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A New Family of Plant Antifungal Proteins

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Plant seeds contain high concentrations of many antimicrobial proteins. These include chitinases, β-1,3-glucanases, proteinase inhibitors, and ribosome-inactivating proteins. We recently reported the presence in corn seeds of zeamatin, a protein that has potent activity against a variety of fungi but has none of the above activities. Zeamatin is a 22-kDa protein that acts by causing membrane permeabilization. Using a novel bioautography technique, we found similar antifungal proteins in the seeds of 6 of 12 plants examined. A polyclonal antiserum was raised against zeamatin and was used in immunoblots to confirm the presence of zeamatinlike proteins in these seeds. N-terminal amino acid sequencing was carried out on the antifungal proteins from corn, oats, sorghum, and wheat, and these sequences revealed considerable homology with each other. Interestingly, these N-terminal sequences are also similar to those of thaumatin, a pathogenesis-related protein from tobacco, and two salt stress-induced proteins. These results indicate that zeamatin is not unique but is a member of a previously unrecognized family of plant defense proteins that may include some species of pathogenesis-related proteins.

Plants have no immune system, and yet they successfully fight infection with both constitutive and induced defense mechanisms. A wide variety of proteins are induced upon pathogen attack, many of which are postulated to play a role in preventing further pathogen invasion. The induced proteins include enzymes involved in lignification and in the synthesis of phytoalexins, hydrolases (including chitinases and β-1,3-glucanases), and proteinase inhibitors. Increased synthesis of several pathogenesis-related (PR) proteins has been best studied in tobacco plants infected by tobacco mosaic virus (TMV) (Gianinazzi *et al.* 1970; Van Loon and Van Kammen 1970; Van Loon 1985). Similar PR proteins have also been found in other plant species, including monocots and dicots (Redolfi 1983; Van Loon 1985).

The functions of many PR proteins are still unknown, but recently it has been shown that some are chitinases (Legrand *et al.* 1987) and others are β-1,3-glucanases (Kauffmann *et al.* 1987). Chitinases and β-1,3-glucanases act to destroy major components of fungal cell walls, and these enzymes have been shown to have antifungal activity in several systems (Schlumbaum *et al.* 1986; Mauch *et al.* 1988; Roberts and Selitrennikoff 1988). Chitinases and β-1,3-glucanases, like other PR proteins, are induced in several plants after viral, bacterial, or fungal attack (Kauffmann *et al.* 1987; Legrand *et al.* 1987; Kombrink *et al.* 1988; Nasser *et al.* 1988; Meins and Ahl 1989; Tuzun *et al.* 1989; Kurosaki *et al.* 1990) and also after treatment of plants with chemicals (Nasser *et al.* 1988) or with the plant stress hormone, ethylene (Boller *et al.* 1983; Vögeli *et al.* 1988; Mauch and Staehelin 1989).

Although not classically considered PR proteins, proteinase inhibitors are also induced by wounding, insect attack, and application of fungal elicitors or plant cell-wall components (Green and Ryan 1972; Walker-Simmons and Ryan 1986; Rickauer *et al.* 1989). They are thought to protect plants against the proteinases of foreign invaders (Richardson 1977). These inhibitors are present at high concentrations in storage organs such as seeds and tubers, where they can account for as much as 10% of the total protein (Ryan 1989). Ribosome-inactivating proteins are also present at high concentrations in plant seeds and have been shown to inhibit fungal growth (Roberts and Selitrennikoff 1986).

Recently, we isolated a protein that has potent activity against a variety of fungi (Roberts *et al.* 1988; Roberts and Selitrennikoff 1990) from corn (*Zea mays* L.) seeds. This protein, zeamatin, has no glucanase or chitinase activity and does not inactivate ribosomes or inhibit trypsin. Instead, zeamatin appears to act by permeabilizing the plasma membranes of fungi (Roberts and Selitrennikoff 1990). Initially, it appeared that this type of antifungal activity might be limited to corn extracts (Roberts *et al.* 1988). However, we now present evidence for the presence of zeamatinlike proteins in seeds from five other plant species. These proteins have anticandidal activity, are all approximately 22 kDa in mass, exhibit cross-reactivity to antizeamatin antiserum, and have similar N-terminal amino acid sequences. We believe that they are members of a novel family of plant antifungal proteins.

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Protein sequence data are to be submitted to GenBank, EMBL, and DDBJ as accession number JO3698.

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MATERIALS AND METHODS

Materials. Sorghum (hybrid 8333) and corn (hybrid 3188) seeds were obtained from Pioneer Hi-Bred International,

Inc. (Des Moines, IA). All other seeds (see Table 1) were organically grown and were obtained from local health food stores either as whole seeds or as flakes. Reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad (Richmond, CA) and other chemicals were from Sigma (St. Louis, MO), unless stated otherwise in the text. Carrot extract (20%, w/v) was prepared by autoclaving peeled, sliced, organically grown carrots in water; the filtrate was stored frozen (−20° C). Distilled, deionized water was used throughout.

Extracting proteins from seeds. Seeds were ground in a coffee grinder (Waring, model 11CG14) at the finest setting. Eighty grams of each resulting ground seed was extracted for 1 hr at 4° C in 160 ml of buffer A (5 mM sodium phosphate, 10 mM NaCl, 1 mM EDTA, pH 7.0). Each suspension was centrifuged at 10,000 × *g* for 20 min at 4° C, and supernatant solutions (S10) were collected. S10 fractions were used for all subsequent experiments. For each extraction, a new source of each seed was used, except in the cases of corn, oats, and sorghum. For *Candida albicans* (C. P. Robin) Berkhout inhibition assays, each S10 fraction was sterilized before use with a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI). Fractions were stored at 4° C for up to 1 wk; for longer storage, aliquots were frozen and stored at −20° C. Protein concentration was measured by the method of Bradford (1976).

***C. albicans* inhibition assays.** Growth inhibition of *C. albicans* by seed extracts was determined as described by Roberts and Selitrennikoff (1990). Briefly, agar assay plates were prepared by autoclaving 80 ml of water, 20 ml of 20% (w/v) carrot extract, and 1.5 g of agar (Difco Laboratories, Detroit, MI), cooling to 45° C, and adding *C. albicans* (B366, ATCC 56884) to a final concentration of 2–4 × 10^5 cells per milliliter. Ten-milliliter aliquots of this warm medium were added to 100-mm-diameter petri dishes and allowed to solidify before placing 0.25-in-diameter sterile paper blanks on the surface of the agar. Thirty microliters of various concentrations of S10 fractions were added to each disk, and plates were incubated overnight at 37° C. Plates were examined for zones of growth inhibition around each disk. The lowest concentration of protein that produced a detectable zone of inhibition was considered the minimum inhibitory dose (MID) and is expressed as micrograms of protein per disk. In plates in which nikkomycin was present, nikkomycin Z (Calbiochem Corporation, San Diego, CA) was added to the agar at 45° C to give a final concentration of 0.2 μg/ml.

Bioautography. SDS-PAGE was performed following the method of Laemmli (1970) using 12% polyacrylamide gels (100 × 140 × 0.75 mm) with a 5% polyacrylamide stacking gel (20 × 140 × 0.75 mm). Samples were boiled for 5 min in sample buffer (15% [w/v] sucrose, 2.5% [w/v] SDS, 125 mM Tris, pH 6.8). Electrophoresis was done for 3 hr at room temperature at 250 V. The resulting gels were then incubated, with shaking, in 250 ml of 1% (v/v) Triton X-100 in water for 20 min at room temperature. After a brief washing in water, gels were incubated in 100 ml of 4% (w/v) carrot extract and gently shaken for 30 min at room temperature. Finally, gels were placed in 150-mm-diameter petri dishes and 30 ml of warm agar (1.5% [w/v] agar, 4% [w/v] carrot extract, 0.2 μg/ml of nikkomycin, and 4–6 × 10^5 *C. albicans* cells per milliliter of final concentration) was poured over the gel and allowed to solidify. After overnight incubation at 37° C, the position of anticandidal activity on the gel was detected as a clear band of growth inhibition against a background of candidal growth.

For comparison of R_f values, gels were run in parallel, stained for 30 min with 0.5% (w/v) Coomassie Brilliant Blue R-250 in ethanol/acetic acid/water 9:2:9 and destained overnight in methanol/acetic acid/water 2:1:7.

Polyclonal antiserum. Polyclonal antibodies were raised in rabbits to a purified preparation of zeamatin. Zeamatin was purified as described previously (Roberts and Selitrennikoff 1990). Briefly, a corn S10 fraction that precipitated between 30 and 55% saturated ammonium sulfate was subjected to carboxy-methyl Sephadex (CM-Sephadex) chromatography and the bound proteins were eluted with a sodium chloride gradient. The active peak (detected by anticandidal assays) from this column was further purified by gel electrophoresis and electroelution. Electrophoresis was done as described in the previous section, and the appropriate band was cut out of the gel by using the sidestrip method (Harlow and Lane 1988). Protein was eluted from the gel with an Elutrap electroseparation device (Schleicher and Scheull, Keene, NH), following the manufacturer's instructions, and this purified preparation was used for the first antigen injection. All subsequent antigen injections used a reverse-phase high-pressure liquid chromatography (HPLC)-purified preparation of zeamatin. The active peak from CM-Sephadex column chromatography was injected onto an Ultrasphere ODS column (Beckman Instruments Inc., Fullerton, CA). The running buffers used were: buffer B, 0.1% trifluoro acetic acid (TFA) in water, and buffer C, 0.1% TFA in acetonitrile. The column was equilibrated with 95% buffer B/5% buffer C. Proteins were eluted from the column with a linear gradient of 5–60% buffer C over a period of 55 min, followed by a gradient of 60–95% buffer C over 5 min. Absorption at A_{214nm} was measured, and peak fractions were collected and evaporated to dryness in a centrifugal evaporator (Gene-Vac, West Nyack, NY). The pellets were resuspended in a five times concentrated buffer A and used for all boost injections. The RIBI adjuvant system (RIBI Immunochem Research Inc., Hamilton, MT) was used for the first two rabbit injections; Freund's incomplete adjuvant was used for subsequent boosts. Approximately 100–200 μg of protein was injected, and the injections were given 30 days apart. Rabbits were bled 10 days after injection. After clot removal, the serum was stored in aliquots at −70° C.

Immunoblots. Extracts to be tested were separated on nonreducing polyacrylamide gels, as described in a previous section. Gels were blotted for 2 hr (0.8 mA/cm^2) to Biotrace NT nitrocellulose (Gelman Sciences) by using a semidry blotter (Gelman Sciences) with transfer buffer (48 mM Tris, pH 8.5, 39 mM glycine, 0.037% [w/v] SDS, and 20% [v/v] methanol) (Kyhse-Andersen 1984). Blots were blocked for 1 hr at room temperature in Tris-buffered saline (TBS, 50 mM Tris, 8.5% [w/v] NaCl, pH 7.4) containing 5% [w/v] nonfat milk powder. All antibody reactions were carried out in TBS-milk buffer (Johnson *et al.* 1984). The

blots were incubated with primary antiserum overnight at room temperature and washed three times (10 min each) with TBS milk. The secondary peroxidase-goat-anti-rabbit antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added and incubated for 1.5 hr followed by three 10 min washes in TBS milk. The color reaction was performed with 4-chloro-1-naphthol for approximately 15 min using the method of Hawkes *et al.* (1982). Preimmune serum from the same rabbit was used as a control in all experiments.

N-terminal amino acid analysis. N-terminal amino acid sequences were determined for anticandidal proteins from corn, sorghum, oats, and wheat. In all purifications, proteins from an S10 fraction were precipitated with ammonium sulfate and then separated by CM-Sephadex column chromatography. Proteins were eluted from CM-Sephadex columns with a gradient of sodium chloride. For each seed, the concentration of ammonium sulfate (AS) used in precipitation and the salt gradient used to elute the CM-Sephadex column were: corn (zeamatin), 30–55% AS, CM-Sephadex eluted with 10–200 mM NaCl; sorghum, 30–65% AS, CM-Sephadex eluted with 10–400 mM NaCl; oats, 30–60% AS, CM-Sephadex eluted with 10–300 mM NaCl; wheat, 30–70% AS, CM-Sephadex eluted with 10–200 mM NaCl.

Zeamatin and the sorghum protein were further purified with a phenyl-Sepharose column (Pharmacia LKB, Piscataway, NJ) (Roberts and Selitrennikoff 1990), and the purified proteins were sequenced in collaboration with Abbott Laboratories. In the case of oats and wheat, proteins in the active CM-Sephadex peak were separated by SDS-PAGE and blotted to ProBlott membranes (Applied Biosystems, Foster City, CA) for 30 min at 50 V in a Transphor electrophoresis unit (Hoeffer Scientific Instruments, San Francisco, CA). The transfer buffer was 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) with 10% (v/v) methanol. Proteins were detected on the blot by Coomassie Blue staining. The position of the antifungal protein band was identified by comparison with previous immunoblotting and bioautography gels of the CM-Sephadex peak; the corresponding band was excised from the blot, and the protein was sequenced on an Applied Biosystems sequencer (model 477A) with online HPLC.

Antifungal assays. The protein from wheat was further purified by using a phosphocellulose column and eluting with a gradient of 50–500 mM NaCl in 50 mM sodium acetate buffer, pH 4.0. Hyphal extension inhibition assays were done essentially as described earlier (Roberts *et al.* 1988). Briefly, hyphae-containing agar plugs were placed in the center of agar plates, and dilutions of protein in water were added to disks surrounding the plugs. Plates were incubated at 23° C for 22 hr (*Neurospora crassa* Shear et Dodge) or 40 hr (*Trichoderma reesei* Simmons). Crescents of hyphal inhibition indicated the presence of antifungal protein.

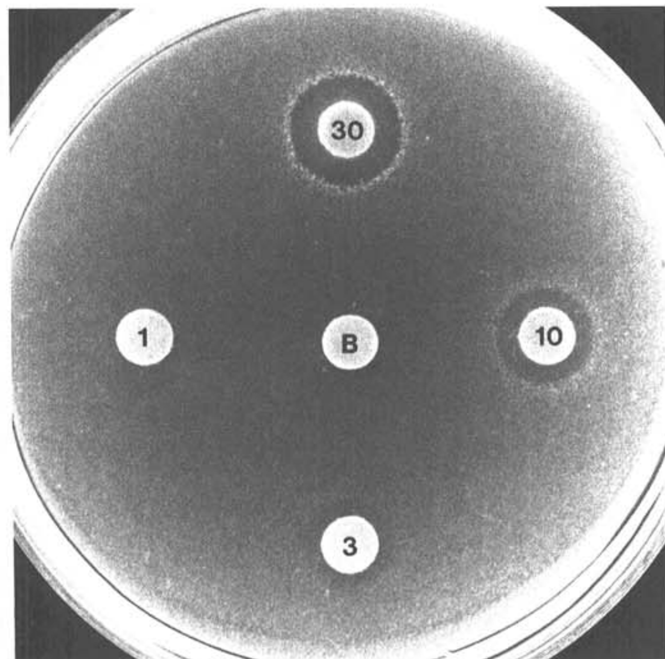

Fig. 1. Synergistic growth inhibition of *Candida albicans* by corn extract and nikkomycin. Decreasing amounts of corn supernatant fraction (S10) were added to paper disks as labeled (micrograms of protein per disk). *C. albicans* and a subinhibitory concentration (0.2 μg/ml) of nikkomycin were present in the agar. The clear dark zones around disks 30, 10, and 3 represent areas of candidal growth inhibition. The center disk, B, is a water blank.

RESULTS

Anticandidal activity in seed extracts. We demonstrated in earlier studies that soluble extracts from corn seeds have potent antifungal activity due to chitinases and a novel antifungal protein, zeamatin (Roberts *et al.* 1988; Roberts and Selitrennikoff 1990). Several fungi were inhibited by zeamatin, including *T. reesei*, *N. crassa*, and the human pathogen, *C. albicans*. Interestingly, although relatively high concentrations of corn extract alone inhibited *C. albicans*, much lower concentrations inhibited growth in the presence of subinhibitory concentrations of antifungal drugs. Synergy was particularly marked with the chitin synthase inhibitor, nikkomycin Z (Roberts *et al.* 1988). As demonstrated in Figure 1, when corn extract was tested against *C. albicans* in the presence of subinhibitory

Table 1. Inhibition of *Candida albicans* by seed extract

Seed		MID[a]
Barley	*Hordeum vulgare*	3
Buckwheat	*Fagopyrum esculentum*	>30
Corn	*Zea mays*	3
Flax	*Linum usitatissimum*	2
Millet	*Panicum miliaceum*	>30
Oats	*Avena sativa*	3
Quinoa	*Chenopodium quinoa*	≥30
Rice	*Oryza sativa*	>30
Rye	*Secale cereale*	≥30
Sorghum	*Sorghum bicolor*	7
Triticale	*Triticosecale*	>30
Wheat	*Triticum aestivum*	7

[a]Minimum inhibitory dose is expressed as micrograms of supernatant solution (S10) protein per disk required to inhibit *C. albicans* in the presence of 0.2 μg/ml of nikkomycin. Values are averages for duplicates from each of two different S10 preparations.

concentrations of nikkomycin, anticandidal activity was detected at 3 μg of protein per disk. In contrast, no inhibition of *C. albicans* was seen in the absence of nikkomycin even when 30 μg of protein was added to the disks (data not shown).

The observed synergy between corn extract and nikkomycin provided a very sensitive assay for identifying and purifying the anticandidal protein zeamatin from corn (Roberts and Selitrennikoff 1990). We have now used this method to detect anticandidal activity in low-speed supernatant fractions (S10s) from other seeds. In total, 12 seed extracts were assayed for anticandidal activity, as described in Materials and Methods. Each extract was tested twice in the *C. albicans* inhibition assay, and the results are shown in Table 1 for two preparations of each seed. In the absence of nikkomycin, none of the 12 extracts tested had anticandidal activity at doses of as much as 30 μg of protein per disk (results not shown). However, in the presence of nikkomycin, eight seed extracts had anticandidal activity. These were barley, corn, flax, oats, quinoa, rye, sorghum, and wheat. Of these, rye and quinoa demonstrated activity only at a dose of 30 μg of protein per disk, whereas the other six had MIDs ranging from 1 to 10 μg of protein. Figure 2 shows typical inhibition assays for flax and wheat, which have MIDs of 3 μg of protein and 10 μg of protein, respectively.

A portion of each extract was boiled for 5 min, then cooled rapidly on ice and tested for anticandidal activity. No boiled extracts had any activity, even when tested in the presence of nikkomycin. This is shown in Figure 2 (disk 30b) for flax and wheat. These results indicate that the antifungal activity present in each extract shows synergy with nikkomycin and is heat-labile.

Bioautography of seed extracts. The anticandidal activity in corn seed extracts is due to a 22-kDa protein, zeamatin, which we have now purified to apparent homogeneity (Roberts and Selitrennikoff 1990). We developed a novel bioautography technique which confirmed that antifungal activity comigrated on a polyacrylamide gel with purified

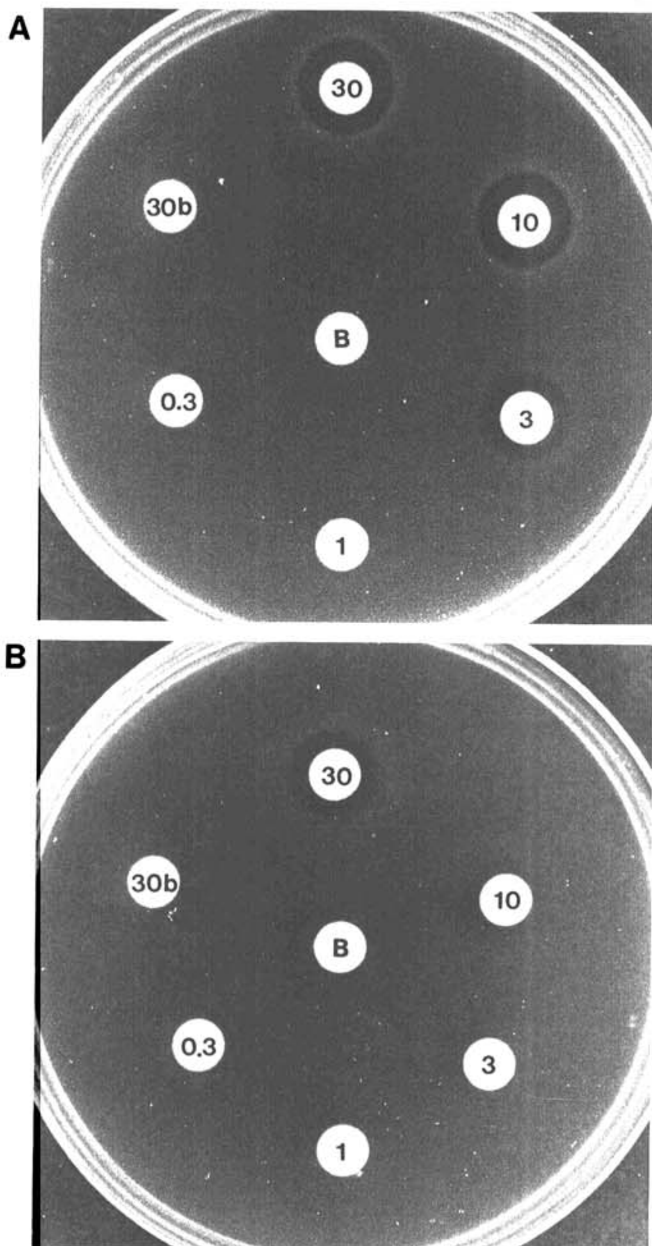

Fig. 2. Growth inhibition of *Candida albicans* by flax (**A**) and wheat (**B**) extracts. **A,** Decreasing amounts of flax supernatant fraction (S10) were added to paper disks on agar containing *C. albicans* and 0.2 μg/ml of nikkomycin. The amounts added were as labeled (micrograms of protein per disk). The minimum inhibitory dose (MID) for flax is 3 μg. Thirty micrograms of protein that was boiled for 5 min and cooled rapidly before addition to the disk is represented by 30b. The center disk, B, is a water blank. **B,** A wheat S10 fraction was used in place of flax S10. The MID for wheat is 10 μg.

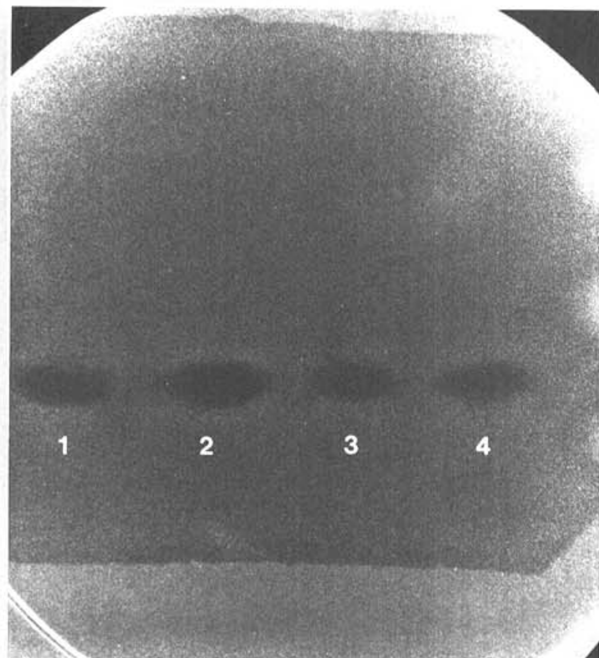

Fig. 3. Anticandidal activity during zeamatin purification is shown in a bioautography of increasingly pure preparations of zeamatin. Agar containing *Candida albicans* and 0.2 μg/ml of nikkomycin was poured over a polycrylamide gel and incubated overnight (see text). The dark bands are areas of candidal inhibition. The lanes in the gel contained: 1) 350 μg of corn S10 fraction; 2) 240 μg 30–55% ammonium sulfate fraction; 3) 20 μg of carboxy-methyl Sephadex peak III; 4) 10 μg of high-pressure liquid chromatography-purified zeamatin.

zeamatin, suggesting that the activity is not due to a trace contaminant.

Using this bioautography technique, the details of which are given in Materials and Methods, the position of clear (antifungal) bands on gels can be measured and compared with a gel stained for protein. Figure 3 shows the results of this bioautography technique with increasingly pure preparations of a corn extract. Note that the clear bands of anticandidal activity are present at the same molecular weight in all steps of the purification, demonstrating that we have indeed purified the anticandidal activity from the original corn S10 fraction. This band of anticandidal activity has the same R_f value as Coomassie Blue-stained HPLC-purified zeamatin (data not shown). With HPLC-purified zeamatin, as little as 0.5 μg of protein gave a visible band of candidal inhibition, while the limit of detection for the S10 fraction was 50 μg of protein per lane (results not shown). Thus, zeamatin is an abundant protein, making up approximately 1% of the extracted corn seed protein.

Bioautography was used to detect similar anticandidal activities in crude supernatant S10 fractions of other seeds without the need for further purification of the extracts. We have used bioautography to study the six seeds (barley, corn, flax, oats, sorghum, and wheat) that had the highest anticandidal activity (Table 1). These results are shown in Figure 4, and it is clear that all six seed extracts have anticandidal activity that migrates with approximately the same molecular mass.

The area of each clear band of inhibition differs widely, and does not necessarily correlate with the MIDs determined in the *C. albicans* inhibition assays (Table 1). This discrepancy is most marked with flax, which has an MID of 1–3 μg of protein, and yet there is only a faint band of candidal inhibition on bioautography. This may be explained by the presence of nonzeamatinlike anticandidal activities in the crude extract or, because bioautography conditions were maximized for zeamatin, it is possible that the proteins from other seeds require different renaturing conditions for optimal anticandidal activity.

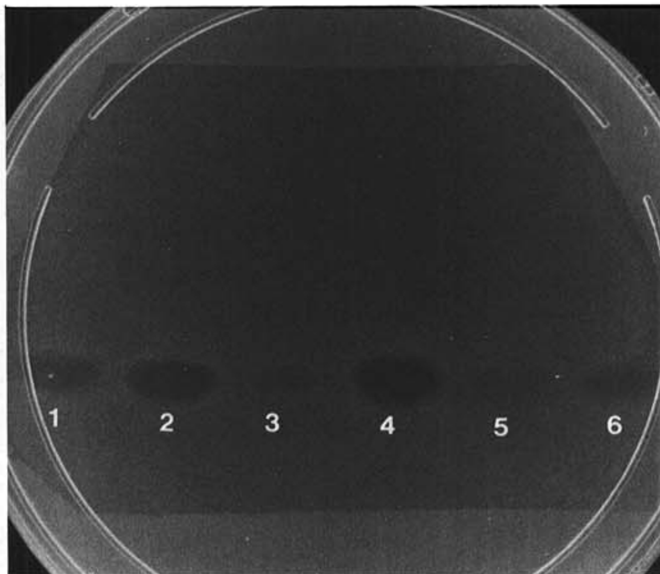

Fig. 4. Zeamatinlike activity in six seed extracts. Bioautography of supernatant (S10) fractions from six seeds (see Fig. 3 and text). Each lane contains 150 μg of protein from 1) barley; 2) corn; 3) flax; 4) oats; 5) sorghum; and 6) wheat.

Immunoblots of seed extracts. Because each of the six seed extracts tested with bioautography contained anticandidal proteins of approximately the same molecular weight and each activity showed synergy with nikkomycin, we wanted to see if these proteins were similar enough to cross-react with antizeamatin antiserum. Polyclonal

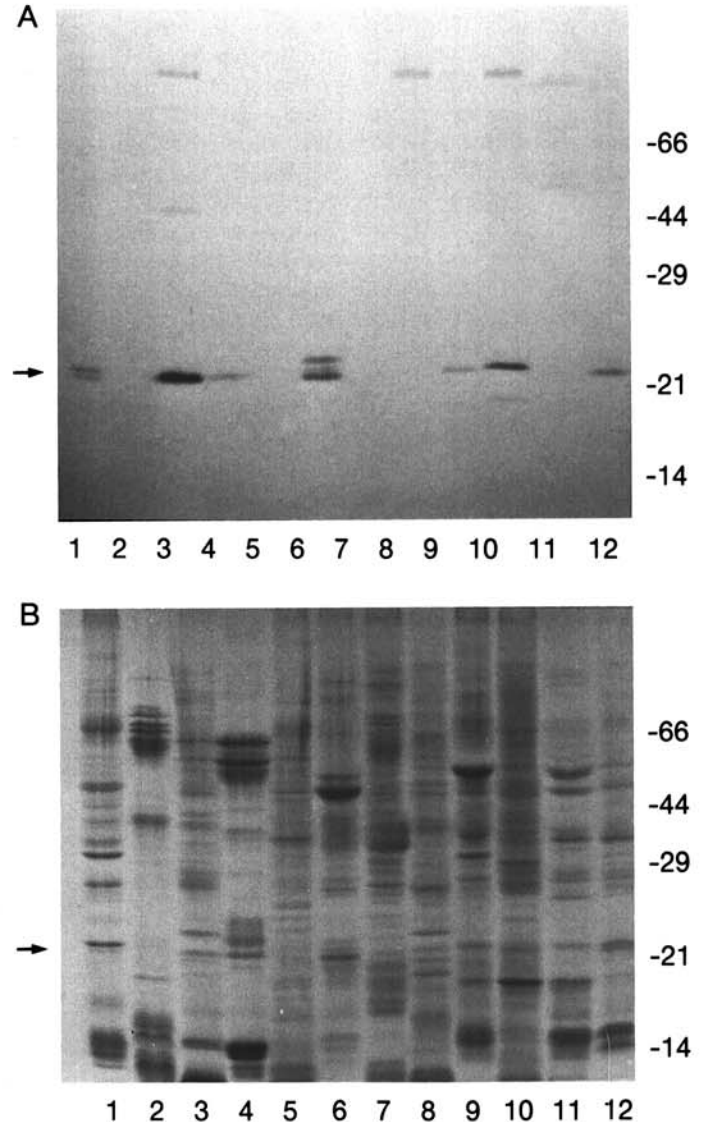

Fig. 5. Zeamatinlike proteins in seed extracts. **A,** Immunoblot of seed extracts stained with antizeamatin serum (1:500 dilution). The lanes contain supernatant (S10) fractions (30 μg of protein) from: 1) barley; 2) buckwheat; 3) corn; 4) flax; 5) millet; 6) oats; 7) quinoa; 8) rice; 9) rye; 10) sorghum; 11) triticale; and 12) wheat. Molecular mass markers (kDa) are shown on the right. The arrow on the left indicates the position of zeamatin. **B,** SDS-PAGE of seed extracts. Each lane is labeled as in 5A and contains 30 μg of S10 protein as in 5A. The proteins were stained with Coomassie Blue R 250. Molecular mass markers are indicated on the right. The arrow on the left indicates the position of zeamatin.

antiserum was raised in rabbits to HPLC-purified zeamatin, and this antiserum was used to probe immunoblots to detect cross-reacting proteins in S10 fractions from different seeds. Figure 5A shows postimmune staining in immunoblots of S10 extracts from each of the 12 seeds. As can be seen, several of the seed extracts have proteins of about 22 kDa that cross-react with antizeamatin antiserum. The seeds that show the most reactivity are barley (a doublet), flax, oats (a triplet), rye, sorghum, and wheat. This cross-reactivity correlates well with the antifungal activities (Table 1) and with the bioautography results (Fig. 4). It is interesting that there is a doublet stained in the barley extract and a triplet stained in the oat extract, suggesting that there are two and three (respectively) similar proteins in these extracts. The triplet in the oats may account for the large clear band on bioautography (Fig. 4), because the three bands are spread over several kilodaltons of molecular mass. In addition to the 22-kDa region, some extracts (corn, rice, and sorghum) showed staining of high molecular weight bands. We do not yet know the significance of these bands, however, they do not show antifungal activity as determined by bioautography (Fig. 4). Neither the high molecular weight nor the 22-kDa regions stained when a preimmune serum was used in place of the postimmune serum (results not shown).

A Coomassie Blue-stained gel of the seed S10 fractions was run under the same conditions as used for immunoblotting and is shown in Figure 5B. Ponceau S staining of the immunoblots revealed the same pattern as the Coomassie stain, indicating that there was efficient transfer of all the S10 proteins onto nitrocellulose (results not shown).

N-terminal amino acid sequences. The anticandidal proteins from corn, oats, sorghum, and wheat were purified as described in Materials and Methods, and the N-terminal amino acid sequences were determined. These sequences are shown in Figure 6. The N-terminal sequences of zeamatin and the sorghum protein show identity in 20 out of 22 amino acids. The proteins from oats and from wheat have sequences more closely related to one another than to either zeamatin or the sorghum protein. This is perhaps not surprising because corn and sorghum belong to the subfamily of Panicoideae and, even within their subfamily, they are closely related, as shown by taxonomic data and DNA sequence analysis of D-ribulose 1,5-bisphosphate carboxylase (Doebley *et al.* 1990). Oats and wheat are both members of the subfamily Pooideae, so are more closely related to one another than to corn or sorghum.

The sequences of these antifungal proteins were compared to other proteins. As shown in Figure 6, thaumatin, PR-R, and osmotin have similar N-terminal sequences to zeamatin and the proteins from sorghum, oats, and wheat. Also, we have now sequenced the first 60 amino acids of zeamatin, and they are identical to the N-terminal region of a bifunctional trypsin/α-amylase inhibitor isolated from corn seeds (sequence shown in Richardson *et al.* 1987). Although the two proteins are very similar, zeamatin does not inhibit either α-amylase or trypsin (data not shown) (A. Blanco-Labra, CINVESTAV, Irapuato, Mexico, personal communication). Zeamatin and the bifunctional inhibitor, therefore, appear to be similar but distinct proteins, having a similar molecular mass and at least the same N-terminal 60 amino acids. We cannot explain the differences in activities between these two proteins, and we are currently sequencing zeamatin further to see if there are indeed differences in amino acid sequences between the two proteins at the carboxy terminus.

Direct antifungal activity of purified proteins. The anticandidal activities described above were assayed indirectly by their ability to facilitate killing of *C. albicans* with a subinhibitory concentration of nikkomycin. This assay was used for identification and purification of zeamatinlike proteins because of its simplicity and sensitivity (as little as 0.1 μg of zeamatin can be detected). Previously, we showed that higher concentrations of purified zeamatin alone (5 μg/ml) inhibited growth of *C. albicans*, *N. crassa*, and *T. reesei* in a suspension culture assay (Roberts and Selitrennikoff 1990). The zeamatinlike protein from wheat was purified to apparent homogeneity and assayed directly for its ability to inhibit hyphal extension of *T. reesei* and *N. crassa* on agar. With this assay, 2.5 μg of wheat protein inhibited growth of *T. reesei* (Fig. 7A) and 7 μg of protein inhibited growth of *N. crassa* (Fig. 7B). Similar results were obtained with purified zeamatin (Fig. 7C,D). In

Protein	N-terminal amino acid sequence
Corn (Zeamatin)	A V F T V V N Q C* P F T V W A A S V P V - - - - - G G G R Q L N
Sorghum	A V F T V V N R C* P Y T V W A A S V P V - - - - - G G
Oats	T T I T V V N K C* S Y T V W P G A L P - - - - - - G G G V V L D
Wheat	A T I T V V N R C S Y T V W P G A L P - - - - - - G G G A
THA	A T F E I V N R C S Y T V W A A A S K G D A A L D A G G R Q L N
PRR	A T F D I V N Q C T Y T V W A A A S P - - - - - - G G G R Q L N
OSM	A T I E V R N N C P Y T V W A A S T P I - - - - - G G G R R L D

Fig. 6. N-terminal amino acid sequences of antifungal proteins from corn, sorghum, oats, and wheat. Also included are the N-terminal sequences for THA, thaumatin II (Edens *et al.* 1982); PRR, pathogenesis-related protein R (Cornelissen *et al.* 1986); and OSM, osmotin I (Singh *et al.* 1987). Dashes were introduced for optimal alignment. * Designates a presumed cysteine residue.

addition, 30 μg/ml of the purified wheat protein was found to inhibit growth of *T. reesei*, *N. crassa*, and *C. albicans* with a suspension culture assay (described in Roberts and Selitrennikoff 1990) (results not shown).

Similar results (not shown) were obtained with partially purified zeamatinlike proteins from sorghum and oats. Moreover, these proteins, as well as the pure wheat protein, at 10 μg of protein per milliliter caused the rapid rupture of *N. crassa* hyphae as observed microscopically with the procedure described earlier for zeamatin (Roberts and Selitrennikoff 1990). We conclude that these proteins resemble zeamatin not only in synergizing with nikkomycin to inhibit growth of *C. albicans*, but also in the ability to directly inhibit fungal growth.

DISCUSSION

In this study, we used a novel bioautography technique to visualize the positions of anticandidal proteins on polyacrylamide gels. This technique provides a good preliminary screen to identify this class of antifungal proteins in crude extracts without the necessity for time-consuming purifications. Using this method, we identified anticandidal proteins of approximately 22 kDa in extracts of barley, corn, flax, oats, sorghum, and wheat. Moreover, immunoblot analysis showed the presence of 22-kDa proteins that cross-reacted with antizeamatin antiserum in those extracts with the highest anticandidal activity. In the cases of corn, oats, sorghum, and wheat, antifungal activity bound tightly to a cation exchange column during purification, thus indicating that the activity is due to a basic protein species. Also, the N-terminal sequences for zeamatin and the proteins from sorghum, oats, and wheat show remarkable homology (Fig. 6). Taken together, these data demonstrate that six seed extracts contain proteins that are similar in size, structure, and activity. We therefore conclude that these proteins are closely related members of a family of antifungal proteins; we propose names for the three proteins for which we have N-terminal amino acid sequences: sormatin (sorghum), avematin (oats), and trimatin (wheat).

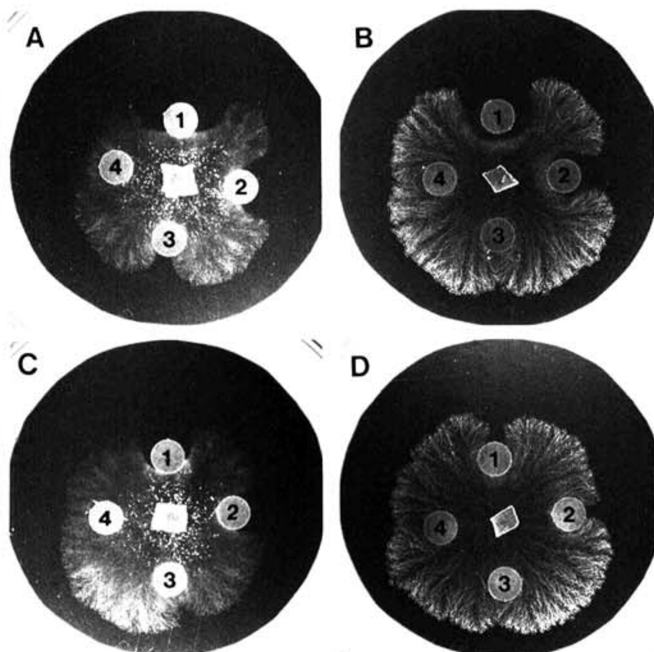

Fig. 7. Direct inhibition of fungal growth by purified proteins. The central portion is an agar plug containing hyphae from *Trichoderma reesei* (**A** and **C**) and from *Neurospora crassa* (**B** and **D**). Disks 1, 2, and 3 contain 25, 7, and 2.5 μg of protein, respectively. Disk 4 is a water blank. Purified protein from wheat was tested in **A** and **B**, while zeamatin was used in **C** and **D**. Dark crescents surrounding disks indicate inhibition of hyphal extension.

In an earlier work (Roberts and Selitrennikoff 1990), we concluded that zeamatin exerts its antifungal effect by a membrane permeabilization mechanism. Evidence for this was the rapid zeamatin-induced release of ultraviolet light-absorbing material from *C. albicans*, release of preloaded amino[1-^{14}C]isobutyric acid from *N. crassa*, and hyphal rupture of *N. crassa* and *C. albicans* observed microscopically in suspension cultures. We believe that relatively high zeamatin concentrations permeabilize fungal membranes causing cell death, whereas lower concentrations do not cause death directly but interact with the membrane to facilitate penetration by nikkomycin, resulting in synergistic killing. If this model is correct, then all six grain extracts that facilitate growth inhibition of *C. albicans* by nikkomycin (Table 1; Fig. 4) would be expected to contain zeamatinlike proteins that can directly inhibit fungal growth. Here we describe fungal growth inhibition by pure zeamatin, pure trimatin, and partially purified avematin and sormatin. In addition, these four proteins induced immediate hyphal rupture of *N. crassa* growing in suspension, suggesting a common membrane-permeabilizing mechanism of action. We suggest the name *permatins* to describe this family of membrane-permeabilizing antifungal proteins.

The N-terminal amino acid sequences of zeamatin, avematin, sormatin, and trimatin have homology with one another and also with a number of other proteins (Fig. 6). These proteins include the sweet protein thaumatin, PR-R protein, two salt stress-induced proteins (osmotin and NP24), and a trypsin/α-amylase inhibitor from corn. PR-R protein is one of the 10 major PR proteins synthesized after TMV infection of tobacco (Kauffmann *et al.* 1990). Osmotin accumulates when tobacco cells are subjected to high osmotic stress (Singh *et al.* 1985). Two forms of osmotin have now been purified and sequenced (Singh *et al.* 1987), and their N-terminal amino acid sequences are identical through position 22. A similar salt-induced protein (NP24) has been detected in tomato cells with an N-terminal amino acid sequence identical to osmotin I through amino acid 25 (King *et al.* 1988). The thaumatins are a group of intensely sweet proteins from the arils of the West African shrub *Thaumatoccus daniellii* (Van der Wel and Loeve 1972). There are at least five forms of thaumatin that have been studied for their sweet-tasting properties but, as yet, no physiological function has been assigned to them. As well as showing sequence similarity to zeamatin, thaumatin also cross-reacts with antizeamatin antiserum (data not shown). Interestingly, despite the similarity between thaumatin, osmotin, and PR-R protein, thaumatin does not cross-react either with anti-osmotin antibodies (Singh

et al. 1987) or with antisera to PR-R protein (Kauffmann *et al.* 1990), nor does the PR-R protein cross-react with antibodies to thaumatin (Cornelissen *et al.* 1986; Pierpoint *et al.* 1987). However, under reducing conditions there is serological cross-reactivity between PR-R protein and thaumatin on dot blots (Cusak and Pierpoint 1988).

The significance of the homologies in N-terminal amino acid sequences among the permatins and these other plant proteins is not clear. Perhaps the termini are involved in regulating transcription, translation, protein modification, or protein targeting, and the sequence similarities reflect a common mechanism of protein synthesis, maturation, or localization. Alternatively, the homologies may reflect similar biological functions among these proteins, such as antifungal activity.

NOTE ADDED IN PROOF

Recent experiments have shown that thaumatin also contains zeamatinlike activity. Using the zeamatin detection assay (Fig. 1), 30 μg of thaumatin per disk inhibited growth of *C. albicans* in the presence of 0.2 μg/ml of nikkomycin Z (Vigers *et al.*, unpublished).

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