Journal of Peptide Science, Vol. 3, 207–215 (1995)

Determination of Disulphide Bridges in PG-2, an Antimicrobial Peptide from Porcine Leukocytes

SYLVIA S.L. HARWIG[1], KRISTINE M. SWIDEREK[2], TERRY D. LEE[2] and ROBERT I. LEHRER[1]

[1]Department of Medicine, Center for the Health Sciences, University of California, Los Angeles, California, USA

[2]Division of Immunology, Beckman Research Institute of City of Hope, Duarte, California, USA

Received 31 October 1994
Accepted 8 December 1994

Abstract: We determined the cysteine connectivity of protegrin PG-2, a leukocyte-derived antimicrobial peptide, by performing sequential enzyme digestions with chymotrypsin and thermolysin, and monitoring each digest by direct liquid chromatography–electrospray mass spectrometric analysis. This approach resolved the disulphide pairing pattern unambiguously with only picomolar amounts of PG-2. The inferred cysteine connectivity was confirmed by traditional amino acid composition analyses using nanomolar amounts of the protegrin. The results suggest that protegrins will assume a tachyplesin-like, disulphide-stabilized antiparallel β-sheet configuration in solution.

Keywords: Antimicrobial; peptides; disulphide pairing analysis; liquid chromatography–electrospray mass spectrometry; protegrins

INTRODUCTION

Protegrins are a family of cysteine-rich cationic antimicrobial peptides that were recently isolated from porcine leukocytes [1] and cloned from porcine bone marrow [2, 3]. Although protegrins show considerable primary sequence homology to defensins [1], they contain only 16–18 amino acid residues and have four cysteines linked in two intramolecular disulphide bonds, whereas defensins contain at least 29 residues with six invariant cysteines linked in three intramolecular disulphide bonds [4, 5]. We have previously reported that protegrins also resemble horseshoe crab haemocyte tachyplesins, a family of small (17–18 residues) antimicrobial peptides with two intramolecular disulphide bonds [6, 7] that, like protegrins [8, 9], also have an amidated C-terminus.

Protegrins exhibit microbicidal activity against a broad range of bacteria, fungi and enveloped viruses. Like other endogenous broad-spectrum peptide antibiotics – for example, defensins, cecropins [10] and magainins [11] – they are likely to play a significant role in the innate resistance of their respective hosts to infection by the myriad organisms encountered during daily life. Because of their small size, relatively simple structure and facile production by direct peptide synthesis, protegrins have considerable potential for a variety of pharmaceutical applications. To foster future drug design studies, we characterized the cysteine connectivities in PG-2 and report the results here.

MATERIALS AND METHODS

Chemicals

Sequence grade phenylisothiocyanate (PITC), pyridine and trifluoroacetic acid (TFA) were purchased from Pierce Chemical Co. Analytical reagent grade acetic acid (HOAc), formic acid (88%), hydrogen peroxide (30%) and HPLC grade water and acetoni-

*Address for correspondence: Professor R. I. Lehrer, Department of Medicine, UCLA Center for the Health Sciences, CHS 37-055, Los Angeles, CA 90095, USA

CCC 1075-2617/95/030207-09

trile were from Fisher. Tosylamide-lysyl chloromethyl ketone (TLCK)-treated chymotrypsin was from Worthington and thermolysin was from Boehringer Mannheim.

PG-2

PG-2 was purified to homogeneity from porcine leukocytes as previously described [1].

Chymotrypsin Digestion

PG-2 (5 nmol) was digested for 4 h at 37 °C with a 0.4 μg of TLCK-treated chymotrypsin in 16 μl of 0.1 M pyridine acetate, pH 6.50. Additional chymotrypsin (0.4 μg) was then added and the digestion was allowed to continue overnight. The reaction was terminated by addition of glacial acetic acid (25% by volume), and a 50 pmol aliquot was removed for liquid chromatography-electrospray mass spectrometric (LC-ESI-MS) analysis. The remaining digest was dried down in a Speed-Vac concentrator (Savant Instruments) and reconstituted in 0.1% TFA for reversed-phase (RP) HPLC purification.

Thermolysin Digestion

Purified chymotryptic fragment 2 (CT-2, ~0.4 nmol) of native PG-2 was digested for 24 h at 37 °C with 0.1 μg of thermolysin in 10 μl of 0.1 M pyridine acetate containing 5 mM $CaCl_2$, pH 6.50. The digestion was terminated by acidification with glacial acetic acid (25% by volume), and an aliquot equal to 20 pmol was removed for LC-ESI-MS analysis. The remaining digest was evaporated to dryness (Speed-Vac) and reconstituted in 0.1% TFA for RP-HPLC.

RP-HPLC

Chymotrypsin and thermolysin digests were purified on a 4.6 × 250 mm Vydac C-18 column (flow rate = 1 ml/min and a 2.0 × 150 mm Vydac C-18 small bore column (flow rate = 0.25 ml/min), respectively, using a linear gradient of acetonitrile that increased in concentration by 1% per minute and contained 0.1% TFA. A small portion of the chymotryptic fragment (~0.12 nmol) was subjected to performic acid oxidation, and the mixture was cleared of residual performic acid by diluting with 100 μl of HPLC grade water and lyophilizing. This step was then repeated twice and the resulting mixture was purified on the Vydac small bore C-18 column.

LC-ESI-MS Analysis

Mass spectrometric analyses were performed at Beckman Research Institute of City of Hope (Duarte, CA) on a triple quadrupole TSQ-700 mass spectrometer (Finnigan-MAT, San Jose, CA) equipped with an electrospray ion source operating at atmospheric pressure. Mass spectra were recorded in positive ion mode. Mass scans were continually acquired every three seconds in a mass range from 500 to 2000 and the data collection was monitored using both the base peak (representing the highest intensity per scan) and the reconstructed ion current profile (representing the continuous collection of the total ion current per scan). Spectra were generated by averaging the scans containing the peak, and the mass assignments were made using the Finnigan MAT BIOMASS data reduction software. A sheath flow of 2-methoxyethanol at a flow rate equal to the flow rate coming from microcapillary HPLC system (typically 2 μl/min during sample elution) was used for all LC-MS experiments. The microcapillary HPLC system, which consisted of a fused silica column with an inner diameter of 250 μm and packed with 3 μm Vydac C18 support, was built at City of Hope. A preformed gradient of 2–72% solvent B in 35 min was used for all LC-MS experiments described here (solvent A = 0.1% TFA in water, solvent B = 0.07% TFA in 90% acetonitrile). A detailed description of the operation has been described previously [12, 13].

Amino Acid Analysis

Selected purified digestion fragments were hydrolysed *in vacuo* at 110 °C for 20 h in 6.0 M HCl. Amino acids were analysed as their phenylthiocarbamyl derivatives [14] following derivatization with PITC.

RESULTS

Chymotrypsin Digestion

As indicated by the RP-HPLC purification profile (Figure 1), chymotryptic digestion of native PG-2 appeared incomplete under the chosen conditions (see Discussion), despite the overnight incubation with two additions of enzyme at an enzyme/substrate mass ratio of 1/20. The major peak that eluted at 38 min had an identical retention time as the native, undigested PG-2. When 50 pmol of this digest were subjected to LC-ESI-MS analysis, both the base peak profile and the reconstructed ion current profile (Figure 2) resembled that of UV tracings

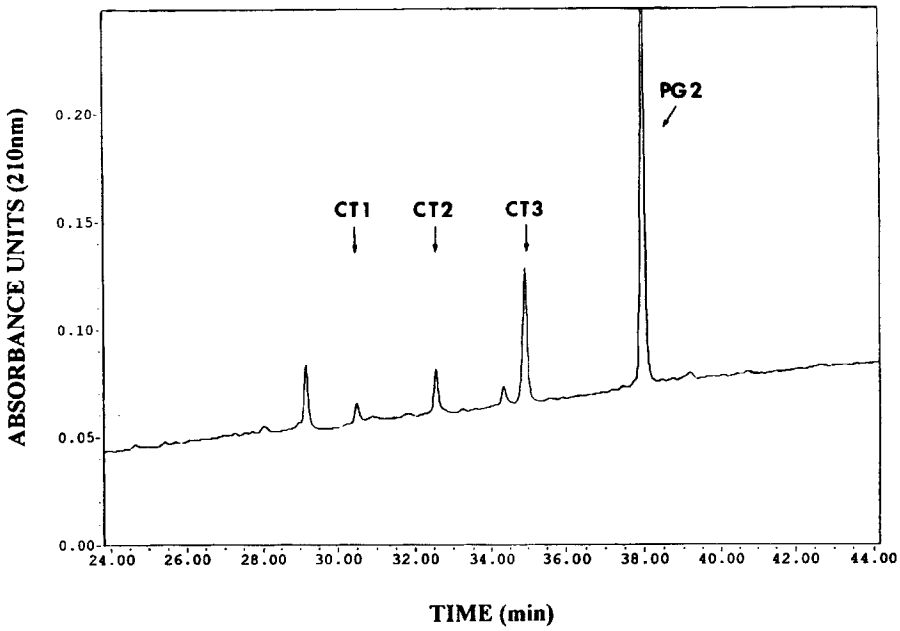

Fig. 1. RP-HPLC purification of PG-2 chymotryptic digest. Approximately 5 nmol of the digest was purified on a 46 × 250 mm Vydac C18 column using a linear gradient of acetonitrile that contained 0.1% TFA and increased in acetonitrile by 1% min^{-1}.

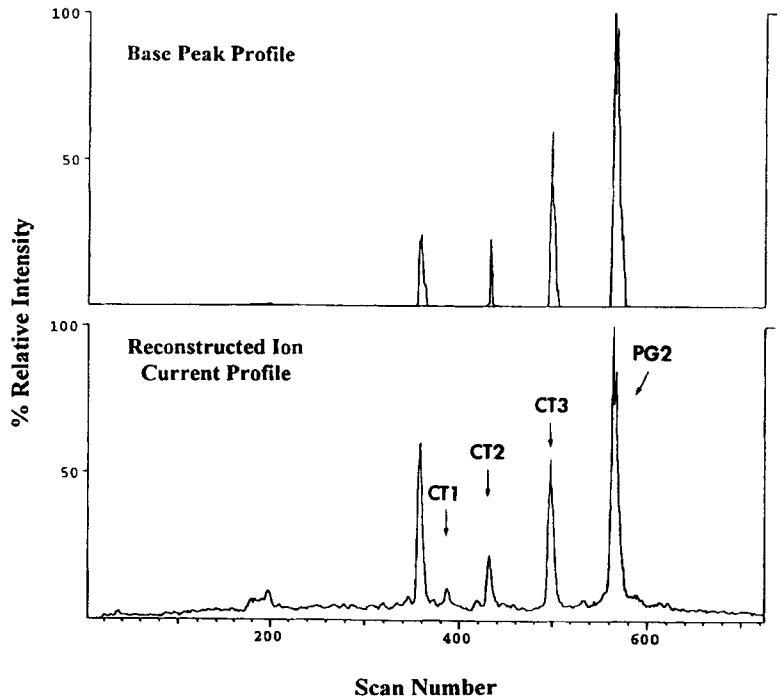

Fig. 2. LC-ESI-MS analysis of PG-2 chymotryptic digest (~50 pmol). Both the base peak profile (top panel) and the reconstructed ion current profile (bottom panel) are shown. The major peak represents the undigested PG-2. Several chymotryptic fragments, labelled as CT1, CT2 and CT3, were generated.

obtained during RP-HPLC purification of the whole digestion mixture (Figure 1). Several chymotryptic fragments were generated, and were designated as CT1, CT2 and CT3. Their average mass values were 1452.0, 1435.0 and 1417.0, respectively, as shown by the corresponding mass spectrum of each peak (Figure 3). The average mass value obtained for the major peak is 1957.0 (data not shown), which agreed with the calculated average mass value of 1956.42 for the parent compound, PG-2, within the instrumentation error of 0.03%.

These mass values of CT1, CT2 and CT3 were compared with the mass values of the predicted chymotryptic fragments generated by MacProMass computer program [15] as shown in Table 1. Because each additional peptide bond cleavage added a water molecule and increased the mass value by 18, it became apparent that CT1, CT2 and CT3 were des-RGGRL derivatives of PG-2 ($C^6YC^8RRRFC^{13}IC^{15}V$) with two, one and no additional chymotryptic cleavage after the potentially chymotrypsin-sensitive tyrosine or phenylalanine residues, respectively (see Figure 5). These des-RGGRL chymotryptic fragments have respective predicted average mass values of 1451.78, 1434.78 and 1416.78. The amino acid composition of each purified fragment also supported this interpretation, as indicated in Table 2.

Based on the mass value and amino acid composition of CT2 fragment, there were only two possible structures for CT2, namely: desRGGRL-PG2 with one additional peptide bond cleavage either after Tyr or Phe. To distinguish between these alternatives, ~0.12 nmol of this CT2 fragment was subjected to performic acid oxidation and the mixture was purified on a 2.0 × 150 mm Vydac small bore C18 column after the removal of excess oxidising agent. Only one peak with significant 210 nm absorbance was observed (data not shown) and its amino acid composition (3.3 Cysteic acid, 3.2 Arg, 1.2 Val, 0.9 Ile and 1.0 Phe) strongly suggested that the additional peptide bond cleavage was at the Tyr site (see also Table 2).

Table 1 Predicted PG-2 chymotrypsin fragments and their corresponding MH+ masses

Fragment	Average MH+ mass	Sequence
Residues 6–7	285.32	C[a]Y
Residues 13–16	436.57	CICV-amide[b]
Residues 1–5	558.64	RGGRL
Residues 8–12	737.89	CRRRF
Residues 1–7	824.96	RGGRLCY
Residues 6–12	1004.21	CYCRRRF
Residues 8–16	1155.46	CRRRFCICV-amide

[a] The average mass value of C in cysteine form is used.
[b] The native protegrin peptides have an amide group on the C-terminus.

Thermolysin Digestion

Although the amount of CT2 fragment generated was much less than CT3, analytic comparison of the two structures suggested that we were more likely to obtain digestion fragments with only one disulphide bond by subjecting CT2 to thermolysin treatment, since peptide bonds that are on the NH_2 side of hydrophobic residues are often sensitive to thermolysin digestion. When approximately 20 pmol of CT2 thermolysin digest was subjected to LC-ESI-MS analysis, the results shown in Figure 4 were obtained. As indicated by the base peak profile (Figure 4(a)) of this digest, two major peaks marked

Table 2 Amino Acid Composition of Chymotrypsin and Thermolysin Digestion Fragments of PG-2

Amino Acid	CT-1	CT-2	CT-3	CT-2(PO)[a]	CT-2,TH1	CT-2,TH2
Cys-acid	ND[b](4)	ND (4)	ND (4)	3.3 (3)	ND (2)	ND (2)
Arg	3.0 (3)	3.3 (3)	3.6 (3)	3.2 (3)	3.0 (3)	
Tyr	1.2 (1)	0.9 (1)	1.1 (1)			1.0 (1)
Val	1.1 (1)	1.2 (1)	1.1 (1)	1.2 (1)		1.1 (1)
Ile	0.8 (1)	0.8 (1)	0.8 (1)	0.9 (1)		1.3 (1)
Phe	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	

[a] CT-2 fragment was subjected to performic acid oxidation, the entire mixture was purified on a 2.0 × 150 mm Vydac small bore C18 column after the removal of the excess oxidising agent. The amino acid composition of the peak eluted at 40 min is shown here.
[b] ND, not determined.
( ), Expected residue number according to structure.

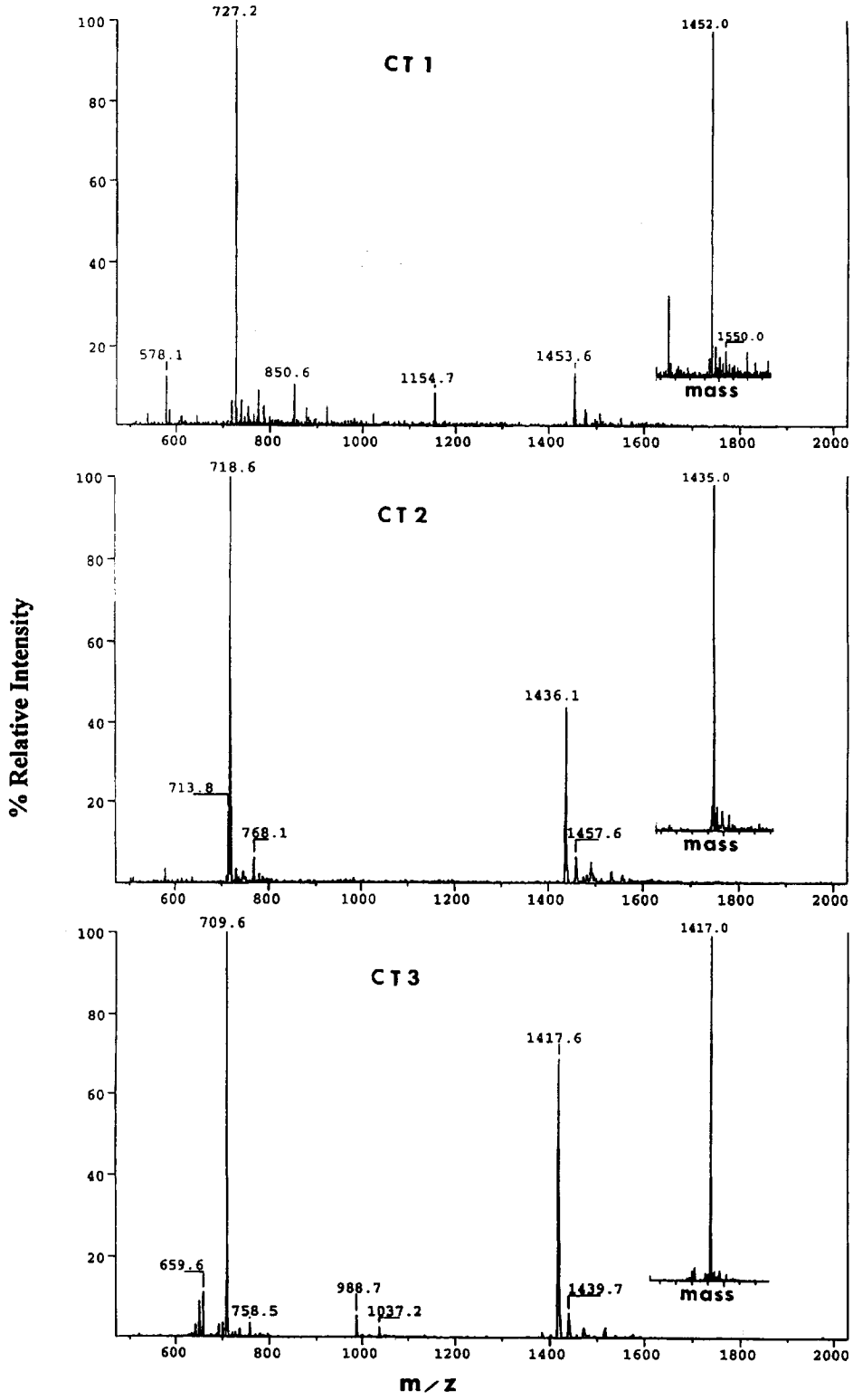

Fig. 3. Mass spectra derived from peaks labelled as CT1, CT2 and CT3 and their corresponding deconvoluted masses (shown in inset).

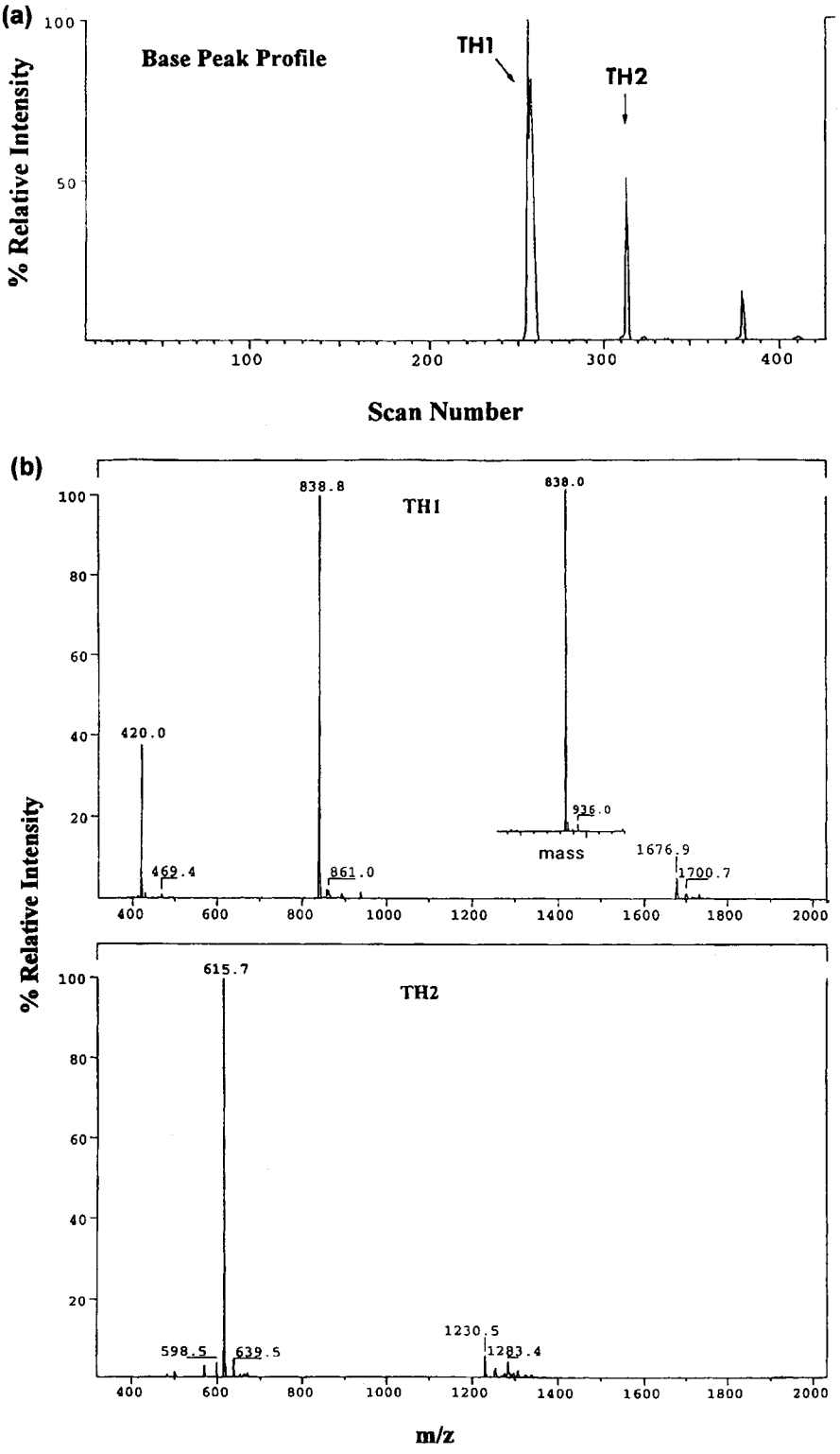

Fig. 4. LC-ESI-MS analysis of thermolysin digest of PG-2 CT2 fragment (~20 pmol). (a). Base peak profile of the digestion mixture. Themolysin digestion of CT2 fragment resulted in two major peaks labelled as TH1 and TH2. (b). Mass spectra derived from peaks labelled as TH1 and TH2. The deconvoluted mass for TH1 is also shown in the inset.

as TH1 and TH2 were obtained, whose corresponding mass spectra are shown in Figure 4(b). The deconvoluted average mass value for TH1 was 838.0, in excellent agreement with the calculated average mass value of 838.03 for thermolysin digestion fragment with $\overline{C^8R^9R^{10}R^{11}F^{12}C^{13}}$ structure. The average mass for MH+ ion of TH2 was 615.7, again in perfect agreement with the calculated average MH+ mass value of 615.75 for thermolysin digestion fragment with $\overline{C^6Y^7\ I^{14}C^{15}}V^{16}$-amide structure. Only two major peaks were obtained during RP-HPLC purification of the remaining thermolysin digest (data not shown), the amino acid composition analysis of the purified fragments shown in Table 2 confirmed the mass spectrometric data. Taken together, the data presented here indicated that the disulphide connectivities in PG2 are Cys^8-Cys^{13} and Cys^6-Cys^{15}.

DISCUSSION

PG-2 contains four cysteine residues, two of which are separated by only one amino acid. Because a disulphide linkage between almost adjacent cysteines is sterically unfavourable, we designed a strategy that could resolve the two remaining possible disulphide pairing patterns (Figure 5). Although it may have been possible to resolve the disulphide bonding pattern in PG-2 by a single step thermolysin digestion, we decided against this approach because of our concern that the relatively non-specific nature of this enzyme, which cleaves peptide bonds on the NH_2 side of hydrophobic residues, would probably have generated many more fragments, and presented a more complex mixture to analyse. On the contrary, chymotrypsin is much more specific and its digestion fragments can generally be predicted. As indicated in this report, although native PG-2 was relatively resistant to chymotrypsin digestion at pH 6.5, a condition intentionally chosen to prevent disulphide interchange [16], sufficient quantities of fragments for analysis were obtained, and their probable structures were ascertained from their respective mass values. One of these fragments, CT2, whose two possible structures are shown in Figure 5, provided an ideal candidate for resolving the disulphide structure. Thermolysin digestion of CT2 yielded fragments with corresponding mass values and amino acid compositions that could only be explained by the disulphide pattern of Cys^6-Cys^{15} and Cys^8-Cys^{13}.

Mass spectrometry is a highly useful technique in determining disulphide connectivities in proteins or

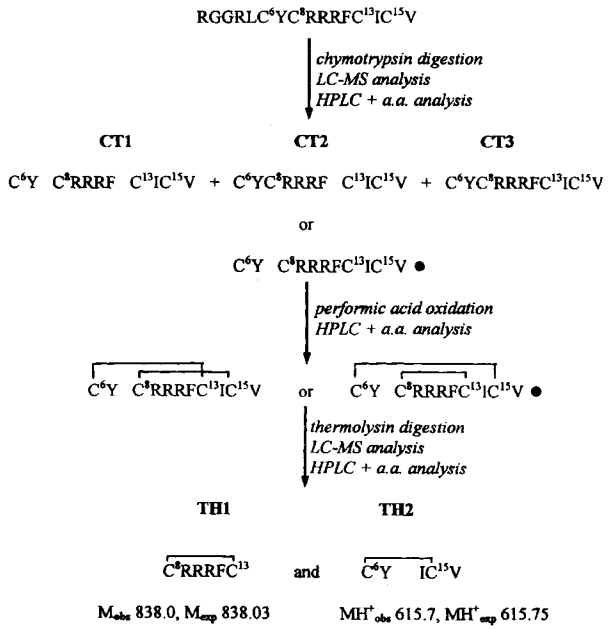

Fig. 5. Strategy for disulphide structure determination of PG-2: Native PG-2 was digested with chymotrypsin and purified on RP-HPLC. Direct LC-ESI-MS analysis was also performed on the whole digest. The CT2 fragment (average mass = 1435.0) has two possible structures as shown in this figure. The correct structure (indicated by ●) was derived after subjecting CT2 to performic acid oxidation followed by HPLC and amino acid analysis. The two possible disulphide connectivities in CT2 are also shown. The one with the correct disulphide linkage, derived from LC-MS analysis and HPLC-amino acid analysis of its thermolysin digest, is also indicated by ●.

peptides [16, 17]. Morris and Pucci [18] demonstrated the potential of fast atom bombardment mass spectrometry (FAB-MAS) as a tool for disulphide linkage analysis in proteins, using insulin as an example. The primary advantage of FAB-MS rests with the fact that disulphide-containing peptide fragments can be identified when present in mixtures. Peaks due to disulphide-containing peptides can be easily identified by the disappearance of these peaks when the mixture is analysed after treatment with either dithiothreitol or mercaptoethanol. The respective mass values of these disulphide-containing peptides allows disulphide pairing pattern assignments, when assisted by computer programs. This technique was successfully applied to determine the disulphide bonding pattern in a variety of peptides [19, 20] including an antimicrobial insect defensin [21] isolated from immune haemolymph of the dipteran *Phormia terranovae*.

The recent development of laser desorption mass

spectrometry (LD-MS) and electrospray ionization mass spectrometry (ESI-MS) techniques allows mass measurements of complex molecules at pmolar levels. By coupling capillary HPLC to electrospray mass spectrometry, one can obtain mass analyses of pmol amounts of complex enzyme digests. The results presented here demonstrate the power of using direct LC-ESI-MS analysis of whole enzyme digests to determine the disulphide connectivities in peptides. Although we further verified each structure by its amino acid composition in this study, it is quite clear from the data presented here that the mass spectrometric data alone would have led to the same unambiguous assignment of disulphide pairing pattern in PG-2, and it would have required only picomolar amounts of the native peptide. Performing disulphide linkage analysis in peptides or proteins is complicated when the enzymatic or chemical reaction fails to generate desired segments of peptides containing only one disulphide bond. This is commonly seen with complexly folded peptides or proteins, or in peptides that contain adjacent cysteine residues. Therefore, rapid identification in a complex digestion mixture of potential candidates for subsequent analyses is highly desirable. This is possible with the technique presented here, since both the base peak profile and the reconstructed ion current profile obtained during LC-ESI-MS analysis resemble the UV tracing during RP-HPLC purification of the whole digestion mixture. Confirmatory amino acid analysis is only necessary on the fragments suggested by the mass spectrometric data.

From the established disulphide structure of PG-2, protegrin molecules are likely to exist as antiparallel β-sheets that are similar to tachyplesins in overall conformation. The structure of protegrins is considerably simpler than that of defensins, larger cysteine-rich antimicrobial peptides with three intramolecular disulphide bonds that stabilise their considerably more complex triple stranded β-sheet configuration [22]. Like defensins, protegrins also possess broad spectrum antimicrobial properties. They differ from defensins, however, in that their antimicrobial activities are completely retained or enhanced in the presence of physiological salt solution and serum [23]. These considerations, when combined with their smaller size and simpler structure than defensin, make protegrins especially promising candidates for pharmaceutical development as broad spectrum peptide antibiotics for chemoprophylaxis or chemotherapy.

Analogous natural peptide antibiotics of similar size and structure to protegrins have also been isolated from haemocytes of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*) and were named tachyplesins and polyphemusins, respectively. These phylogenetically ancient endogenous antibiotics differ from the protegrins by having three amino acids between their first and the second Cys residues, and between the third and fourth Cys residues. Nevertheless, their disulphide connectivities, structures and their microbicidal properties resemble that of protegrins. Studies are currently in progress to determine the contribution of each disulphide bond in protegrins with respect to its microbicidal properties and structural stability.

Acknowledgement

The authors thank Vladimir N. Kokryakov for performing preliminary purification steps and Leonie Tan for final HPLC purifications of protegrins. This work was supported, in part, by research grants AI 22839, RR 06217 and CA 33572 from the National Institute of Health.

REFERENCES

1. V. N. Kokryakov, S. S. L. Harwig, E. A. Panyutich, A. A. Shevchenko, G. M. Aleshina, O. V. Shamova, H. A. Korneva and R. I. Lehrer (1993). Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS lett. 327*, 231–236.
2. P. Storici and M. Zanetti (1993). A novel cDNA sequence encoding a pig leukocyte antimicrobial peptide with a cathelin-like pro-sequence. *Biochem. Biophys. Res. Commun. 196*, 1363–1368.
3. C. Zhao, L. Liu and R. I. Lehrer (1994). Identification of a new member of the protegrin family by cDNA cloning. *FEBS lett. 346*, 285–288.
4. R. I. Lehrer, A. K. Lichtenstein and T. Ganz (1993). Defensins: antimicrobial and cytotoxic peptides of the mammalian cells. *Annu. Rev. Immunol. 11*, 105–128.
5. R. I. Lehrer, T. Ganz and M. E. Selsted (1991). Defensins: endogenous antibiotic peptides of animal cells. *Cell 64*, 229–230.
6. T. Nakamura, H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao and Y. Shimonishi (1988). Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). *J. Biol. Chem. 263*, 16709–16713.
7. T. Miyata, F. Tokunaga, T. Yoneya, K. Yoshikawa, S. Iwanaga, M. Niwa, T. Takao and Y. Shimonishi (1989). Antimicrobial peptides, isolated from horseshoe crab

haemocytes, tachyplesin II and polyphemusins I and II: chemical structure and biological activity. *J. Biochem. 106*, 663–668.

8. O. A. Mirgorodskaya, A. A. Schevchenko, K. O. Adballa, I. V. Chernushevich, T. A. Egorov, A. X. Musoliamov, V. N. Kokryakov and O. V. Shamova (1993). Primary structure of three cationic peptides from porcine neutrophils: sequence determination by the combined usage of electrospray ionization mass spectrometry and Edman degradation. *FEBS lett. 330*, 339–342.
9. S. S. L. Harwig, K. M. Swiderek, T. D. Lee and R. I. Lehrer (1994). Posttranslational covalent modifications of protegrins. *Protein Science 3*, Suppl. 1, Abstract 349.
10. H. G. Boman (1991). Antibacterial peptides: key components needed in immunity. *Cell 65*, 205–207.
11. M. Zasloff (1992). Antibiotic peptides as mediators of innate immunity. *Current Opinion Immunol. 4*, 3–7.
12. K. M. Swiderek, T. D. Lee and J. E. Shively. Trace structural analysis of proteins. *Methods Enzymol.* (in press).
13. K. M. Swiderek, P. S. K. Deng, T. D. Lee, J. E. Shively, Y. Hatefi and S. Chen in: *Techniques in Protein Chemistry V*, J. Crabb Ed., p. 49–57 1994.
14. R. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin (1984). Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr. 336*, 93–104.
15. T. D. Lee and S. Vemuri (1990). MacProPass: a computer program to correlate mass spectral data to peptide and protein structures. *Biomed. Environ. Mass Spectrom. 19*, 639–645.
16. T. E. Creighton (1984). Disulphide bond formation in proteins. *Methods Enzymol. 107*, 305–329.
17. D. L. Smith and Z. Zhou (1990). Strategies for locating disulphide bonds in proteins. *Methods Enzymol. 193*, 374–389.
18. H. R. Morris and P. Pucci (1985). A new method for rapid assignment of s-s bridges in proteins. *Biochem. Biophys. Res. Commun. 126*, 1122–1128.
19. M. O. Glocker, B. Arbogast, J. Schreurs and M. Deinzer (1993). Assignment of the inter- and intramolecular disulphide linkages in recombinant human macrophage colony stimulating factor using fast atom bombardment mass spectrometry. *Biochemistry 32*, 482–488.
20. E. Poerio, C. Caporale, L. Carrano, P. Pucci and V. Buonocore (1991). Assignment of the five disulphide bridges in α-amylase inhibitor from wheat kernel by fast-atom-bombardment mass spectrometry and Edman degradation. *Eur. J. Biochem. 199*, 595–600.
21. P. Lepage, F. Bitsch, D. Roecklin, E. Keppi, J-L. Dimarcq, J-M. Reichhart, J. A. Hoffmann, C. Roitsch and A. Van Dorsselar (1991). Determination of disulphide bridges in natural and recombinant insect defensin A. *Eur. J. Biochem. 196*, 735–742.
22. C. P. Hill, J. Yee, M. E. Selsted and D. Eisenberg (1991). Crystal structure of defensin HNP-3, an amhiphilic dimer: mechanisms of membrane permeabilization. *Science 251*, 1481–1485.
23. R. I. Lehrer, S. S. L. Harwig, E. Panyutich and V. N. Kokryakov (1994). Structural determinants of antimicrobial activity and LPS-binding by leukocyte protegrins. *Clin. Res. 42*, Abstract 151A.