

## Structural and Antifungal Properties of a Pathogenesis-Related Protein from Wheat Kernel

Carla Caruso,<sup>1,2</sup> Carlo Caporale,<sup>1</sup> Gabriele Chilosi,<sup>3</sup> Francesco Vacca,<sup>1</sup> Laura Bertini,<sup>1</sup> Paolo Magro,<sup>3</sup> Elia Poerio,<sup>1</sup> and Vincenzo Buonocore<sup>1</sup>

Received September 11, 1995

We have purified and characterized a protein from the water-soluble fraction of wheat kernel (*Triticum aestivum* cv. S. Pastore) consisting of a single polypeptide chain blocked at its N-terminus by a pyroglutamate residue; the complete amino acid sequence has been determined by automated sequence analysis performed on peptide fragments obtained by enzymatic hydrolyses of the protein. Homology studies have shown that this protein is very similar (97% sequence identity) to the previously characterized wheatwin1 as well as to other members of the pathogenesis-related (PR) proteins of class 4; in analogy with wheatwin1, we have termed this protein wheatwin2. Both wheatwin1 and wheatwin2 have specific antifungal activity toward the wide-host-range pathogen *Botrytis cinerea* and the wheat-specific pathogenic fungi of wheat *Fusarium culmorum* and *Fusarium graminearum* of groups 1 and 2. On the basis of their structural and functional properties, wheatwin1 and wheatwin2 can be classified as members of the PR4 protein family; this represents the first report concerning the presence of this kind of protein in wheat.

**KEY WORDS:** Antifungal activity; pathogenesis-related proteins; primary structure; wheat kernel.

### 1. INTRODUCTION

During evolution, plants have developed constitutive and inducible mechanisms to respond to infection by viruses, fungi, and bacteria (Hahlbrock and Scheel, 1987) or mechanical and chemical treatments (Davies, 1987; Boller, 1988; Hildmann *et al.*, 1992); these defense reactions are considered to play an important role in disease resistance. The most extensively characterized defense reactions are the activation of the phenylpropanoid metabolism (Lawton *et al.*, 1983; Cramer *et al.*, 1985; Friend, 1985), the synthesis of antimicrobial compounds such as phytoalexins (Bowles, 1990)

and proteinase inhibitors (Ryan, 1990), and the synthesis and accumulation of a set of host-encoded pathogenesis-related (PR) proteins (Van Loon, 1985; Boller, 1987; Carr and Klessing, 1989; Bol *et al.*, 1990; Dixon and Lamb, 1990; Linthorst, 1991).

PR proteins are widely distributed in the plant kingdom and are induced by different stress situations; they were first described in tobacco plants infected with tobacco mosaic virus (TMV) (Gianinazzi *et al.*, 1970; Van Loon and Van Kammen, 1970); since then, several PR proteins have been described in other plant species challenged with many biotic and abiotic stresses (Van Loon, 1989).

While the molecular characterization of PR1, PR2, PR3, and PR5 protein families has been extensively reported, only a little is known about members of group 4. Two PR4 proteins have been isolated from tobacco and tomato plants infected with TMV and *Cladosporium fulvum*, respectively

<sup>1</sup>Dipartimento di Agrobiologia e Agrochimica, Università della Toscana, I-01100 Viterbo, Italy.

<sup>2</sup>To whom correspondence should be addressed. E-mail: Caruso@tusmx1.utover.it.

<sup>3</sup>Dipartimento di Protezione delle Piante, Università della Toscana, I-01100 Viterbo, Italy.

(Friedrich *et al.*, 1991; Linthorst *et al.*, 1991). Their cDNA sequences were found to be homologous to the C-terminal domain of both win1 and win2 genes from potato (Stanford *et al.*, 1989) and to the region of hev1 gene encoding the precursor of hevein, a small protein isolated from rubber tree (*Hevea brasiliensis*) latex (Broekaert *et al.*, 1990); interestingly, the N-terminal hevein-like domain of win1, win2, and hev1 genes is missing in both tobacco and tomato PR4 genes, while it is present in genes coding lectins from monocots (Raikhel and Wilkins, 1987; Wright and Raikhel, 1989; Lerner and Raikhel, 1989; Chrispeels and Raikhel, 1991) and dicots (Chapot *et al.*, 1986) as well as in genes coding some basic chitinases; these last have the hevein-like domain fused to an unrelated C-terminal portion (Broglic *et al.*, 1986; Shinshi *et al.*, 1987, 1990; Parson *et al.*, 1989; Laflamme and Roxby, 1989).

A mature protein, called barwin, from barley seeds has been isolated and completely sequenced (Svensson *et al.*, 1992); this protein was found to be homologous to the tobacco and tomato PR4 proteins as well as to the C-terminal domain of the win1, win2, and hev1 proteins; barwin, which is also present in primary leaves challenged with *Erysiphe graminis*, shows an antifungal activity toward *Trichoderma harzianum* (Hejgaard *et al.*, 1992).

Recently we reported the structural characterization of a protein from wheat kernel, named wheatwin1, which was highly homologous to the PR4 protein family (Caruso *et al.*, 1993); on the basis of this structural evidence we assumed for wheatwin1 a possible role in the mechanisms of plant defense. In this paper we report the isolation and structural characterization of a new protein from wheat kernel, named wheatwin2, closely related to wheatwin1, and the antifungal activity of both wheatwin1 and wheatwin2 toward a wide-host-range fungal pathogen and isolates of pathogenic fungi of wheat.

## 2. MATERIALS AND METHODS

### 2.1. Materials

*Triticum aestivum*, cultivar San Pastore, was kindly supplied from Istituto Sperimentale per la Cerealicoltura (S. Angelo Lodigiani, Italy). HPLC

was carried out on a Beckman GOLD apparatus equipped with a variable-wavelength monitor model 166. HPLC-grade acetonitrile and methanol were from Baker (Holland). Bovine serum albumin, trifluoroacetic acid, dithiothreitol, and 4-vinylpyridine were from Sigma Chemical Co. (St. Louis, MO). Sequencing-grade trypsin (EC 3.4.21.4) and endoproteinase Lys-C from *Lysobacter enzymogenes* were from Boehringer Mannheim Italia SpA. The  $\mu$ -Bondapak C<sub>18</sub> column (0.39  $\times$  30 cm) was from Waters-Millipore (Milford, MA). Reagents for SDS-PAGE, including low-molecular-weight markers, were from Bio-Rad (Italy); reagents for isoelectric focusing were from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity commercially available.

### 2.2. Protein Purification

Wheatwin1 and wheatwin2 were purified from the water-soluble albumin fraction of wheat kernel following the procedure previously described for the purification of the 13-kD monomeric iso-inhibitors of  $\alpha$ -amylase (Carrano *et al.*, 1989) with slight modifications: the last chromatographic step on a Q-Sepharose Fast Flow column using an FPLC apparatus was replaced by reverse phase (RP) HPLC using a  $\mu$ -Bondapak C<sub>18</sub> column. Eluent A was aqueous 0.1% trifluoroacetic acid and eluent B was 0.07% trifluoroacetic acid in acetonitrile. Elution was accomplished at a flow rate of 1 ml min<sup>-1</sup> monitoring the absorbance at 220 nm. The elution program was isocratic elution for 5 min at 28% B, followed by a linear gradient from 28% to 36% B in 55 min. Fractions were manually collected and freeze-dried. The protein concentration was determined by the Bio-Rad assay, following the manufacturer's instructions and using bovine serum albumin as a standard.

### 2.3. Electrophoresis

SDS-PAGE was carried out on a mini-Protean apparatus (Bio-Rad) using a discontinuous system according to Laemmli (1970) on a 12% acrylamide gel; both Coomassie and silver staining procedures were used following the instruction of the manufacturer. IEF was performed in the pH range 3.5–10 using both an LKB apparatus (Pharmacia,

Uppsala, Sweden) and a mini-IEF apparatus from Bio-Rad.

#### 2.4. Protein Reduction and Pyridylethylation

Wheatwin2 (10 nmol) was treated with 0.5  $\mu$ mol of dithiothreitol in 0.1 ml of 0.25 M Tris-HCl buffer, pH 8.0, 2 mM EDTA, 6 M guanidine-HCl, for 2 hr at 37°C under nitrogen atmosphere. Alkylation was performed with 4-vinylpyridine (15  $\mu$ mol) for 2 hr at room temperature in the dark under nitrogen atmosphere. The reaction was stopped by freezing and reagents in excess were removed by RP-HPLC on the  $\mu$ -Bondapak C<sub>18</sub> column. Eluents A and B were those described above. The elution was carried out at a flow rate of 1 ml min<sup>-1</sup> by the following program: isocratic elution for 5 min at 15% B followed by a linear gradient from 15% to 40% B in 30 min. The S-pyridylethylated (PyEt) wheatwin2 was manually collected and freeze-dried.

#### 2.5. Digestion with Trypsin

PyEt-wheatwin2 (5 nmol) was digested with trypsin (enzyme:substrate 1:50, w/w) for 6 hr at 37°C in 0.1 ml of 0.1% ammonium bicarbonate buffer, pH 8.0. The digest was freeze-dried and dissolved in 0.1 ml of aqueous 0.1% trifluoroacetic acid. The fragment mixture was resolved by RP-HPLC on the  $\mu$ -Bondapak C<sub>18</sub> column. Eluents A and B were those described in the previous section. The elution was performed at a flow rate of 1 ml min<sup>-1</sup> monitoring the absorbance at 220 nm by using the following program: isocratic elution for 10 min at 10% B followed by a linear gradient from 10% to 26% B in 56 min and then from 26% to 34% B in 54 min. Peaks were manually collected and freeze-dried.

#### 2.6. Digestion with Endoproteinase Lys-C

PyEt-wheatwin2 (10 nmol) was digested with endoproteinase Lys-C from *Lysobacter enzymogenes* (enzyme:substrate 1:50, w/w) for 8 hr at 37°C in 0.1 ml of 0.1% ammonium bicarbonate buffer, pH 8.0; the digestion was terminated by acidification with trifluoroacetic acid (1 mM final concentration) and the peptide mixture was freeze-dried and dissolved in 0.1 ml of aqueous 0.1% trifluoroacetic acid. The fragment mixture was resolved by RP-HPLC on the  $\mu$ -Bondapak C<sub>18</sub>

column. Eluents A and B were those described in the previous section. The elution was performed at a flow rate of 1 ml min<sup>-1</sup> monitoring the absorbance at 220 nm and using the following program: isocratic elution for 5 min at 5% B followed by a linear gradient from 5% to 40% B in 85 min. Peaks were manually collected and freeze-dried.

#### 2.7. Sequence Analyses

The purified tryptic and endoproteinase Lys-C peptides of wheatwin2 were dissolved in aqueous 0.1% trifluoroacetic acid (10–40  $\mu$ l); portions (200–500 pmol) were submitted to automated sequence analysis using a pulsed liquid-phase automatic sequencer (Applied Biosystems model 477A) equipped on-line with a phenylthiohydantoin amino acid derivative analyzer (Applied Biosystems model 120A). Samples were loaded onto a trifluoroacetic acid-treated glass-fiber filter, coated with polybrene, and washed according to manufacturer's instructions. The relative reagents were from Applied Biosystems.

#### 2.8. Deblocking of the N-Terminal Residue

Deblocking of the N-terminal residue was achieved by incubating the N-terminal tryptic fragment (200 pmol) of wheatwin2 in 50  $\mu$ l of 1 M HCl in methanol for 1 hr at 45°C.

#### 2.9. In Vitro Antifungal Assay

To assess the antifungal properties of wheatwin1 and wheatwin2 the two proteins were extensively dialyzed against distilled water and lyophilized. Inhibition bioassays of fungal growth were carried out with the pathogenic fungi of wheat *Fusarium culmorum* (Smith) Sacc., *Fusarium graminearum* Schwabe of groups 1 and 2, and *Botrytis cinerea* Pers. (isolate 91/3). The *F. culmorum* (isolate 485) and *F. graminearum* group 1 (isolate 218) and group 2 (isolate 511) were obtained from wheat and kindly supplied by Istituto Sperimentale per la Patologia vegetale (Rome, Italy); the isolates were grown on carnation leaf agar (CLA). *B. cinerea* (isolate 91/3), obtained from grape and grown on potato dextrose agar

(PDA) (OXOID, Hampshire, England), was maintained as a stock culture on PDA slants at 4°C in our culture collection. Macroconidia from sporodochia of *Fusarium* cultures and conidia from *B. cinerea* cultures were collected from 10-day cultures grown at 20°C and suspended in sterile potato dextrose broth. In the growth inhibition assay 10- $\mu$ l aliquots of suspension containing  $10^4$  ml<sup>-1</sup> spores of each isolate were placed on sterile microscope slides and 90  $\mu$ l of protein solution was added to each spore suspension (final protein concentration 5, 10, and 25  $\mu$ g ml<sup>-1</sup>); the microscope slides were kept in a moist chamber at 21°C in the dark for 8 hr. Antifungal activity was determined microscopically by measuring hyphal growth; two sets of spore suspensions in sterile distilled water and in bovine serum albumin solution represented the controls. Assays were performed in duplicate and both the average of at least 100 measurements of hyphal growth for each sample and the standard deviations were calculated. The effective dose for 50% inhibition (ED<sub>50</sub>) of each sample was obtained by least-square linear regression of the dosage response.

### 3. RESULTS

#### 3.1. Protein Purification

Wheatwin2 has been purified to homogeneity following basically the purification procedure of  $\alpha$ -amylase and protease inhibitors from wheat seeds (Carrano *et al.*, 1989); after the gel-filtration step on the Sephadex G-100 superfine column the fractions corresponding to a molecular weight of about 13,000 were pooled and the last step of ion-exchange chromatography on a Q-Sepharose column was replaced with RP-HPLC, which allowed us to isolate this new protein together with wheatwin1 in higher yields (Fig. 1). SDS-PAGE in the presence and absence of mercaptoethanol shows that wheatwin2 is a monomeric protein with a molecular mass of about 13 kD, according with the value previously determined by gel filtration; the isoelectric point of wheatwin2 is 8.3, which is slightly higher than the value of 7.3 determined for wheatwin 1. Both SDS-PAGE and IEF confirmed that wheatwin1 and wheatwin2 appear as pure

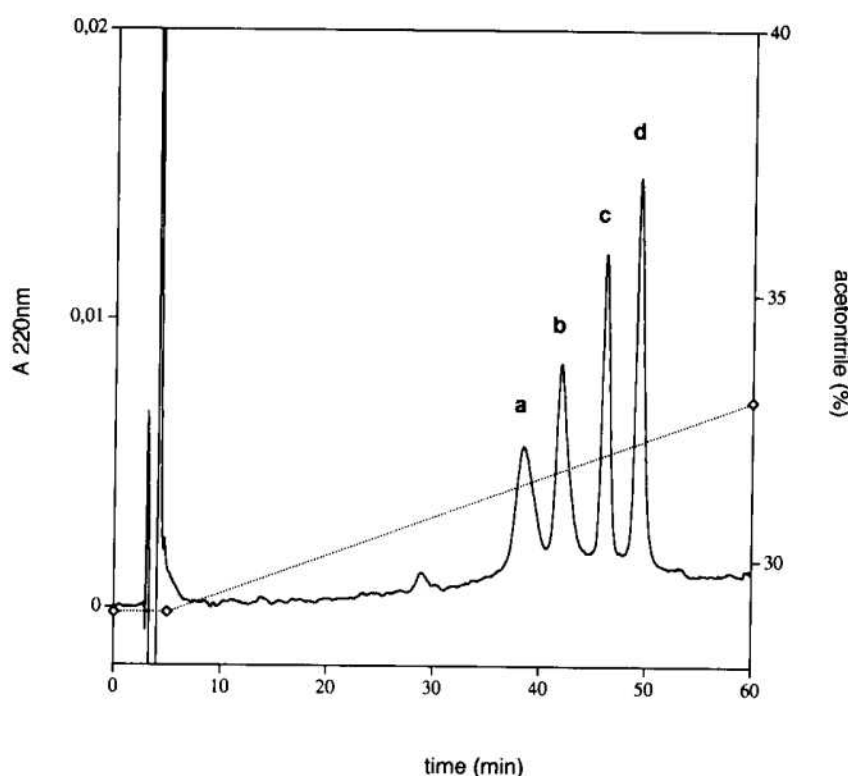


Fig. 1. Purification of wheatwin1 and wheatwin2 by RP-HPLC.  $\alpha$ -Amylase monomeric inhibitors (a) 0.28 and (b) 0.39; (c) wheatwin2; and (d) wheatwin1.

proteins after RP-HPLC and do not contaminate each other. Their total grain concentration is about  $10\text{--}15\ \mu\text{g g}^{-1}$  grain.

### 3.2. Sequence Analysis

The Edman degradation of the reduced and pyridylethylated wheatwin2 was unsuccessful due to the presence of a blocking group at the N-terminus. The pyridylethylated protein was separately digested with trypsin and endoproteinase Lys-C; the two peptide mixtures were resolved by RP-HPLC, which allowed the purification of all peptides through a single chromatographic run (Fig. 2). The

purified tryptic (T1–T8; Fig. 2A) and endoproteinase Lys-C (K1–K4; Fig. 2B) peptides of the S-pyridylethylated wheatwin2 were submitted to automated sequence analysis. Fragment T1 (Fig. 2A) failed to show any amino acid derivative, thus suggesting the presence of a blocked N-terminal residue; it was deblocked by acid treatment with HCl–methanol and its sequence was then assessed. The presence of glutamic acid at the N-terminus of the deblocked T1 fragment suggested that the masking group was pyroglutamate, analogous to the situation for wheatwin1 (Caruso *et al.*, 1993). As determined by the sequencer software, the average and combined repetitive amino acid yields were not lower than 90% for each sequenced peptide; the

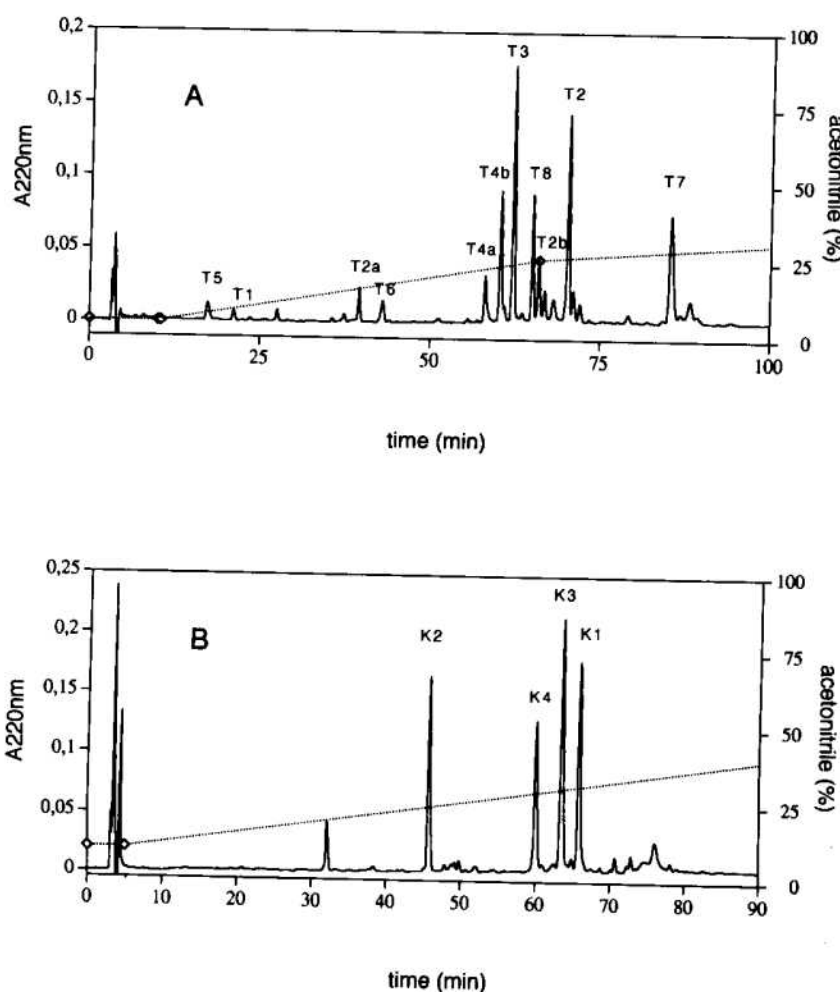


**Fig. 2.** RP-HPLC of (A) tryptic and (B) endoproteinase Lys-C peptides of S-pyridylethylated wheatwin2. All peptides were subjected to sequence analysis. Peptides T1 and K1 were found to be blocked at their N-terminus.

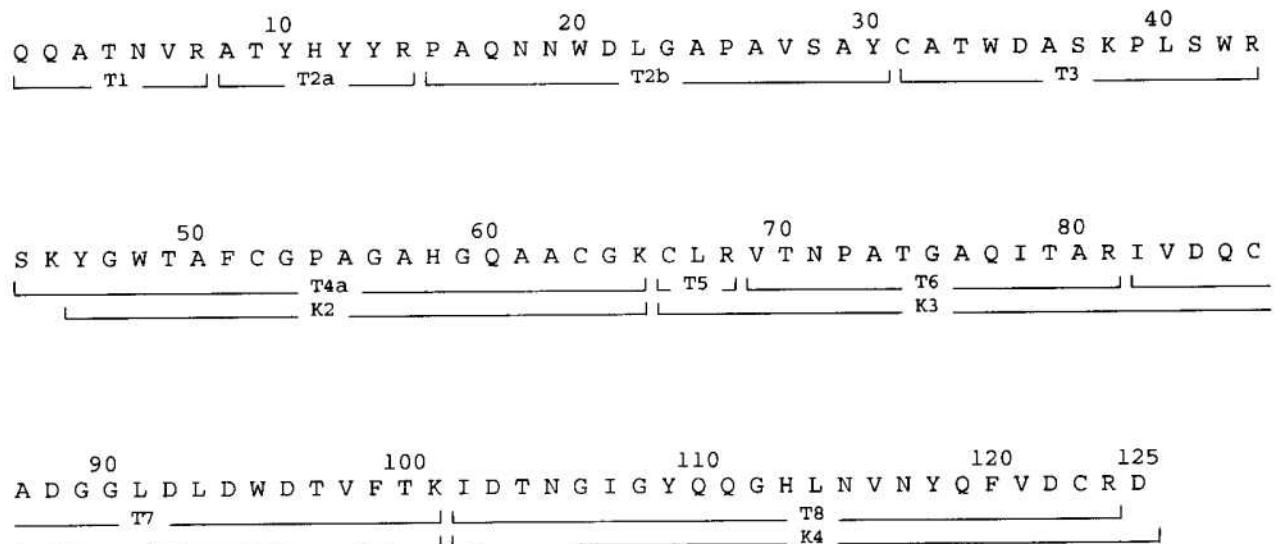


Fig. 3. Amino acid sequence of wheatwin2. Tryptic (T1-T8) and endoproteinase Lys-C (K2-K4) peptides utilized for establishing the sequence are indicated.

theoretical initial yields were not lower than 60%. The complete amino acid sequence of wheatwin2, obtained by overlapping useful tryptic and endoproteinase Lys-C peptides, is shown in Fig. 3. The

alignment of the tryptic peptides in the N-terminal region was achieved by homology with the corresponding region of wheatwin1, since peptide K1 from endoproteinase Lys-C digestion was

|             |                                                                                 |
|-------------|---------------------------------------------------------------------------------|
| HEV1        | -N- -F- -VV- -C-L-TGV I E Q A K P N W D Y DHN N KDS -EGVG-G                     |
| WIN2        | - CGP A V CISLTSV - R GN Y Q S -PD GQG                                          |
| WIN1        | MVKLISNSTILLSLFLFSIAAI-ANAQQ-CGRQKGGALCSGNLCCSQFGWCGSTPEFCSPSQGCQSRCTGTGGSTPTPS |
| PR4 Tobacco | ERVNNYKLCVAL IISMVM M A-----                                                    |
| PR4 Tomato  | ERVNK--LCVAFVINMM V A-----                                                      |

|             |                                                                                 |
|-------------|---------------------------------------------------------------------------------|
| HEV1        | S L L S DH -- A Y S AH QS S G K                                                 |
| WIN2        | -- A Y S                                                                        |
| WIN1        | GSAQNV RATYHIYNPQNVGWDLN--AVSAYCSTWDANKPLSWRKKYGTAF CGPVGPRGRDSCGKCLRVNTNRTGAQT |
| PR4 Tobacco | Q T S L IN R-- A F A D A Q A QV R G T                                           |
| PR4 Tomato  | Q T L IN R--TA V A D E RR A T QA R G T E                                        |
| Barwin      | QQ ND Y R AQNN GAP A S S A QAA PA I                                             |
| Wheatwin1   | QQ TN Y R AQNN GAP A S SG A AH QA Q PA I                                        |
| Wheatwin2   | QQ TN Y R AQNN GAP A S S A AH QAA PA I                                          |

|             |                                                  |
|-------------|--------------------------------------------------|
| HEV1        | - L K YER IT SFN-PLFSVMKSSVIN                    |
| WIN2        | I - Q V Q N VNPILLSV-DK--E                       |
| WIN1        | TVRIVDQCSNGGLDLVDN-VFRQIDTDGNGNHQGHILVNYQFVDCGDN |
| PR4 Tobacco | - N L N V YQ T E N N -                           |
| PR4 Tomato  | - NRL N L YQR N N E N -                          |
| Barwin      | A A WDT TK N I YQ N R -                          |
| Wheatwin1   | A A WDT TK N I YQ N R -                          |
| Wheatwin2   | A AD WDT TK N I YQ N R -                         |

Fig. 4. Sequence comparison of wheatwin1, wheatwin2, barwin, tobacco PR4 protein, tomato PR4 protein, and the proteins encoded by win1, win2, and hev1 genes. All comparisons refer to the sequence of win1, which is comprehensive of all domains. The amino acid residues of the other proteins different from those in the win1 sequence are indicated. Gaps (—) are introduced to maximize the homology.

deduced to be the N-terminal blocked fragment by the evidence that its sequence analysis was unsuccessful. The calculated molecular weight of 13,645 is in good agreement with the mass value estimated from SDS-PAGE.

Figure 4 shows the sequence of wheatwin2 in comparison with that of wheatwin1 and other PR4 proteins as well as with the gene-deduced sequences of win1 and win2 from potato and hev1 from rubber tree. Wheatwin2 is highly homologous to wheatwin1 (Caruso *et al.*, 1993), differing in only 3 residues out of 125, and both proteins are very closely related to barwin (94–97% sequence

identity) isolated from barley (Svensson *et al.*, 1992) and to the two PR4 mature proteins purified from tobacco and tomato plants (69–71% sequence identity) (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991). All these mature proteins were found to be homologous to the C-terminal domain of the gene-deduced sequences of win1 and win2 (70% sequence identity) (Stanford *et al.*, 1989) and hev1 (68% sequence identity) (Broekaert *et al.*, 1990). However, these putative proteins contain an N-terminal cysteine-rich domain which is missing in the PR4 proteins. This domain of 43 amino acid residues, encoding the mature hevein (Walujono *et*

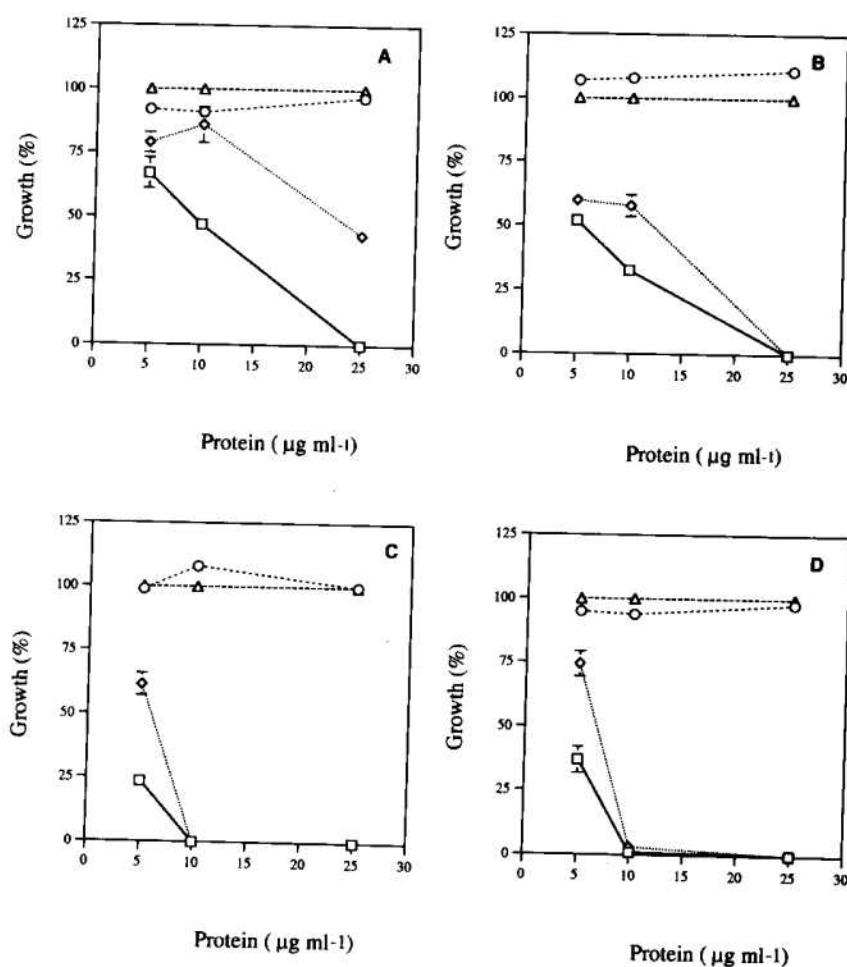


**Fig. 5.** Antifungal activity of wheatwin1 and wheatwin2. Hyphal growth was measured at varying concentrations of the proteins using the following test fungi: (A) *Botrytis cinerea*, (B) *Fusarium culmorum*, (C) *Fusarium graminearum* of group 1, and (D) *Fusarium graminearum* of group 2. Relative hyphal growth is expressed as percentage of the hyphal growth of control cultures and data are means of two independent experiments. Bars represent SDs; SD values not indicated were not detectable under the unit scale used. (Δ) Control; (○) BSA; (□) wheatwin1; (◇) wheatwin2.

**Table 1.** ED<sub>50</sub> Values of Germ Tube Elongation in Phytopathogenic Fungi *Botrytis cinerea*, *Fusarium culmorum*, and *Fusarium graminearum* Groups 1 and 2 Treated with Wheatwin1 and Wheatwin 2<sup>a</sup>

| Pathogen                            | ED <sub>50</sub> (μg ml <sup>-1</sup> ) |           |
|-------------------------------------|-----------------------------------------|-----------|
|                                     | wheatwin1                               | wheatwin2 |
| <i>Botrytis cinerea</i>             | 12.1                                    | 14.7      |
| <i>Fusarium culmorum</i>            | 9.6                                     | 12.2      |
| <i>Fusarium graminearum</i> group 1 | 3.2                                     | 9.7       |
| <i>Fusarium graminearum</i> group 2 | 10.4                                    | 11.3      |

<sup>a</sup> ED<sub>50</sub> values were obtained by least-square linear regression of the dosage response.

*al.*, 1975), is found in other chitin-binding proteins (Shinshi *et al.*, 1990) and some lectins (Chrispeels and Raikhel, 1991).

### 3.3. Antifungal Activity

The antifungal activity was determined by measuring the effect of increasing protein concentrations (5–25 μg ml<sup>-1</sup>) on the growth of fungal hyphae. Both proteins are effective in inhibiting the hyphal growth of the wide-host-range pathogen *B. cinerea* (Fig. 5A) and the three wheat-specific pathogenic fungi *F. culmorum* and *F. graminearum* of group 1 and of group 2 (Fig. 5B–D). The inhibitory abilities of the two proteins toward the four fungi, compared on the basis of ED<sub>50</sub> values, are reported in Table I.

## 4. DISCUSSION

Wheatwin1 and wheatwin2 are two highly homologous components (97.6% sequence identity) of the albumin family of wheat kernel, differing in only 3 amino acid residues out of 125; the difference in the basic amino acid content (higher in wheatwin2) is in agreement with the different isoelectric points of the two proteins.

Given the sequence similarity, the two proteins are likely to have a global fold similar to that of barwin, whose three-dimensional structure in solution has been determined by NMR (Ludvigsen and Poulsen, 1992a,b). It is of great interest to compare the similar primary structure of proteins, especially when three-dimensional data are available; in particular, the comparison can shed some light on the molecule sites where the structure is

conserved and similarly on the sites where mutations have occurred. Ludvigsen and Poulsen (1992b) reported that barwin differs from win1, win2, and hev1 proteins in sites on the protein surface located in the loop regions (Arg 14–Ala 29 and Gly 53–Gly 64) and in the small  $\alpha$ -helix Trp95–Gly111. On the other hand, wheatwin1 and wheatwin2 differ from barwin only in the loop region Gly 53–Gly 64 and in the  $\beta$ -sheet Gln2–Thr9, which is also located on the surface of the protein. These findings show that wheatwin1 and wheatwin2 are very similar to barwin also in the three-dimensional structure; this is not surprising considering that wheat and barley are very closely related. Furthermore, the observed differences of wheatwin1 and wheatwin2 from the proteins from potato and rubber tree are located in the surface regions where random mutations are more likely to be expected, confirming that the global fold of all these PR4 proteins is likely to be more conserved during evolution than their primary structures.

In comparison with the gene-deduced sequences of win1, win2, and hev1, wheatwin1 and wheatwin2 lack the hevein-like domain, analogous to the other PR4 mature proteins from tobacco, tomato, and barley (Friedrich *et al.*, 1991; Linthorst *et al.*, 1991; Svensson *et al.*, 1992). Therefore, the PR4 family is a structurally distinct example of a phenomenon already described in chitinases. These enzymes, belonging to the PR3 family, have been grouped into three classes on the basis of their primary structures (Shinshi *et al.*, 1990). Class I chitinases are basic proteins of about 32–34 kD mainly located in the vacuole, characterized by the presence of the hevein-like domain, followed by the presence of a C-terminal chitinolytic, sugar-binding domain; in addition, the vacuolar chitinases contain a C-terminal extension of seven amino acids necessary for vacuolar sorting that is missing in the mature protein (Neuhaus *et al.*, 1991; Bednarek and Raikhel, 1992; Nakamura and Matsuoka, 1993). Class II chitinases are structurally similar to the chitinolytic domain of class I enzymes, but are acidic proteins of about 27–28 kD that lack the hevein-like domain and are extracellularly located (Payne *et al.*, 1990). Class III chitinases also lack the hevein-like domain and are structurally unrelated to class I and class II enzymes. It has been proposed that class I chitinases have evolved by gene fusion of the chitin-binding domain with the DNA encoding the hevein domain, whereas class II enzymes could have evolved from class I chitinases

by excision of this domain (Shinshi *et al.*, 1990). The sugar-binding domain, found in several lectins, could represent a building block of many proteins, all of which have similar sugar-binding specificities (Chrispeels and Raikhel, 1991). During evolution, this cysteine-rich domain could have been duplicated several times producing lectins, such as wheat germ agglutinin (Raikhel and Wilkins, 1987), or becoming incorporated into genes that now encode different proteins, such as chitinases and PR4 proteins. This combination of fusion and duplication of building blocks represents a model of evolution that yields mosaic structures with different biological functions.

It has been demonstrated that mature plants respond to microbial infection by synthesizing a group of pathogenesis-related proteins, all of which possess antifungal activity with the exception of the PR1 family, which has been characterized only at DNA level. In order to determine the biological role of wheatwin1 and wheatwin2, assays for antifungal activity were performed utilizing a number of pathogenic fungi. Wheatwin1 was found to be more effective than wheatwin2 toward all tested fungi; for instance, the inhibition of *B. cinerea* growth is complete with wheatwin1 and is about 60% with wheatwin2 using a concentration of  $25 \mu\text{g ml}^{-1}$ . Both proteins were more effective toward *F. culmorum* growth; this is not surprising considering that *F. culmorum* is a pathogen specific for wheat, whereas *B. cinerea* is a wide-host-range pathogen. This trend is confirmed by the experiments performed on two other isolates of wheat: *F. graminearum* groups 1 and 2. Both wheatwin1 and wheatwin2 show a more pronounced antifungal effect against these latter two isolates; in fact, the inhibition is complete for both proteins at  $10 \mu\text{g ml}^{-1}$ . Control experiments with BSA confirmed that the observed inhibition patterns were not due to the general effect on cell growth found for many basic proteins in the  $10\text{--}100 \mu\text{g ml}^{-1}$  range (Jacobsen *et al.*, 1990). The two proteins, in particular wheatwin1, appear to possess higher antifungal activity toward *F. graminearum* G1 than *F. graminearum* G2 as revealed by  $\text{ED}_{50}$  values. Members of the two groups differ essentially because the former is a soil-borne pathogen which causes crown rot of temperate cereals, while the latter is an air-borne pathogen which causes head blight of temperate cereals (Francis and Burgess, 1977).

It is worth pointing out that the two proteins

differ in only three residues, all located in the surface region, which probably play an important role in determining their different antifungal activity. The functional significance of the structural differences between wheatwin1 and wheatwin2 is not fully understood, but the existence of two proteins with different specific antifungal activity might represent a selective advantage toward a wide range of potential pathogens.

Our study provides strong evidence that wheatwin1 and wheatwin 2, which accumulate in wheat seeds, play a protective role against attack by pathogens and seed rots. The aim of our future projects is to gain more insight into the molecular characterization of these PR4 proteins; to this end, cloning of wheatwin1 is in progress in order to study the gene organization and the regulation of its expression. Moreover, we are producing monoclonal antibodies raised against wheatwin 1 to elucidate the tissue and subcellular localizations of the two proteins by immunocytochemical techniques.

## ACKNOWLEDGMENTS

This research was supported by the National Research Council of Italy, Special Project RAISA, Sub-project N.2. Paper N.2461.

## REFERENCES

- Bednarek, S. K., and Raikhel, N. V. (1992). *Plant Mol. Biol.* **20**, 133–150.
- Bol, J. F., Linthorst, J. M., and Cornelissen, B. J. C. (1990). *Annu. Rev. Phytopathol.* **28**, 113–138.
- Boller, T. (1987). In *Plant-Microbe Interactions: Molecular and Genetic Perspectives* (Kosuge, T., and Nester, E. W., eds.), Macmillan, New York, Vol. 2, pp. 385–413.
- Boller, T. (1988). In *Oxford Surveys of Plant Molecular and Cell Biology*, Vol. 5 (Mifflin, B. J., ed.), Oxford University Press, Oxford, pp. 145–174.
- Bowles, D. J. (1990). *Annu. Rev. Biochem.* **59**, 873–907.
- Brockaert, W., Lee, H., Kush, A., Chua, N. H., and Raikhel, N. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 7633–7637.
- Broglic, K. E., Gaynor, J. J., and Broglic, R. M. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 6820–6824.
- Carr, J. P., and Klessing, D. F. (1989). In *Genetic Engineering: Principles and Methods* (Setlow, J. K., ed.), Plenum Press, New York, pp. 65–109.
- Carrano, L., Nitti, G., Buonocore, V., Caporale, C., and Poerio, E. (1989). *Plant Sci.* **65**, 25–31.
- Caruso, C., Caporale, C., Poerio, E., Facchiano, A., and Buonocore, V. (1993). *J. Protein Chem.* **12**, 379–386.
- Chapot, M. P., Peumans, W. J., and Strosberg, A. D. (1986). *FEBS Lett.* **195**, 231–234.

- Chrispeels, M. J., and Raikhel, N. V. (1991). *Plant Cell* **3**, 1-9.
- Cramer, C. L., Bell, J. N., Ryder, T. B., Bailey, J. A., Schuch, W., Bolwell, G. P., Robbins, M. P., Dixon, R. A., and Lamb, C. J. (1985). *EMBO J.* **4**, 285-289.
- Davies, E. (1987). In *The Biochemistry of Plants*, Vol. 12 (Davis, E., ed.), Academic Press, pp. 243-264.
- Dixon, R. A., and Lamb, C. J. (1990). *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 339-367.
- Francis, R. G., and Burgess, L. W. (1977). *Trans. Br. Mycol. Soc.* **68**, 421-427.
- Friedrich, L., Moyer, M., Ward, E., and Ryals, J. (1991). *Mol. Gen. Genet.* **230**, 113-119.
- Friend, J. (1985). *Ann. Proc. Phytochem. Soc.* **25**, 367-392.
- Gianinazzi, S., Martin, C., and Vallee, J. C. (1970). *C. R. Acad. Sci. D* **270**, 2383-2386.
- Hahlbrock, K., and Scheel, D. (1987). In *Innovative Approaches to Plant Disease Control* (Chet, I., ed.), Wiley, New York, pp. 229-254.
- Hejgaard, J., Jacobsen, S., Bjorn, S. E., and Kragh, K. M. (1992). *FEBS Lett.* **307**, 389-392.
- Hildmann, T., Ebnet, M., Pena-Cortés, H., Sanchez-Serrano, J., Willmitzer, L., and Prat, S. (1992). *Plant Cell* **4**, 1157-1170.
- Jacobsen, S., Mikkelsen, J. D., and Hejgaard, J. (1990). *Physiol. Plant.* **79**, 554-562.
- Laemmli, U. K. (1970). *Nature* **227**, 680-685.
- Laflamme, D., and Roxby, R. (1989). *Plant Mol. Biol.* **13**, 249-250.
- Lawton, M. A., Dixon, R. A., Hahlbrock, K., and Lamb, C. (1983). *Eur. J. Biochem.* **129**, 593-601.
- Lerner, D. R., and Raikhel, N. V. (1989). *Plant Physiol.* **91**, 124-129.
- Linthorst, H. J. M. (1991). *Crit. Rev. Plant Sci.* **10**, 113-150.
- Linthorst, H. J. M., Danhash, N., Brederode, F. T., Van Kan, J. A. L., De Witt, P. J. G. M., and Bol, J. F. (1991). *Mol. Plant-Microbe Interact.* **4**, 585-592.
- Ludvigsen, S., and Poulsen, F. M. (1992a). *Biochemistry* **31**, 8771-8782.
- Ludvigsen, S., and Poulsen, F. M. (1992b). *Biochemistry* **31**, 8783-8789.
- Nakamura, K., and Matsuoka, K. (1993). *Plant Physiol.* **101**, 1-5.
- Neuhaus, J.-M., Sticher, L., Meins, F., and Boller, T. (1991). *Proc. Natl. Acad. Sci. USA* **88**, 10362-10366.
- Parson, T. J., Bradshaw, H. D., and Gordon, M. P. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 7895-7899.
- Payne, G., Ahl, P., Moyer, M., Harper, A., Beck, J., Meins, F., and Ryals, J. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 98-102.
- Raikhel, N. V., and Wilkins, T. A. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 6745-6749.
- Ryan, C. A. (1990). *Annu. Rev. Phytopathol.* **28**, 425-449.
- Shinshi, H., Mohnen, D., and Meins, F. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 89-93.
- Shinshi, H., Neuhaus, J. M., Ryals, J., and Meins, F. (1990). *Plant Mol. Biol.* **14**, 357-368.
- Stanford, A., Bevan, M., and Northcote, D. (1989). *Mol. Gen. Genet.* **215**, 200-208.
- Svensson, B., Svendsen, I., Hojrup, P., Roepstorff, P., Ludvigsen, S., and Poulsen, F. M. (1992). *Biochemistry* **31**, 8767-8770.
- Van Loon, L. C. (1985). *Plant Mol. Biol.* **4**, 111-116.
- Van Loon, L. C. (1989). In *Plant-Microbe Interactions: Molecular and Genetic Perspectives* (Kosuge, T., and Nester, E. W., eds.), McGraw-Hill, New York, Vol. 3, pp. 198-237.
- Van Loon, L. C., and Van Kammen, A. (1970). *Virology* **40**, 199-211.
- Walujono, K., Scholma, R. A., Beintema, J. J., Mariono, A., and Hahn, A. M. (1975). In *Proceedings of the International Rubber Conference*, Kuala Lumpur, Malaysia, Vol. 2, pp. 518-531.
- Wright, C. S., and Raikhel, N. V. (1989). *J. Mol. Evol.* **28**, 327-336.