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Characterization and Structural Role of Disulfide Bonds in a Highly Knotted Thionin from *Pyrularia pubera*

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Abstract: *Disulfide bonds play a crucial role in the stabilization of the amphipathic folding of the diverse families of cysteine-rich antimicrobial peptides. The determination of cysteine pairings in these peptides has largely depended on sequence homology criteria, since the classical methods of disulfide bond characterization, which usually require proteolysis as a first step, encounter serious drawbacks derived from the tight folding and the presence of vicinal cysteines. We have chosen the* Pyrularia pubera *thionin, a 47-residue peptide with four internal disulfides and a remarkable resistance to most proteases, as a representative member of this type of cysteine-rich peptides and have shown that a combination of partial reduction and cyanylation readily allows the determination of its disulfide bonds. We have also studied by molecular dynamics and a combination of partial reduction and proteolysis the role of disulfide bonds in the stabilization of the tridimensional structure of this thionin and found a good agreement with our partial reduction data, suggesting that removal of only one disulfide bond is enough to significantly alter the folding of the peptide.* © 2005 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 80: 697–707, 2005

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Keywords: *disulfide bonds; amphipathic; cysteine-rich antimicrobial peptides; molecular dynamics; partial reduction; proteolysis*

INTRODUCTION

Thionins[1] were the first family of peptides for which activity against plant pathogens was demonstrated[2] and a defensive role postulated. Despite some sequence diversity, they are highly homologous, usually 45–47 amino acids in length, with minimum net charge of +2 and a well-conserved tridimensional structure stabilized by 3 or 4 disulfide bonds. After their early discovery, several other families of cysteine-rich peptides (see Ref. 3 to 5 for reviews) have been isolated from almost all organisms, including plants, insects, amphibians, and humans, and their significance in the innate immunity system has been well established.

The crucial role of disulfide bonds in the stabilization of membrane-active conformations of cysteine-rich antimicrobial peptides is now well acknowledged. The development of analytical methods to characterize these structures, and of synthetic strategies to access them, is a permanent challenge for peptide chemists.[6,7]

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The cysteine connectivities of thionins (PF00321 accession number at the Protein Families Database of the Sanger Institute http://www.sanger.ac.uk/Software/Pfam and IPR001010 at the European Bioinformatics Institute http://www.ebi.ac.uk/index.html) have been established by homology from high-resolution NMR and X-ray structures of other plant defensins such as crambin[8,9] and α-1-purothionin.[10] However, to the best of our knowledge, no direct determination of cysteine pairings has been reported for most members of the family.

The most usual methodology for disulfide linkage determination consists of enzymatic cleavage of the peptide backbone and identification by mass spectrometry (MS) of fragments containing or linked by disulfide bonds. These conventional protocols have several important limitations: (a) protease resistance of some highly folded peptides and proteins, (b) difficulty in characterizing disulfide bonds involving adjacent Cys residues, and (c) disulfide scrambling at the basic pH usually required for enzymatic digestion. Recent advances based on chemical cleavage and MS analysis have been reported,[7,11] but they still retain some of the above drawbacks and/or require computationally demanding data analysis. X-ray crystallography and NMR can also be used to generate high-resolution structures that define disulfide pairing, but their application is restricted to the availability of large amounts of sample.

In the course of our research on *Pyrularia pubera* thionin (PpTH),[12,13] we attempted the characterization of its disulfide bonds by the classical approach of enzymatic digestion and MS analysis. However, PpTH proved to be resistant to most proteases, due to its very compact folding, so we resorted to its cleavage by chemical methods. We describe here the determination of the *Pyrularia pubera* thionin disulfide pattern using the novel methodology of partial reduction and cyanylation[14,15] and demonstrate its suitability for the characterization of this family of highly knotted and compact peptides containing vicinal cysteines. Optimization of the reduction chemistry was particularly crucial, since removal of any of the disulfide bonds quickly led to extensive reduction. The relevance of disulfide linkages for thionin structure has been further studied by molecular dynamics simulations.

MATERIALS AND METHODS

Chemicals

High performance liquid chromatography (HPLC)-grade acetonitrile and trifluoroacetic acid (TFA) were from SDS (Peypin, France). 1,4-Dithio-DL-threitol (DTT), tris(2-carboxyethyl)-phosphine hydro-choloride (TCEP), and 1-cyano-4-(dimethyl-amino)-pyridinium tetrafluoroborate (CDAP) were from Sigma (Madrid, Spain) and *N*-ethylmaleimide (NEM) from Fluka (Madrid, Spain). TCEP and CDAP solutions in aqueous buffers were freshly prepared prior to use. Sequencing-grade trypsin was from Promega (Madison, WI), Arg-C endoproteinase from Roche (Barcelona, Spain), and pepsin and thermolysin from Sigma (Madrid, Spain). Poros R2 was from Applied Biosystems (Foster City, CA). Water used in all procedures was purified using a Millipore (Bedford, MA) Milli-Q system.

Table I Attempts to Cleave PpTH by Enzymatic Digestion[a]

Enzyme	Buffer	pH	T (°C)
Trypsin	50 m*M* Tris-HCl	8.0	37
Arg-C	50 m*M* Tris-HCl	8.0	37
Pepsin	50 m*M* citrate	1.5	37
Thermolysin	20 m*M* Tris-HCl 1 m*M* $CaCl_2$	7.0	80

[a] None of the proteases tested (1:20, enzyme/peptide ratio) was able to cleave PpTH to a detectable extent.

Chromatography and Mass Spectrometry

Analytical reversed-phase HPLC was done on a Shimadzu LC-2010A system using a Phenomenex Luna C8 column (3 μm, 0.46 × 5 cm). Separations were performed using a gradient of buffer B into buffer A at a flow rate of 1 mL/min. Buffer A = 0.045% TFA in water. Buffer B = 0.036% TFA in acetonitrile.

Peptide samples for MS analysis were desalted and concentrated using home-packed Poros R2 micro columns. Poros R2 beads were thoroughly washed with acetonitrile and equilibrated with 0.1% TFA in water; peptide sample was applied and desalted by aqueous washes and eluted with a solution of the appropriate matrix for matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) analysis. Mass spectrometric analysis of peptides was performed on a Voyager DE-STR instrument (Applied Biosystems, Foster City, CA) on the linear positive mode using α-cyano-4′-hydroxycinnamic acid as matrix (1 mg/mL in 70% acetonitrile, 0.1% TFA in water). Peptide mass fingerprint data was filtered using the PeakErazor software. Peptide sequencing was carried out on a Q-Star-Pulsar nanospray instrument (Applied Biosystems, Foster City, CA) interfaced to an Ultimate II nano-LC chromatograph (LC Packings, Sunnyvale, CA). Peptide digests were loaded onto a C18 PepMap 100 column (3 μm, 100 Å, 75 μm × 15 cm, LC packings) and eluted with a linear gradient from 5 to 50% acetonitrile and 0.1% formic acid in water at a 0.2 μL/min flow rate prior to MS/MS. Fragmentation spectra were used to determine peptide sequence by means of the Analyst software package.

Source of Thionins

Synthetic PpTH was obtained as previously described[12] and natural PpTH was a kind gift of Dr. Antonio Molina (ETISA-UPM, Madrid).

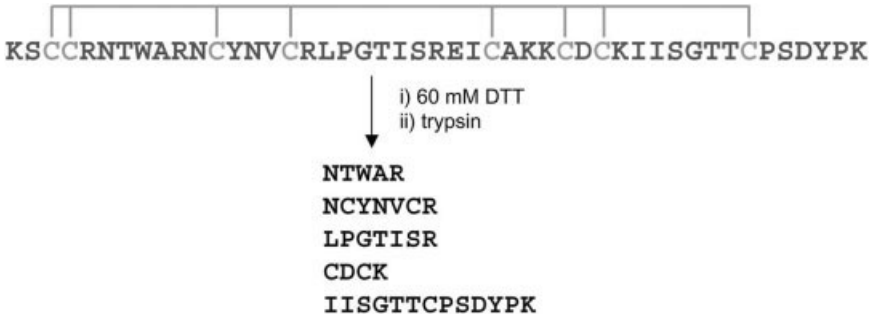

FIGURE 1 Sequence of *Pyrularia pubera* thionin and peptides identified by MALDI–TOF MS after complete reduction and trypsin digestion.

Peptide Digestion

Fifty micrograms of peptide were dissolved in 20 μL of 50 m*M* Tris-HCl, 6*M* HCl · Gn, pH 8.0 and denaturated for 1 h at 50°C. Denaturant agent was diluted below 1*M* with the appropriate buffer (depending on the protease used, Table I); the enzyme was added (1:20, protease/peptide ratio) and the sample was incubated for 18 h at 37°C (80°C for digestions with thermolysin). Enzyme cleavage was quenched by addition of 0.1*M* AcOH. Samples were desalted and concentrated as above, then analyzed by MALDI–TOF. Alternatively, peptides (20 μg) were completely reduced prior to enzymatic digestion by incubation in 200 μL of 50 m*M* Tris-HCl, 60 m*M* DTT, 6*M* HCl · Gn, pH 8.0, for 2 h at 37°C and then purified using Sep-Pack cartridges (Waters, Milford, MA).

Partial Reduction and Alkylation

Ten nmol of peptide were dissolved at 1 m*M* concentration in 0.1*M* citrate buffer containing 6*M* HCl · Gn at pH 3.0 and denaturated for 20 min at 65°C; in the optimal protocol 1 eq. TCEP/cystine (10 m*M* TCEP solution in 0.1*M* citrate, 6*M* HCl · Gn, pH 3.0) was then added and partial reduction allowed to proceed for 5 min at 65°C (several other stoichiometries, temperatures, and reaction times were tested, see Results and Discussion). The reaction mixture was cooled to room temperature (RT). Twenty equivalents of CDAP/cysteine (0.1*M* CDAP solution in 0.1*M* citrate buffer, pH 3) were added to cyanylate-reduced Cys residues and the reaction mixture was incubated for 30 min at RT. Partially reduced and cyanylated peptides were separated by reverse-phase (RP-HPLC) (5 min of isocratic elution at 15% B followed by a 20–27% B linear gradient in 30 min) lyophilized and cleaved by addition of 7 μL of 1*M* NH_4OH, 6*M* HCl · Gn, pH 12, for 1 h at RT; excess NH_4OH was removed by lyophilization and the resulting cleaved peptides completely reduced by incubation with 2 μL of 0.1*M* TCEP in 0.1*M* citrate, pH 3.0, for 30 min at 37 °C. Samples were desalted and concentrated as above, then analyzed by MALDI–TOF and/or MS/MS.

Molecular Modeling

The InsightII/ Discover3 software package (Accelrys, San Diego, CA) was used to perform and analyze molecular dynamics calculations with the consistent valence force field (CVFF) and $\eta = 4r_{ij}$.[16] PpTH tridimensional structure was

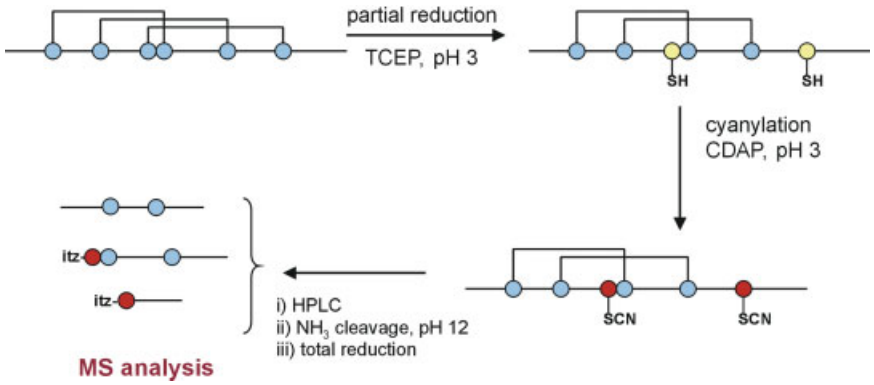

FIGURE 2 Schematic representation of partial reduction/cyanylation methodology. Peptide is partially reduced at pH 3 (thus avoiding disulfide scrambling) with TCEP and the nascent sulfhydryl groups are cyanylated in situ with CDAP. The resulting singly reduced and cyanylated peptides are isolated and cleaved using aqueous ammonia, and the molecular weight of the iminothiazolidine (itz-) peptide fragments obtained is directly related to the cysteines that were paired prior to partial reduction.

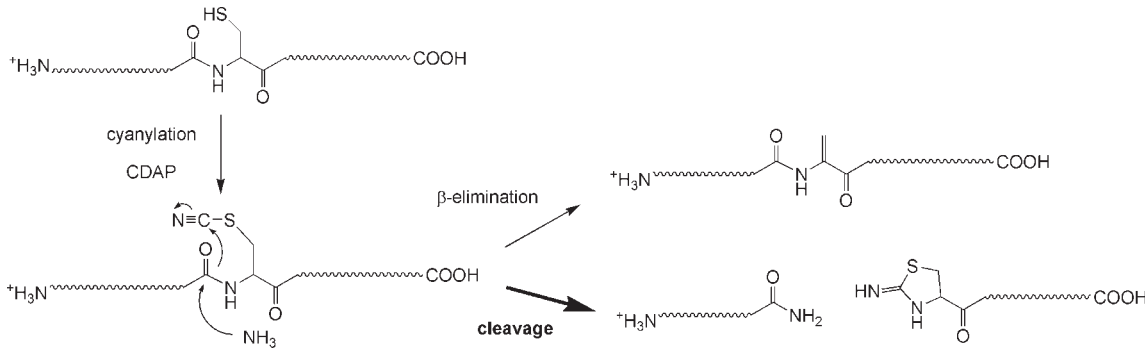

FIGURE 3 Mechanism of free thiol cyanylation with CDAP and CN-induced cleavage at the N-terminus of cyanylated cysteines by ammonia. The β-elimination side reaction is also shown.

obtained by homology modeling using the SWISS-MODEL server (www.expasy.org/swissmod/SWISS-MODEL.html).

Four different partially reduced thionin constructs were generated using the Biopolymer module, by removing each one of the disulfide bonds of the native thionin. Fully oxidized and singly reduced disulfide thionin structures were then minimized, equilibrated at 298 K for 5 fs and submitted to unrestricted molecular dynamics for 5 ns. Structures were sampled out every 10 ps.

Table II Calculated MH^+ (Average Values) of Peptide Fragments and β-Elimination Products from CN-Induced Cleavage for all Possible PpTH Disulfide Pairings

C_x–C_y[a]	*itz*-[*x* - (y - 1)][b]	*itz*-[*y* - 47][c]	β-@y[d]	β-@x[e]	[1 - (*x* - 1)][f]
31–33	262.28	1638.92	1823.15	3660.30	3475.14
27–31	474.61	1857.15	2253.72	3442.07	3044.57
12–16	523.6	3514.19	3959.74	1785.03	1338.54
27–33	692.85	1638.92	2253.72	3660.30	3044.57
33–41	848.02	834.94	1604.92	4464.28	3693.37
4–12	1046.17	3993.74	4961.87	1305.47	336.42
31–41	1066.25	834.94	1823.15	4464.28	3475.14
3–12	1149.31	3993.74	5065.01	1305.47	233.28
16–27	**1270.51**	**2287.72**	3480.19	3011.50	**1817.11**
27–41	1496.83	834.94	2253.72	4464.28	3044.57
4–16	1525.72	3514.19	4961.87	1785.03	336.42
3–16	1628.87	3514.19	5065.01	1785.03	233.28
16–31	1701.08	1857.15	3480.19	3467.16	1818.1
12–27	1750.07	2287.72	3959.74	3011.50	1338.54
16–33	1919.31	1638.92	3480.19	3660.30	1818.1
12–31	**2180.64**	**1857.15**	**3959.74**	3442.07	1338.54
12–33	2398.87	1638.92	3959.74	3660.30	1338.54
16–41	2723.29	834.94	3480.19	4464.28	1818.1
4–27	2752.19	2287.72	4961.87	3011.50	336.42
3–27	2855.34	2287.72	5065.01	3011.50	233.28
4–31	3182.76	1857.15	4961.87	3467.16	336.42
12–41	3202.85	834.94	3959.74	4464.28	1338.54
3–31	3285.91	1857.15	5065.01	3442.07	233.28
4–33	**3401.00**	**1638.92**	**4961.87**	3660.30	336.42
3–33	3504.14	1638.92	3470.14	3660.30	233.28
4–41	4204.97	834.94	4961.87	4464.28	336.42
3–41	**4308.12**	**834.94**	**5065.01**	4464.28	233.28

[a] Disulfide bond.
[b] Iminothiazolidine (itz-) fragment from residue *x* to residue (*y* - 1).
[c] The itz- fragment from residue *y* to the last residue (47).
[d] The itz-peptide from Cys^x residue to the last one with β-elimination at Cys^y.
[e] Peptide fragment from residue 1 to (*y* - 1) with β-elimination at Cys^x.
[f] Peptide fragment from residue 1 to residue (*x* - 1).

Table III Optimization of Partial Reduction Conditions for PpTH

TCEP (eq./Cys)	T (°C)	Time (min)	Denaturant	Result
18	65	30	6*M* HCl · Gn	100% $PpTH(SH)_8$
18	65	15	6*M* HCl · Gn	100% $PpTH(SH)_8$
18	65	5	6*M* HCl · Gn	100% $PpTH(SH)_8$
8	65	30	—	100% PpTH
8	65	15	6*M* HCl · Gn	72% $PpTH(SH)_8$[a]
8	65	5	6*M* HCl · Gn	25% $PpTH(SH)_8$[a]
8	37	30	6*M* HCl · Gn	48% $PpTH(SH)_8$[a]
8	37	15	6*M* HCl · Gn	36% $PpTH(SH)_8$[a]
4	65	10	6*M* HCl · Gn	5% $PpTH(SH)_2$[b] 11% $PpTH(SH)_8$
4	37	15	6*M* HCl · Gn	2% $PpTH(SH)_2$[b] 11% $PpTH(SH)_8$
0.5	37	15	6*M* HCl · Gn	100% PpTH
0.5	65	5	6*M* HCl · Gn	10% $PpTH(SH)_2$[b]

[a] Only completely reduced and fully oxidized thionin were obtained. Percentage of the fully reduced isoforms is indicated.

[b] Overall yield of singly reduced thionin isoforms detected.

RESULTS AND DISCUSSION

Our first attempts to characterize cysteine connectivity in *Pyrularia pubera* thionin (both natural and synthetic[12]) by the classical methodology of proteolytic cleavage and MS analysis failed due to PpTH imperviousness to representative proteases (Table I), even under highly denaturant conditions such as pepsin at pH 2 or thermolysin at 80°C. However, they were easily digested by trypsin after complete reduction with 60 m*M* DTT and the expected fragments could be identified by MALDI–TOF (Figure 1), thus suggesting that proteloytic resistance was due to the highly compact folding stabilized by disulfide bonds.

We decided to use the novel strategy of partial reduction and cyanylation.[14,17] This method (Figure 2) is based on the partial reduction of a multiple disulfide-containing peptide with the water-soluble reducing agent TCEP, in such optimized conditions that only singly reduced isoforms are obtained and then cyanylated in situ by CDAP. Singly reduced and cyanylated species are separated by HPLC and subsequently cleaved by aqueous ammonia at the N-terminus of modified cysteine residues (mechanism of CN-induced cleavage shown in Figure 3). The resulting peptide fragments are then fully reduced, to remove any residual disulfide bonds, and analyzed by MS: the molecular weights of the peptides thus obtained can be directly related to the cysteines that were connected prior to partial reduction. The use of TCEP and CDAP as reducing and cyanylating agents, respectively, is advantageous in that they are compatible with acidic pH, thus avoiding disulfide scrambling. It should be noted that some undesirable reactions such as β-elimination might compete with cleavage; however, this side reaction yields overlapping peptides that are helpful during disulfide assignment (see below).

Pyrularia pubera thionin, as well as other peptides of the same family, have 8 cysteine residues, so 105 different disulfide pairings are possible, and 28 singly reduced isomers could be expected (actually, only 27 should be considered, since a disulfide bond between the adjacent cysteines 3 and 4 is geometrically unfavorable). Calculated MH^+ values for all possible fragments resulting from cleavage at cyanylated Cys residues are summarized in Table II.

First attempts to reduce PpTH using nondenaturing buffers yielded the intact protein. However, denaturing conditions previously described for other proteins (18 eq. TCEP/Cys, 30 min at 65°C in the presence of 6*M* HCl · Gn)[14,18] produced completely reduced thionin. On the other hand, milder conditions (8 eq. TCEP/Cys at 37°C for 15 min) gave a mixture of nonreduced and completely reduced peptides, with no singly reduced species detected. These results suggested that the resistance of folded PpTH to reducing agents was completely eliminated upon reduction of some of its disulfide bonds, allowing all remaining disulfides to be readily reduced. Accordingly, several reduction conditions were carefully explored in order to obtain significant amounts of singly reduced isoforms (Table III). Partially reduced test samples were also cyanylated with CDAP (or alkylated with NEM)

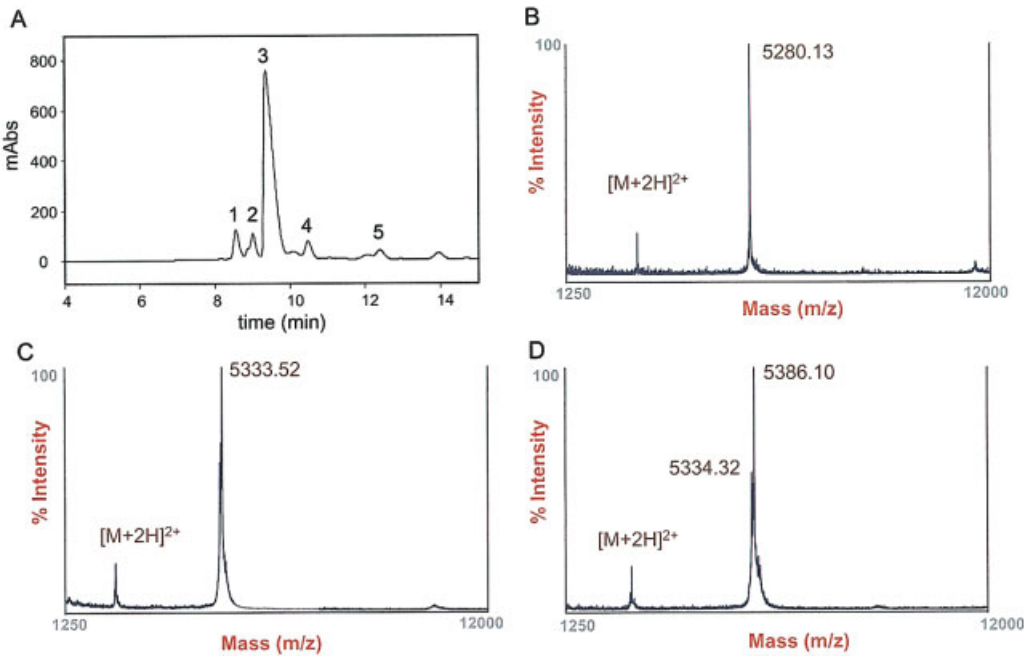

FIGURE 4 Partial reduction and cyanylation of PpTH. Panel A: RP-HPLC analysis of the reaction mixture; panel B: MALDI–TOF spectrum of isolated peak 3, corresponding to intact PpTH ($MH^+ = 5280.13$); panel C: peaks 1, 2, and 4 give all three a $MH^+ = 5333.52$ Da main peak, characteristic of a singly reduced and cyanylated peptide; panel D: peak 5 contains a mixture of singly and doubly reduced and cyanylated ($MH^+ = 5386.10$ Da) thionin.

to simplify MALDI–TOF MS detection, as mass differences between fully oxidized and singly reduced species (2 Da) might be difficult to observe in the linear mode of the MALDI–TOF spectrometer suitable for the analysis of large peptides and proteins.

In our optimal protocol, PpTH was denaturated and reduced by 0.5 eq. TCEP/Cys for 5 min at 65°C and subsequently cyanylated by addition of 20 eq. CDAP/Cys at RT. After RP-HPLC separation of the reaction mixture (Figure 4), three peaks (labeled 1, 2,

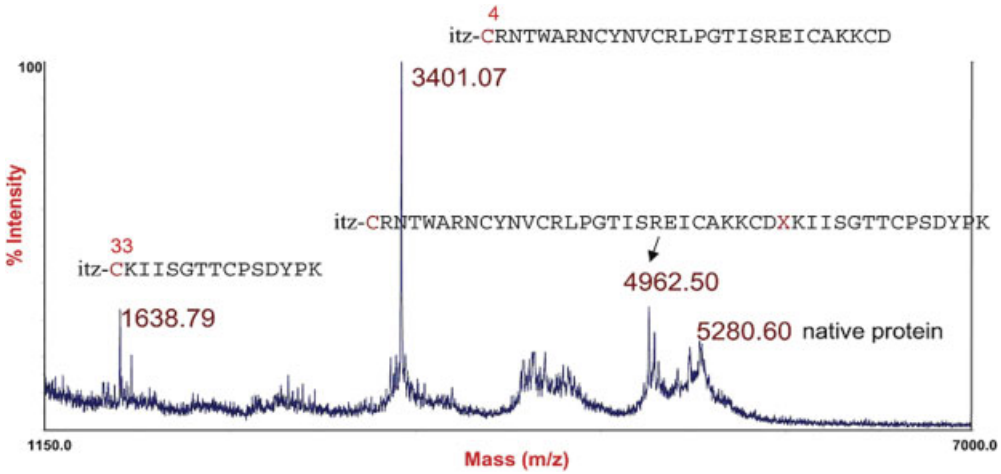

FIGURE 5 MALDI–TOF MS of the cleavage of peak 1. The peak at *m/z* 3401 can be assigned to the iminothiazolidine derivative from CN-induced cleavage at Cys^4 and Cys^{33} (itz-[4–32] fragment, Table III); peak at *m/z* 1638.79 matches the expected MH^+ value for the iminothiazolidine derivative from cleavage at Cys^{33} (itz-[33–47], Table III) and peak at *m/z* 4962.50 corresponds to the product of cleavage at Cys^4 and β-elimination at Cys^{33}. These data allow assignment of the Cys^4 and Cys^{33} disulfide.

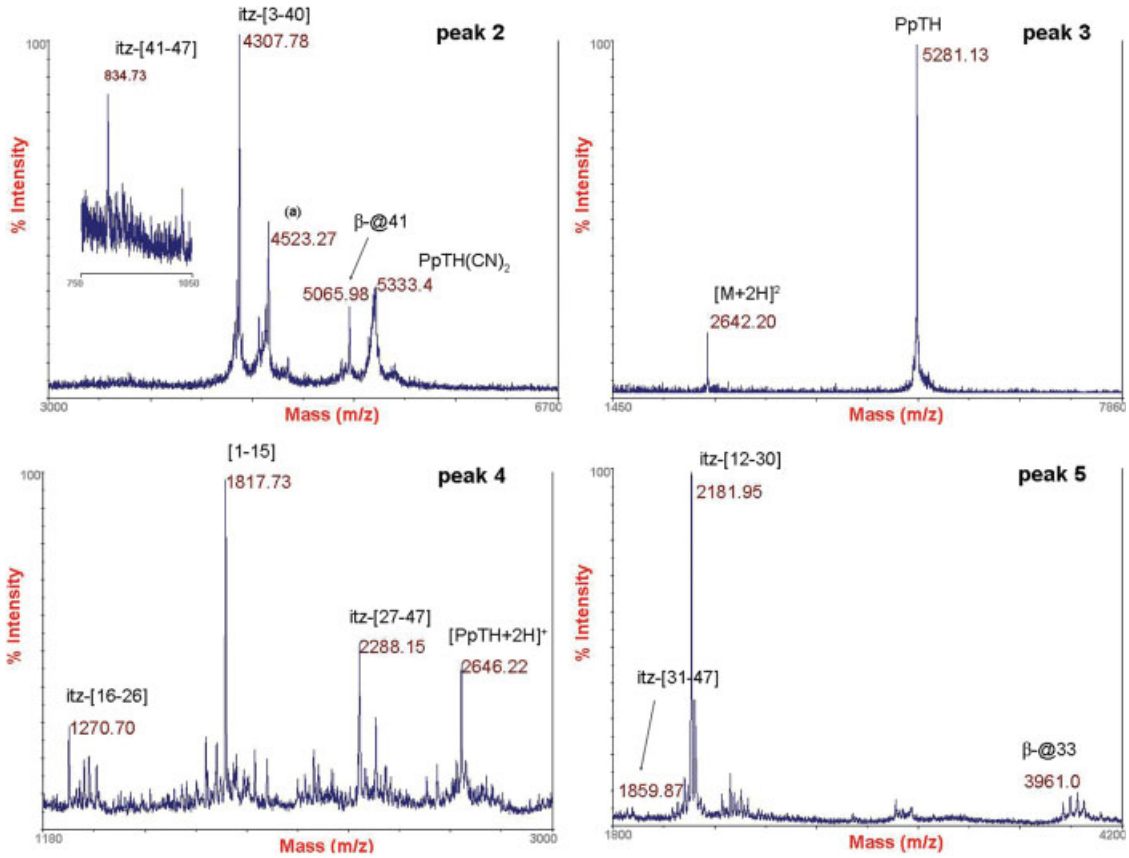

FIGURE 6 MALDI–TOF MS of the chemical cleavage of HPLC peaks 2, 3, 4, and 5 (Figure 4). $[M + H]^+$ signals significant for disulfide assignment are labeled, other minor peaks corresponding to miscleavage products can be identified: peak (a) can be assigned to a peptide spanning residues 1– 41, with a noncleaved cyanylated Cys residue.

and 4 in Figure 4), corresponding to singly reduced and cyanylated peptides, were identified by MALDI–TOF MS analysis ($MH^+ = 5333.2$ Da) and an additional peak containing a mixture of singly and doubly reduced thionin ($MH^+ = 5386.10$ Da) was also detected (peak 5). After lyophilization, each cyanylated peptide was dissolved in 2 μL of 1*M* NH_4OH, 6*M* HCl · Gn and cleaved by addition of 5 μL of 1*M* NH_4OH, pH 12. Although cleavage conditions were carefully optimized, some peaks corresponding to incomplete cyanylation, incomplete CN-induced cleavage, and β-elimination could be also observed. After evaporation of excess NH_4OH, samples were completely reduced to remove all residual disulfide bonds by incubation with 0.1*M* TCEP for 1 h at 37°C and analyzed by MALDI-TOF after desalting and concentrating (Figures 5 and 6).

Figure 5 shows in detail the MS spectrum of the cleavage of peak 1 with the assignment of its main peaks. Several intense *m*/*z* signals can be assigned to

Table IV Disulfide Assignment by Peptide Mapping of Cleavage Mixtures of Singly Reduced and Cyanylated Peptides

HPLC Peak	$[MH^+]$[a]	Peptide Fragment[b]	Disulfide Assignment
1	3401.07	itz-[4–32]	Cys^4–Cys^{33}
	1638.79	itz-[33–47]	
	4962.50	β-@33	
2	4307.78	itz-[3–40]	Cys^3–Cys^{41}
	834.73	itz-[41–47]	
	5065.98	β-@41	
4	2288.15	itz-[27–47]	Cys^{16}–Cys^{27}
	1817.73	[1–15]	
	1270.70	itz-[16–26]	
5	1859.87	itz-[31–47]	Cys^{12}–Cys^{31}
	2181.95	itz-[12–30]	

[a] Average values.

[b] Peptide fragment corresponding to the experimentally determined MH^+.

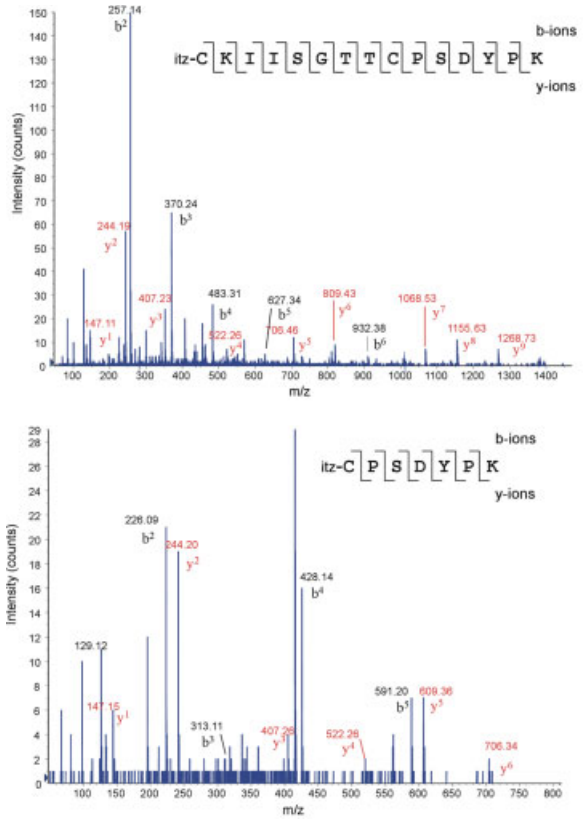

FIGURE 7 MS/MS analysis of itz-peptide fragments of $MH^+ = 1637.86$ (itz-[33–47]) from cleavage of HPLC peak 1 (top panel) and $MH^+ = 833.39$ from cleavage of peak 2 (bottom panel). Desalted samples from ammonium cleavage were analyzed by nanoLC–MS (LC: liquid chromatography) and desired peaks were sequenced.

products of CN-induced cleavage at Cys residues 4 and 33 ($MH^+ = 3401.07$, corresponding to itz-[4–32]; $MH^+ = 1638.79$, corresponding to itz-[33–47], see Table II for nomenclature and Table IV for a summary of disulfide assignment) as well as fragments from cleavage at Cys 4 and β-elimination at Cys 33 ($MH^+ = 4962.50$, henceforth β-@33 peptide), all of them consistent with the presence of a disulfide bond between Cys^4 and Cys^{33}. Other less intense peaks can be assigned to products of side reactions such as incomplete CN-induced cleavage and *trans* thio-cyanylation.[14] From the analysis of the cleavage products of HPLC peaks 1 (Figure 5), 2, and 4 (Figure 6), three disulfide bonds could be easily assigned: Cys^4–Cys^{33}, Cys^3–Cys^{41}, and Cys^1–-Cys^{27}. The fourth disulfide pairing could be deduced from this data and also confirmed by analyzing the cleavage mixture of peak 5 (Figure 6), which contained the fragments expected from CN-induced cleavage at Cys^{12} and Cys^{31}. Although the identity of all peaks significant for disulfide assignment was readily established by MALDI–TOF MS, we also performed MS/MS analysis of some relevant itz-peptides in the crude cleavage mixtures, which confirmed the previous assignments (Figure 7).

The need for an extremely careful optimization of the reduction chemistry in thionins seems to be related to the important role of disulfide bonds in the stabilization of its tightly folded structure, which becomes a lot more accessible after partial reduction. In order to study the effect of a single disulfide reduction on thionin structure, we performed molecular dynamics simulations for fully oxidized PpTH and each one of its four possible singly reduced peptides. From the variation of the RMSD (root mean square deviation) backbone values vs. time (Figure 8), one can see that thionin disulfides (particularly Cys^{16}–Cys^{27} and Cys^{12}–Cys^{31}) play a crucial role in the stabilization of PpTH tridimensional structure, as their in silico reduction promotes a fast deviation of the corresponding singly reduced peptides from the native structure of fully oxidized PpTH. These results suggest that after partial reduction PpTH quickly unfolds, leading to a structure (Figure 9) that can easily be further reduced.

In order to confirm the structural loosening of PpTH after removal of any of its disulfide bonds, we performed enzymatic cleavage of fully oxidized thionin and of partially reduced peptides, under (i) denaturing conditions, with cyanylation followed by pepsin digestion at pH 3.0; and (ii) nondenaturing conditions, using trypsin at pH 8.0. In this last procedure,

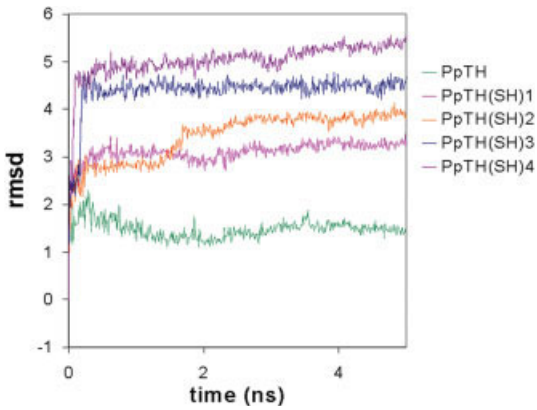

FIGURE 8 RMSD backbone values vs. time during 5-ns molecular dynamics simulation at 298 K for PpTH and each one of the four possible singly reduced isoforms [PpTH(SH)1, PpTH(SH)2, PpTH(SH)3, PpTH(SH)4. A rapid and significant (RMSD > 2) deviation from the initial (folded) structure is observed after removal of any of the PpTH disulfide bonds.

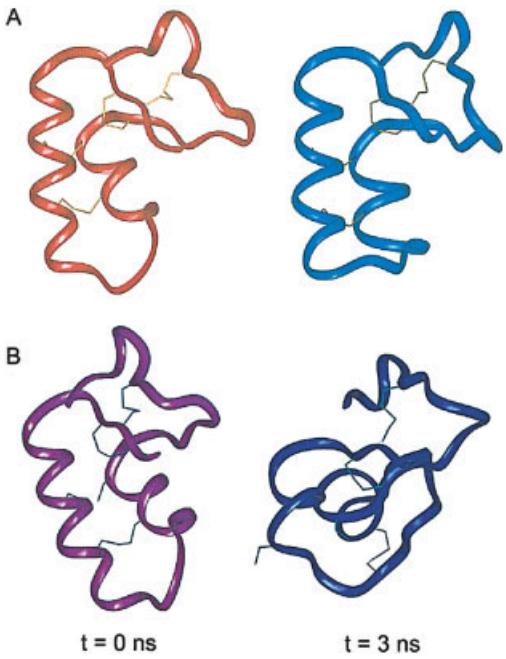

FIGURE 9 Modeled tridimensional structure of fully oxidized (A) and singly reduced (Cys^{12}–Cys^{31}) PpTH (B) at time 0 and after 3 ns of unrestricted molecular dynamics at 298 K.

the free Cys residues generated after partial reduction were modified with NEM instead of CDAP, as cyanylated peptides undergo undesired base-mediated cleavage on incubation at pH 8.0. As previously shown, completely folded PpTH was impervious to enzymatic cleavage (even at pH 3.0, Figure 10), but partially reduced peptides were easily digested. In order to facilitate MS interpretation, partially reduced and S-modified peptides were isolated by HPLC, digested with trypsin, then fully reduced and analyzed by MALDI–TOF (Figure 11), which allowed us to identify characteristic thionin fragments in the resulting mixtures.

Partially reduced thionin isomers show increased susceptibility to enzymatic cleavage, compared to folded PpTH, thus proving the crucial role of thionin disulfide bonds in maintaining its tight folding. These partially reduced thionins might also be considered as starting points for disulfide assignment by classical enzymatic methods. However, as the proteolysis is necessarily not exhaustive, and peptides containing modified Cys residues are difficult to detect by MALDI–TOF over other more intense fragments, inconclusive information often results (i.e., peptides of identical mass with more than one Cys), which makes the determination of disulfide bonds by this classical strategy rather more difficult than by the method of CN-induced cleavage we have used.

CONCLUSIONS

We have produced evidence for the cysteine connectivites for *Pyrularia pubera* thionin to be Cys^4–Cys^{33}, Cys^3–Cys^{41}, Cys^{12}–Cys^{31}, and Cys^{16}–Cys^{27}. We have also proved that optimization of reduction

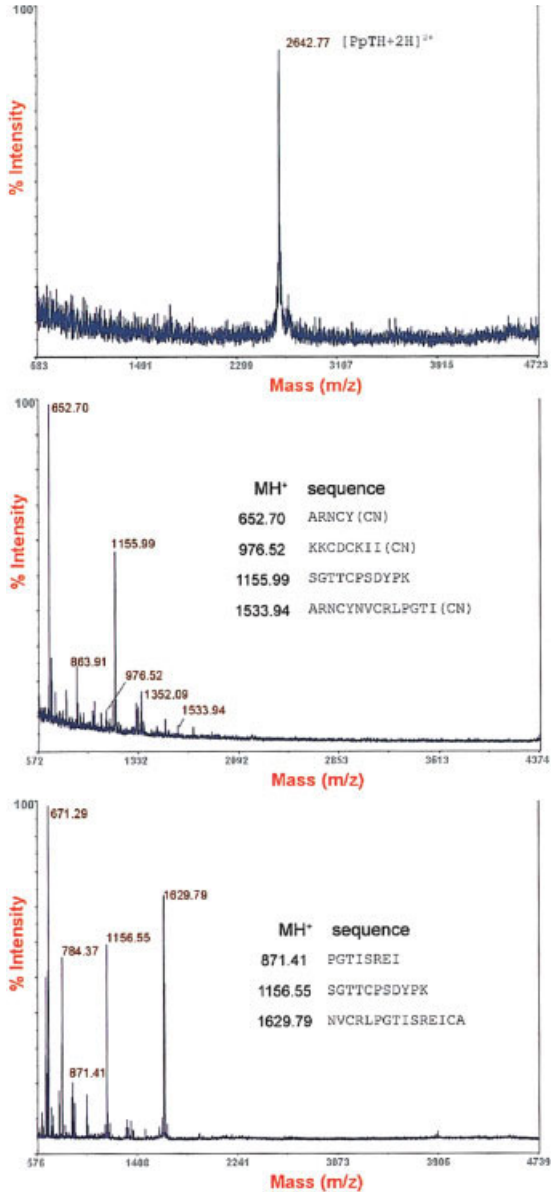

FIGURE 10 MALDI–TOF analysis of folded PpTH (top) and singly reduced and cyanylated thionin isomers (middle and bottom; reduced Cys^{12}–Cys^{31} and Cys^4–Cys^{33} disulfides, respectively) after digestion with pepsin. Singly reduced and cyanylated peptides were isolated by HPLC, lyophilized, and digested with pepsin (8 h, 37°C). Expected MH^+ signals were obtained for all partially reduced isomers, indicating increased enzymatic susceptibility upon single disulfide reduction. Identified peptide fragments are shown and cyanylated peptides are indicated as CN.

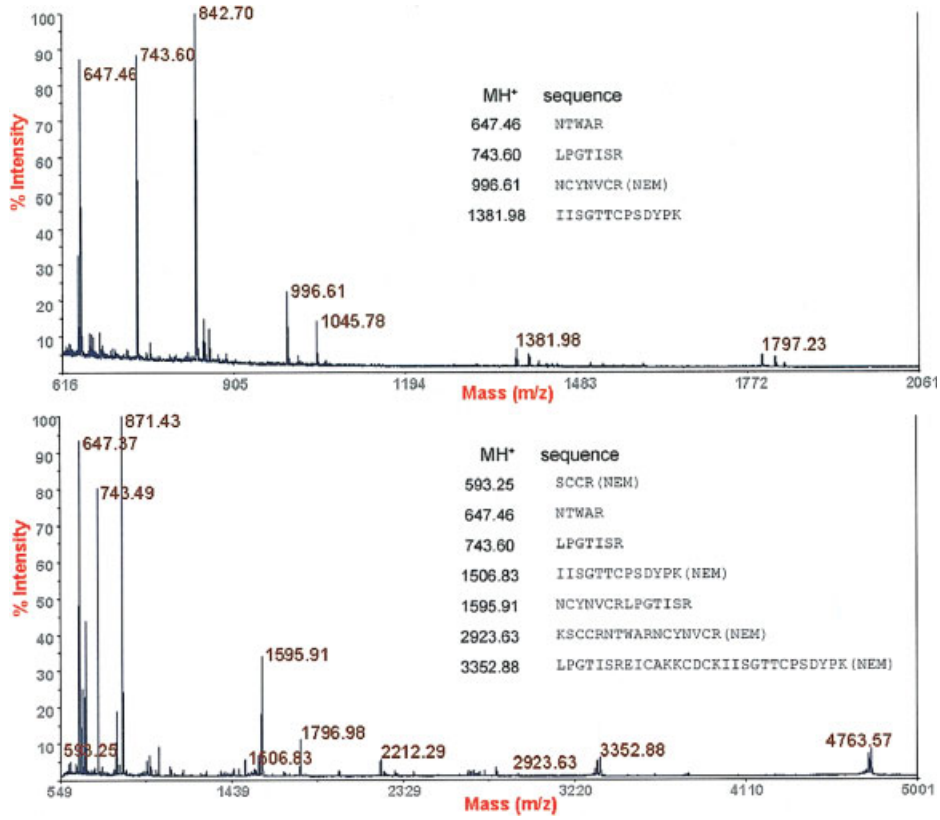

FIGURE 11 MALDI–TOF analysis of thionin isomers with Cys^{12}–Cys^{31} (top) and Cys^{4}–Cys^{33} (bottom) disulfides singly reduced and alkylated after tryptic digestion and complete reduction. Singly reduced and alkylated peptides were isolated by HPLC, lyophilized, and digested with trypsin (8 h at 37°C), then fully reduced with 25 m*M* DTT (45 min at 56°C). Identified thionin fragments are shown (NEM-derivatized peptides are labeled), other peaks can be assigned to trypsin autolysis (842.70, 1045.78, 2212.29 Da) and to keratins present on the sample.

conditions is extremely important when dealing with peptides and proteins such as thionins, for which disulfide bonds are essential to maintain native folding. Remarkably, very mild reducing conditions (1 eq. TCEP/cystine) are desirable, as tiny amounts of singly reduced peptides are enough for disulfide bond characterization.

Partially reduced thionin isomers were susceptible to proteolysis, in contrast to fully oxidized PpTH, which turned out to be protease resistant. Molecular dynamics simulations are in good agreement with data from enzymatic digestion and partial reduction, suggesting that reduction of one of the four disulfide bonds can lead to a fast relaxation of the native folding, and thus promote the observed vulnerability to both reducing agents and proteases. In summary, we have shown that partial reduction followed by cyanylation and ammonia cleavage is an optimal approach for determining disulfide connectivities of highly folded peptides such as PpTH. We are currently exploring the applicability of this approach to other highly folded structures, including plant defensins and the recently described snakins.[19]

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