

The Amino Acid Sequence of a Protein from Wheat Kernel Closely Related to Proteins Involved in the Mechanisms of Plant Defence

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Received February 1, 1993

The amino acid sequence of wheatwin1, a monomeric protein of 125 residues isolated from wheat kernel (variety S. Pastore), is reported. Wheatwin1 is highly homologous (95%) to barwin, a protein from barley seed, which was shown to be related to the C-terminal domain of two proteins encoded by the wound-induced genes *win1* and *win2* in potato and to a protein encoded by the same domain of the hevein gene (*hev1*) in rubber tree. Similarly to barwin, wheatwin1 contains six cysteine residues all linked in disulfide bridges and the N-terminal residue is pyroglutamate. Moreover, structural studies performed on wheatwin1 and *win1* protein by predictive methods demonstrated that these proteins and barwin are closely related in the secondary structure also. The high level of homology found with the product of *win1*, *win2*, and *hev1* genes strongly suggests that barwin and wheatwin1 play a common role in the mechanism of plant defence.

KEY WORDS: Amino acid sequence; wheat; wounding; plant defence.

1. INTRODUCTION

The biological responses in higher plants after wounding have been widely studied. In 1978, Kahl reported extensive changes in the protein synthesis pattern; many of these changes, also induced by fungal elicitors and/or chemicals (Darvill and Albersheim, 1984), may be involved in defence mechanisms, resulting in an increased synthesis of different proteins such as chitinases and β -1,3-glucanases degrading fungal cell wall (Legrand *et al.*, 1987; Kauffmann *et al.*, 1987; Kombrink *et al.*, 1988; Bolter, 1985), enzymes of phenylpropanoid metabolism leading to lignification of the cell wall (Lawton *et al.*,

1983; Cramer *et al.*, 1985a; Friend, 1985), several pathogenesis-related (PR) proteins (Van Loon, 1985), glycoproteins (Chrispeels *et al.*, 1974; Esquerre-Tugaye and Mazau, 1974), and proteinase inhibitors (Ryan, 1973).

Several classes of chitin-binding proteins have been reported to contain a sugar-binding region often referred as hevein-like domain (Chrispeels and Raikhel, 1991). This domain, consisting of 43 amino acids, represents the sequence of hevein, a small protein of 5 K_d isolated from luteoid body-enriched fraction of rubber tree latex (*Hevea brasiliensis*) (Archer *et al.*, 1969; Walujono *et al.*, 1975), which has been shown to bind chitin and to inhibit the growth of several chitin-containing fungi (Van Parijs *et al.*, 1991). Chitinases from bean (Broglie *et al.*, 1986; Lucas *et al.*, 1985), tobacco (Shinshi *et al.*, 1987), and poplar (Parson *et al.*, 1989) show one hevein-like domain located at the N-terminus, whereas lectins from some monocotyledonous or dicotyledonous contain four and two isostructural domain, respectively (Wright *et al.*, 1984; Raikhel and

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Wilkins, 1987; Lerner and Raikhel, 1989; Wilkins and Raikhel, 1989; Chapot *et al.*, 1986). Moreover, hevein is highly homologous to the N-terminal region of two proteins encoded by the wound-induced genes *win1* and *win2* in potato (Stanford *et al.*, 1989). The C-terminal domain of *win1* and *win2* proteins differs from the chitinase carboxyl region, and very recently the sequence of barwin, a protein isolated from barley seed which is homologous to this region and lacking in the hevein-like domain, has been reported (Svensson *et al.*, 1992).

Furthermore, the isolation and sequencing of a cDNA clone (*hev1*) encoding for a putative signal sequence followed by a polypeptide of 187 amino acids has been reported (Broekaert *et al.*, 1990). This polypeptide presents two distinct regions: the N-terminal domain of 43 amino acids corresponding to mature hevein and the C-terminal domain of 144 amino acids showing a high degree of identity both with barwin and the corresponding region of *win1* and *win2* proteins.

In 1989, we reported the isolation and preliminary characterization of a new protein from wheat kernel coded 0.14 on the basis of its electrophoretic mobility relative to bromophenol blue (Carrano *et al.*, 1989). In this paper, we report the whole sequence of this protein showing a high degree of identity both with barwin and the C-terminal domain of the proteins encoded by *win1*, *win2*, and *hev1* genes. On the basis of this correlation, we decided to rename protein 0.14 as wheatwin1. It is reasonable to claim that wheatwin1 and barwin play a common biological role that should be related to the mechanism of plant defence.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

Triticum aestivum, pure variety San Pastore, was kindly supplied from Istituto Nazionale per la Cerealicoltura (Rome, Italy). HPLC procedures were carried out on a Beckmann GOLD apparatus equipped with a variable-wavelength monitor model 166. Sequencing grade trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and pyroglutamic aminopeptidase (EC 3.4.19.3) were from Boehringer Mannheim Italia SpA. The μ -Bondapak C₁₈ column (0.39 $\times$ 30 cm) was from Waters-Millipore (Milford, MA, USA). All other reagents were of analytical grade.

2.2. Protein Purification

Wheatwin1 was purified as previously reported (Carrano *et al.*, 1989). 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. Reduction and Pyridylethylation

Wheatwin1 (60 nmol) was treated with 3 μ mol of dithiothreitol in 0.2 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 (90 μ 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 reversed-phase HPLC (RP-HPLC) on the μ -Bondapak C₁₈ column. Eluent A was aqueous 0.1% trifluoroacetic acid and eluent B was 0.07% trifluoroacetic acid in acetonitrile. The elution was carried out at a flow rate of 1 ml/min by the following program: 5 min at 10% B followed by a linear gradient from 10–70% B in 55 min. The s-pyridylethylated protein (PyEt-wheatwin1) was manually collected and freeze-dried.

2.4. Digestion with Trypsin

PyEt-wheatwin1 (10 nmol) was digested with trypsin (enzyme/substrate 1:50 w/w) for 5 hr at 37°C in 0.2 ml of 0.1% ammonium bicarbonate buffer, pH 8.0. The digest was then freeze-dried and dissolved in 0.2 ml of aqueous 0.1% trifluoroacetic acid. The fragment mixture was resolved by RP-HPLC on the μ -Bondapak C₁₈ column. Eluents A and B were those described in the previous section. The elution was performed at a flow rate of 1 ml/min by using the following program: 5 min 10% B followed by a linear gradient from 10–35% B in 90 min. Peaks were manually collected and freeze-dried.

2.5. Digestion with Chymotrypsin

PyEt-wheatwin1 (10 nmol) was digested with chymotrypsin (enzyme/substrate 1:60 w/w) for 40 min at 30°C in 0.2 ml of 1% ammonium bicarbonate buffer, pH 7.8. The digestion was stopped by freezing. The fragment mixture was resolved by RP-HPLC on the μ -Bondapak C₁₈ column. Eluents A and B were those described in previous sections.

The elution was performed at a flow rate of 1 ml/min by using the following program: 2 min 10% B followed by a linear gradient from 10–30% B in 90 min. Peaks were manually collected and freeze-dried.

2.6. Sequence Analyses

The purified tryptic and chymotryptic peptides of wheatwin1 were dissolved in aqueous 0.1% trifluoroacetic acid (30–60 μ l); aliquots (200–500 pmol) were submitted to automated sequence analysis using a pulsed liquid-phase automatic sequencer (Applied Biosystems model 477A) equipped on-line with phenylthiohydantoin amino acid derivatives analyzer (Applied Biosystems model 120A). Samples were loaded onto a trifluoroacetic-acid-treated glass-fiber filter, coated with polybrene and washed according to manufacturer instructions. The relative reagents were from Applied Biosystems.

2.7. Deblocking of N-Terminal Residue and Identification of N-Terminal Sequence of Wheatwin1

The N-terminal blocked tryptic fragment of wheatwin1 (200 pmol) was digested by pyroglutamate aminopeptidase (enzyme/substrate 1:10 w/w) for 1 hr at 37°C in 0.1 M potassium phosphate, pH 8.0, 10 mM EDTA, 5 mM dithiothreitol, 5% (v/v) glycerol; deblocking was also achieved dissolving and incubating the tryptic peptide (200 pmol) in 50 μ l of 1 M HCl in methanol for 1 hr at 37°C. Sequence analyses of the N-terminal tryptic peptide deblocked by both methods were performed. Furthermore, a portion (100 pmol) of the N-terminal blocked tryptic fragment was analyzed by fast atom bombardment mass spectrometry (FAB-MS), using a Kratos MS 50 double focusing mass spectrometer fitted with a MScan Xenon gun operating at 8 KV (2 mA).

2.8. Predictive Methods

Secondary structure predictions were obtained by the package SECSTR developed by Hamodrakas (Hamodrakas, 1988). The package executes a joint prediction scheme, making use of several secondary structure prediction methods (Nagano, 1977a, b; Garnier *et al.*, 1978; Burgess *et al.*, 1974; Chou and Fasman, 1974a, b; Lim, 1974a, b; Dufton and Hider, 1977). The results of the individual prediction methods are combined to produce joint prediction histograms, reported separately for each type of secondary structure.

3. RESULTS AND DISCUSSION

3.1. Sequence Analyses

Wheatwin1 was purified to homogeneity as previously described (Carrano *et al.*, 1989). In order to better characterize the protein and get some insight into its biological role, we determined its complete amino acid sequence and then compared it with sequences of other proteins stored in the available data banks. The PyEt-protein was separately digested with trypsin and chymotrypsin. HPLC of the two peptide mixtures resulted in the purification of all peptides through a single chromatographic run (Fig. 1). All tryptic (Fig. 1a) and chymotryptic (Fig. 1b) peptides were submitted to automatic sequence analysis and their sequences were completely elucidated. As determined by the instrument software, the average and combined repetitive amino acids yields were not lower than 90% for each sequenced peptide. The theoretical initial yields were not lower than 50%. Only fragment T1 (Fig. 1a) failed to manifest any amino acid derivative, thus suggesting the presence of a blocked N-terminal residue. Peptide T1 was then submitted to acid treatment and analyzed by automatic sequencing. Several amino acid residues were identified at each sequencing step, thus indicating the formation of several peptides shorter than T1 deriving from partial hydrolysis at susceptible bonds; the longest sequence reconstructed for peptide T1 was EQATNVR. Appearing glutamate as the first residue of acid-treated peptide T1, it was supposed the presence at the N-terminus of the whole protein and of the peptide T1 of a pyroglutamate residue. To confirm this hypothesis, the blocked peptide T1 was treated with pyroglutamate aminopeptidase and submitted to sequence analysis; due to the loss of the pyroglutamate residue, the sequence QATNVR of the truncated peptide was unambiguously identified. Finally, the untreated peptide T1 was submitted to FAB-MS analysis; a single signal at MH^+ 799, corresponding to the mass of the peptide Glp-QATNVR, confirmed that the N-terminal residue of peptide T1 and, consequently, of the intact protein is pyroglutamate.

The complete sequence of wheatwin1, obtained by overlapping useful tryptic and chymotryptic peptides, is shown in Fig. 2. Both residues Asn and Asp were found at position 88 (tryptic peptides T6 and T6* in Fig. 1a). The calculated molecular weight is 13,563 D.

3.2. Homology Studies

In order to get information about the biological function of wheatwin1, we performed a search for sequence homology with proteins stored in the PIR databank. The degree of identity of wheatwin1 with the C-terminal domain of two proteins encoded by the wound-induced *win1* and *win2* genes in potato (Stanford *et al.*, 1989) was 70% and 69%, respectively. Furthermore, 77% identity was found when comparing the sequences of wheatwin1 and of the same region of the protein encoded by the hevein gene (*hev1*) in rubber tree (Broackaert *et al.*, 1990). In addition, wheatwin1 shows 95% identity with barwin, a basic protein from barley seed blocked at its N-terminus by a pyroglutamate residue (Svensson *et al.*, 1992) like wheatwin1.

The comparison of the sequences of wheatwin1,

barwin, the proteins encoded by *hev1*, *win1*, and *win2* genes, and the N-terminal domain of chitinase is shown in Fig. 3. The most evident feature of this comparison is the presence of two distinct domains each corresponding to different mature proteins. While the function of the N-terminal domain is to recognize chitin, so that a role in the mechanisms of plant defence can be pointed out, the significance and function of the C-terminal domain, completely unrelated to the carboxyl region of chitinase (Lucas *et al.*, 1985), remain still unclear. It has been supposed that the chitin binding domain has become incorporated into different defense genes encoding a variety of proteins in widely divergent plants, and that the combination of their fusion and duplication may have given plants an evolutionary advantage by creating new

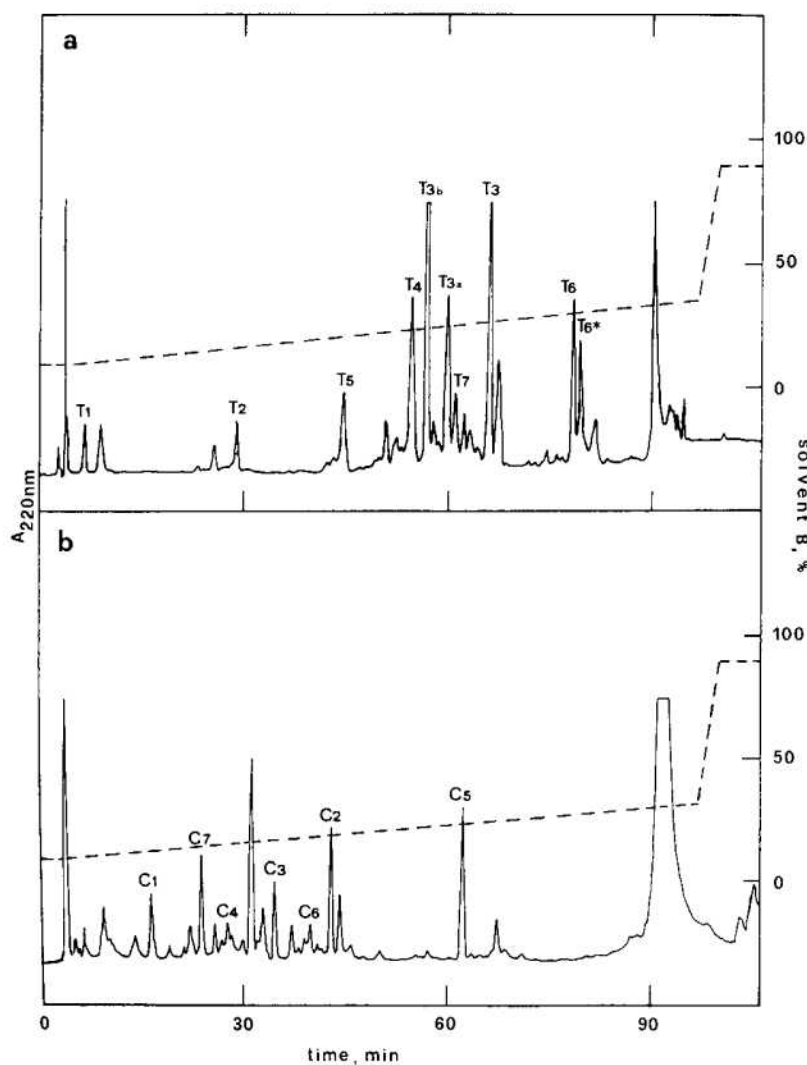


Fig. 1. RP-HPLC purification of (a) tryptic and (b) chymotryptic digests of S-pyridylethylated wheatwin1. All peptides were submitted to sequence analysis; only those utilized for the overlaps have been numbered.

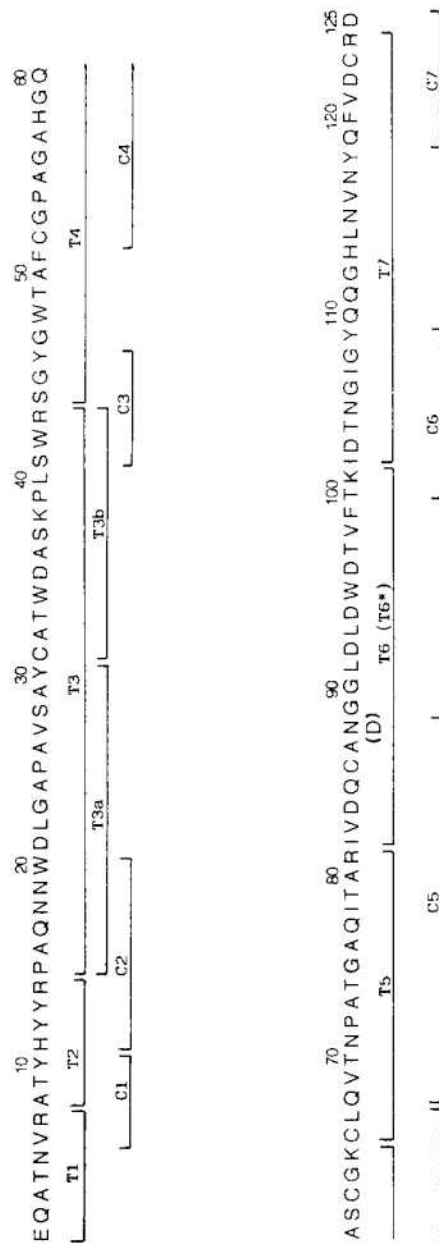


Fig. 2. Amino acid sequence of wheatwin1. Tryptic (T) and chymotryptic (C) fragments utilized for establishing the sequence are indicated.

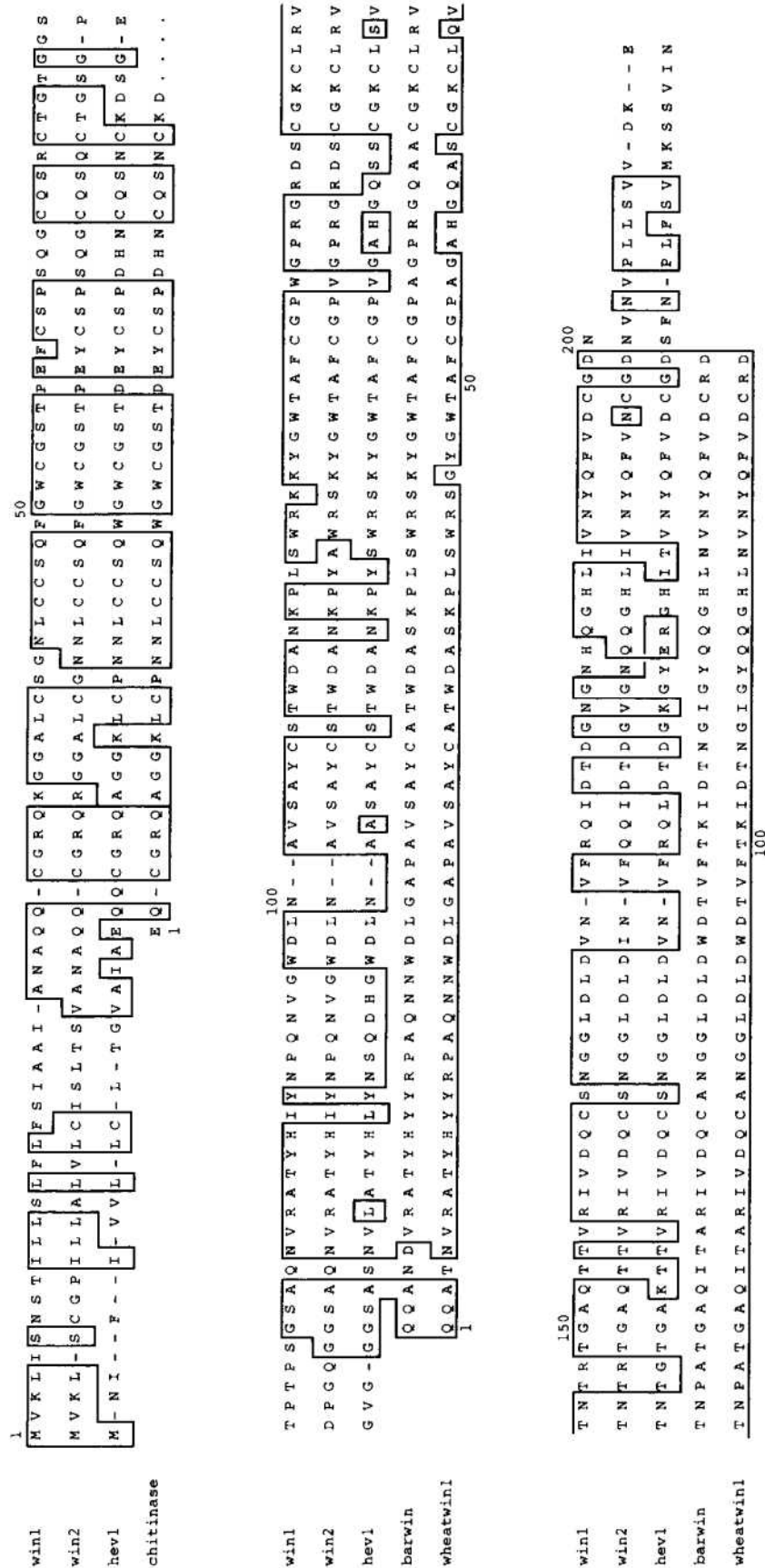


Fig. 3. Sequence comparison of wheatwin1, barwin, the proteins encoded by *win1*, *win2*, and *hev1* genes and the N-terminal domain of chitinase. Conserved residues are boxed.

proteins with diverse specificities (Chrispeels and Raikhel, 1991).

It has been demonstrated that the hevein gene (*hev1*) product undergoes co- and posttranslational processing *in vivo*, leading to two distinct mature proteins: a 5 *K_d* protein encoded by the N-terminal domain (mature hevein) and a 14 *K_d* protein encoded by the C-terminal domain, strictly related to wheatwin1 and barwin, whose function is unknown (Lee *et al.*, 1991).

Moreover, the isolation was reported of two N-terminal blocked proteins (coded component 3 and 4) from wheat varieties Chinese spring (*Triticum aestivum*) and Senatore Capelli (*Triticum turgidum*), which appeared to be very similar. In fact, the sequences of two internal fragments of 10 and 15 residues from each protein were identical (Gomez *et al.*, 1991). These two sequences show a degree of identity of 90% and 93% with the sequence of regions 46–55 and 102–116 of wheatwin1, respectively.

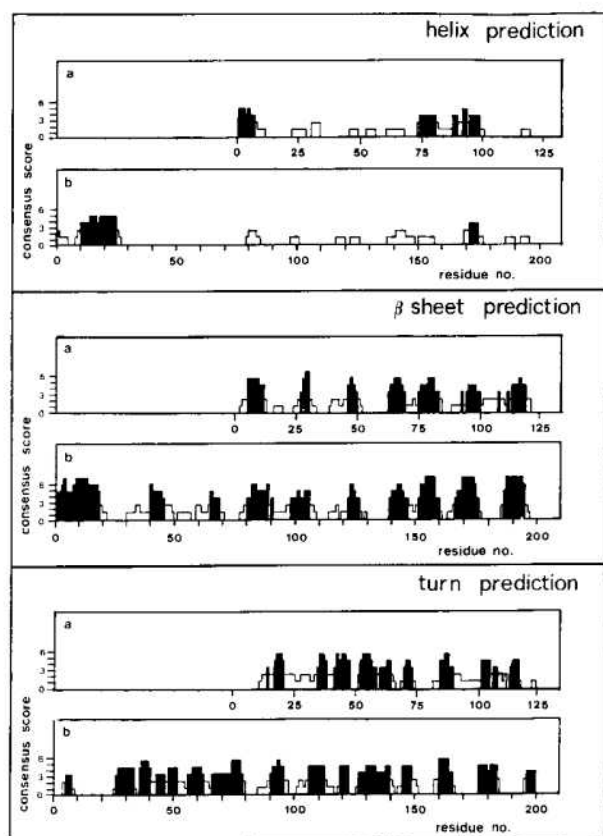


Fig. 4. Secondary structure prediction of wheatwin1 (a) and of the protein encoded by *win1* gene (b). The prediction was performed according to Hamodrakas (1988). Black histograms represent residues with at least three consensus. Proteins are aligned by C-terminal residues.

These findings strongly suggest that wheatwin1, barwin, components 3 and 4, and the 14 *K_d* protein from rubber tree belong to the same class of molecules, likely involved in plant defence, although their identity remains a matter for speculation.

3.3. Secondary Structure Prediction

The amino acid sequences of wheatwin1 and the protein encoded by *win1* gene were submitted to a comparative analysis of their secondary structures. To obtain reliable results, we used a predictive method which integrates six different methods of analysis (Hamodrakas, 1988). This joint method ascribes a consensus score for secondary structure to each residue; scores are shown by a histogram. A threshold (at least three consensus) selects residues with the highest consensus. The high consensus allows for a better prediction. Figure 4 reports the prediction pattern for helices, β -sheets, and turns of wheatwin1 as well as of *win1* protein. The two sequences have been paired by the C-terminal residues. The analysis shows that the helix structure is very poor; the few segments shown as helices are very short and difficult to be correctly predicted because of the equally high potential for helix and sheet (Chou and Fasman, 1978). Both proteins are dominated by the presence of beta structure with numerous turns and/or large unordered segments. The high local correspondence for identical stretches of secondary structure within the two homologous regions indicates that the sequence homology reflects a similar secondary structure for the two proteins. The N-terminal region of *win1*, missing in wheatwin1, is poorly organized. Only the first 17–19 residues seem to possess an organized structural core. The remaining part of this region, which has already been correlated to pathogenesis-related proteins (Fig. 3), shows long unordered segments with numerous turns alternating with a few short beta segments.

Three recent articles (Svensson *et al.*, 1992; Ludvigsen and Poulsen, 1992a, b) devoted to the structural analysis of barwin from barley seed have shown that numerous features of its secondary structure, found in solution by NMR, are common to those predicted for wheatwin1 in this study. This is not surprising, given the large sequence identity between the two proteins. In fact, the topology of the major secondary structure components of barwin, i.e., beta sheets and turns is well retained by wheatwin1, while the very short helices are not very predictable. However, numerous residues such

as glycines, involved in maintaining the backbone folding by turn formation, are conserved in the same key positions throughout the two sequences. This suggests that the two proteins are likely to have a similar global fold.

Unfortunately, the function of wheatwin1 and its homologous proteins still remains unclear, although the high degree of sequence identity and structural organization with products of wound-induced genes may lead to the hypothesis that the synthesis of these protein is related to mechanisms of plant defence. Wheatwin1, barwin, components 3 and 4 are mature proteins blocked at the N-terminus and lacking in the hevein-like domain. They should represent the products of a posttranslational processing of the proteins encoded by genes closely related to *win1*, *win2*, and *hev1*. It should be of great interest to verify this hypothesis, determining the nucleotide sequence and the expression of the gene encoding wheatwin1 in wheat. In this way, the existence of a 5 K_d chitin-binding protein encoded by the hevein-like domain of this gene could be ascertained.

ACKNOWLEDGMENTS

The authors greatly acknowledge Prof. P. Pucci and the Servizio di Spettrometria di Massa C.N.R., Naples, Italy, for FAB-MS facilities and Prof. G. Colonna for the helpful discussion. Research was supported by the National Research Council of Italy, Special Project RAISA, Sub-project N.2. Paper N. 820.

REFERENCES

- Archer, B. L., Audley, B. G., McSweeney, G. P., and Hong, T. C. (1969). *J. Rubber Res. Inst. Malaya* **21**, 560-569.
- Boller, T. (1985). In *Cellular and Molecular Biology of Plant Stress* (Key, J. L. and Krsuge, T., eds), Liss, New York, pp. 247-262.
- Broekaert, W., Lee, H., Kush, A., Chua, N. H., and Raikhel, N. (1990). *Proc. Natl. Acad. Sci. USA* **87**, 7633-7637.
- Brogie, K. E., Gaynor, J. J., and Brogie, R. M. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 6820-6824.
- Burgess, A. W., Ponnuswamy P. K., and Scheraga H. A. (1974). *Isr. J. Chem.* **12**, 239-286.
- Caporale, C., Carrano, L., Nitti, G., Poerio, E., Pucci, P., and Buonocore, V. (1991). *Protein Seq. Data Anal.* **4**, 3-8.
- Carrano, L., Nitti, G., Buonocore, V., Caporale, C., and Poerio, E. (1989). *Plant Science* **65**, 25-31.
- Chapot, M. P., Peumans, W. J., and Strosberg, A. D. (1986). *FEBS Lett.* **195**, 231-234.
- Chou, P., and Fasman, G. D. (1974a). *Biochemistry* **13**, 211-221.
- Chou, P., and Fasman, G. D. (1974b). *Biochemistry* **13**, 222-245.
- Chou, P. V., and Fasman, G. (1978). *Adv. Enzymol. Relat. Areas Mol. Biol.* **47**, 45-148.
- Chrispeels, M. J., Sadava, D., and Chou, Y. P. (1974). *J. Exp. Bot.* **25**, 1157-1166.
- 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.
- Darvill, A. G., and Albersheim, P. (1984). *Annu. Rev. Plant Physiol.* **35**, 243-298.
- Dufton, M. J., and Hider, R. C. (1977). *J. Mol. Biol.* **115**, 117-193.
- Esquerre-Tugaye, M. T., and Mazau, D. (1974). *J. Exp. Bot.* **25**, 509-513.
- Friend, J. (1985). *Ann. Proc. Phytochem. Soc.* **25**, 367-392.
- Garnier, J., Osguthorpe, D. J., and Robson, B. (1978). *J. Mol. Biol.* **120**, 97-120.
- Gomez, L., Sanchez-Monge, R., Lopez-Otin, C., and Salcedo, G. (1991). *Plant Physiol.* **96**, 768-774.
- Hamodrakas, S. (1988). *Comp. Applic. Biosci.* **4**, 473-477.
- Hedrick, S. A., Bell, J. N., Boller, T., and Lamb, C. J. (1988). *Plant Physiol.* **86**, 182-186.
- Kahl, G. (1978). In *Biochemistry of Wounded Plant Tissue*. De Gruyter, Berlin.
- Kauffmann, S., Legrand, M., Geoffroy, P., and Fritting, B. (1987). *EMBO J.* **6**, 3209-3212.
- Kombrink, E., Schroder, M., and Halbrock, K. (1988). *Proc. Natl. Acad. Sci. USA* **85**, 782-786.
- Lawton, M. A., Dixon, R. A., Hahlbrock, K., and Lamb, C. (1983). *Eur. J. Biochem.* **129**, 593-601.
- Lee, H., Broekaert, W. F., and Raikhel, N. V. (1991). *J. Biol. Chem.* **266**, 15,944-15,948.
- Legrand, M., Kauffmann, S., Geoffroy, P. and Fritting, B. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 6750-6754.
- Lerner, D. R., and Raikhel, N. V. (1989). *Plant Physiol.* **91**, 124-129.
- Lim, V. I. (1974a). *J. Mol. Biol.* **88**, 857-872.
- Lim, V. I. (1974b). *J. Mol. Biol.* **88**, 873-894.
- Lucas, J., Henschen, A., Lottspeich, F., Voegeli, U., and Boller, T. (1985). *FEBS Lett.* **193**, 208-210.
- Ludvigsen, S., and Poulsen, F. M. (1992a). *Biochemistry* **31**, 8771-8782.
- Ludvigsen, S., and Poulsen, F. M. (1992b). *Biochemistry* **31**, 8783-8789.
- Nagano, K. J. (1977a). *J. Mol. Biol.* **109**, 235-250.
- Nagano, K. J. (1977b). *J. Mol. Biol.* **109**, 251-274.
- Parson, T. J., Bradshaw, H. D., and Gordon, M. P. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 7895-7899.
- Raikhel, N. V., and Wilkins, T. A. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 6745-6749.
- Ryan, C. A. (1973). *Annu. Rev. Plant Physiol.* **24**, 173-196.
- Shinshi, H., Mohnen, D., and Meins, F. (1987). *Proc. Natl. Acad. Sci. USA* **84**, 89-93.
- 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.
- VanParijs, J., Broekaert, W. F., Goldstein, I. J., and Peumans, W. J. (1991). *Planta (Berl.)* **183**, 258-264.
- Walujono, K., Scholma, R. A., Beintema, J. J., Mariono A., and Hahn, A. M. (1975). In *Proc. Internatl. Rubber Conf.* 518-531.
- Wilkins, T. A., and Raikhel, N. V. (1989). *Plant Cell* **1**, 541-549.
- Wright, C. S., Garilanes, F., and Peterson, D. L. (1984). *Biochemistry* **23**, 280-287.