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Communication

In Vitro Antifungal Activity of a Radish (*Raphanus sativus* L.) Seed Protein Homologous to Nonspecific Lipid Transfer Proteins[1]

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ABSTRACT

A basic 9-kD protein was purified from seeds of radish (*Raphanus sativus* L.). The 43 amino-terminal amino acids show extensive sequence identity with nonspecific lipid transfer proteins from other plant species. The radish seed nonspecific lipid transfer protein-like protein inhibits the growth of several fungi in vitro.

Plant seeds contain many proteins that may be involved in the protection of the dormant seeds and the developing young seedlings against microbial infection. Among these proteins are glycosidases (13, 16), thionins (10), permatins (22), and ribosome-inactivating proteins (16), all of which exert antifungal activity in vitro (4).

In a previous report (21), we described two novel classes of antifungal proteins isolated from radish (*Raphanus sativus* L.) seeds: the storage 2S albumins and two proteins, *Rs*-AFP1 and *Rs*-AFP2, that are related to γ-type thionins (14) and pea pod proteins induced upon fungal attack (8). A protein that copurified with *Rs*-AFP2 upon cation exchange chromatography could be separated from the latter by reversed-phase chromatography. This protein was found to exert antifungal activity on *Fusarium culmorum* in a low ionic strength medium but not in a medium containing 1 mM $CaCl_2$ and 50 mM KCl (21). We have now further analyzed the antifungal properties of this protein in vitro. Protein sequencing shows that it belongs to the class of ns-LTPs[2].

MATERIALS AND METHODS

Microorganisms

Filamentous fungi were grown on six-cereal agar (6), and spores were harvested as previously described (6). The following fungal strains were used: *Alternaria brassicola* (MUCL 20297), *Ascochyta pisi* (MUCL 20164), *Botrytis cinerea* (MUCL 30158), *Colletotrichum lindemuthianum* (MUCL 9577), *Fusarium culmorum* (IMI 180420), *Fusarium oxysporum* f. sp. *lycopersici* (MUCL 909), *Fusarium oxysporum* f. sp. *pisi* (IMI 236441), *Nectria haematococca* (Collection Van Etten 260–2–2), *Phoma betae* (MUCL 9916), *Pyricularia oryzae* (MUCL 30166), *Trichoderma hamatum* (MUCL 29736), and *Verticillium dahliae* (MUCL 6963).

Antifungal Activity Assay

Antifungal activity was measured by a microspectrophotometric assay (6). In a microplate well, 20 μL of the test solution was combined with 80 μL of a suspension of 2×10^4 fungal spores per mL of a synthetic fungal growth medium (7).

Electrophoresis

SDS-PAGE was performed with precast Phastgel high-density gels using a PhastSystem electrophoresis system (Pharmacia, Uppsala, Sweden). Fourfold concentrated sample buffers contained 200 mM Tris-HCl (pH 8.3), 1% (w/v) SDS, 1 mM EDTA, 0.005% (w/v) bromophenol blue, and 1% (w/v) DTE. DTE was omitted for the analysis of unreduced proteins. Silver staining of separated proteins was done by the method of Heukeshoven and Dernick (11) using 12.5% (v/v) glutaraldehyde as a fixative. Precast Immobiline Dry Strips (Pharmacia) rehydrated in 8 M urea were used to perform isoelectric focusing. Marker proteins in the isoelectric point range from 4.7 to 10.5 (Pharmacia) were applied to estimate the isoelectric point of the sample protein.

Purification of the Radish Seed ns-LTP

An ns-LTP-like protein was purified from the basic heat-stable protein fraction from radish (*Raphanus sativus* L.) seeds (21). Briefly, this fraction was obtained by collecting the proteins precipitating between 30 and 70% relative $(NH_4)_2SO_4$ saturation, heating for 15 min at 80°C, and passing the nondenatured material over an anion-exchange col-

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[2] Abbreviations: ns-LTP: nonspecific lipid transfer protein; *Rs*-AFP: *R. sativus* antifungal protein; IC_{50}: protein concentration required for 50% inhibition of fungal growth.

umn (Q-Sepharose Fast Flow; Pharmacia) equilibrated at pH 9. The unbound proteins, representing the basic heat-stable protein fraction, were subsequently separated on a cation-exchange column (S-Sepharose High Performance; Pharmacia) at pH 6 as described previously (21). The second desorbed peak (eluting at 180 mM NaCl) was finally loaded on a reversed-phase column (C_2/C_{18} 15-μm porous silica 25 × 0.93 cm; Pharmacia) and separated into two components by applying a linear gradient from 0 to 40% acetonitrile in 0.1% TFA. The first peak elutes at 30% acetonitrile and consists of *Rs*-AFP2 (21), and the second peak eluting at 33% acetonitrile represents the ns-LTP-like protein of *R. sativus* seeds. Protein fractions were vacuum dried to remove the solvents.

Analytical Methods

Protein determination was performed using the bicinchoninic acid assay (17). Amino-terminal amino acid sequence analysis was done by automated Edman degradation in a 477A Protein Sequenator (Applied Biosystems Inc., Foster City, CA) with on-line detection of phenylthiohydanthoin derivatives in a 120-A PTH Analyzer (Applied Biosystems). Before amino acid sequence analysis, cysteine residues were modified by *S*-carboxyamido-methylation as previously described (21).

RESULTS

The first 43 amino acids of the purified protein (see "Materials and Methods") were determined by automated Edman degradation (Fig. 1A). By comparing this NH_2-terminal region with sequences in the SwissProt databank (release 18), we found homology with ns-LTPs (consisting of 91–94 amino acids) from different plant sources (Fig. 1B).

SDS-PAGE analysis of the radish ns-LTP-like protein is shown in Figure 2. The reduced ns-LTP-like protein appears as a 9-kD band. However, in its unreduced state, the ns-LTP-like protein migrates with an apparent molecular mass of 18 kD, suggesting that it is composed of two 9-kD subunits. All basic plant ns-LTPs characterized to date have 9- to 10-kD polypeptides (1) and, at least for the ns-LTP purified from maize seedlings, a dimeric structure was reported (9). At higher concentrations, aggregation of this type of proteins was also observed (12). An isoelectric point higher than 10.5 was deduced after isoelectric focusing of the radish ns-LTP-like protein (results not shown).

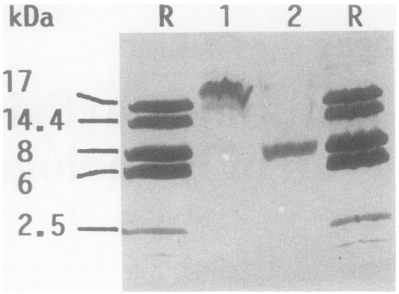

Figure 2. SDS-PAGE analysis of the purified radish seed ns-LTP-like protein. Electrophoresis of 500 ng of the unreduced (lane 1) and reduced protein (lane 2) was performed on Phastgel high-density gels (Pharmacia). The gel was silver stained after fixing with 12.5% glutaraldehyde. Lane R, Myoglobin fragments with molecular masses indicated in kD at the left.

Using a microspectrophotometric antifungal assay (6), we assessed the inhibitory effect of the ns-LTP-like protein on the growth of 12 phytopathogenic fungi. Because the activity of many antimicrobial proteins is sensitive to the presence of cations (6, 7, 21), the IC_{50} was determined in a low ionic strength synthetic fungal growth medium (7) and in the same medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl. With the latter medium, physiological ionic strength conditions are approximated. The results of these tests are summarized in Table I, which, for comparative purposes, also includes the IC_{50} values of the radish seed 2S albumins and of *Rs*-AFP2 (21). In the low ionic strength medium, IC_{50} values ranging from 7 to 100 μg/mL were obtained for the radish ns-LTP-like protein. In the medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl, five of the 12 tested fungi were not affected at concentrations below 1 mg/mL, whereas the other seven fungi were inhibited at IC_{50} values ranging from 135 to 900 μg/mL. This places the antifungal potency of the radish ns-LTP-like protein in between that of the 2S albumins and of *Rs*-AFP2.

Figure 3 shows how the antifungal activity of the radish ns-LTP-like protein is affected by different concentrations of $CaCl_2$ (0, 1, and 5 mM). It is clear that all antifungal activity is abolished when 5 mM $CaCl_2$ is added to the synthetic low ionic strength medium. In the presence of 1 mM $CaCl_2$, the activity is reduced 3-fold.

```
A
     1    5    10   15   20   25   30   35   40
Rs   A L S C G T V N S N L A A C I G Y L T Q N A P L A R G C C T G V T N L N N M A X T T P

B
     1    5    10   15   20   25   30   35   40   45
Rs   A L S C G T V N S N L A A C I G Y L T Q N - A - - - P L A R G C C T G V T N L N N M A X T T P
So   G I T C G m V s S k L A P C I G Y L k g g - - - - - - P L G g G C C G G I k a L N a a A a T T P
Hv   A L n C G q V D S k M k P C L T Y V q g g p G - - - P s G e - C C n G V r D L h N q A q S S G
Dc   v L T C G q V t G a L A P C L G Y L r s Q v n v p v P L T - - C C n v V r g L N N a A r T T l
Zm   A I S C G q V a S a I A P C I S Y a r g Q - G s g - P s A - G C C S G V r s L N N a A r T T A
Rc   - V d C G q V N S s L A S C I P F L T g g v A s - - P s A s - C C A G V q N L k t L A p T S A
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Figure 1. A, Amino-terminal amino acid sequence of the radish seed ns-LTP-like protein. B, Alignment of the NH_2-terminal radish seed ns-LTP-like protein with NH_2-terminal regions of ns-LTPs from *Spinacia oleraceae* (So; 3), *Hordeum vulgare* (Hv; 15), *Daucus carota* (Dc; 18), *Zea mays* (Zm; 20), and *Ricinus communis* (Rc; 19). Amino acids identical with the radish ns-LTP-like protein are indicated by uppercase letters, conserved residues are marked in italics, and nonconserved changes are indicated by lowercase letters. Conserved changes are considered as substitutions within the amino acid homology groups FWY, MILV, RKH, NQDE, and PAGST. Unidentified amino acids are denoted by X. Gaps (–) are introduced for maximum alignment.

To test whether the ns-LTP-like protein influences spore germination, *B. cinerea* spores were germinated in the low ionic strength synthetic fungal growth medium in either the presence or absence of the protein at 50 μg/mL. However, no differences in germination percentages were observed, although the protein-treated germlings had much shorter hyphae relative to the controls (results not shown).

DISCUSSION

A highly basic protein (isoelectric point higher than 10.5) was purified to homogeneity from radish (*R. sativus* L.) seeds. In its unreduced form, it exists as a dimer of a 9-kD protomer. These characteristics together with the protein sequence data suggest that the isolated protein is a true ns-LTP. Indeed, the 43 determined NH_2-terminal amino acids are 57 to 70% homologous (or 38–53% identical) to the NH_2-terminal regions of five known plant ns-LTPs.

ns-LTPs are capable of translocating phospholipids and other apolar compounds between two different phospholipid membranes (1, 23). It has, therefore, often been speculated that ns-LTPs play a role in the biogenesis and/or maintenance of intracellular membranes (1, 23). However, this putative function was recently questioned because of the fact that the mature protein is derived from a precursor molecule containing a signal peptide, thus suggesting an extracellular localization of the ns-LTPs (2, 3, 18, 20).

Thorough analysis of the expression pattern of the ns-LTP gene of carrot revealed that the biosynthesis of the corresponding protein is confined to the epidermal layers of young tissues and organs only (18). Based on the time- and cell-specific expression of the carrot ns-LTP, Sterk et al. (18) suggested the involvement of the ns-LTP in the formation of the cutin layer. This hypothesis is also compatible with the extracellular localization of carrot ns-LTP, as determined by immunochemistry.

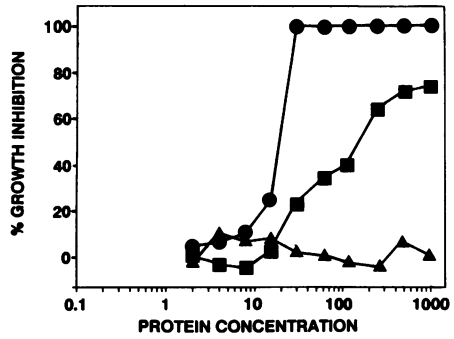

Figure 3. Influence of $CaCl_2$ on the antifungal activity of the radish seed ns-LTP-like protein. Dose-response curves determined after 48 h of incubation of *F. culmorum* spores in the synthetic low ionic strength medium (●) and the same medium supplemented with 1 mM $CaCl_2$ (■), and 5 mM $CaCl_2$ (▲). Protein concentration is in μg/mL.

The α-amylase inhibitor protein I-2 isolated from Indian finger millet, which is also an ns-LTP-like protein, inhibits enzymic activities of several animal α-amylases (2). As in the case of the barley ns-LTP, previously known as a probable amylase/protease inhibitor (5), the radish ns-LTP does not have inhibitory activity against α-amylases from porcine pancreas or *Bacillus* species or against chymotrypsin (F.R.G. Terras, unpublished results). Instead, we were able to attribute antifungal activity to this protein. Until now, antifungal activity of any of the known plant ns-LTPs has not been described. The inhibition seems to be the result of the restriction of hyphal growth rather than of the prevention of germination. The in vivo level of the ns-LTP-like protein in radish seeds can presently only be assessed in an indirect way. The amount of the radish ns-LTP-like protein that can be purified by our isolation procedure approximates 100 μg/

Table I. *Antifungal Activity of the Radish Seed ns-LTP, the Radish 2S Albumins (21), and Rs-AFP2 (21)*

Protein concentrations required for IC_{50} after 48 h of incubation were determined from the dose-response curves (percentage of growth inhibition versus protein concentration).

Fungus	IC_{50} values					
	Medium A[a]			Medium B[b]		
	ns-LTP	2S	*Rs*-AFP2	ns-LTP	2S	*Rs*-AFP2
	μg/mL					
A. brassicola	48	10	2	500	>1000	20
A. pisi	41	75	4	700	>1000	50
B. cinerea	45	>500	2	680	>1000	>100
C. lindemuthianum	25	15	3	>1000	>1000	>100
F. culmorum	20	35	2	520	>1000	5
F. oxysporum f. sp. *lycopersici*	54	>500	2	>1000	>1000	>100
F. oxysporum f. sp. *pisi*	58	200	2	900	>1000	>100
N. haematococca	100	33	2	>1000	>1000	30
P. betae	18	500	1	750	>1000	6
P. oryzae	10	10	0.4	>1000	>1000	7
T. hamatum	30	30	2	>1000	>1000	4
V. dahliae	7	3.3	1.5	135	>1000	50

[a] Medium A, Synthetic low ionic strength growth medium (7). [b] Medium B, Medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCl.

g of seeds. When the specific weight of the seeds (1.2 g/mL) is taken into account, this would mean an in vivo level of approximately 120 μg/mL, thus exceeding the IC_{50} values in the low ionic strength medium (Table I).

If we consider all the data, a model in which ns-LTPs play a role in defense (at least against fungi) can be proposed. This type of protein could confer to defense in two ways: indirectly by its involvement in the formation of a mechanical cutin barrier and directly by its intrinsic antifungal activity following deposition of the transported cutin monomers. The putative role of seed ns-LTPs in protection of seeds or seedlings against microbial attack is consistent with the observation that barley ns-LTP is synthesized de novo in the aleurone cells at the onset of water uptake by the seeds and is secreted from the aleurone layer into the incubation medium (15).

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LITERATURE CITED

1. **Arondel V, Kader J-C** (1990) Lipid transfer in plants. Experientia **46:** 579–585
2. **Bernhard WR, Somerville CR** (1989) Coidentity of putative amylase inhibitors from barley and finger millet with phospholipid transfer proteins inferred from amino acid sequence homology. Arch Biochem Biophys **269:** 695–697
3. **Bernhard WR, Thoma S, Botella J, Somerville CR** (1991) Isolation of a cDNA clone for spinach lipid transfer protein and evidence that the protein is synthesized by the secretory pathway. Plant Physiol **95:** 164–170
4. **Bowles DJ** (1990) Defense-related proteins in higher plants. Annu Rev Biochem **59:** 873–907
5. **Breu V, Guerbette F, Kader J-C, Kannangara CG, Svensson B, von Wettstein-Knowles P** (1989) A 10 kD barley basic protein transfers phosphatidylcholine from liposomes to mitochondria. Carlsberg Res Commun **54:** 81–84
6. **Broekaert WF, Terras FRG, Cammue BPA, Vanderleyden J** (1990) An automated quantitative assay for fungal growth inhibition. FEMS Microbiol Lett **69:** 55–60
7. **Cammue BPA, De Bolle MFC, Terras FRG, Proost P, Van Damme J, Rees SB, Vanderleyden J, Broekaert WF** (1992) Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. J Biol Chem **267:** 2228–2233
8. **Chiang CC, Hadwiger LA** (1991) The *Fusarium solani*-induced expression of a pea gene family encoding high cysteine content proteins. Mol Plant-Microbe Interact **4:** 324–331
9. **Douady D, Grosbois M, Guerbette F, Kader J-C** (1982) Purification of a basic phospholipid transfer protein from maize seedlings. Biochim Biophys Acta **710:** 143–153
10. **Fernandez de Caleya R, Gonzalez-Pascual B, Garcia-Olmedo F, Carbonero P** (1972) Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro. Appl Microbiol **23:** 998–1000
11. **Heukeshoven J, Dernick R** (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. Electrophoresis **6:** 103–112
12. **Kader J-C., Julienne M, Vergnolle C** (1984) Purification and characterization of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. Eur J Biochem **139:** 411–416
13. **Manners DJ, Marshall JJ** (1973) Some properties of a β-1,3-glucanase from rye. Phytochemistry **12:** 547–553
14. **Mendez E, Moreno A, Collila F, Pelaez F, Limas GG, Mendez R, Soriano F, Salinas M, DeHaro C** (1990) Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, τ-hordothionin, from barley endosperm. Eur J Biochem **194:** 533–539
15. **Mundy J, Rogers JC** (1986) Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: comparison to the barley amylase/subtilisin inhibitor. Planta **169:** 51–63
16. **Roberts WK, Selitrennikoff CP** (1986) Isolation and partial characterization of two antifungal proteins from barley. Biochim Biophys Acta **880:** 161–170
17. **Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujitomo EK, Goeke NM, Olson BJ, Klenk DC** (1985) Measurement of protein using bicinchoninic acid. Anal Biochem **150:** 76–85
18. **Sterk P, Booij H, Schellekens GA, Van Kammen A, De Vries SC** (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3:** 907–921
19. **Takishima K, Watanabe S, Yamada M, Mamiga G** (1986) The amino-acid sequence of the nonspecific lipid transfer protein from germinated castor bean endosperms. Biochim Biophys Acta **870:** 248–255
20. **Tchang F, This P, Stiefel V, Arondel V, Morch M-D, Pages M, Puigdomenech P, Grellet F, Delseny M, Bouillon P, Huet J-C, Guerbette F, Beauvais-Cante F, Duranton H, Pernollet J-C, Kader J-C** (1988) Phospholipid transfer protein: full length cDNA and amino acid sequence in maize. Amino acid sequence homologies between plant phospholipid transfer proteins. J Biol Chem **263:** 16849–16855
21. **Terras FRG, Schoofs HME, De Bolle MFC, Van Leuven F, Rees SB, Vanderleyden J, Cammue BPA, Broekaert WF** (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. J Biol Chem **267:** 15301–15309
22. **Vigers AJ, Roberts WK, Selitrennikoff CP** (1991) A new family of plant antifungal proteins. Mol Plant-Microbe Interact **4:** 315–323
23. **Wirtz KWA** (1991) Phospholipid transfer proteins. Annu Rev Biochem **60:** 73–99