

Purification and Characterization of Tobacco Pathogenesis-Related Protein PR-5d, an Antifungal Thaumatin-like Protein

Hisashi Koiwa¹, Hiroaki Kato², Toru Nakatsu², Junichi Oda², Yasuyuki Yamada³ and Fumihiko Sato^{1,4}

¹ Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto, 606-01 Japan

² Institute for Chemical Research, Kyoto University, Uji, Kyoto, 611 Japan

³ Graduate School of Biological Science, Nara Institute of Science and Technology, Ikoma, Nara, 630-01 Japan

Cultured tobacco cells accumulate several pathogenesis-related proteins. A neutral PR-5 protein, PR-5d, was purified to homogeneity from such cells. PR-5d has highly hydrophobic characteristics, but hydropathy analysis of its primary structure did not show a hydrophobic domain. In a series of bioassays, purified PR-5d showed inhibitory activity against several phytopathogenic and non-phytopathogenic fungi as do other members of the PR-5 protein family. To study the antifungal mechanism based on three dimensional structure of PR-5d, purified PR-5d was crystallized. The preliminary X-ray analysis of the crystal revealed that the crystals belong to space group C2, with cell dimensions $a=80.2 \text{ \AA}$, $b=63.8 \text{ \AA}$, $c=45.7 \text{ \AA}$, and $\beta=107.2^\circ$, and diffract at least 1.8 \AA resolution.

Key words: Antifungal activity — Crystallization — *Nicotiana tabacum* cv. Samsun NN — Osmotin — Pathogenesis-related protein PR-5d — Thaumatin.

Plants accumulate several proteins when attacked by a pathogen. These pathogenesis-related (PR) proteins were originally grouped into five families (Bol and van Kan 1988, van Loon 1985, van Loon et al. 1987), but an expansion into eleven families has now been proposed (van Loon et al. 1994). Recent investigations have shown that many PR-proteins have antifungal activities (Alexander et al. 1993, Hejgaard et al. 1991, Malehorn et al. 1994, Mauch et al. 1988, Niderman et al. 1995, Ponstein et al. 1994, Roberts, and Selitrennikoff 1990, Schlumbaum et al. 1986, Sela-Buurlage et al. 1993, Vigers et al. 1990, 1992, Vu and Huynh 1994, Woloshuk et al. 1991). PR-2 and PR-3 are known to encode the hydrolytic enzymes β -1,3-glucanase, and chitinase, which can degrade fungal cell walls (Kauffman et al. 1987, Legrand et al. 1987). The actual antifungal mechanisms of many PR-proteins, however, remain unknown.

Several proteins of different origins (e.g., permatin, osmotin, thaumatin), are grouped into the PR-5 protein

family. Permatin has been purified from the seeds of several crops (Roberts and Selitrennikoff 1990) and osmotin, from salt-adapted tobacco cells (Singh et al. 1987). These proteins have potent antifungal activity in vitro and in vivo. Because permatin causes the release of intracellular materials and hyphal rupture of target microorganisms, permeabilization of the fungal plasmamembrane has been proposed as the action mechanism of these proteins (Roberts and Selitrennikoff 1990). However, thaumatin, a PR-5 protein of *Thaumatococcus danielli*, has no antifungal activity. The three dimensional structures of zeamatin and thaumatin (Batalia et al. 1996, de Vos et al. 1985, Ogata et al. 1992) verify their structural similarity despite their functional differences. Thus, more detailed information is needed to identify the structural basis for the antifungal activity of PR-5 proteins as well as sweet taste of thaumatin.

PR-5d, previously called neutral osmotin-like protein (OLP), accumulates in cultured tobacco cells and roots (Koiwa et al. 1994, Sato et al. 1992, 1995, Takeda et al. 1990, 1991). Although PR-5d protein has been reported in TMV-infected leaves, cultured tobacco cells, and protoplasts (Grosset et al. 1990, Singh et al. 1987), this protein has had little attention. It was not purified to date because of its low levels in TMV-infected leaves and salt-adapted tobacco cells, the major sources of acidic and basic PR-5 proteins. IEF-immunoblot analysis, a method suitable for the detection of individual isoforms, shows that PR-5d is the major PR-5 protein in the root, particularly in the cortex tissues, and that the expression of these neutral isoforms differs from those of known PR-5s (Koiwa et al. 1994). We report the characteristics of PR-5d protein purified from cultured tobacco cells and a preliminary crystallographic study of the PR-5d crystal.

Materials and Methods

Biological materials—Cultured tobacco cells (*Nicotiana tabacum* cv. Samsun NN) were maintained as described elsewhere (Takeda et al. 1990). Three non-phytopathogenic fungi (*Candida albicans*, *Trichoderma reesei* and *Neurospora crassa*) were provided by Dr S. Shimizu of Kyoto University. Rice blast (*Pyricularia oryzae*), rice brown spot (*Cochliobolus miyabeanus*), anthracnose of cucurbits (*Colletotrichum lagenarium*) and early blight of potato (*Alternaria solani*) were gifts from Dr. Y. Kubo of Kyoto

Abbreviations: CD, circular dichroism; PR, pathogenesis related; TMV, tobacco mosaic virus.

⁴ Correspondence should be addressed.

Prefectural University. Fusarium wilt (*Fusarium oxysporum*) of cucumber or of tomato and potato stem rot (*Rhizoctonia solani*) were provided by the Takeda Chemical Industries (Kyoto). Black shank (*Phytophthora parasitica* var *nicotiana*) and black root rot (*Thielaviopsis basicola*) of tobacco were obtained from the Japan Tobacco Corporation (Iwata). These fungi were maintained on potato dextrose agar (Difco, MI) plates at 25°C in the dark.

Purification of PR-5d—PR-5d was extracted from tobacco cells cultured for one month. The cells were homogenized in ice-cold extraction buffer [20 mM potassium phosphate buffer (pH 6.0), containing 20 mM β -mercaptoethanol], and the homogenate was filtered through Miracloth (Calbiochem, U.K.). The following steps (Fig. 1) were done at 4°C: After centrifugation at $10,000 \times g$ for 20 min, proteins were precipitated from the supernatant by 40% saturation with ammonium sulfate. The precipitates were redissolved in 20 mM potassium phosphate buffer (pH 6.0) (buffer A) and fractionated by centrifugation at $20,000 \times g$ for 10 min. The supernatant was dialyzed against buffer A. The pellet that was not dissolved in buffer A was extracted with 0.2% NP-40 in buffer A to recover PR-5d, which strongly bound to the pellet. The desalted proteins were combined, applied to an S-Sepharose

(Pharmacia, Uppsala) column ($\phi 2.5 \times 15$ cm) equilibrated with buffer A, and eluted with a 0–0.5 M linear gradient of NaCl. The positive fractions were dialyzed against 2-fold diluted buffer A and applied to a hydroxyapatite (BIO-RAD, CA) column ($\phi 2.5 \times 5$ cm). Fractions that passed through the column were pooled, adjusted to 1 M ammonium sulfate, and applied to a butyl-Sepharose (Pharmacia) column ($\phi 1 \times 7$ cm). After the column has been washed with a 3 column volume of buffer A containing 1 M ammonium sulfate, PR-5d was eluted with 10% glycerol in buffer A. PR-5d in the eluted fractions was identified by SDS-PAGE, 2D-PAGE analysis, or both.

Protein analysis—The DC protein assay (BIO-RAD) was used to quantify the protein. SDS-PAGE analysis was done on 15% SDS-polyacrylamide gels using the method of Laemmli (1970). Protein obtained was blotted on a PVDF membrane and sequenced in a gas phase sequencer (470 A protein sequencer, Applied Biosystems), as described previously (Takeda et al. 1990), to determine the N-terminal amino acid sequence.

Bioassays—Antifungal activity was measured by several bioassay systems essentially as described by Roberts et al. (1990). Proteins were dissolved in 20 mM potassium phosphate (pH 6.0) con-

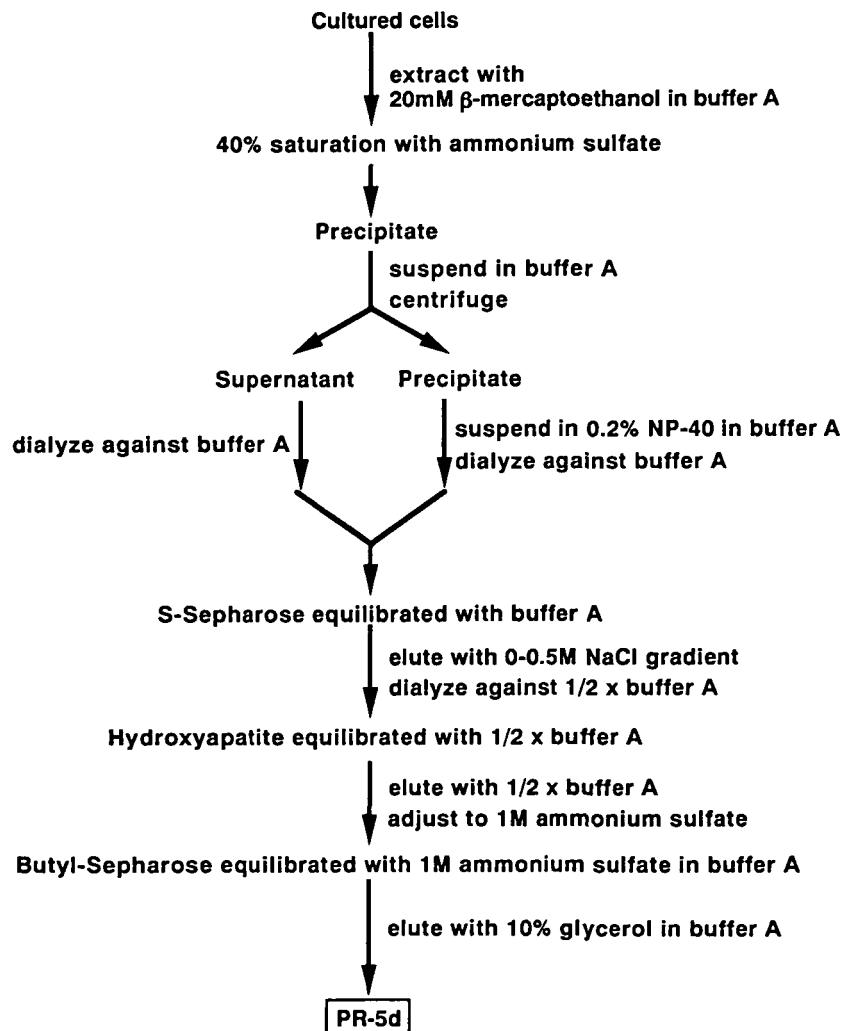


Fig. 1 Purification procedure.

taining 10% glycerol and filter sterilized. In the hyphal rupture assay with *Trichoderma reesei*, 0.18 M mannitol was included in the reaction to avoid PR-5d independent rupture.

Circular dichroism measurement—CD spectra of the purified PR-5 proteins were measured with J-720W spectropolarimeter (JUSCO) using 0.1 cm cells. Proteins were dissolved in 10 mM potassium phosphate buffer (pH 6.2) and passed through a PD-10 column (Pharmacia) equilibrated with the same buffer to remove low molecular weight compounds which would interfere with the measurements.

Structural prediction—A Kyte & Doolittle hydrophobicity profile ($H(i)$) (Kyte and Doolittle 1982) and a sided-hydrophobicity profile (Vogel and Jähnig 1986) of amphipathic β -strands ($H_{\beta}(i)$) were calculated on the basis of the amino acid sequence of PR-5d cDNA (Takeda et al. 1991). $H_{\beta}(i)$ was determined using the following equation and the slightly modified method of Vogel et al. (1986).

$$H_{\beta}(i) = h(i \pm 4)/2 + h(i \pm 2) + h(i) \quad h: \text{hydrophobicity index}$$

Crystallization of PR-5d—The first experiment seeking crystallization conditions was performed with Crystal Screen® (Hampton Research Laboratory). Crystallization was performed by hanging-drop vapor diffusion method. The best crystallizing conditions were the following: Two microliters of protein solution containing 10 mg ml⁻¹ PR-5d, 10% glycerol, and 10 mM MES buffer (pH 6.0) were mixed with 2 μ l of reservoir solution containing 0.7 M MgCl₂ and 50 mM HEPES (pH 7.5); then the drops were equilibrated at 20°C for 3 to 4 weeks against 1 ml of the reservoir solution.

X-ray data collection—The PR-5d crystals were mounted in quartz capillaries coated with prosil® 28 (PCR, FL). To take the precession photographs, we used a Huber precession goniometer and Ni-filtered CuK α radiation from a Rigaku RU300 rotataring-anode operated at 40 kV and 100 mA. The diffraction data were also collected on an R-AXIS IIC imaging plate detector (Rigaku) with monochromatized CuK α radiation. The space group and unit cell parameters were determined from the precession photographs and refined with the diffraction data collected by R-AXIS IIC.

Results

Purification and chemical properties of the PR-5d—PR-5d was purified by a combination of ammonium sulfate precipitation and conventional column chromatography (Fig. 1). PR-5d was precipitated with a relatively low concentration of ammonium sulfate. When the pellet was redissolved with buffer, half the PR-5d remained undissolved in the pellet. Treatment with mild detergent successfully recovered PR-5d from this insoluble precipitate. The recovered fraction was loaded onto an S-Sepharose column. PR-5d was eluted as a rather broad peak as expected from the 2D-PAGE results (Koiwa et al. 1994). The PR-5d fractions were combined and applied to a hydroxyapatite column. All the proteins except PR-5d and 21 kDa protein were retained on the column. PR-5d and 21 kDa protein were successfully separated in a butyl-Sepharose column. Phenyl-Sepharose (or Superose), used to purify osmotin (data not shown) and other thaumatin-like proteins, could not be used because PR-5d bound to this ligand too tightly

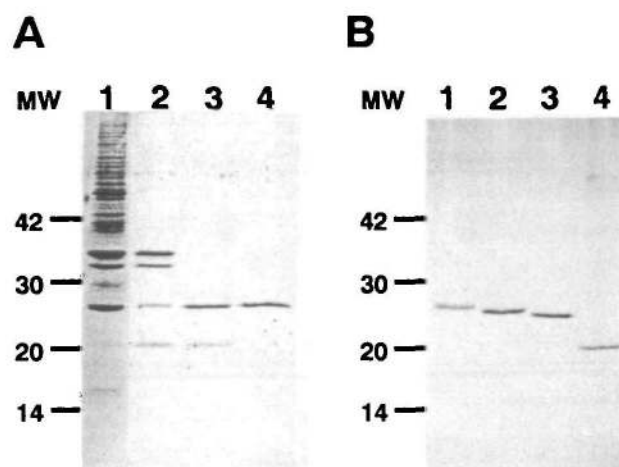


Fig. 2 A: Purity of PR-5d analyzed by SDS-PAGE. Lane 1: total soluble proteins from cultured tobacco cells. After ammonium sulfate precipitation, PR-5d was purified on S-sepharose (lane 2), hydroxylapatite (lane 3), and butyl-sepharose (lane 4) column. B: SDS-PAGE of commercial thaumatin (lane 1), purified PR-5d (lane 2), osmotin (lane 3) and 21 kDa protein (lane 4). Each lane contains 0.5 μ g protein. Proteins were stained with Coomassie Brilliant Blue.

even in buffer without salts, and protein recovery with 50% ethyleneglycol was low. These results clearly show that PR-5d is much more hydrophobic than osmotin and the other relatively hydrophobic thaumatin-like proteins.

The purities of the PR-5d were confirmed by SDS-PAGE (Fig. 2A, B). As shown in Fig. 3B, careful SDS-PAGE analysis can distinguish between PR-5d, osmotin, and thaumatin. Analysis of the N-terminal amino acid sequence confirmed the identity of the purified PR-5d (data not shown).

Antifungal activity of PR-5d—A series of assays was used to test the antifungal activities of the purified PR-5d. Hyphal extension analysis showed antifungal activity as crescent-shaped growth retardation of the mycelial front (Fig. 3). PR-5d effectively inhibited the growth of non-phytopathogenic fungi (*N. crassa* and *T. reesei*) in this assay, as has been reported for purified zeamatin (Roberts and Selitrennikoff 1990). Growth of some phytopathogenic fungi (*C. miyabeanus*, *F. oxysporum* and *Al. solani*) were also considerably inhibited. The protein showed similar inhibition of spore germination in liquid media (Table 1).

PR-5d induced a rapid burst of hyphal tips in the fungi *N. crassa*, *T. reesei*, and *C. miyabeanus* (Fig. 4). When 0.6 M mannitol was added to the hyphal rupture test, the PR-5d-induced burst was suppressed (data not shown). The *Trichoderma* spore germination assay showed that the growth inhibition produced by 50 μ g ml⁻¹ PR-5d was maintained at concentrations of up to 0.6 M mannitol, although hyphal rupture was suppressed. On the other hand, the ad-

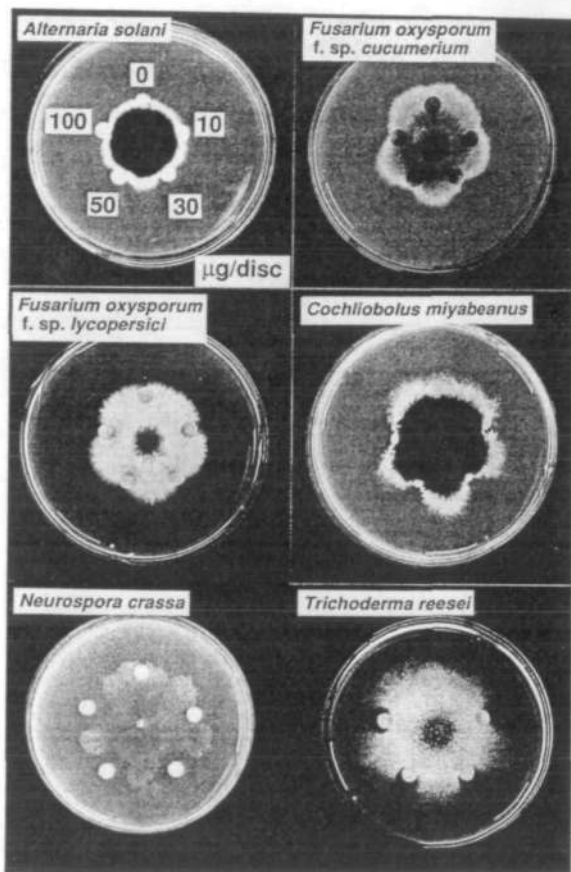


Fig. 3

Fig. 3 Inhibitory effects of PR-5d on hyphal growth of fungi. Filter papers (clockwise) contain 0 (top), 10, 30, 50 and 100 μg protein. Fungi were cultured on potato dextrose agar plates (90 mm diameter) for 2 d.

Fig. 4 PR-5d-induced hyphal rupture of mycelia. The fungi *N. crassa*, *T. reesei*, and *C. miyabeanus* were treated with buffer alone, 10 $\mu\text{g ml}^{-1}$ PR-5d, or 50 $\mu\text{g ml}^{-1}$ PR-5d. Hyphal rupture of mycelia was observed within one minute after addition of PR-5d solution.

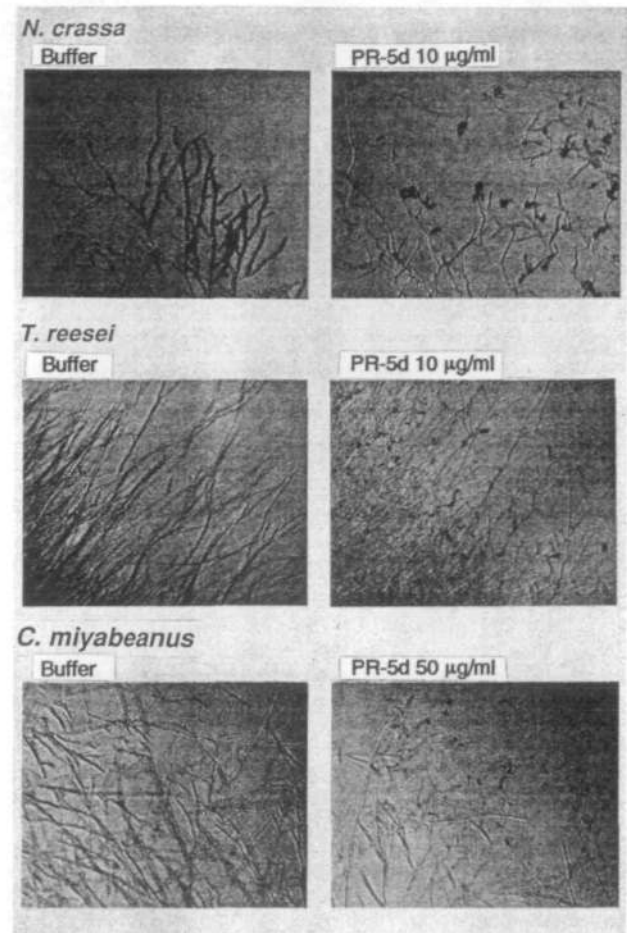


Fig. 4

dition of salts to the spore germination medium inhibited the antifungal activity of PR-5d (Table 2), although this treatment did not inhibit the hyphal rupture activity of PR-5d (data not shown). The effect of salts depended on the concentration and was saturated at 50 mM. These results indicate that hyphal rupture was not the primary cause of the antifungal activity in some cases and that the ionic interaction between PR-5d and the target molecule(s) is also important for the activity.

Our results show that PR-5d inhibits the growth of a variety of fungi at different titers. To clarify the antifungal mechanism of PR-5d, the antifungal activities of the PR-5 proteins, osmotin and thaumatin, as well as the activity of the 21 kDa protein [identified as CBP20 by peptide sequencing (data not shown)] were determined. Synergistic growth inhibition of *C. albicans* with nikkomycin Z, a inhibitor of chitin synthase, is a characteristic of antifungal PR-5 pro-

teins. As shown in Fig. 5A, osmotin was less inhibitory than PR-5d to *C. albicans* in the synergistic inhibition assay, but more inhibitory than PR-5d in the liquid suspension assay without nikkomycin Z (Fig. 5B). We sometimes observed precipitation of osmotin around the paper disc in the agar plate assay, which may explain the difference in the effectiveness of osmotin under the assay conditions used. These results suggest that osmotin may be more potent in its antifungal function but less stable than PR-5d. Thaumatin and the 21 kDa protein showed no antifungal activity against these fungi.

Circular dichroism—The CD spectra of PR-5d and osmotin were analyzed and compared with thaumatin to analyse their secondary structures. Figure 6 shows a single negative peak at about 210 nm, indicating that the β -structure is the dominant secondary structure of these PR-5 proteins, as it is for thaumatin. Furthermore, all parts of the

Table 1 Antifungal activity of PR-5d

Fungal species	Effective concentration		
	Hyphal extension ($\mu\text{g disc}^{-1}$)	Spore germination ($\mu\text{g ml}^{-1}$)	Hyphal rupture ($\mu\text{g ml}^{-1}$)
<i>Alternaria solani</i>	10	NT	NT
<i>Candida albicans</i>	5 ^a	50	NT
<i>Cochliobolus miyabeanus</i>	10	NT	50
<i>Collectotrichum lagenarium</i>	>100	NT	NT
<i>Fusarium oxysporum</i> f. sp. <i>cucumerium</i>	30	100	NT
<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	30	100	NT
<i>Neurospora crassa</i>	10	10	10
<i>Phytophthora parastica</i> var. <i>nicotianae</i>	>100	NT	NT
<i>Piricularia oryzae</i>	100	100	NT
<i>Thilaviopsis basicola</i>	>100	300	NT
<i>Trichoderma reesei</i>	10	10	5 ^b

In vitro antifungal activity of PR-5d was determined on agar plates (hyphal extension and hyphal rupture) and in liquid media (spore germination). Details are described in 'Materials and Methods'.

^a Synergistic with nikkomycin Z ($0.2 \mu\text{g ml}^{-1}$).

^b In the presence of 0.18 M mannitol.

NT: not tested.

CD spectra were similar. On the basis of the X-ray crystallographic results for thaumatin and zeamatin (Batalia et al. 1996, de Vos et al. 1985, Ogata et al. 1992), we speculate that these PR-5 proteins fold in a β -barrel structure.

Characterization of PR-5d crystal—First screening with Crystal Screen[®] showed that salt precipitation is effective for crystallization of PR-5d. Several crystal forms were grown from ammonium sulfate, sodium acetate, and sodium chloride. The best PR-5d crystal was obtained with magnesium chloride and grew reproducibly to at least $0.6 \times 0.2 \times 0.2$ mm (Fig. 7). Figure 8 shows a 15° precession photograph of the *0kl* zone. The crystals belong to the monoclinic space group *C2* with $a=80.2$ Å, $b=63.8$ Å, $c=45.7$ Å and $\beta=107^\circ$. Assuming a PR-5d molecule in the asymmetric unit gives a solvent content of 50% and V_M pf 2.4 Å³ Da⁻¹ (Table 3). This specific value is well within the normal range of V_M values for protein crystals (Matthews 1968). The crystals are stable in the X-ray beam and diffract to at least 1.8 Å resolution.

Discussion

PR-5d is the major PR-5 isoform in roots and in cultured cells (Koiwa et al. 1994). We therefore purified this protein from cultured tobacco cells and investigated its antifungal activity in vitro. Successful enrichment of PR-5d by cation-exchange chromatography indicated that it has a positive charge in its native state. This cationic character of native proteins also is common to osmotin, acidic PR-5s (Kauffmann et al. 1990, Woloshuk et al. 1991), and maize thaumatin-like proteins (Roberts and Selitrennikoff 1990, Malehorn et al. 1994).

Another common chemical property of PR-5 proteins is hydrophobicity. Woloshuk et al. (1991) reported the hydrophobic character of AP24 (osmotin) and suggested that hydrophobicity is important for its antifungal activity. Although the Kyte & Doolittle hydrophobicity profile suggests that PR-5d has no hydrophobic region (Fig. 9A), PR-5d is very hydrophobic. We suppose that its character is bestowed by its three dimensional structure. Results of CD measurements and the high homology of PR-5d to

Table 2 Effect of solutes on PR-5d antifungal activity

Solute	NaCl	NaNO ₃	NH ₄ Cl	NH ₄ NO ₃	CaCl ₂	Mannitol
Inhibitory concentration (mM)	50	50	50	50	50	>600

Spore germination assays with *T. reesei* and $50 \mu\text{g ml}^{-1}$ PR-5d were compared for various solutes. The inhibitory concentration of each solute determined optically was the lowest concentration at which fungal growth was not affected by the presence of PR-5d.

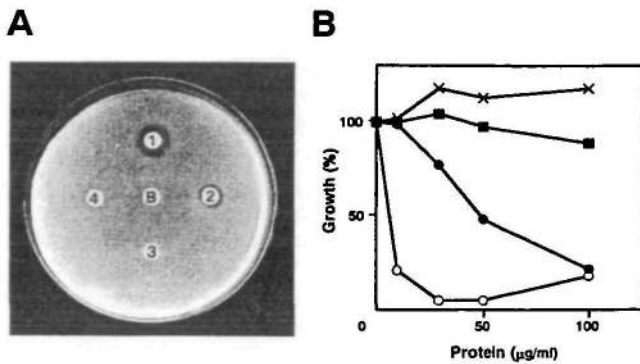


Fig. 5 Inhibitory effects of PR-5d and some PR-proteins on the growth of *Candida albicans*. A: In the presence of nikkomycin Z. The filter papers contain 30 μg of (1) PR-5d, (2) osmotin, (3) thaumatin, and (4) 21 kDa protein and (B) an equal volume of buffer. B: Growth inhibition of *Candida albicans* by PR-proteins in liquid culture. Symbols: PR-5d (●), osmotin (○), thaumatin (×), 21 kDa protein (◆). Growth of *Candida* was monitored as the change of absorbance of 600 nm after 24 hours.

thaumatin and zeamatin (Fig. 10) suggest that PR-5d also folds into a β -barrel structure (Batalia et al. 1996, de Vos et al. 1985, Ogata et al. 1992). This amphiphilic structure could produce a hydrophobic region on the surface of the molecule. Analysis of the amphipathic β -structure of PR-5d (Fig. 9B) showed oscillation of the $H_{(\beta)}$ value from hydrophobic to hydrophilic every second residue, indicative of the high probability of the existence of this structure. Crystallographical findings from homologous proteins, thaumatin and zeamatin (Batalia et al. 1996, de Vos et al. 1985, Ogata et al. 1992), showed that the position of the predicted β -strands of PR-5d match this oscillation well.

The proposed mechanism of PR-5 proteins is the for-

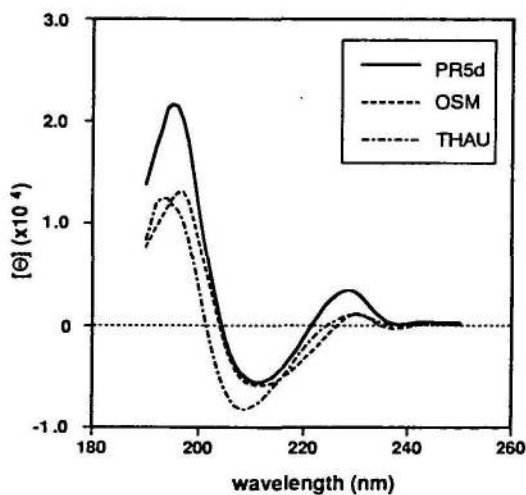


Fig. 6 Circular dichroism spectra of PR-5d (PR5d), osmotin (OSM), and thaumatin (THAU).

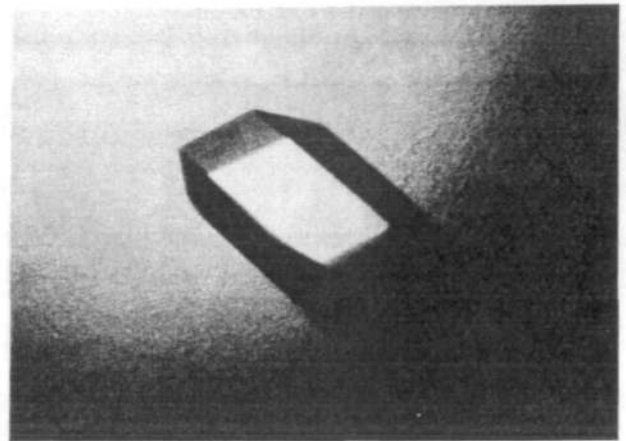


Fig. 7 Photograph of PR-5d crystal on a microscope.

mation of transmembrane pores in the fungal plasma membrane. Woloshuk et al. (1991) speculated that hydrophobic interaction is important for antifungal activity because the more hydrophobic tobacco AP24 was more antifungal than was tomato AP24. However, the interference in PR-5d antifungal activity by salts indicates that an ionic interaction rather than a hydrophobic interaction between PR-5 and the fungal membrane component is probably the primary factor for antifungal activity. The crystal structure of zeamatin and our recent data for PR-5d (Batalia et al. 1996, Koiwa et al. in preparation), showed a highly acidic cleft in these molecular surfaces, which is not present in thaumatin. It is still possible that a subsequent hydrophobic interaction acts as the common antifungal mechanism after the specific ionic interaction. The final determination of the PR-5d structure will be a great help in studying the antifungal activity of various PR-5 proteins.

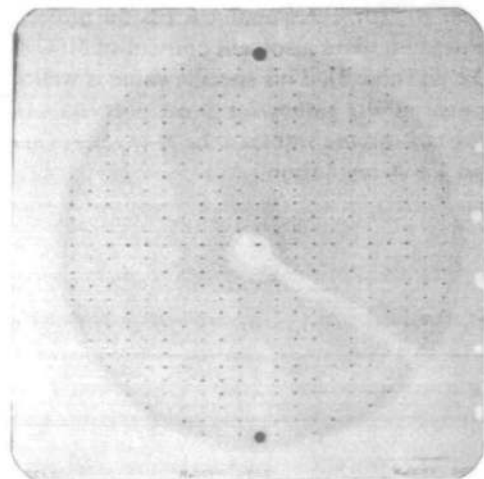


Fig. 8 A 15° precession photograph of the 0kl zone of PR-5d crystal.

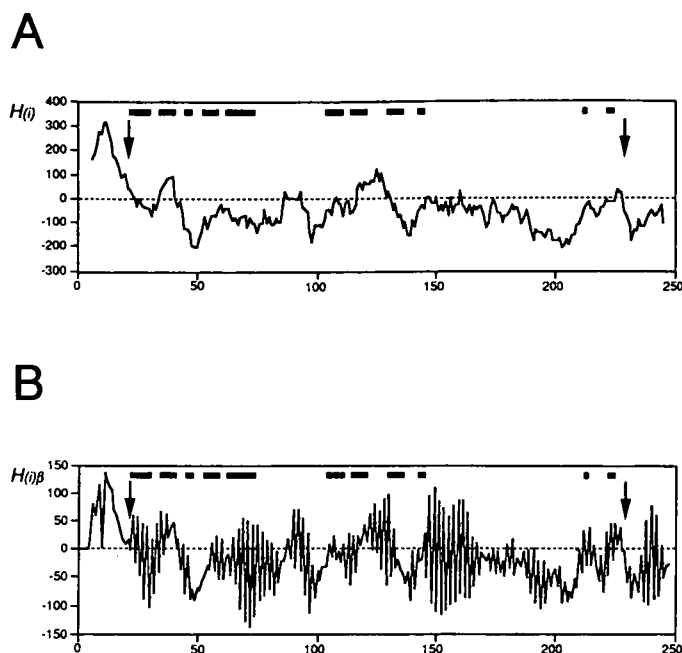


Fig. 9 A: Hydropathy plot of the primary PR-5d structure according to the Kyte-Doolittle method. Span setting: ± 5 residues. B: Side-hydropathy profile of PR-5d. Arrows: post-transcriptional processing site. Solid bars: predicted β -strand positions based on the structure of thaumatin.

THAU	-ATFEIVNRC	SYTVWAAASK	GDAALDAGGR	QLNSGESWTI	NVEPGTNGGK	49
ZEAM	-AVFTVVNQC	PFTVWAAS--	--VPV-GGGR	QLNRGESWRI	TAPAGTTAAR	44
PR5d	SGVFEVENNC	PYTVWAAA--	--TPV-GGGR	RLERGSWWF	WAPPGTKMAR	45
THAU	IWARTDCYFD	DSGSGICKTG	DCGGLLRCKR	FGRPPTLAE	FSLNQYGK-D	98
ZEAM	IWARTGCKFD	ASGRGSCRTG	DCGGVLDCTG	YGRAPNTLAE	YALKQFNILD	94
PR5d	IWGRINCDFD	GAGRGWCQTG	DCGGVLECKG	WCKPPNTLAE	YALNQFSNLD	95
THAU	YIDISNIKGF	NMPMDESEPT	RG---CRGVR	CAADIVGQCP	AKLKAPGGGC	145
ZEAM	FFDISLIDGF	NMPMSELE-D	GGSGCSRQPR	CAVDVNARCP	AELRQDGV-C	143
PR5d	FWDISVIDGF	NMPMSEGETK	PGPGKCHGIQ	CTANINGECP	GALRVFPG-C	145
THAU	NDACTVFQTS	EYCCITGK--	-CGPTEYSRF	FKRLCPDAFS	YVLDKPT-IV	191
ZEAM	NNACPVFKKD	EYCCVGSAAAN	DCEPTNYSRY	FKGQCPDAYS	YPKDDATSTF	192
PR5d	NNECTTFGGQ	QYCCITQGP--	-CGPTELSRW	FKQRCPDAYS	YPODDPTSTF	191
THAU	TCPG-SSNYR	VIFCPA				207
ZEAM	TCPA-GTNYK	VVFCP--				206
PR5d	TCISWTTDYK	VVFCPYG				208

Fig. 10 Sequence alignment of *Nicotiana tabacum* PR-5d (PR5d), *Zea mays* zeamatin (ZEAM), and *Thaumatococcus daniellii* thaumatin A (THAU). Identical amino acids are boxed.

Table 3 Summary of crystallographic data of PR-5d crystal determined by RAXIS IIC

Crystal system: monoclinic
Space group: C2
$a = 80.2$ (Å)
$b = 63.8$ (Å)
$c = 45.7$ (Å)
$\beta = 107.2$ (°)
$Z = 4$ (molecule)
$V_M = 2.45$ (Å ³ Da ⁻¹)
$V_{prot} = 50$ (%)

Our results indicate that PR-5d also functions in the defense mechanism against fungi, as do other PR-5s in tobacco and permatins in plant seeds. The accumulation of PR-5d in healthy plant roots suggests that it is an active defense protein. Rapid mRNA accumulation of the PR-5d in response to ethylene (Sato et al. 1992) supports this. However, tobacco pathogenic fungi might also develop the tolerance to this antifungal activity (Table 1). Therefore, overexpression of PR-5d in heterologous plant species, like cucumber, is being conducted to evaluate the in vivo function of this PR-5d.

We thank Dr. I. Furusawa and Dr. T. Okuno of Kyoto University for their valuable advice and discussions. We appreciate Dr. H. Ueno and Dr. S. Ozawa of Kyoto University for their help in CD measurement. We also thank Dr. S. Shimizu of Kyoto University for his helpful advice on manipulating the fungi. Thanks also due Dr. Y. Kubo, Japan Tobacco Co., and the Takeda Chemical Industries for their kind gifts of fungi. This research was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (No. 04273103) from the Ministry of Education, Science and Culture, Japan, by NEDOs International Joint Research Program (FS), and by a JSPS Fellowship for Japanese Junior Scientists (HK).

References

- Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E. and Ryal, J. (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. USA* 90: 7328-7331.
- Batalia, M.A., Monzingo, A.F., Ernst, S., Roberts, W. and Robertus, J.D. (1996) The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, PR-5 protein family. *Nat. Struct. Biol.* 3: 19-23.
- Bol, J.F. and van Kan, J.A.L. (1988) The synthesis and possible function of virus-induced proteins in plants. *Microbiol. Sci.* 5: 47-52.
- de Vos, A.M., Hatada, M., Wel, H.v.d., Krabbendam, H., Peerdeman, A.F. and Kim, S.-H. (1985) Three-dimensional structure of thaumatin I, an intensely sweet protein. *Proc. Natl. Acad. Sci. USA* 82: 1406-1409.
- Grosset, J., Meyer, Y., Chartier, Y., Kauffmann, S., Legrand, M. and Fritig, B. (1990) Tobacco mesophyll protoplasts synthesize 1,3- β -glucanase, chitinase, and "osmotins" during in vitro culture. *Plant Physiol.* 92: 520-527.
- Hejgaard, J., Jacobsen, S. and Svendsen, I. (1991) Two antifungal thaumatin-like proteins from barley grain. *FEBS Lett.* 291: 127-131.
- Kauffmann, S., Legrand, M. and Fritig, B. (1990) Isolation and characterization of six pathogenesis-related (PR) proteins of Samsun NN tobacco. *Plant Mol. Biol.* 14: 381-390.
- Kauffman, S., Legrand, M., Geoffrey, P. and Fritig, B. (1987) Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3- β -glucanase activity. *EMBO J.* 6: 3209-3212.
- Koiwa, H., Sato, F. and Yamada, Y. (1994) Characterization of accumulation of PR-5 proteins by IEF-immunoblot analysis. *Plant Cell Physiol.* 35: 821-827.
- Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* 157: 105-132.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- Legrand, M., Kauffmann, S., Geoffroy, P. and Fritig, B. (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. *Proc. Natl. Acad. Sci. USA* 84: 6750-6754.
- Malehorn, D.E., Borgmeyer, J.R., Smith, C.E. and Shah, D.M. (1994) Characterization and expression of an antifungal zeamatin-like protein (Zlp) gene from *Zea mays*. *Plant Physiol.* 106: 1471-1481.
- Matthews, B.W. (1968) Solvent content of protein crystals. *J. Mol. Biol.* 33: 491-497.
- Mauch, F., Mauch-Mani, B. and Boller, T. (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combination of chitinase and β -1,3-glucanase. *Plant Physiol.* 88: 936-942.
- Niderman, T., Genetet, I., Bruyère, T., Gees, R., Stinzi, A., Legrand, M., Fritig, B. and Mösinger, E. (1995) Pathogenesis-related PR-1 proteins are antifungal. *Plant Physiol.* 108: 17-27.
- Ogata, C.M., Gordon, P.F., Vos, A.M.D. and Kim, S.-H. (1992) Crystal structure of a sweet tasting protein, thaumatin I, at 1.65 Å resolution. *J. Mol. Biol.* 228: 893-908.
- Ponstein, A.S., Bres-Vloemans, S.A., Sela-Buurlage, M.B., van den Elzen, P.J.M., Melchers, L.S. and Cornelissen, B.J.C. (1994) A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiol.* 104: 104-118.
- Roberts, W.K. and Selitrennikoff, C.P. (1990) Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. *J. Gen. Microbiol.* 136: 1771-1778.
- Sato, F., Koiwa, H., Sakai, Y., Kato, N. and Yamada, Y. (1995) Synthesis and secretion of tobacco neutral PR-5 protein by transgenic tobacco and yeast. *Biochem. Biophys. Res. Commun.* 211: 909-913.
- Sato, F., Takeda, S. and Yamada, Y. (1992) Accumulation of stress-proteins in cultured tobacco cells and ethylene. In *Plant Tissue Culture and Gene Manipulation for Breeding and Formation of Phytochemicals*. Edited by Oono, K., Hirabayashi, T., Kikuchi, S., Handa, H. and Kajihara, K. pp. 377-389. National Institute of Agrobiological Resources, Tsukuba, Japan.
- Schlumberg, A., Mauch, F., Vogeli, U. and Boller, T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* 324: 365-367.
- Sela-Buurlage, M.B., Ponstein, A.S., Vloemans, S.A., Melchers, L.S., Elzen, P.J.M.V.D. and Cornelissen, B.J.C. (1993) Only specific tobacco chitinases and β -glucanases exhibit antifungal activity. *Plant Physiol.* 101: 857-863.
- Singh, N.K., Bracker, C.A., Hasegawa, P.M., Handa, A.K., Hermodson, M.A., Pfankoch, E., Regnier, F.E. and Bressan, R.A. (1987) Characterization of osmotin. *Plant Physiol.* 85: 529-536.
- Singh, N.K., LaRosa, C., Handa, A.K., Hasegawa, P.M. and Bressan, R.A. (1987) Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. *Proc. Natl. Acad. Sci. USA* 84: 739-743.
- Takeda, S., Sato, F., Ida, K. and Yamada, Y. (1990) Characterization of polypeptides that accumulated in cultured *Nicotiana tabacum* cells. *Plant Cell Physiol.* 31: 215-221.
- Takeda, S., Sato, F., Ida, K. and Yamada, Y. (1991) Nucleotide sequence of a cDNA for osmotin-like protein from cultured tobacco cells. *Plant Physiol.* 97: 844-846.
- van Loon, L.C. (1985) Pathogenesis-related proteins. *Plant Mol. Biol.* 4: 111-116.
- van Loon, L.C., Gerritsen, Y.A.M. and Ritter, C.E. (1987) Identification, purification, and characterization of pathogenesis-related proteins from

- virus-infected tobacco leaves. *Plant Mol. Biol.* 9: 593-609.
- van Loon, L.C., Pierpoint, W.S., Boller, T. and Conejero, V. (1994) Recommendations for naming plant pathogenesis-related proteins. *Plant Mol. Biol. Rep.* 12: 245-264.
- Vigers, A.J., Roberts, W.K. and Selitrennikoff, C.P. (1990) A new family of plant antifungal proteins. *Mol. Plant-Microbe Interact.* 4: 315-323.
- Vigers, A.J., Wiedemann, S., Roberts, W.K., Legrand, M., Selitrennikoff, C.P. and Fritig, B. (1992) Thaumatin-like pathogenesis-related proteins are antifungal. *Plant Sci.* 83: 155-161.
- Vogel, H. and Jähnig, F. (1986) Models for the structure of outer-membrane proteins of *Escherichia coli* derived from Raman spectroscopy and prediction methods. *J. Mol. Biol.* 190: 191-199.
- Vu, L. and Huynh, K. (1994) Isolation and characterization of a 27-kDa antifungal protein from the fruits of *Diospyros texana*. *Biochem. Biophys. Res. Commun.* 202: 666-672.
- Woloshuk, C.P., Meulenhoff, J.S., Sela-Buurlage, M., van den Elzen, P.J.M. and Cornelissen, B.J.C. (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. *Plant Cell* 3: 619-628.

(Received August 9, 1996; Accepted April 14, 1997)