

**EXPRESSION OF JUMPER ANT (*MYRMECIA PILOSULA*) VENOM ALLERGENS:
POST-TRANSLATIONAL PROCESSING OF ALLERGEN GENE PRODUCTS**

G.R. Donovan¹, M.D. Street¹, T. Tetaz², A.I. Smith², D., Alewood³, P. Alewood³, S.K. Sutherland⁴ and B.A. Baldo^{1,5}*

Molecular Immunology Unit, Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards NSW 2065¹; Baker Medical Research Institute, Prahran, Victoria²; Centre for Drug Design and Development, University of Queensland, St Lucia, Queensland³; Australian Venom Research Unit, Department of Pharmacology, University of Melbourne, Parkville, Victoria⁴; Department of Medicine, University of Sydney NSW⁵, Australia.

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SUMMARY

N-terminal analyses of electrophoretically-separated allergenic polypeptides of the venom of the jumper ant *M. pilosula* showed that five out of the six allergenic polypeptides identified are homologous with the cloned major allergen *Myr p I* and may be derived from a single precursor polypeptide. The sixth polypeptide is homologous with a second cloned major allergen, *Myr p II* which is expressed as a single precursor polypeptide but exists in its native form as a disulphide bond-linked complex.

Keywords: Allergens, venom, electrophoresis, post-translational processing, gene expression

INTRODUCTION

The sting of the jumper ant, *Myrmecia pilosula*, found in the sandy-soiled high country of south-eastern Australia and Tasmania, causes severe allergic reactions, including anaphylaxis, in sensitised individuals. Previous attempts by us to characterise the allergenic components of the ant venom by SDS-PAGE of 2-mercaptoethanol-reduced native venom revealed four major IgE antibody-binding bands located at approximately 8.5, 7.5, 5.5 and 2 kDa and a minor band located at approximately 4 kDa [1]. Charge-based electrophoretic separation of the venom, however, revealed up to 8 IgE antibody-binding components [2]. Immunoscreening of a jumper ant cDNA library with sera from ant venom-allergic individuals yielded only two classes of IgE-binding clones [3]. Full-length cDNAs of each have been named *Myr p I* and *Myr p II* respectively according to the International Union of Immunological Societies (IUIS) convention. The expressed *Myr p I* polypeptide showed IgE antibody-binding identity with the two major bands at 7.5 and 5.5 kDa on SDS-PAGE blots of 2-mercaptoethanol-reduced whole venom [1], whereas, the expressed *Myr II* polypeptide

* Correspondence:

Dr Brian A. Baldo, Molecular Immunology Unit, Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards NSW 2065, Australia Telephone: +612 9926 7272; Facsimile: +612 9926 8484; Email: Bbaldo@blackburn.med.su.oz.au

showed IgE-binding identity with the major band centred at 8.5 kDa and a diffuse band between 2-4 kDa [3]. N-terminal analyses of the 2-mercaptoethanol-reduced IgE-binding polypeptides separated by SDS-PAGE and blotting to polyvinylidene difluoride (PVDF) membrane [3] indicated that the 5.5 kDa band was homologous with the 45 residue C-terminal component of *Myr p I* (residues 68-112; calculated molecular mass of 4,932 daltons). The 7.5 kDa band although showing immunological identity with *Myr p I*, gave an N-terminal sequence that was more consistent with residues 49-75 of *Myr p II* (unpublished results). N-terminal sequencing of the 8.5 kDa band indicated that it was comprised of a polypeptide homologous with the 27 C-terminal residues 49-75 of *Myr p II* having a calculated molecular mass of 3,208 daltons [3]. The calculated molecular mass of the 45 C-terminal residues of *Myr p I* of 4,932 daltons approximated the molecular mass of 5.5 kDa estimated by SDS-PAGE. The immunological identity of *Myr p II* with the 8.5 kDa band, however, was not at all consistent with the calculated molecular mass of 3,208 daltons of the 27 C-terminal residues of *Myr p II* identified by N-terminal analysis. Blots of reduced and alkylated whole venom after electrophoretic separation demonstrated a substantial shift of IgE-binding activity from the 5-10 kDa region to a major band at approximately 3 kDa [3], however, the band definition was insufficient to identify the remaining IgE-binding bands in the 5-10 kDa region. The presence of two cysteines in the 27 C-terminal residues of the derived amino acid sequence of *Myr p II* suggested that such a molecule might form disulfide bonds with itself or with other non-allergenic venom polypeptides.

This paper reports the unambiguous molecular separation by SDS-PAGE of the venom components as a result of reduction and alkylation and the inclusion of 6M urea in the separating gels. N-terminal analyses of the separated polypeptides provides evidence that a set of five immunologically identical polypeptides in the 5-8 kDa molecular mass range are homologous with *Myr p I* and are possibly derived from a single precursor gene product whereas *Myr p II* is expressed as a single precursor gene product, but assumes complex disulphide bond-linked forms.

MATERIALS AND METHODS

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

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 tricine buffer system described by Schagger and von Jagow [4] with a 16.5% total acrylamide (6% bis-acrylamide) separating gel containing 6M urea (Pharmacia, Uppsala, Sweden).

M. pilosula venom sac extracts were prepared from ants collected at several locations in Victoria as previously described [5]. Venom was reduced with 2-mercaptoethanol (ME) [1] or reduced with dithiothreitol (DTT) and then alkylated with iodoacetamide (IAA) as previously described [3]. The ME-reduced samples and the DTT-reduced and IAA-alkylated samples were mixed with equal volumes of 2X loading buffer, heated at 50°C for 30 min, cooled to room temperature and electrophoretically separated by SDS-PAGE by loading 50 µg of treated venom across the full width of the stacking gel of the slab gels. Polypeptide markers (Pharmacia) were run in lanes at the edges of the stacking gel. Electrophoretic separation in the Hoefer SE400 was at 4°C for 3000 volt hours at a constant current of 30mA until the voltage reached 200 volts and then at constant voltage. Mini-gel separations were carried out at room temperature at a constant current of 45mA.

Immunoblotting of ant venom allergens

After electrophoresis the separated polypeptides in large slab gels were transferred to nitrocellulose membrane (0.1µM pore size, Schleicher and Schull, Dassel, Germany) or to PVDF membrane (Immobilon-psq, Millipore Corp, Bedford, MA, USA) in a semi-dry blotter (Milliblot Graphite Electroblotter II, Millipore Corp.) using the discontinuous buffer system described by Lauriere [6] with an applied current of 0.8 milliamperes/cm². Mini-gels were transferred to nitrocellulose membrane (0.1µM, Schleicher and Schull) in a Hoefer Mighty SmallTM Transfer Unit (Hoefer Scientific Instruments) using the system described by Towbin [7]. Strips were cut from the sides of the blots to include both the polypeptide molecular weight markers and the separated venom components and were stained with amido black for subsequent alignment of the IgE-binding regions. The remaining blot was blocked by incubation for 1h in 0.1% (v/v) Tween 20 in 0.01M Tris-HCl pH8.0, 0.15M NaCl. Blot strips (5mm wide) were cut from the blot and incubated overnight at room temperature with sera from ant venom-allergic subjects as previously described [1]. IgE-binding bands were identified on the strips by incubation overnight with ¹²⁵I-labelled anti-human IgE antibody and autoradiography.

Immunological identification of cDNA clones encoding Myr p I and Myr p II

A rapid method of immunological analysis of λ gt11 cDNA clones was employed to identify the IgE antibody-binding components recognised by both the expressed cDNA clones and the reduced and alkylated venom polypeptides separated by SDS-PAGE. Briefly, lysogens of the λ gt11 Sfi-Not cDNA clones encoding *Myr p I* and *Myr p II* were induced to express fusion polypeptides and crude lysates prepared [8]. A crude lysate from an induced non-recombinant lysogen of λ gt11 Sfi-Not was also prepared. Nitrocellulose membrane discs (85mm dia.; 0.45µM pore size, Bio-Rad) were coated with expressed fusion polypeptides by incubation for 2h in a 90mm petri dish with the crude lysates of the induced lysogens of *Myr p I*, *Myr p II* and non-recombinant λ gt11 Sfi-Not. The discs were then washed three times with TBS (0.01M Tris-HCl pH8.0, 0.15M NaCl) containing 0.05% (v/v) Tween 20 (TBST) and blocked by incubation for 1h with 0.1% (v/v) Tween 20 in TBS. Single discs coated with each cloned fusion polypeptide were then incubated for 4h with serum from ant venom-allergic individuals diluted 1:10 with TBS, 0.005% (v/v) Tween 20, 0.5% bovine serum albumin in a total volume of 10mL. The discs were washed three times for 10 min each wash with TBST and the adsorbed IgE antibodies were eluted from the discs by vigorous agitation for 10 min with 10mL 0.1M glycine-HCl pH 2.5, 0.5% bovine serum albumin. The eluted antibodies were immediately neutralised with 3M Tris base. SDS-PAGE blot strips of reduced and alkylated native venom from large slab gels were incubated with the eluted antibodies, as described above, to identify polypeptides recognised by both the expressed cDNA clones and whole venom components.

N-terminal analyses of native venom polypeptides separated by SDS-PAGE

SDS-PAGE separation was carried out on the large slab gels as described above except a high sample loading (200 µg) of reduced and alkylated venom was applied to the gels. The electrophoretically-separated venom polypeptides were blotted onto PVDF membrane and washed several times with MilliQ deionised water. Strips were excised from the edges of the blot to include both the polypeptide markers and the separated venom and stained with amido black for subsequent alignment of the IgE-binding regions. Strips (5mm) were cut from the left and right hand sides of the remaining blot for immunoprobings with a serum pool of ant venom allergic individuals. The remainder of the blot was stained for five minutes with 0.1% amido black (Sigma) dissolved in destaining solution (acetic acid-methanol-water; 67:28:5) and vigorously destained for five minutes with several changes of destaining solution. The stained gel was washed several times in deionised MilliQ water and air dried. Bands were excised from the stained blot and N-terminal sequenced for 8 cycles on an Applied Biosystems 470A Gas-phase Sequenator (Baker Medical Research Institute, Prahran, Victoria).

Electrophoretic separation of a synthetic peptide based upon Myr p II

A 27 residue synthetic peptide (free N- and C- termini) that corresponded to the 49-75 residue C-terminal domain of the derived amino acid sequence of *Myr p II* (>70% purity) was prepared as previously described [9]. Reduction and alkylation were as previously described [3]. DTT-reduced peptide (0.7µg) and DTT-reduced and IAA-alkylated peptide were loaded in

the wells of a Mini Protean II Dual Slab Cell (Bio-Rad) and electrophoretically separated and blotted as described above.

RESULTS

SDS-PAGE separation of native venom polypeptides Figures 1 A, B and C show the electrophoretic separations of ant venom after reduction with ME and separation in the standard separating gel (Fig 1A); reduction with ME and separation in a separating gel containing 6M urea (Figure 1B); and reduction with DTT and alkylation with IAA and separation in a separating gel containing 6M urea (Fig 1C). Reduction and alkylation of the venom (Fig. 1C) resulted in a substantial shift of IgE-binding activity from the diffuse 5-8kDa and 2-4 kDa regions observed in blots of ME-reduced venom (Fig. 1A and 1B) with a strong enhancement of the 3kDa band. Polypeptides in the 5-8kDa region, after reduction and alkylation, resolved into a group of at least five IgE-binding bands. The presence of 6M urea in the separating gels of ME-reduced venom (Fig 1B) resulted in a loss of band definition and a downward molecular weight shift of IgE-binding. However, with reduced and alkylated venom consistently sharper band separations and a superior signal to background IgE-binding were obtained compared to non-urea gels.

Immunological identification of cDNA clones encoding Myr p I and Myr p II The IgE-binding profiles of the sera of two ant venom-allergic individuals having different venom polypeptide specificities are shown in the SDS-PAGE blot strips of reduced and alkylated venom (Figure 2, tracks a and e). The IgE antibodies that specifically bound to expressed Myr p I (see Materials and Methods) recognised the group of polypeptides between 5 and 8

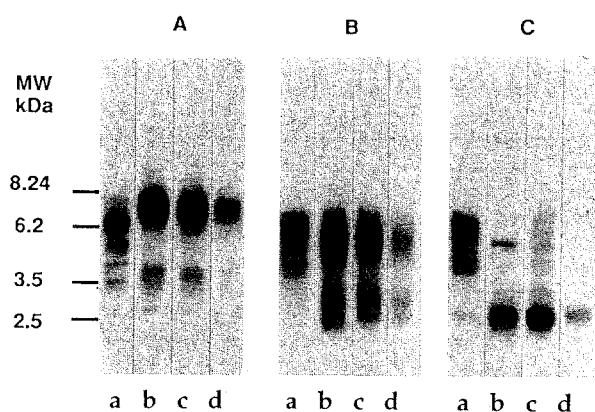


Figure 1. SDS-PAGE of *M. pilosula* venom and immunoblotting with sera from venom-allergic subjects. (A) ME-reduced venom separation in standard separating gel, (B) ME-reduced venom in 6M urea separating gel, (C) DTT-reduced and IAA-alkylated venom in 6M urea separating gel. (a-d) Sera from four ant venom-allergic subjects. Blot strips were incubated with sera from allergic subjects as described in Materials and Methods and IgE-binding was detected by incubation of the strips with ^{125}I -labelled antihuman IgE and autoradiography.

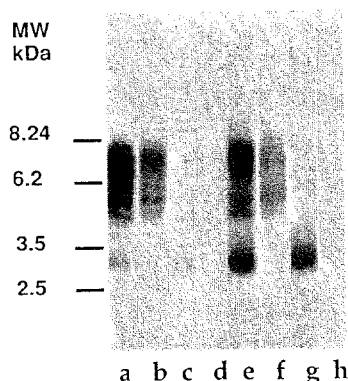


Figure 2. Immunological characterisation of the cDNA clones *Myr p I* and *Myr p II*. Sera from two ant venom-allergic subjects (A,B) were reacted with expressed lysates of λ gt11 Sfi-Not lysogens of *Myr p I* and *Myr p II* coated on nitrocellulose membranes. The antibodies were eluted as described in Materials and Methods and reacted with 6M urea SDS-PAGE blot strips of reduced and alkylated venom. IgE-binding was determined as described for Fig 1. (a) IgE-binding to serum A, (b) IgE antibodies eluted after serum A was adsorbed to expressed *Myr p I*, (c) IgE antibodies eluted after serum A was adsorbed to expressed *Myr p II*, (d) IgE antibodies eluted after serum A was adsorbed to expressed non-recombinant λ gt11 Sfi-Not, (e) IgE-binding to serum B, (f) IgE antibodies eluted after serum B was adsorbed to expressed *Myr p I*, (g) IgE antibodies eluted after serum B was adsorbed to expressed *Myr p II*, (h) IgE antibodies eluted after serum B was adsorbed to expressed non-recombinant λ gt11 Sfi-Not.

kDa (tracks b and f) but not the band at 3 kDa. IgE antibodies that specifically bound to expressed *Myr p II*, however, identified the dominant 3kDa IgE-binding band (tracks c and g) and a single minor band at 5.7 kDa (see track g). Expressed non-recombinant λ gt11 Sfi-Not lysate did not bind any ant venom-specific IgE (tracks d and h).

Electrophoretic separation of synthetic *Myr p II* The 27 residue synthetic peptide corresponding to residues 49-75 of the derived amino acid sequence of *Myr p II* was reduced with DTT and reduced and alkylated with DTT and IAA as described in Materials and Methods. The treated peptides were electrophoretically separated in a mini-gel and the gel blotted onto nitrocellulose membrane and reacted with a serum pool prepared from ant venom-allergic subjects that recognised all of the allergenic components of the venom (Figure 3). The DTT-reduced 27 residue synthetic *Myr p II* peptide (track a) separated as 3 bands. After both reduction and alkylation (track b) only a single band was observed and this corresponded in size to the 3kDa band identified in the large slab gel blots of reduced and alkylated venom. The results indicate that DTT-reduced synthetic *Myr p II* was able to form disulphide bonds in the separating gel during the course of electrophoresis.

N-terminal analyses of native venom allergens separated by SDS-PAGE Reduction and alkylation of venom resulted in a substantial shift of IgE-binding from the 5-8kDa region to the 3 kDa region (see Fig 1) giving a clearer band definition, in particular, for the residual

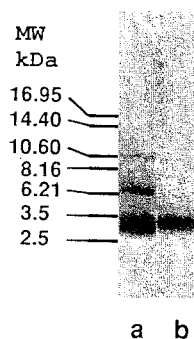


Figure 3. Electrophoretic separation of synthetic *Myr p II*. A synthetic peptide corresponding to residues 49-75 of the deduced amino acid sequence of *Myr p II* was prepared. The peptide was reduced and alkylated and electrophoretically separated and blotted as described in Materials and Methods. The blot was reacted with a serum pool of ant venom-allergic subjects recognising all the allergenic components of the venom (a) DTT-reduced synthetic *Myr p II*, (b) DTT-reduced and IAA-alkylated synthetic *Myr p II*. IgE-binding was determined as described for Fig 1.

polypeptides in the 5-8 kDa region (see Fig. 4). N-terminal amino acid sequences for the IgE-binding bands 1-6 indicated that bands 1,2,3,4 and 5 had exact sequence homology with the derived amino acid sequence of *Myr p I* from residues 57 (band 1), 65 (band 2), 68 (bands 3 and 4) and 71 (band 5). A previous study defined a single *Myr p I* IgE antibody combining site between residues 93-106 [9] and it is therefore presumed that IgE-binding bands subjected to N-terminal analysis extend at least to residue 106 or beyond. On the assumption that all the *Myr p I*-related bands extend to residue 112, the C-terminal residue of the derived amino acid sequence of *Myr p I*, band 1 corresponds to a 56 residue polypeptide (residues 57-112, calculated molecular mass 6,045 daltons), band 2 to a 48 residue polypeptide (65-112, calculated molecular mass 4,649 daltons), bands 3 and 4 to 45 residue polypeptides (68-112, calculated molecular mass 4,932 daltons) and band 5 to a 42 residue polypeptide (71-112, calculated molecular mass 4,649 daltons). The dominant IgE-binding band 6 had exact N-terminal sequence homology with the C-terminal domain of *Myr p II* (residues 49-75). A minor signal was also located in band 6 that appears to correspond to a sequence commencing at residue 86 of *Myr p I*.

DISCUSSION

The alkylation of the exposed thiol groups with IAA after reduction of venom and the inclusion of 6M urea in the SDS-PAGE separating gels produced superior separations to those obtained previously by reduction of venom with ME and SDS-PAGE in non-urea gels [1,2,3]. It is clear that IgE-binding polypeptide bands observed in the ME-reduced venom separations were diffuse because they included disulphide-bonded components that upon

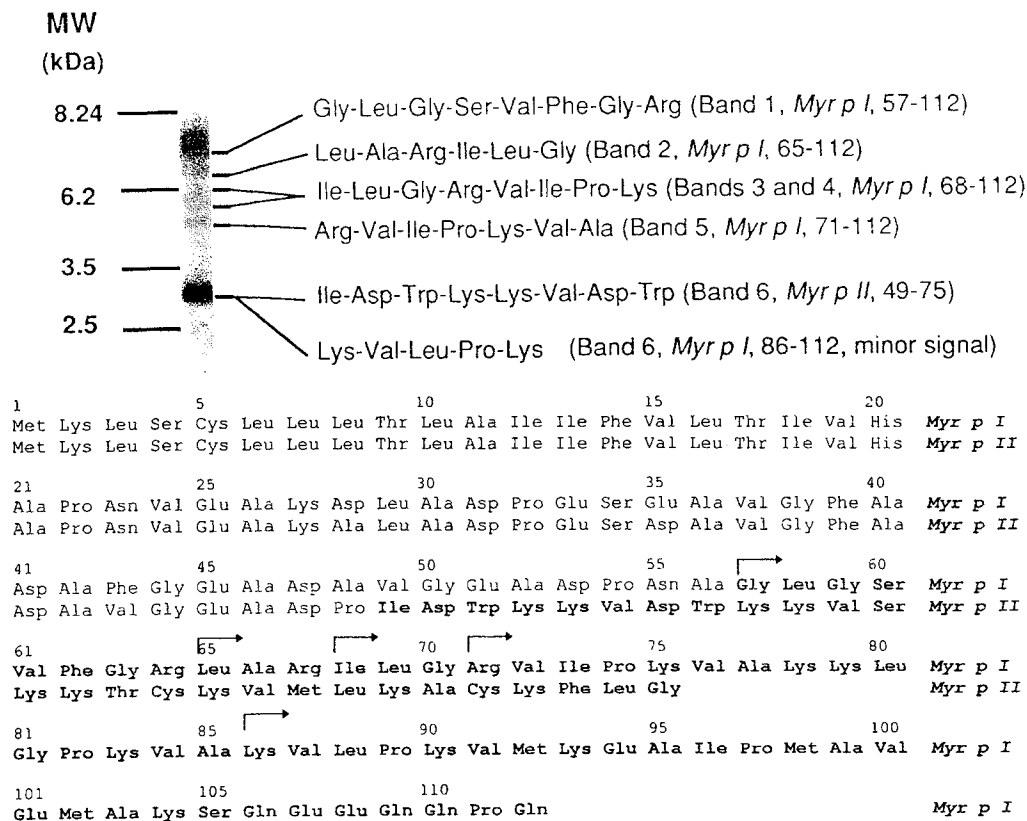


Figure 4. N-terminal analyses of ant venom polypeptides separated by SDS-PAGE. Ant venom polypeptides were separated by SDS-PAGE and blotted onto PVDF membrane. IgE-binding bands were excised and microsequenced as described in Materials and Methods. The derived amino acid sequences of *Myr p I* and *Myr p II* are shown. The sequences identified by N-terminal analyses are highlighted. Putative proteolytic cleavage points in *Myr p I* are arrowed.

both reduction and alkylation underwent a molecular weight shift to become the major IgE-binding band at 3 kDa. The presence of 6M urea, a protein denaturant, further improved the clarity of the separations and reduced background.

Rapid immunological analysis of the cDNA clones encoding the major allergens carried out by selectively adsorbing IgE antibodies to the expressed cloned allergens, eluting them and reacting them with blots of reduced and alkylated venom, established that the cDNA clone *Myr p I* has immunological identity with a group of at least five IgE-binding bands on SDS-PAGE blots with molecular masses between 5 and 8 kDa. *Myr p II* has immunological identity on SDS-PAGE blots with a major polypeptide of molecular mass of approximately 3 kDa and a minor 5.7 kDa polypeptide band.

N-terminal analyses of the major 3 kDa IgE-binding band identified a sequence homologous with the C-terminal 27 residues of *Myr p II* (see Fig.4, band 6). SDS-PAGE and

blotting of a 27 residue synthetic peptide based upon the C-terminal 27 residues of the derived amino acid sequence of *Myr p II* (residues 49-75) confirmed the identity of this peptide as the major 3 kDa polypeptide identified by SDS-PAGE of reduced and alkylated native venom. Its calculated molecular mass of 3,208 daltons agrees well with the molecular mass estimated by SDS-PAGE. Its behaviour in SDS-PAGE after chemical reduction alone, indicated that it re-formed disulphide bond-linked molecules. This presumably occurs through the cysteine residues located at positions 64 and 72 of the 27 residue C-terminal polypeptide of *Myr p II*. From blotting studies *Myr p II* appears to be expressed in the ant venom sac as a single gene product and subsequently forms disulphide-bonded structures. N-terminal analyses of bands 1-5 from the PVDF blots making up the 5-8 kDa group showed that the N-terminal sequences of these bands were all exactly homologous with *Myr p I* and have predicted N-termini at residues 57, 65, 68, 71 and 86 of its derived 112 amino acid sequence. Previous N-terminal analyses of SDS-PAGE PVDF blots of ME-reduced whole venom carried out by us identified the 45 residue fragment (68-112) of *Myr p I* as the largest IgE-binding fragment having a calculated molecular mass of 4,932 daltons [3]. N-terminal sequencing identified bands 1 and 2 as larger *Myr p I*-related polypeptides than previously identified. Band 1 appears to be the major IgE-binding component of the *Myr p I*-related group. Bands 3 and 4, although having different electrophoretic mobilities, gave identical *Myr p I*-related N-terminal sequence data, both commencing from residue 68. Doublets have been reported by the original authors of the tricine SDS-PAGE separation system [4] and it is therefore possible that these bands are a doublet. Alternatively, bands 3 and 4 may differ at their C-termini giving rise to different molecular masses and different electrophoretic mobilities.

Southern analyses previously carried out by us indicated that *Myr p I* and *Myr p II* are both encoded by multiple genes [1,3]. *Myr p II*, although encoded by multiple genes, appears in SDS-PAGE blots of reduced and alkylated venom as a single gene product, a 27 amino acid polypeptide. Cleavage of the 75 residue nascent *Myr p II* polypeptide to produce a 27 residue C-terminal peptide suggests a 48 amino acid leader sequence. Helix breaking residues (Gly or Pro) or large polar residues, frequently occur 4-8 residues before the cleavage point of a leader sequence [10]. Consensus with this structural arrangement is found in the 8 residue sequence Asp-Ala-Val-Gly-Glu-Ala-Asp-Pro preceding the cleavage point, where Glu and Asp, both polar residues, are located at 4 and 8 residues before the cleavage point. The different sized *Myr p I*-related polypeptides may arise as a result of the expression of multiple genes or alternatively they may arise by proteolytic cleavage of a single *Myr p I* gene product. Expression as a single gene product would require cleavage of the nascent *Myr p I* between residues 56-57 (ala-gly), 64-65 (arg-leu), 67-68 (arg-ile), 70-71 (gly-arg) and 85-86 (ala-lys). Cleavage to generate the 56 residue fragment (residues 57-112) suggests a polypeptide leader sequence of 56 residues. Large polar amino acids are found in the 8 amino acid sequence Val-Gly-Glu-Ala-Asp-Pro-Asn-Ala preceding this putative signal peptide cleavage point at 4 (Asp) and 6 (Glu) residues from the cleavage

point and a glycine to generate a turn is found 7 residues from the cleavage point which fits the consensus signal peptide cleavage motif described above. Cleavage between residues 64-65, 67-68, 70-71 and 86-87 of *Myr p I* may involve a different mechanism. Cleavage of the peptide hormones derived from the pituitary precursor pro-opiomelanocortin (POMC) which gives rise to the peptide hormones adrenocorticotropin (ACTH), β -endorphin and α -melanocyte stimulating hormone (α -MSH) appear to occur via a group of mammalian proteases having specificities similar to the bacterial subtilisins [11]. These enzymes cleave at basic residues (Arg and Lys) occurring singly, in pairs, or in a group of 4 having the motif lys/arg-X-X-arg (X- basic or hydroxylated residues). Cleavage after residues 64 and 67 of *Myr p I* may occur via this mechanism since both these amino acids are arginines. Similarly, cleavages after residues 70 (residue 71 is an Arg) and 85 (between Ala and Lys) may also involve this mechanism. Although Southern analysis indicates that *Myr p I* is encoded by a multigene family, the generation of a family of *Myr p I*-related polypeptides also fits with a model of endo-proteolytic cleavage of a single gene product involving the removal of a signal peptide leader sequence and then subtilisin-like random cleavage of the remaining polypeptide.

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