



Proteomic analysis of *Myrmecia pilosula* (jack jumper) ant venom

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Abstract

Ant sting allergy in Australia is predominantly due to the *Myrmecia pilosula* species complex. Gel separation of *M. pilosula* venom is necessary so that the allergenic importance of each component can be defined by western blotting. However, previous PAGE methods produced suboptimal resolution and the components of each band were not precisely defined. Venom was resolved in both non-reduced and reduced form by one-dimensional acid urea PAGE, SDS-PAGE and two-dimensional acid urea-SDS PAGE. Resolved peptides were extracted and analysed by HPLC-MS. Acid urea PAGE and acid urea-SDS PAGE proved more effective than SDS-PAGE for resolution of peptides smaller than 10 kDa. All of the major peptides previously observed in *M. pilosula* venom were observed in gel resolved venom. Venom was found to primarily consist of peptides with molecular weight < 10 kDa, most of which contain disulfide bridges. SDS-PAGE of non-reduced venom clearly defined six higher molecular weight proteins between 26 and 90 kDa. An 8546 Da dimer named pilosulin 5 was observed, but pilosulin 4, a peptide recently proposed to be present in venom was not. A variant of pilosulin 4 here named pilosulin 4.1a, existing as an 8198 Da dimer, was observed and has been characterised.

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1. Introduction

Ant sting allergy within Australia is predominantly due to ants of the *Myrmecia* genus — with around 90% of

life-threatening allergy (anaphylaxis) thought to be due to the *Myrmecia pilosula* species complex (*M. pilosula*, jack jumper ant, JJA) (Brown et al., 2003a; Douglas et al., 1998; Street et al., 1994). JJA are found in South-Eastern Australia, including Tasmania, Victoria, New South Wales, southern Queensland and cooler areas of South Australia and Western Australia. (<http://anic.ento.csiro.au/bcbmapquery/>) It has been reported that 1.9% of adults living in rural Victoria and 2.7% of the entire Tasmanian population are allergic to JJA venom (Brown et al., 2003a;

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Douglas et al., 1998). Desensitisation with whole body extract is no longer available due to concerns regarding efficacy (Weiner et al., 1995), but our group has demonstrated the effectiveness of venom immunotherapy in preventing anaphylaxis to JJA stings (Brown et al., 2003b).

Previous analysis of JJA venom by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was followed by the cDNA cloning of two allergenic peptides named Myr p 1 and Myr p 2 (Donovan et al., 1993; Street et al., 1996). Myr p 1 and Myr p 2 have been shown to contain a common protein leader sequence (Street et al., 1996). The main product of Myr p 1 (Myr p 1 57→112) has been named pilosulin 1 (Wu et al., 1998) and the main product of Myr p 2 (Myr p 2 49→75) was named pilosulin 2 (Donovan and Baldo, 1997). Both pilosulin 1 and pilosulin 2 are highly basic peptides, with theoretical isoelectric points of 9.94 and 10.45, respectively. Using HPLC–mass spectrometry (MS), our group reported the presence of more than 50 peptides in JJA venom with molecular weight <10 kDa, with two peptides comprising around 80% of total venom content (Davies et al., 2004). The two main peptides were identified by a combination of tandem mass spectrometry (MS–MS) and Edman degradation sequence analysis to be [Ile⁵]pilosulin 1 and a previously undescribed heterodimer. Pilosulin 1 was detected in limited quantities. Pilosulin 2 was not detected in whole venom; rather it was present in this heterodimer as des-Gly²⁷-pilosulin 2 (Myr p 2 49→74), linked head to tail by two disulfide bridges to a previously unreported 23 amino acid residue peptide with a mass of 2457 Da (peptide masses are given as isotopically averaged unless otherwise specified). This heterodimer was named pilosulin 3, while des-Gly²⁷-pilosulin 2 was named pilosulin 3a and the 2457 Da peptide named pilosulin 3b.

Recently, Inagaki et al. (2004) obtained novel cDNA clones, which contained the same common protein leader sequence as Myr p 1 and Myr p 2. These cDNA clones were obtained from homogenised whole body preparations of *Myrmecia banksi*, a member of the *M. pilosula* species complex. The clones encoded two peptides, which Inagaki et al. named pilosulin 3 (encoded by the cDNA Myr p 3) and pilosulin 4 (encoded by Myr p 4). Inagaki's 'pilosulin 3' is a variant of the peptide that had previously been described as pilosulin 3b (Davies et al., 2004), in which a glycine is added to the C-terminus of pilosulin 3b and isoleucine replaces leucine at the N-terminus. As these findings were not correlated with an examination of venom, the described sequences have uncertain relevance. Therefore, to avoid confusion, the terminology previously proposed by our group shall be used (Davies et al., 2004), where pilosulin 3 refers to the heterodimer composed of pilosulin 3a and pilosulin 3b. The reported sequences of JJA venom peptides are shown in Table 1.

In 2001, it was reported that serum from a man who died of JJA venom anaphylaxis showed poor recognition for known peptide allergens when compared to whole venom

(Brown et al., 2001). Further investigations found that approximately 20% of allergic subjects had a similar pattern of serological reactivity (Wu, 2000). SDS-PAGE immunoblots using individual allergic patient sera and 2-mercaptoethanol reduced venom suggested four additional allergens with molecular weight 11.7–43.5 kDa (Wu, 2000).

Previously, Ford et al. (1991) detected several higher molecular weight proteins, but at that time, they were not thought to be allergenic. The IgE binding activity of these proteins, as well as [Ile⁵]pilosulin 1, pilosulin 3 and other peptides in JJA venom is unknown. The identity of all major venom components and an appreciation of their allergenic importance is essential if we are to develop a standardised venom preparation that is suitable for routine diagnostic and therapeutic use.

Initial studies used 2-mercaptoethanol to reduce disulfide bonds (Donovan et al., 1993, 1995; Street et al., 1994; Wu, 2000), and it was reported that SDS-PAGE separation of this reduced JJA venom was inadequate. Previous attempts to perform two-dimensional (2D) SDS-PAGE with 2-mercaptoethanol reduced venom have also proved unsuccessful (Donovan et al., 1995). Donovan et al. (1996) found that synthetic pilosulin 2 created artificial multimers if it was not alkylated with iodoacetamide prior to electrophoresis. Street et al. (1996) then demonstrated superior SDS-PAGE resolution using reduction and alkylation of venom prior to electrophoresis. Whether peptides other than pilosulin 2 form aberrant multimers during electrophoresis when they are reduced but not alkylated is unclear.

Our aim was to develop gel separation methodologies for the analysis of JJA venom and to identify the peptides present in resolved gel bands by mass spectrometry. These investigations are a prelude to the development of robust quality control methods and western blot studies to characterise the important venom allergens.

2. Materials and methods

Polyacrylamide gels were cast in a Hoefer Mighty Small gel caster (Amersham Biosciences, Uppsala, Sweden). Acrylamide and molecular weight markers (broad range and polypeptide) were purchased from Bio-Rad (Hercules, California, USA). Microtubes for peptide extraction were obtained from Axygen (Union City, California, USA) and silanised according to the manufacturer's instructions with AquaSil™ siliconizing fluid, obtained from Pierce (Rockford, Illinois, USA). All chemicals and consumables used were analytical grade.

2.1. JJA venom preparation

JJA venom was collected from a variety of locations around Tasmania as previously described (Davies et al., 2004). Venom was separated on all types of gels in both non-reduced and reduced/alkylated form. Prior to

Table 1
Previously published primary amino acid sequence of JJA venom peptides

	1	6	11	16	21	26	31	36	41	46	51	Molecular weight (Da)	pI
Pilosulin 1 (Donovan et al., 1993)	GLGSV	FGRLA	RILGR	VIPKV	AKKLG	PKVAK	VLPKV	MKEAI	PMAVE	MAKSQ	EEQQPQ	6052.36 ^a	10.45 ^a
[Ile ⁵] pilosulin 1 (Davies et al., 2004)	GLGSI	FGRLA	RILGR	VIPKV	AKKLG	PKVAK	VLPKV	MKEAI	PMAVE	MAKSQ	EEQQPQ	6066.41 ^b	10.45 ^b
Pilosulin 2 (Street et al., 1996)	IDWKK	VDWK-K	VSKKT	CKVML	KACKF	LG						3212.02 ^a	9.94 ^a
Pilosulin 3a (Davies et al., 2004)	IDWKK	VDWK-K	VSKKT	CKVML	KACKF	L ^c						3153.97 ^b	9.94 ^b
Pilosulin 3b (Davies et al., 2004)	LIGLV	SKGTC	VLVKT	VCKKV	LKQ ^c							2457.15 ^b	9.90 ^b
Pilosulin 3 ^d (Inagaki et al., 2004)	IIGLV	SKGTC	VLVKT	VCKKV	LKQG							2515.20 ^a	9.90 ^a
Pilosulin 4 (Inagaki et al., 2004)	FDITK	LNIKK	LTKAT	CKVIS	KGASM	CKVLF	DKKKQ E					4087.01 ^a	9.87 ^a

^a Calculated from sequences published on ExPASy (http://au.expasy.org/tools/pi_tool.html).

^b Calculated by the authors, using sequence entered onto ExPASy (http://au.expasy.org/tools/pi_tool.html).

^c Amidated C-terminal residue.

^d Inagaki's pilosulin 3 (Inagaki et al., 2004) is equivalent to [Ile¹]pilosulin 3b, as per Davies et al. (2004) with addition of a C-terminal glycine residue.

electrophoresis, reduced venom was prepared by reduction with dithiothreitol and alkylation with iodoacetamide using the protocol of [Street et al. \(1996\)](#).

2.2. One-dimensional SDS-PAGE

Gradient mini-gels (containing 10–20% acrylamide and *N,N'*-methylenebisacrylamide (Bis), 37.5:1) were run as per the method of [Laemmli \(1970\)](#), and tris–tricine SDS-PAGE gels were run as per the method of [Schagger and von Jagow \(1987\)](#). Gels were stained with either Coomassie blue or zinc ([Castellanos-Serra et al., 1999](#)). For Coomassie blue, gels were stained for 30 min in Coomassie blue G (0.1% in 40% methanol and 10% acetic acid), then destained in boiling water for 20 min. Further destaining (with 20% methanol and 7.5% acetic acid) was used until the gel background was colourless. For zinc staining, gels were incubated in 0.1 M imidazole/0.1% SDS for 15 min. Bands were developed with 0.2 M zinc sulphate until the background became opaque. Development was stopped using three 1-min washes in distilled water ([Castellanos-Serra et al., 1999](#)). Gels were immediately visualised and individual bands excised for extraction and subsequent MS analysis.

2.3. Acid urea PAGE

One-dimensional acid urea PAGE (AU PAGE) was performed on 15% acrylamide (19:1 Bis) mini-gels containing 6 M urea as per the method of [Rabilloud et al. \(1996\)](#) except that Triton X100 was not added. Gel polymerisation was catalysed using the ammonium persulphate–TEMED system and gels pre-run overnight at 90 V. Sample buffer contained a final concentration of 6 M urea, 250 mM acetic acid, 100 mM potassium acetate, 15% (w/v) sucrose and methyl green as the tracking dye. Voltage was applied (200 V) until the methyl green tracking dye had migrated through and left the separating gel. Gels were stained with Coomassie blue (as for one-dimensional SDS-PAGE) or zinc stain. For zinc staining, it was necessary to increase the pH by washing the gels three times for 5 min in 192 mM tris/25 mM glycine prior to staining as described for SDS-PAGE.

2.4. AU-SDS PAGE

Venom was separated using AU PAGE as described above. When complete, the gel was removed from the plates and a thin vertical section (approximately 2 mm in width) cut from the centre of each lane, incubated for 20 min in equilibration buffer (containing 6 M urea, 2% SDS, 50 mM Tris pH=8.8, 20% (v/v) glycerol and a trace amount of bromophenol blue) and placed on top of a 16.5% SDS-PAGE separating gel (19:1 Bis) overlaid with a 1 cm 10% acrylamide (33.3:1 Bis) 'spacer' gel ([Schagger and von Jagow, 1987](#)). The acrylamide strip was held in place with 1% low-melting point agarose containing 0.1% SDS and

50 mM tris, pH=8.8. The second dimension was then run with a tris–tricine running buffer, initially at 30 V for 30 min then 120 V until the bromophenol blue reached the bottom of the separating gel. Gels were stained with Coomassie blue, as described above.

2.5. Peptide extraction for mass spectrometry

Peptides were extracted from zinc and Coomassie blue stained bands using a modification of the method of [Stone and Williams \(2002\)](#). Briefly, gel bands were excised with a scalpel, frozen, crushed and destained with two 10-min washes with 25 mM ammonium bicarbonate in 50:50 acetonitrile:water. Gel pieces were dried with a vacuum centrifuge and then incubated overnight at 37 °C in 100 µl 25 mM ammonium bicarbonate. The following morning, gel pieces were sonicated for 5 min and peptides extracted with two 10-min incubations in 0.5% trifluoroacetic acid (TFA) in 50:50 acetonitrile:water. The extract was evaporated to dryness in a vacuum centrifuge and stored at –20 °C pending analysis by HPLC–MS.

When peptides were reduced and alkylated following electrophoresis, destained and dried gel bands were incubated in 10 mM dithiothreitol in 25 mM ammonium bicarbonate at 56 °C. After 1 h, the reducing agent was replaced by 55 mM iodoacetamide in 25 mM ammonium bicarbonate and the sample incubated in the dark for 45 min at room temperature. The processed peptides were then extracted as above.

2.6. Trypsin digestion

Trypsin digestion was employed to investigate selected bands; extracted and dried peptides were reconstituted in 100 µl of 25 mM ammonium bicarbonate solution (pH=8.9). Trypsin was added at a molar ratio of approximately 1:20 to the extracted peptide, and the solution incubated at 37 °C for 6 h. Digestion was stopped by the addition of 5 µl of 1% formic acid, and the digested peptide mixture was stored at –20 °C pending analysis by HPLC–MS.

2.7. HPLC–MS analysis

For HPLC–MS analysis, extracted peptides were reconstituted in 50 µl of 50:50 acetonitrile:water and 20 µl was injected into the HPLC column. HPLC–MS and MS–MS analyses were performed using an ion-trap mass spectrometer (Finnigan LCQ) with a standard electrospray source, as per the methods of [Davies et al. \(2004\)](#). Relative ion current was determined by adding the area under the curve of the three most abundant ions for any given peptide. Theoretical fragment ion data, used for MS–MS and peptide sequence analysis, was calculated using software available from the University of California San Francisco website ([Clauser et al., 1999](#)). When artefacts following mass spectrometry of gel extracted peptides were observed,

the 'real' peptide was identified as the species with the lowest molecular mass. This was confirmed from HPLC–MS observation of both non-reduced and reduced/alkylated venom (Davies et al., 2004). The 'observed molecular weight' refers to the molecular weight of a peptide observed following deconvolution of mass spectra, whereas 'calculated molecular weight' refers to the weight after subtraction of the mass added by alkylation (if this process was undertaken).

3. Results

3.1. One-dimensional SDS-PAGE

SDS-PAGE of non-reduced venom, independent of the running buffer used, resulted in poor separation of the major venom peptides (Fig. 1). These did not migrate according to molecular weight and as the load was increased above 1 µg/lane, there was significant streaking of major bands, which severely limited this technique. Reduced venom showed improved separation, but again, as the load increased there was increased band streaking and loss of resolution. Gradient SDS-PAGE, however, provided excellent resolution of higher molecular weight species (Fig. 1, lane A). Predominant proteins with a molecular weight > 20 kDa included those with masses of 26, 31, 33, 35, 45 and 90 kDa. When JJA venom was reduced and alkylated (Fig. 1, lane B), there was a considerable change to the distribution of

proteins, with all of the above, other than the 90 kDa protein, affected by the process.

3.2. One-dimensional AU PAGE

AU PAGE resulted in good separation of the two main peptides in non-reduced venom (Fig. 2, lane A) and no streaking of bands was observed with loads of up to 100 µg/lane. The calculated molecular weights of peptides extracted from each band, as determined by HPLC–MS, are shown in Table 2. HPLC–MS confirmed that the two main bands resolved by AU PAGE from non-reduced venom contained [Ile⁵]pilosulin 1 (Fig. 2, lane A, band 1) and pilosulin 3 (Fig. 2, lane A, band 5). The band containing [Ile⁵]pilosulin 1 also contained a peptide of mass 5545 Da, which created approximately 3% of the ion current of [Ile⁵]pilosulin 1. Pilosulin 1 (Fig. 2, lane A, band 2) was clearly separated from [Ile⁵]pilosulin 1. The band containing pilosulin 3 (Fig. 2, lane A, band 5) also contained peptides with masses of 5665, 5723, 5779 and 5835 Da that co-eluted on HPLC. Each of these contributed 16, 12, 3 and 1%, respectively, towards the total ion current of this HPLC–MS peak. The 8546 Da peptide was well separated by HPLC from the peak containing pilosulin 3 and produced approximately 15% of the ion current compared to this pilosulin 3 containing peak.

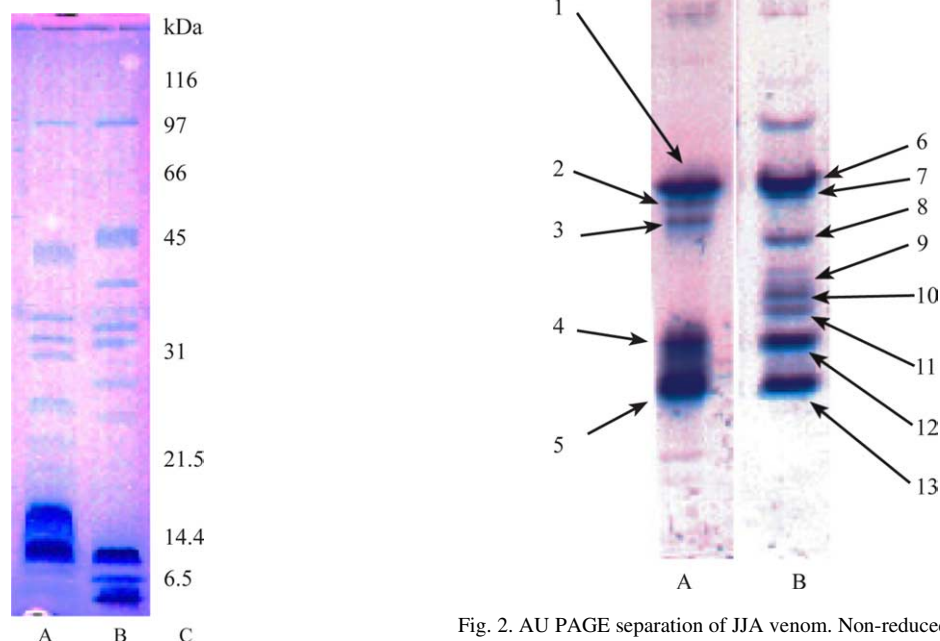


Fig. 1. Gradient SDS-PAGE separation of JJA venom. Non-reduced JJA venom (Lane A), reduced JJA venom (Lane B) and broad range molecular weight markers (Lane C) were subject to gradient SDS-PAGE and bands visualized with zinc stain.

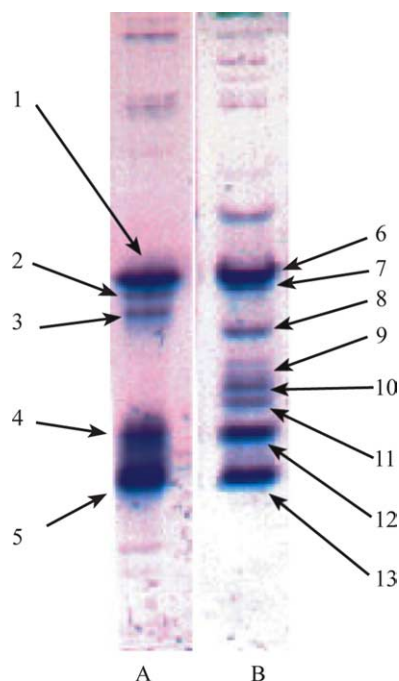


Fig. 2. AU PAGE separation of JJA venom. Non-reduced (Lane A) or reduced (Lane B) JJA venom was subject to AU PAGE and stained with Coomassie blue. Resolved bands are labelled 1–13. These bands were excised, extracted and subject to HPLC–MS analysis—the identity of the peptides comprising the labelled bands are shown in Table 2.

Table 2
Peptides identified by mass spectrometry from AU PAGE resolved bands

Band label ^a	Non-reduced or reduced	Molecular weight (Da)		Peptide identity
		Observed	Calculated (non-alkylated)	
1	Non-reduced	6066	NA	[Ile ⁵]pilosulin 1
		5545	NA	Unidentified monomer
2	Non-reduced	6052	NA	Pilosulin 1
3	Non-reduced	8198	NA	Pilosulin 4.1
4	Non-reduced	5624	NA	Oxidised pilosulin 3
5	Non-reduced	5608	NA	Pilosulin 3 heterodimer ^b
		5665	NA	Pilosulin 3.1 heterodimer ^b
		5723	NA	Unidentified peptide
		5779	NA	Unidentified peptide
		5835	NA	Unidentified peptide
		8546	NA	Pilosulin 5
6	Reduced	6066	NA	[Ile ⁵]pilosulin 1
7	Reduced	6052	NA	Pilosulin 1
8	Reduced	4331 ^c	4274 ^d	Pilosulin 5a
9	Reduced	2571 ^c	2457 ^d	Alternate form of pilosulin 3b
10	Reduced	4215 ^c	4101 ^d	Pilosulin 4.1a
11	Reduced	2628 ^c	2514 ^d	Pilosulin 3.1b ^b
12	Reduced	2571 ^c	2457 ^d	Pilosulin 3b ^b
13	Reduced	3269 ^c	3155 ^d	Pilosulin 3a

NA, not applicable as these peptides were either not reduced or non-reducible.

^a Band labels correspond to those stated in Fig. 2.

^b Two forms of this peptide are evident upon separation by HPLC–MS. One of these forms comprise 90–95% of the peptide content.

^c The molecular weight deduced from the mass spectra when cysteine residues were alkylated.

^d Represent the molecular weight of the peptide after subtraction of the mass added by alkylation of cysteine residues.

Other peptides, as outlined in Table 2, were detected in non-reduced venom.

Reduced and alkylated venom was well resolved by AU PAGE (Fig. 2, lane B) and subsequent HPLC–MS analysis did not detect any aberrant multimers. [Ile⁵]pilosulin 1 was the only predominant peptide not affected by the reduction process. The monomers identified from AU PAGE resolved reduced venom extracts are shown in Table 2.

3.3. Identification of monomer peptides

Reduction and alkylation of the 8198 Da peptide, extracted from band 3 (Fig. 2, lane A), resulted in the formation of a single 4215 Da peptide, which was identical to the peptide extracted from band 10 (Fig. 2, lane B). After consideration of the mass shift caused by alkylation, this is consistent with the 8198 Da peptide being a homodimer of a 4101 Da peptide, linked by two disulfide bonds.

Reduction and alkylation of band 5 (Fig. 2, lane A), which contained pilosulin 3 and peptides with masses 5665, 5723, 5779, 5835 and 8546 Da (Table 2), formed several new peptides. Pilosulin 3a and pilosulin 3b (in two forms) were identified, as were peptides with alkylated molecular weight 4331 Da (identical to the peptide extracted from band 8 (Fig. 2, lane B)) and 2628 Da (identical to the peptides extracted from band 11 (Fig. 2, lane B), in two

forms). Monomer chains from the 5723, 5779 and 5835 Da peptides were not apparent.

After considering the reduction products of the other peptides, it was clear that reduction and alkylation of the 8546 Da peptide resulted in formation of a single peptide with mass 4331 Da. Allowing for the possible mass shifts due to alkylation, this was consistent with the 8546 Da peptide being composed of a homodimer of a 4274 Da peptide with one disulfide linkage. This 8546 Da dimer shall be referred to from this point as pilosulin 5 and the monomer pilosulin 5a. MS–MS analysis of pilosulin 5a did not suggest that it was related to any of the sequences listed in Table 1.

3.4. Mass spectrometry analysis of the 2628 Da peptide

Theoretical 'y' and 'b' fragment ion data (Roepstorff and Fohlman, 1984) for pilosulin 3b with an additional C-terminal glycine were generated, and MS–MS was undertaken on the 2628 Da peptides extracted from band 11 (Fig. 2, lane B). Every singly charged 'b' ion from b₅ through to b₁₈, and every singly charged 'y' ion from y₅ through to y₁₇ was observed. This was consistent with both our previously identified variant of pilosulin 3b, (Davies et al., 2004) which shall now be referred to as pilosulin 3.1b and Inagaki's 'pilosulin 3' (Inagaki et al., 2004) (Table 1—molecular mass 2514 Da). Pilosulin 3.1b is present in non-reduced venom as a disulfide-linked

heterodimer with pilosulin 3a. This heterodimer has a molecular mass of 5665 Da and has been named pilosulin 3.1. Both PAGE and ion trap mass spectrometry were unable to detect if the N-terminal residue was leucine or isoleucine, or indeed, if both forms were present.

3.5. Isomass variants of pilosulin 3b

When analysed by HPLC–MS and MS–MS, pilosulin 3b and pilosulin 3.1b each appeared as two well separated, but otherwise indistinguishable peaks (i.e. isomass variants). In each case, one of the isomass variants predominated, generating 90–95% of the total ion current. Consistent with these findings, the corresponding heterodimers (5608 and 5665 Da) appeared as two well-separated peaks following HPLC–MS, with one isomass variant of each heterodimer predominating.

3.6. Mass spectrometry analysis of the 4215 Da peptide

MS analysis of the 4215 Da peptide demonstrated that it was a previously unreported variant of pilosulin 4, with a molecular mass 4101 Da ([Glu³¹]pilosulin 4 according to IUPAC-IUB naming guidelines). This 4101 Da peptide shall be referred to from this point forward as pilosulin 4.1a, and the 8198 Da dimer pilosulin 4.1. This was supported by MS–MS data from pilosulin 4.1a showing identical singly charged ‘b’ ions from b₄ to b₉ and singly charged ‘y’ ions from y₃ to y₅ as predicted for pilosulin 4. This was suggestive that the five C-terminal and nine N-terminal residues of pilosulin 4.1a and pilosulin 4 were identical. The MS–MS data for a series of possible variant sequences differing by 14 and 15 Da were examined in detail. From this set, substitution of glutamic acid for aspartic acid at position 31 then extended the ‘y’ ion sequence to y₈. Multiply charged ‘b’ and ‘y’ ions were then observed for the entire length of the peptide, suggesting that there was only

one residue different between pilosulin 4 and pilosulin 4.1a, although the MS–MS data from the ion trap MS was too complex for *a priori* sequencing to be unequivocal.

Further confirmation of the sequence was obtained by HPLC–MS–MS analysis of a trypsin digest. A peptide with monoisotopic mass of 634 Da was observed, and MS–MS analysis of this, the correct mass for residues 28–32 (Val–Leu–Phe–Glu–Lys) of the proposed peptide was performed. Fragment ions b₂–b₄ and y₂–y₄ for the predicted peptide were all observed, as was the internal fragment ion for Phe–Glu. This was consistent with pilosulin 4.1a being pilosulin 4 modified by the substitution of glutamic acid for aspartic acid at position 31. Other trypsin digest fragments were consistent with the pilosulin 4 sequence, as given by Inagaki et al. (2004).

3.7. Two-dimensional PAGE

Two-dimensional SDS-PAGE was attempted using immobilised pH gradient strips of pI 3–10 and pI 6–11 but, as was experienced by Donovan et al. (1995) these techniques were mostly unsuccessful (data not shown). This was due to the peptides migrating to the extreme basic end of the strip, causing poor first dimensional resolution. Significant band streaking during SDS-PAGE also contributed to sub-optimal peptide separation.

AU-SDS PAGE was therefore employed to resolve peptides co-eluting in AU PAGE resolved venom bands (Fig. 3). A number of peptides from non-reduced venom that co-migrated throughout AU PAGE appeared to be well separated by this two-dimensional method. This technique resolved peptides <10 kDa in non-reduced and reduced venom into seven and 12 bands, respectively. AU-SDS PAGE achieved excellent separation with reduced JJA venom and band streaking during SDS-PAGE was minimal with loads of up to 20 µg. Peptides were extracted from major bands in non-reduced and reduced JJA venom and subject to HPLC–MS analysis, but the sensitivity of

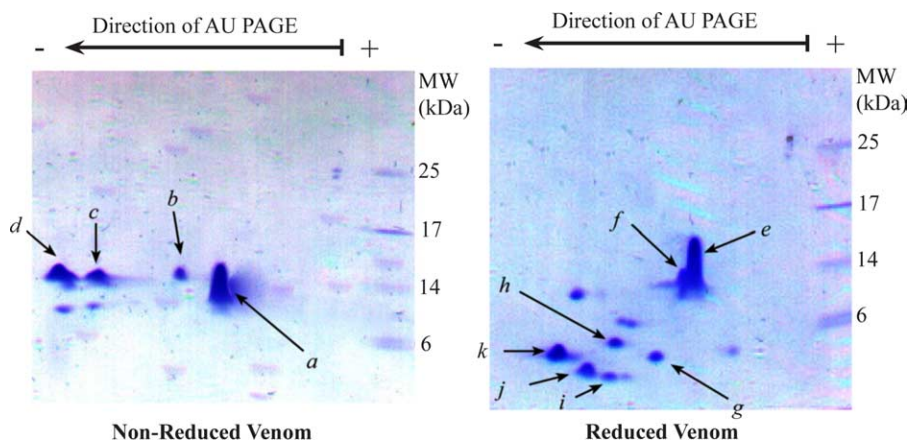


Fig. 3. Two-dimensional AU-SDS PAGE separation of JJA venom. Non-reduced (left) or reduced (right) JJA venom was subject to 2D AU-SDS PAGE and stained with Coomassie blue. Resolved bands are labelled a–k. These bands were excised, extracted and subject to HPLC–MS analysis. The identity of the peptides comprising the labelled bands is shown in Table 3.

Table 3

Peptides identified by mass spectrometry from 2D AU-SDS PAGE resolved gel bands

Band label ^a	Non-reduced or reduced	Molecular weight (Da)		Peptide identity
		Observed	Calculated (non-alkylated)	
a	Non-reduced	6066	NA	[Ile ⁵]pilosulin 1
b	Non-reduced	8198	NA	Pilosulin 4.1
c	Non-reduced	5624	NA	Oxidised pilosulin 3
d	Non-reduced	5608	NA	Pilosulin 3 heterodimer ^b
		5665	NA	Pilosulin 3.1 heterodimer ^b
e	Reduced	6066	NA	[Ile ⁵]pilosulin 1
f	Reduced	6052	NA	Pilosulin 1
g	Reduced	4331 ^c	4274 ^d	Pilosulin 5a
h	Reduced	4215 ^c	4101 ^d	Pilosulin 4.1a
i	Reduced	2628 ^c	2514 ^d	Pilosulin 3.1b ^b
j	Reduced	2571 ^c	2457 ^d	Pilosulin 3b ^b
k	Reduced	3269 ^c	3155 ^d	Pilosulin 3a

NA, not applicable as these peptides were either not reduced or non-reducible.

^a Band labels correspond to those stated in Fig. 3.^b Two forms of this peptide are evident upon separation by HPLC–MC. One of these forms comprise 90–95% of the peptide content.^c The molecular weight deduced from the mass spectra when cysteine residues were alkylated.^d Represent the molecular weight of the peptide after subtraction of the mass added by alkylation of cysteine residues.

the HPLC–MS method was poor, so peptides were not observed from all Coomassie blue stained bands. However, those that were are detailed in Table 3.

4. Discussion

JJA venom is composed of a number of disulfide-linked peptides and the majority of these have a molecular weight under 10 kDa. AU PAGE and AU-SDS PAGE were more effective than SDS-PAGE for separating peptides smaller than 10 kDa in non-reduced and reduced venom. Peptides that were well separated include the previously described [Ile⁵]pilosulin 1, pilosulin 1, and the heterodimer pilosulin 3. As well as successfully separating the main venom components, evidence has been provided for the structure and composition of three new disulfide-linked dimers named pilosulin 3.1, pilosulin 4.1 and pilosulin 5. Furthermore, SDS-PAGE clearly defined six higher molecular weight proteins between 26 and 90 kDa in non-reduced venom.

SDS-PAGE displayed limited ability to separate the lower molecular weight venom peptides, presumably because they had similar molecular weights and also because of their small size and highly basic nature. AU PAGE, in contrast to SDS-PAGE, permitted larger venom loads onto individual lanes without any band streaking. This was significant, as large Coomassie blue stained gel bands were required to detect venom peptides by HPLC–MS.

Several steps were taken in an attempt to improve the sensitivity of the HPLC–MS. The limit of detection was tested with human insulin, and approximately 1 pmol was clearly visible. Alternative extraction methods were attempted without success (Galvani et al., 2000), so silanised microtubes and carrier proteins (BSA and human

insulin) were introduced to decrease peptide adsorption. This appeared to increase recovery (as determined by running extracts back on gels), but did not greatly improve HPLC–MS detection. This suggested that protein recovery was adequate, so HPLC mobile phases that avoided the use of TFA (which has a strong propensity to suppress electrospray ionisation (Garcia, 2005)), including replacing it with formic acid or formic acid/formate buffers, were tested (McCalley, 2004). Even at high concentrations, these mobile phases resulted in complete disappearance of almost all venom peptides, so there was no option but to persist with TFA. Their highly basic nature proved troublesome during PAGE and may have contributed to these difficulties with MS. As a result, MS was unable to identify peptides in all resolved bands. Regardless, all major peptides previously identified by HPLC–MS in non-reduced venom were identified in extracts from AU PAGE resolved venom.

When sequencing pilosulin 3b, Davies et al. (2004) used Edman degradation to predict an N-terminal leucine, whilst Inagaki et al. (2004) used cDNA sequence analysis to determine that pilosulin 3b with a C-terminal glycine had an N-terminal isoleucine. Pilosulin 3b and pilosulin 3.1b were each identified as two isoforms that were well separated by HPLC, but had apparently identical MS and MS–MS data. Electrospray MS was unable to determine if the N-terminal residue was leucine or isoleucine and the exact nature of the isomass variation could not be identified. As a result, the presence of Inagaki's pilosulin 3 (2004) in JJA venom could not be confirmed or rejected.

We did not observe pilosulin 4 as postulated by Inagaki et al. (2004) which has a theoretical molecular weight of 4087 Da (4201 Da after alkylation of both cysteine residues). In our previous investigations with MS (Davies

Table 4
Updated nomenclature for JJA venom peptides

Previous name	Molecular weight (Da)	Description	Updated peptide name
pilosulin 1	6052		Pilosulin 1
[Ile ⁵]Pilosophulin 1	6066		[Ile ⁵]pilosophulin 1
Pilosulin 2	3212	Proposed peptide not observed in JJA venom	Pilosulin 2
Pilosulin 3	5608	Heterodimer of pilosulin 3a and pilosulin 3b	Pilosulin 3
–	5665	Pilosulin 3 heterodimer with an additional glycine on the 3b chain	Pilosulin 3.1
Pilosulin 3a	3155	Des-Gly ²⁷ -pilosulin 2	Pilosulin 3a
Pilosulin 3b	2457		Pilosulin 3b
Variant of pilosulin 3b	2514	Pilosulin 3b with an additional C-terminal glycine	Pilosulin 3.1b
Pilosulin 4	4087	Proposed peptide not observed in JJA venom	Pilosulin 4
–	8198	Homodimer of [Glu ³¹]pilosulin 4	Pilosulin 4.1
–	4101	[Glu ³¹]pilosulin 4	Pilosulin 4.1a
–	8546	Homodimer of pilosulin 5a	Pilosulin 5
–	4274		Pilosulin 5a

'Pilosophulin 3' as described by Inagaki et al. (2004), (equivalent to [Ile¹] pilosulin 3b.1), is not included as this name was first used by Davies et al. (2004) to describe the heterodimer of pilosulin 3a and pilosulin 3b.

et al., 2004), pilosulin 1 was found to be mostly present as a variant, [Ile⁵]pilosophulin 1. It is possible that there is a single nucleotide polymorphism in Myr p 4 amongst *M. pilosula* and that pilosulin 4 is present in tiny amounts that we were unable to detect. The presence of pilosulin 4.1a could be due to genetic variation (*M. banksi* used by Inagaki et al. (2004) was obtained from Nowra in New South Wales, whereas our JJA venom was pooled from ants collected from a variety of locations around the island state of Tasmania), or could represent the typical situation for all populations of the *M. pilosula* species complex.

Different isoforms of allergenic peptides may have variable IgE binding capacity and relative amounts may vary between sibling species and across geographical areas. With this in mind, the optimum composition of immunotherapy vaccines to satisfy the needs of all patients must be defined. The work of Donovan et al. (1994), suggests that IgE binding is not dependant on the variation between pilosulin 1 and [Ile⁵]pilosophulin 1, but we do not yet have similar data for the different forms of pilosulin 3.

Separation of non-reduced venom by AU PAGE is adequate so that venom resolved in this form is suitable to investigate allergenic species by western blotting. Previous studies investigating venom allergens were performed using 2-mercaptoethanol reduced venom, as at this time, it was thought to be the best method to separate known allergens. Unfortunately, these methods may have led subsequent investigators to conclude that seemingly novel allergens were actually artefacts caused by disulfide bond scrambling. When investigating reduced venom, reduction and alkylation prior to electrophoresis would seem essential, as we found no evidence of disulfide bond scrambling using this technique. The presence of allergens proposed by Wu (2000) must be confirmed using reduced and alkylated venom.

A consistent nomenclature is needed for JJA venom peptides. After a comprehensive study of native venom, the term 'pilosulin 3' was first used by Davies et al. (2004) to describe the heterodimer of pilosulin 3a and pilosulin 3b, so we believe that it should be used to describe this heterodimer. Subsequent to this, the term 'pilosulin 3' was used by Inagaki et al. (2004) to describe [Ile¹]pilosophulin 3b with an additional C-terminal glycine. The appropriate naming of Inagaki's pilosophulin 3 (2004) will require attention if its presence is confirmed in JJA venom. Furthermore, pilosulin 3.1b should be used to describe pilosulin 3b with an additional C-terminal glycine. Novel peptides Pilosophulin 4.1 and 5 have now been described and represent dimers of 8198 and 8546 Da, respectively. A summary of the updated nomenclature of JJA venom peptides is located in Table 4.

As well as providing an improved methodology for the gel separation of JJA venom peptides, and thus, a framework for quality control regimes and western blot studies, this work increases our knowledge of JJA venom composition, including the description of two new peptides. Further characterisation by peptide sequencing, investigation of the higher molecular weight species and IgE binding studies using synthetic peptides may now be performed. These studies will need to be performed on venoms obtained from a variety of locations and sibling species around Australia, and will form an important basis for standardising therapeutic JJA venom extracts.

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