$_{81}$ | 1666.0526 | 1 |
| $_{82}$ YYLDGENTPQLYNNHFFAK $_{100}$ | 2334.5232 | 1 |
| $_{101}$ GVSDPYMVSVA NGTVPDDVVDSVGEK $_{125}$ | 2636.8601 | 1 |
| $_{126}$ LQQAFGYYK $_{134}$ | 1117.2665 | 1 |
| $_{135}$ EKLAED FDK $_{143}$ | 1094.1835 | 1 |
| $_{144}$ GFDELMLVDDIATREYLKR $_{162}$ | 2284.6057 | 1 |
| $_{158}$ EYLKRGGPKGEAPK $_{171}$ | 1529.7549 | 1 |
| $_{172}$YDYFAIQWMETQNTGTNLFDQAFSESVID SFDFDNPTK $_{209}$ | 4486.7881 | 2 |
| $_{228}$ ETLVHKVQNNKR $_{239}$ | 1465.6713 | 1 |
| $_{240}$ VDAISIDLDAP DDGNMSVR $_{258}$ | 2003.1631 | 1 |
| $_{286}$ GLNLHPTQADAIRCLHYDNSTKVALK $_{311}$ | 2879.2783 | 2 |
| $_{308}$ VALKFSYPWWIK $_{319}$ | 1537.8632 | 1 |
| $_{320}$ DCGITCGGAASTDLPLR $_{336}$ | 1649.8500 | 1 |
| $_{337}$ TCVY PSYNLDDGDG EAVLLASYTW SQDATR $_{366}$ | 3311.5291 | 1 |
| $_{367}$ IGSLVKEAPPQPPK $_{380}$ | 1460.7323 | 1 |
| $_{381}$ EDELIELILQNL $_{394}$ | 1328.4783 | 1 |
| $_{395}$ ARLHAEHI TYEKIK $_{406}$ | 1708.9762 | 1 |
| $_{452}$ FHIVGEASSVHHAWIIGSL $_{470}$ | 2060.3369 | 1 |

[a] The conserved dinucleotide-binding motif and GG motif are shaded.

**Measurement of LAAO Activity.** LAAO activity was assayed in a 96-well microplate using an enzyme-coupled assay.[13] The 200 $\mu$L reaction mixture contained 20 mM sodium phosphate buffer (pH 7.5), 50 mM L-phenylalanine as substrate, 0.5 mg $mL^{-1}$ *o*-dianisidine (Sigma-Aldrich, St. Louis, MO), 4.7 U of horseradish peroxidase (Sigma-Aldrich St. Louis, MO), and predetermined amounts of the eluted fraction (purified Th-LAAO) or crude extract. One unit of Th-LAAO was defined as the production of 1 $\mu$mol of $H_2O_2$ per minute. After 17 h at 37 °C, the $A_{450}$ was recorded using a microplate reader (model 680; Bio-Rad, Hercules, CA). The substrate specificity of Th-LAAO was examined using other L-amino acids (L-amino acid kit; Sigma-Aldrich St. Louis, MO) under identical assay conditions. The concentration of Th-LAAO in the reaction mixture was 0.3 $\mu$M and the concentration of the substrate was 1.0−50 mM. All experiments were performed at least three times. The kinetic parameters $K_m$ and $k_{cat}$ for the substrates were calculated using the nonlinear least-squares fit of the experimental data to the Michaelis−Menten equation, using Micro-Origin v7.0 (Microcal Software, Northampton, MA).

**Effects of Th-LAAO on *R. solani* and *T. harzianum* ETS 323 Hyphal Growth.** Two-millimeter-diameter discs of the fungal hyphae of either *T. harzianum* ETS 323 or *R. solani* were obtained from the edges of a 5-day-old PDA fungal culture. Afterward, two discs of the fungus under investigation were placed at the opposite sides of a 9 cm PDA plate. A sterile filter paper disk (6 mm) impregnated with one of several concentrations (3, 6, or 12 $\mu$g per disk) of Th-LAAO was placed on the advancing fungal hyphae (*T. harzianum* ETS 323 and *R. solani*), while a disk saturated with sterile distilled water was positioned on the other to serve as a negative control. The plates were further incubated at 28 °C for a period of 6 days. The growth of the hyphae was photographed daily, with the growth diameters also recorded on a daily basis. The percentage of hyphal growth reduction was calculated using the following equation:

$$\text{hyphal growth reduction}(\%) = \frac{D_{(c)} - D_{(t)}}{D_{(c)}} \times 100$$

where $D_{(c)}$ and $D_{(t)}$ are the diameter of the *R. solani* growth on the control plate and the Th-LAAO-containing plate, respectively. All experiments were performed at least three times.

**Effects of Th-LAAO on the Sporulation of *T. harzianum* ETS 323.** For determining the effect of Th-LAAO on the sporulation of *T. harzianum* ETS 323, the fungus was cultivated (with or without Th-LAAO) as above for 7 days. The conidia were harvested from the culture plate by adding 2 mL of sterile distilled water, spun down, and resuspended in 10 mL of distilled water. The number of conidia was determined by a Neubauer hemacytometer slide (Neubauer, West Germany).

**Microscopic Observation.** For light microscopic observations, a thin layer of PDA was spread on a sterile microscope coverslip and placed in the middle of a sterile Petri dish. One disk (2 mm in diameter) of the fungal hyphae (*T. harzianum* ETS 323 or *R. solani*) was cut from the growing edges of 5-day-old fungal culture and transferred to the center of the coverslip. One sterile filter paper disk impregnated with 6 $\mu$g of Th-LAAO was placed on the fungal hyphae. The glass coverslips were stained with cotton blue and observed daily under a light microscope for a period of 5 days. All experiments were performed at least three times.

## RESULTS

**Antagonism between *T. harzianum* ETS 323 and *R. solani*.** For examining the effects of *T. harzianum* ETS 323 antagonism on *R. solani*, events such as mycoparasitism, coiling, antibiosis, and hyphae lysis were observed. While growing near the *R. solani* hyphae, *T. harzianum* ETS 323 produced branches oriented toward the *R. solani* hyphae. After 2−3 days of coculturing, *T. harzianum* ETS 323 hyphae had made contact with those of *R. solani* and were observed coiling around them (Supporting Information (SI) Figure S1 A). After 4−5 days, more extensive coiling was observed and had lead to growth inhibition of *R. solani* (SI Figure S1 B). After 6 days, *T. harzianum* ETS 323

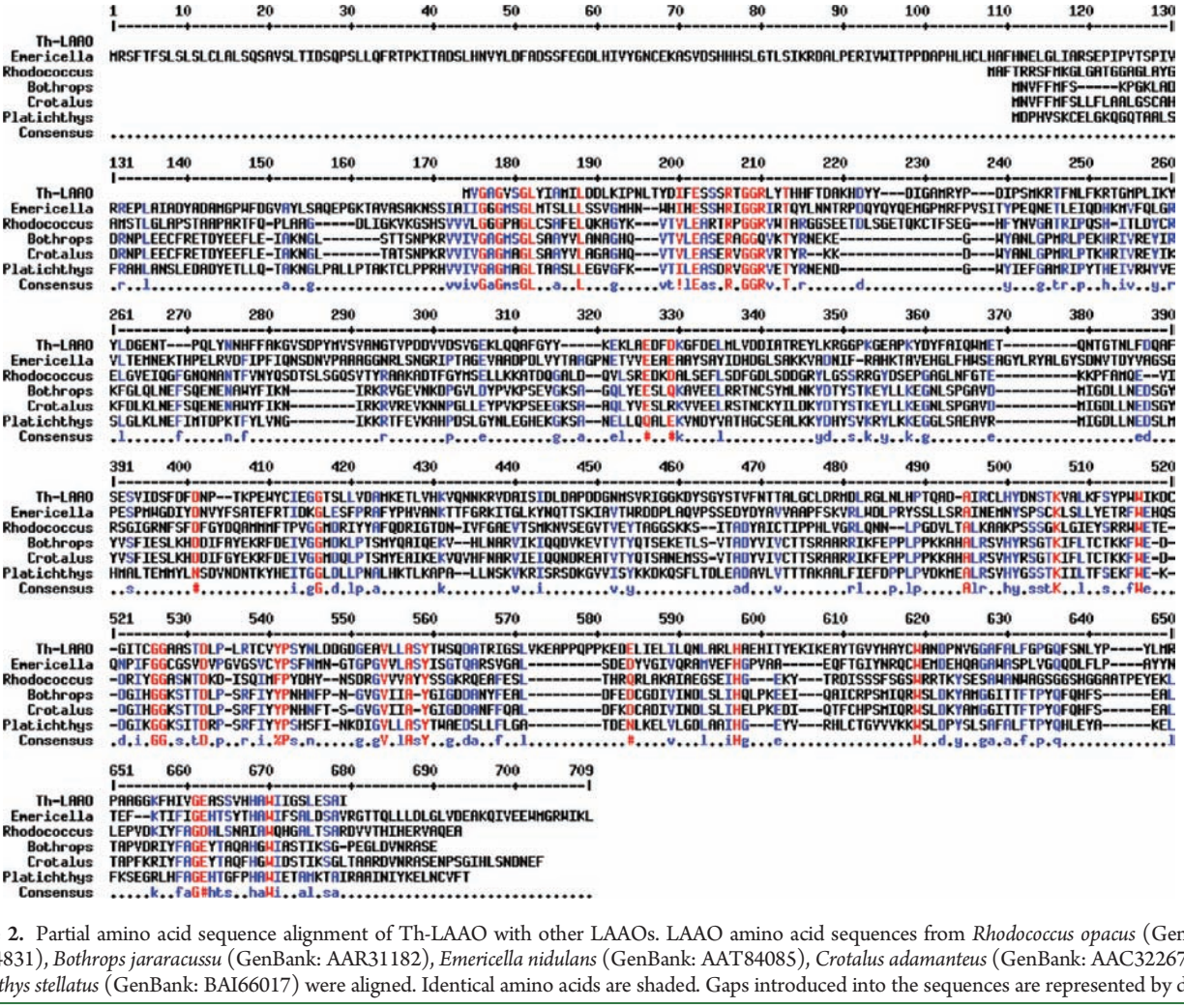


**Figure 2.** Partial amino acid sequence alignment of Th-LAAO with other LAAOs. LAAO amino acid sequences from *Rhodococcus opacus* (GenBank: AAL14831), *Bothrops jararacussu* (GenBank: AAR31182), *Emericella nidulans* (GenBank: AAT84085), *Crotalus adamanteus* (GenBank: AAC32267), and *Platichthys stellatus* (GenBank: BAI66017) were aligned. Identical amino acids are shaded. Gaps introduced into the sequences are represented by dashes.

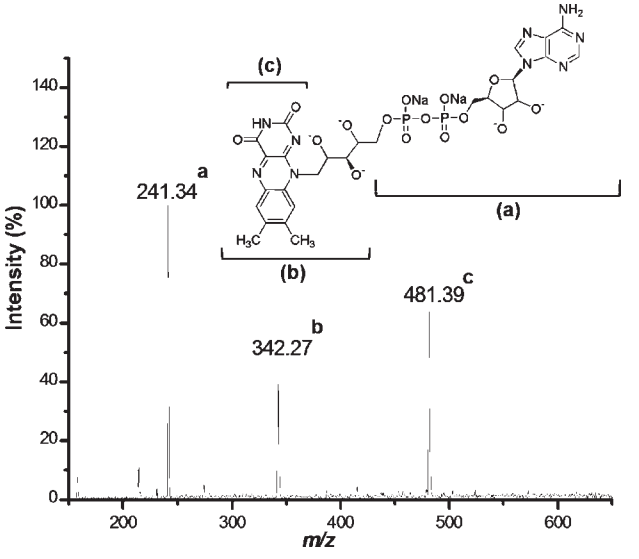


**Figure 3.** Mass spectrum of the chromophore from Th-LAAO. The scheme illustrates the structure of FAD-$Na_2$.

hyphae had lysed those of *R. solani*, resulting in the death of the fungus (SI Figure S1 C).

**Table 2. Kinetic Parameters for Th-LAAO with Substrates**

| L-amino acid[a] | $V_{max}$ (nmol $min^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ ($min^{-1}$ $mM^{-1}$) |
|---|---|---|---|
| L-phe | 454.97 | 11.82 | 0.127 |
| L-lys | 204.95 | 9.95 | 0.068 |
| L-glu | 189.26 | 19.66 | 0.032 |
| L-ala | 134.65 | 6.01 | 0.044 |

[a] Th-LAAO was inactive against L-gly, L-leu, L-his, L-val, L-arg, L-pro, L-met, L-thr, L-iso, L-gln, L-trp, L-ser, and L-asn substrates.

**Purification of a Putative LAAO from Extracellular Proteins of *T. harzianum* ETS-323.** The putative LAAO from extracellular protein extracts of *T. harzianum* ETS 323 grown in medium I (in the absence of deactivated *R. solani* hyphae) and medium II (in the presence of deactivated *R. solani* hyphae) were purified to homogeneity with a ConA-Sepharose column and gel filtration column chromatography. The purification process was monitored by SDS-PAGE (SI Figure S2 A, B and Figure 1C). Purified Th-LAAO generated two peaks at a retention volume of 6.7 mL (15 mAU) and 8.5 mL (27 mAU) for medium I and medium II, respectively, in gel filtration (Figure 1A.). Rechromatography of the active fraction from medium II via the gel filtration column is shown in SI Figure S2C, which showed that the Th-LAAO afforded 1 major peak at 8.5 mL (25 mAU) of the

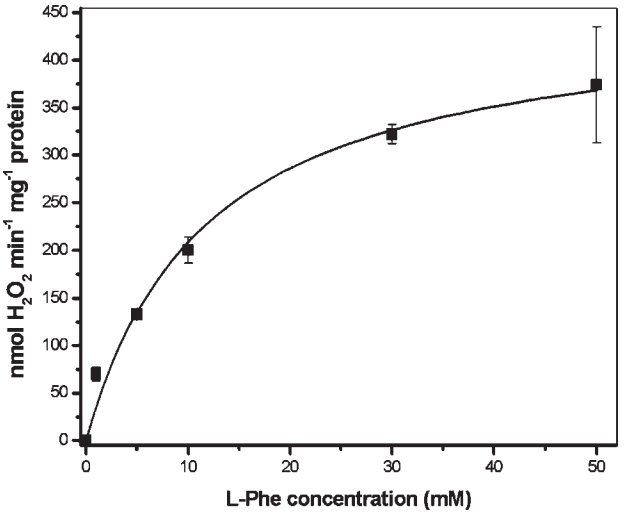


**Figure 4.** Determination of the kinetic parameters of the substrate for Th-LAAO. LAAO activity was measured using an $H_2O_2$-generation assay. Reaction rate is defined as the generation of $H_2O_2$ per minute.

retention volume. Purified Th-LAAO presented 1 band on the SDS-PAGE from both media conditions (data not shown for medium I) (Figure 1C, b, lane 1). The mass spectrum from Th-LAAO MALDI-TOF in the positive mode gave a main peak at $m/z$ 67912.3773. These results demonstrated homogeneity of the purified Th-LAAO. In this 2-step purification, 6.5 mg of LAAO (77% yield) was obtained from 2500 mL of crude extract from medium II containing 240 mg protein. These values were calculated in relation to the LAAO activity in the crude extract, which had a specific activity of up to 0.014 U $mg^{-1}$. The specific activity of the homogeneous Th-LAAO was 0.402 U $mg^{-1}$ against the substrate L-phenylalanine.

**Molecular Mass and Glycosylation.** Based on the results of gel filtration chromatography, the molecular masses of Th-LAAO were estimated to be approximately 130 kDa and 67 kDa in the absence and presence of deactivated *R. solani* hyphae (Figure 1B), respectively, despite the fact that 130 kDa is higher than the expected resolution of the Superdex 75 column. On the other hand, SDS-PAGE analysis revealed a molecular mass of 67 kDa (Figure 1C, lane 1) for both medium conditions. These data suggested that Th-LAAO was a homodimeric protein but that the monomeric form of Th-LAAO was predominant when *T. harzianum* ETS 323 was grown in the presence of *R. solani* hyphae. In addition, the results showed that the amount of Th-LAAO increased in the culture medium, whereas *T. harzianum* ETS 323 grew in the presence of deactivated *R. solani* hyphae (Figure 1A and SI Figure S2B). Moreover, a sugar moiety was effectively liberated by the digestion of Th-LAAO with glycopeptidase F (Figure 1C, lane 2), indicating the presence of an N-linked carbohydrate side chain. The molecular weight of deglycosylated Th-LAAO was estimated to be 60 kDa.

**Amino Acid Sequence Analysis.** Th-LAAO was digested with sequencing-grade trypsin, and the peptide mass fingerprint was analyzed by MALDI-TOF-MS. Tryptic peptides were identified (Table 1) when aligned with the deduced amino acid sequence of *Hypocrea lixii* (*T. harzianum* ETS 323) L-amino acid oxidase mRNA as derived from partial cDNAs (GenBank: GU902953, which was submitted by us previously), with an estimated molecular weight of 52 kDa. The MS-derived amino acid sequence of the Th-LAAO fragments covered 80% of the *H. lixii* LAAO sequence. Therefore, this indicated that the deduced amino acid sequence of the *H. lixii* LAAO mRNA was a partial sequence of Th-LAAO.

A partial amino acid sequence alignment analysis using ClustalW2 revealed that Th-LAAO showed limited homology to other LAAOs (e.g., those from microorganisms and animals), such as that from *Rhodococcus opacus* (16%), *Bothrops jararacussu* (19%), *Emericella nidulans* (16%), *Crotalus adamanteus* (19%), and *Platichthys stellatus* (24%). In addition, a highly conserved dinucleotide-binding motif (G-X-G-X-X-G-X-X-X-G/A, Gly3-Ala11) and GG motif (R-X-G-G-R-X-X-S/T, Arg33-Thr40) were identified in the N-terminus of Th-LAAO (Figure 2). These two motifs are present in many families of flavoproteins, including FAD-binding LAAOs.[14,15] These results supported the conclusion that Th-LAAO was a novel L-amino acid oxidase from *T. harzianum* ETS 323.

**Flavoprotein Analysis.** A conserved FAD-binding motif was identified in the N-terminal region of Th-LAAO. For verifying that Th-LAAO was a FAD-containing protein, a yellow chromophore was isolated from the protein and submitted to mass spectrum analysis, which supported the involvement of flavin in Th-LAAO. As supported by the ESI-MS result, Figure 3 shows the mass spectrum of the chromophore that dissociated from Th-LAAO by heating. The ESI-MS in the positive mode gave the ion peak at $m/z$ 241.34, 342.27, and 481.39, consistent with those of the FAD-$Na_2$ standard (SI Figure S3). The former corresponded to the ion peaks at $m/z$ 481.39, 342.27, and 241.34, which were assignable to fragment a (carrying two sodium ions), fragment b, and fragment c, respectively. These results provided evidence that FAD is the chromophore associated with Th-LAAO.

**Substrate Specificity.** The substrate specificity of Th-LAAO was determined among the 17 tested L-amino acids (Table 2). The specificity constant, $k_{cat}/K_m$, of L-phe, L-lys, L-glu, and L-ala were 0.127, 0.068, 0.032, and 0.044 $min^{-1}$ $mM^{-1}$, respectively, but the other amino acids had no detectable activity. Therefore, the LAAO from *T. harzianum* ETS 323 was dubbed an L-phenylalanine oxidase because L-phe demonstrated the best substrate specificity constant (Figure 4).

**Antagonistic Effect of Th-LAAO on *R. solani*.** A 6-$\mu$g $disk^{-1}$ of purified monomeric Th-LAAO from medium II was tested for its antagonistic effect on the hyphal growth of *R. solani* (Figure 5 A). On the fourth day of incubation, hyphal lysis of *R. solani* was observed in the presence of Th-LAAO (Figure 5A, a). After 6 days, the fungal hyphae along the edge of the colony facing the paper disk appeared to be thinning and revealed an inhibition zone with a diameter of 20 mm (Figure 5A, c). However, the fungal hyphae showed regular growth along the edge of the control disk. These results indicated a lytic effect on *R. solani* hyphae in the presence of Th-LAAO.

The hyphal growth of *R. solani* with respect to the different concentrations of Th-LAAO showed effectiveness at suppressing the growth of *R. solani* (Figure 5B). In comparison with the control treatment, the percentage of hyphal growth reduction was 62.5% and 87.5% on the third day of incubation in the presence of a 3-$\mu$g $disk^{-1}$ and 6-$\mu$g $disk^{-1}$ of Th-LAAO, respectively. The minimum concentration of Th-LAAO for inhibition of hyphal extension was 3-$\mu$g $disk^{-1}$, demonstrating a 50% hyphae growth reduction on the fifth day of incubation. Overall, a 6-$\mu$g $disk^{-1}$ of Th-LAAO was the most efficient at inhibiting hyphal growth of *R. solani*, demonstrating a 75% hyphae growth reduction. The antagonistic activity of Th-LAAO at 6 $\mu$g was the same as that at 12 $\mu$g, with both concentrations completely inhibiting hyphae extension after 5 days of incubation. These results indicated the positive correlation of *R. solani* hyphal growth inhibition to the concentration of Th-LAAO.

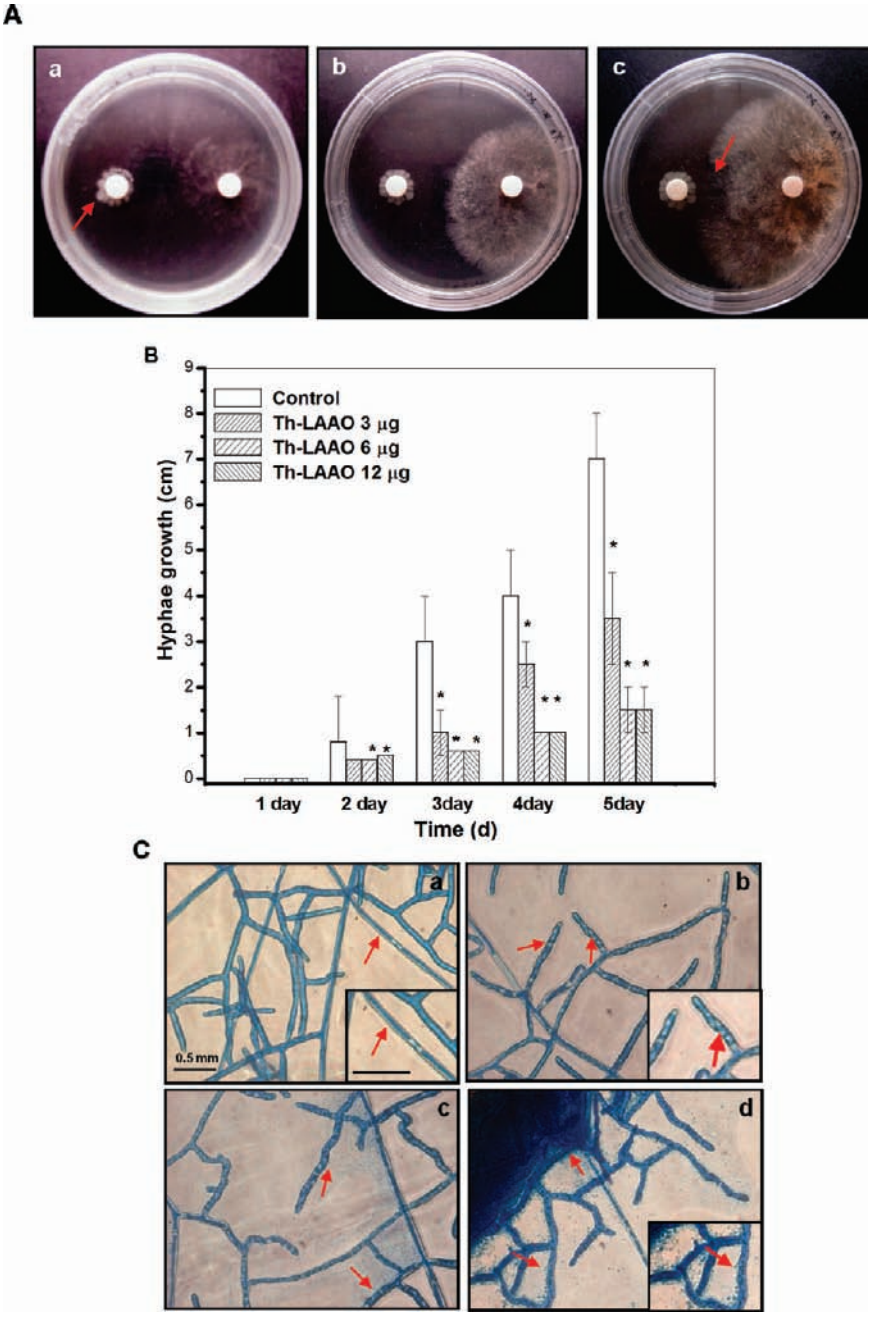


**Figure 5.** Effect of Th-LAAO on the hyphal growth of *R. solani*. (A) Paper disk method: growth of *R. solani* on PDA media with (left) or without (right) Th-LAAO (6 $\mu$g disk$^{-1}$) on PDA plates were photographed on the 4th (a), 5th (b), and 6th (c) days. The arrow in panel (a) indicates a large amount of hyphal lysis. In panel c, the arrow indicates the antifungal zone of Th-LAAO against *R. solani*. The asterisk in plot B indicates hyphal lysis. (C) Microscopy observations: all panels are presented in the same magnification. Panel (a) and its inset indicate the control treatment after the 5th day of incubation. The arrows in panel (b) and its inset indicate the malformed and vacuolated hyphae of *R. solani* after the 5th day of incubation with Th-LAAO. The arrows in panel (c) indicate the lysis of malformed hyphae after vacuolation. In panel (d) and its inset, the arrow indicates large amounts of hyphal lysis. Scale bar for inset panels = 0.5 mm, and all inset panels are presented in the same magnification.

When observed under a light microscope, results from the PDA-coverslip showed some additional hyphal growth of *R. solani* after treatment with Th-LAAO. These mycelia demonstrated hyphal distortion (Figure 5C), including swelling, malformed hyphae, and vacuolation; however, these were not observed in the control group (Figure 5C, a). After vacuolation, hyphae lyses occurred in the malformed hyphae (Figure 5C, b and c), whereas control hyphal walls were smooth and showed no vacuolation.

**Effect of Th-LAAO on *T. harzianum* ETS 323.** The profound hyphal damage observed in *R. solani* in the presence of Th-LAAO raised the possibility that this protein might have an effect on *T. harzianum* ETS 323 itself. However, the effect of Th-LAAO on the hyphal growth of *T. harzianum* ETS 323 was opposite to that of *R. solani*. Both hyphal density and sporulation of *T. harzianum* ETS 323 increased in the presence of Th-LAAO relative to those of the control (Figure 6A). Production of conidia by both $H_2O$- and

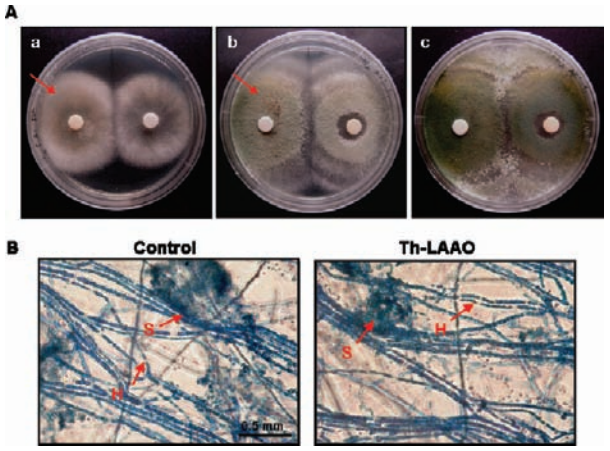


**Figure 6.** Effect of Th-LAAO on the hyphal growth and subsequent sporulation of *T. harzianum* ETS 323. (A) Paper disk method: growth of *T. harzianum* ETS 323 on PDA media with (left) or without (right) Th-LAAO (6 $\mu$g disk$^{-1}$) was photographed on the 3rd (a), 4th (b), and 5th (c) days. The arrows in panels (a) and (b) indicate that the addition of Th-LAAO enhanced the hyphal density and sporulation of *T. harzianum* ETS 323. (B) Light microscopy: the photomicrographs reveal that there was no significant difference in the morphology of hyphae and spores with no treatment (left) and treatment (right) of Th-LAAO. H: hyphae, S: spore.

Th-LAAO-treated colonies was quantified after 5 and 7 days of incubation (Table 3). The number of conidia produced at the fifth day differed significantly between the two groups, with sporulation higher in Th-LAAO-treated *T. harzianum* ETS 323. By the seventh day, the difference was even greater, with a 3-fold increase in the conidia of Th-LAAO-treated *T. harzianum* ETS 323 when compared with the control treatment. Moreover, the hyphal morphology at the fifth day indicated normal and extensive hyphal growth on both control and Th-LAAO-treated colonies (Figure 6B).

## DISCUSSION

A novel L-amino acid oxidase from the extracellular proteins of *T. harzianum* ETS 323 was first reported here. Th-LAAO is a homodimeric glycoprotein like other LAAO family members,[14,16] with a monomeric molecular weight of 67 kDa. Based on previous reports, LAAO family members possess flavin as a coenzyme and two highly conserved motifs, including a dinucleotide-binding motif and a GG motif.[10,14] In this study, Th-LAAO possessed the two conserved motifs in its N-terminal portion, and a FAD cofactor was identified, indicating high homology between Th-LAAO and other LAAOs in the N-terminus; however, the amino acid sequence alignment of Th-LAAO showed limited homology to other LAAOs outside this region.

LAAOs have been reported to be present in the secretions of many organisms, such as the venom of snake,[17] the body surface mucus of the giant African snail,[18] the skin mucus of rockfish,[19] and the ink of the sea hare.[8] However, their biological purposes and mode(s) of action have not been fully clarified. In this study, in the presence of *R. solani* hyphae, there was an increase in the concentration of Th-LAAO in the culture medium of *T. harzianum* ETS 323. Moreover, coculture with deactivated *R. solani* hyphae increased the levels of the monomeric form of Th-LAAO relative to the homodimeric one. However, interestingly, the homodimeric form of Th-LAAO from medium I had lower effect of inducing *R. solani* hyphal lysis (data not shown) but no discernible effect on the hyphal growth of *T. harzianum* ETS 323 (data not shown). A possible explanation is that large amounts of monomeric Th-LAAO increase the contact area between Th-LAAO and *R. solani* hyphae and subsequently improve the hyphal lysis of the fungus. Th-LAAO may play an important defense role in the biocontrol mechanism in that *R. solani* may alter the secretion amount and dimerization state of Th-LAAO and ultimately affect the ability of Th-LAAO to defend itself while enhancing the growth of *T. harzianum* ETS 323. Furthermore, in vitro assays revealed a possible role for Th-LAAO in the antagonism between *T. harzianum* ETS 323 and *R. solani*. This is the first evidence that an LAAO from *T. harzianum* ETS 323 might be associated with the mycoparasitic mechanism against *R. solani*. However, it is not clear if other LAAOs from different organisms share similar biological purpose and modes of action.

**Table 3. Effect of Th-LAAO on the Amount of Conidia Produced by T. Harzianum ETS 323 after 5 and 7 Days of Incubation**

| | conidia yield (spore mL$^{-1}$)[a] | |
|---|---|---|
| treatment | 5 days | 7 days |
| $H_2O$ | $1.9 \times 10^7 \pm 0.13$ | $2.3 \times 10^7 \pm 0.12$ |
| Th-LAAO | $5.1 \times 10^7 \pm 0.21$ | $7.1 \times 10^7 \pm 0.11$ |

[a] Values shown are mean ± SEM of three replicates. Treatment with highest growth was compared to other treatments using the Student's *t*-test, where $^*P < 0.05$.

It has been reported that venom LAAOs [20,21] can associate with cell surfaces such as those of human vein endothelial cells and mouse lymphocytic leukemia cells, inducing $H_2O_2$-mediated damage that initiates apoptosis. Nonetheless, cell viability was not fully recovered with catalase. This result indicated that the LAAO-induced apoptosis is not fully explained by the effect of $H_2O_2$ production at the cell membrane. Therefore, it will be intriguing to discover whether Th-LAAO associates with the cell wall of *R. solani*. For addressing this question, a fluorescent isothiocyanate-labeled Th-LAAO was prepared. The preliminary results showed that Th-LAAO did attach to the cell wall of *R. solani*. In light of this, 10 $\mu$M $H_2O_2$ was applied directly to *R. solani*, but no expected growth inhibition was observed. Again, exogenous application of $H_2O_2$ cannot fully explain the effect of Th-LAAO on the hyphal lysis of *R. solani*. Therefore, based on previous reports,[20,21] we propose that Th-LAAO may bind to apoptosis (hyphal lysis)-related cell wall proteins of *R. solani* and that Th-LAAO may alter the structures of these proteins and thereby cause their dysfunction. Later, Th-LAAO may also induce the oxidation of the target proteins and produce a local $H_2O_2$ concentration increase that induces the apoptosis (hyphae lysis) of *R. solani*. Further study is now under progress to search for cell wall components that correlate with the Th-LAAO-induced hyphal lysis and the apoptotic features of *R. solani*.

LAAOs have been widely found in snake venoms and are among the most abundant proteins in venoms, particularly hemorrhagic venoms.[10,22] In previous reports, venom LAAOs have been shown to affect platelet aggregation,[23,24] induce apoptosis,[15] have antimicrobial effects,[24] and effect hemolysis[25] in addition to catalyzing the oxidation of amino acids. However, the venom of snakes is usually employed for both prey capture and defense. Even though *T. harzianum* ETS 323 has limited sequence homology to other LAAO family members and is in a different kingdom of classification from the snake, considering that Th-LAAO has a similar

defensive role for *T. harzianum* ETS 323 itself, this may suggest the importance of conservation and genetic diversification of LAAOs contributing to the survival of diverse organisms.

Further investigations will be needed to determine whether Th-LAAO has antagonistic activity toward other plant pathogens besides *R. solani*. We anticipate that further research into the function of Th-LAAO and other LAAOs will establish important paradigms in the field of biocontrol and provide insight into the biological function of these enzymes.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Three additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Authors

*(J.-W.L.) Tel: 886-3-8565301; Fax: 886-3-8572526; E-mail: jwlee@mail.tcu.edu.tw. (K.-C.P.) Tel: 886-3-8633635; Fax: 886-3-8633630; E-mail: kcpeng@mail.ndhu.edu.tw.

### Funding Sources

The work was supported by the NSC, Taiwan, under Grant No. NSC-99−2324-B-259−001.

## ■ ACKNOWLEDGMENT

We thank Dr. Yen-Peng Ho of Chemistry Department, National Dong Hwa University, Tawain, for technical assistance of ESI-MS.

## ■ ABBREVIATIONS USED:

*T. harzianum* ETS 323, *Trichoderma harzianum* ETS 323; *R. solani*, *Rhizoctonia solani*; LAAO, L-amino acid oxidase; Th-LAAO, *T. harzianum* ETS 323 L-amino acid oxidase; FAD, flavin adenine dinucleotide.

## ■ REFERENCES

(1) Chet, I. *Trichoderma*: application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In *InnoVatiVe Approaches to Plant Disease Control*; Chet, I., Ed.; Wiley: New York, 1987; pp 137−160.

(2) Benitez, T.; Rincon, A. M.; Limon, M. C.; Codon, A. C. Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* **2004**, *7*, 249–260.

(3) Almeida, F. B.; Cerqueira, F. M.; Nascimento, R. N.; Ulhoa, C. J.; Lima, A. L. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: Evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Lett.* **2007**, *29*, 1189–1193.

(4) Antal, Z.; Manczinger, L.; Szakács, G.; Tengerdy, R. P.; Ferenczy, L. Colony growth, *in vitro* antagonism and secretion of extracellular enzymes in cold tolerant strains of *Trichoderma* species. *Mycol. Res.* **2000**, *104*, 545–549.

(5) Tseng, S. C.; Liu, S. Y.; Yang, H. H.; Lo, C. T.; Peng, K. C. Proteomic study of biocontrol mechanisms of *Trichoderma harzianum* ETS 323 in response to *Rhizoctonia solani*. *J. Agric. Food Chem.* **2008**, *56*, 6914–6922.

(6) Samel, M.; Vija, H.; Rönnholm, G.; Siigur, J.; Kalkkinen, N.; Siigur, E. Isolation and characterization of an apoptotic and platelet aggregation inhibiting L-amino acid oxidase from *Vipera berus berus* (common viper) venom. *Biochim. Biophys. Acta* **2006 1764**, 707–714.

(7) Ehara, T.; Kitajima, S.; Kanzawa, N.; Tamiya, T.; Tsuchiya, T. Antimicrobial action is mediated by L-amino acid oxidase activity. *FEBS Lett.* **2002**, *531*, 509–512.

(8) Yang, H.; Johnson, P. M.; Ko, K. C.; Kamio, M.; Germann, M. W.; Derby, C. D.; Tai, P. C. Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink the sea hare Aplysia californica. *J. Exp. Biol.* **2005**, *208*, 3609–3622.

(9) Skarnes, R. C. L-Amino acid oxidase, a bactericidal system. *Nature* **1970**, *225*, 1072–1073.

(10) Du, X. Y.; Clemetson, K. J. Snake L-amino acid oxidases. *Toxicon* *40*: 659-665.

(11) Takatsuka, H.; Sakurai, Y.; Yoshioka, A.; Kokubo, T.; Usami, Y.; Suzuki, M.; Matsui, T.; Titani, K.; Yagi, H.; Matsumoto, M.; Fujimura, Y. Molecular characterization of Lamino acid oxidase from *Agkistrodon halys blomhoffii* with special reference to platelet aggregation. *Biochim. Biophys. Acta* **2001**, *1544*, 267–277.

(12) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(13) Nishizawa, T.; Aldrich, C. C.; Sherman, D. H. Molecular analysis of the rebeccamycin L-amino acid oxidase from *Lechevalieria aerocolonigenes* ATCC 39243. *J. Bacteriol.* **2005**, *187*, 2084–2092.

(14) Vallon, O. New sequence motifs in Flavoproteins: Evidence for common ancestry and tools to predict structure. *Proteins* **2000**, *38*, 95–114.

(15) Torii, S.; Yamane, K.; Mashima, T.; Haga, N.; Yamamoto, K.; Fox, J. W.; Naito, M.; Tsuruo, T. Molecular cloning and functional analysis of apoxin I, a snake venom-derived apoptosis inducing factor with L-amino acid oxidase activity. *Biochemistry* **2000**, *39*, 3197–3205.

(16) Rodrigues, R. S.; da Silva, J. F.; Boldrini França, J.; Fonseca, F. P.; Otaviano, A. R.; Henrique, S.; Hamaguchi, A.; Magro, A. J.; Braz, A. S.; dos Santos, J. I.; Homsi Brandeburgo, M. I.; Fontes, M. R.; Fuly, A. L.; Soares, A. M.; Rodrigues, V. M Structural and functional properties of Bp-LAAO, a new L-amino acid oxidase isolated from *Bothrops pauloensis* snake venom. *Biochimie* **2009**, *91*, 490–501.

(17) Stiles, B. G.; Sexton, F. W.; Weinstein, S. A. Antibacterial effects of different snake venoms: purification and characterization of antibacterial proteins from *Pseudechis australis* (Australian king brown or mulga snake) venom. *Toxicon* **1991**, *29*, 1129–1141.

(18) Ehara, T.; Kitajima, S.; Kanzawa, N.; Tamiya, T.; Tsuchiya, T. Antimicrobial action is mediated by L-amino acid oxidase activity. *FEBS Lett.* **2002**, *531*, 509–512.

(19) Kitani, Y.; Tsukamoto, C.; Zhang, G.; Nagai, H.; Ishida, M.; Ishizaki, S.; Shimakura, K.; Shiomi, K.; Nagashima, Y. Identification of an antibacterial protein as L-amino acid oxidase in the skin mucus of rockfish Sebastes schlegeli. *FEBS J.* **2007**, *274* (1), 125–36.

(20) Suhr, S. M.; Kim, D. S. Identification of the snake venom substance that induces apoptosis. *Biochem. Biophys. Res. Commun.* **1996**, *224*, 134–139.

(21) Torii, S.; Naito, M.; Tsuruo, T. Apoxin I, a novel apoptosis-inducing factor with L-amino acid oxidase activity purified from western diamondback rattlesnake venom. *J. Biol. Chem.* **1997**, *272*, 9539–9542.

(22) Sun, M. Z.; Guo, C.; Tian, Y.; Chen, D.; Greenaway, F. T.; Liu, S. Biochemical, functional and structural characterization of Akbu-LAAO: A novel snake venom L-amino acid oxidase from *Agkistrodon blomhoffii* ussurensis. *Biochimie* **2010**, *92*, 343–349.

(23) Sakurai, Y.; Takatsuka, H.; Yoshioka, A.; Matsui, T.; Suzuki, M.; Titani, K.; Fujimura, Y. Inhibition of human platelet aggregation by L-amino acid oxidase purified from *Naja naja kaouthia* venom. *Toxicon* **2002**, *39*, 1827–1833.

(24) Stábeli, R. G.; Marcussi, S.; Carlos, G. B.; Pietro, R. C.; Selistre-de-Araujo, H. S.; Giglio, J R.; Oliveira, E. B.; Soares, A. M. Platelet aggregation and Antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom. *Bioorg. Med. Chem.* **2004**, *12*, 81–2886.

(25) Ciscotto, P.; Machado de Avila, R. A.; Coelho, E. A.; Oliveira, J.; Diniz, C. G.; Farías, L. M.; Carvalho, M. A.; Maria, W. S.; Sanchez, E. F.; Borges, A.; Chávez-Olórtegui, C. Antigenic, microbicidal and antiparasitic properties of an L-amino acid oxidase isolated from *Bothrops jararaca* snake venom. *Toxicon* **2009**, *53*, 330–341.