Analytical methods for differentiating minor sequence variations in related peptides

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ABSTRACT

A proline-rich peptide was isolated and purified to homogeneity from an extract of bovine neutrophil granules using semi-preparative RP-HPLC. The relative molecular mass of the peptide (called Bac-X) was determined by ionspray MS to be 5149 ± 0.5. The amino acid composition of the peptide was characterized by its limited number of amino acid types, which included a high proline (43.3%) and arginine content (20.3%), and hydrophobic residues. Bac-X had similar characteristics to Bac-5, a previously characterised bactenecin of bovine neutrophil granules, with respect to its proline, arginine and hydrophobic amino acid content, molecular mass and antibacterial specificity. Tryptic and N-bromosuccinimide digestion of Bac-X produced fragments with masses (M_r 785 and 4224 and 3100 respectively) consistent with those expected from a peptide with the reported sequence of Bac-5. Bac-X differed from Bac-5 in the number of amino acid residues (43 for Bac-X *versus* 42 for Bac-5) and contained glycine which Bac-5 did not. However, the calculated molecular mass of the peptide, based on the amino acid compositional data, did not match the experimental value. The purified peptide could not be sequenced by Edman degradation due to apparent blockage of the N-terminus. Partial sequence information, obtained by LC–MS and collision induced dissociation MS–MS analysis of a M_r 785 tryptic fragment of Bac-X, showed that this peptide contained a six residue sequence (–RFPPIR–) not found in Bac-5 which, based on its reported sequence, contained a M_r 785 tryptic fragment with the sequence –FRPPIR–. This difference in sequence of Bac-X compared with Bac-5 illustrates the application of electrospray (ionspray) MS techniques to the detection and identification of minor differences in related protein/peptide forms.

INTRODUCTION

Multicellular organisms contain specialised cells (granulocytes, macrophages, cytotoxic lymphocytes, natural killer cells etc.) that can selectively destroy invading organisms such as bacteria. Such cells, including the bovine neutrophil, contain a variety of lytic enzymes and antimicrobial/cytotoxic proteins stored in cytoplasmic granules [1].

The term "bactenecin" has been ascribed to antibacterial proteins/peptides isolated from bovine neutrophils. To date three bactenecins have been isolated from the large granules of the bovine neutrophil and fully characterised. These include a defensin-like, cyclic dodecapeptide [2] and two unique proline- and arginine-rich peptides Bac-5 and Bac-7 which do not resemble the reported sequences of other mammalian antimicrobial peptides [3].

The two bactenecins Bac-5 and Bac-7 ($M_r \approx$ 5000 and ≈ 7000, respectively) were characterised by their limited number of amino acid types, including a high proline (>45%) and arginine (>23%) content and hydrophobic amino acids. Bac-5 and Bac-7 are highly antibacterial towards Gram-negative organisms (*Escherichia coli*) but

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not Gram-positive organisms (*Staphylococcus aureus*) [4].

This investigation reports the purification and characterisation of a proline- and arginine-rich peptide of similar molecular mass and amino acid composition to the bactenecin, Bac-5. Evidence is presented, based on partial sequence information (obtained by ionspray mass spectrometry (MS), liquid chromatography (LC)–MS and collision induced dissociation (CID) MS–MS analysis of the peptide and its enzyme and chemical digestion products), that this peptide (called Bac-X) is different to Bac-5.

EXPERIMENTAL

Source of antibacterial peptides

The crude antibacterial peptide mixture (crude Bac-X), used as the source of bactenecins in this study, was isolated from extracts of granules of bovine neutrophils, using gel permeation chromatography and cation-exchange chromatography as described previously [5].

Antibacterial assay

Antibacterial activity of Bac-X was determined by a direct plate count method described previously [5]. Briefly, bacterial pathogens (*E. coli* B117 and *S. aureus* GLAXO), cultivated to the mid-logarithmic growth phase, were incubated with the peptide at 37°C for 30 min, in 10 mM sodium phosphate buffer, pH 7.4. The number of viable organisms in assay mixtures was determined by a pour-plate count method. Bacterial killing was expressed as a percentage of the initial colony count.

Peptide purification

Lyophilised peptide extract (8.6 mg of crude Bac-X) was reconstituted in 500 μl 0.1% trifluoroacetic acid (TFA) and subjected to semi-preparative reversed-phase (RP) high-performance liquid chromatography (HPLC) with a Waters C_{18} DeltaPak column (300 Å pore size, 30 cm × 3.9 mm) connected to a Waters 625 LC system (Waters Chromatography Division, Millipore Australia, Lane Cove, Australia). Elution of the bound material, monitored at 220 nm, was performed with a linear 30–50%B gradient over

25 min at a flow-rate of 1 ml/min. Solvent A: 0.1% TFA. Solvent B: 90% acetonitrile in 0.1% TFA.

Collected fractions (*ca*. 1 ml) containing the peptide of interest, as determined by analytical RP-HPLC and ionspray MS, were pooled and lyophilised. Except for the 30–50%B gradient being run over 30 min instead of 25 min, analytical RP-HPLC of samples was carried out using the same column, LC system and separation conditions as used for the semi-preparative RP-HPLC. In some cases, peak purity was assessed by spectral analysis of the eluting peptide(s) in a Waters 990 photodiode array detector. Minor contaminants in the pooled fraction(s) were removed by re-subjecting the fractions to the semi-preparative RP-HPLC protocol.

Capillary zone electrophoresis (CZE)

CZE of the purified peptide was carried out in a fused silica capillary connected to a Waters Quanta 4000 capillary electrophoresis system. Electrolyte solution was 10 mM phosphoric acid, pH 2. Peptide solution (0.167 μg/μl) in electrolyte solution was loaded into the capillary under hydrostatic pressure using a 10-s sampling time. CZE was carried out at 12 kV, which generated a current of *ca*. 80 mA under these conditions. Migration of the peptide was monitored at 214 nm. The migration time of the peptide was compared with lysozyme which was used as a basic reference protein.

Ionspray mass spectrometry

A Sciex AP111 triple quadrupole mass spectrometer (Sciex, Ontario, Canada) was used for MS and MS–MS analysis of the peptide samples and tryptic digests. The MS was operated in the positive ion mode (ionspray voltage + 5.5 kV). Sample, typically prepared in 0.1% TFA with or without an organic modifier (normally acetonitrile), was infused into the ionspray (via a fused-silica capillary) at a flow-rate of *ca*. 2–3 μl/min with an infusion pump (Syringe Infusion Pump 22, Harvard Apparatus).

Ions generated from the ionspray interface via ion evaporation mechanisms [6] were sampled into the mass spectrometer by a potential difference set between the sprayer and the sample

orifice. Ultrapure nitrogen was applied at the atmospheric side of the skimmer to minimise solvent clusters and particulate matter entering the mass spectrometer. The third quadrupole (Q-3) was scanned over the required mass range. Typical scan conditions were m/z 300–2400 in 5 s with a scan step of 0.5 a.m.u. Spectra were collected in the multichannel averaging (MCA) mode at orifice potentials typically set between 60 and 130 V. Molecular masses of charged species were calculated using the HyperMass software (Sciex).

For MS–MS ultrapure argon was used as the collision gas and was introduced into the collision cell (Q-2) at a target gas thickness of $350 \cdot 10^{12}$ atoms/cm². The collision energy was controlled by the Q-2 offset voltage and was typically set at -20 V. The daughter ion mass spectra were acquired by scanning the third quadrupole (Q-3) while the first quadrupole (Q-1) was set for transmitting the parent ion. The Q-3 offset was programmed to provide optimum ion-transmission efficiency.

In LC–MS experiments Q-3 was scanned, typically from m/z 300–2400 and the total ion current (TIC) chromatogram acquired.

Microbore RP-HPLC

Microbore RP-HPLC was carried out with an Aquapore RP-300 column (50 × 1 mm; ABI, USA). In tryptic digest LC–MS experiments the column was connected to a Waters 600-MS analytical LC system controller and pump in which the tubing dead-space was minimised by connecting the Rheodyne injector directly to the mixer "T-piece" at the pump. However, even with this modification the fluid system still had a significant dead-volume which resulted in long analysis times (*ca.* 120 min). Microbore RP-HPLC of tryptic digests was also carried out using UV detection at 214 nm for comparison with the mass spectrometric TIC chromatograms. In these analyses the Aquapore column was connected to a Waters 625-LC system which was also modified to minimise tubing dead-space. The Rheodyne injector was connected directly to the pump junction/transducer head and tubing connections kept to a minimum length. The injection (10 μl) of samples in 0.05% TFA (2 μg/ul) in both systems was accomplished with a Rheodyne injector fitted with a 50-μl sample loop. Elution of bound material was performed with a linear, 0–90%B gradient over 60 min. Solvent A: 0.1% TFA. Solvent B: 90% acetonitrile in 0.1% TFA. The column flow-rate was maintained at 50 μl/min and in the case of LC–MS experiments, the exit of the column was connected directly to the ionspray interface via the fused silica capillary tubing. There was no presplit (before the column) or postsplit (after the column) of the mobile phase.

Amino acid analysis

Dried portions of the peptide (*ca.* 25 nmol) were hydrolysed for 24 h at 105°C *in vacuo* with 6 M HCl in the vapour phase. Amino acids in the peptide hydrolysate were derivatised to their phenylthiocarbamyl (PTC) derivatives using Pico-Tag chemistry (Waters, Chromatography Division, Millipore, Australia). PTC-amino acids were separated on a Beckman Ultrasphere PTH column (25 cm × 4.6 mm) at 1 ml/min at 45°C with a gradient of 0–46%B over 18 min, and monitored at 254 nm. Buffer A: 0.14 M sodium acetate, pH 6.45 containing 0.02% EDTA and 0.05% triethylamine. Buffer B: 60% acetonitrile in water. Standard amino acids (Pierce Amino Acid Standard H) were derivatised and run under identical conditions to the samples.

Edman sequencing

Purified peptide (*ca.* 2 nmol) was applied to Polybrene-treated glass fibre filters for automated Edman degradation on a gas-phase sequencer (Model 470A; Applied Biosystems, Foster City, CA, USA).

Tryptic digestion of Bac-X

Tryptic digestion was based on the method of Deibler *et al.* [7] as described by Milne *et al.* [8]. Briefly, purified peptide (*ca.* 200 μg) was dissolved in 100 μl 0.1 M ammonium hydrogencarbonate, pH 8.0 in a 1.5-ml capacity Eppendorf tube. N-Tosyl(Tos)-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) was added to the peptide solution at a 1:50 enzyme/substrate mass ratio, vortexed and incu-

batcd at room temperature for 24 h. Enzyme and substrate blanks were treated in a similar manner. After incubation the digest and blanks were lyophilised and reconstituted in 0.05% TFA prior to analysis by microbore LC–MS and MS–MS.

Carboxypeptidase Y (CP-Y) cleavage

Peptide (*ca.* 5 μg) was dissolved in 50 μl 50 m*M* ammonium acetate buffer, pH 5.5 and stock CP-Y solution (0.4 μg CP-Y/μl) was prepared by reconstituting one vial of CP-Y (Boehringer, sequencing grade) (20 μg) in 50 μl deionised water. The digestion was initiated by the addition of stock CP-Y solution (1 μl) to the peptide solution (enzyme/substrate ratio, 1:125). The reaction mixture was infused directly into the ionspray source of the mass spectrometer at a flow-rate of 3 μl/min at selected intervals. The ionspray mass spectra were acquired using the MCA mode as previously described.

Cleavage of Bac-X with N-bromosuccinimide

Bac-X was cleaved at the tyrosyl-peptide bond by treatment with N-bromosuccinimide (NBS; Aldrich) [3,9]. The peptide (1.94 nmol) was incubated for 16 h at room temperature with 8 nmol NBS in 100 μl of 50% acetic acid. The resulting fragments were analysed by microbore LC–MS on a Spherisorb 5 μm C_{18} column (SGE, Australia) using a linear 0–65% gradient of acetonitrile in 0.1% TFA over 65 min.

Peptide synthesis

The peptide fragment sequence RFPPIR, deduced from LC–MS and MS–MS analysis of a tryptic fragment of the parent peptide, was synthesised essentially by a rapid manual technique [10] on a 0.5 m*M* scale. Briefly, *tert.*-butyloxycarbonyl (Boc)-arginine(Tos) coupled phenylacetamidomethyl (PAM) resin (polystyrene, 1% cross-linked) was exposed to a repeated cycle of deprotection with TFA; washing with dimethylformamide (DMF); reaction with hydroxybenzotriazyltetramethyluronium pentafluorophosphate (HBTU)-activated amino acid neutralised *in situ* with diisopropylethylamine (DIEA); and DMF washes until the final amino acid was coupled to the peptide

chain. Coupling efficiencies, as determined by quantitative ninhydrin analysis were 99.85%, 99.97% and 99.97% for Ile, the first Pro and the N-terminal Arg, respectively. Coupling efficiencies of the second Pro and Phe, were qualitatively assessed using the Isatin test for N-terminal proline residues.

Deprotected, resin-coupled peptide (275 mg) was cleaved using a HF protocol [11] and yielded 25 mg crude peptide. Crude synthetic peptide was reconstitutated in *ca.* 20 ml 5% acetic acid and subjected to preparative RP-HPLC on a Waters DeltaPak C_{18} column (39 cm × 7.8 mm). The crude peptide solution was loaded directly onto the column via the solvent reservoir line C of the Waters 625 LC system. Elution of peptide material, monitored at 220 nm, was effected with a 0–67% B gradient over 60 min at a flow-rate of 3 ml/min. Solvent A: 0.1% TFA. Solvent B: 90% acetonitrile in 0.1% TFA. Collected fractions were analysed by analytical RP-HPLC and ionspray MS. Fractions containing the peptide were pooled and lyophilised. Preparative RP-HPLC of crude peptide yielded 13.3 mg pure product. Peptide purity was confirmed by ionspray MS.

RESULTS AND DISCUSSION

Analytical RP-HPLC of the crude Bac-X extract (Fig. 1) achieved reasonable resolution of the several components present in this fraction, including the peak containing Bac-X. Bac-X was purified from the crude antibacterial fraction by semi-preparative RP-HPLC. The purified peptide was active (100% inhibition) towards *E. coli* B117 at a concentration of 13 μ*M* in the assay but was not active towards *S. aureus* GLAXO at this peptide concentration. This apparent antibacterial specificity of Bac-X towards Gram-negative bacteria was consistent with that reported for Bac-5 [3]. Evidence for the homogeneity of Bac-X was provided by analytical RP-HPLC, including photodiode array detection and spectral analysis of the peptide peak (see insets in Fig. 1), CZE (Fig. 2) and ionspray MS (Fig. 3). Analytical RP-HPLC of Bac-X showed a single symmetrical peak. Spectral analysis (210–300 nm) of the peak maximum and the right and
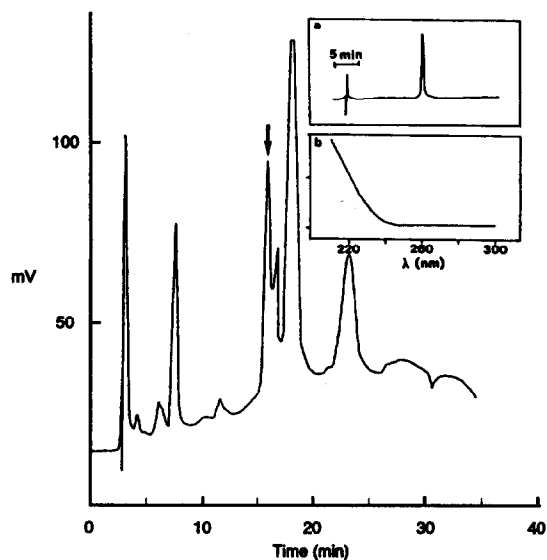
Fig. 1. Analytical RP-HPLC of antibacterial granule protein (crude Bac-X). Lyophilised protein was reconstituted in 0.1% TFA (1 µg/µl) and 50 µl injected onto a Waters DeltaPak C_{18} column (30 cm × 3.9 mm) connected to a Waters 625 LC system. Solvent A, 0.1% TFA; solvent B, 90% acetonitrile in 0.1% TFA; gradient, 30–50% B in 25 min; flow-rate, 1 ml/min; detection, 220 nm. The peak containing Bac-X is indicated by the arrow. Inset: (a) Homogeneity of Bac-X, purified by semi-preparative RP-HPLC, as determined by analytical RP-HPLC using the same conditions described above except a 30–50% gradient was run over 30 min. (b) Overlaid spectra (210–300 nm) of the peak maximum and the right and left peak slopes normalised to 210 nm illustrating spectral coincidence.

left slope were coincident when normalised to 210 nm indicating peak homogeneity. The UV spectrum of Bac-X revealed little absorbance at 254 and 280 nm, indicating a lack or absence of tryptophan or tyrosine residues in the peptide. CZE analysis of the peptide (Fig. 2) also revealed a single peak which had a longer migration time (7.04 min) than lysozyme (6.2 min), a reference cationic protein. The average molecular mass of the peptide (±S.D.), as determined by ionspray MS (calculated by the HyperMass routine from the MS data in Fig. 3) was 5149 ± 0.5 and was almost identical to the calculated molecular mass of 5150.5 for Bac-5, based on the reported sequence data for this peptide [3].

The amino acid composition of Bac-X is shown in Table I. Also included for comparison purposes are the reported amino acid composi-
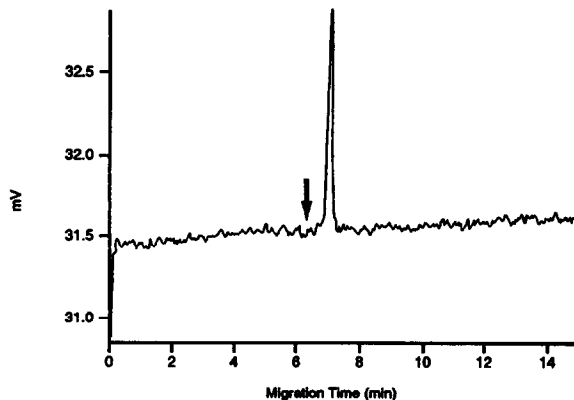

Fig. 2. Capillary zone electrophoresis of the purified bactenecin Bac-X. CZE of the purified peptide in electrolyte solution (0.167 µg/µl) was carried out in a fused-silica capillary (47 cm) fitted to a Waters Quanta 4000 capillary electrophoresis system. Conditions: electrolyte, 10 mM phosphoric acid, pH 2; sampling mode/time, hydrostatic/10 s; voltage, 12 kV; current, ca. 80 µA; detection, 214 nm. The migration time of lysozyme, used as a cationic reference protein, is indicated by the arrow.

tions of the bactenecins Bac-5 and Bac-7 [3]. Bac-X was characterised by a limited number of amino acid types, including a high proline and arginine content (43.3 and 20.3%, respectively) and hydrophobic residues. The amino acid composition (mol/mol) of Bac-X differed slightly from the reported composition of Bac-5 in its
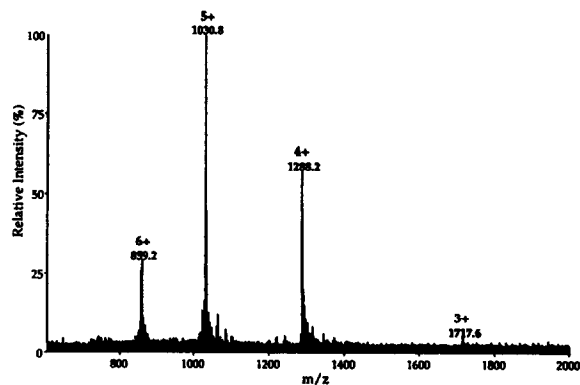

Fig. 3. Ionspray mass spectrometry of purified bactenecin Bac-X. The mass spectrum of the peptide was acquired as described in Experimental. The mass assigned peaks represent the series of protonated molecular ion charge states, $[M + nH]^{n+}$ of the peptide. Each peak includes the unresolved isotopic contributions to each molecular ion charge state. The average molecular mass of the peptide (±S.D.), calculated by the HyperMass routine, was 5149 ± 0.5.

TABLE I

AMINO ACID COMPOSITION OF BAC-X

The amino acid compositions of Bac-5 and Bac-7 were also included for comparison with the data for Bac-X. Values in parentheses are from the sequencing data [3].

Amino acid	Composition (mol/mol)		
	Bac-X	Bac-5	Bac-7
Pro	18.7	20.1 (19)	28.9 (28)
Gly	1.1	–	2.6 (3)
Ile	4.7	5.0 (5)	4.1 (4)
Leu	1.6	1.2 (1)	4.2 (4)
Phe	5.8	6.1 (6)	3.3 (3)
Arg	8.8	8.8 (10)	16.3 (17)
Tyr	0.6	0.8 (1)	–

glycine, and possibly leucine content. However, the calculated M_r of Bac-X (5164.7), based on the amino acid compositional data, was not consistent with the experimentally determined molecular mass of the peptide. This indicated a degree of uncertainty in the accuracy of the amino acid compositional data. The amino acid composition and molecular mass data, however, indicated that Bac-X and Bac-5 were closely related, if not identical peptides.

The peptide could not be sequenced by automated Edman degradation. Hence it was concluded that the major peptide was N-terminally blocked. Also, attempts to determine the C-terminal sequence of the peptide by carboxypeptidase Y digestion and analysis by ionspray MS were unsuccessful. Molecular ions, associated with C-terminally truncated peptides of Bac-X were not detected even after 24 h digestion of the peptide with carboxypeptidase Y. This resistance to cleavage by the carboxypeptidase was unexpected in view of the amino acid compositon of Bac-X and the broad amino acid specificity of carboxypeptidase Y and may indicate that there is a confirmational restraint to exopeptidase action on the C-terminus of the peptide.

Partial sequence information on Bac-X was obtained by LC–MS and CID–MS–MS analysis of tryptic digests and LC–MS analysis of NBS cleavage of the peptide. The total ion current

profile obtained from LC–MS analysis of the tryptic digest of Bac-X is shown in Fig. 4. As confirmed by microbore LC using UV detection at 220 nm (data not shown), the LC–MS profile also showed the presence of three peaks. Full scan ionspray mass spectra of these peaks are shown in (Fig. 5. Peak 1 contained low intensity ions in the low m/z range (300–600), several of which were also present in the trypsin blank, such as m/z 355 and 429 (data not shown). However, no molecular ion series were identified in this peak when analysed by the HyperMass routine. Peak 2 contained only one peptide fragment of molecular mass 785.0, identified by the presence of the singly and doubly charged molecular ions, m/z 786 and 393.5, respectively. Peak 3 also contained only one fragment of molecular mass of 4223.7 as calculated from the protonated molecular ion charge states, [M +
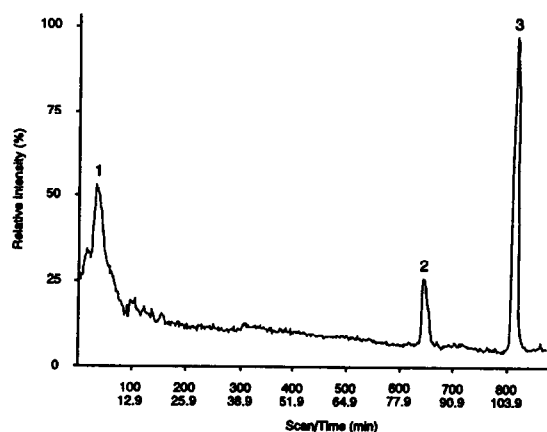

Fig. 4. LC–MS total ion current (TIC) profile from the analysis of a tryptic digest of Bac-X. Lyophilised tryptic digest was reconstituted in 0.05% TFA (2 μg/μl) and 10 μl fractionated on an Aquapore RP–300 column (50 × 1 mm) connected to a Waters 600-MS system controller and pump. Solvent A, 0.05% TFA; solvent B%, 90% acetonitrile in 0.05% TFA; gradient (see Note below), 0–90%B in 60 min; flow-rate, 50 μl/min. The column outlet was connected directly to the ionspray interface of the MS system via the fused-silica capillary. The mass range scanned was from m/z 300–2400 and the scan data acquired as the TIC chromatogram. Note: Microbore LC analysis of the tryptic digest, using UV detection, had revealed only three components. Therefore, due to the excessively long analysis time, caused by the excessive volume between the gradient mixer and column of the modified LC–MS system, the gradient was terminated after elution of the second peak and the third component eluted isocratically with 90%B.
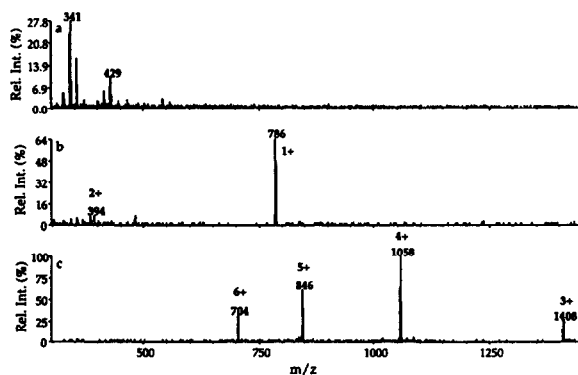
Fig. 5. Full scan ionspray MS of tryptic peptide fragments of Bac-X. The component(s) present in each of the peaks was identified by extracting the mass spectrum of the scan corresponding to the peak maximum from the TIC profile. The protonated molecular ion charge state(s), $[M + nH]^{n+}$ of the peptide fragment associated with each peak, as determined by the HyperMass routine, were identified. The molecular mass of tryptic fragments calculated by the HyperMass routine were: (a) peak 1, no molecular ion series identified; (b) peak 2, M_r 785; (c) peak 3, M_r 4224.

$nH]^{n+}$ of the peptide fragment. After subtracting 18 for the loss of a water molecule the combined masses of the two fragments is 4991. This combined mass accounts for 96.7% of the mass (M_r 5149) of the parent peptide. The mass difference between the mass of Bac-X and the combined mass of the two peptides (4991) was 158 which approximates to the mass of an Arg residue (156.2).

The molecular masses of the fragments produced by trypsin digestion of Bac-X were consistent with the masses of fragments that would be expected to be generated by tryptic digestion of a peptide with the sequence of Bac-5. Trypsin cleavage of Bac-5 would potentially produce a free N-terminus Arg, a M_r 785 peptide fragment with the sequence FRPPIR and a M_r 4228 fragment containing the C-terminus of the peptide. Further evidence that Bac-X and Bac-5 were closely related peptides was that cleavage of Bac-X with NBS produced a fragment with an average molecular mass of 3100 (data not shown) which is very close to the reported molecular mass (M_r 3104) of the C-terminal fragment released by NBS cleavage of the tyrosyl-peptide bond of Bac-5 [3]. The release of a M_r 4224 tryptic fragment from Bac-X, with almost identi-

cal mass to a C-terminal fragment of Bac-5, indicated that this fragment may contain the C-terminal portion of Bac-X. Hence it is probable (although not confirmed in this study) that the M_r 785 fragment and free arginine, released by tryptic digestion, originate from the N-terminal portion of the peptide.

The results of LC–MS analysis of tryptic and NBS digests of Bac-X were further evidence that Bac-X and Bac-5 were indeed identical peptides. However, subsequent MS–MS analysis of spectra generated by CID of the molecular ions associated with the tryptic peptide fragments revealed a different sequence for the M_r 785 fragment of Bac-X than that expected for Bac-5.

CID of the doubly and singly charged molecular ions of the M_r 785 tryptic fragment (Fig. 6a, b) yielded both Y-series and B-series fragment ions that were rich in sequence information. These data (also see Table II), together with the amino acid composition data of Bac-X, and the knowledge that trypsin cleaves peptide bonds on the C-terminal side of Arg residues, enabled the sequence of the M_r 785 tryptic fragment to be deduced. As the C-terminal residue of this small tryptic fragment was assumed to be Arg the Y_1 ion of the Y-series ions ($^+NH_3CHRCOOH$) would be Arg. This was confirmed by the presence of an m/z 174.5 fragment ion in the m/z 786 $[M + H]^+$ and m/z 393.5 $[M + 2H]^{2+}$ CID spectra. Successive mass increments between the Y_1–Y_5 ions were 112.5, 97, 98, and 147 and corresponded to the masses of amino acid residues Leu(Ile), Pro, Pro and Phe, respectively. Hence the preliminary sequence proposed for the tryptic fragment was XFPPL(I)R where X was an unassigned residue. The calculated difference in mass between the M_r 785 tryptic fragment and the mass of the assigned residues was 156. This is equivalent to the mass of an Arg residue and indicated that the unidentified N-terminal residue X, was Arg. The identity of this residue was confirmed by the presence of the B_1 fragment ion (B-series ions have the general formula NH_2CHRCO^+) of Arg (m/z 157) in the m/z 393.5 $[M + 2H]^{2+}$ CID spectra. Therefore the proposed sequence for this tryptic fragment is RFPPL(I)R. As the sequence of Bac-5 also contained a potential M_r 785 tryptic fragment
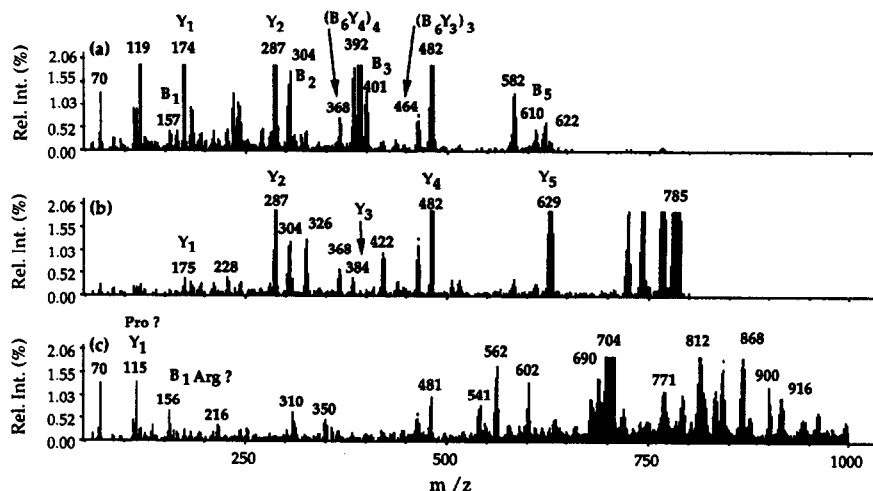
Fig. 6. Collision induced dissociation spectra of parent ions of tryptic peptide fragments derived from Bac-X. Tryptic digest was infused into the ionspray source at a flow-rate of 2 μl/min. MS–MS analysis was carried out on singly and multiply charged parent ions of the tryptic peptide identified by LC–MS (Fig. 5). MS–MS spectra are: (a) the $[M + 2H]^{2+}$ parent ion of peak 2 at m/z 393.5; (b) the $[M + H]^+$ parent ion of peak 2 at m/z 786; (c) the $[M + 6H]^{6+}$ parent ion of peak 3 at m/z 704. The fragment ions used to deduce the sequence of the M_r 785 tryptic peptide of Bac-X are identified (see Table II).

with the sequence FRPPIR, it was imperative that there were no ambiguities in the interpretation of the MS–MS sequencing data for the M_r 785 fragment of Bac-X. The only difference between the CID-MS–MS spectra of this tryptic fragment and that of Bac-X are in the masses of the B_1 and Y_5 ions. There was no evidence of the presence of a m/z 148 B_1 ion or a m/z 638 Y_5 ion, indicative of the sequence FRPPIR, in the CID-MS–MS spectra of the Bac-X tryptic fragment.

Further evidence for the proposed sequence of this tryptic peptide was the presence of proline associated ions of the type $(B_xY_y)_z$, where B_x and Y_y indicate the points of cleavage to produce the carboxyl terminus and amino terminus of the fragment respectively and the number (z) outside the parentheses describes the number of amino acid residues present in the ion. Such ions are common in peptides that contain proline, as protonation and cleavage of the proline amide bond reportedly produces a highly favoured Y-type ion [12]. Intramolecular transfer of the proton to the other amide linkages in the ion and subsequent cleavage results in the formation of $(B_xY_y)_z$ type ions. Therefore, if the M_r 785 tryptic fragment sequence was as proposed, a (B_6Y_4) ion $[PPL(I)R^+]$ and a $(B_6Y_3)_3$ ion

$[PL(I)R^+]$ with m/z values of 464 and 367, respectively, would be expected in the CID spectra. Reasonably strong signals were detected at m/z 464 and m/z 367.5 in the m/z 393.5 $[M + 2H]^{2+}$ CID spectrum (Fig. 6a).

The Y- and B-type fragment ions do not allow differentiation of leucine from isoleucine as both have the same mass (113.2). Two side-chain-specific fragmentation processes have been reported [13], observed at high collision energies, that produce a W-type ion with the formula $[R'CH = CHCO\text{-}(NHCHRCO)_{n-1}OH + H]^+$ where $R' = H$ for Leu but CH_3 for Ile. However, such ions are reportedly not produced under the lower energies used in ionspray MS.

The proposed sequence of RFPPL(I)R was confirmed by peptide synthesis and analysis of the synthetic analogue (RFPPIR) by ionspray MS and MS–MS (data not shown). The synthetic peptide gave identical MS and MS–MS spectra to that shown in Fig. 5 and Fig. 6a and b, respectively.

Little sequence information was obtained from the CID-MS–MS spectra of the multiply charged molecular ions of the M_r 4224 tryptic fragment of Bac-X. The doubly charged molecular $[M + 2H]^{2+}$ ion at m/z 2112 from the M_r 4224 tryptic fragment of Bac-X was resistant to CID frag-

TABLE II

MS–MS ANALYSIS OF THE M_r 785 TRYPTIC FRAGMENT OF BAC-X

Daughter ions generated by CID of the singly and doubly charged molecular ions of the M_r 785 tryptic fragment of peak 2 (Fig. 6a, b). Ions Y_1, Y_3 and the B-series ions were detected in the m/z 393.5 $[M + 2H]^{2+}$ CID spectrum (Fig. 6a). Ions Y_1–Y_5 were detected in the m/z 786 $[M + H]^+$ CID spectrum (Fig. 6b).

Fragment ion series[a]	m/z	$\Delta m/z$ ($Y_n - Y_{n+1}$ or $B_n - B_{n+1}$)	Identified residue(s)
Y_1	174.5		Arg
		112.5	Leu/Ile
Y_2	287.0		
		97.0	Pro
Y_3	384.0		
		98	Pro
Y_4	482.0		
		147	Phe
Y_5	629.0		
B_1	157.0		Arg
		147.0	Phe
B_2	304.0		
		97	Pro
B_3	401.0		
		210 (97 + 113)	Pro-Leu/Ile[b]
B_5	611.0		

[a] Y-series and B-series ions have the general formula $^+NH_3CHRCOOH$ and NH_2CHRCO^+, respectively [12,15].
[b] The B_4 ion (m/z 498) was not detected in the m/z 786 $[M + H]^+$ CID spectrum. The difference in mass between B_3 and B_5 (m/z 210) fits with the combined mass of Pro and Leu/Ile which would be expected based on the preliminary sequence deduced from the Y-series fragmentation [XFPPL(I)R].

mentation, yielding only a few daughter ions of relatively low intensities (data not shown). There were no obvious fragmentation ion-series identified in this CID spectrum. The m/z 704 $[M + 6H]^{6+}$ molecular ion of this large tryptic fragment produced a large number of CID fragment ions (Fig. 6c). CID spectra of precursor ions containing multiple charges are complex and assignment of ion-types to the fragments is difficult due to the uncertainty as to the number of charges on the fragment ions [14]. However, examination of the low m/z region of the spec-

trum revealed possible Arg, B_1 and Pro, Y_1 fragment ions which were consistent with the reported sequence of Bac-5 [3].

CONCLUSIONS

Initial biochemical characterisation of Bac-X indicated that this proline-rich peptide was identical to Bac-5, a well characterised member of the bactenecin family of antibacterial peptides. Both peptides had similar amino acid compositions and identical relative molecular masses. Also, tryptic and NBS digestion of Bac-X released fragments with relative molecular masses consistent with the masses of fragments expected from a peptide with the sequence of Bac-5. However, the sequence [RFPPL(I)R] of the small (M_r 785) tryptic fragment of Bac-X (deduced from CID-MS–MS spectral data of the doubly and singly charged molecular ions of this fragment) did not match any six residue segment of Bac-5.

This partial sequence information is evidence that Bac-X is a new, although closely related, form of Bac-5 and illustrates the application of electrospray (ionspray) MS, LC–MS and CID-MS–MS techniques to the detection and identification of minor differences in protein/peptide forms.

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