

An antifungal peptide from passion fruit (*Passiflora edulis*) seeds with similarities to 2S albumin proteins

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Abstract

An actual worldwide problem consists of an expressive increase of economic losses and health problems caused by fungi. In order to solve this problem, several studies have been concentrating on the screening of novel plant defence peptides with antifungal activities. These peptides are commonly characterized by having low molecular masses and cationic charges. This present work reports on the purification and characterization of a novel plant peptide of 5.0 kDa, *Pe-AFP1*, purified from the seeds of passion fruit (*Passiflora edulis*). Purification was achieved using a Red-Sepharose Cl-6B affinity column followed by reversed-phase chromatography on Vydac C18-TP column. In vitro assays indicated that *Pe-AFP1* was able of inhibiting the development of the filamentous fungi *Trichoderma harzianum*, *Fusarium oxysporum*, and *Aspergillus fumigatus* with IC₅₀ values of 32, 34, and 40 μg ml⁻¹, respectively, but not of *Rhizoctonia solani*, *Paracoccidioides brasiliensis* and *Candida albicans*. This protein was also subjected to automated N-terminal amino acid sequence, showing high degree of similarities to storage 2S albumins, adding a new member to this protein-defence family. The discovery of *Pe-AFP1* could contribute, in a near future, to the development of biotechnological products as antifungal drugs and transgenic plants with enhanced resistance to pathogenic fungi.

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1. Introduction

Several plant proteins capable of inhibiting the growth of agronomically important pathogens have been isolated during the last few years [1,2]. There are five main families of peptides

that seem to be essential for plant defence processes, acting toward pathogens by diverse ways [1]. The first one, grouped as pathogen-related proteins or simply PR proteins, have been isolated from several plant species [3–5] as tobacco, barley, beans, *Arabidopsis*, and so far have been divided in seventeen subfamilies that includes chitinases, β-1,3-glucanases, thaumatin-like (TL) proteins, proteinase inhibitors, endoprotease, peroxidase, ribonuclease-like, γ-thionin or plant defensins, oxalate oxidase, oxalate-oxidase-like and others proteins of unknown biological properties [6–12]. Two of these PR subfamilies, chitinases and β-1,3-glucanases, comprise genuine antifungal and antibacterial proteins which act on the cell wall structure hydrolysing chitin and/or peptidoglycans [1,13]. In addition, these enzymes are involved in the recognition processes of the attacking pathogen by the host when they hydrolyse defence-activating signal molecules from the walls of involving pathogens [10]. Plant defensins or γ-thionins form the second group. This class is composed of cationic peptides synthesized in leaves, roots, flowers and seeds and are

Abbreviations: Cp-thionins, thionins from cowpea; CW-1, cheeseweed antifungal protein; HPLC, high-performance liquid chromatography; LTP, lipid-transfer proteins; MYG, malt yeast glucose medium; NaD1, antimicrobial peptide from *Nicotiana glauca*; *Pe-AFP1*, antifungal peptide from *Passiflora edulis*; *Pp-AMP1*, antimicrobial peptide from *Pyrularia pubera* 1; *Pp-AMP2*, antimicrobial peptide from *Pyrularia pubera* 2; PR protein, pathogen-related proteins; Ra-AFP2, *Raphanus sativus* antifungal protein isotype 2; RIP, ribosome-inactivating proteins; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TL, thaumatin-like; Tu-AMP1, antimicrobial peptide from *Tulipa gesneriana* 1; Tu-AMP2, antimicrobial peptide from *Tulipa gesneriana* 2; VrD1, antimicrobial peptide from *Vigna angularis*

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typically characterized by their low molecular masses and high disulfide bond content [14]. Defensins probably are able to inhibit fungi growth by a direct interaction with a sphingolipid at cell membrane surface [2]. The third family, known as lipid-transfer protein (LTP), shows also low molecular mass and is strongly stabilized by four disulfide bonds [15]. They are usually found in cereal grains but are also present in onions, spinach and *A. thaliana* [16] and exert their growth inhibition effects probably by acting on the traffic of phospholipids across membranes [17]. The fourth group encompasses the ribosome-inactivating proteins (RIP) that cause irreversible damages of ribosomes by depuration of rRNA [18]. RIPs are expressed in response to various pathogens, as virus and fungi, mechanical wounding, jasmonic and abscisic acid treatments and abiotic stresses [19–21]. The well-conserved fifth family comprises the cyclophilin-like proteins and has been isolated from several plant species [22]. These proteins work as intracellular receptors for cyclosporins leading to remarkable fungus lethality [23].

There is a body of evidence pointing out that these above-mentioned five groups of proteins play important roles in the plant protection against microbial infections. However, a sixth class of plant defence peptides showing structural similarities to the 2S-storage proteins has recently gained more attention. Although most studies on 2S peptides have addressed their allergenic properties, as those reported for Brazil nut (*Bertholletia excelsa*), cashew nut (*Anacardium occidentale* L.), oilseed rape and turnip rape [24–26], some reports deal with the antimicrobial activity of 2S proteins against filamentous fungi and bacteria [27,28,56].

The 2S family consists of storage proteins found both in mono- and dicotyledonous seeds that display several similarities to plant defence peptides including characteristic low molecular masses, high disulfide bond content that confer temperature and pH stabilities, and other common structural features [26,29]. Hitherto only a few number of 2S antifungal peptides has been described as those from seeds of *Raphanus sativum* [56], *Passiflora edulis* [28], *Malva parviflora* [30] and *Brassica* species [31].

This current work reports on the purification and biochemical characterization of a novel 2S antimicrobial peptide, *Pe*-AFP1, from passion fruit seeds that show close similarities to other members of the 2S albumin family. Additionally, the ability of *Pe*-AFP1 to inhibit the growth of filamentous fungi, including a species that is the main causative agent of human pulmonary infection, is demonstrated.

2. Material and methods

2.1. Extraction and isolation of *Pe*-AFP1

P. edulis mature seeds were air dried, macerated to a fine flour and extracted with 0.6 M NaCl+0.1% HCl solution in a proportion of 1:3 (w/v). The suspension was centrifuged at 6000×g, for 20 min, 4 °C, and the supernatant (crude extract) precipitated with ammonium sulfate at 100% saturation, at 25 °C. After centrifugation at the same conditions described above, the precipitate material was resuspended and dialyzed (cut off 3.0 kDa) against distilled water. This fraction was lyophilized, resuspended with equilibration buffer (0.15 M Tris-HCl buffer, pH 7.0 containing 0.005 M CaCl₂) and applied onto a Red-Sepharose affinity column. Chromatography was carried out at 0.5 ml min⁻¹ flow rate and the eluted fractions (2.0 ml) read at 280 nm in a spectrophotometer. The non-

retained protein fraction was displaced from the column with the equilibration buffer and the retained peak eluted in a single step with 3.0 M NaCl dissolved in the equilibration buffer. After dialyses (cut off 3.0 kDa) and lyophilization, 1.0 mg of the retained fraction was diluted with 0.1% trifluoroacetic acid (TFA) and applied onto a reversed-phase Vydac C-18TP column coupled to a HPLC system and equilibrated with 0.1% TFA from where the retained proteins were eluted with a linear acetonitrile gradient (0–100%) at a flow rate of 1.0 ml min⁻¹.

2.2. SDS-PAGE analyses

The protein fractions eluted from the reversed-phase chromatography were analysed by 15% SDS-PAGE according to Laemmli [32] with minor modifications using bromophenol blue as tracking dye. Samples were run for 45 min at 200 V, and gels were silver stained.

2.3. Amino acid sequencing and in silico analyses

The N-terminal amino acid sequence of *Pe*-AFP1 was determined on a Shimadzu PPSQ-23A Automated Protein Sequencer performing Edman degradation.

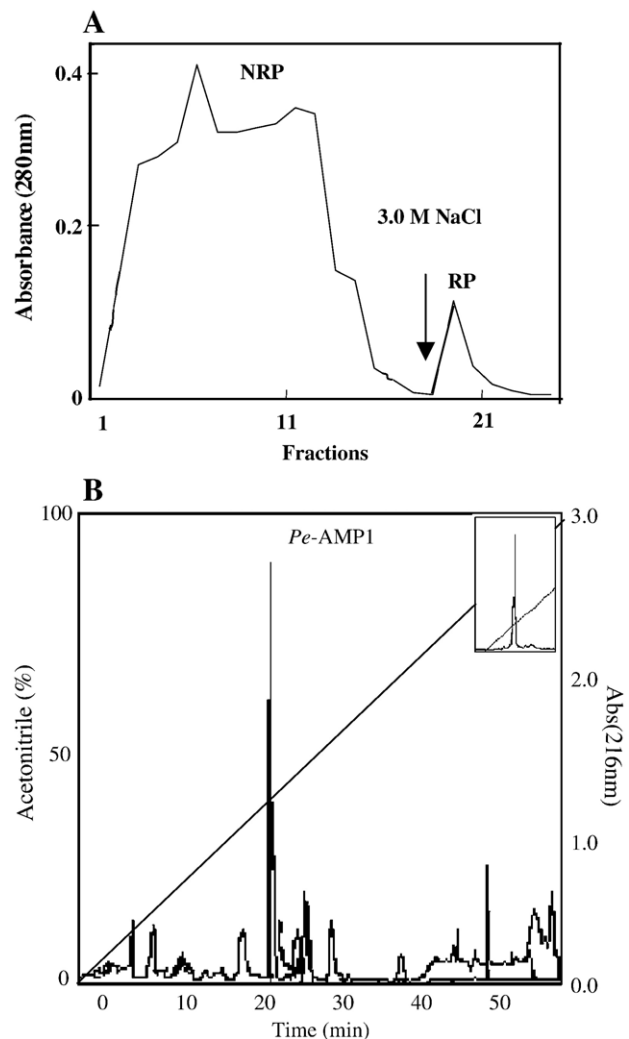


Fig. 1. (A) Red-Sepharose Cl-6B chromatography profile of *Pe*-AFP1 enriched fraction from passion fruit (*Passiflora edulis*) seeds. NRP and RP correspond to non-retained and retained peaks, respectively. Black arrow indicates the retained proteins eluted with 0.15 M Tris-HCl buffer, pH 7.0, containing 3.0 M NaCl and 0.05 M CaCl₂. (B) Reversed-phase chromatogram profile (Vydac C18-TP column) of Red-Sepharose retained fraction. The diagonal line indicates the linear acetonitrile gradient. Insert: re-chromatography of *Pe*-AFP1 on Vydac C18-TP column.

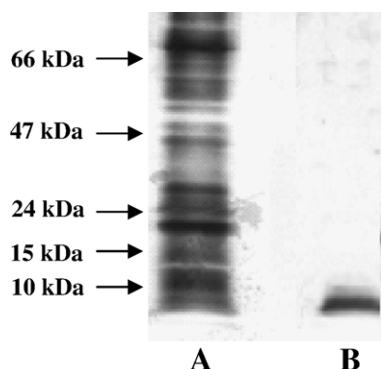


Fig. 2. SDS-PAGE of Red-Sepharose retained proteins (lane A) and *Pe*-AFP1 (lane B) obtained from passion fruit seeds.

Sequences were determined from 5 pmol samples. PHT-amino acids were detected at 269 nm after separation on a reversed phase C18 column (4.6 × 2.5 mm) under isocratic conditions, according to the manufacturer's instructions. Amino acid sequences were then compared to SWISSPROT Data Bank, using FASTA3 Program [33–35]. An alignment using ClustalW Program [36] was performed in order to analyse primary sequence similarities into each group.

2.4. Antifungal bioassays

The effect of *Pe*-AFP1 on the growth of the filamentous fungi *Trichoderma harzianum*, *Aspergillus fumigatus*, *Fusarium oxysporum* and *Rhizoctonia solani* was assessed according to Oard et al. [37]. These microorganisms were grown in 20 ml MYG medium (0.5% malt extract, 0.25% yeast extract, 1.0% glucose, pH 6.0) for 48 h, at 25 °C, in the presence and absence of *Pe*-AFP1 at concentrations of 25, 50, 75 and 100 µg ml⁻¹. Distilled water was used as negative control and 0.5% Capitan as positive control. Evaluation of growth inhibition in comparison with both controls was obtained by measuring the fungus dry weight 48 h after incubation with the test and control substances.

Yeast bioassay was performed according to Galgiani et al. [38]. *Paracoccidioides brasiliensis* and *Candida albicans* were grown in Sabouraud dextrose agar and incubated at 35 °C for 24–48 h. *Pe*-AFP1, at concentrations varying from 0.125 to 150 mg ml⁻¹, was incubated with the yeast cells in 12 × 75 mm tubes at 35 °C for 46 to 50 h. Amphotericin B and fluconazole were used at standard concentration of 20 µg ml⁻¹, as positive controls and a solution of 1% dimethylsulfoxide (DMSO) as the negative control. The growth inhibition of the yeast cells promoted by *Pe*-AFP1, in relation to negative and positive controls, was assessed by direct comparison of the number of spores formed as counted under optical microscopy using a Newbauer camera. All assays were carried out in triplicate.

2.5. Bacteria bioassays

In vivo analyses against two human pathogenic bacteria, *Klebsiella* sp. and *Proteus* sp. were performed using 1.0 ml Luria-Bertani medium (10 g l⁻¹ NaCl, 5 g l⁻¹ yeast extract, 45 g l⁻¹ bacto-peptone). Bacteria were pre-incubated with LB medium during 18–24 h at 37 °C before, starting peptides challenge. Distilled water was used as negative control and 40 µg ml⁻¹ cloranfenicol, as positive control. Peptides from fruit seeds were incubated with bacteria for 4 h at 37 °C at standard concentration of 40 µg ml⁻¹. Evaluation of bacterial growth was done in triplicate at each hour by measurement of optical density of 595 nm.

3. Results

3.1. Purification and molecular characterization of *Pe*-AFP1

To isolate the novel antimicrobial peptide from passion fruit (*P. edulis*) seeds, the ammonium sulfate precipitated rich fraction was applied onto a Red-Sepharose Cl-6B affinity column that has been often utilized to purify positive charged proteins, especially plant defence peptides [39–41]. As the retained peak (Fig. 1A) was contaminated with other numerous proteins of molecular masses in a range of 5.0 to 80.0 kDa (Fig. 2, lane A), it was submitted to reversed-phase chromatography on Vydac C-18TP column from which a major protein peak was eluted at 40% acetonitrile concentration (Fig. 1B). This peak was re-chromatographed in the same Vydac C-18TP column, but now eluted with a linear gradient of 20–50% acetonitrile (Fig. 1B, top right side) when a highly homogenous purified peptide (*Pe*-AFP1) migrating at approximately 5.0 kDa was obtained (Fig. 2, lane B).

3.2. Amino acid sequencing and alignment

Partial sequencing of *Pe*-AFP1 revealed an N-terminal sequence consisting of 25 amino acid residues in this order: Q S E R F E Q Q M Q G Q D F S H D E R F L S Q A A (Fig. 3). *Pe*-AFP1 showed various conserved residues as compared with the 2S albumin family (Fig. 3). For example, it was found 84% and 82% identity of *Pe*-AFP1 to the 2S albumin proteins from *Sesamum indicum* and *Vitis vinifera*, respectively, which are both storage molecules [42,43] whose antifungal activities have

Code number	Species	Alignment	Reference
This report	<i>P. edulis</i>	QSERFEQQMQGQD-FSHDERFLSQAA-----	This report
-	<i>P. edulis</i>	PSERCRRMQGQD-FS-----	[28]
AAP31048	<i>V. vinifera</i>	QSQRCRQQIQGQQ-FQQCERYIRQQA-----	[43]
AAK15088	<i>S. indicum</i>	QSQQCRQQQLGRQ-FRSCQRYLSQGR-----	[42]
P01089	<i>R. communis</i>	SSQQCRQEVQRKD-LSSCERYLRQSS-----	[55]
CAA38362	<i>B. excelsa</i>	NQEECREQMQRQMLSHCRMVMRQQM-----	[53]
AAD00178	<i>G. max</i>	QQDSCRKQLQGVN-LTPCEKHIMEKI-----	[54]
P83137	<i>M. parviflora</i>	---PEDPQ-----RRYQEEQRRE-----	[30]
Q9S8Y8	<i>R. sativum</i>	---PAGPFR---I--PRCRREFQQAQWLRACQQLHRQ-	[56]
P24565	<i>B. napus</i>	-----QPQ-----KCQREFQQEQHLRACQQWIRQQL	[48]
AAD10958	<i>P. glauca</i>	QRRSCEEQ---R-LSSCREYME-RRR-----	[52]
AAC34614	<i>P. glauca</i>	QRRSCDPQRDPQR-LSSCRDYLE-RRR-----	[51]
P83141	<i>M. parviflora</i>	---VAGPFR---I-PPLRREFQ-----	[27]
		* * *	

Fig. 3. Alignment of the N-terminal sequences of *Pe*-AFP1 and other members of the 2S albumin family possessing antifungal activity. Cysteine residues are marked with asterisk (*).

not yet been well established. In addition, *Pe*-AFP1 and the 2S protein from *P. edulis* have only 50% identical amino acid residues. Although the 2S peptide of *P. edulis* presents conserved Cys₅ and Cys₁₃ residues compared with other similar proteins, *Pe*-AFP1 shows different amino acids at these positions, Phe and Asp residues instead (Fig. 3, asterisks). Moreover, *Pe*-AFP1 and the previous described 2S protein from *P. edulis* show different molecular masses, suggesting that this plant species has at least two antifungal proteins belonging to the 2S family.

3.3. Antifungal activity

The partially purified *Pe*-AFP1 obtained by affinity chromatography on Red-Sepharose column caused growth inhibition of *T. harzianum* (80%), *A. fumigatus* (60%) and *F. oxysporum* growth (70%) but not of *R. solani*, *P. brasiliensis* and *C. albicans* (Fig. 4A)

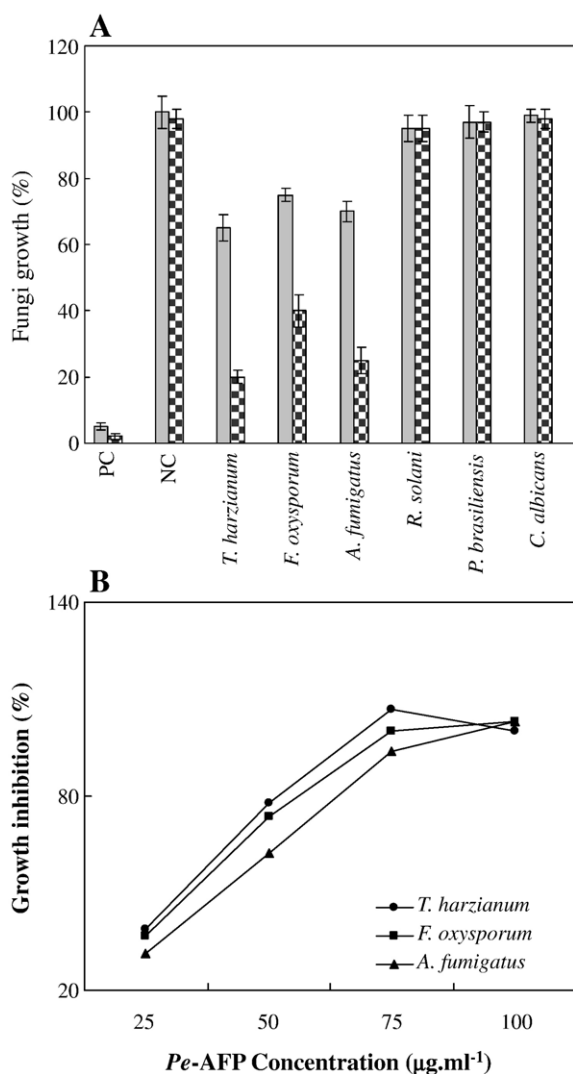


Fig. 4. (A) Antifungal activity of Red-Sepharose retained peak (■) and *Pe*-AFP1 (□) obtained from passion fruit. Vertical bars represent standard deviation. Each assay was carried out in triplicate. (B) In vitro assays by using *Pe*-AFP1 toward the filamentous fungi *Trichoderma harzianum*, *Fusarium oxysporum*, and *Aspergillus fumigatus*, showing IC₅₀ values of 32, 34, and 40 μg ml⁻¹, respectively. Vertical bars represent standard deviation. Each assay was carried out in triplicate. Each replicate do not differed more than 12%.

as compared to the corresponding positive controls. These last were able to totally inhibit fungi growth (data not shown). When the highly homogenous *Pe*-AFP1 peptide was tested against the filamentous fungi *T. harzianum*, *F. oxysporum*, and *A. fumigatus* (Fig. 4B), the IC₅₀ values obtained in the bioassays were 32, 34, and 40 μg ml⁻¹, respectively, indicating that the antifungal activity verified in the retained peak eluted from the Red-Sepharose column was at least partly due to *Pe*-AFP1.

4. Discussion

This report described the purification and biochemical characterization of a novel plant peptide, *Pe*-AFP1, purified from the seeds of passion-fruit with similarities to 2S albumin proteins. The purification steps of *Pe*-AFP1, which include affinity chromatography on Red-Sepharose and reversed-phase chromatography on Vydac C-18TP column, were very similar to those employed for purification of plant defence peptides [41]. Additionally similar acetonitrile concentrations in identical column were used to elute antimicrobial peptides from Japanese bamboo shoots (*Phyllostachys pubescens*), *Pp*-AMP1 and *Pp*-AMP2. Indeed, in these cases, these defensins were eluted at 41% acetonitrile concentration [44]. Furthermore, defensins from tobacco (*Nicotiana glauca*) and petunia (*Petunia hybrida*) were eluted at 30.7% and 29.5% acetonitrile, respectively [45]. A chitin-binding peptide from sugar beet leaves also showed a similar elution pattern for the major peaks, when compared to *Pe*-AFP1 chromatograms [46]. Nevertheless, in spite of these above similarities, further results indicated that *Pe*-AFP1 does not belong to these classes of peptides, but to the 2S family. All together, these above data provide clear evidence that not only plant defensins could be purified following a similar protocol but also several other classes of plant defence peptides.

The N-terminal amino acid sequence of *Pe*-AMP1 (Fig. 3) showed high conserved alignment compared with peptides of the 2S albumin family but not with those of other plant defence-related protein families. Using the FASTA3 program, *Pe*-AFP1 was shown 50%, 84% and 82% similarities with 2S albumin proteins from *P. edulis*, *V. vinifera* and *S. indicum*, respectively [42,43]. Therefore, *Pe*-AFP1 also showed clear similarity, which range from 50 to 70%, to 2S proteins from *Ricinus communis*, *B. excelsa* and *G. max* [53–55] and low similarities (less than 45%) to *M. parviflora*, *R. sativum*, *Picea glauca* and *Brassica napus* [27,30,48,51,52,56]. However, the 2S albumins are a major group of storage proteins presented in various dicotyledonous plant species that have been involved in some allergic reactions, such as the 2S albumin from Brazil nut, cashew nut, *B. napus* and *Brassica rapa* [25,26,29,47]. Nevertheless, *Pe*-AFP1 has not or shows low similarities to these allergenic peptides. The allergenicity of passion fruit peptide probably will be assessed in further studies, relating structure and biological properties. Alignment of *Pe*-AFP1 fragment with the plant 2S albumins revealed another intriguing data. Although almost all aligned proteins presented conserved Cys₅ and Cys₁₃ residues (Fig. 3, asterisks), *Pe*-AFP1 shows Phe₅ and Asp₁₃ instead. This particularity was only shared by CW-1 from *M. parviflora*, which showed Phe₅ and an acidic residue, Glu, in position 13. These data suggest that distinct

disulfide patterns could be found in antifungal 2S albumins, once that two-conserved Cys residues, marked by asterisks in Fig. 3, in formation of two distinct disulfide bond. In fact, *P. edulis* amino acid fragment here sequenced represent the first and second helices of 2S protein, in comparison to napin structures. Additional comparisons to other plant 2S proteins, such as BnIb from *B. napus* and SFA-8 from *Helianthus annuus*, revealed that these helices positions are wide variable with an enhanced diversity of amino acids [48,49].

Previous studies have proposed that proteins belonging to the 2S family are mainly storage molecules to which the major researcher interests are on their allergen characteristics [29]. The first member of 2S albumin family, which showed the ability to reduce fungi growth, was isolated from *R. sativum* [56]. Further studies, however, have revealed that other 2S proteins could also display a similar activity or possess the ability of inhibiting proteinases [31,50] as that (CW-1) with 5.0 kDa purified from *M. parviflora* seeds [27,30]. Furthermore, two 12 kDa proteins from *P. edulis* showed high similarity to 2S albumins and also antifungal activity, strengthening the idea that storage proteins could have, in addition, a secondary function as plant defence molecules [28]. In this current report, a 5 kDa peptide from *P. edulis*, *Pe*-AFP1, showed high similarity to 2S albumin storage proteins, a group known by its ability to serve as sulfur and nitrogen sources for plant germination [28]. Moreover, *Pe*-AFP1 caused considerable growth inhibition of three filamentous fungi, *T. harzianum*, *A. fumigatus*, and *F. oxysporum*. Conversely, *Pe*-AFP1 was incapable of inhibiting any kind of bacteria and the two yeasts tested (data not shown). These are not pioneering data since other 2S albumins were demonstrated to possess antifungal activity. For instance, peptides belonging to this family have been isolated from *M. parviflora* and showed inhibitory effects on the growth of *F. graminearum* and *Phytophthora infestans* [30]. Several other similar 2S proteins from *Brassicaceae* species also have antifungal properties and are able of inhibiting the development of *F. oxysporum*, *F. culmorum*, *Alternaria brassicola*, *Botrytis cinerea*, *Pyrularia oryzae*, and *Verticillium dahliae* [50]. Possibly, 2S albumins might be involved in an interesting plant defence network since in vitro synergic effects of plant γ -thionins and 2S albumins were described to occur on fungus growth [50].

In conclusion, we demonstrate the presence of a novel peptide in passion fruit seeds, named *Pe*-AFP1, with close similarity to 2S albumins, which possesses antifungal activity towards fungi that cause disease in human and plants. Therefore, *Pe*-AFP1 could contribute, in a near future, to the production of antifungal drugs to control human infection and to the development of transgenic plants with enhanced resistance to phytopathogenic fungi. Additionally, it may contribute for the establishment of a consistent classification system for the 2S albumin family based on the disulfide bridge patterns and for a better understanding of plant defence mechanisms.

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References

- [1] C.P. Selitrennikoff, Antifungal proteins, *Appl. Environ. Microbiol.* 67 (2001) 2883–2884.
- [2] P.B. Pelegrini, O.L. Franco, Plant gamma-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins, *Int. J. Biochem. Cell Biol.* 37 (2005) 2239–2253.
- [3] L.C. Van Loon, W.S. Pierpoint, T. Boller, V. Conejero, Recommendations for naming plant pathogenesis-related proteins, *Plant. Mol. Biol. Report.* 12 (1994) 245–264.
- [4] L.C. Van Loon, E.A. Van Strien, The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins, *Physiol. Mol. Plant Pathol.* 55 (1999) 85–97.
- [5] X. Liu, B. Huang, J. Lin, J. Fei, Z. Chen, Y. Pang, X. Sun, K. Tang, A novel pathogenesis-related protein (SsPR10) from *Solanum surattense* with ribonucleolytic and antimicrobial activity is stress- and pathogen-inducible, *J. Plant Physiol.* 163 (2006) 546–556.
- [6] A. Muradov, L. Petrasovits, A. Davidson, K.J. Scott, A cDNA clone for a pathogenesis-related protein 1 from barley, *Plant Mol. Biol.* 23 (1993) 439–442.
- [7] T. Bryngelsson, J. Sommer-Knudsen, P.L. Gregersen, D.B. Collinge, B. Ek, H. Thordal-Christensen, Purification, characterization, and molecular cloning of basic PR-1-type pathogenesis-related proteins from barley, *Mol. Plant-Microb. Interact.* 7 (1994) 267–275.
- [8] A. Molina, J. Gorch, S. Volrath, J. Ryals, Wheat genes encoding two types of PR-1 proteins are pathogen inducible, but do not respond to activators of systemic acquired resistance, *Mol. Plant-Microb. Interact.* 12 (1999) 53–58.
- [9] M. Rauscher, A.L. Adam, S. Wirtz, R. Guggenheim, K. Mendgen, H.B. Deising, PR-1 protein inhibits the differentiation of rust infection hyphae in leaves of acquired resistant broad bean, *Plant J.* 19 (1999) 625–633.
- [10] L.C. Van Loon, Occurrence and properties of plant pathogenesis-related proteins, in: S.K. Datta, S. Muthukrishnan (Eds.), *Pathogenesis-related Proteins in Plants*, CRC Press, Boca Raton, 1999, pp. 1–19.
- [11] G.K. Agrawal, N.S. Jwa, R. Rakwal, A novel rice (*Oryza sativa* L.) acidic PR1 gene highly responsive to cut, phytohormones, and protein phosphatase inhibitors, *Biochem. Biophys. Res. Commun.* 274 (2000) 157–165.
- [12] A.B. Christensen, B. Ho Cho, M. Naesby, P.L. Gregersen, J. Brandt, K. Madriz-ordenana, D.B. Collinge, H. Thordal-Christensen, The molecular characterization of two barley proteins establishes the novel PR-17 family of pathogenesis-related proteins, *Mol. Plant Pathol.* 3 (2002) 135–144.
- [13] F. Meuriot, C. Noquet, J.-C. Avicé, J.J. Volenc, S.M. Cunningham, T.G. Sors, S. Caillot, A. Ourry, Methyl jasmonate alters N partitioning, N reserves accumulation and induces gene expression of a 32-kDa vegetative storage protein that possesses chitinase activity in *Medicago sativa* taproots, *Physiol. Plant.* 120 (2004) 113–123.
- [14] F.R.G. Terras, K. Eggermont, V. Kovaleva, N.V. Raikel, R.W. Osborn, A. Kester, S.B. Rees, S. Torreken, F. Van-Leuven, K. Vanderleyden, B.P.A. Cammue, W.F. Broekaert, Small cysteine-rich antifungal proteins from radish: their role in host defense, *Plant Cell* 7 (1995) 573–588.
- [15] V. Arondel, J.C. Kader, Lipid transfer in plants, *Experientia* 46 (1990) 579–585.
- [16] M.S. Castro, W. Fontes, Plant defense and antimicrobial peptides, *Prot. Peptide Letters* 12 (2005) 13–18.
- [17] B.P. Cammue, K. Thevissen, M. Hendriks, K. Eggermont, I.J. Goderis, I. Proost, J. Van Damme, R.W. Osborn, F. Guerbet, J.C. Kader, A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins, *Plant Physiol.* 109 (1995) 445–455.
- [18] L. Hwu, C.C. Huang, D.T. Chen, A. Lin, The action mode of the ribosome-inactivating protein a-sarcin, *J. Biomed. Sci.* 7 (2000) 420–428.
- [19] B. Desvoves, J.L. Poyet, J.L. Schlick, P. Adami, M. Jouvenot, P. Dulieu, Identification of a biological inactive complex form of pokeweed antiviral protein, *FEBS Lett.* 410 (1997) 303–308.
- [20] F.R. Joerg, B.M. Christine, E.N. Donald, J.B. Hans, Induction of a ribosome-inactivating protein upon environmental stress, *Plant Mol. Biol.* 35 (1997) 701–709.
- [21] S.K. Song, Y.H. Choi, Y. Moon, S.G. Kim, Y.D. Choi, J.S. Lee, Systemic induction of a *Phytolacca insularis* antiviral protein gene by mechanical wounding, jasmonic acid, and abscisic acid, *Plant Mol. Biol.* 43 (2000) 439–450.

- [22] X.Y. Ye, T.B. Ng, Mungin, a novel cyclophilin-like antifungal protein from the mung bean. *Biochem. Biophys. Res. Commun.* 273 (22) 1111–1115.
- [23] P. Ostoa-Saloma, J.C. Carrero, P. Petrossian, P. Herion, A. Landa, J.P. Laclette, Cloning, characterization and functional expression of a cyclophilin of *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* 107 (2000) 219–225.
- [24] F.J. Moreno, J.A. Jenkins, F.A. Mellon, N.M. Rigby, J.A. Robertson, N. Wellner, E.N. Clare-Mills, Mass spectrometry and structural characterization of 2S albumin isoforms from Brazil nuts (*Bertholletia excelsa*). *Biochim. Biophys. Acta* 1698 (2004) 175–186.
- [25] J.M. Robotham, F. Wang, V. Seamon, S.S. Teuber, S.K. Sathe, H.A. Sampson, K. Beyer, M. Seavy, K.H. Roux, Ana o 3, an important cashew nut (*Anacardium occidentale* L.) allergen of the 2S albumin family. *J. Allergy Clin. Immunol.* 115 (2005) 1284–1290.
- [26] T.J. Puumalainen, S. Poikonen, A. Kotovuori, K. Vaali, N. Kalkinen, T. Reunala, K. Turjanmaa, T. Palosuo, Napins, 2S albumins, are major allergens in oilseed rape and turnip rape. *J. Allergy Clin. Immunol.* 117 (2006) 427–732.
- [27] X. Wang, G.J. Bunkers, Potent heterologous antifungal proteins from cheeseweed (*Malva parviflora*). *Biochem. Biophys. Res. Commun.* 279 (2000) 669–673.
- [28] A.P. Agizzio, A.O. Carvalho, F.F. Ribeiro, O.L.T. Machado, E.W. Alves, L.A. Okorokov, S.S. Samarão, C. Bloch Jr., M.V. Prates, V.M. Gomes, A 2S albumin-homologous protein from passion fruit seeds inhibits the fungal growth and acidification of the médium by *Fusarium oxysporum*. *Arch. Biochem. Biophys.* 416 (2003) 188–195.
- [29] H. Breiteneder, C. Radauer, A classification of plant food allergens. *J. Allergy Clin. Immunol.* 113 (2004) 821–830.
- [30] X. Wang, G.J. Bunkers, M.R. Walters, R.S. Thoma, Purification and characterization of three antifungal proteins from cheeseweed (*Malva parviflora*). *Biochem. Biophys. Res. Commun.* 282 (2001) 1224–1228.
- [31] F.R.G. Terras, S. Torrekens, F.V. Leuven, R.W. Osborn, J. Vanderleyden, B.P.A. Cammue, W.F. Broekaert, A new family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* species. *FEBS Lett.* 316 (1993) 233–240.
- [32] U.K. Laemmli, Cleavage of structural proteins using assembly of the head of the bacteriophage T4. *Nature* 227 (1970) 680–685.
- [33] W.R. Pearson, D.J. Lipman, Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U. S. A.* 85 (1988) 2444–2448.
- [34] W.R. Pearson, Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods Enzymol.* 183 (1990) 63–98.
- [35] A. Bairoch, R. Apweiler, The SWISS-PROT protein sequence database: its relevance to human molecular medical research. *J. Mol. Med.* 75 (1997) 312–316.
- [36] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22 (1994) 4673–4680.
- [37] S. Oard, M.C. Rush, J.H. Oard, Characterization of antimicrobial peptides against a US strain of the rice pathogen *Rhizoctonia solani*. *J. Appl. Microbiol.* 97 (2004) 169–180.
- [38] J.N. Galgiani, M.S. Bartlett, M.A. Ghannoum, A. Espinel-Ingroff, M.V. Lancaster, F.C. Odds, M.A. Pfaller, J.H. Rex, M.G. Rinaldi, T.J. Walsh, Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard. *Nac. Comit. Clin. Lab. Stand.* 17 (1997) 1–29.
- [39] C. Bloch Jr., M. Richardson, A new family of small (5 kDa) protein inhibitors of insect α -amylases from seeds of sorghum (*Sorghum bicolor* L.) Moench has sequence homologies with wheat γ -purothionins. *FEBS Lett.* 279 (1991) 101–104.
- [40] F.R. Melo, M.P. Sales, L.S. Pereira, C. Bloch Jr., O.L. Franco, M.B. Ary, α -Amylase inhibitors from cowpea seeds. *Prot. Peptide Letters* 6 (1999) 385–390.
- [41] F.R. Melo, D.J. Ridgen, O.L. Franco, L.V. Mello, M.B. Ary, M.F. Grossi-de-Sá, C. Bloch Jr., Inhibition of trypsin by cowpea thionin: characterization, molecular modeling, and docking. *Proteins* 48 (2002) 311–319.
- [42] S.S.K. Tai, T.T.T. Lee, C.C.Y. Sai, T.-J. Yui, J.T.C. Tzen, Expression pattern and deposition of three storage proteins, 11S globulin, 2S albumin and 7S globulin in maturing sesame seeds. *Plant Physiol. Biochem.* 39 (2001) 981–992.
- [43] Z.T. Li, D.J. Gray, Isolation by improved thermal asymmetric interlaced PCR and characterization of a seed-specific 2S albumin gene and its promoter from grape (*Vitis vinifera* L.). *Genome* 48 (2005) 312–320.
- [44] M. Fujimura, I. Ideguchi, Y. Minami, K. Watanabe, K. Tadera, Amino acid sequence and antimicrobial activity of chitin-binding peptides, *Pp*-AMP 1 and *Pp*-AMP 2, from Japanese bamboo shoots (*Phyllostachys pubescens*). *Biosci. Biotechnol. Biochem.* 69 (2005) 642–645.
- [45] F.T. Lay, F. Brugliera, M.A. Anderson, Isolation and properties of floral defensins from ornamental tobacco and petunia. *Plant Physiol.* 131 (2003) 1283–1293.
- [46] K.K. Nielsen, J.E. Nielsen, S.M. Madrid, J.D. Mikkelsen, Characterization of a new antifungal chitin-binding peptide from sugar beet leaves. *Plant Physiol.* 113 (1997) 83–91.
- [47] S.J. Koppelman, W.F. Nieuwenhuizen, M. Gaspari, L.M.J. Knippels, A.H. Penninks, E.F. Knol, S.L. Hefle, H.H.J. de Jongh, Reversible denaturation of Brazil nut 2S albumin (Bere1) and implication of structural destabilization on digestion by pepsin. *J. Agric. Food Chem.* 53 (2005) 123–131.
- [48] M. Rico, M. Bruix, C. González, R.I. Monsalve, R. Rodríguez, H NMR assignment and global fold of napin Bn1b, a representative 2S albumins seed protein. *Biochemistry* 35 (1996) 15672–15682.
- [49] D. Pantoja-Uceda, P.R. Shewry, M. Bruix, A.S. Tatham, J. Santoro, M. Rico, Solution structure of a methionin-rich 2S albumin from sunflower seeds: relationship to its allergenic and emulsifying properties. *Biochem. J.* 43 (2004) 6976–6986.
- [50] F.R.G. Terras, H.M.E. Shoofs, K. Thevissen, R.W. Osborn, J. Vanderleyden, B.P.A. Cammue, W.F. Broekaert, Synergistic enhancement of the antifungal activity of wheat and barley thionins by radish and oilseed rape 2S albumins and by barley trypsin inhibitors. *Plant Physiol.* 130 (1993) 1311–1319.
- [51] J.-Z. Dong, D.I. Dunstan, Cloning and characterization of six embryogenesis-associated cDNAs from somatic embryos of *Picea glauca* and their comparative expression during zygotic embryogenesis. *Plant Mol. Biol.* 39 (1999) 859–864.
- [52] S.M. McInnis, C.H. Newton, B.S.C. Sutton, Molecular characterization of a white spruce (*Picea glauca*) 2S albumin gene and related pseudogene. Unpublished. (Swissprot direct submission).
- [53] E.S. Gander, K.O. Holmstroem, G.R. De Paiva, L.A. De Castro, M. Carneiro, M.F. Grossi de Sa, Isolation, characterization and expression of a gene coding for a 2S albumin from *Bertholletia excelsa* (Brazil nut). *Plant Mol. Biol.* 16 (1991) 437–448.
- [54] R. Jung, C. Hastings, S.J. Coughlan, W.-N. Hu, Sulfur- and lysine-rich napin-type 2S albumins from soybean seed. Unpublished. (Swissprot direct submission).
- [55] S.D. Irwin, J.N. Keen, J.B.C. Findlay, J.M. Lord, The *Ricinus communis* 2S albumin precursor. A single preproprotein may be processed into two different heterodimeric storage proteins. *Mol. Gen. Genet.* 222 (1990) 400–408.
- [56] F.R. Terras, H.M. Schoofs, M.F. De Bolle, F. Van Leuven, S.B. Rees, J. Vanderleyden, B.P. Cammue, W.F. Broekaert, Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.* 267 (1992) 15301–15309.