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Purification and Characterization of an Ethylene-Induced Antifungal Protein from Leaves of Guilder Rose (*Hydrangea macrophylla*)

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An ethylene-induced, chitin-binding protein (designated as HM30) from leaves of *Hydrangea macrophylla* was identified and purified to apparent homogeneity by chitin affinity chromatography followed by FPLC on a Superose 12 column. The molecular mass of HM30 was 30,010.0 Da determined by mass spectrometry and its isoelectric point of 8.4 was estimated by isoelectric focusing. The amino acid composition of HM30 was also determined. The initial 15 amino acid residues of the N-terminal were found to be N-S-M-E-R-V-E-E-L-R-K-K-L-Q-D by automatic Edman degradation. This chitin-binding protein showed antifungal activity toward several crop fungal pathogens. Knowledge of properties of HM30 should be useful for its potential application as a plant fungicidal agent.

Key Words: **ethylene; antifungal protein; chitin.**

Chitin, together with β-1,3-glucan, is the major component of fungal cell walls. Many plant proteins are capable of binding native chitin and/or oligomers of GlcNAc and thus retaining on chitin affinity columns (1). Among them, class I chitinases, PR-4 (pathogenesis-related) proteins, and some nonenzymatic chitin-binding lectins have an inhibitory effect on fungal growth, so they are considered to be part of plant defense systems against fungi and other plant pathogens (2).

Class I chitinases, which consist of a N-terminal chitin-binding domain (referred as hevein domain) linked through a short and variable hinge domain to a catalytically active chitinase domain, inhibit the fungal growth independently or synergistically with β-1,3-glucanases or thaumatin-like proteins (2).

Chitin-binding lectins, such as hevein (3), stinging nettle lectin (4), and some antimicrobial peptides purified from *Amaranthus candatus* seeds (5), exhibit antifungal activities. All these chitin-binding lectins lack detectable levels of chitinase. In contrast to the lectins mentioned above, wheat germ agglutinin, consisting of four hevein domains in tandem (6), was reported to lack antifungal activity (7).

PR-4 proteins, such as CBP20 (2), wheatwin2 (8), and three antifungal proteins from barley grain and stressed leaf (9), consist of a hevein domain and a domain with unknown activity. These proteins can inhibit fungal growth independently or synergistically with class I chitinases or β-1,3-glucanases.

These proteins are constitutively expressed or induced in different organs and tissues of higher plants and are regulated by normal developmental processes, ethylene, and other plant hormones (10). They are also induced in plants after pathogen attack and exposure to various biotic and abiotic elicitors (2).

In this paper, we report the extraction, purification, and characterization of an ethylene-induced, chitin-binding protein with antifungal activity from leaves of *Hydrangea macrophylla*.

MATERIALS AND METHODS

Materials

Mature plants of *H. macrophylla* were collected from the yard of our Institute in September. The plant fungal pathogens, including *Thanatephorus cucumeris, Collectotrichum gossypii, Fusarium oxysporum, Alternria*

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alternate (Fries) Keissler, *F. oxysporum* Sch. f. p. *melonis, Fusarium moniliforme, Alternaria cucumerina, Verticillium dahliae, Aspergillus niger,* and *Pyricularia oryzae Cav.*, were maintained by our laboratory. Regenerate chitosan was prepared from 3.0 g of chitosan according to the method of Molano *et al.* (11), autoclaved, equilibrated with 20 mM $NaHCO_3$, and filled in a chromatography column (2.6 × 25 cm). The resulting bed volume was about 51 ml. Other reagents were of analytical grade.

Ethylene Induction of Explant Materials

The explants of *H. macrophylla* were prepared by cutting from the bottom of stems of mature plants, rinsed twice with distilled water, and then bundled loosely together. The explant bundle was placed into a bulky beaker containing 7 mM 2-chlorothylphosphonic acid solution that would be absorbed into explants and release ethylene. The beaker containing the explants was kept in room temperature for 48 h. After induction, leaves of explants were collected for the purification of antifungal protein. Meanwhile, another explant bundle was placed into a bulky beaker containing distilled water and kept in room temperature for 48 h as the control for ethylene induction.

Purification of Antifungal Protein

Unless otherwise stated, all steps were performed at 4°C. Five hundred grams of induced leaves of *H. macrophylla* was fully homogenized in 2–3 vol of 20 mM NaPi–citrate buffer (pH 5.0) containing 1 mM phenylmethylsulfonyl fluoride. The homogenate was filtered through three layers of gauze and the filtrate was centrifuged at 10,000*g* for 30 min. Solid ammonium sulfate was added to the supernatant up to 80% saturation. After stirring overnight, the crude proteins were collected by centrifugation at 38,000*g* for 30 min, resuspended in 30 ml of 20 mM $NaHCO_3$ (pH 8.3), and dialyzed against the same buffer overnight. The dialyzed solution was centrifuged at 20,000*g* for 10 min to remove the insoluble materials and then loaded on a chitin affinity column at a flow rate of 0.3 ml/min. The column was washed with 20 mM $NaHCO_3$ (pH 8.3) and 20 mM sodium–acetic acid buffer (pH 5.6) consecutively. Finally, the chitin-binding proteins were released from the column by washing with 20 mM acetic acid (pH 3.3) and the eluent was immediately adjusted to pH 7.0. The protein solution was dialyzed against distilled water and dried in a vacuum concentrator. Then the protein samples were dissolved in 200 mM $NaHCO_3$ and subjected to FPLC (Pharmacia Biotech FPLC system) on a Superose 12 column (Pharmacia Biotech) equilibrated with 200 mM $NaHCO_3$. The column was washed with 200 mM $NaHCO_3$ at a flow rate of 0.5 ml/min and the eluted proteins were monitored by measurement of the absorbance at 280 nm. Each fraction was tested for antifungal activity and analyzed for purity and molecular weight by SDS–PAGE (12). The protein concentration of each fraction was determined by the method of Bradford (13) with bovine serum albumin as the standard.

Assay of Antifungal Activity

Hyphal extension–inhibition assays were performed under sterile condition as described by Roberts and Selitrennikoff (14). Fungal mycelia were inoculated in the center of petri dishes (90 mm) containing potato dextrose agar and incubated at 28°C. When the fungal mycelia were expanding to desirable size, sterile filter paper disks were placed on the agar surface in front of the fungal mycelia. Various amounts of purified proteins dissolved in 20 mM NaPi–citrate buffer (15 μl) were added to the paper disks. The plates continued to be incubated at 28°C for 24–48 h and then photographed. In this manner, if the material being tested was antifungal, a crescent-shaped zone of inhibition was observed around the disk.

Spore germination–inhibition assays were done under sterile condition as described by Hao *et al.* (15). The fungal spores were washed from the culture plates with potato dextrose solution, rinsed once with distilled water, suspended in distilled water, and finally adjusted to the concentration of 4×10^4/ml. The spore suspension (5 μl) was mixed with 20 μl of various concentrations of protein solution and added to punctured holes in petri dishes containing potato dextrose agar. After incubation at 28°C for 24–48 h, the plates were photographed for the examination of inhibitory results.

Mass Spectrometry (MS)[2] and Isoelectric Focusing (IEF) Analysis

The accurate molecular weight of the purified protein was determined by mass spectrometry with Finnigan LCQ-ESI mass spectrometer. The purified protein (100 pmol) was dissolved in water/methanol (50/50, v/v) containing 1% (v/v) acetic acid at a protein concentration of 5 μmol/L and then applied on the MS instrument.

Isoelectric focusing was performed with Model III mini IEF cell (Bio-Rad) as described by instruction manual. The Bio-Rad's IEF standards, a mixture of nine natural proteins with isoelectric points (p*I*s) ranging from 4.45 to 9.6, were used.

[2] Abbreviations used: GlcNAc, *N*-acetylglucosamine; FPLC, fast protein liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MS, mass spectrometry; IEF, isoelectric focusing; PVDF, polyvinylidene difluoride.

Amino Acid Composition Determination and N-Terminal Amino Acid Sequencing

The purified protein (100 μg) was hydrolyzed under vacuum with 5.7 M HCl containing 2% (w/v) phenol at 110°C for 24 h by the Waters Pico-tag Workstation. The one-third resulting amino acid composition was colorized by ninhydrin and analyzed with amino acid analyzer (Beckman system 6300).

N-terminal sequence analysis of the protein electroblotted to PVDF membrane was carried out by the method of automated Edman degradation on a pulse liquid automatic sequenator (Applied Biosystems Model 491) (16).

Chitinase Activity and Agglutination Assays

The chitinase activity was identified by modified colorimetric method as described by Cabib *et al.* (17) and Ohta *et al.* (18). The reaction solution (0.2 ml) containing 5 μg purified protein and 0.5% regenerated colloidal chitin were incubated at 37°C for 30 min. The soluble sugar products were measured colorimetrically under 550 nm using GlcNAc as the standard. One unit of chitinase was defined as the amount of enzyme that released 1 μM of soluble sugar (calculated as GlcNAc) per minute.

The agglutination assays were carried out in the way described by Van Damme *et al.* (19). The protein solution (10 μl) was mixed with 40 μl of 1% suspension of rabbit red blood cell and placed into small glass tubes. To determine the agglutination titer, the protein was serially diluted with twofold increments. Agglutination was assessed visually after 1 h at room temperature.

RESULTS

Purification of Antifungal Protein

At first, leaves of *H. macropylla* without ethylene induction were used for the purification of antifungal proteins. However, after ammonium sulfate precipitation and chitin affinity chromatography, no protein band was detected by SDS–PAGE. The experiments were repeated for two more times, but the results were the same. It suggested that leaves of *H. macrophylla* contained almost no or very few chitin-binding protein in normal physiological condition. It was reported that many antifungal proteins, such as chitin-binding lectin, chitinase, and β-1,3-glucanase, were regulated by ethylene and other plant hormones (20). Based on the report, ethylene was employed for the induction of antifungal proteins. Explants of *H. macrophylla* were dipped into 7 mM 2-chlorothylphosphonic acid solution and induced for 48 h. With the use of induced leaves as starting materials, several chitin-binding proteins were detected by SDS–PAGE after chitin affinity chromatography (Fig. 1B).

Among the purified chitin-binding proteins, the antifungal protein HM30 was purified to homogeneity following the three steps: ammonium sulfate precipitation, affinity chromatography on a chitin column, and FPLC on a Superose 12 column (Fig. 1A). At the second step, proteins, which could bind chitin specifically, were released from chitin matrix with 20 mM acetic acid (pH 3.3) and exhibited antifungal activity. SDS–PAGE analysis showed that the whole protein peak contained

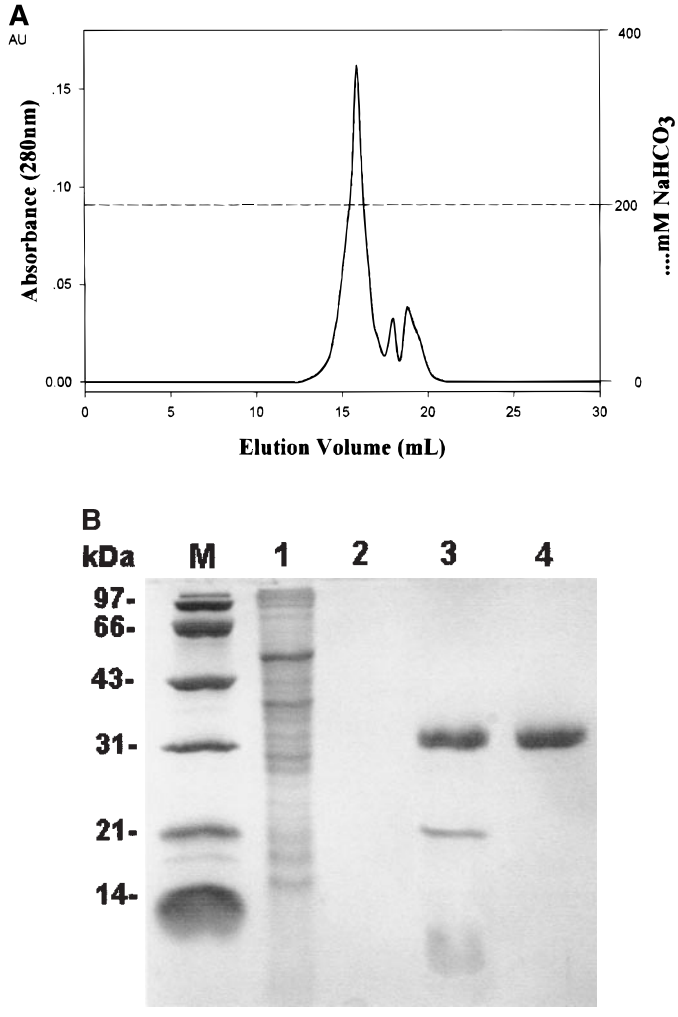

FIG. 1. Purification of HM30. (A) Separation of the chitin-binding proteins by FPLC on a Superose 12 column. About 4.3 mg of chitin-binding protein released from chitin affinity column was loaded on the Superose 12 column. (B) 15% SDS–PAGE analysis of protein. Lane M, SDS–PAGE molecular weight standard (kDa); lane 1, the total proteins from induced leaves of *H. macrophylla* precipitated by 80% saturation ammonium sulfate; lane 2, no protein band was detected after chitin affinity chromatography when the control leaves were used as starting materials; lane 3, the chitin-binding proteins released from the chitin affinity column when induced leaves were used as starting materials; lane 4, HM30 in the peak 1 of FPLC on the Superose 12 column.

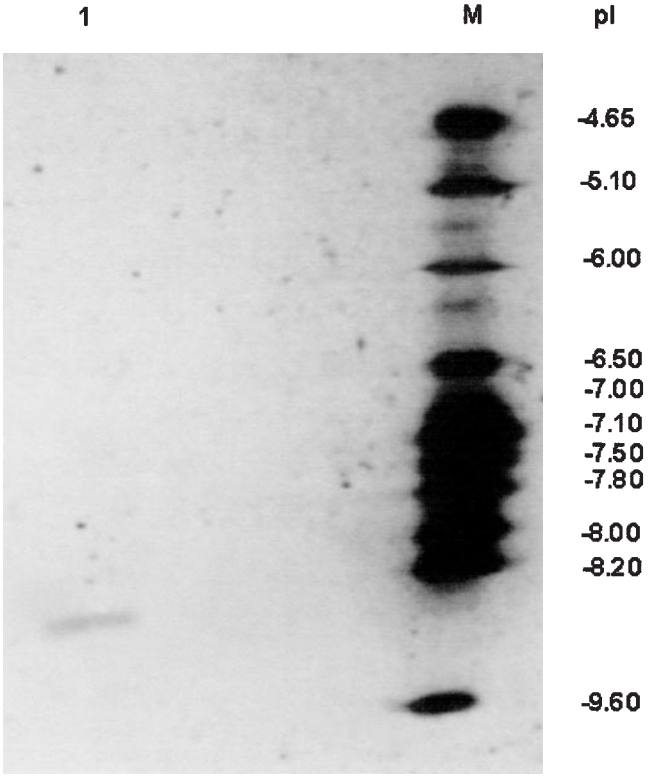

FIG. 2. IEF pattern of HM30. Lane 1, HM30; lane M, IEF standard marker.

two proteins and a peptide with the molecular mass of 33, 20, and 4 kDa, respectively (Fig. 1B). The whole protein peak was then subjected to FPLC. A protein peak eluted at 16 ml showed strong antifungal activity while only very weak antifungal activities were detected in other protein peaks. This protein peak was proved to be the pure 33-kDa protein by SDS–PAGE and IEF analysis (Figs. 1B and 2). As shown in Table 1, from 500 g induced leaves of *H. macropylla*, 2.2 mg of HM30 was acquired.

Accurate Molecular Mass and pI

HM30, which was shown as a 33-kDa protein band on SDS–PAGE, was subjected to mass spectrometry analysis. The accurate molecular mass obtained was 30,010.0 Da. The p*I* of HM30 calculated by isoelectric focusing was estimated to be 8.4 as shown in Fig. 2.

TABLE 1

Purification of HM30

Purification step	Total protein (mg)	Yield (%)
Extracted protein solution	1863	100
Ammonium sulfate precipitation	1240	66.6
Chitin affinity chromatography	4.3	0.23
FPLC on a Superose 12 column	2.2	0.12

Note. The total weight of materials is 500 g.

TABLE 2

Amino Acid Composition of HM30

Amino acid	Residues per molecule	% Residues
Asp/Asn	18.28	7.57
Thr	10.01	4.13
Ser	17.42	7.20
Glu/Gln	16.61	6.87
Gly	38.04	15.72
Ala	13.52	5.58
Cys	Not determined	Not determined
Val	6.57	2.73
Met	11.21	4.63
Ile	49.48	20.50
Leu	20.26	8.48
Tyr	8.02	3.31
Phe	8.03	3.31
Lys	4.72	1.94
His	2.51	1.03
Arg	9.68	4.01
Pro	5.78	2.40

Amino Acid Composition and N-Terminal Amino Acid Sequence

In amino acid composition analysis, Gln and Asn were hydrolyzed to Glu and Asp, respectively, and meanwhile, Cys was oxygenized and could not be detected. As shown in Table 2, HM30 had high percentage of Gly and Ile, i.e., 15.72 and 20.50%, respectively. The high content of Gly in HM30 was also a general property of chitin-binding proteins (21).

The 15 N-terminal amino acid residues of HM30 were N-S-M-E-R-V-E-E-L-R-K-K-L-Q-D, which showed no sequence homology with other plant chitin-binding proteins and antifungal proteins. The result suggested that HM30 was a novel antifungal protein. However, NH_2-terminal sequence of HM30 was highly homologous with human parathyroid hormone as shown in Fig. 3.

Lack of Chitinase and Agglutination Activity

As HM30 could bind chitin, it seemed natural to assay this protein for chitinase activity. To this end, a modified colorimetric method as described by Cabib *et al.* (17) and Ohta *et al.* (18) was used. HM30 revealed no detectable chitinase activity. The occurrence of chitin-binding lectins with antifungal activities promoted us to test agglutination activity for HM30 as described by Van

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HM30  1- NSMERVEELRKKLQD -15
PH   15- NSMERVEWLRKKLQD -29
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FIG. 3. Alignment of N-terminal sequence of HM30 with amino acid sequence of human parathyroid hormone that was abbreviated as PH. Identical residues are boxed.

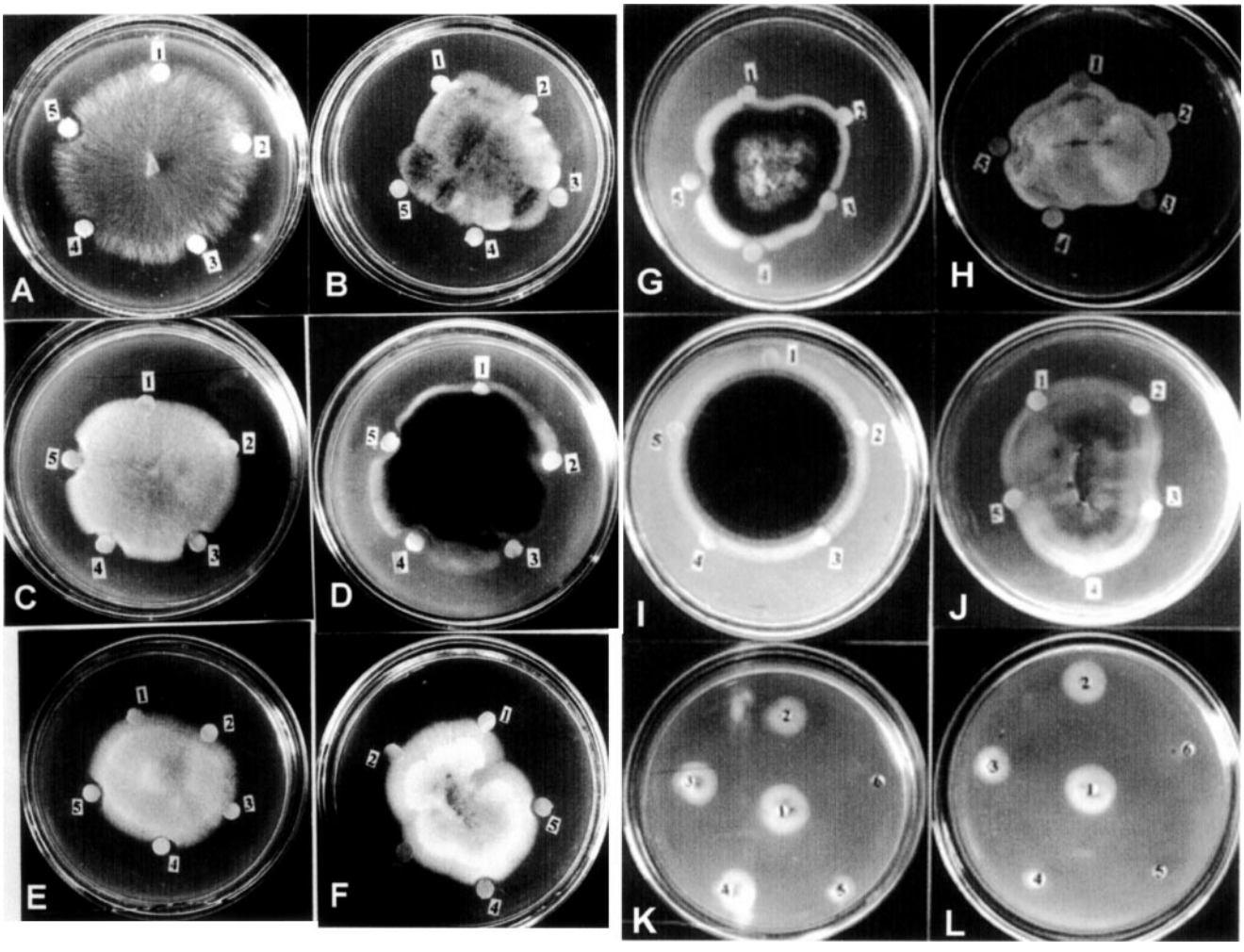

FIG. 4. Antifungal activity of HM30. (A–J) Hyphal extension inhibition assays. Ten phytopathogenic fungi were tested: A, *Thanatephorus cucumeris*; B, *Collectotrichum gossypii*; C, *Fusarium oxysporum*; D, *Alternria alternate* (Fries) Keissler; E, *Fusarium oxysporum* Sch. f. p. *melonis*; F, *Fusarium moniliforme*; G, *Alternaria cucumerina*; H, *Verticillium dahliae*; I, *Aspergillus niger*; J, *Pyricularia oryzae Cav.* Disk 1, 15 μg of bovine serum albumin; disk 2, 15 μl of 20 mM sodium citrate buffer (pH 5.0); disk 3, 5 μg of HM30; disk 4, 10 μg of HM30; Disk 5, 15 μg of HM30. (K, L) Spore germination inhibition assays. K, *Aspergillus niger;* L, *Alternria alternate* (Fries) Keissler. Disk 1, 20 mM sodium citrate buffer (pH 5.0); disk 2, 2 μg of HM30; Disk 3, 4 μg of HM30; disk 4, 6 μg of HM30; disk 5, 8 μg of HM30; disk 6, 10 μg of HM30.

Damme *et al.* (19). The result was also negative: no agglutination activity was detected for HM30 even when 50 μg of the purified protein was added to the testing system.

Antifungal Activity

The antifungal activity of HM30 was tested against 10 phytopathogenic fungi. The fungi used in this study were *T. cucumeris, C. gossypii, F. oxysporum, A. alternate* (Fries) Keissler, *F. oxysporum* Sch. f. p. *melonis, F. moniliforme, A. cucumerina, V. dahliae, A. niger,* and *P. oryzae Cav.* HM30 showed potent inhibitory effect on hyphal extension of four fungi: *T. cucumeris, C. gossypii, F. oxysporum,* and *A. alternate* (Fries) Keissler, when only 5 μg/disk of purified protein was applied (Figs. 4A–4D). HM30 also inhibited the hyphal extension of *F. oxysporum* Sch. f. p. *melonis, F. moniliforme, A. cucumerina,* and *V. dahliae* (Figs. 4E–4H). Purified HM30 had no effect on the hyphal growth of *A. niger* and *P. oryzae Cav.* at 15 μg of HM30 per disk (Figs. 4I and 4J).

HM30 inhibited the spore germination of *A. niger* and *A. alternate* (Fries) Keissler (Figs. 5K and 5L) as well. The IC_{50} values of HM30 for 50% spore germination inhibition were found to be 1.0 μM for *A. niger* and 0.5 μM for *A. alternate* (Fries) Keissler.

DISCUSSION

We have identified an antifungal protein from ethylene-induced leaves of *H. macrophylla*. Because this protein binds to chitin, it is referred to as chitin-binding

protein (HM30). Neither chitinase activity nor agglutination activity is detected with this basic protein. However, HM30 inhibits the hyphal extension of eight phytopathogenic fungi and spore germination of two fungi.

Similar to other chitin-binding proteins, HM30 is rich in Gly (21). This protein is devoid of chitinase and agglutination activity as PR-4 protein is. Members of PR-4 family have not any known catalytic activity. They contain only one carbohydrate-binding domain and thus lack the agglutination activity of lectins which have at least two carbohydrate-binding domains (2). The fact that HM30 does not agglutinate rabbit red blood cell may suggest this protein contains only one chitin-binding domain as PR-4 protein. HM30 is likely to be a member of PR-4 family or a new antifungal protein family. N-terminal sequence of HM30 bearing no homology with any other known antifungal protein also supports this presumption.

It is very interesting that 15 NH_2-terminal amino acid residues of HM30 exhibit 93.3% identity to the center sequence of human parathyroid hormone. This hormone, consisting of 84 amino acid residues, raises plasma levels of calcium by promoting the differentiation of preosteoblasts into osteoblasts that resorb bone and liberate calcium (22). There seems so far to be no connection between function of parathyroid hormone and antifungal activity of HM30. Whether the close identity between the two proteins is merely a coincidence or unknown inherent relationship should be elucidated after the primary structure of HM30 is clarified.

HM30 shows a wide range of antifungal spectrum. Among 10 phytopathogenic fungi tested, HM30 can inhibit hyphal growth of eight fungi. Among them, *T. cucumeris, C. gossypii, F. oxysporum*, and *A. alternate* (Fries) Keissler are most sensitive to HM30. HM30 can not inhibit the hyphal growth of *A. niger* but can inhibit its spore germination. This phenomenon may be of great importance for understanding the mechanism by which HM30 and other plant antifungal proteins inhibit fungal growth.

Ethylene is a gaseous plant hormone that is reported to be implicated in a range of physiological processes including control of plant defense response to microbial pathogens (10). Our study of ethylene-induced antifungal protein from leaves of *H. macrophylla* also confirmed this conclusion.

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REFERENCES

1. Van Damme, E. J. M. Chitin-binding lectins. *Crit. Rev. Plant Sci.* 605–625.
2. Ponstein, A. S., Bres-Vloemans, S. A., Sela-Buurlage, M. B., Van den Elzen, P. J. M., Melchers, L. S., and Cornelissen, J. C. (1994) A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiol.* **104,** 109–118.
3. Van Parijis, J., Broekaert, W. F., Goldstein, I. J., and Peuman, W. J. (1991) Hevien: An antifunal protein from rubber-tree (*Hevea brasiliensis*) latex. *Planta* **183,** 258–264.
4. Brokaert, W. F., Van Parijs, J., Leyns, F., Joos, H., and Peumans, W. J. (1989) A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. *Sciences* **245,** 1100–1102.
5. Brokaert, W. F., Marien, W., Terras, F. R. G., de Bolle, M. F. C., Proost, P., Van Damme, J., Dillen, L., Claerys, M., Rees, S. B., Vanderleyden, J., and Cammue B. P. A. (1992) Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine rich domain of chitin-binding proteins. *Biochemistry* **31,** 4308–4314.
6. Raikhel, N. V., and Wikins, T. A. (1987) Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. *Proc. Natl. Acad. Sci. USA* **84,** 6745–6749.
7. Chrispeels, M. J., and Raikhel, N. V. (1992) Lectin genes and their role in plant defense. *Plant Cell* **3,** 1–9.
8. Caruso, C., Caporale, C., Chilosi, G., Vacca, F., Bertini, L. Magro, P., Poerio, E., and Buonocore, V. (1996) Structural and antifungal properties of a pathogenesis related protein from wheat kernel. *J. Protein Chem.* **15**(1), 35–44.
9. Hejgaard, J., Jacobsen, S., Bjorn, S. E., and Kragh, K. M. (1992) Antifunal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf. *FEBS Lett.* **307**(3), 389–392.
10. Thomma, T., Eggermont, K., Tirens, F. M. J., and Broekaert, W. F. (1999) Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea. Plant Physiol.* **121,** 1093–1101.
11. Molano, J., Duran, A., and Cabib, E. (1977) A rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* **83,** 648–656.
12. Lammli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
13. Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.
14. Roberts, W. K., and Selitrennikoff, C. P. (1986) Isolation and partial characterization of two antifungal proteins from barley. *Biochim. Biophys. Acta* **880,** 161–170.
15. Hao, J. J., Xu, Y. Z., Geng, C. D., Liu, W. Y., Wang, E. D., Gong, Z. Z., and Ulbrich, N. (1998) Purification of alpha-sarcin and an antifungal protein from *Aspergillus giganteus* by blue sepharose CL-6B affinity chromatography. *Protein Express. Purif.* **14,** 295–301.
16. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto poyvinylidene difluoride membranes. *J. Biol. Chem.* **262**(21), 10035–10038.
17. Cabib, E., and Keller, F. A. (1971) Chitin and yeast budding: Allosteric inhibition of chitin synthetase by a heat-stable protein from yeast. *J. Biol. Chem.* **246**(1), 167–173.
18. Ohta, M., Yamagami, T., and Funatsu, G. (1995) Purification and characterization of two chitinases from leaves of pokeweed (*Phytolacca americana*). *Biosci. Biotech. Biochem.* **59**(4), 656–661.
19. Van Damme, E. J. M., Barre, A., Rougè, P., Van Leuven, F., Balzarini, J., and Peumans, W. J. (1996) Molecular cloning of the lectin and a lectin related protein from common Solomon's seal (*Polygonatum multiflorum*). *Plant Mol. Biol.* **31,** 657–672.

20. Pozo, M. J., Azcon-Aguilar, C., Dumas-Gaudot, E., and Barea, J. M. (1999) β-1,3-Glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. *Plant Sci.* **141,** 149–157.
21. Van Damme, D. J. M., Charels, D., Roy, S., Tierens, K., Barre, A., Martins, J. C., Rougè, P., Van Leuven, F., Does, M., and Peumans. W. J. (1999) A gene encoding a hevein-like protein from elderberry fruits is homologous to PR-4 and class V chitinase genes. *Plant Physiol.* **119,** 1547–1556.
22. Kroll, M. H. (2000) Parathyroid hormone temporal effects on bone formation and resorption. *Bull. Math. Biol.* **62**(1), 163–188.