View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS 28782 FEBS Letters 575 (2004) 71–76

Wheat pathogenesis-related proteins of class 4 have ribonuclease activity

Carlo Caporale, Iris Di Berardino, Luca Leonardi, Laura Bertini, Annunziata Cascone, Vincenzo Buonocore, Carla Caruso*

Dipartimento di Agrobiologia ed Agrochimica, Università della Tuscia, via S. Camillo de Lellis, 01100 Viterbo, Italy

Received 7 July 2004; revised 23 July 2004; accepted 23 July 2004

Available online 28 August 2004

Edited by Richard Cogdell

Abstract We have demonstrated that wheatwin1, a wheat pathogenesis-related protein of class 4 (PR4), has ribonuclease activity. Both native and recombinant proteins hydrolyse RNA from wheat coleoptils and have antifungal activity. Sepharose-bound wheatwin1 is able to interact with either wheat or *Fusarium culmorum* RNA. 3D modelling studies showed that, like ribonucleases A and T1, the action mechanism should involve two His residues, an Arg residue and an Asp residue.
© 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.

Keywords: PR protein; Antifungal activity; Ribonuclease activity; Homology modelling; *Triticum aestivum*; *Fusarium culmorum*

1. Introduction

Plants respond to pathogens by defence strategies leading to the synthesis of several protective compounds such as the pathogenesis-related (PR) proteins; these molecules were first described in tobacco plants infected with tobacco mosaic virus (TMV) and then in a variety of species submitted to various kinds of biotic and abiotic injury [1]. At present, a large number of PR-proteins have been characterised and grouped into 17 families based on their primary structure, serological relationships, and biological activities [2]. Most of them show antifungal activity against specific pathogens, while just a few of them possess enzymatic or inhibitory activity such as chitinases (PR3, PR8, PR11), glucanases (PR2), peroxidases (PR9), ribonuclease-like (PR10) and proteinase inhibitors (PR6). It is thought that the wide-ranging modes of action of these molecules make them an ideal tool in defence strategies against "all comers". Among the less extensively studied PR proteins are those belonging to the number 4 family. Up to now, although their effect in inhibiting the pathogen hyphal growth and spore germination has been reported [3], their action mechanism and interaction with pathogen molecular targets are still unknown. In the last years, we have isolated and sequenced four PR4 proteins from wheat kernels, named wheatwin1 to wheatwin4, that inhibit phytopathogenic fungi with a wide host range (e.g., *Botrytis cinerea*) and host specific pathogens (e.g., *Fusarium culmorum*, *F. graminearum*) [3–5]. We demonstrated that wheatwin1 and wheatwin2 and their genes are specifically induced in wheat seedlings infected with *Fusarium culmorum* and upon treatment with systemic acquired resistance activators [6,7]; the cDNAs have been cloned and the recombinant proteins expressed in *E. coli* [8,9]. The three-dimensional model of wheatwin1 has been already designed (PDB code 1C2Z) and experimentally validated [14], based on the knowledge of the tertiary structure in solution of barwin [10,11, PDB code 1BW3], a highly homologous protein from barley [12] showing a six-stranded double-psi ß barrel [13]. We also compared the 3D structures of the four wheatwin proteins by homology modelling and related their micro differences to the different antifungal activity [15]. In this paper, we report that both native and recombinant wheatwin1 have a ribonuclease activity that cannot be related to the action mechanism of the PR10 family proteins, but rather to the classical acid–base mechanism of ribonucleases A and T1 involving two His residues [16, and references therein].

2. Materials and methods

2.1. Recombinant protein production

pGEM®-4Z vector containing the full-length wPR4a gene coding for wheatwin1 (win1) [8] was engineered to introduce *Eco*RI sites at both 5′ and 3′ ends of the coding regions by PCR using the oligonucleotides 5′-CCGGAATTCATCAGCAGGCGACC-3′ and 5′-CCGGAATTC-CTAGTCGCGGCA-3′ as forward and reverse primer, respectively. The PCR-amplified DNA fragment was subcloned into the *Eco*RI site of pGEX-2T plasmid in frame. The recombinant vector, containing the coding sequence corresponding to the mature wheatwin1 linked to glutathione-S-transferase (GST), was named pGEX-win1. Sequence analyses performed on the recombinant vector confirmed the addition of *Eco*RI sites at both 5′ and 3′ ends of the mature protein. The expression construct was transformed into *E. coli* strain BL21 for production of recombinant protein.

E. coli strain BL21 transformed with pGEX-win1 was grown in 2× YT medium containing ampicillin (100 μg/ml) at 37 °C to an absorbance of 0.5–0.6 at 600 nm. The protein expression was induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Cells were harvested two hours after induction by centrifugation (8000 rpm, 4 °C, 10 min). Bacterial cell pellets containing recombinant fusion protein (GST-win1) were resuspended in 50 mM Tris–HCl, pH 8.0, containing 1 mM EDTA (TE buffer), 100 μg/ml lysozyme and a protease inhibitor cocktail. After incubation on ice for 15 min, the mixture was lysed by sonication and treated with DNase at 37 °C for 60 min, afterwards the inclusion bodies were collected by centrifugation at 12 000 rpm, 4 °C for 30 min. The pellet was resuspended in 100 mM Tris–HCl, pH 8.0, containing 5 mM EDTA, 6 M guanidine and 30 mM dithiothreitol and fully reduced under nitrogen atmosphere at 37 °C for 1 h. After centrifugation at 12 000 rpm at 4 °C for 10 min, the supernatant was diluted into the refolding buffer (Tris–HCl 100 mM, pH 8, containing 2 mM oxidised glutathione (GSSG), 3 mM reduced glutathione (GSH), 0.5 M L-arginine and 5 mM EDTA), to a final protein concentration of

* Corresponding author. Fax: +39-0761-357242.
E-mail address: caruso@unitus.it (C. Caruso).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.07.091

30 μg/ml, allowing the renaturation of recombinant GST-win1 for two days at 15 °C.

2.2. Purification of recombinant proteins

Refolded GST-wheatwin1 was purified on preparative scale by gel filtration chromatography on a Superdex 75 column at a flow rate of 1.0 ml/min using an FPLC apparatus and monitoring the absorbance at 280 nm. The fraction containing recombinant GST-win1 was analysed by SDS–PAGE and immunoblot analysis using either anti-wheatwin1 or anti-GST polyclonal antibodies. The protein concentration was determined according to Bradford [17] using bovine serum albumin as a standard.

Removal of the N-terminal GST tag was achieved by digesting the fusion protein with thrombin in 50 mM Tris–HCl buffer, pH 8.0, containing 100 mM NaCl for 18 h using one enzyme unit for 100 μg of protein. GST tag and recombinant wheatwin1 (r-win1) were purified on a preparative scale by gel filtration chromatography on a Superdex 75 column as described above and on analytical scale by RP-HPLC. In the latter case, a reverse phase μ-Bondapak C_{18} column was utilised using aqueous 0.07% trifluoroacetic acid as eluent A and 0.05% trifluoroacetic acid in acetonitrile as eluent B. Elution was accomplished at a flow rate of 1 ml/min according to the following program: isocratic elution for 5 min at 25% B, followed by linear gradient from 25% to 60% B in 60 min. The eluate was monitored measuring the absorbance at 220 nm.

2.3. Mass spectrometry analysis

Molecular mass of both GST-win1 and r-win1, as well as the absence of molecules containing mixed disulfides with glutathione, were confirmed by electrospray mass spectrometry (ES-MS) using a ZQ single quadrupole mass spectrometer (Micromass, Waters) at CEINGE Biotecnologie Avanzate (Naples, Italy).

2.4. In vitro antifungal assay

In vitro antifungal activity assays were performed using either the GST fusion protein or the thrombin-cleaved r-win1. Native wheatwin1, purified as already described [3], was used as control. Germinating spores of *F. culmorum* were grown in the presence of native and recombinant PR4 proteins (40 μg/ml) using sterile water as control. Hyphal growth inhibition was evaluated after 8 h at 21 °C and expressed as IC_{50}.

2.5. Affinity chromatography

CH-Sepharose 4B (Amersham Biosciences) (1 ml) was used to couple native wheatwin1 (1 mg) in 50 mM Tris–HCl, pH 7.2, allowing the production of an affinity matrix (Sepharose-win1) to be used to isolate putative ligands of PR4 proteins. Affinity chromatography was carried out using a crude proteic extract from coleoptils (50 mg) harvested seven days after germination of salycilic acid (SA) treated wheat seeds prepared as previously described [7]. A crude proteic extract was also prepared from *F. culmorum* mycelium grown in liquid potato dextrose broth for 7 days at 22 °C under stirring. The cultural broth was homogenised with an UltraTurrax mixer TP-18N (Ika-Werk Janke and Kunfel, Germany) and total proteins were extracted with 50 mM Tris–HCl, pH 7.2, containing 1% polyvinylpyrrolidone and 0.5 mM phenylmethylsulfonylfluoride. Sepharose-win1 (1 ml) was equilibrated with 50 mM Tris–HCl, pH 7.2, and either wheat (50 mg) or fungal extract (1 mg) was dissolved in the same buffer. Elution of the unbound material was accomplished using the same buffer at a flow rate of 0.4 ml/min, monitoring the absorbance at 280 nm. Elution of the bound material was accomplished with 100 mM Tris–HCl, pH 8.0, containing 500 mM NaCl. All fractions eluted from the affinity chromatography were further characterised by SDS–PAGE and agarose gel electrophoresis after digesting the samples either with bovine pancreas RNase (50 μg/ml) or DNase (80 μg/ml) at 37 °C for 1 h in 100 mM Tris–HCl, pH 8.0, containing 500 mM NaCl.

2.6. Ribonuclease activity

Total RNA was isolated from wheat coleoptils according to Prescott and Martin [18]. RNase activity of native wheatwin1, recombinant fusion protein (GST-win1) and recombinant protein purified after removal of the GST tag (r-win1) was assayed at room temperature. The reaction mixture (20 μl) contained 12 μg of wheat coleoptil RNA and 4 μg of each protein in10 mM Tris–HCl, pH 7.5, containing 10 mM imidazole and 5 mM NaCl. After 1 h incubation, the proteins were removed by phenol-chloroform (1:1) extraction and the results were observed on 1.2% agarose gel. Heat-inactivated native wheatwin1 was used as control.

2.7. Molecular modelling

3D structure models of wheatwin1 were based on the availability of the NMR three-dimensional co-ordinates of the homologous protein barwin [10–12] (PDB code 1BW3) and performed as previously described [14,15]. The alignment of wheatwin1 and barwin did not require deletion or insertion of gap. The program MODELLER [19] and Quanta (Accelrys, Inc.) were used to build protein models according to the comparative protein modelling methodology. Figures were drawn with Swiss PDB Viewer program [20].

3. Results and discussion

3.1. Isolation and characterisation of recombinant wheatwin1

We already described a procedure to produce recombinant wheatwin1 [9]. The new approach reported in this paper allowed a faster and higher-yield production (15 mg/100 ml of bacterial culture) of the GST-fusion protein (GST-win1). When analysed by ES-MS, purified GST-win1 showed a molecular mass of 40363.13 ± 0.38 Da, corresponding to the fusion protein. The GST tag was efficiently removed with thrombin and the recombinant protein (r-win1) and GST were separated by RP-HPLC (Fig. 1A) and analysed by SDS–PAGE (Fig. 1B). The final yield of purified r-win1 was about 2.0 mg/100 ml of culture. The mass spectrometry analysis of r-win1 showed a molecular weight of 14214.69 ± 0.40 Da, corresponding to the processed wheatwin1 containing the spacer peptide GSPGIH at its N-terminus.

3.2. Antifungal and ribonuclease activity

GST-win1 and r-win1 were characterised for their biological activities by in vitro antifungal assays. The hyphal growth of *Fusarium culmorum* was evaluated in their presence and absence using the native protein as control. Fig. 2 shows that both wheatwin1 (panel C) and r-win1 (panel D) display antimicrobial activity toward *F. culmorum* hyphal growth. The antifungal effect is exerted either on spore germination or germ tube elongation combined with morphological alterations like swelling and wrinkling; r-win1 was able to inhibit the fungal growth with the same efficiency with respect to the native protein showing IC50 values of about 40 μg/ml (data not shown). On the contrary, GST-win1 was found to be fully inactive either on spore germination or hyphal growth (panel B) as no alterations were evident like in the control (panel A).

Affinity chromatography on Sepharose-win1 was carried out using either wheat or *F. culmorum* crude extracts in order to isolate putative wheat or fungal ligands, respectively. In both cases, one species was bound to the matrix and subsequently eluted as a single peak with the elution buffer (data not shown). SDS–PAGE analysis performed on both ligands indicated that they were not proteins; the A260/A280 nm ratio indicated that the ligands could be nucleic acids (not shown). In order to verify whether the ligands were deoxyribonucleic or ribonucleic acid, DNase and RNase digestions were performed on both of them. Results presented in Fig. 3A clearly indicate that the fungal ligand is a small RNA as it is digested only by RNase; similar results were obtained using the wheat ligand (not shown). With the aim of ascertaining whether wheatwin1 was not only able to bind RNA but also to degrade it, its

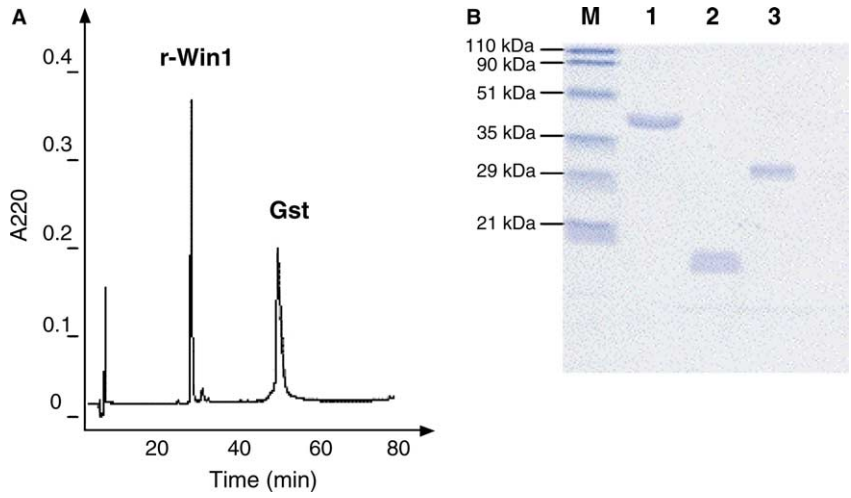

Fig. 1. (A) Separation of r-win1 and GST tag by RP-HPLC; (B) SDS–PAGE of recombinant proteins; M: prestained molecular markers (phosphorylase B – 110 kDa; bovine serum albumin – 90 kDa; ovalbumin – 51 kDa; carbonic anhydrase – 36 kDa; soybean trypsin inhibitor – 29 kDa; lysozyme – 21 kDa); 1: GST-win1; 2: r-win1; 3: GST tag.

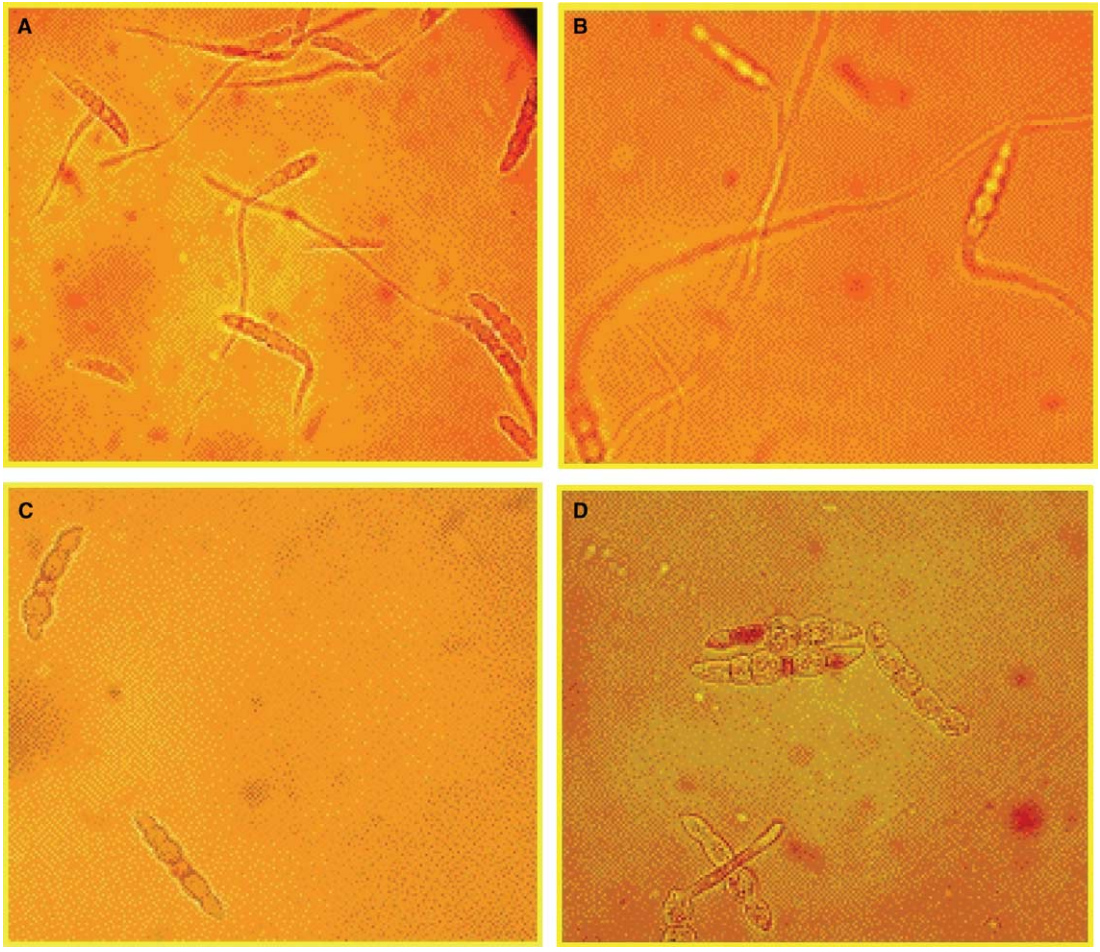

Fig. 2. Antifungal effect of wheatwin1 on *Fusarium culmorum* growth revealed by optical microscopy (40× objective). Fungal spores germinated in the presence of sterile water (A), GST-win1 (B), wheatwin1 (C) and r-win1 (D).

RNase activity was examined on wheat total RNA. As shown in Fig. 3B, both native and r-win1 are able to degrade RNA, whereas GST-win1 is inactive. In addition, heat-inactivated wheatwin1 is no longer able to exhibit ribonucleolytic activity suggesting that RNase activity requires a correctly folded protein. Preliminary results indicated that, as expected, native

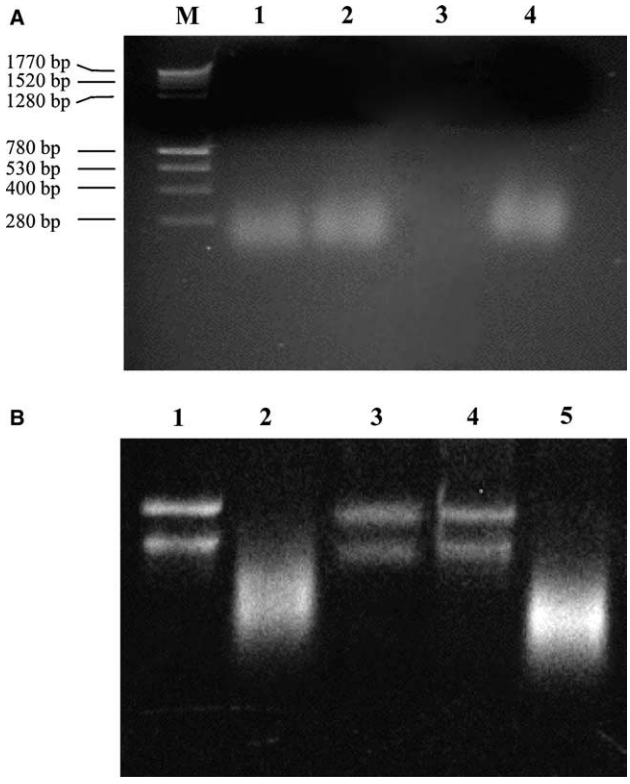

Fig. 3. Panel A: Agarose gel electrophoresis of the *F. culmorum* ligand eluted from the affinity column. M: Gibco BRL RNA Ladder markers; DNase-digested (1) and undigested (2) sample; RNase-digested (3) and undigested (4) sample. Panel B: Ribonuclease activity tested against wheat total RNA. Control (1), incubation with native wheatwin1 (2), heat-inactivated wheatwin1 (3), GST-win1 (4), r-win1 (5).

wheatwin2 was also able to degrade RNA (not shown). We are going to produce GST recombinant wheatwin2, wheatwin3 and wheatwin4 to fully characterise the RNase activity of all these proteins. In fact, further studies are needed to determine if the differences in the antifungal activity, that have been already related to micro diversities in the 3D structure [15], are also endowed with dissimilar enzymatic efficiency.

3.3. Structural studies

Plant PR proteins with ribonuclease activity have been classified in the PR10 family [1]. The amino acid sequences (about 150 residues) of most of them are known [21] together with some 3D structures allowing the identification of highly conserved residues and regions involved in the action mechanism. These proteins consist of a seven-stranded beta-sheet wrapped around a long C-terminal helix [22,23]. Fig. 4A shows the sequence of the lupine PR10 protein Lipr10.1A, whose 3D structure has been determined by X-ray diffraction (PDB code 1ICX) [23]. The conserved residues with side chains that can be involved in the catalytic reaction are Glu95, Glu146 and Tyr148. Furthermore, PR10 proteins conserve a P-loop structure with the sequence GXGGXGXXK (positions 45–53 in Fig. 4A), which should be the binding site for a phosphate group of RNA [22,23]; another surface loop, showing an unusual structural conservation and rigidity, has been supposed to play a role in ligand binding [23]. There is no similarity with the 3D structures of other ribonucleases, as can be also deduced from the absence of conserved histidine residues in the PR10 family. Wheatwin1, although showing a ribonuclease activity, is completely unrelated to the PR10 proteins. In fact, its amino acid sequence is 125 residues long, no Glu residue is present, and the P-loop is lacking (Fig. 4B). Furthermore, like the highly homologous protein barwin (95, 2% identity, Fig. 4C), its structure consists of a main beta-sheet of four anti-parallel strands, two short parallel beta strands constituting a little independent beta-sheet, and few short helices [10,11,14]. Consequently, the ribonuclease activity of wheatwin1 can be explained by the classical acid–base mechanism common to other ribonucleases involving two His residues [16, and references therein]. Ribonucleases A (124 residues, PDB

A PR10 - protein Lipr10.1A - PDB 1ICX
```
  1 GIFAFENEQS STVAPAKLYK ALTKDSDEIV PKVIEPIQSV EIVEGNGGPG
 51 TIKKIIAIHD GHTSFVLHKL DAIDEANLTY NYSIIGGEGL DESLEKISYE
101 SKILPGPDGG SIGKINVKFH TKGDVLSETV RDQAKFKGLG LFKAIEGYVL
151 AHPDY
```

B PR4 - Weatwin1 - PDB 1C2Z
```
  1 QQATNVRATY HYYRPAQNNW DLGAPAVSAY CATWDASKPL SWRSGYGWTA
 51 FCGPAGAHGQ ASCGKCLQVT NPATGAQITA RIVDQCANGG LDLDWDTVFT
101 KIDTNGIGYQ QGHLNVNYQF VDCRD
```

C PR4 – Barwin – PDB 1BW3
```
  1 QQANDVRATY HYYRPAQNNW DLGAPAVSAY CATWDASKPL SWRSGYGWTA
 51 FCGPAGPRGQ AACGKCLRVT NPATGAQITA RIVDQCANGG LDLDWDTVFT
101 KIDTNGIGYQ QGHLNVNYQF VDCRD
```

D Ribonuclease A - PDB 5RSA
```
  1 KETAAAKFER QHMDSSTSAA SSSNYCNQMM KSRNLTKDRC KPVNTFVHES
 51 LADVQAVCSQ KNVACKNGQT NCYQSYSTMS ITDCRETGSS KYPNCAYKTT
101 QANKHIIVAC EGNPYVPVHF DASV
```

E Ribonuclease T1 – PDB 1RLS
```
  1 ACDYTCGSNC YSSSDVSTAQ AAGYQLHEDG ETVGSNSYPH KYNNYEGFDF
 51 SVSSPYYEWP ILSSGDVYSG GSPGADRVVF NENNQLAGVI THTGASGNNF
101 VECT
```

Fig. 4. Amino acid sequences of (A) lipr10.1A; (B) wheatwin1; (C) barwin; (D) ribonuclease A; (E) ribonuclease T1. The residues involved in the action mechanism are underlined and in boldface. The five different residues in the sequences of wheatwin1 and barwin are in boldface.

code 5RSA, Fig. 4D) [24] and T1 (104 residues, PDB code 1RLS, Fig. 4E) [25] are the most similar in size to wheatwin1. The mechanism proposed for the catalytic action of bovine pancreatic RNase A postulates that four amino acid residues (His12, Lys41, His119, and Asp121) are involved in the acid–base catalytic process [16]. The relative positions of these residues in the 3D structure of Bos Taurus Ribonuclease A, determined by X-ray diffraction (PDB code 5RSA) [24], are shown in Fig. 5A. The minimal distance between the side chains of the His residues is 6.31 Å, while that of the same residues in the backbone is 8.87 Å. The distances His119–Asp121 and His12–Lys41 in the backbone are 6.02 and 12.72 Å, respectively. Although the primary structure of ribonuclease T1 (Fig. 4E) is completely different from that of RNase A,

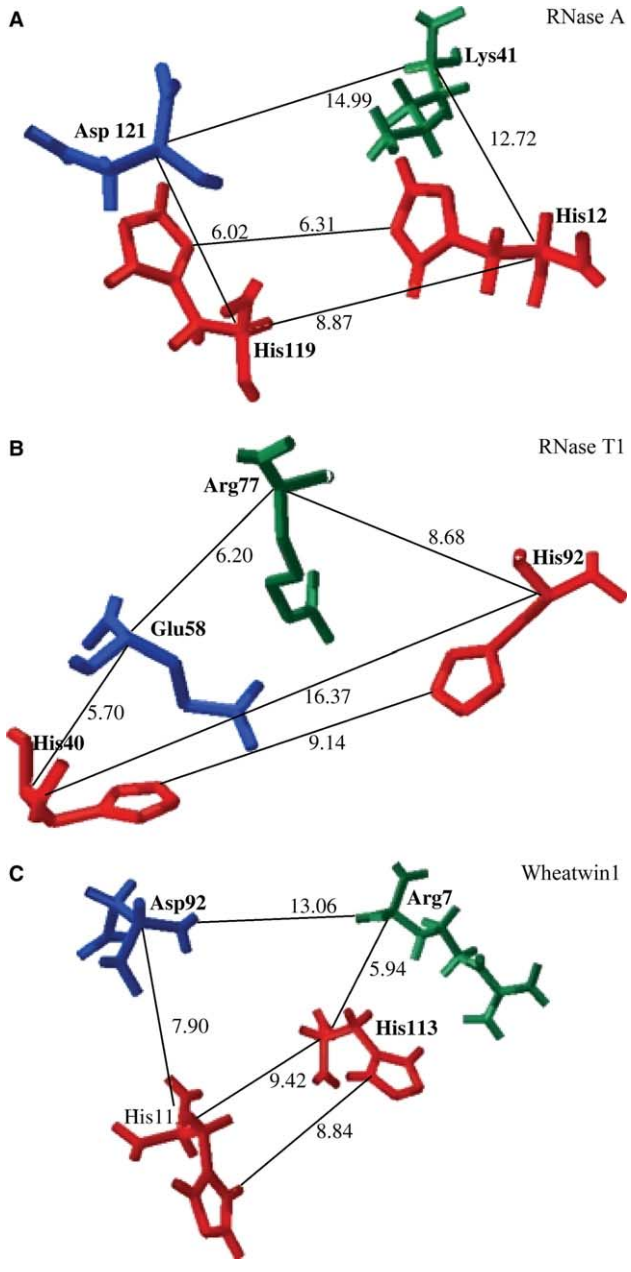

Fig. 5. Relative spatial positions of the residues involved in the action mechanism; (A) ribonuclease A; (B) ribonuclease T1; (C) wheatwin1. The distances are measured in angstrom (Å).

the residues involved in the action mechanism are conserved and efficiently positioned in the 3D structure determined by X-ray diffraction (PDB code 1RLS, Fig. 5B) [25]. Similarly to RNase A, the amino acid residues involved in the mechanism are two His residues (His40 and His92), an acid residue (Glu58) and a basic residue (Arg 77). In this case, the minimal distance between the His side chains is quite higher (9.14 Å) as well as that of these residues in the backbone (16.37 Å). The distances His40–Glu57 and His92–Arg77 in the backbone are 5.70 and 8.68 Å, respectively. A similar situation occurs in wheatwin1 and barwin. In fact, although their amino acid sequences are unrelated to that of both ribonucleases A and T1 (Fig. 4), the residues conserved in the 3D structure and involved in the acid–base mechanism should be His11, His113, Asp92 and Arg7. It should be noted that the 3D structure of barwin has been determined in solution by NMR [10,11]. Using this technique, the side chains are likely to be less well resolved than in X-ray diffraction, and errors in the initial side chain placement might alter the results of the refinement. As a matter of fact, 20 similar models of wheatwin1 can be constructed using the 20 NMR structures of barwin (PDB code 1BW3) as template; like in the reference protein, the minimal distance between His11 and His113 side chains in all of them is variable from 13.56 Å (not shown) to 8.84 Å (Fig. 5C). In this case, the distance of these residues in the backbone is 9.42 Å, while the distances His11–Asp92 and His113–Arg7 in the backbone are 7.90 and 5.94 Å, respectively. The 3D structure similarity of RNase A, RNase T1 and wheatwin1 in regions involving residues able to participate to an acid–base action mechanism should be the key to explain the ribonuclease activity of wheatwin1. It should be remarked that the recombinant protein r-win1 with the N-terminal spacer peptide is also active, while the fusion protein GST-win1 is not. Since the His residues are very accessible [14] and close to the N-terminus in the 3D structure (not shown), it is reasonable to suppose that the presence of the spacer peptide GSPGIH does not affect their interaction with the phosphate group, while the whole GST tag does. This observation is in agreement with our previous results showing that the N-terminal region 15–21 could play a role in the biological activity of PR4 proteins [15].

4. Conclusion

To our knowledge, this is the first report of a PR4 protein with RNase activity; both native and recombinant wheatwin1 show enzymatic and antifungal activities, degrading wheat coleoptil RNA and inhibiting hyphal growth and spore germination of *F. culmorum*. Up to now, the action mechanism of PR4 proteins has been completely unknown, since no enzymatic activity has been ascribed to these molecules. Besides, they have been classified as chitinases on the basis of the presence of a chitin binding domain found only in a few of them [1]. Actually, the chitin binding domain has been found also in several lectins and in other chitin-unrelated proteins [26]. In our opinion, PR4 proteins should not be considered as belonging to the chitinase superfamily but could constitute a distinct protein group. In fact, wheatwin1 has been shown to possess ribonuclease activity even though its action mechanism is different from that of PR10 proteins. Probably, this is not the only activity related to its biological function; anyway, it

should be possible that PR4 proteins operate on the invading pathogen by a translation-inhibitory process that could be ascribed to their ribonuclease activity.

Acknowledgements: This research was supported by the Ministero dell'Istruzione, Università e Ricerca Scientifica (MIUR), project MIUR-FIRB (RBNE01KZE7). The authors thank Dr. Maria Pia Aleandri for her skilful assistance in antifungal assays.

References

[1] Datta, S.K. and Muthukrishnan, S. (1999) Pathogenesis-related Proteins in Plants. CRC Press, Boca Raton, FL.
[2] Christensen, A.B., Cho, B.H., Naesby, M., Gregersen, P.L., Brandt, J., Madriz-Ordenana, K., Collinge, D.B. and Thordal-Christensen, H. (2002) Mol. Plant Pathol. 3, 135–144.
[3] Caruso, C., Caporale, C., Chilosi, G., Vacca, F., Bertini, L., Magro, P., Poerio, E. and Buonocore, V. (1996) J. Protein Chem. 15, 35–44.
[4] Caruso, C., Caporale, C., Poerio, E., Facchiano, A. and Buonocore, V. (1993) J. Protein Chem. 12, 379–386.
[5] Caruso, C., Nobile, M., Leonardi, L., Bertini, L., Buonocore, V. and Caporale, C. (2001) J. Protein Chem. 20, 327–335.
[6] Caruso, C., Chilosi, G., Caporale, C., Bertini, L., Magro, P. and Buonocore, V. (1999) Plant Sci. 140, 87–97.
[7] Bertini, L., Leonardi, L., Caporale, C., Tucci, M., Cascone, A., Di Berardino, I., Buonocore, V. and Caruso, C. (2003) Plant Sci. 164, 1067–1078.
[8] Caruso, C., Bertini, L., Tucci, M., Caporale, C., Leonardi, L., Saccardo, F., Bressan, R., Veronese, P. and Buonocore, V. (1999) DNA Seq. 10, 301–307.
[9] Caruso, C., Bertini, L., Tucci, M., Caporale, C., Nobile, M., Leonardi, L. and Buonocore, V. (2001) Protein Expr. Purif. 23, 380–388.
[10] Ludvigsen, S. and Poulsen, F.M. (1992) Biochemistry 31, 8771–8782.
[11] Ludvigsen, S. and Poulsen, F.M. (1992) Biochemistry 31, 8783–8789.
[12] Svensson, B., Svendsen, I., Hojrup, P., Roepstorff, P., Ludvigsen, S. and Poulsen, F.M. (1992) Biochemistry 31, 8767–8770.
[13] Castillo, R.M., Mizuguchi, K., Dhanaraj, V., Albert, A., Blundell, T.L. and Murzin, A.G. (1999) Structure Fold Des. 15, 227–236.
[14] Caporale, C., Caruso, C., Facchiano, A., Nobile, M., Leonardi, L., Bertini, L., Colonna, G. and Buonocore, V. (1999) Proteins 36, 192–204.
[15] Caporale, C., Facchiano, A., Bertini, L., Leonardi, L., Chilosi, G., Buonocore, V. and Caruso, C. (2003) J. Mol. Model. 9, 9–15.
[16] D'Alessio, G. and Riordan, F. (1997) Ribonucleases: Structures and Functions. Academic Press, New York.
[17] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[18] Prescott, A. and Martin, C. (1987) Plant Mol. Biol. Rep. 4, 219–224.
[19] Sali, A. and Blundell, T.L. (1993) J. Mol. Biol. 234, 779–815.
[20] Guex, N. and Peitsch, M.C. (1997) Electrophoresis 18, 2714–2723.
[21] Wu, F., Yan, M., Li, Y., Chang, S., Song, X., Zhou, Z. and Gong, W. (2003) Biochem. Biophys. Res. Commun. 312, 761–766.
[22] Gajhede, M., Osmark, P., Poulsen, F.M., Ipsen, H., Larsen, J.N., Joost van Neerven, R.J., Schou, C., Lowenstein, H. and Spangfort, M.D. (1996) Nat. Struct. Biol. 3, 1040–1045.
[23] Biesiadka, J., Bujacz, G., Sikorski, M.M. and Jaskolski, M. (2002) J. Mol. Biol. 319, 1223–1234.
[24] Wlodawer, A. and Sjolin, L. (1983) Biochemistry 22, 2720–2728.
[25] Gohda, K., Oka, K., Tomita, K. and Hakoshima, T. (1994) J. Biol. Chem. 269, 17531–17536.
[26] Wilkins, T.A. and Raikhel, N.V. (1989) Plant Cell 1, 541–549.