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Full Length Research Paper

Purification of an elicitor from *Magnaporthe oryzae* inducing defense resistance in rice

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Inducible defenses that contribute to overall resistance in plant can be triggered by elicitors. A novel elicitor, derived from the mycelia of the blast fungus *Magnaporthe oryzae*, was purified to homogeneity by HiPrep 16/20 DEAE-Sepharose FF, Concanavalin A-Sepharose 4B and HiPrep 16/60 Sephacryl S-100 column chromatography. The purified elicitor appeared as single band corresponding to a molecular weight of 48.53 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophresis (SDS-PAGE) and a pI of 6.01 on isoeletric focusing (IEF) gel. Treatment with the purified elicitor increased the activities of phenylalanine ammonium-lyase (PAL**) and peroxidase (**POD**) in rice susceptible cultivar CO39. Time-course analysis showed peak accumulation of PAL appeared at 24 h after treatment, and it was higher in challenge-inoculated plants than non-challenge plants. POD accumulation showed similar kinetics with PAL, but the largest peak appeared at 36 h after treatment. Compared to the untreated control plants, pretreatment of rice leaves with the purified elicitor provided an enhanced level of protection against *M. oryzae*. *N*-terminal blocked elicitor was identified as hypothetical protein MG 05155.4 with 26.28% mass fingerprint coverage by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The results suggest that the purified elicitor is involved in inducing resistance against blast fungus.**

Key words: *Magnaporthe oryzae*, elicitor, purification, induced resistance.

INTRODUCTION

Plant innate immunity is generally divided into two branches that efficiently wards off dangerous micro-organism. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs), resulting in PAMP-triggered immunity (PTI). However, pathogens deploy effectors that interfere with PTI and ultimately enable a successful infection. In turn, plants evolved the ability to recognize effectors either directly or indirectly through NB-LRR proteins that activate effector-triggered immunity (ETI). These two forms formerly called basal or horizontal disease resistance and resistance(*R*) gene-based or vertical disease resistance (Boller and Felix, 2009; Jones and Dangl, 2006).

Rice blast, caused by the fungus *Magnaporthe oryzae*, is one of the most destructive diseases of rice and often reduces yields greatly in rice-growing countries (Ou, 1980; Rossman et al., 1990). Annual losses caused by *M. oryzae* vary between 10 and 30% of the harvest (Skamnioti and Gurr, 2009). Genetic resistance is the major method of disease control for blast (Martin et al., 2002). However, due to rapid adaptability and high variability of the fungal population in the field, frequent loss of resistance of rice cultivars is a major restraint in sustainable rice production. In practices, farmers usually go for chemical control which is comparatively expensive and environmentally unsafe. Concomitantly, increased public concerns to the possible negative health effects of chemical residues have forced policy changes in many countries. Therefore, it has become increasingly important to develop the efficient and environment-

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Abbreviations: SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophresis; **IEF,** isoeletric focusing; **PAL,** phenylalanine ammonium-lyase; **POD,** peroxidase; **MALDI-TOF MS,** matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

friendly control strategy against rice blast.

PAMPs usually represent highly conserved structures which carry essential functions. It is clear that “general elicitors” are conceptually equivalent to PAMPs (Nurnberger et al., 2004). Plant cells exposed to elicitors, whether crude fungal cell wall fragments or defined molecular, respond with a battery of cellular changes such as changes in ion fluxes, the generation of reactive oxygen species, the synthesis of phytoalexins, the accumulation of defense-related enzymes that including Peroxidase (POD) and Phenylalanine Ammonium-Lyase (PAL), and the activation of defense gene expression (Dixon and Lamb, 1990; Yang et al., 1997; Zhao et al., 2005). As an alternative and ecologically-friendly approach for plant protection, induced disease resistance has been shown to occur in plants in response to a localized pretreatment with elicitors thus making them resistant to subsequent pathogen infection (Hammerschmidt, 1999; Mohammadi and Kazemi 2002). In recent years, understanding the molecular basis of plant responses to PAMPs is an active area of research in the field of plant-microbe interactions. The rice-*M. oryzae* pathosystem has become a model system for genetic and molecular biology studies in plant pathology (Valent, 1990). Some biotic elicitors originating from fungi, oomycetes and bacteria have been described in a wide range of plant species (Garcia-Brugger et al., 2006). In this paper, a novel elicitor was purified from the mycelia of *M. oryzae* and characterized by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Treatment of rice leaves with the purified elicitor induced the accumulation of defense-related enzymes (POD and PAL), and increased resistance to blast fungus.

MATERIALS AND METHODS

Plant and fungal material

Rice blast fungus, *M. oryzae*, race ZC_{13}, one of the primary blast physiological races found in Guangdong Province, P. R. China, and the susceptible indica cultivar CO39 were used in this study.

Crude elicitor preparation

The preparation of the crude elicitor was performed according to the method described by Schaffrath (1995) with some modifications. Mycelia were incubated in yeast liquid medium (yeast extract 5.0 g, sucrose 20 g, distilled water 1 L). Vegetative mycelia were harvested from 7-day-old liquid cultures. After thawing in a Waring-Blender, the material was stirred at 4°C overnight and centrifuged at 10,000 g for 30 min. The supernatants were filtered successively through Whatman No.1 qualitative filter paper and a 0.22-µm Millipore filter. The filtrates were concentrated by ultra-filtration (Amicon 8400 ultrafiltration system) using a PM-30 membrane (Millipore) and the biologically active residue (>30 kDa) as crude elicitor fractions (CEF).

Column chromatography

HiPrep 16/20 DEAE-Sepharose FF column, HiPrep 16/60 Sephacryl S-100 column, XK 16/20 column and Concanavalin A-Sepharose 4B media were purchased from Pharmacia. The following described purification procedure was performed on an AKTA purifier system 100 (Amersham Pharmacia Biotech, USA).

All operations were performed at 0 to 4°C. The crude elicitor solutions were loaded onto a HiPrep 16/20 DEAE-Sepharose FF column, which was equilibrated with 20 mM Tris- HCl buffer, pH7.4. Unbound material was washed out with 2 columns of Tris-HCl buffer. The bound fraction was eluted with 10 columns of a linear gradient of 0 to 0.75M NaCl at a flow rate of 3 ml/min. The fractions with the highest elicitor activity collected from the anion-exchange column were injected to Concanavalin A-Sepharose 4B column (2.6 × 20 cm), which was equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$. The non-binding material was washed with equilibration buffer, and ConA-binding material was subsequently displaced by 0.15 M α-methyl-D-Mannoside in equilibration buffer at a flow rate of 0.2 ml/min. The highest elicitor active fractions collected from the ConA affinity column were subjected to a HiPrep 16/60 Sephacryl S-100 column. The column was eluted with 20 mM Tris-HCl buffer (pH 7.4) at a flow rate of 0.5 ml/min. The highest elicitor active fractions obtained from several separate gel filtration runs were pooled, lyophilized, and stored at -80°C until use.

Protein concentration was determined according to the Bradford (1976) method using bovine serum albumin as a standard.

Assay for elicitor activity

Elicitor activity was assayed by the method of Schaffrath (1995) using POD activity as a biomarker with some modifications. The rice seedlings (cv. CO39) were grown in the greenhouse at 26～22°C (day/night) with 70 to 80% relative humidity. At the four-leaf stage, the fully expanded fourth leaves were injected with the tested solutions using a syringe without needle. Control plants were infiltrated with distilled water. Treated rice seedlings were kept in a humidity chamber and sampled 36 h after treatment. The POD activity was assayed as described by Ryan et al., (1982) except that the 3-ml reaction mixture contained sodium phosphate buffer (20 mM, pH5.6), 18 mM guaiacol, 3.3 mM H_2O_2 and 20 µl crude enzyme. An increase in the absorbance at 470 nm ($\triangle A_{470}$) of 2 min was recorded using a spectrophotometer. One unit of the POD activity was defined as the amount of enzyme that increased the absorbance by 0.01 per min at 470 nm. One unit of elicitor activity was defined as the amount of the sample required to induce one unit of POD activity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and *N*-terminal amino acid sequence analysis

Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 3% acrylamide stacking and 10% resolving gels on a mini-PROTEAN III gel system (Bio-Rad). After electrophoresis, gels were rinsed and subsequently silver stained (Oakley et al., 1980). The molecular masses of the purified elicitor were measured by SDS-PAGE.

The purified elicitor was separated by SDS-PAGE and electro-blotted onto polyvinylidene difluoride (PVDF) membranes. The membrane stained and protein band cut out for *N*-terminal sequence analysis with Procise 491 automatic sequencer (Applied Biosystems Inc., USA).

Isoelectric focusing

The obtained purified elicitor was analyzed by isoeletric focusing (IEF) as described by Garfin (1990) with some modifications. 10 mM phosphoric acid and 20 mM sodium hydroxide were used as anolyte and catholyte respectively. The sample focusing was conducted under a constant voltage of 100 V for 15 min, 250 V for an additional 15 min, and finally 450 V for 1.5 h. After focusing, the samples were stained with Coomassie brilliant blue R-250. The isoeletric point of the sample was determined by comigration with protein-pI markers.

Mass spectrometry

The Coomassie G-250 stained protein band was excised from the SDS-PAGE gel and subjected to in-gel digestion with trypsin. Mass spectra were recorded on a Bruker Biflex III MALDI-TOF mass spectrometer in reflector mode. Protein identification was performed with Mascot search engine (http://www.matrixscience.com and SWISS-PROT database (http://www.expasy.ch/tools/peptident.html).

Rice infection assays

M. oryzae was cultured in Petri dishes containing 20 ml of medium composed of 20 g l^{-1} rice bran powder, 2.5 g l^{-1} yeast extract, 1.5% agar. The cultures were placed in a growth chamber with a 12 h photoperiod for 7 to 9 d. Conidia were harvested from plates by rinsing with sterile distilled water and filtering through two layers of gauze.

Rice seedlings of susceptible cultivar CO39 at four-leaf-stage were pretreated with the purified elicitor or distilled water as described above, and challenge inoculated with *M. oryzae* 1 d later by spraying conidial suspensions at a concentration of 2×10^5 conidia per ml. The non-inoculated plants were sprayed with the same amount of distilled water. Inoculated and non-inoculated rice seedlings were kept in a moist chamber at 25℃ for 24 h and then transferred to the glasshouse. Disease index of each replicate (containing 30 seedlings) and a total 90 seedlings in three replicates of each treatment were evaluated 5 to 9 d after inoculation according to IRRI (2002). Disease index and inducing effect (%) were calculated according to the formula:

$$\text{Disease index} = [\Sigma(r \times n_r)/(9 \times N_r)] \times 100$$

Where, r is the score of scale; n_r is the number of infected leaves with a rating of r; N_r is the total number of investigated leaves; and Σ is the sum numbers of infected leaves of different scores.

Inducing effect (%) = (Control - Treated)/Control ×100.

Enzyme assays

In order to elucidate the mechanism of induced resistance in elicitor-pretreated rice leaves, the time course of defense-related enzymes including POD and PAL activities was investigated. There were four treatment groups with three replications: (1) challenge inoculated with *M. oryzae* in elicitor-treated plants, (2) challenge inoculated with *M. oryzae* in water-treated plants, (3) elicitor-treated plants, (4) water-treated plants. The elicitor infiltration and pathogen inoculation were carried out as described above. At various times after treatment, the fresh leaves were pooled, frozen in liquid nitrogen and stored at -20℃ until used. All enzyme extract procedures were conducted at 4℃. POD activity was carried out as described above. PAL activity was determined according to the method of Mozzetti (1995) with slight modifications. One gram (1 g) of the leaves was ground with 2 ml extracting buffer (20 mM potassium borate buffer containing 0.2 g PVPP, pH 8.8). The extracts were homogenized and centrifuged at 12,000 × g for 30 min, the supernatant was collected as crude enzyme extract. The reaction mixture consisted of 100 µl of crude enzyme, 3.65 ml of 100 mM potassium borate buffer (pH 8.8), and 1.25 ml of 20 mM L-phenylalanine solution. After incubation at 40℃ for 30 min, the reaction was stopped by addition of 250 µl of 5 N HCl. One unit of PAL activity was defined as the amount causing an increase of 0.01 in A290 per min. PAL activity was expressed as enzyme units per mg protein (U mg $protein^{-1}$).

Statistical analysis

All statistical analyses were performed using SPSS software version 10.0. Data were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was performed to determine significant difference between treatments. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Purification of a 49 kDa elicitor from *M. oryzae*

Vegetative mycelia of *M. oryzae* were harvested from 7-day-old liquid culture. After centrifugation and ultrafiltration, the >30 kDa fractions were active and served as CEF for the further purification. CEF was applied to a HiPrep 16/20 DEAE-Sepharose FF column, the anion-exchange chromatographic profile is shown in Figure 1a. The elution of the bound fractions, including D3, D4, were pooled and tested for elicitor activity respectively. All these two fractions (D3, D4) were biologically active, while the highest active fraction was yielded in the peak D4. The active peak D4 was injected onto an affinity column (Concanavalin A-Sepharose 4B). The activity assay indicated that the non-binding fraction (peak C2) contained elicitor-active components (Figure 1b). Further purification of active peak C2 by the gel filtration column (Figure 1c) resulted in one sharp peak (S1) and one small diffused peak (S2), two peak fractions (S1，S2) were pooled and analyzed for elicitor activity, the peak S1 fraction showed the highest biological activity. The pooled peak S1 fractions showed only one band on a silver-stained SDS-polyacrylamide gel (Figure 2). The apparent molecular weight of peak S1 fraction estimated by SDS-PAGE was about 48.53 kDa. SDS-PAGE with or without the reducing agent *β*-mercaptoethanol had no effect on the migration of the peak S1 fraction, indicating there is no existence of the disulfide bridges in peak S1 fraction. Thus, one 48.53 kDa elicitor was purified to homogeneity from the mycelia of *M. oryzae*. A balance sheet of the elicitor purification is summarized in Table 1. Elicitor was purified with a recovery of 10.13% to a final specific activity of 35 U mg $protein^{-1}$. The isoelectric point of the elicitor was estimated to be 6.01 by IEF (Figure 3).

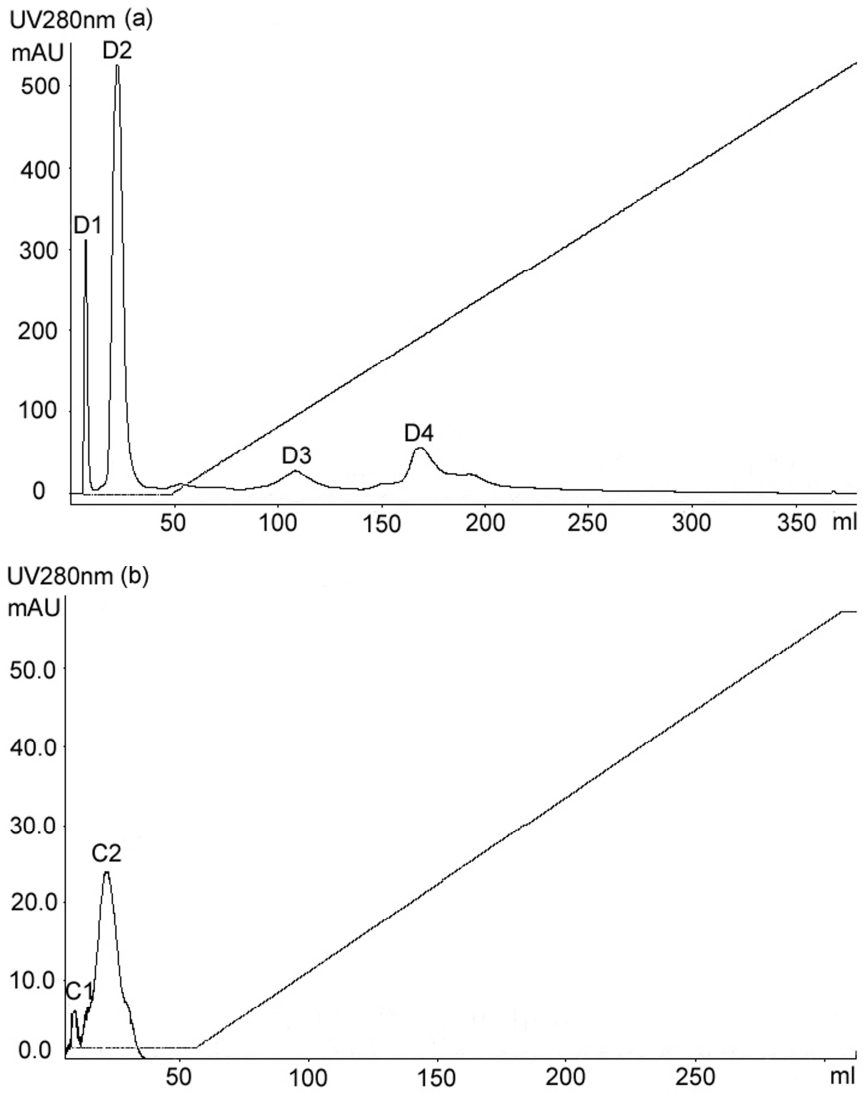
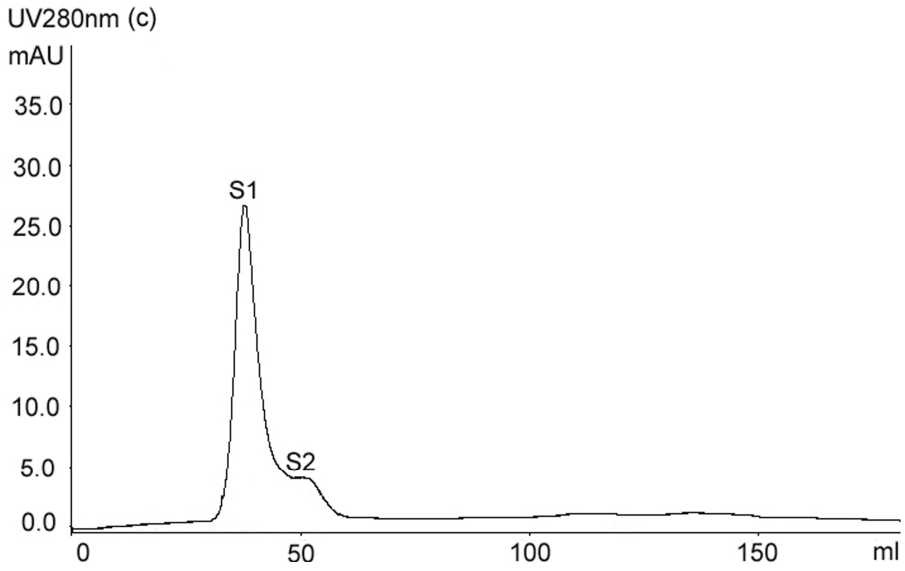


Figure 1. Purification of elicitor prepared from the crude CEF. CEF derived from the mycelia of *M. oryzae* was further purified by the column chromatography. The elution profiles were monitored at 280 nm. Each peak fraction was collected according to the elution profile and assayed for elicitor activity by detecting the ability to induce the accumulation of POD at 36h post-inoculation in susceptible cultivar CO39. (a): Elution profile of crude elicitor fraction on DEAE-Sepharose FF column. Four distinct peaks (D1, D2, D3, and D4) were obtained and tested for biological activity. Peak D4 was the main activity fraction. (b): Elution profile of DEAE-Sepharose FF fraction D4 on Concanavalin A-Sepharose 4B column. Two peaks (C1, C2) were obtained and assayed for biological activity. Unbounded fraction C2 showed the elicitor activity. (c): Elution profile of Concanavalin A-Sepharose 4B fraction C2 on Sephacryl S-100 column. Gel filtration yielded two peaks (S1, S2) and assayed for biological activity, Peak S1 showed the highest elicitor activity.

N-terminal sequencing and MALDI-TOF MS analysis

Edman degradation is the conventional method for the *N*-terminal sequencing. Unfortunately, sequence analysis indicated the elicitor was *N*-terminal blocked.

The genome of *M. oryzae* has been fully sequenced, proteomics approaches are most effective when supported by complete genome sequence databases. The purified elicitor band from the Coomassie bule R-250 staining gel was excised and followed by MALDI-TOF MS analysis (Figure 4). The purified elicitor was identified as hypothetical protein MG 05155.4 with 26.28% mass fingerprint coverage (Table 2). Furthermore, search for sequence similarity using the BLAST program did not



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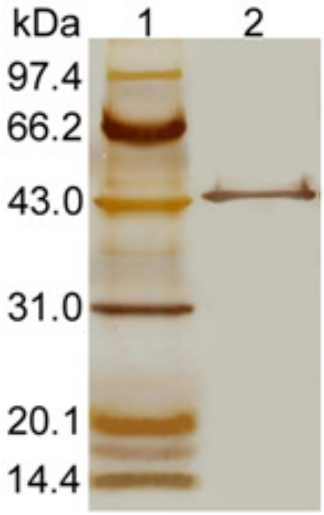

Figure 2. SDS-PAGE analysis of the purified elicitor. The active peak S1 (obtained from Sephacryl S-100 gel filtration) was examined on silver-stained SDS-polyacrylamide gel and displayed a clear single band with apparent relative molecular weight of 48.53 kDa. Lane 1: Low molecular weight standard molecular weight marker (Qiangen): 97,400, 66,200, 43,000, 31,000, 20,100 and 14,400. Lane 2: Purified elicitor.

reveal any significant sequence similarity to any known proteins.

The purified elicitor increased resistance to *M. oryzae*

In order to evaluate whether the application of elicitor affected rice blast disease development, the fully expanded fourth leaves of susceptible cultivar CO39 were injected with the purified elicitor or distilled water and subsequently challenged with *M. oryzae*. As shown in Table 3, the Disease index in water-treated control plants was higher than in plants pretreated with elicitor. In comparison to control plants, the application of elicitor reduced the Disease index of rice blast by 16.65, 38.99 and 44.65% at 5, 7 and 9 days post-inoculation, respectively. The results suggested the purified elicitor treatment induced resistance in rice plants against *M. oryzae*.

The accumulation of defense related enzyme activities induced by the purified elicitor

POD activity has been reported to be a biochemical marker for resistance (Hiraga et al., 2001). PAL is the first enzyme in the phenylpropanoid pathway which has important functions in plants following exposure to environmental stresses and pathogen attack (Hahlbrock and Scheel, 1989). The time course measurement of POD and PAL accumulation induced by the purified elicitor against *M. oryzae* were carried out using leaf tissues of susceptible cultivar CO39. As shown in Figure 5a, in inoculated elicitor-treated plants, assays of POD activity revealed the highest peak appeared at 36 h after challenge inoculation, and the activity was nearly 1.19 times higher than that in inoculated water-treated plants. A similar pattern of increased activity of POD was observed in uninoculated elicitor-treated plants, the activity reached maximum at 36 h, then to decline gradually. There was no marked change in POD activity in uninoculated water-treated plants during the time

Table 1. Purification of elicitor from the mycelia of *M. oryzae*.

Purification step	Total protein (mg)	Total activity (units)[a]	Specific activity (U mg protein^{-1})[b]	Yield (%)
Crude elicitor fraction	512	2355	4.6	100
DEAE	97.5	2033.9	20.86	86.36
ConA	30.6	736.5	24.07	31.27
S-100	6.82	238.7	35	10.13

a: One unit of elicitor activity is defined as the amount of the sample required to induce one unit of POD activity at 36 h post-inoculation in cultivar CO39, one unit of POD activity was defined as the increase of 0.01 Δ470 per min. *b*: The specific elicitor activity of the sample is defined as the number of units per mg protein of the sample.

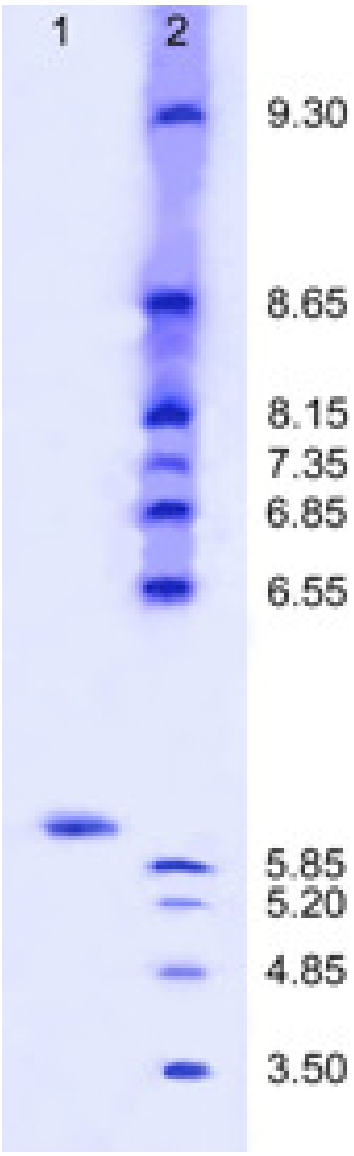

Figure 3. Isoelectric focusing of the purified elicitor. Isoelectric focusing was performed in pH gradient 3.5 to 9.3, the purified elicitor migrated as a single band and focused at apparent isoelectric point (pI) 6.01. Line 1: purified elicitor. Line 2: pI standard marker, 3.5 to 9.3 pI range (Pharmacia).

course of experimental period.

As shown in Figure 5b, in inoculated elicitor-treated plants, PAL activity increased quickly and reached its maximum level at 24 h after challenge inoculation, and was about 1.38 times more than that in inoculated water-treated plants. PAL activity was also significantly higher than that of the control plants and reached its maximum at 24 h after treatment with elicitor alone.

DISCUSSION

To better understand the molecular mechanism of how plant cells perceive and transduce the elicitor signal, pure elicitor molecules are preferred to rule out effects of contaminants in the crude preparation. In the present paper, a novel elicitor, which has a molecular mass of 48.53 kDa and pI of 6.1, was purified to homogeneity form the mycelia of *M. oryzae* by HiPrep 16/20 DEAE-Sepharose FF, Concanavalin A-Sepharose 4B and HiPrep 16/60 Sephacryl S-100 column chromatography. The availability of this pure elicitor offers more possibilities to further study the downstream defense reactions in rice host plants. Affinity chromatography separates proteins on the basis of a reversible interaction between a protein and a specific ligand coupled to a chromatography matrix. It offers high selectivity, and high capacity for the protein of interest. Unfortunately, the purified elicitor did not bind to Con-A Sepharose 4B column during the entire chromatography process, thus, in order to minimize loss of elicitor activity, it might be possible to establish a more rapid purification procedure for further study by eliminating any steps such as Con-A Sepharose 4B that might unfavorably affect yield.

Different types of elicitors including oligosaccharides, glucans, glycoproteins and sphingolipids have been reported to induce defense responses in rice plants (Kishimoto et al., 2010; Koga et al., 1998; Matsumura et al., 2003; Peng et al., 2011; Schaffrath et al., 1995; Yamaguchi et al., 2002). Among them, chitin oligo-saccharides elicitor like *N*-acetylchitooligosaccaride was given an extensive study, which could induce various cellular responses including phytoalexin production, oxidative burst, gene expression and HR induction. A high affinity binding site for *N*-acetylchitooligosaccaride

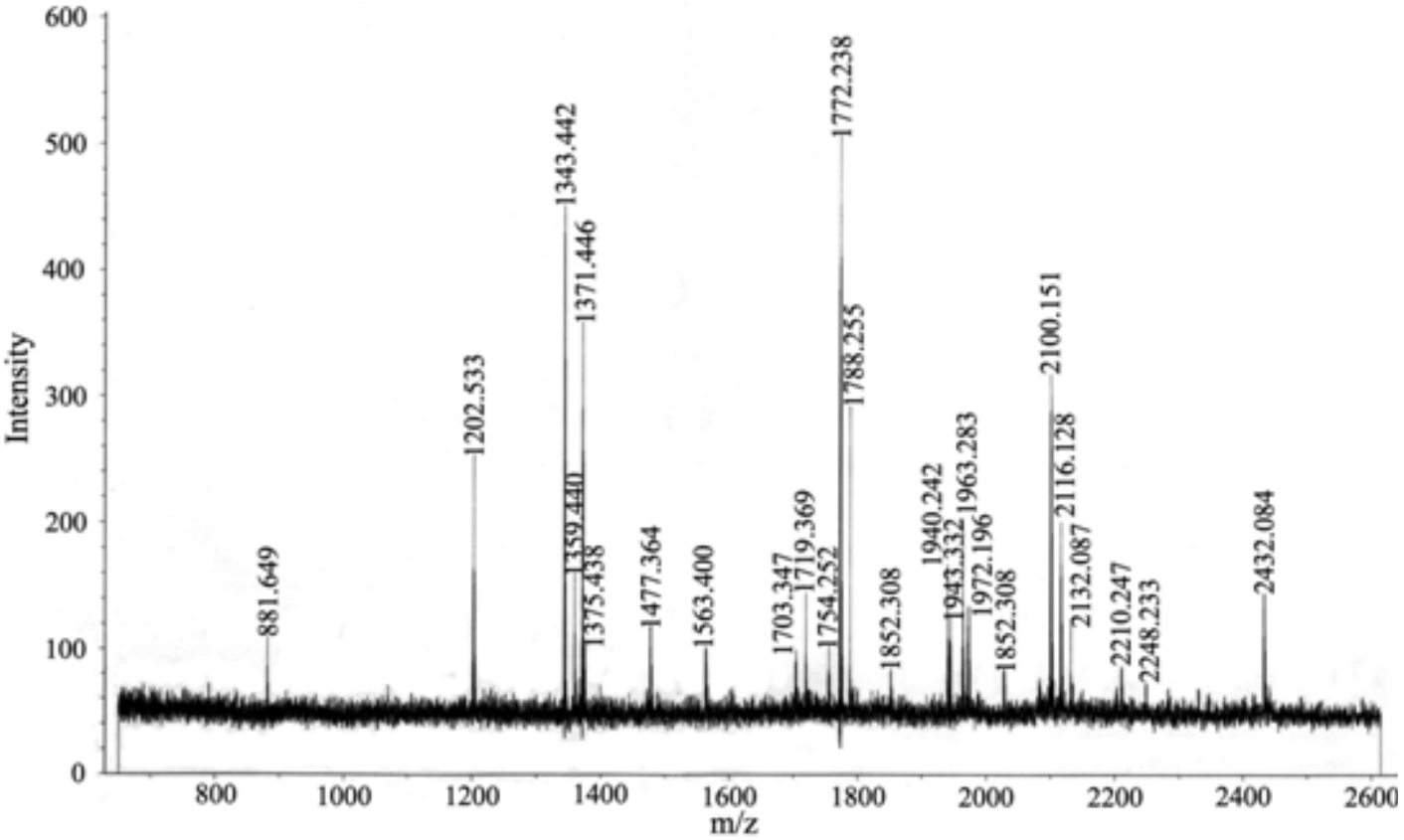

Figure 4. MALDI-TOF MS analysis of the purified elicitor.

Table 2. Identification of the purified elicitor by MALDI-TOF MS analysis.

Protein	Accession no.[a]	MW/pI[b]	Sequence coverage (%)[c]	Score	Peptide hit	Peptides identified
Hypothetical protein MG05155.4	gi38106113	48.9523kDa/5.94	26.28	106	12	FKVADLSLAAFGR;VADLSLAAFGR;VADLSLAAFGRK;EIELAENEMPGLMQTR;GETEEEYNWCLEQQLLAFK;LNLILDDGGDLTHLVHDK;SKFDNLYGCR;FDNLYGCR;AASVGQIFVTTTGCR;AASVGQIFVTTTGCRDILVGK;SVQNIKPQVDR;HIILLAEGR

a: Accession number in the NCBInr database. *b*: MW, molecular weight; pI, isoelectric point. *c*: Amino acid sequence coverage of the protein with respect to matched tryptic digest fragments

Table 3. Effects of the purified elicitor application on rice blast disease.

Treatment	5th day		7th day		9th day	
	Disease index	Inducing effect (%)	Disease index	Inducing effect (%)	Disease index	Inducing effect (%)
Water-treated	71.57 ± 1.63		82.94 ± 2.39		86.10 ± 1.61	
Elicitor-treated	59.65 ± 1.64*	16.65	50.60 ± 1.32*	38.99	47.65 ± 0.65*	44.65

The fully expanded fourth leaves (susceptible cv. CO39) were inoculated with *M. oryzae* at 2×10^5 conidia per ml 1 d after infiltrating with purified elicitor (100 µg/ml) or distilled water (as a control). The disease index was evaluated using a 0 to 9 scale standard at 5 to 7 days post-inoculation. Each experiment was performed with 30 plants and repeated three times with similar results. Each value represents the mean of three exprements±SD, Asterisks (*) show significant differences from the control at $p < 0.05$.

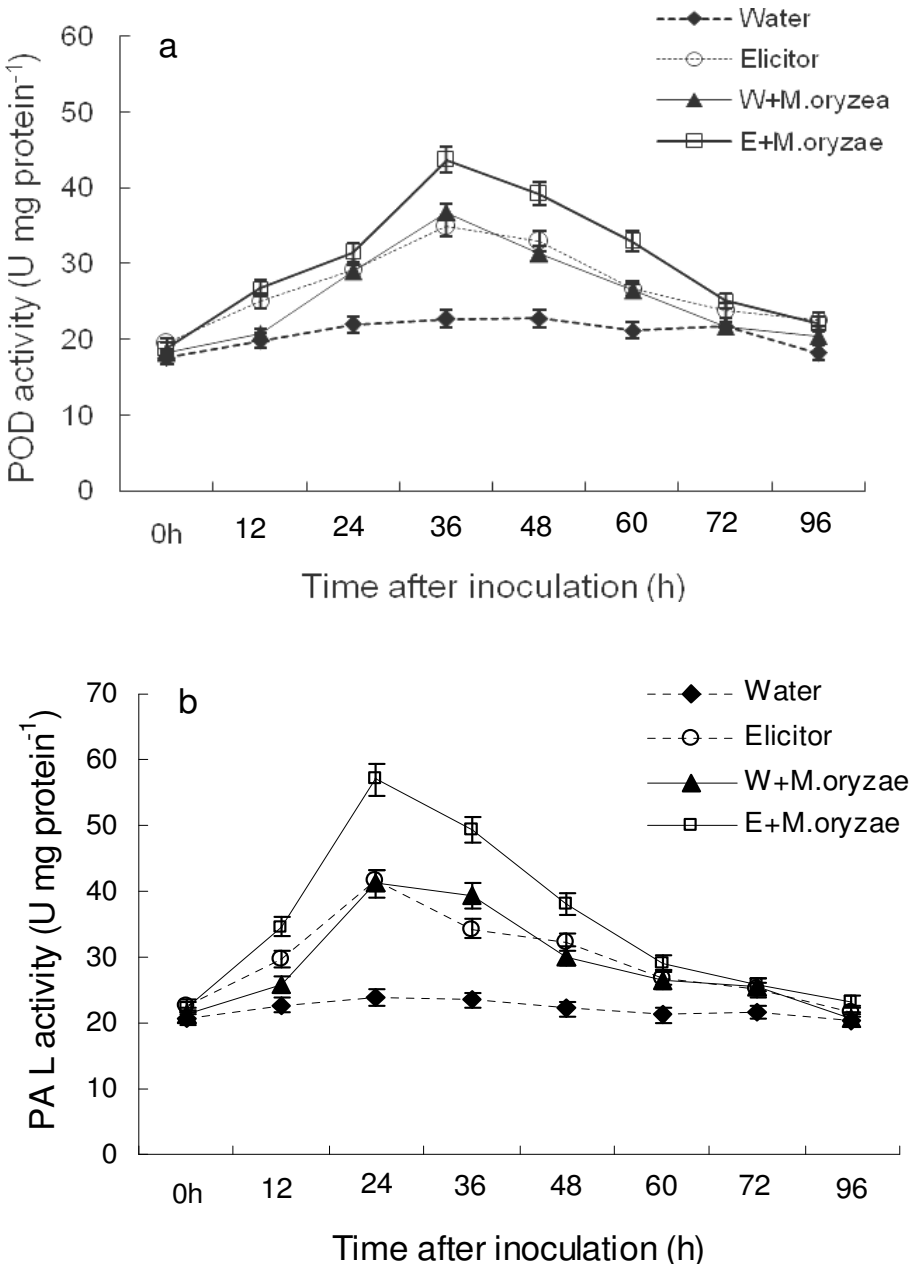

Figure 5. Time-course curves for the induction of POD (a) and PAL (b) activities in the leaves of rice. The fully expanded fourth leaves of rice plants (cv. CO39) at the four-leaf-stage were infiltrated with elicitor (100 µg/ml) alone or elicitor at 1 d before challenge inoculated with *M. oryzae* (2×10^5 spores per ml), and infiltrated with water alone or water before challenge inoculation as controls. Rice leaves were harvested for enzyme assay at different time intervals after treatment. Vertical bars on the line represent standard deviations of the means.

was detected in the plasma membrane of rice plants (Yamada et al., 1993; Shibuya et al., 1996; Ito et al., 1997; Okada et al., 2002). Furthermore, CEBiP, a binding glycoprotein, for this elicitor was isolated from the plasma membrane of suspension cultured rice cells, knock-down experiment suggested that CEBiP was a functional receptor for the chitin oligosaccharide elicitor (Kishimoto et al., 2010). Two oligosaccharide elicitors (*N*-acetylchito-heptaose and a tetraglucosyl glucitol) synergistically activated phytoalexin biosynthesis in rice cultured cells (Yamaguchi et al., 2002; Kaku et al., 2006). A *β*-glucan elicitor and two sphingolipid elicitors were found to cause cell death in rice suspension cells (Matsumura et al., 2003; Takahashi et al., 2008; Koga et al., 1998). To date,

there are a few reports on potential protein elicitor derived from *M. oryzae*. A glycoprotein elicitor with a molecular weight of 15.6 kDa was purified from the culture filtrates of *M. oryzae*, and elicitor activity was due to the carbohydrate moiety (Schaffrath et al., 1995). A protein elicitor PemG1 from *M. oryzae* was recently found to improve resistance of rice and Arabidopsis to pathogen attack, and the PemG1-mediated systemic acquired resistance was modulated by SA^- and $Ca^{(2+)}$-related signaling pathways (Peng et al., 2011). In this paper, The *N*-terminal-blocked elicitor was identified as a hypothesis protein MG05155.4 by MALDI-TOF MS. The active part of elicitor appeared to be the protein moiety since the water-soluble elicitor was sensitive to heat treatment. The elicitor lost its biological activity after 10 min incubation under 100°C. Cloning the gene that encodes the purified elicitor is currently being pursued by using the MALDI-TOF MS information; meanwhile, the corresponding expression system is expected to be successfully constructed for further downstream research.

POD and PAL enzymes play important roles in the plant defense reaction against pathogen. POD, a group of heme-containing glycosylated proteins, is involved in the scavenging of reactive oxygen species and the biosynthesis of cell wall. The last step in the synthesis of lignin and suberin has been proposed to be catalyzed by POD (Bolwell and Wojtaszek 1997; Quiroga et al., 2000). The phenylpropanoid pathway is one of the most important plant metabolic pathways. PAL represents the key enzyme of phenylpropanoid metabolism leading to biosynthesis of phytoalexins, precursor of lignin, and phenolic compounds (Jones, 1984; Hahlbrock and Scheel, 1989). The activities of POD and PAL enzymes in plants may be enhanced under the influence of various factors, such as pathogen attack and treatment with elicitors (Dixon and Lamb, 1990). Enhanced POD and PAL activities were reported in rice leaves infected by *M. oryzae* (Sekizawa et al., 1990). The partially *N*-acetylatedchitosan elicited both POD and PAL activities and led to lignin deposition in wheat leaves (Vander et al., 1998); Tobacco leaves infiltrated with a protein elicitor (PB90) exhibited an enhanced POD and PAL activities (Wang et al., 2003). The incompatible flagellin of *Acidovorax avenae* was a specific proteinaceous elicitor in rice, PAL activity was increased by the purified incompatible flagellin (Tanaka et al., 2003), The accumulation of POD and PAL enzymes was observed in eggplants after elicitors such as chitosan, salicylic acid, methyl salicylate and methyl jasmonate treatment (Mandal, 2010). In our experiment, time-course analysis showed peak accumulation of PAL appeared at 24 h after treatment, and it was higher in challenge-inoculated plants than non-challenge plants. POD accumulation showed similar kinetics with PAL, but the largest peak appeared at 36 h after treatment. Our results suggest that POD and PAL may be involved in elicitor-mediated induced resistance against rice blast. Infection assay indicated the purified elicitor was able to protect rice from infection by *M. oryzae*. In water-treated controls, the disease progressed and finally caused dead tissues, whereas, the disease development was significantly reduced in elicitor-treated rice plants compared to the control plants.

In conclusion, a novel elicitor, purified from the mycelia of *M. oryzae* was involved in the induction of resistance in rice plants against blast fungus. Clearly, much work such as how this purified elicitor is perceived by the rice plant and leading to the activation of the defense responses need to be thoroughly investigated in future.

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