

Molecular cloning and characterization of the major allergen *Myr p II* from the venom of the jumper ant *Myrmecia pilosula*: *Myr p I* and *Myr p II* share a common protein leader sequence

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Abstract

A major allergen *Myr p II* of the Australian jumper ant *Myrmecia pilosula* has been cloned, immunocharacterized and nucleotide sequenced. An open reading frame of 225 bases was identified and found to encode a deduced amino acid sequence of 75 residues which contained a typical hydrophobic peptide leader sequence. Expressed fusion proteins of *Myr p II* in both phage and plasmid vectors bind high levels of ant venom-specific IgE and the expressed clones are recognised by 35% of ant venom-allergic individuals. IgE antibodies that recognise the expressed clone have been shown to recognise IgE-binding bands in blots of native venom after separation by SDS-PAGE. The amino acid sequence of *Myr p II* shares close structural homology with the other major jumper ant allergen *Myr p I*, differing by only three amino acids in the first 47 residues of both sequences. However, N-terminal analysis of IgE-binding bands derived from Tricine-SDS-PAGE gel blots indicates that both *Myr p I* and *Myr p II* undergo extensive post-translational proteolytic processing to unique peptides of 45 and 27 residues, respectively.

Keywords: Leader sequence; Venom allergen; IgE-antibody; Allergy; (*M. pilosula*)

1. Introduction

The sting of the Australian jumper ant *Myrmecia pilosula* causes severe IgE antibody-mediated reactions, including anaphylaxis in sensitized individuals [1,2]. Blotting studies with sera from allergic individuals have shown that the venom contains four major IgE antibody-binding components having molecular masses of 8.5, 7.5, 5.5, and 2 kDa and a minor IgE-binding component of 4 kDa [3,4]. One of the major allergens, *Myr p I*, has already been cloned and nucleotide sequenced. This allergen has a deduced amino acid sequence of 112 residues and IgE antibodies that recognise it bind to both the 7.5 and 5.5 kDa polypeptides identified in the electrophoretically separated venom [3]. 50% of the IgE antibody binding can be attributed to *Myr p I* in subjects that recognise all the allergenic components of this venom. Subsequent studies showed that *Myr p I* contained a single 14 amino acid residue IgE-binding determinant located between residues

93–106 which appeared to be invariant for all allergic subjects that recognised this allergen [5].

This paper describes the cloning and characterisation of a second allergen that is immunologically cross-reactive with the 8.5 and 2–4 kDa major IgE-binding bands. This sequenced allergenic polypeptide has been named *Myr p II* according to the convention recommended by the International Union of Immunological Societies (IUIS). Results of comparative homology studies between the two jumper ant venom allergens are also described.

2. Materials and methods

2.1. Radioimmunoassay (RIA) for IgE antibodies to *M. pilosula* venom

Sera from *M. pilosula* venom-allergic individuals were tested for the presence of venom-specific IgE-antibodies using nitrocellulose (Bio-Rad Laboratories, Hercules, CA, USA) discs (6 mm discs, 0.45 µm pore size), impregnated with 2.5 µg of *M. pilosula* venom proteins/disc, as

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previously described [2], or 20 μg of total λ or plasmid lysates (see below), in a RIA. Protein concentrations were determined using the Pierce BCA protein determination kit (Pierce, Rockford, IL, USA). Bound IgE antibodies were detected using ^{125}I -labelled anti-human-IgE antibody (Bioclone, Sydney, Australia). Test results are expressed as the percentage uptake of the total amount of anti-IgE antibody (30000 cpm per tube) used in the assay. Routine controls included an IgE-free control (human cord serum) and an IgE-positive control (a serum with IgE-antibodies to allergens other than ant venom).

2.2. SDS-polyacrylamide gel electrophoresis and Western blotting of the venom allergens

M. pilosula venom sac extracts were prepared from ants collected in several locations in Victoria and New South Wales, as previously described [2].

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of jumper ant venom was carried out in a Sturdiel SE400 (Hoefer Scientific Instruments, San Francisco, CA, USA), or a Mini Protean II Dual Slab Cell (Bio-Rad, Richmond, CA, USA), using the *N*-tris(hydroxymethyl)methylglycine-SDS-PAGE system [6] with a 16% total, 6% crosslinker separating gel as previously described [3,6]. Briefly, freeze dried venom samples were prepared in loading buffer (4% (w/v) SDS, 12% (v/v) glycerol, 50 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 0.01% Serva Blue G) at 50 $\mu\text{g}/\text{ml}$ protein concentration. The samples were heated (100°C for 10 min), cooled to room temperature and 300 μl (15 μg) of venom preparation was loaded across the full width of the stacking gel (4% T, 3% C) of a large vertical slab gel. Polypeptide low molecular weight calibration markers (Pharmacia, Uppsala, Sweden) were run on either side of the main well.

Separated venom proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher and Schull, Dassel, Germany, 0.1 μm pore size) as described previously [7,8]. The venom electroblots were blocked by incubation for 1 h in 0.1% Tween 20 in TBS (0.01 M Tris-HCl pH 8.0, 0.15 M NaCl) and then cut into 4 mm wide strips and probed with sera from ant venom-allergic individuals as has been previously described [3]. Venom polypeptides which bound IgE were detected using a ^{125}I -labelled anti-human-IgE antibody (Bioclone) and autoradiography [2]. The controls used in the before-mentioned RIA tests were routinely used in all protein blotting experiments. Molecular weight estimations of IgE-binding bands were calculated from the position of their relative mobility (R_f) value on the polypeptide markers molecular weight calibration curve.

2.3. Denaturation, reduction and alkylation of *M. pilosula* venom

Alkylation of reduced jumper ant venom was carried out using a modified method of Hollecker [9]. Venom

protein (200 μg) in 100 μl of reduction buffer (10 mM Tris-HCl pH 8.0, 1 mM di-Na EDTA, 10 mM dithiothreitol (DTT), 8 M urea) was incubated for 30 min at 37°C. 80 μg (40 μl) of reduced venom was gently mixed with 10 μl of a 0.25 M iodoacetamide (Sigma, St. Louis, MO, USA) solution prepared in 0.25 M Tris-HCl pH 8.0. The solution was incubated at room temperature for 15 mins and stored on ice until required. 6.4 μg (4 μl) of the alkylated sample was mixed with an equal volume of 2 $\times$ loading buffer and electrophoretically separated by Tricine-SDS-PAGE on minigels as described above. Samples were then either stained with amido black as described elsewhere [4], or transferred to nitrocellulose membrane and probed with serum from jumper ant venom-allergic individuals as described above.

To ensure that reduction of disulphide bonds and alkylation of the thiol groups on the now exposed cysteine residues was occurring, samples were also prepared as above but in modified reduction buffer containing no DTT.

2.4. Isolation and characterisation of Myr p II cDNA

A $\lambda\text{gt}11$ Sfi-Not cDNA library was previously prepared from poly(A)⁺ mRNA isolated from the abdomens of *M. pilosula* ants collected at Springwood, NSW [3].

Sera from *M. pilosula* venom-allergic individuals whose IgE bound to the higher molecular weight IgE-binding component (8.5 kDa) of *M. pilosula* venom (Fig. 1), were pooled and used at a 1:10 dilution in 0.005% (v/v) Tween 20, 0.5% (w/v) bovine serum albumin, 0.1% (w/v) sodium azide, 0.01 M Tris-HCl (pH 8.0) buffer to immunoscreen the cDNA library for IgE-binding clones. Immunoscreening of the cDNA library was carried out as previously described [3]. A modified method for the rapid immunological analysis of recombinant phage clones [10] as described previously [3] was used to immunocharacterize the isolated $\lambda\text{gt}11$ clones.

RIA discs were prepared from the crude lysates of the induced lysogens of nominated IgE-binding clones in the bacterial strain Y1089 [11]. Individual sera from jumper ant venom-allergic subjects were incubated with these discs in IgE-RIAs (see above) to determine the frequency of recognition of the expressed IgE-binding clones. Control discs were prepared from lysates of expressed $\lambda\text{gt}11$ Sfi-Not in the strain Y1089 and used in all assays with expressed lysate discs.

2.5. Nucleotide sequencing of cDNA clones

The cDNA inserts of IgE-binding clones were subcloned into the vector pGEMEX-1 (Promega, Madison, WI, USA) and transformed into the *E. coli* strain JM109, as previously described [3].

Sequencing of the coding and non-coding DNA strands was carried out using the T3 and SP6 dye-labelled primers of a dye primer cycle sequencing kit (Applied Biosystems,

Foster City, CA, USA) on a Perkin Elmer Cetus thermal cycler. The sequencing reaction products were analysed using an Applied Biosystems 373A DNA Sequencer (Applied Biosystems), and the generated nucleotide sequences were analysed using DNASIS™ (Hitachi Software Engineering, Yokohama, Japan). Hydropathicity studies using the methods of Hopp and Woods [12] and Kyte and Doolittle [13], were performed using a public domain software package PROFILEGRAPH. Nucleotide and amino acid sequence homology searches were performed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service, accessed via the Australian National Genomic Information Service (ANGIS). Isoelectric point estimations were performed using the University of Wisconsin Genetics Computer Group's set of programs (GCG Programs) accessed through ANGIS.

2.6. Generation of unidirectional deletions in the cDNA

For the production of unidirectional deletion subclones, the DNA of interest was initially digested with two restriction enzymes to generate an Exonuclease III-resistant 3' overhang and either a 5' overhang or a blunt end that are both Exonuclease III-digestible [11]. The *Myr p II* clone pMp37, in the expression vector pGEMEX-1 (Promega), contained no useful restriction sites for the deletion of the DNA insert's poly(A)⁺ tail. Consequently, the DNA insert was directionally subcloned into another expression vector pGEM-7Zf(+) (Promega), through the *EcoRI* and *HindIII* restriction sites [11]. The pGEM-7Zf(+) subclone DNA, was prepared using a scaled-up procedure of the mini-prep protocol [11]. The plasmid DNA was purified by CsCl-ethidium bromide ultracentrifugation as described previously [14]. Closed circular plasmid DNA isolated from the CsCl gradient was extracted 5 times with an equal volume of H₂O-saturated 1-butanol and dialysed for 48 h at 4°C against several changes of distilled water and a final dialysis against several changes of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer. The purified pGEM-7Zf(+) plasmid DNA was digested with *SacI* and *HindIII* as previously describe [11], generating a 5' overhang at the poly(A)⁺ tail of the insert DNA. Unidirectional deleted clones were generated thorough the *HindIII* Exonuclease III-susceptible restriction site as previously described [11] and these clones were nucleotide sequenced using the T7 and SP6 dye-labelled primers (Applied Biosystems) as described above.

2.7. Northern and Southern hybridization

The materials and methods for Northern and Southern hybridizations of *M. pilosula* RNA and DNA have been described in detail elsewhere [3]. The probe used in these hybridizations was generated by a *ClaI*-*NotI* double-digest of the pGEMEX-1 subclone and the insert recovered as previously described [3].

2.8. N-terminal analysis of venom polypeptides

M. pilosula venom, prepared at a protein concentration of 0.5 mg/ml was reduced with 2-mercaptoethanol and separated by Tricine-SDS-PAGE, as mentioned above, in a vertical mini-gel system (Bio-Rad, Mini PROTEAN II Dual Slab Cell, Richmond, CA, USA). 10 µl (5 µg) of venom protein was loaded into each 7 mm wide well of the stacking gel, with polypeptide markers (Pharmacia) loaded in both end wells of the stacking gel. The electrophoretic transfer of the separated polypeptides to polyvinylidene difluoride (PVDF) membrane was carried out in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 11.0, 10% methanol as described by Matsudaira [15]. Bands corresponding to IgE-binding proteins of interest were identified by immunoprobings (as described above), of sample strips from the PVDF transfer with serum from subjects having a restricted pattern of recognition that identified either *Myr p I* or *Myr p II*. The IgE-binding bands were identified on the remaining Coomassie Blue stained PVDF transfer [15] by alignment with autoradiographs of the probed strips, excised and N-terminal sequenced by Macquarie University Centre for Analytical Biotechnology (MUCAB) (Macquarie University, Sydney) on a Applied Biosystems 470A Gas Phase Sequenator.

3. Results

3.1. SDS-PAGE and immunoblotting

Sera from a number of allergic subjects with RIA values (see Section 2) of > 2% uptake of the ¹²⁵I anti-human-IgE antibody, were used to probe 4 mm wide strips of nitrocellulose protein blots of *M. pilosula* venom separated by Tricine-SDS-PAGE. Protein blotting identified four IgE-binding bands centred around 8.5, 7.5, 5.5 and < 2 kDa and a large diffuse band from 2 to < 4 kDa (Fig. 1). IgE-binding profiles were identified on these *M. pilosula* venom strips, using the individual sera from a number of ant venom-allergic subjects. Profiles shown in Fig. 1 are representative serological IgE-binding patterns. The IgE-binding profiles commonly identified were either all five IgE-binding bands (Fig. 1, track c), the 7.5 and 5.5 kDa bands (Fig. 1, track b) and the 8.5 kDa band and 2 to < 4 kDa diffuse band (Fig. 1, tracks d and e).

The major allergen *Myr p I* isolated from the *M. pilosula* λgt11 Sfi-Not cDNA library expressed a polypeptide which was immunologically cross-reactive to IgE-binding bands at 7.5 and 5.5 kDa identified by Tricine-SDS-PAGE and protein blotting [3]. Thus, individual sera with IgE-binding profiles to *M. pilosula* venom which identified the higher molecular weight IgE-binding band of 8.5 kDa (Fig. 1), were used to screen the same *M. pilosula* cDNA library for new IgE-binding clones.

3.2. Denaturation, reduction and alkylation of venom polypeptides

Iodoacetamide was used to alkylate the ionized thiol groups on reduced proteins, blocking cysteine residues and preventing any possible reassociation of the proteins during electrophoresis. Alkylation of reduced jumper ant venom resulted in a shift in protein staining intensity from the molecular mass region of ~10 kDa to a lower molecular mass ~2–3 kDa region (Fig. 2A). However, a band of protein was still detectable in the higher molecular mass domain.

The shift in apparent molecular mass of the venom proteins was observed when using the mini-gel apparatus. It was clear that the band observed at ~10 kDa corresponded to the 8.5 kDa band observed on blot strips prepared from separations using the larger gel in the Hoefer apparatus. It was presumed that variations in electrophoretic conditions between large and mini-gel apparatuses caused the 8.5 kDa band to run at an apparent molecular mass of approx. 10 kDa in the mini-gel.

Protein blotting of the alkylated jumper ant venom revealed a dramatic change in the IgE-binding profile of

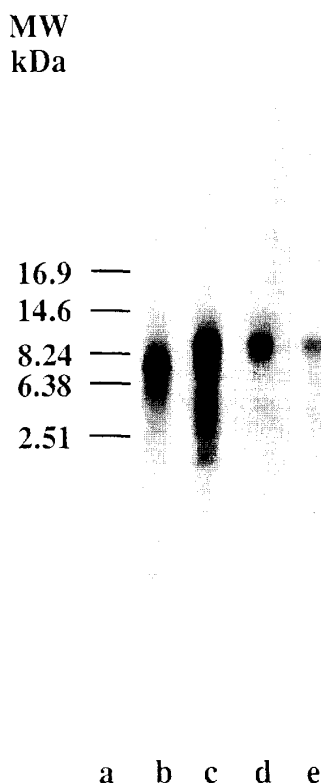


Fig. 1. SDS-PAGE of *Myrmecia pilosula* venom and immunoblotting with sera from ant venom-allergic subjects. (a) Polypeptide molecular weight markers; (b–e) IgE-binding polypeptides recognised by ant venom-allergic sera.

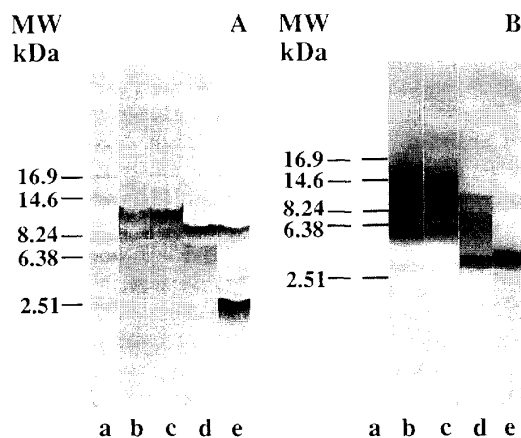


Fig. 2. Effects of alkylation on *Myrmecia pilosula* venom proteins. Reduced and unreduced *M. pilosula* venom was incubated with iodoacetamide to alkylate the thiol groups on cysteine residues (see Section 2). Treated samples were separated by Tricine-SDS-PAGE and either (A) stained in gel with amido black or (B) transferred to nitrocellulose membrane and probed with a pool of serum from jumper ant venom-allergic individuals, to identify IgE-binding bands. (a) Polypeptide low molecular weight markers; (b) unreduced venom in the presence of urea; (c) unreduced venom in the presence of urea and iodoacetamide; (d) DTT reduced venom; (e) venom reduced with DTT and alkylated with iodoacetamide.

M. pilosula venom (Fig. 2B). Only 2 IgE-binding bands were visible, one of approx. 10 kDa and the other approx. 2–3 kDa. The staining intensity of IgE-binding also shifted from the 10 kDa molecular mass region to the lower molecular mass areas of the gel. No shifts in molecular mass were detected in those samples where reducing agent was omitted (Fig. 2A and B, track c).

3.3. Isolation and characterisation of cDNA clones encoding Myr p II

Three cDNA clones were selected from the *M. pilosula* library by immunoscreening with a pool of 10 sera from venom-allergic individuals selected to recognise the 8.5 kDa molecular mass IgE-binding band. Three clones λ Mp37, λ Mp41 and λ Mp42 were plaque purified and immunocharacterized using the rapid method for the immunological analysis of recombinant phage clones [10]. The three expressed clones were used to adsorb IgE-antibodies from the diluted serum pool. The bound antibodies were eluted and used to probe nitrocellulose blots of *M. pilosula* venom separated by Tricine-SDS-PAGE (Fig. 3). The three clones all of similar size, λ Mp37 (495 bp), λ Mp41 (465 bp) and λ Mp42 (485 bp), each expressed a polypeptide that bound IgE-antibodies which were cross-reactive with the 8.5 and the diffuse 2–4 kDa bands in the native venom (Fig. 3). An expressed control, λ gt11 Sfi-Not, did not adsorb any IgE from the serum pool and the eluted material did not bind to native venom.

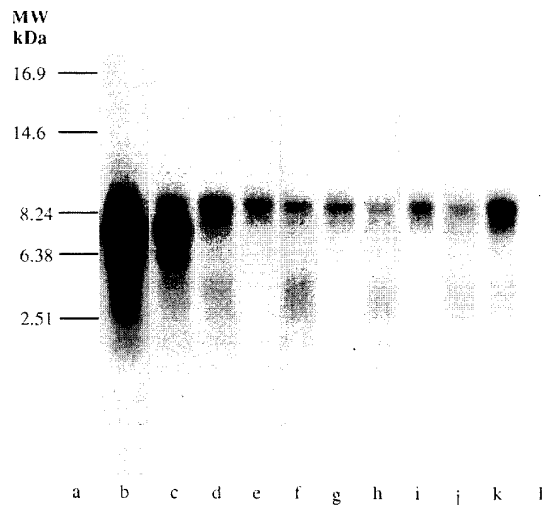


Fig. 3. Immunocharacterization of λ gt11 Sfi-Not cDNA clones. Expressed λ gt11 Sfi-Not cDNA clones bound to nitrocellulose membrane were used to adsorb IgE-antibodies from a pooled serum which predominantly recognised the 8.5 kDa IgE-binding band of jumper ant venom. The adsorbed IgE antibodies were subsequently eluted and neutralized. The eluted antibodies and depleted serum were used to probe native venom SDS-PAGE Western blot strips (see Section 2). (a) Polypeptide molecular mass markers; (b) Profile of all the IgE-binding venom components identified by an individual's serum; (c) IgE-binding profile from an individual's serum that predominantly recognises the 7.5 and 5.5 kDa bands; (d) Diluted pooled serum (see tracks d and e, Fig. 1); (e), (g) and (i) Serum adsorbed by expressed λ Mp37 (e), λ Mp41 (g), λ Mp42 (i); (f), (h) and (j) IgE antibodies eluted from λ Mp37 (f), λ Mp41 (h), λ Mp42 (j); (k) Expressed λ gt11 Sfi-Not adsorbed serum; (l) IgE eluted from expressed λ gt11 Sfi-Not.

The lysate from expressed λ Mp37 was used to prepare test discs for use in IgE-RIAs. In a study involving 57 subjects using the expressed λ Mp37 lysates, 35% of these subjects recognised this expressed clone. The cDNA insert of λ Mp37 was subcloned into the expression vector pGEMEX-1 for nucleotide sequencing. To ensure that the pGEMEX-1 subclone of λ Mp37 retained the IgE-binding

characteristics of λ Mp37 the *E. coli* strain JM109DE3 was transformed and expressed. Lysates prepared from the expressed material were recognised in an IgE-RIA by all subjects who recognised the expressed λ Mp37 (results not shown). We were therefore satisfied that nucleotide sequencing of the subclone would generate a deduced amino acid sequence that contained the IgE-binding determinants of λ Mp37.

3.4. Nucleotide sequence analysis of Myr p II

Sequencing reactions of pMp37 carried out using the T3 dye-labelled primer revealed the presence of a *Cla*I restriction site in the insert DNA. This *Cla*I site was used to prepare two subclones pMp37.1 and pMp37.2 (Fig. 4), for further sequencing of the pMp37 insert.

Sequencing of the non-coding strand using the SP6 dye-labelled primer through the poly(A)⁺ tail of the insert cDNA in pMp37 and pMp37.2 produced very poor sequence data. As no unique restriction sites could be found near the poly(A)⁺ tail, unidirectional deleted subclones of pMp37 were prepared in pGEM-7Zf(+) as described in Section 2. The poly(A)⁺ tail was deleted in these subclones using Exonuclease III after a double digestion with *Hind*III and *Sac*I restriction enzymes. Multiple subclones were generated by this method, but only three pMp37.3, pMp37.4 and pMp37.5 (Fig. 4), were selected for sequencing based on their size as determined by agarose gel electrophoresis (results not shown). Sequencing of these clones through the 3' tail of the cDNA insert generated usable sequence data for the completion of the sequencing of the pMp37 cDNA insert. Unidirectional deleted clone pMp37.3 (450 bp) contained only 5 adenosines of the poly(A)⁺ tail of pMp37, whereas pMp37.5 (314 bp) and pMp37.4 (375 bp) contained none of the poly(A)⁺ tail of pMp37 (Fig. 4).

Sequencing of the coding and non-coding strands of the various subclones of pMp37 was performed a number of times to generate overlapping sequence data in the 5' to 3'

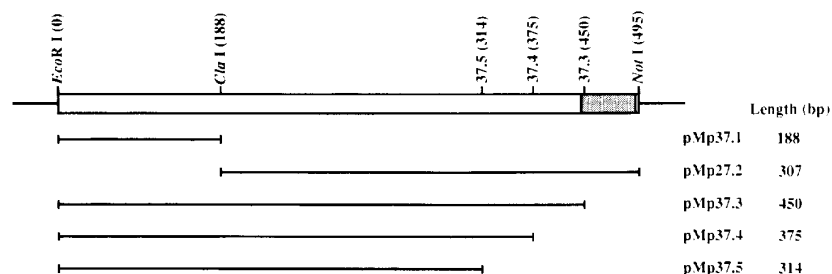


Fig. 4. Combined restriction and deletion map of pMp37 cDNA insert. The cDNA insert from λ Mp37 was subcloned into pGEMEX-1 and nucleotide sequenced. Sequence data for the full length clone was obtained by constructing subclones pMp37.1 and pMp37.2 through the *Cla*I restriction site and by the generation of Exonuclease III deletion subclones pMp37.3, pMp37.4 and pMp37.5 in the plasmid vector pGEM-7Zf(+). All subclones were sequenced at least twice in both directions.

and the 3' to 5' directions for verification of the pMp37 nucleotide sequence (Fig. 5). Sequence data generated were analysed using the DNA sequence input and analysis software DNASIS™ (Hitachi Software Engineering). The insert cDNA of pMp37 was verified as 495 base pairs in length with an open reading frame of 225 base pairs which translates an unprocessed polypeptide of 75 amino acids in length (Fig. 5).

A methionine residue identified at nucleotides 44–46 is followed by a sequence of 25 largely hydrophobic amino acid residues that correspond to a possible signal peptide leader sequence [16–18]. A signal peptide cleavage site is predicted at amino acid residues 22–26 (Pro-Asn-Val-Glu-Ala) (nucleotides 107–121). Cleavage of this signal peptide at the alanine residue, nucleotides 119–121 would produce a polypeptide of 52 amino acids with a lysine as the first residue of this processed molecule (Fig. 5). This predicted processed form of the expressed pMp37 has a molecular mass of 5333 Da and a predicted isoelectric point of 8.65. A second possible signal peptide cleavage site Pro-Glu-Ser-Asp-Ala is present at residues 32–36 (nucleotides 136–150). Cleavage between the Ala and Val at residues 36 and 37 would produce a polypeptide of molecular mass 4336 and predicted pI value of 9.93.

M. pilosula venom was separated by Tricine-SDS-PAGE and transferred to PVDF membrane in CAPS buffer pH 11.0 as described above. The membrane was stained with Coomassie Blue and the 8.5 kDa and 7.5 kDa IgE-binding bands corresponding to *Myr p II* and *Myr p I*, respectively (Fig. 1), were excised and N-terminal sequenced. Six sequencing cycles were completed on the 8.5 kDa band, and the following sequence generated, Ile-Asp-Trp-Lys-Lys-Val. The isoleucine (Ile) residue at position 49 of the deduced amino acid sequence of *Myr p II* (nucleotide positions: 188–190) (Fig. 5), was thus identified as the first amino acid of the fully processed form of the encoded full-length allergenic molecule. This IgE-binding molecule, when expressed in its fully post-translationally processed form, is a peptide of 27 amino acids in length with a calculated molecular mass of 3208 Da and predicted pI value of 10.70. Sixteen sequencing cycles were completed on the 7.5 kDa band corresponding to *Myr p I* and the following sequence generated, Ile-Leu-Gly-Arg-Val-Ile-Pro-Lys-Val-Ala-Lys-Lys-Leu-Gly-Lys-Thr. The isoleucine (Ile) residue at position 68 of the deduced amino acid sequence of *Myr p I* (Fig. 9), was identified as the first amino acid of the fully processed form of the encoded allergenic molecule. *Myr p I* when

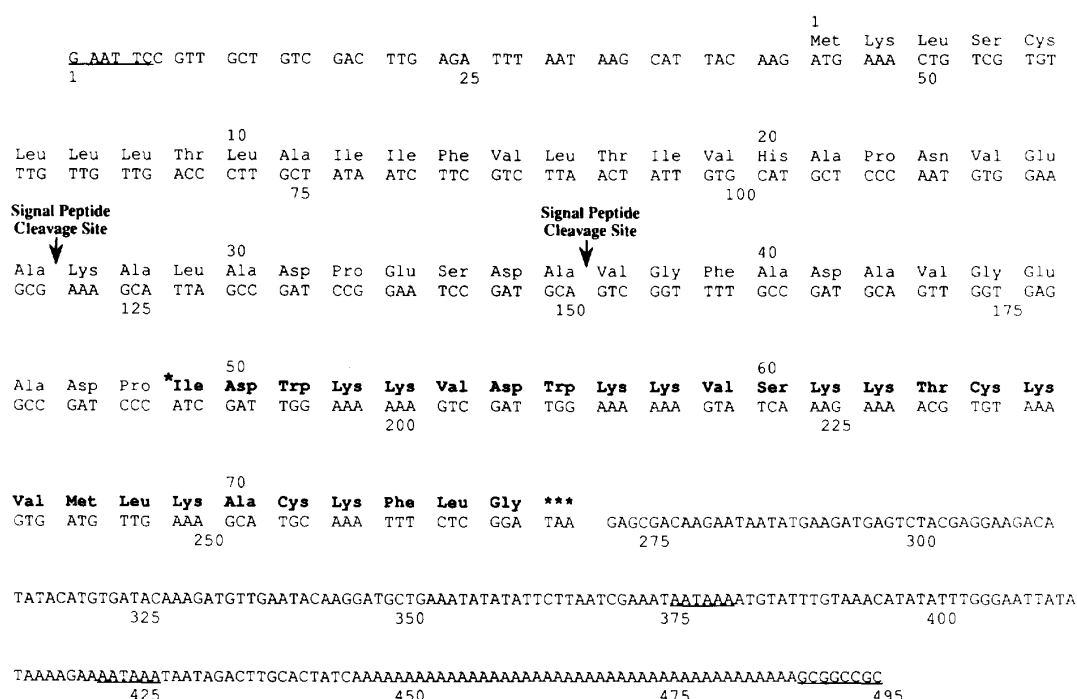


Fig. 5. Nucleotide sequence and deduced amino acid sequence of the IgE-binding *M. pilosula* clone pMp37. The initiation codon (ATG) of the open reading frame in the amino acid sequence encoded by pMp37 begins at nucleotide 44. This open reading frame ends with the termination codon (TAA) at nucleotide 269. Two putative polyadenylation signals (AATAAA), and the *EcoRI* (GAATTC) and *NotI* (GCGGCCGC) restriction sites are underlined. The nucleotide base pairs are numbered below the nucleotide sequence and the amino acid residues are numbered above the derived amino acid sequence. Ile-49 (*), the first amino acid residue of the fully processed native form of *Myr p II* was determined by N-terminal analysis of the native venom polypeptides (see Section 2).

expressed in its fully processed form is a peptide of 45 amino acids in length with a calculated molecular mass of 4932 Da and predicted *pI* value of 10.79.

3.5. Northern and Southern hybridizations

The cDNA insert of the *ClaI* subclone pMp37.2 (Fig. 4), corresponding to the unique 307 bases of the clone pMp37, hybridized to a single mRNA band of approx. 500 bases in length (Fig. 6). This would indicate that pMp37 with a cDNA insert of 495 bp, as determined by nucleotide sequencing, contains the complete full-length cDNA of this new IgE-binding clone. The subclone pMp37.2 was used in these hybridizations as it lacked 5' sequence homology with *Myr p I* as was seen with the full length clone pMp37 (see below).

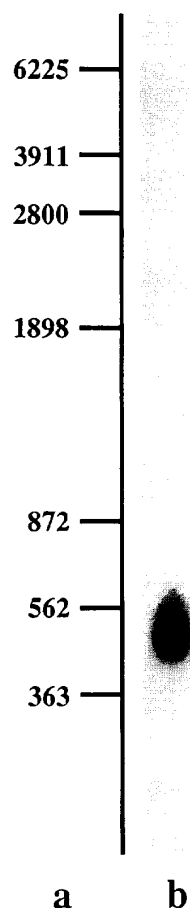


Fig. 6. Northern blot analysis of the cDNA insert of the jumper ant clone pMp37. The cDNA insert was digested with both *ClaI* (ATCGAT) and *NotI* (GCGGCCGC) restriction enzymes to excise the unique portion of this IgE-binding clone (see Fig. 7), from nucleotides 188 to 480 of the full nucleotide sequence (see Fig. 3). The restricted insert of pMp37 was radioactively labelled with ^{32}P and used to probe a Northern blot of *Myrmecia pilosula* poly(A) $^{+}$ mRNA. (a) RNA size markers (bases); (b) hybridization profile for *ClaI/NotI* digested pMp37 cDNA.

Multiple bands of restriction enzyme-digested genomic DNA were identified by hybridization of the ^{32}P -labelled cDNA insert of pMp37.2 with an *M. pilosula* genomic DNA Southern blot (Fig. 7A). Eight restriction enzymes were used to digest the *M. pilosula* genomic DNA, and none of these enzymes contained restriction sites within the pMp37.2 cDNA insert.

3.6. Hydropathicity analysis of *Myr p II*

A highly hydrophobic domain was identified in the N-terminal 20 amino acids of *Myr p II* using both algorithms [12,13] (Fig. 8). Several potential antigenic domains; residues 22–25, 30–35, 47–64 (Kyte and Doolittle [12]) and residues 23–36, 42–64 (Hopp and Woods [13]) were also identified (Fig. 8).

3.7. Database searches

Comparison of the nucleotide and derived amino acid sequences of pMp37 against current DNA and protein databases using ANGIS, revealed a 62% homology with the other allergen of *M. pilosula* venom, *Myr p I*. As this allergen has as yet no identified biological function, the function of the cloned allergen pMp37 could not be predicted.

3.8. Comparison of *Myr p I* and *Myr p II*

The sequence homology between *Myr p I* and *Myr p II* revealed an overall homology of 62% due to localised regions of high homology. Identification of these highly homologous regions revealed a 94% homology between the first 47 amino acid residues of each polypeptide (Fig. 9) (nucleotides 33–184 of pMp37) (Fig. 4). Also a 91% homology between the final 171 base pairs of the 3' non-coding regions of both these allergens was identified. However, from amino acid residues 49 to 75 of the *Myr p II* sequence and from residues 68 to 112 of the *Myr p I* sequence (Fig. 9), no homology exists between these two sequences.

Within the first 47 amino acid residues of both allergens, single base changes in the nucleotide sequences of both clones are present at residues 11, 15, 20, 35, 43, 44 and a two base change is present at residue 28 (Fig. 9). Of these variations in DNA sequence, only changes at residues 28, 35 and 43 have resulted in a change in the amino acid sequence of the proteins. However, from amino acid residue 48 onwards in both sequences, the DNA and amino acid homologies vary greatly (Fig. 9).

High sequence homology in the 3' non-coding regions of each sequence is reestablished at nucleotide positions 346 in *Myr p II* and 420 in *Myr p I* by realignment of the two nucleotide sequences.

Comparison of Southern blots of pMp37 with the previously published *Myr p I* blot, shows three genomic DNA

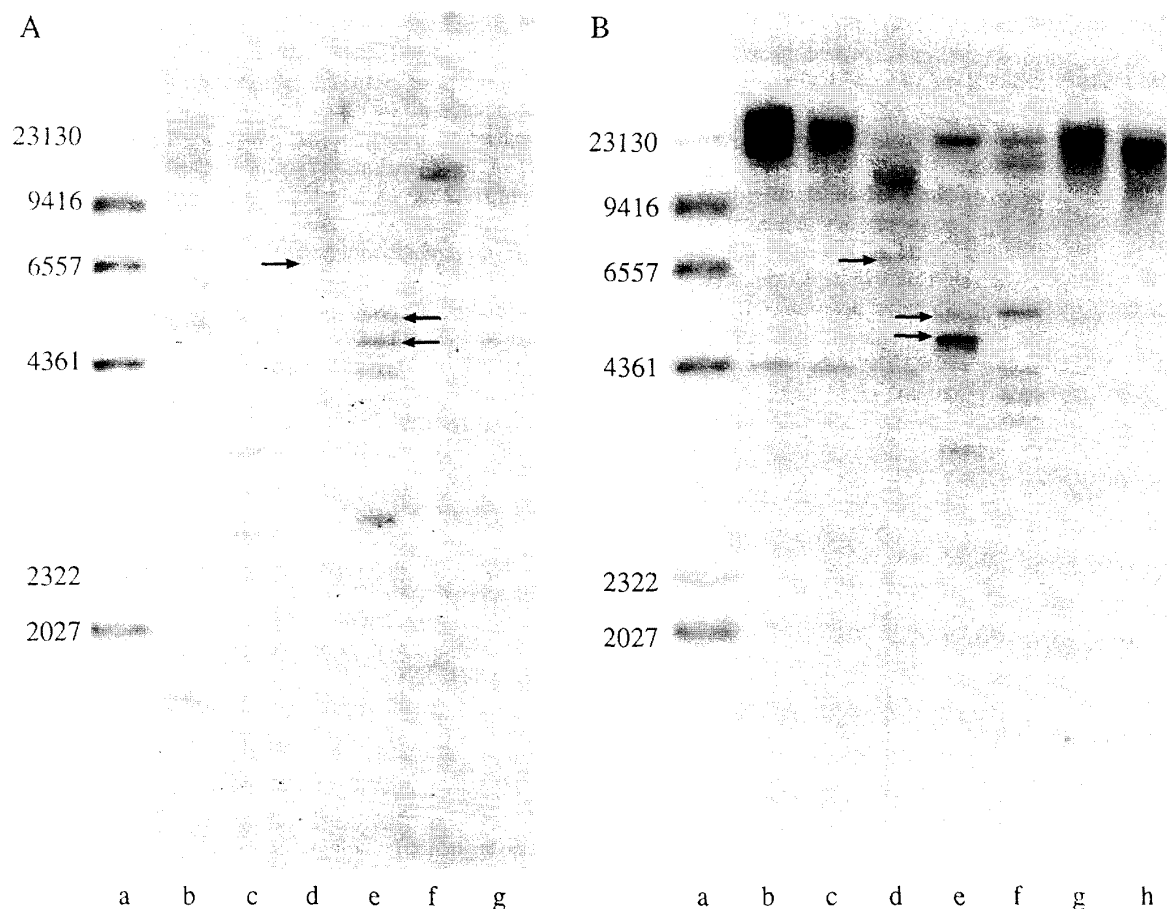


Fig. 7. Southern blots of restriction enzyme digests of *M. pilosula* (jumper ant) genomic DNA probed with ^{32} P-labelled cDNA inserts of clones pMp37.2 (*Myr p II*) (A) and pMp2 (*Myr p I*) (B). (a) *HindIII* digested lambda DNA markers (bp) and restriction digests of 20 μ g of genomic DNA with (b) *SmaI*, (c) *BamHI*, (d) *HindIII*, (e) *XbaI*, (f) *XhoI*, (g) *PstI*, (h) *BglI*. Arrows indicate DNA bands recognised by both cDNA clones. (*Myr p I* Southern blot reproduced from Donovan et al. [3]).

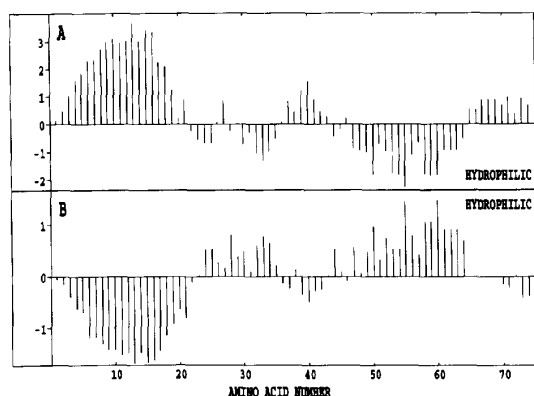


Fig. 8. Hydropathicity profiles of the derived amino acid sequence of the complete cDNA insert pMp37. Hydropathicity was calculated using two different algorithms; Kite and Doolittle (A), and Hopp and Woods (B). Calculated regions of hydrophilicity are negative (below the central mid-line) in the Kite and Doolittle representation and positive (above the line) in the Hopp and Woods representation.

bands in common (Fig. 7A and B). However, the remaining 15 bands on the pMp37 blot are unique to this new IgE-binding clone.

4. Discussion

In this paper we have described the isolation and characterisation of a second major IgE-binding clone from the *M. pilosula* λ gt11 Sfi-Not cDNA library [3]. This clone is immunologically cross-reactive with IgE-binding bands of 8.5 kDa and a diffuse 2–4 kDa band identified on a Tricine-SDS-PAGE-separated *M. pilosula* venom protein blot. The expressed clone is recognised by the sera of 35% of *M. pilosula* venom allergic individuals.

Immunological recognition patterns of Tricine-SDS-PAGE-separated *M. pilosula* venom proteins can be categorized into three profiles. Individuals' sera appear to recognise either all IgE-antibody binding bands, both the

Fig. 9. A comparison of the nucleotide and derived amino acid sequences of the IgE-binding cDNA clones pMp2 (*Myr p I*, Donovan et al. [3]) and pMp37 (*Myr p II*). Sequences have been aligned to show the high degree of sequence homology in the 47 amino acid residue peptide leader sequence of each IgE-binding clone. Base changes in the nucleotide sequences resulting in amino acid changes have been double underlined. Base changes in nucleotide sequence not effecting the derived amino acid are singly underlined. Ile-49 (*), the first amino acid residue of the fully processed native form of *Myr p II* was determined by N-terminal analysis of the native venom (see Section 2). Ile-68 (#), the first amino acid residue of the fully processed native form of *Myr p I* was also determined by N-terminal analysis of the native venom (see Section 2). Arrows indicate the start of the 3' non-coding region where high homology has been identified between both DNA sequences. (*Myr p I* sequence reproduced from Donovan et al. [3]).

7.5 and 5.5 kDa bands or both the 8.5 band and 2–4 kDa diffuse band.

Three clones were isolated from the *M. pilosula* cDNA library by screening with a serum pool from subjects whose sera recognised the higher molecular mass 8.5 kDa IgE-binding band. Immunocharacterization of these clones demonstrated that each clone expressed a polypeptide that corresponded immunologically to both the 8.5 and diffuse 2 to 4 kDa IgE-binding bands on *M. pilosula* venom protein blots. Nucleotide sequencing of the three isolated clones revealed that they each contained a different sized insert of identical sequence. The λ clone Mp37 with a complete cDNA insert, containing an initiator codon (ATG) and a poly(A)⁺ tail, was selected for further studies.

Sequencing of this cDNA clone revealed an open reading frame of 225 base pairs which encodes a protein with a predicted molecular mass of 8.1 kDa in its unprocessed form. This protein has a classic hydrophobic leader sequence (Fig. 8) with two putative signal peptide cleavage sites which could give rise to substantially smaller polypeptides. N-terminal analysis however, indicated that the protein underwent substantial post-translational modifications and was expressed in the venom gland as a polypeptide of 27 amino acid residues with a predicted molecular mass of 3.2 kDa and a predicted pI of 10.70.

N-terminal analysis of *Myr p I* indicated that this molecule also underwent similar significant post-translational modifications to be expressed in the venom gland as a 45 amino acid residue polypeptide with a predicted molecular mass of 4.9 kDa.

IgE-antibodies isolated from the serum of jumper ant venom-allergic individuals, by adsorption and subsequent elution from the expressed clone λ Mp37, bound to both the 8.5 and diffuse 2–4 kDa bands in *M. pilosula* venom protein blots.

A number of plausible explanations exist for this dual recognition of the different molecular mass proteins. It is possible that two independent genes may be producing different proteins that are immunologically identical. However, the single mRNA found of 500 bases in length, identified by Northern blot analysis of the *Myr p II* plasmid clone pMp37.2 strongly suggests that this dual recognition of the two IgE-binding bands is not the result of two separate genes expressing immunologically similar proteins. The two IgE-binding bands could be due to different post-translational modifications of *Myr p II* in the venom sac extract. Their presence in our venom extracts may be the result of cellular lysis of the 'venom-producing' cells and release of preproteins during the venom extraction procedure. Subjects allergic to the native venom would recognise fully processed venom proteins and their IgE responses would also be directed towards these processed forms of the allergen. The same IgE-binding site present on the fully processed allergen would also be present on the nascent allergen polypeptide and the different post-translationally modified forms of this protein.

Thus, the IgE antibodies would also bind to these forms, giving rise to the dual recognition pattern observed for IgE-antibodies that recognise expressed *Myr p II*.

While dual recognition may be due to the different post-translationally modified forms of the allergens, alkylation of reduced venom proteins with iodoacetamide suggested the existence of multimeric forms of *Myr p II* in the electrophoretically separated venom sample as the basis of the dual recognition. Venom samples not reduced but incubated in the presence of urea and iodoacetamide (Fig. 2A and B, track c), showed no change in the protein or IgE-binding profiles. This indicated that alkylation only occurred when the proteins were reduced and the cysteine thiol groups exposed. Further, all changes observed in the reduced and alkylated venom samples were due to the action of iodoacetamide on the exposed thiol groups and not the action on any other amino acid residues. These results indicated that some form of reassociation of venom proteins was occurring during electrophoresis of the reduced venom samples. Oxidative conditions used to polymerize the SDS-polyacrylamide gel may be sufficient to influence the reassociation of this small polypeptide. Recombination of identical allergenic peptides may be occurring giving rise to different multimeric forms of the same allergen. Reassociation could also occur between allergenic and non-allergenic venom proteins. This phenomenon of reassociation appears to be the most probable cause of dual recognition of native venom immunoblots by IgE-antibodies binding to the expressed *Myr p II*. The diffuse nature of the IgE-binding bands under routine reduction and electrophoresis could well be the result of associations between a number of different non-allergenic molecules or reassociation with itself to form multimers. Although alkylation with iodoacetamide does not markedly change the overall charge of the molecule, it does chemically alter the cysteine residue side chains which could affect IgE-binding to this molecule. This may account for the observed loss of IgE-binding, however, protein staining of the electrophoresed alkylated venom sample clearly shows a shift down in the molecular mass of some of the reduced venom proteins after alkylation.

A comparison of the *Myr p II* nucleotide and derived amino acid sequences with current nucleotide and protein data bases revealed a 62% homology between the sequenced *Myr p II* clone and the major jumper ant (*M. pilosula*) allergen *Myr p I*. On closer examination of this homology, it was revealed that homology of > 90%, existed between the first 47 amino acid residues of the deduced amino acid sequence of each allergen and also between the nucleotide sequence of the non-coding tail regions of each clone. However no sequence homology was observed between the fully processed forms of the 27 and 45 amino acid residues respectively of *Myr p II* and *Myr p I*.

Hybridization of the *Myr p II* cDNA of pMp37.2 encoding the unique portion of the clone pMp37 to South-

ern blots of ant genomic DNA (Fig. 7) indicated that the expression of this allergen is probably controlled by a multigene family. This finding was also true for the major allergen *Myr p I* [3]. Although strong sequence homology exists between these two allergens, the unique Southern blots obtained for each allergen indicate that their expression is controlled by different gene families.

These two allergens, although expressed by different multigene families, share substantial homology within their signal peptide leader sequences. Little similarity between peptide leader sequences of proteins from the same organism has been observed previously. However, conservation of leader sequences of homologous proteins in different organisms is common [17].

Leader sequences are important in directing the proteins to their final destinations [19]. These jumper ant venom proteins may require a highly specific targeting sequence to ensure that they are released only into the gland space and not into another extracellular space which may have detrimental consequences for the jumper ant. The highly homologous leader sequences would ensure this process occurred.

One could speculate that the genes encoding the venom allergens *Myr p I* and *Myr p II* arose from a common ancestral gene that underwent a number of DNA splicing and fusion events to generate the precursor gene for each allergen. Then, through a series of gene amplification events, and under the influence of selection pressures to maintain each gene in the expressed genome of the ant, the present day multigene families may have arisen.

Acknowledgements

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