Synthetic and structural studies on *Pyrularia pubera* thionin: a single-residue mutation enhances activity against Gram-negative bacteria

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Abstract The thionin from *Pyrularia pubera* (Pp-TH), a 47-residue peptide with four internal disulfide bonds, was efficiently produced by chemical synthesis. Its antimicrobial activity in vitro against several representative pathogens (EC\(_{50}\) = 0.3–3.0 \(\mu\)M) was identical to that of natural Pp-TH. This peptide has a unique Asp\(^{32}\) instead of the consensus Arg found in other thionins of the same family. In order to evaluate the effect of this mutation, the Arg\(^{32}\) analogue (Pp-TH(D32R)) was also synthesized and showed a significant increase in antibiotic activity against several Gram-negative bacteria, whereas it retained the same activity against other pathogens. The overall structure of Pp-TH(D32R) was maintained, though a slight decrease in the helical content of the peptide was observed.

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Key words: Thionin; Antimicrobial peptide; Oxidative folding; Plant

1. Introduction

All organisms, from plants and insects to humans, produce gene-encoded cationic antimicrobial peptides [1-3] as constituents of their innate immune systems. In recent years these peptides have been proposed as a potential solution to the widespread appearance of resistance to classical antibiotics in clinically relevant bacteria [4-6]. Such expectations are based on the fact that these peptides exert their antimicrobial action through fairly simple mechanisms of membrane disruption [7-9] which are unlikely to promote the appearance of resistance [3].

In the course of our studies on antimicrobial peptides we have focused our attention on the thionins, the first family of plant peptides [10,11] for which activity against plant pathogens was demonstrated in vitro [12]. Thionins are basic peptides (minimum net charge of +2), usually 45-47 amino acids in length, with a high rate of sequence homology [10,11]. They have been classified into five structural types (I-V) based on their amino acid sequences and disulfide bridge patterns. The secondary structure of thionins is well conserved and consists of one \(\beta\)-sheet and two antiparallel \(\alpha\)-helices, with hydrophobic and hydrophilic residues segregated at the outer and inner helix surfaces, respectively. Such an amphipathic distribution gives thionins their ability to disrupt microbial membranes and phospholipid liposomes [13]. Despite sequence differences, the 3D structure of thionins of types I-IV is almost identical, resembling a capital gamma (\(\Gamma\)) [14].

The thionin from *Pyrularia pubera* (Pp-TH), a cytotoxic and hemolytic type II thionin [15], is remarkable for the presence of an Asp residue at position 32 instead of the consensus Arg found in most family I and II thionins with the same disulfide pattern (Fig. 1) [10,11]. In order to evaluate the effect of Asp\(^{32}\), and the ensuing loss of cationic character, on the activity of this peptide, we decided to synthesize both the native sequence and its Arg\(^{32}\) (D32R) analogue, with a view to expanding the spectrum of antimicrobial activity of this thionin.

We report here the chemical synthesis of Pp-TH and its D32R analogue, their study by circular dichroism (CD) and the in vitro antimicrobial activities against bacterial and fungal plant pathogens. The antibiotic activities of the synthetic and natural Pp-TH were found to be identical against the pathogens tested. In contrast, the D32R mutation selectively increased the activity against the Gram-negative species analyzed, whereas it did not affect the activity against other pathogens. The D32R substitution has only minor effects in the overall structure of the thionin.

2. Materials and methods

2.1. Peptide synthesis

The 47-amino acid sequence of Pp-TH (KSCCRNTWARN-CYNVCRLPFTISREIKKCDCKIKSGTCTPSDYPK) and its D32R analogue were assembled on a 9-fluorenylmethoxycarbonyl (Fmoc)-Lys(\(\text{\textsuperscript{Boc}}\))-polyethyleneglycol polystyrene resin (0.18 mmol/g, Applied Biosystems) by Fmoc solid-phase synthesis protocols using an Applied Biosystems 433A instrument. Side chain protection was as follows: Lys(Boc), Ser(\(\text{\textsuperscript{Boc}}\)-butyl), Arg(\(\text{\textsuperscript{Boc}}\)-butyl), Asp(\(\text{\textsuperscript{Boc}}\)-butyl ester), Cys(Trt), Trp(Boc), Tyr(\(\text{\textsuperscript{Boc}}\)-butyl), Glu(\(\text{\textsuperscript{Boc}}\)-butyl ester), Asp(\(\text{\textsuperscript{Boc}}\)-butyl ester) and Cys(Trt). Couplings (10 equivalent of Fmoc-amino acid) were mediated by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (PyBop). Couplings (10 equivalent of Fmoc-amino acid) were mediated by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (PyBop).
2.4 Modeling studies

2.3 Circular dichroism

2.2 Oxidative folding of octathiol precursors

Several oxidative folding conditions (see Section 3 and Table 1) were tested. In the optimal protocol, the purified octathiol was dissolved in 0.1 M NH₄OAc, pH 7.8, at 5 mM concentration, under Ar atmosphere, in the presence of 1 M Gn-HCl and reduced and oxidized glutathione (GSH, GSSG) to give a peptide GSH·GSSG ratio of 1:100:1. After gentle stirring for 48 h at 25°C, and since the Ellman test [16] of the major HPLC product was negative, the reaction mixture was quenched by TFA addition to pH 1.5-2, then directly loaded into a preparative reverse-phase HPLC system (see above). After eluting the salts with 0.1% TFA/water, the folded peptide was purified by a linear 40-80% gradient of 0.1% TFA/acetonitrile into 0.1% TFA/water over 60 min at 25 ml/min. Fractions judged to be homogeneous by analytical HPLC were pooled and their identity was further confirmed by amino acid analysis and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra, both of them consistent with the expected compositions.

2.5 Antimicrobial activity

Inhibition tests were carried out in sterile microtiter plates by mixing different amounts of the peptide dissolved in 66.7 μl of sterile water with either 33.3 μl of bacterial suspensions (final concentration 10^8 cfu/ml) in nutrient broth (Oxoid, Basingstoke, UK) or TY medium (Rothobium radiuli [17]), or 33.3 μl of fungal spore suspensions (final concentration 10^4 spores/ml) in potato dextrose (Difeo, Detroit, MI, USA). Growth of microorganisms was recorded 24-48 h after incubation at 28°C, by measuring absorbance at 490 nm in an ELISA plate reader. Spores used in the inhibition experiments were harvested and stored as previously described [18]. The bacterial pathogens Clavibacter michiganensis subsp. sepedonicus, strain S, Xanthomonas campestris pv. campestris, X. campestris pv. translucens and R. meliloti, as well as the fungal pathogen Botrytis cinerea (strain 1) were from the ETSA collection (Madrid, Spain). The fungi Plectosphaerella cucumerina and Fusarium oxysporum (strain 1) were from the EPM collection (Madrid, Spain). The fungi P. pustulosa, P. pustulosa (strain 1), B. cinerea and B. cinerea (strain 1) were from the ETSA collection (Madrid, Spain). The fungi P. pustulosa, P. pustulosa (strain 1), B. cinerea and B. cinerea (strain 1) were from the ETSA collection (Madrid, Spain). The fungi P. pustulosa, P. pustulosa (strain 1), B. cinerea and B. cinerea (strain 1) were from the ETSA collection (Madrid, Spain). The fungi P. pustulosa, P. pustulosa (strain 1), B. cinerea and B. cinerea (strain 1) were from the ETSA collection (Madrid, Spain).

3. Results

3.1 Synthetic thinning

Pp-TH and Pp-TH(D32R) were readily obtained by Fmoc/Bu solid-phase peptide synthesis protocols [19,20], with all eight Cys residues protected with the Trt group. HPLC purification of the products resulting from TFA treatment gave the fully reduced octathiol precursors in highly pure form, in 15-20% yield (relative to the original resin substitution). MALDI-TOF mass spectrometry (MS) analysis gave molecular masses of 5288.15 and 5330.64 Da for the Pp-TH and Pp-TH(D32R) precursors, respectively, in full agreement with the theoretical values (5288.24 and 5330.34). Different temperature and denaturant conditions (Table 1) were tested for the folding [21,22] of these precursors, the optimal preparative protocol consisting in anaerobic (Ar atmosphere) oxidation of a 5 μM reduced peptide solution in 0.1 M NH₄OAc buffer (pH 7.8) in the presence of GSH (500 μM), GSSG (50 μM) and Gn-HCl (1 M) for 48 h at 25°C. The reactions were stopped by freezing or adding 20 μl of 2 M TFA/water and analyzing the products in a reverse-phase HPLC, with a 0.1% TFA/acetonitrile gradient into 0.1% TFA/water over 60 min at 25 ml/min. Fractions judged to be homogeneous by analytical HPLC were pooled and their identity was further confirmed by amino acid analysis and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra, both of them consistent with the expected compositions.

![Fig. 1. Amino acid sequence alignment (A) and disulfide bridge pattern (B) of thionins, types II and I. Conserved positions are shaded in black. The Asp residue of native Pp-TH (SwissProt accession number P07509) mutated to Arg in Pp-TH(D32R) is indicated by an arrow. The following sequences (SwissProt accession numbers in parentheses) were used in the alignment algorithm: Hv-THD (P01072), Hv-THB (P09616), Ta-THB (P01543), Hv-THB (P21742) and Hv-THA (P01545).](image-url)
3.2. Structural studies

Estimated by HPLC peak integration of main oxidation product.

Time required for maximum extent of oxidative reaction.

Experiments showed the helical pattern to be fully preserved for the natural peptide under several HPLC conditions as in Fig. 2.

Table 1

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>1 M HCl</th>
<th>1 M HCl</th>
<th>2 M HCl</th>
<th>2 M HCl</th>
<th>1 mM EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>25°C</td>
<td>5°C</td>
<td>25°C</td>
<td>5°C</td>
<td>25°C</td>
</tr>
<tr>
<td>Time</td>
<td>48 h</td>
<td>96 h</td>
<td>48 h</td>
<td>72 h</td>
<td>48 h</td>
</tr>
</tbody>
</table>

Assuming these changes to be related to the presence in the former peptide of the Asp residue, which in the latter is replaced by Arg, we obtained 3D structures for both peptides by molecular modeling, using the SWISS-MODEL homology server [25]. The modeled structures were generated from 11 available Protein Data Bank templates, with an homology range of 50-70%, and were refined by energy minimization with Gromos 96. The resulting structures were further evaluated by molecular dynamics using the package MOLARIS [26]. Results from the computational study (Fig. 5) were in agreement with the CD observation of decrease in helical content upon Arg-for-Asp replacement.

3.3. Antimicrobial activity

The antibiotic activities of natural and synthetic P. puhera thionin [Pp-TH(nat)] and [Pp-TH(synt)], respectively, and the synthetic D32R analogue [Pp-TH(D32R)] were evaluated in vitro against bacterial and fungal plant pathogens (Table 2). Pp-TH(synt) and Pp-TH(nat) were similarly active against all the microorganisms tested (EC50 = 0.3-3.1 μM), which further reinforced the previous chemical (HPLC) and structural (CD) evidence for the correct folding of the synthetic thionin. The D32R analogue was significantly more active than Pp-TH against several Gram-negative bacteria (R. meliloti, X. campestris pv. translucens and X. campestris pv. campesiris) but comparable to Pp-TH against the Gram-positive bacterium C. michiganensis and the fungal plant pathogens P. cucumerina, F. oxyxporum and B. cinerea (Table 2). As previously described [18,27,28], inhibition of microbial growth was abolished by salt (1 mM CaCl2+50 mM KCl; data not shown).

Table 1

<table>
<thead>
<tr>
<th>Conditions for oxidative folding of Pp-TH and Pp-TH(D32R) precursors</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturant</td>
<td>1 M HCl</td>
<td>1 M HCl</td>
<td>2 M HCl</td>
<td>2 M HCl</td>
<td>1 mM EDTA</td>
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<tr>
<td>Temperature</td>
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<td>5°C</td>
<td>25°C</td>
<td>5°C</td>
<td>25°C</td>
</tr>
<tr>
<td>Time</td>
<td>48 h</td>
<td>96 h</td>
<td>48 h</td>
<td>72 h</td>
<td>48 h</td>
</tr>
</tbody>
</table>

*a* Reduced octathiol precursor at 5 μM in 0.1 M NH4OAc, pH 7.8.

*b* Time required for maximum extent of oxidative reaction.

*c* Estimated by HPLC peak integration of main oxidation product.
Fig. 4. CD spectra of natural (Pp-TH(nat)) and synthetic Pp-TH (Pp-TH(synt)) and of the D32R analogue (Pp-TH(D32R)). See conditions in text.

4. Discussion

Thionins were the first plant antimicrobial peptides to show activity against plant pathogens in vitro and the first eukaryotic peptides for which a defense role was postulated [12]. The present study had a two-fold purpose: (i) to provide useful amounts of *P. púbera* thionin for a complete study of its antimicrobial spectrum, and (ii) to investigate the role of Asp$^{32}$, an unusual substitution at one of the best conserved positions in the thionins (Fig. 1), and in particularly in those of subfamilies I and II, which share an identical disulfide pattern.

The efficient synthesis of both Pp-TH and its D32R analogue described above has been crucial for the success of our approach. The linear precursor, assembled in high purity by stepwise Fmoc solid-phase synthesis, could be cleanly oxidized under highly optimized conditions to provide the folded peptides in reasonable yields. Despite the resistance of the peptide (synthetic and natural) to most proteases even under denaturing conditions, which prevented us from determining its disulfide connectivity by proteolysis and mass spectrometry, we are sure of having achieved the native folding in both synthetic Pp-TH and its D32R analogue, based on several criteria: (i) synthetic Pp-TH coelutes with the natural peptide under several HPLC conditions; (ii) CD spectra of both peptides are superimposable, and (iii) their antimicrobial activities against several representative plant pathogens are indistinguishable.

Interestingly, the Asp to Arg replacement in the D32R analogue significantly enhanced activity against several Gram-negative bacteria, while no relevant effects against Gram-positives or fungi were found. This fact can be plausibly related to the presence of two additional positive charges in the mutant peptide, cationic character being well known to favor antimicrobial activity of peptides [29,30]. The different effect of this mutation on the activity of Pp-TH against Gram-negative and -positive bacteria is probably related to differences in membrane composition. In line with this hypothesis, it has been shown that the permeabilization of model membranes by thionins was dependent on their phospholipid composition [13], and thionin-sensitive mutants of the Gram-negative bacterium *Ralstonia solanacearum* have been described to display alterations in their membrane lipopolysaccharide structure [31].

On the other hand, the mutant D32R shows a surprising decrease in helicity, unpredicted by Chou-Fasman algorithms [32,33], which may be explained (as suggested by molecular modeling, Fig. 5) by the newly created repulsion between Arg$^{32}$ and Lys$^{29}$ (i, i+3 position), compounded by the possible loss of the salt bridge between Asp$^{32}$ and Lys$^{29}$, a potentially

Table 2

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<thead>
<tr>
<th>Pathogen</th>
<th>EC50 (μM)$^b$</th>
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<tr>
<td><strong>Bacteria</strong></td>
<td>Pp-TH(nat)</td>
</tr>
<tr>
<td>Gram-negative</td>
<td></td>
</tr>
<tr>
<td><em>R. meliloti</em></td>
<td>$&gt;20$</td>
</tr>
<tr>
<td><em>X. campestris pv. campestris</em></td>
<td>$3.1 \pm 0.1$</td>
</tr>
<tr>
<td><em>X. campestris pv. translucens</em></td>
<td>$1.7 \pm 0.4$</td>
</tr>
<tr>
<td>Gram-positive</td>
<td></td>
</tr>
<tr>
<td><em>C. michiganensis</em></td>
<td>$0.48 \pm 0.1$</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td><em>P. oxydoruni</em> sp. conglutinum*</td>
<td>$0.43 \pm 0.15$</td>
</tr>
<tr>
<td><em>P. cucumerina</em></td>
<td>$0.7 \pm 0.17$</td>
</tr>
<tr>
<td><em>B. cinerea</em></td>
<td>$2.2 \pm 0.1^c$</td>
</tr>
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$a$ Effective concentration for 50% inhibition (mean of two/three experiments ± S.D.).

$b$ EC50 values of Pp-TH(D32R), Pp-TH(nat) and Pp-TH(synt) are statistically different ($P=0.05$, $t$-test).

$^c$ nd, not done.
The helix-stabilizing feature of the native form. Despite this slight difference in helicity, both Pp-TH and Pp-TH(D32R) showed remarkable conformational imperviousness to different environments (pH, temperature), as well as resistance to proteolysis, all in all suggesting a very compact folding highly stabilized by the four disulfide bridges. Taken together, these results clearly show that even compact and complex structures such as thionins are susceptible to considerable improvements in their activity by judicious choice of structural modifications.

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