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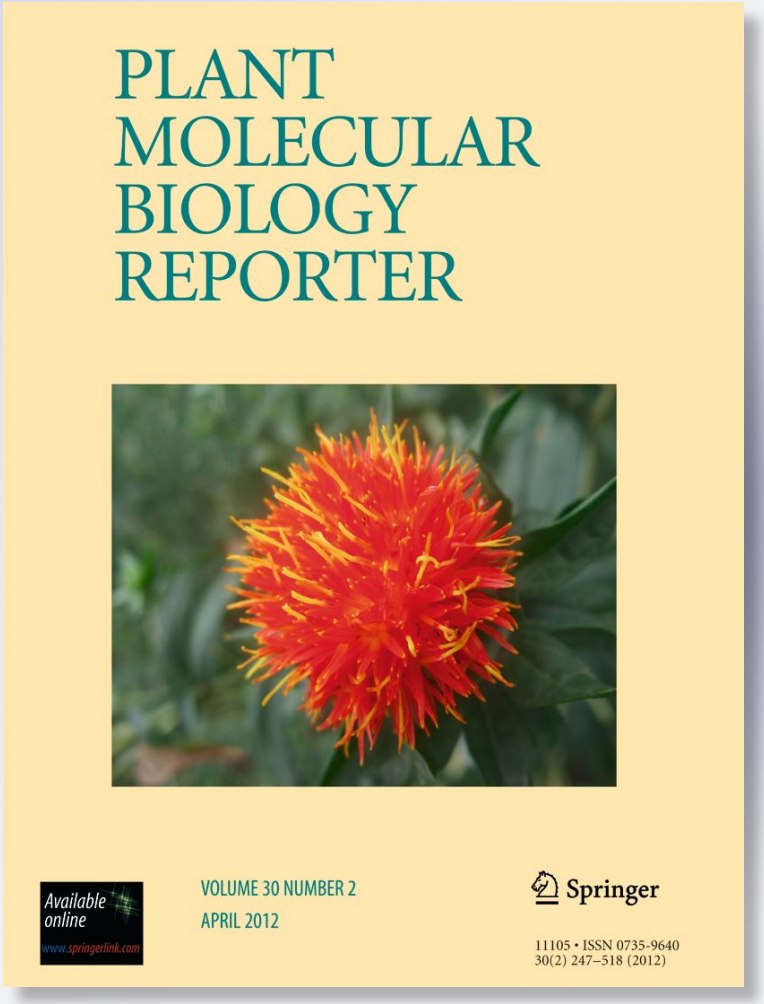

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Structural and Functional Features of a Wheat Germin-Like Protein that Inhibits Trypsin

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Abstract The wheat leaf apoplast contains a protein that inhibits trypsin and belongs to the family of germin-like proteins called germin-like protease inhibitor (GLPI). Since it was first described in our laboratory, the objective of this study was to find out if GLPI is a new germin-like protein and to identify the molecular site responsible for its inhibitory action. Amino acid sequence fragments of GLPI have been determined using mass spectrometry and used to synthesize complementary DNA by reverse transcription PCR. This has allowed recovery of the amino acid sequence of the mature form of GLPI, which is indistinguishable from barley GLP and having pyrophosphatase/phosphodiesterase activity. Using chemical modifiers of amino acids, the unique Arg of GLPI is found to be necessary for preserving its protease inhibition activity. Furthermore, structural homology modeling has allowed prediction that Arg is located along the GLPI surface, which could aid in its activity on proteases. Given that GLPI acts as a superoxide dismutase and as pyrophosphatase/phosphodiesterase, it is deemed to be a multifunctional protein.

Keywords Germin-like protein · Leaf apoplast · Multifunctionality · Serine protease inhibitor · Wheat

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Introduction

Previous search for proteins involved in germination has lead to identify a wheat protein named Germin (Thompson and Lane 1980). Germin is an apoplastic and glycosylated protein coupled to either Mn or Cu, resistant to temperature and proteolysis, and holding oxalate oxidase activity. Some years later, many proteins presenting different percentages of identity with germin were described. They are grouped as germins and germin-like proteins (GLPs) with around 90% and 30–70% of sequence identity with the original germin, respectively. Both germins and GLPs are composed of three conserved oligopeptides or boxes designated as A, B, and C (Bernier and Berna 2001). The sequence of the box A contains one of the two Cys forming an internal disulfide bridge that stabilizes the N-terminus conformation. The boxes B and C, containing one Glu and three His residues for metal binding, form the β-barrel structure characteristic of the cupin superfamily, which both germins and GLPs belong to (Dunwell et al. 2004). Like germins, GLPs have been described as homohexamers of a molecular mass nearly 100 kDa (Woo et al. 2000). The GLPs have diverse enzymatic activities, such as superoxide dismutase (SOD), oxalate oxidase, and pyrophosphatase/phosphodiesterase (AGPPase) (Bernier and Berna 2001; Rodríguez-López et al. 2001; Chen et al. 2011). Thus, proteins belonging of the cupin family share a structural homology but carry out many enzymatic activities. This diversity remarks the need to characterize the site for each particular activity into the protein. They also act as receptors, as described for the GLP from *Prunus persica* L. cv. Akatsuki that binds to auxins. In addition, a GLP from *Pisum sativum* with an RGD sequence acts as receptor for rhicadhesin, a protein that mediates the binding to plant cells of both *Rhizobium* and *Agrobacterium* (Bernier and Berna 2001). Other studies

consider the GLPs as key pieces of basal resistance to biotic stress in wheat and barley (Davidson et al. 2009). It has also been found that eight GLPs promoter sequences are induced by biotic stress in both, wheat, and barley (Himmelbach et al. 2010). Furthermore, it has been described that GLPs act against stress caused by salt, presence of heavy metals (Bernier and Berna 2001), cold (Zimmermann et al. 2006), and wounding (Tabuchi et al. 2003).

In previous studies, we found that a serine-type proteolytic activity of the intercellular fluid of wheat leaf increases in cultivars resistant to the fungal pathogen *Septoria tritici* (Segarra et al. 2002). We also identified that this proteolytic activity is controlled by a serine protease inhibitor with an N-terminal sequence identical to that of box A of the GLPs (Segarra et al. 2003). Like other GLPs, this germin-like protease inhibitor (called GLPI) is an oligomeric glycoprotein of 66 kDa with SOD activity (Segarra et al. 2003). The ability of GLPI to inhibit serine proteases was the first to be found within both germins and GLPs (Segarra et al. 2003). Subsequently, we found that GLPI takes part in the biological control of septoriosis in wheat by prior inoculation of seeds with the biocontroller fungus *Trichoderma* spp. (Cordo et al. 2007)

Based on the mentioned background, the present study was designed to find out both the GLPI amino acid sequence and its reactive center for trypsin inhibition.

Materials and Methods

Plant Material

Wheat (*Triticum aestivum* L.) cv. Relmo Centinela 12-day-old seedlings were obtained. For this, 400 seeds were put by pot (30×15 cm and 5 cm depth) filled with 0.2 kg of vermiculite soaked in 800 ml of the nutrient developed by Hoagland and Arnon (1950). Then, they were grown at 25°C in a chamber illuminated by Grolux lamps (Sylvania) at an average photosynthetically active radiation of 250 μmol m^{-2} s^{-1} for 14 hday^{-1}. Each pot was watered with 200 ml of the nutrient solution each 48 h.

Preparation of IWF-70

The leaf intercellular washing fluid (IWF) was obtained from leaves as described by Segarra et al. (2003). Five grams of leaf fresh weight (about 80–100 leaves) yielded nearly 0.25 ml of IWF. Then, it was heated at 70°C for 30 min and cleared by centrifugation at 16,000×*g* for 15 min to obtain the IWF-70 fraction. The protein present in IWF-70 was precipitated with acetone 90% (*v*/*v*) at −20°C and dried. This material contains more than 90% of the GLPI as determined by both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Segarra et al. 2003).

Two-dimensional gel electrophoresis

The first dimension gel solution contained 6.25% (*v*/*v*) ampholytes, pH range 3–10 (Pharmacia). Dried IWF-70 (20 μg protein) was dissolved in 10 μl of a solution containing 4% (*w*/*v*) CHAPS, 0.2 M DTT, and 18 M urea and deposited under 10 μl of ampholytes, pH range 3–10. Gels were run at 4°C for 10 min at 500 V and then for 3.5 h at 700 V. Gel was 1 mm thick and 60 mm long, with an acrylamide concentration of 5.4%. The second dimension was performed as in the conventional 2D-PAGE described by Garrels (1983). Briefly, 1D gels were extruded from the basic end and equilibrated for 4-min periods with buffer C (3% SDS, 50 mM DTT, 0.5 M Tris–HCl, pH 6.8) followed by buffer D (3% SDS, 0.2 M iodoacetamide, 0.5 M Tris–HCl, pH 6.8). Then, they were layered onto a 12% SDS-PAGE slab (1 mm thick, 90 mm wide, and 75 mm long) and electrophoresed at 4°C for 1 h. The protein spots were revealed using the colloidal Coomassie Brilliant Blue staining technique developed by Neuhoff et al. (1988).

Mass spectrometry

The GLPI monomer was obtained by SDS-PAGE as previously reported by Segarra et al. (2003). The liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis was performed using an Agilent 1100 series nano-high-performance liquid chromatography chromatograph online with a spectrometer, ion trap XCT Plus (Agilent) equipped with a nano-ESI source at the University of Alicante, Spain.

MS data from tryptic digestion of 2D SDS-PAGE spots were obtained using a matrix assisted laser desorption ionization–time of flight (MALDI-TOF) spectrometer, Ultraflex II (Bruker), in the MS facility CEQUIBIEM in Argentina, according to the following protocol: the stained protein spots were excised from the gel and destained with 10 mM ammonium bicarbonate–50% acetonitrile; gel pieces were air-dried and further reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide, both in 50 mM ammonium bicarbonate. Gel pieces were dehydrated with acetonitrile and swollen in a minimum volume of digestion buffer containing 10 ng $μl^{-1}$ trypsin in 50 mM ammonium bicarbonate. Digestion was performed by overnight incubation at 37°C. The peptides were recovered from the digestion mixture followed by a further extraction and sonication with 30% acetonitrile–0.1% trifluoracetic acid (TFA). The resulting peptide extracts were pooled and concentrated in a Speed Vac, redisolved by sonication in a matrix solution of a-cyano-4-hydroxycinnamic acid (3 mg ml^{-1}) and dissolved in 70% acetonitrile–0.1% TFA. When no consistent hit was

found, protein identification was achieved by tandem mass spectrometry analysis (Bian et al. 2010). Search for *T. aestivum* proteins was done in the latest version of the non-redundant National Center for Biotechnology Information database using the Mascot search engine (Perkins et al. 1999).

All mass spectrums were externally calibrated using bovine serum albumin (Sigma) and internally calibrated with trypsin autolysis peaks (Promega Sequencing Grade Modified Trypsin).

Initial search parameters were as follows: carbamidomethyl cysteine and oxidation of methionine as a variable modification, one missed cleavage site, peptide mass tolerance of ±50 ppm, and MS/MS tolerance of 0.1–2.5 Da.

cDNA cloning of GLPI

First leaves of 12-day-old wheat seedlings were ground in a mortar in the presence of liquid nitrogen, and total RNA isolation was done with the TRIzol Reagent (Invitrogen). The wheat RNA was reverse transcribed and PCR amplified using the primers 5′-ACCCAGGACTTCTGCGTC-3′ and 5′-CTTCACCTGCGCGTC GTC-3′ as sense and anti-sense primers, respectively. The sense primer was deduced from the N-terminus peptide sequence (ltqdfcvadl), previously obtained by Edman sequencing (Segarra et al. 2003) and highly conserved in the GLPs. The other sequence used, near the C-terminus and obtained by mass spectrometry (vtflddaqvk) is not conserved in all the proteins of the family. The reverse translation was performed using the most frequent codons code in wheat (program Reverse Translate) (Stothard 2000). The PCR amplification was performed using the following procedure: 95 C for 300 s (once), followed by 95°C for 60 s, 56°C for 60 s, and 72°C for 60 s (30 cycles), and finally 72°C for 300 s (once). A PCR fragment of about 540 bp was purified from agarose gel (Kit Wizard, Promega). This fragment was cloned into a pGEM-T Easy vector (Promega) and introduced into competent *Escherichia coli* DH5α using chemical transformation. Two recombinant clones were automated sequenced (ABI310) using forward and reverse M13 universal primers. The obtained DNA sequence was translated according to the six possible frames. The deduced protein sequence from the forward frame 1 showed the longest amino acidic sequence.

Chemical modification of amino acids

Arginyl residues were modified with ninhydrin (100 mM final concentration) for 30 min in 100 mM sodium phosphate buffer (pH 9.1) at 37°C as described by Chaplin (1976). Dried IWF-70 (GLPI) was incubated with different concentrations of 2,3-butanedione in 100 mM phosphate buffer (pH 8.5) in the dark at 37°C according to Kumar et al. (2004).

Diethylpyrocarbonate (DEPC), a reagent that modifies the imidazole of histidine in proteins, was used at 10 mM final concentration for 30 min in 100 mM sodium phosphate buffer (pH 6.8) at 37°C as described by Romaniouk and Vijay (1997). Controls without modifiers were run simultaneously.

Gel electrophoresis and in-gel activity assays

GLPI was analyzed by SDS-PAGE according to Laemmli (1970) in a Mini Protean III cell (Bio-Rad). Slab gels containing 12% acrylamide were 1 mm thick. When indicated, samples were heated at 100°C for 3 min. Analysis in SDS-PAGE slabs containing 0.1% (*w*/*v*) gelatin was performed as described by Heussen and Dowdle (1980). Samples containing 10–20 μg protein were run on slab gels 1 mm thick. After washing with 25 mM Tris–HCl (pH 8.0) containing 1% Triton X-100 and subsequently 10 mM $CaCl_2$ to remove SDS, gels were incubated in 1 mg ml^{-1} trypsin solution (Trypsin SIGMA 1426, from bovine pancreas, TPCK Treated, 13,960 U/mg protein) for 12 h at 37°C. Then, they were washed with distilled water and stained with Coomassie Brilliant Blue. Blue-stained bands revealed a protease inhibitor activity, while transparent background showed the proteolytic digestion of gelatin.

For the phosphodiesterase assay, the gel was incubated with 5 mM bis(4-nitrophenyl) phosphate (Sigma) and 5 mM $MgCl_2$ until the 4-nitrophenyl product appeared as a yellow band (Rodríguez-López et al. 2001). The ImageQuant 5.2 computer program was used to obtain spot densitograms of scanned gels.

Immunization and antiserum preparation

Dried IWF-70 (800 μg protein) was dissolved in SDS sample buffer at 100°C for 3 min. Then, it was run on SDS-PAGE and stained with imidazole-zinc (Fernández-Patrón et al. 1992). The main 18–21 kDa band was cut and blended with 2 ml of 25 mM sodium phosphate buffer (pH 7.4). The suspension was mixed with 2 ml of Freund's adjuvant. Four 1-ml injections of the antigen mixed with complete adjuvant were given subcutaneously in an adult white rabbit. Two booster injections, in incomplete adjuvant, were given at 30 and 60 days after the first injection, both with 600 μg of the antigen. Four weeks later, blood (30 ml) was collected from the femoral vein. The serum was separated by centrifugation at 2,000×*g* for 15 min and stored frozen at −20°C. Titre was tested by dot immunobinding according to Hawkes (1986).

Immunoblot assays

After SDS-PAGE, proteins were transferred onto nitrocellulose paper (BIO-RAD; pore size is 0.45 μm). The paper was washed with water and blocked with Tris-buffered

saline solution (pH 7.5) containing 0.1% (*v*/*v*) Tween-20 (TBST) and 5% (*w*/*v*) dried milk for 12 h. Then, it was incubated for 1 h with a 1:2,000 dilution of rabbit antibody against the 18–21 kDa germin, washed with TBST solution, and incubated for 2 h with Cy TM 5 Goat Anti-rabbit IgG (Invitrogen) diluted 1:2,000. Signals were then scanned by a Storm 840 instrument and analyzed by Image-Quant 5.2 software (Molecular Dynamics).

Protein determination

The protein content of samples was measured by the bicinchoninic method (Smith et al. 1985), using bovine serum albumin as standard.

Modeling of GLPI

The 3D structure of GLPI was built in Swiss-model workspace (Arnold et al. 2006) using as template the crystal structure of oxalate oxidase from barley (PDB ID number 2ET7, Uniprot accession number P45850) (Opaleye et al. 2006). This protein has about 40% identity with GLPI and *E* value of 3.84041*e*−27. The resulting model was pictured using PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.).

Results

Amino acid sequence of GLPI

The GLPI monomer obtained by 1D SDS-PAGE and subsequently digested with trypsin was analyzed by mass spectrometry (LC/MS/MS). Data MS/MS analyzed with the Mascot program revealed a peptide of mass/charge ratio (*m*/*z*) of 568.17 Da of sequence VTFLDDAQVK. This sequence is present in the adenosine diphosphate glucose pyrophosphatase/phosphodiesterase protein (AGPPase) of wheat (GenBank accession number CAC85479), which belongs to the GLPs. It is placed close to the C-terminus of the AGPPase and outside the three oligopeptides (boxes A, B, and C) conserved in the family of germins and GLPs (Bernier and Berna 2001).

To confirm the identity of GLPI, it was further separated by 2D-PAGE. Six main spots were observed at kilodalton and p*I* coordinates as follows: 18, 5.5; 18, 6.2; 20, 5.1; 20, 5.7; 20, 6.2; and 21, 6.2 (Fig. 1).

These six main spots underwent MALDI-TOF analyses. The masses of tryptic fragments showed that they share at least four peptides (Fig. 2). However, it was not possible to find out any spot identity by peptide fingerprint analyses in databases. Therefore, since it was the most intense and common within the six spots, we analyzed the fragment of

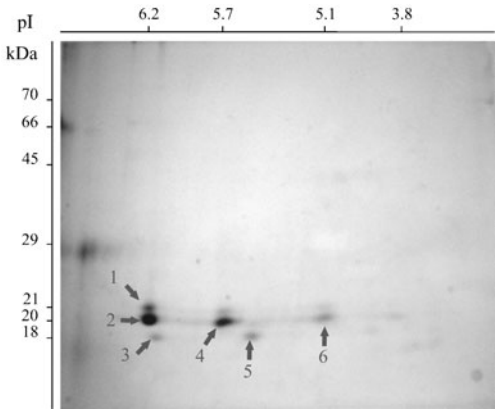

Fig. 1 2D-PAGE of GLPI stained with colloidal Coomassie Brilliant Blue

2,201 Da by MS/MS. The result obtained with the program Mascot identified an internal peptide of 23 amino acids (AAVTPAFVGQFPGVNGLGISAAR), which is present in the GLP with AGPPase activity. These results suggest that the six spots are isoforms of GLPI. For spots of equal mass and different p*I*, the difference may be because of modification of some of its amino acids. For spots of the same p*I* but different molecular weight, this could be because of different degree of glycosylation.

To get the complete sequence of GLPI from its complementary DNA (cDNA), we designed the respective primers. One of the sequences used matches the N-terminus of GLPI, which is conserved in the GLPs (box A). The other sequence used was that of amino acids 170–180 at the C-terminus of the mature protein, which contains 190 amino acids. Amplification of a cDNA fragment of nearly 540 bp by reverse transcription PCR (RT-PCR) allowed to obtain a DNA sequence showing 99% identity with wheat GLP with AGPPase activity, which confirms the results of mass spectrometry (Fig. 3).

Because the sequence of GLPI was found to be 99% identical the GLP described by Rodríguez-López et al. (2001), it was examined whether it contains the AGPPase activity. Therefore, we conducted an in-gel trial for phosphodiesterase activity after separation of the proteins from the IWF by gel electrophoresis. A band of activity with a relative mobility similar to that of GLPI oligomer (66 kDa) was detected. To confirm its identity, this band was eluted with sample buffer and boiled 3 min to obtain the monomeric form of GLPI. Western blot assay with the specific primary antibody against GLPI gave a positive reaction with this phosphodiesterase activity eluted from the gel (Fig. 4).

Search of the reactive center of GLPI as a protease inhibitor

Beyond the SOD activity previously described (Segarra et al. 2003) and the phosphodiesterase now identified, GLPI is

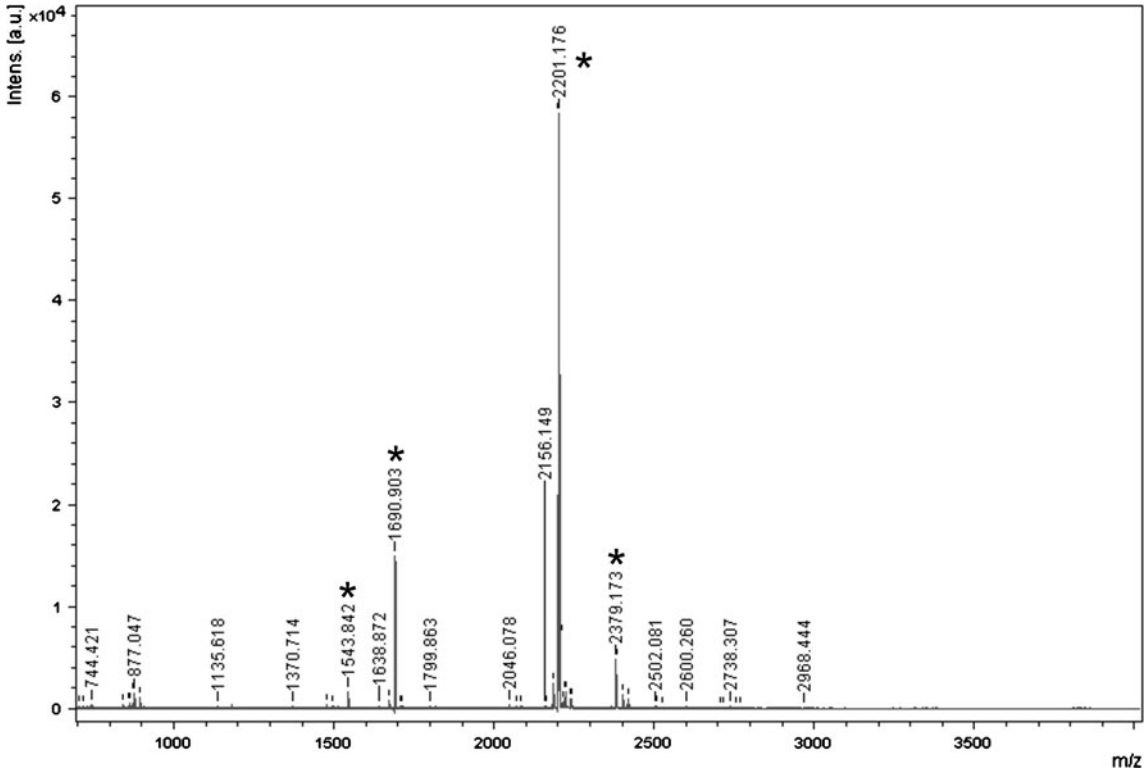

Fig. 2 MALDI-TOF spectrum of the 2D-PAGE spot 2 depicted in Fig. 1. *Asterisks* mark the peaks *m/z* that it shares with the other five spots observed

```
1:  CTGACCCAGGACTTCTGCGTCGCCGACCTGTCCTGCAGCGACACACCGGCG
      L  T  Q  D  F  C  V  A  D  L  S  C  S  D  T  P  A

52:  GGGTACCCGTGCAAGGCCGGCGTCAGCGCAGGGGACTTCTACTACCACGGC
      G  Y  P  C  K  A  G  V  S  A  G  D  F  Y  Y  H  G

103: CTCGCCGCCGCGGGCAACACCAGCAACCTCATCAAGGCGGCCGTGACCCCG
      L  A  A  A  G  N  T  S  N  L  I  K  A  A  V  T  P

154: GCCTTCGTCGGGCAGTTCCCCGGCGTGAACGGGCTCGGCATCTCCGCAGCG
      A  F  V  G  Q  F  P  G  V  N  G  L  G  I  S  A  A

205: AGGCTCGACATCGCCATGGGCGGCGTCGTGCCGCTGCACACCCACCCGGCC
      R  L  D  I  A  M  G  G  V  V  P  L  H  T  H  P  A

256: GCCTCTGAGCTCCTGTTTGTCACCGAGGGCACCATCCTGGCGGGCTTCATC
      A  S  E  L  L  F  V  T  E  G  T  I  L  A  G  F  I

307: AGCTCCTCCTCCAACACCGTGTACACCAAGACACTCTACAAGGGCGACATC
      S  S  S  S  N  T  V  Y  T  K  T  L  Y  K  G  D  I

358: ATGGTGTTCCCCCAGGGCCTGCTCCACTACCAGTACAACGGCGGCAGCTCG
      M  V  F  P  Q  G  L  L  H  Y  Q  Y  N  G  G  S  S

409: GCGGCGGTGGCGCTCGTTGCGTTCAGCGGCCCCAACCCCGGCCTGCAGATC
      A  A  V  A  L  V  A  F  S  G  P  N  P  G  L  Q  I

460: ACTGACTACGCGCTCTTCGCCAACAACCTGCCGTCCGCCGTCGTTGAGAAG
      T  D  Y  A  L  F  A  N  N  L  P  S  A  V  V  E  K

511: GTCACCTTCTTGGACGACGCGCAGGTGAAG
      V  T  F  L  D  D  A  Q  V  K
```

Fig. 3 Amino acid sequence of GLPI deduced from its cDNA. The *arrows* indicate both the sequence and orientation (forward and reverse) of the DNA primers used for the RT-PCR reaction. The amino acidic sequences of conserved boxes within GLPs are shown in *bold characters* (Bernier and Berna 2001)

the first GLP having activity for serine protease inhibition. Since no sequence homology with any of the families of protease inhibitors described was found, knowledge of the reactive center is important to characterize this novel role.

Therefore, two different GLPI amino acids were chemically modified before measuring in gel its inhibitory capacity. To confirm that GLPI displays the same size after the chemical treatment, an SDS-PAGE stained with Coomassie Brilliant Blue was done in parallel to the activity test (Fig. 5a). The GLPI oligomer (66 kDa) treated with DEPC kept its inhibitory activity, pointing out that it does not depend on histidines (Fig. 5b, lane 2). On the other

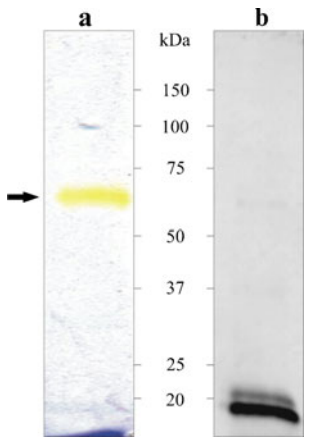

Fig. 4 Phosphodiesterase activity of GLPI. **a** An IWF aliquot was subjected to SDS-PAGE under non reducing condition and tested in gel for enzyme activity. The *arrow* indicates a yellow activity band. **b** Western blot of the band identified in **a**, which was eluted with a sample buffer containing 2-mercaptoethanol and heated at 100°C

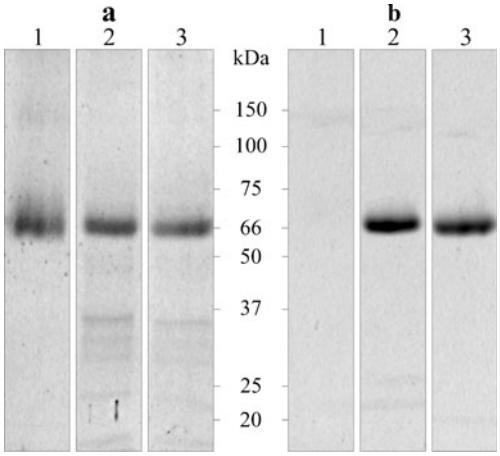

Fig. 5 Evaluation of the protease inhibitor activity of GLPI after the following conditions: *1* preincubation with 100 mM ninhydrin; *2* preincubation with 10 mM DEPC; *3* untreated. **a** SDS-PAGE of GLPI stained with Coomassie Blue; **b** SDS-PAGE with copolymerized gelatin of GLPI stained with Coomassie Blue after digestion with trypsin

hand, treatment with ninhydrin under experimental conditions that change specifically the guanidinium group of arginine (Chaplin 1976) resulted in complete loss of GLPI activity (Fig. 5b, lane 1). To confirm this result, we used the 2,3-butanedione that also changes specifically arginine, using increased concentrations of this reactive. In the same manner as for both DEPC and ninhydrin tests, the size control was also performed by a SDS-PAGE stained with Coomassie Brilliant Blue (Fig. 6b). This new result showed an inverse relationship between the GLPI inhibitory action and the reagent concentration (Fig. 6a). It suggests that the unique arginine at place 69 of GLPI is required to inhibit trypsin.

Considering that arginine is involved in the specific association with trypsin (Laskowski and Qasim 2000), the spatial location of the GLPI Arg69 was explored (Fig. 7) by modeling GLPI using as template a germin, the barley

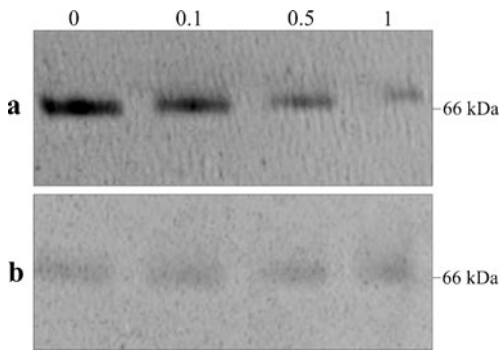

Fig. 6 Effect of 2,3-butanedione on the protease inhibitor activity of GLPI. **a** GLPI preincubated with 0–1 M 2,3-butanedione was subjected to SDS-PAGE with copolymerized gelatin and stained with Coomassie Blue after digestion with trypsin. **b** GLPI analyzed in SDS-PAGE and stained with Coomassie Blue

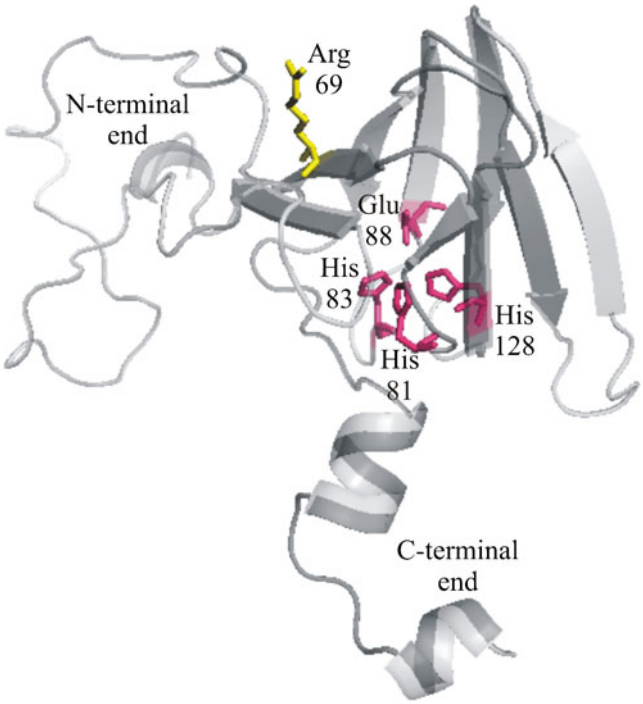

Fig. 7 Prediction of the tertiary structure of GLPI. The amino acids Arg69, His81, His83, His128, and Glu88 are highlighted

oxalate oxidase (PDB code 2et7; Uniprot accession number P45850). This protein was used because has the highest amino acid sequence identity with GLPI and a known 3D structure (Opaleye et al. 2006). The model obtained shows that Arg69 is peripheral, which would justify his role in the interaction with trypsin. About the SOD activity, the GLPI model shows that Glu88 and three histidines (His81, 83, and 128) involved in binding manganese are found on antiparallel β-strands forming a cluster of imidazole groups like in other germins (Woo et al. 2000).

Discussion

Most GLPs have been discovered indirectly during the search and molecular characterization of proteins with certain enzymatic activities and biological roles. Many of them have the peculiarity of presenting more than one biological role (Bernier and Berna 2001). For GLPI, a GLP identified in a search for protease inhibitors (Segarra et al. 2003), we determined its primary structure and identified the boxes A, B, and C that characterize both germins and GLPs (Bernier and Berna 2001). In addition, the amino acid sequence of GLPI does not show any likeness with the families of protease inhibitors described so far. Since a GLP with capacity for protease inhibition had never been described, at first, we thought that GLPI was a new protein. However, we found that GLPI is the barley GLP with

AGPPase activity described by Rodríguez-López et al. (2001), which is also similar to the protein HvGER2a previously characterized by Vallelian-Bindschedler et al. (1998). Then, the phylogenetic distance between GLPI and other GLPs is the same that of HvGER2a as shown by El-Sharkawy et al. (2010). The oligomeric form of GLPI also displays SOD activity (Segarra et al. 2003). Moreover, as shown in Fig. 7, GLPI's manganese binding site is identical with that described for other germins and GLPs with SOD activity (Woo et al. 2000). However, the recombinant protein HvGER2a does not show SOD activity (Zimmermann et al. 2006). A possible explanation for this difference could be that HvGER2a almost does not form oligomers.

In recent years, many proteins were found to have more than one function or enzymatic activity that may or may not be part of the same metabolic pathway (Huberts and van der Klei 2010). GLPI has at least three activities linked to responses to different types of stress, namely, serine protease inhibitor, AGPPase, and SOD. Indeed, it has been described that, besides controlling proteolysis and serving as storage proteins in seeds, protease inhibitors may take part in defense responses against phytopathogenic micro-organisms and insects (Mosolov and Valueva 2005). In addition, the AGPPase regulates the synthesis of cell wall and, therefore, plays an important role in its strengthening (Rodríguez-López et al. 2001). Finally, in addition to protecting cells from the toxic effects of superoxide anion caused by an oxidative stress (Chen et al. 2009; Xu et al. 2011), SOD generates hydrogen peroxide that can act as a signaling molecule in defense mechanisms (Kovtun et al. 2000), catalyze cross-linking reactions in the synthesis of lignin (Nakata et al. 2002), or act as an antimicrobial agent in defense responses (Shetty et al. 2008).

Shetty et al. (2009) have recently shown that the leaf intercellular fluid of a wheat resistant to septoriosis accumulates β-1,3-glucanase isoforms after inoculation with the fungus *S. tritici*. Further mass spectrometry analysis of one of them proved that it was the barley GLP as described by Rodríguez-López et al. (2001). Therefore, this would be a new role of this protein in the wheat defense against septoriosis. We have already shown that the activity of GLPI decreases in a cultivar resistant to septoriosis after infection with *S. tritici* permitting the antifungal action of the serine protease of the intercellular fluid (Segarra et al. 2002). These examples let us hypothesize that the same protein can activate two different pathways against the same stress.

Since the inhibitor activity of serine-type proteases GLPI was first described in our laboratory, the objective of this study was to find the molecular site responsible for this activity. The strategy used was to block that activity by changing some amino acid residues with specific chemical reagents. Thus, we modified histidine with DEPC to show the inhibitory action on serine proteases does not coincide with that of SOD (Woo et al. 2000). In addition, we modified the arginine due to its specificity in binding to trypsin within serine protease inhibitors (Laskowski and Qasim 2000). Since the inhibitory activity of GLPI did not change with DEPC, the idea that histidines take part in it was dismissed. In contrast, GLPI has no action against trypsin when its unique arginine is modified. Although GLPI has no sequence similarity with proteins of different families of protease inhibitors, this finding is interesting because most Kunitz-type inhibitors have arginine at its reactive center (Joubert et al. 1985; Ceciliani et al. 1994; Haldar et al. 1996). Furthermore, like in the tertiary structures of *Ascaris* trypsin inhibitor (Grasberger et al. 1994), ragi bifunctional trypsin/α-amylase inhibitor (Strobl et al. 1995) and pancreatic trypsin inhibitor (Bode and Huber 1992), arginine appears in an exposed loop of the GLPI model obtained. In addition, the Arg76 of each monomer of the germin oxalate oxidase from barley hexamer appears in an external loop (Opaleye et al. 2006), which is like the Arg69 of GLPI. Besides, as shown by El-Sharkawy et al. (2010), it is possible to note the Arg69 of the mature protein is conserved in several GLPs. Thus, these proteins could also display trypsin inhibitory activity.

To advance in characterizing the GLPI responsible site for protease inhibition, future studies must be carried both directed mutagenesis and use of synthetic peptides. Another approach could be the use of GLPI fragments containing the Arg69 obtained by expression in *E. coli* (Marcus et al. 2008).

In conclusion, GLPI is a multifunctional GLP having at least three enzymatic activities: serine protease inhibitor, AGPPase, and SOD. The multifunctionality of proteins is an emerging paradigm that extends the traditional concept that an amino acid sequence settles a unique structure and folding for a single role. While this view allowed a great progress in developing modern biochemistry, the vast amount of structure and function information gathered proves the plasticity of proteins. So far, we know that GLPI has at least three biological activities, its complete amino acid sequence and molecular parts responsible for both trypsin inhibition and SOD activity. Thus, GLPI is a suitable model for studying multifunctional proteins.

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