Vol. 182, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1992 Pages 1-5

ISOLATION AND CHARACTERIZATION OF A 22 kDa PROTEIN WITH ANTIFUNGAL PROPERTIES FROM MAIZE SEEDS

Quang Khai Huynh[1], Jeffry R. Borgmeyer and James F. Zobel

Department of Protein Biochemistry, Monsanto Corporate Research,
The Monsanto Company, St. Louis, MO 63198

Received November 21, 1991

We have purified a 22 kDa protein from maize seeds to homogeneity by ammonium sulfate precipitation, chitin extraction and Mono-S column chromatography. The purified protein inhibited the growth of the agronomically important pathogens of potato wilt (*Fusarium oxysporum*) and tomato early blight (*Alternaria solani*). Sequence analysis of the purified protein showed that it has 52% homology with the sweet protein thaumatin (Edens, L., Hselinga, L., Klok, R., Ledeboer, A. M., Maat, J., Toonen, M. Y., Visser, C., and Verrips, C. (1982) *Gene* **18**, 1-12), 57 % homology with the pathogenesis-related protein (Cornelissen, B. J. C., Huijsduijnen, R. A. M., and Bol, J. F. (1986) *Nature* **321**, 531-532) and 99 % homology with the 22 kDa trypsin/α-amylase inhibitor (Richardson, M., Valdes-Rodriguez, S., and Blanco-Labra, A. (1987) *Nature* **327**, 432-434).

Plants do not contain an immune system and thus must rely on other mechanisms to defend themselves from infection by a variety of pathogens. In the case of fungal infection, these mechanisms include synthesis of inhibitory compounds such as phenols, melanins, tannins or phytoalexins and an accumulation of proteins which have the capability of acting directly on fungi to inhibit growth (1-2). These proteins are called pathogenesis-related proteins, often protease inhibitors or pathogen targeted hydrolases (1-2). In addition to the unique defense mechanism of these pathogenesis-related proteins, much of the current interest followed the concept that transgenic plants which have been tranfected with proteins with antifungal properties will be resistant to fungal infection. As an approach to this concept, recently we have isolated and characterized a chitinase from *Arabidopsis thaliana* (3). In the present communication, we describe the isolation and characterization of a 22 kDa protein

[1]To whom correspondence should be addressed. FAX: 314-537-7264.

The abbreviations used are: HPLC, high performance liquid chromatography; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone and SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

0006-291X/92 $1.50

with antifungal properties against the agronomically important pathogens of tomato early blight (*A. solani*) and potato wilt (*F. oxysporum*) from the seeds of maize. The purified protein showed 52% of sequence homology to a sweet protein thaumatin (4), 57% of homology to the pathogenesis-related protein from tobacco (5) and a near perfect 99% of sequence homology to the maize 22 kDa trypsin/α-amylase inhibitor (6).

MATERIALS AND METHODS

Materials - Seeds of maize (*Zea mays* L.) were obtained from the Illinois Foundation Seed Co. TPCK -treated trypsin and cyanogen bromide were purchased from Sigma Chemicals. Colloidal chitin was prepared from acetylation of chitosan as described by Hirano *et al.*, (7). The Mono-S column (HR 5/5) was from Pharmacia LKB Biotechnology. Plant pathogens *F. oxysporum, A. solani* were provided by Monsanto Agricultural Company. The other reagents used were of the highest grade commercially available.

Purification of a 22 kDa Antifungal Protein - All purification procedures were carried out at 4°C unless otherwise noted. Two kg of maize seed was extracted in 2 liters of 10 mM sodium acetate buffer, pH 5.0. The precipitate from the extract was discarded by centrifugation and solid ammonium sulfate was added to the supernatant to 60% of saturation. After stirring overnight, the precipitate was collected by centrifugation and extensively dialyzed against 20 mM sodium bicarbonate buffer, pH 8.4. The dialyzed fraction was then incubated with 200 g of colloidal chitin in 200 ml of 20 mM sodium bicarbonate buffer, pH 8.4 for 1 hour at room temperature to separate antifungal chitinases and other possible chitin binding proteins. The fraction which was not bound to chitin was collected by centrifugation, dialyzed against a 20 mM sodium acetate buffer, pH 5.0 and fractionated on a Mono-S column equilibrated with the above buffer. All fractions were collected and assayed for antifungal activity. A fraction which eluted at 120 mM NaCl showed strong antifungal activity. SDS-PAGE and subsequent amino acid sequencing of this fraction showed that it contained a homogeneous protein which has a molecular weight of about 22 kDa.

Assay of Antifungal Activity - Antifungal activity was carried out using a hyphal extension-inhibition assay as described by Roberts and Selittennikoff (10). Fungal mycelium were harvested from actively growing fungal plates and placed into the center of Petri dishes containing the nutrient agar. After incubation of these dishes for 20 to 24 hrs at room temperature to allow for mycelial growth, sterile filter paper discs were laid on the agar surface in front of the advancing fungal mycelium and then 35 μl of the protein solutions were applied to the discs. The plates were then further incubated at room temperature for 20 hrs. In this manner, if the protein being tested is an antifungal protein, a crescent shaped zone of inhibition is observed around the disc.

Other Methods - The procedures for carboxymethylation, tryptic digestion, cyanogen bromide cleavage, peptide isolation by HPLC, amino acid analysis and peptide sequencing were described in our previous papers (3, 11-13). Protein concentration was determined by the method of Bradford (14). Purity of the purified protein was confirmed by SDS-PAGE (15) with silver staining and by amino acid sequencing. The isoelectric point of the purified protein was determined as described by Righetti and Drysdale (16).

Details of this work will be reported elsewhere.

RESULTS AND DISCUSSION

In the present study, we purified an antifungal protein from maize seed to homogeneity by ammonium sulfate precipitation, chitin and Mono-S column chromatographies. The purified protein has an isoelectric point of about 9.3 and exhibited a molecular weight of about 22 kDa as determined by SDS-PAGE (Fig. 1)

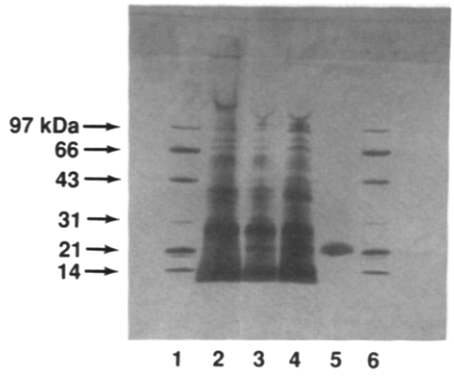

Fig. 1. SDS-PAGE of the purified antifungal protein. As indicated: lanes 1 and 6, molecular weight standards; lane 2, maize seed extract; lane 3, ammonium sulfate fraction; lane 4, chitin unbound fraction and lane 5, purified protein after Mono-S column chromatography.

and gel filtration on a Sephadex G-75 column (data not shown). From 2 kg of maize seed, about 27 mg of the protein was obtained. The purified protein strongly inhibited the growth of *A. solani* (tomato early blight) and *F. oxysporum* (potato wilt) at the amount below 10 μg per disc (Fig. 2), indicating that the gene for this protein may be a promising candidate for the genetic engineering of fungal resistant crops.

To obtain sequence information for gene cloning purposes, the purified protein was subjected to sequence analysis and its NH_2-terminal amino acid sequence of 15 residues was established as shown in Fig. 3. Results from a computer search for homology with other published protein sequences showed

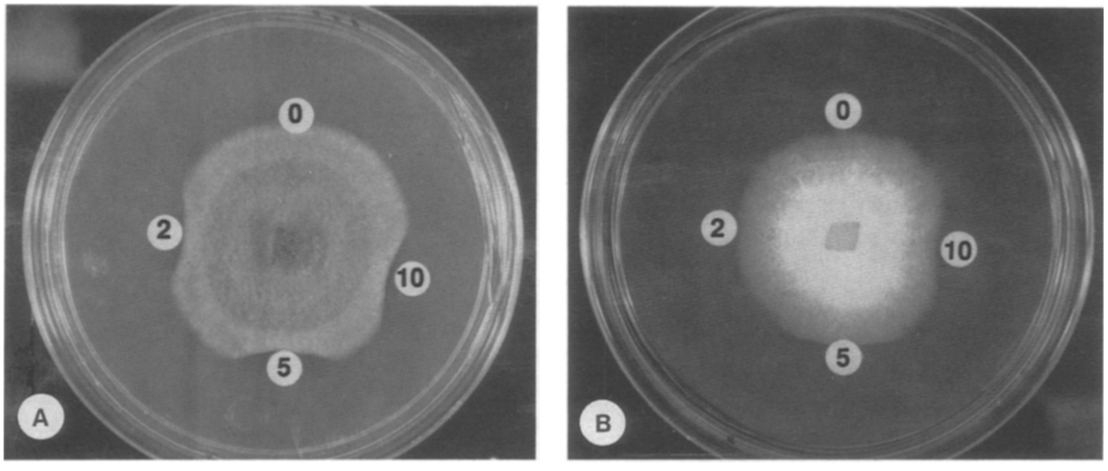

Fig. 2. In vitro antifungal activity of the purified 22 kDa protein against *A. solani* (A) and *F. oxysporum* (B). Appropriate amounts (μg) of the purified protein in 35 μl of 10 mM sodium acetate buffer, pH 5.0 were applied in the discs as indicated. See "Materials and Methods" for details of the *in vitro* antifungal assay.

```
(a)  AVFTVVNQCP FTVWAASVPV .....GGGRQ LNRGESWRIT APAGTTAARI WARTGCQFDA SGRGSCRTGD CGGVVQCTGY GRAPNTLAEY ALKQFNNLDF
(b)  AVFTVVNQCP FTVWAASVPV .....GGGRQ LNRGESWRIT APAGTTAARI WARTGCQFDA SGRGSCRTGD CGGVVQCTGY GRAPNTLAEY ALKQFNNLDF
(c)  ATFEIVNRCS YTVWAAASKG DAALDAGGRQ LNSGESWTIN VEPGTNGGKI WARTDCYFDD SGSGICKTGD CGGLLRCKRF GRPPTTLAEF SLNQYGK.DY
(d)  ATFDIVNQCT YTVWAAASPG ......GGRQ LNSGQSWSIN VNPGTVQARI WGRTNCNFDG SGRGNCETGD CNGMLECQGY GKPPNTLAEF ALNQPNQ.DF

(a)  FDISLIDGFN VPMSFLPDGG SGCSRGPRCA VDVNARCPAE LR.QDGVCNN ACPVFKKDEY CCVGSAANNC HPTNYSRYFK GQCPDAYSYP KDDATSTFTC
(b)  FDISILDGFN VPYSFLPDGG SGCSRGPRCA VDVNARCPAE LR.QDGVCNN ACPVFKKDEY CCVGSAANNC HPTNYSRYFK GQCPDAYSYP KDDATSTFTC
(c)  IDISNIKGFN VPMNFSPTTR G..CRGVRCA ADIVGQCPAK LKAPGGGCND ACTVFQTSEY CCT..TG.KC GPTEYSRFFK RLCPDAFSYV LDKPTTV.TC
(d)  VDISLVDGFN IPMEFSPTNG G..CRNLRCT APINEQCPAQ LKTQ.GGCNN PCTVIKTNEF CCTNGPG.SC GPTDLSRFFK ARCPDAYSYP QDDPPSLFTC

(a)  PAGTNYKVVF CP..
(b)  PAGTNYKVVF CP..
(c)  PGSSNYRVTF CPTA
(d)  PPGTNYRVVF CP..
```

Fig. 3. Amino acid sequence of the purified antifungal protein (a) in comparison to those of the maize 22 kDa trypsin/α-amylase inhibitor (b), the sweet protein thaumatin (c) and the pathogenesis-related protein from tobacco (d). The dots denote spaces required for optimal alignment.

that the purified protein has no homology with any antifungal β-1,3-glucanase or chitinase sequences published to date (20-22) The purified protein, however, exhibited a high degree of homology with a sweet protein thaumatin (4) and a pathogenesis-related protein from tobacco (5). Furthermore, it showed a perfect 100% homology with a 22 kDa trypsin/α-amylase inhibitor from maize (6). The antifungal acitivities these three proteins have not been reported. To obtain the complete sequence, the purified protein was then carboxymethylated, cleaved with trypsin and cyanogen bromide. The peptides were isolated by C_{18} reversed phase HPLC and their amino acid compositions and amino acid sequences were determined. Based on the homology to these published protein sequences, especially to that of the 22 kDa trypsin/α-amylase inhibitor, the complete amino acid sequence of the purified protein was established as shown in Fig. 3. Recently the cDNA clone of the purifief protein has been isolated[2] using oligonucleotide probes based on the above sequence. The amino acid sequence deduced from the nucleotide sequence of the isolated cDNA clone[2] is in complete agreement with our sequence (Fig. 3). Comparison of the four sequences indicated that the purified protein has 52% homology with the sweet thaumatin protein (4), 57% homology with the pathogenesis-related protein (5) and a near perfect 99% homology with the 22 kDa trypsin/α-amylase inhibitor (6) in which Tyr at position 108 was replaced by Met in our purified protein (Fig. 3). It is worth to noting that in preparation of this manuscript, Vigers *et al.* (23) reported the isolation of a 22 kDa antifungal protein from maize which has the NH_2-terminal 60 amino acids identical with that of the maize 22 kDa trypsin/α-amylase inhibitor (6).

In conclusion, the results in this study provide evidence for the existence of a novel class of antifungal proteins which show a high degree of homology with the sweet protein thaumatin (4), the pathogenesis-related protein from tobacco (5) and especially with the trypsin/α-amylase inhibitor from maize (6). Since the maize seeds contain large amounts of this protein, it is likely that it play a significant role in protecting seeds against attack from pathogens during germination.

---

[2]Malehorn, D. and Shah, D. M., Manuscript in preparation.

Acknowledgments - We would like to thank Drs. L. D. Bell, D. M. Shah, G. R. Galluppi and R. T. Fraley for their strong encouragement and support of this project. In addition, we wish to thank C. E. Smith for peptide sequencing.

REFERENCES

1. Bowles, D. J. (1990) *Annu. Rev. Biochem.* **59**, 873-907.
2. Linthorst, H. J. M. (1991) *Critical Reviews in Plant Sciences*, 10, 123-150.
3. Verburg, J.G., and Huynh, Q. K. (1991) *Plant Physiol.* **95**, 450-455.
4. Eden, L., Heslinga, L., Klok, R., Ledeboer, A. M., Maat, J., Toonen, M. Y., Visser, C. and Verrips, C. (1982) *Gene* **18**, 1-12.
5. Cornelissen, B. J. C., Hooft van Huijsduijnen, R. A. M. and Bol, J. F. (1986) *Nature* **321**, 531-532.
6. Richardson, M., Valdes-Rodriguez, S. and Blanco-Labra, A. (1987) *Nature* **327**, 432-434.
7. Hirano, S., Ohe, Y. and Ono, H. (1987) *Carbohydr. Res.* **47**, 315-320.
8. Mauch, F., Hadwiger, L. A. and Boller, T. (1988) *Plant Physiol.* **87**, 325-333.
9. Boller, T. and Mauch, F. (1988) *Methods Enzymol.* **161**, 430-435.
10. Roberts, W. K. and Selitrennikoff, C. P. (1986) *Biochim. Biophys. Acta* **880**, 161-170.
11. Huynh, Q. K., Kishore, G. M., and Bild, G. S. (1988) *J. Biol. Chem.* **263**, 735-739.
12. Huynh, Q. K. (1988) *J. Biol. Chem.* **263**, 11631-11635.
13. Huynh, Q. K. (1990) *J. Biol. Chem.* **265**, 6700-6704.
14. Bradford, M. M. (1986) *Anal. Biochem.* **72**, 248-254.
15. Laemmli, U. K. (1970) *Nature* **227**, 680-685.
16. Righetti, P., and Drysdale, J. W. (1971) *Biochim. Biophys. Acta* **236**, 17-28.
17. Wong. Y. S., and Maclachlan, G. A. (1979) *Biochim. Biophys. Acta* **571**, 244-255.
18. Ballance, G. M., and Mannaers, D. J. (1978) *Phytochemistry* **17**, 1539-1543.
19. Van Den Buike, M., Bauw, G., Castresana, C., Van Montagu, M. and Vandekerckhove, J. (1989) *Proc. Natl. Acad. Sci.* U. S. A. **86**, 2673-2677.
20. De Loose, M., Alliotte, G., Gemetello, C., Gielen, J., Soetaert, P., Van Montagu, M. and Inze, D. (1988) *Gene* **70**, 13-23.
21. Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991) *J. Biol. Chem.* **266**, 1564-1573.
22. Fincher, G. B., Lock, P.A., Morgan, M.M.,Lingelbach, K., Wettenhall, R. E., Mercer, J. F. B., Brandt, A., and Thomsen, K. K. (1986) *Proc. Natl. Sci. Acad.* U. S. A. **83**, 2081-2085.
23. Vigers, A. J., Roberts, W. K., and Selitrennikoff, C. P. (1991) *Molecular Plant-Microbe Interaction* **4**, 315-323.