Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae

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Abstract **From seeds of *Aesculus hippocastanum*, *Clitoria ternatea*, *Dahlia merckii* and *Heuchera sanguinea* five antifungal proteins were isolated and shown to be homologous to plant defensins previously characterised from radish seeds and γ-thionins from Poaceae seeds. Based on the spectrum of their antimicriobial activity and the morphological distortions they induce on fungi the peptides can be divided into two classes. The peptides did not inhibit any of three different α-amylases.**

Key words: Peptide; Amino acid sequence; γ-Thionin; Antifungal; Antimicrobial

1. Introduction

Cysteine-rich antimicrobial peptides have been isolated from a wide range of organisms including microorganisms themselves (reviewed in [1]). More recently it has become increasingly clear that these types of peptide have an important role to play in the protection of plants. At least four different classes of cysteine-rich antimicrobial peptide have been isolated from seeds of barley and wheat [2], *Mirabilis jalapa* [3], *Amaranthus caudatus* [4] and five Brassicaceae species [5,6]. This latter class of peptide appears to be widely distributed in the plant kingdom and homologous peptides have been isolated from seeds [7–10] or identified via sequencing of cDNA clones [11–14] in both monocot and dicot species. A number of different in vitro activities have been attributed to this class of peptide including inhibition of in vitro protein synthesis [8,9], inhibition of α-amylases [10] and antimicrobial activity [5–7]. Based on the inhibitory activity of at least some members of this peptide family to plant pathogenic fungi, their expression patterns in planta [11,12] and their three-dimensional structures, which show homology to that of insect defensins [15], we have proposed the name plant defensin to describe this family [16].

In this paper we describe the isolation of five new members of the plant defensin family from seeds of *Aesculus hippocastanum* (Hippocastanaceae), *Clitoria ternatea* (Fabaceae), *Dahlia merckii* (Asteraceae) and *Heuchera sanguinea* (Saxifragaceae). Comparative assays for antifungal, antibacterial and α-amylase-inhibition activity between these peptides and other members of this family are reported.

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Abbreviations: AFP, antifungal protein; AMP, antimicrobial protein; Ah, *Aesculus hippocastanum*; Ct, *Clitoria ternatea*; Dm, *Dahlia merckii*; Hs, *Heuchera sanguinea*; Rs, *Raphanus sativus*; IC_{50}, protein concentration required for 50% growth inhibition; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

2. Materials and methods

2.1. Biological materials

Seeds were purchased from Chiltern Seeds (Cumbria, UK) or collected locally (*A. hippocastanum*). Fungi were maintained and spores harvested as previously described [17]. Bacteria were pre-cultured in 1% tryptone at 28°C on a rotary shaker. The following fungal and bacterial strains were used: *Botrytis cinerea* (K1147), *Cladosporium sphaerospermum* (K0791), *Fusarium culmorum* (K0311), *Leptosphaeria maculans* (LM36uea), *Neurospora crassa* (CBS 32754), *Penicillium digitatum* (K0879), *Trichoderma viride* (K1127), *Septoria tritici* (K1097), *Verticillium albo-atrum* (K0937); *Bacillus subtilis* (JHCC 553331), *Escherichia coli* (HB101), *Micrococcus luteus* (ATCC 9341), *Proteus vulgaris* (JHCC 558711), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus faecalis* (ATCC 29212).

2.2. Extraction of seed proteins

The purification of antimicrobial peptides from seeds was carried out essentially as described previously [5,6]. 500 g amounts were ground in a coffee-mill and protein extacted by stirring overnight at 4°C in extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA, 1.5% PVPP). *A. hippocastanum* seeds were peeled and lyophilised prior to extraction. Following a 70% (relative saturation) ammonium sulphate precipitation the pellets were resuspended in distilled water, extensively dialysed against distilled water using 2000 Da cut-off dialysis tubing (Sigma) and finally adjusted to 50 mM NH_4Ac (pH 9). Extracts were passed over a Q-Sepharose Fast Flow column (12 × 5 cm, Pharmacia) equilibrated in 50 mM NH_4Ac (pH 9) and the unbound proteins collected. This flow-through fraction was adjusted to pH 6 with acetic acid and passed over an S-Sepharose Fast Flow column (10 × 2.6 cm, Pharmacia) equilibrated in 50 mM NH_4Ac (pH 6). Bound proteins were eluted with a linear gradient of 50 mM–1 M NH_4Ac (pH 6) over 400 min at 3 ml/min. Proteins were monitored by on-line measurement of the absorbance at 280 nm.

Fractions with the highest antifungal activity were pooled for each peak and further purified by RP-HPLC on a Pep-S column (C_2/C_{18} silica, 25 × 0.93 cm, Pharmacia). Peptides were eluted with linear gradients of 0.1% TFA to 100% acetonitrile/0.1% TFA over 100 min at 3 ml/min and monitored by on-line measurement of the absorbance at 214 nm.

Samples of SIα1, SIα2 and SIα3 from *Sorghum bicolor* and γ1-purothionin from wheat were gifts from Carlos Bloch and Enrique Mendez, respectively. Rs-AFP1 and Rs-AFP2 were purified from radish seeds as described [5].

2.3. Electrophoresis and amino acid sequencing

SDS-PAGE was carried out using Pharmacia's PhastSystem on precast high-density gels. Proteins were diffusion blotted onto nitrocellulose and visualised by silver staining as described [18].

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N-terminal amino acid sequences were determined by automatic Edman degradation on a 477A Protein Sequencer (Applied Biosystems, CA). Cystine residues were reduced and cysteines subsequently alkylated with 4-vinylpyridine prior to sequencing [5].

2.4. Antifungal and antibacterial assays

Antifungal and antibacterial assays were carried out as previously described [5,17]. Unless otherwise indicated, the growth medium for the antifungal assays was either half-strength potato dextrose broth (1/2 PDB, from Difco) or 1/2 PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl. Assays to determine effects of temperature and cations on antifungal activity were carried out in a synthetic low salt medium (SMF) [3]. The growth medium for antibacterial activity assays was 1% tryptone, 0.5% low melting point agarose. Growth was assessed after the appropriate incubation period (48 h at 24°C for fungi and 24 h at 28°C for bacteria).

2.5. α-Amylase inhibition assay

A crude α-amylase extract was prepared from dissected guts of adult cockroaches (*Blatta orientalis*) by homogenising in 20 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$ and removal of cell debris by centrifugation. Human and porcine α-amylases were purchased from Sigma. Type 1-α-amylase inhibitor from wheat (purchased from Sigma) was used as a positive control. Amylase extracts were incubated with peptides for 20 min at 30°C prior to addition of starch and enzyme activity was detected using the method of Bernfeld [19].

3. Results

3.1. Purification and characterisation of seed antifungal peptides

The basic protein fractions from the four different plant seeds were fractionated on an S-Sepharose cation exchange column and fractions with antifungal activity identified by assaying for inhibition of *F. culmorum* spore germination. Each of the extracts yielded a single major peak of activity eluting around 300 mM NH_4Ac in the gradient. Fractions from each peak were pooled and further purified by RP-HPLC to obtain homogeneous active peptides. The pooled active fractions from S-Sepharose fractionation of the *D. merckii* extract yielded two peaks on RP-HPLC as monitiored by the absorbance at 214 nm. Both peaks contained antifungal activity and the active material from each peak was designated Dm-AMP1 and Dm-AMP2. The S-Sepharose-purified active fraction from *H. sanguinea* also separated as two peaks upon RP-HPLC, only the smallest and most polar of which, designated Hs-AFP1, showed significant antifungal activity. The active peaks from the cation exchange chromatography of the *A. hippocastanum* and *C. ternatea* extracts gave single peaks of absorbance at 214 nm on RP-HPLC, each of which possessed antifungal activity. The purified active fractions for the *A. hippocastanum* and *C. ternatea* extracts were designated Ah-AMP1 and Ct-AMP1, respectively. SDS-PAGE was performed to estimate the molecular masses of the purified peptides. Peptides were analysed under reducing conditions and each gave a single band of approx. 5 kDa (Fig. 1). Under non-reducing conditions the peptides migrated as single bands of between 15 and 20 kDa (data not shown). This apparent oligomerisation under non-reducing conditions has been reported for the Brassicaceae plant defensins [5,6] and it is not known whether this represents their native state or is an artifact of the gel system used.

Peptides were subjected to automated N-terminal sequencing and the deduced sequences are given in Fig. 2. Complete amino acid sequences were obtained for all the peptides except for Dm-AMP2 where only the first 20 residues were sequenced. Within this region Dm-AMP2 differs from Dm-AMP1 at only one position. The length of the fully sequenced peptides varies from 49 amino acids for Ct-AMP1 to 54 amino acids for Hs-AFP1. All the peptides show a high degree of homology to each other as well as to the plant defensin Rs-AFP2 from radish seeds [5], the N-terminal 20 amino acids of PTh-St1 from potato tubers [7], the α-amylase inhibitor from *Sorghum* seeds, SIα2 [10] and the γ-thionins from wheat and barley seeds [8,9] (Fig. 2). Residues conserved in all sequences are all eight cysteines, two glycines at positions 13 and 34, a serine at position 8, an aromatic residue at position 11 and a glutamic acid at position 29 (numbering relative to Rs-AFP2).

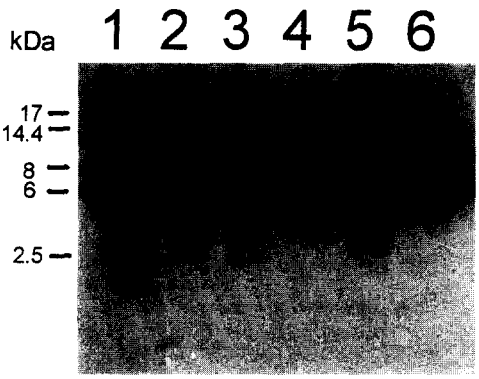

Fig. 1. SDS-PAGE analysis of the purified peptides. 200 ng of the different peptides were dissolved in reducing sample buffer, separated on a high-density Phastgel and blotted onto nitrocellulose membrane prior to silver-staining. Lanes: 1 = myoglobin fragments with molecular masses in kDa as indicated; 2 = Dm-AMP1; 3 = Dm-AMP2; 4 = Ct-AMP1; 5 = Hs-AFP1; 6 = Ah-AMP1.

3.2. Antimicrobial properties of the purified peptides

The antifungal properties of the five purified peptides were compared by determining protein concentrations required for 50% inhibition of fungal growth (IC_{50} values). Eight fungi were included and tests were carried out in two media: 1/2 PDB and 1/2 PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl. The results are shown in Table 1. In the medium without the addition of salt, IC_{50} values were generally in the 1–25 μg/ml range except on *T. viride* where only Hs-AFP1 showed any significant inhibition. Addition of salts to the medium severely decreased the activity of the peptides although the sensitivity of their antifungal activity largely depended on the fungus. For instance on many of the fungi activity was no longer detected even at 100 μg/ml whereas the IC_{50} values on *Sep. tritici* for all the peptides except Ct-AMP1 were still below 10 μg/ml. Sensitivity of antifungal activity to inorganic cations has been reported for

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Ah-AMP1      LCNERPSQTWSGNCGNTAHCDKQCQDWEK-ASHGACHKRENBWKCFCYFNC
Ct-AMP1     NLC-ERASLTWTGNCGNTGHCDTQCRNWES-AKHGACHKR-GNWKCFCYFNC
Dm-AMP1     ELC-EKASKTWSGNCGNTGHCDNQCKSWEG-AAHGACHVRNGKEMCFCYFNC
Dm-AMP2     EVC-EKASKTWSGNCGNTGHC
Hs-AFP1  DGVKLC-DVPSGTWSGHCGSSSKCSQQCKDREHFAYGGACHYQFPSVKCFCKRQC

Rs-AFP2    ZKLC-QRPSGTWSGVCGNNNACKNQCIRLEK-ARHGSCNYVFPAHKCICYFPC
PTh-St1     RNC-ESLSHRFKGPCTRDSNC
SIα2        RVC-MGKSAGFKGLCMRDQNCAQVCL-QEG-WGGGNCDGVM--RQCKCIRQCW
γ1-P        KIC-RRRSAGFKGPCMSNKNCAQVCQ-QEG-WGGGNCDGPF--RRCKCIRQC
γ1-H        RIC-RRRSAGFKGPCVSNKNCAQVCM-QEG-WGGGNCDGPL--RRCKCMRRC
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Fig. 2. Alignment of amino acid sequences of the purified peptides with those of Rs-AFP2 [5], PTh-St1 [7], SIα2 [10], γ1-purothionin from wheat [8] and γ1-H from barley [9]. All amino acid sequences are full length except Dm-AMP2 and PTh-St1. Dashes indicate gaps introduced to maximise homology.

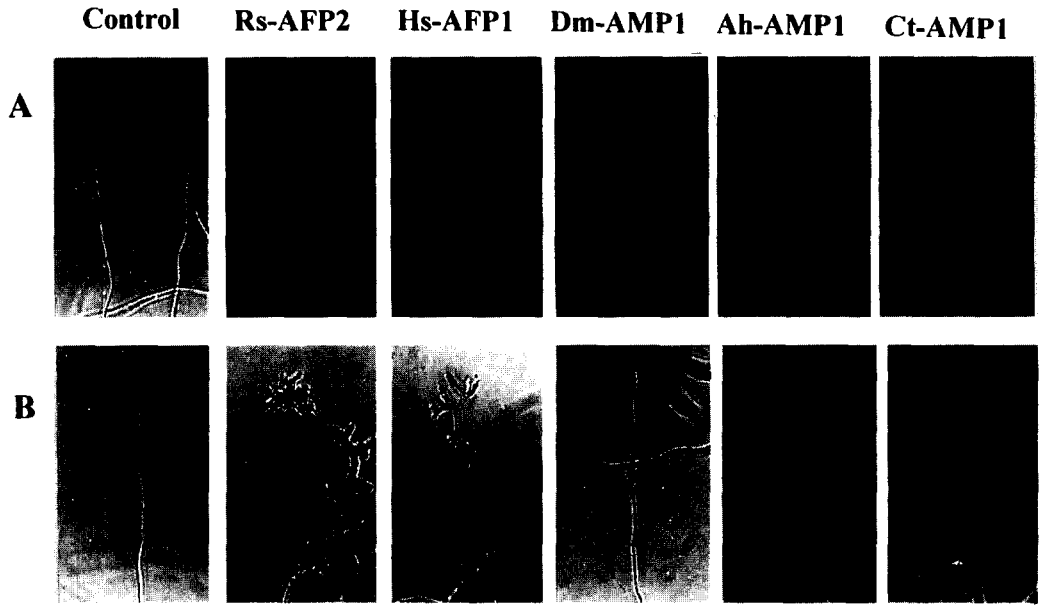

Fig. 3. Morphological changes induced by the peptides on *Fusarium culmorum*. (A) Fungal spores were germinated and grown in 1/2 PDB in the presence of the peptides and observed after 24 h of incubation. The concentration of the peptides was 1.25, 0.3, 2.5, 1.25 and 2.5 μg/ml for Rs-AFP2, Hs-AFP1, Dm-AMP1, Ah-AMP1 and Ct-AMP1, respectively. (B) Fungal spores were germinated and grown in 1/2 PDB. After 24 h of preincubation, the peptides were added and the hyphae observed 6 h later. The concentration of peptides was 1.25, 0.3, 5, 5 and 5 μg/ml for Rs-AFP2, Hs-AFP1, Dm-AMP1, Ah-AMP1 and Ct-AMP1, respectively.

a number of proteins including the plant defensin homologues from Brassicaceae seeds [3–6]. The radish plant defensin Rs-AFP2 [5] was included in these assays for comparison and on the range of fungi tested its activity and sensitivity to inorganic cations is similar to that of the other peptides (Table 1). Comparative antifungal assays were also carried out with other members of the family, namely the three homologues from *S. bicolor* (SIα1–3) [10] and γ1-purothionin from wheat [8]. On the five fungi tested only SIα1 was found to possess any antifungal activity and then only on three of the fungi and at rates higher than 50 μg/ml (Table 1).

When inhibited fungi were visualised under the microscope the peptides can be seen to cause distinct morphological changes (Fig. 3). On some fungi, typified by *F. culmorum*, Hs-AFP1 and Rs-AFP2 cause germ tubes and hyphae to swell and form multiple hyphal buds. Dm-AMP1, Ah-AMP1 and Ct-AMP1 inhibit the rate of elongation of *F. culmorum* germ tubes but do not cause the swelling and budding seen with the other

Table 1
Antifungal activity of the different peptides

Fungus	IC_{50} values (μg/ml)						
	Ah-AMP1	Ct-AMP1	Dm-AMP1	Dm-AMP2	Hs-AFP1	SIα1	Rs-AFP2
Medium A[a]							
Botrytis cinerea	25	20	12	10	6	100	10
Cladosporium sphaerospermum	0.5	6	3	3	1	80	3
Fusarium culmorum	12	10	5	3	1	>200	1.5
Leptosphaeria maculans	0.5	6	1.5	1	25	nt	12
Penicillium digitatum	6	20	2	2	1	>200	1.5
Trichoderma viride	>100	>100	>100	>100	15	50	30
Septoria tritici	0.5	2	1	1	0.5	nt	1.5
Verticilium albo-atrum	6	2	4	2	12	nt	12
Medium B[b]							
Botrytis cinerea	>100	>100	>100	>100	25	>200	100
Cladosporium sphaerospermum	12	100	12	12	3	>200	10
Fusarium culmorum	>100	50	8	12	3	>200	6
Leptosphaeria maculans	6	20	15	20	>100	nt	>100
Penicillium digitatum	25	80	70	50	3	>200	12
Trichoderma viride	>100	>100	>100	>100	>100	>200	>100
Septoria tritici	1.5	80	4	2	3	nt	6
Verticilium albo-atrum	>100	>100	>100	>100	30	nt	>100

Protein concentrations required for 50% growth inhibition after 48 h at 24°C were determined from dose–response curves. nt = not tested.
[a]1/2 strength potato dextrose broth.
[b]Medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCl.

Table 2
Effect of temperature on the antifungal activity of the peptides

Protein	IC_{50} (μg/ml)							
	Fusarium culmorum				*Neurospora crassa*			
	10°C	16°C	20°C	30°C	10°C	16°C	20°C	30°C
Ah-AMP1	0.3	0.5	0.9	20	0.5	0.25	0.3	0.6
Ct-AMP1	0.2	0.5	0.8	2.3	0.2	<0.3	<0.3	0.5
Dm-AMP1	0.5	1.3	1.3	4	1.5	1.3	1.5	4
Hs-AFP1	0.6	0.7	0.8	1	0.2	0.4	0.8	2
Rs-AFP2	2	5	4.5	4.5	5	3.5	4.2	4.5

IC_{50} values for the peptides were determined on *F. culmorum* and *N. crassa* in a synthetic growth medium (SMF) after 3 days of incubation at 20 and 30°C, 4 days of incubation at 16°C and 8 days of incubation at 10°C.

peptides (Fig. 3A). On hyphae from pregerminated spores of this fungus, Dm-AMPs, Ah-AMP1 and Ct-AMP1 cause a reduction in hyphal thickness and an apparent collapse of the plasmamembrane leading to an apparent fragmentation of the cytoplasm (Fig. 3B). On *N. crassa* hyphae, however, there was no apparent morphological distinction in the action of Rs-AFP2, Hs-AFP1, Dm-AMP1, Ah-AMP1 and Ct-AMP1. All five peptides reduced hyphal thickness when added to pregerminated *N. crassa* spores (not shown).

The antibacterial activities of the peptides were measured on four Gram-positive bacteria (*B. subtilis*, *M. luteus*, *Staph. aureus* and *Strep. faecalis*) and two Gram-negative bacteria (*E. coli* and *P. vulgaris*). Hs-AFP1 and Rs-AFP2 did not inhibit the growth of any of the bacteria at rates of 200 μg/ml. Dm-AMP1, Ct-AMP1 and Ah-AMP1 inhibited the growth of *B. subtilis* at 150, 15 and 100 μg/ml, respectively, but did not affect the growth of the other bacteria tested.

3.3. Effects of temperature and divalent cations on antifungal activity

To measure the effect of temperature on antifungal activity, IC_{50} values were determined for each of the peptides on *F. culmorum* and *N. crassa* in a synthetic growth medium (SMF) after incubation at different temperatures (Table 2). No significant effect of the incubation temperature on the antifungal activities of Hs-AFP1 and Rs-AFP2 could be detected for either fungal species. For Dm-AMP1, Ah-AMP1 and Ct-AMP1 their antifungal activity on *F. culmorum* was reduced at higher incubation temperatures. These peptides are approx. 10-fold more active at 10°C than at 30°C on *F. culmorum*. However, this effect was less pronounced with *N. crassa*.

The effect of the addition of Mg^{2+} and Ca^{2+} to the fungal growth medium was likewise investigated (Table 3). The antifungal activities of Hs-AFP1 and Rs-AFP2 on *N. crassa* were practically unaffected by 1 or 5 mM Ca^{2+} or Mg^{2+}. On *F. culmorum*, the antifungal activity of RsAFP2 is decreased by a factor of 2 by the addition of 5 mM Ca^{2+} or Mg^{2+} and that of Hs-AFP1 by a factor of 2 and 10 in the presence of 5 mM Mg^{2+} and Ca^{2+}, respectively. In contrast, the antifungal activities of Dm-AMP1 and Ah-AMP1 on both *N. crassa* and *F. culmorum* are reduced in the presence of both 1 and 5 mM Ca^{2+} or Mg^{2+}. This is also the case with Ct-AMP1 on *F. culmorum* although its activity on *N. crassa* is practically unaffected by the cations. The inhibition of activity of Dm-AMP1, Ah-AMP1 and Ct-AMP1 is markedly greater in the presence of Ca^{2+} than of Mg^{2+} (1–3- and 3–5-fold more on *N. crassa* and *F. culmorum*, respectively).

3.4. Inhibition of α-amylase activity

The α-amylase-inhibition activities of Dm-AMP1, Ah-AMP1, Ct-AMP1, Hs-AFP1 and Rs-AFP2 were compared on α-amylase preparations from three sources to that of the *S. bicolor* homologue SIα3, previously reported to inhibit insect gut α-amylases [10]. SIα3 inhibited the activity of the enzymes from insect gut and human saliva to greater than 70% at rates as low as 5 μg/ml. Comparable inhibition was achieved with 10

Table 3
Effect of different cations on the antifungal activity of the peptides

Protein	IC_{50} (μg/ml)				
	SMF	1 mM Ca^{2+}	5 mM Ca^{2+}	1 mM Mg^{2+}	5 mM Mg^{2+}
Fusarium culmorum					
Ah-AMP1	0.7	>50	>50	22	>50
Ct-AMP1	0.6	20	>40	7	>40
Dm-AMP1	1	23	>50	4	11
Hs-AFP1	0.9	1.5	20	1	2.5
Rs-AFP2	5	5.1	11	5	11
Neurospora crassa					
Ah-AMP1	0.5	3.3	18	0.8	4
Ct-AMP1	0.2	0.9	0.9	0.8	1.8
Dm-AMP1	1	4.5	22	3.4	9
Hs-AFP1	0.8	0.3	0.3	0.6	0.6
Rs-AFP2	5	5	5	5	5

IC_{50} values for the peptides were determined on *F. culmorum* and *N. crassa* in a synthetic growth medium (SMF) and in SMF supplemented with 1 or 5 mM of the chloride salts of the indicated cations.

U/ml of a commercial preparation of type 1 α-amylase inhibitor from wheat. SIα3 was essentially inactive on the enzyme from porcine pancreas as previously reported [10]. No inhibition of α-amylase activity was observed with any of the other peptides tested on the three enzymes even when included at rates as high as 200 μg/ml.

4. Discussion

We have isolated five novel peptides from seeds of four different dicotyledonous plant species belonging to four different families. The peptides are members of a plant peptide family grouped together based on amino acid sequence homologies but to which a number of different in vitro activities have been attributed. The peptides isolated in this study share similar biological activities to their homologues from Brassicaceae seeds [5,6] and Pth-St1 from potato tubers [7]. All these peptides have been shown to be inhibitory to a range of fungi but do not inhibit insect gut α-amylases. In contrast, other members of the family, namely those from Poaceae seeds, have been shown to inhibit either insect gut α-amylases [10] or protein synthesis in cell-free systems [8,9]. Examples of proteins to which these other activities have been attributed were tested for their antifungal activity on five different fungi and shown to be either devoid of activity or only very weakly active. A wider range of fungi would need to be tested to ascertain whether the lack of substantial antifungal activity detected in these tests is a generalisation, but it may well be that despite the sequence homology amongst the plant defensins inhibition of fungal growth is not a general property of this peptide family. It is conceivable, however, that the differences in the biological activities of the different members of the peptide family are a consequence of the specialisation of their task in host defence, which may be either directed towards phytophagous insects (for the peptides with α-amylase inhibitor activity) or towards phytopathogenic microorganisms (for the peptides with antimicrobial activity).

The five purified peptides cause very distinct morphological changes in some but not all fungi and can be divided into two classes based on these observations. Hs-AFP1 causes multiple budding and swelling of *F. culmorum* germ tubes and hyphae which is also seen with the Brassicaceae peptides as typified by Rs-AFP2. In contrast, the peptides purified from *D. merckii*, *C. ternatea* and *A. hippocastanum* inhibit the rate of germ tube elongation but cause few alterations to the morphology of *F. culmorum* germ tubes. On hyphae from pregerminated spores they cause hyphal collapse, probably reflecting a loss in turgescence. Whilst the observed morphological changes are not consistent on all fungi, the finding that there are clear differences on particular fungi would suggest that the two sets of peptides inhibit fungal growth by different mechanisms. This is also supported by the differential sensitivity of their antifungal activity to temperature and their ability to inhibit the growth of some Gram-positive bacteria. The antifungal activity of Ah-AMP1, Ct-AMP1 and Dm-AMP1 on *F. culmorum* is higher at lower temperatures and these peptides all inhibit to some extent growth of the Gram-positive bacteria *B. subtilis*. On the other hand, the antifungal activity of Hs-AFP1 and Rs-AFP2 is not affected by temperature and these peptides do not inhibit the growth of any of the six bacterial species tested.

All the purified peptides share a high degree of homology at the amino acid level to each other and to other members of the plant defensin family. Interestingly, the highest homologies are found between peptides which also share the same biological activities. Dm-AMP1, for instance, is 68% and 72% identical to Ah-AMP1 and Ct-AMP1, but only 46% identical to Hs-AFP1. Based on these homologies it may be possible to identify conserved residues which are important to the biological activities of the different classes of peptide. Recently, we have successfully expressed functional Rs-AFP2 in *Saccharomyces cerevisiae* [20] and this system is being used to express variants of the peptide which have single amino acid substitutions based on differences with γ1-purothionin.

Insect and human defensins, which have been shown to form voltage-gated ion channels in bacterial membranes, are more active at higher temperatures [20,21]. This supposedly reflects the greater fluidity of the membrane at higher temperatures facilitating protein insertion. However, the activity of the plant peptides studied here is either not affected by temperature or decreased at higher temperatures, which would argue against the membrane being the primary target site. If, on the other hand, the peptides are interacting with fungal receptors then identifying these receptors may provide an important insight into the mechanisms by which fungi control hyphal growth.

The peptides described in this paper have all been isolated from seeds but other members of the family have been shown to be either constitutively expressed in some vegetative cell-types or induced in others by fungal infection [7,11,12,16]. The occurrence of the described peptides in tissues of the plants other than their seed has yet to be investigated.

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