JBC Papers in Press. Published on February 26, 2002 as Manuscript M201387200

Proteins from *Mucuna pruriens* and enzymes from *Echis carinatus* venom: characterization and cross-reactions

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Running title: Antibodies against snake venom raised by *Mucuna pruriens*

Key words: snake venom, plant extract, western blot, protein purification, immunization, electrospray ionization

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SUMMARY

Mucuna pruriens seeds have been widely used against snake bite in traditional medicine. The antivenin property of a water extract of seeds was assessed *in vivo* in mice. The serum of mice treated with extract was tested for its immunological properties. Two proteins of *Echis carinatus* venom with apparent molecular weights of 25 and 16 KDa were detected by western blot analysis carried out using IgG of mice immunized with extract or its partially purified protein fractions. By enzymatic in gel digestion and ESI-MS/MS analysis of immunoreactive venom proteins, phospholipase A_2, the most toxic enzyme of snake venom, was identified. These results demonstrate that the observed antivenin activity has an immune mechanism. Antibodies of mice treated with non lethal doses of venom reacted against some proteins of *Mucuna pruriens* extract. Proteins of *Echis carinatus* venom and *Mucuna pruriens* extract have at least one epitope in common as confirmed by immunodiffusion assay.

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INTRODUCTION

Snake bite is a considerable problem in certain tropical and subtropical countries. According to WHO estimates, 40000 out of 5 million cases of snakebite are fatal. Antivenins obtained from horses treated with snake venom are one of the principal remedies against snakebite. This therapy has the disadvantage that antivenins must be given immediately and snakebite victims may develop an adverse reaction including anaphylactic shock (1). The use of endogenous plants with a reputation against snake bite is therefore worth considering (2).

In preliminary experiments (3, 4) we demonstrated that extract of *Mucuna pruriens* (MPE)[1], a medicinal plant widely used in Nigeria for its chemical and pharmacological properties, protects mice against the lethal effect of *Echis carinatus* venom (EV). Both MPE and EV are heterogenous mixtures, their interaction represents a complex phenomenon and there is no information about its biochemical mechanism. EV contains proteins with different toxic properties including opposite effects on blood clotting. Well known proteins are disintegrins EC3 (5), EC6 (6) and echistatin (7) that inhibit the interaction of fibrinogen with GpIIb-IIIa receptor on the platelet surface; echicetin (8) and ECLVIX/Xbp (9) with an opposite effect on platelet aggregation; two metalloproteases, ecarin (EC) (10, 11) and carinactivase (CA) (12), are prothrombin activators and act as procoagulant enzymes; phospholipase A_2 (PLA_2) (13, 14, 15), the most abundant enzyme, has many effects including inhibition of prothrombin activation by EC and CA (16). When injected to mice, this complex

[1] *Abbreviations*: MPE, *Mucuna pruriens* extract; EV, *Echis carinatus* venom; MLD, minimal lethal dose; EC, Ecarin; CA, carinactivase; PLA_2, phospholipase A_2; anti-MPE: serum of mice treated with MPE; anti-P1: serum of mice treated with P1; anti-P2: serum of mice treated with P2; anti-P3: serum of mice treated with P3; anti-EV: serum of mice treated with EV; P: protein fraction of *Mucuna pruriens* extract; NP: non protein fraction of *Mucuna pruriens* extract; EV25: 25 KDa EV protein; EV16: 16 KDa EV protein; MPE 22-28: 22-28 KDa *Mucuna pruriens* proteins; ESI-MS: electrospray ionization mass spectrometry; CLPs: Ca-dependent type lectin-related proteins; CTL: C-type lectin domain; CRD: carbohydrate-recognition domain

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mixture of proteins, induces disseminated intravascular coagulation (DIC) leading to death in less than a day. The composition of *Mucuna Pruriens* seed is also complex and variable, with 20-30% protein (lectins, globulins, protease inhibitors), 1-10% fat, 4-5 % ash, 4-9 % water, 4-7% fiber (17, 18, 19, 20) and L-DOPA (21) an interesting non-protein component.

The aim of the present study was to study the mechanism, the factors of MPE, the proteins of EV involved in the observed phenomenon.

EXPERIMENTAL PROCEDURES

Materials – Crude venom of *Echis carinatus* (common name: saw-scaled viper, family: *viperidae,* subfamily: *viperinae,* genus: *echis*, species: *carinatus*, subspecies: *sochureki,* Taxonomy ID:124223) was purchased from Sigma. Authenticity was certified by Miami Serpentarium Laboratories for its quality and representation of species; PBS, ammonium persulfate, Temed, DTT and peroxidase-conjugated goat anti-mouse IgG were from Sigma; non fat powdered milk was from Humana Milchunion eG; LMW protein electrophoresis standards, PlusOne^{TM} Silver Staining Kit Protein, Hi Prep 26/10, Hi-Trap Protein G column, Sephacryl S-200 HR, agarose and ECL detection kit were from Amersham Pharmacia Biotech; nitrocellulose membrane, 30% acrylamide/bis solution and tris/glycine SDS buffer were from Bio-Rad.

Plant material - Mucuna pruriens (family: *fabaceae,* subfamily: *papilionoideae,* genus: *mucuna*, species: *pruriens*) seeds were collected in the Rukuba area in Jos, Nigeria, with the aid of a traditional healer. They were authenticated by Prof S.W.H. Hussini of the Department of Botany, University of Jos. Voucher specimen Number A102 is deposited in the Pharmacy Herbarium of the University of Jos.

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Animals - CDI-ICR mice (30 g) from Nossan were kept at temperature 22 ± 1°C, relative humidity 60 ± 5%, 12 h light/dark cycle, standard diet and water ad libitum.

Preparation and partial purification of Mucuna pruriens seed extract - Sun dried seeds of *Mucuna pruriens* were ground to a paste of uniform consistency, 50 g of which was soaked in 100 ml H_2O, extracted for 24 h at 4°C, centrifuged at 10,000 x g for 20 min, and the supernatant lyophilized to a powder (24% protein) which was stored at - 4°C. Separation of the protein (P) and non-protein (NP) fractions was achieved by gel filtration on HiPrep 26/10 column: 5 ml MPE solution (5.4 mg/ml) was loaded and eluted with 50 mM tris buffer pH 7 (flow rate of 5 ml/min). E_{280}, E_{254} nm and conductivity were monitored. For partial purification of MPE proteins, 5 ml MPE solution (5.4 mg/ml) was applied to a Sephacryl S-200 HR column (2.6 x 80 cm), eluted with 50 mM Na_2HPO_4 pH 7.5 at a flow rate of 1.5 ml/min and read at 280 nm. P1, P2 and P3 were obtained.

In vivo protective effect of MPE, P and NP against EV - Groups of 8 mice were injected with MPE, P or NP fractions or saline (control). At different times afterwards, the mice were injected with a minimum lethal dose of EV (MLD 2 mg/Kg). The percentage of survivors was assessed 24 h later. Control group was injected with saline before EV. All fractions were injected intraperitoneally (i.p.) at doses proportional to body weight, calculating dilution after separation.

Preparation of anti-sera and purification of IgG - Six groups of 8 mice were treated once a week for 3 weeks with MPE, P1, P2, P3, EV (non lethal dose) and saline. After 28 days they were sacrified, blood was withdrawn, anti-MPE, anti-P1, anti-P2, anti-P3, anti-EV and preimmune sera were obtained. All anti-sera were purified by affinity chromatography with an AKTA liquid chromatography system. 10 ml of each anti-serum diluted in binding buffer (20 mM Na_2HPO_4 pH 7) and filtered on 0.22 µm membrane, was adsorbed on a 5 ml protein G column (1.6 x 2.5 cm) equilibrated in binding buffer until all unbound material was washed

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out. IgG fractions were then eluted at a flow rate of 2.5 ml/min with 0.1 M glycine-HCl pH 2.7 (elution buffer). The fractions were neutralized with 1M tris pH 9 and concentrated with centriplus membrane (final concentration 1.5 mg/ml).

SDS-PAGE and Western Blot analysis - Proteins in all fresh samples were determined by Bio-Rad assay (22) and separated by SDS–PAGE 12% according to Laemmli (23). They were transferred to a nitrocellulose membrane (0.45 µm) at 100 V for 1 h at 4°C and stained with Ponceau S. The membrane was blocked with 0.3% non fat powdered milk in PBS 1X containing 0.1% Tween 20 and incubated overnight at 37°C with treated mouse IgG. IgG of untreated mice was used as negative control. The membrane was washed in PBS 1X containing 0.1% (v/v) Tween 20, incubated with peroxidase-conjugated goat anti-mouse IgG (1:2000) and developed by enhanced chemiluminescence (ECL).

Detection of total proteins after SDS-PAGE 12% was achieved by silver staining using PlusOne™ Silver Staining Kit Protein.

Neutralization of lethal potency of EV– The minimal lethal dose of EV was preincubated with 100 µl each of anti-MPE, anti-EV, anti-P1, anti-P2, anti-P3 and preimmune IgG fractions at 37°C for 1 h. The preincubated mixtures were then injected into six groups of 8 mice. Control groups were injected with EV mixed with saline or purified IgG from serum of preimmune mice. The number of deaths in the subsequent 24 h was recorded.

Immunodiffusion assay –Antigenic relationships between the various antigens were studied by double diffusion test, according to Ouchterlony (24). Holes 5 mm in diameter were punched in horizontal gels containing 1% agarose in PBS 1X. Protein fractions of MPE or EV (20 µl) were placed in peripheral and IgG in central well. Diffusion was allowed to proceed for 24 h at 37°C. The gel was then washed with saline and dried. Precipitine line was visualized with Coomassie Brilliant Blue.

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Enzymatic in gel digestion – Coomassie blue-stained EV and MPE protein bands separated by SDS-PAGE were excised from the gel and digested with trypsin according to known procedures (25) with slight modifications. Briefly, gel slices were washed for at least 1 h in 100 mM NH_4HCO_3 pH 8.0, then for 1 h with 50% acetonitrile,100 mM NH_4HCO_3 pH 8.0 under shaking. Acetronitrile was added to shrink the gel pieces, and after 10-15 min of incubation, the solvent was removed, and the samples were dried in a speed-vac. Gel slices were reswollen with 25 mM NH_4HCO_3 pH 8.0, containing modified trypsin (Promega) and incubated for 4 h at 37°C. The supernatant was acidified with trifluoroacetic acid to a final concentration of 1%. Peptides were extracted from the gel slices twice with 60% acetonitrile 0.1% trifluoroacetic acid for 20 min. All supernatants were combined, and after evaporation to near dryness, peptide fragments were reconstituted in 20 µl of 0.1% trifluoroacetic acid.

Liquid Chromatography/Electrospray Ionization-Tandem Mass Spectrometry (LC/ESI-MS/MS) Analyses – mixture of peptides was separated using a Nucleosil C 8 column (4.6x 250 mm, 5-µm particle size, 300 A) and analyzed with a Finnigan LCQ ion trap mass spectrometer (San Jose, CA) with an electrospray ionization (ESI) source. A detailed scheme of the experimental setup for this type of analyses is described elsewhere (26). Briefly, a positive voltage of 3 kV was applied to the electrospray needle, and a N_2 sheath flow was applied to stabilize the ESI signal. The LC/MS analysis was conducted using a Perkin Elmer HPLC system coupled to the LCQ. The mobile phase was split before the injector by a Tee-connector, and a flow rate of 2 µl/min was established through the capillary C8 column. The enzymatically digested peptides were eluted from the column using 0.5% formic acid in water (mobile phase A) and 0.5% formic acid in acetonitrile (mobile phase B) with a three-step linear gradient of 5 to 10% B in the first 10 min, 10 to 35% B in the next 40 min, and 35 to 40% B in the last 5 min. The LC/ESI-MS/MS analysis was accomplished using an automated data acquisition procedure, in which a cyclic series of three different scan modes was

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performed. Data acquisition was conducted using the full scan mode (*m*/*z* 300-2000) to obtain the most intense peak (signal > 1.5×10^5 counts) as the precursor ion, followed by a high resolution zoom scan mode to determine the charge state of the precursor ion and MS/MS scan mode to determine the structural fragment ions of the precursor ion. The resulting MS/MS spectra were then matched against a protein database (Owl) by Sequest software to confirm the sequence of tryptic peptides.

RESULTS

Antivenin activity of MPE - We first found that the protective effect of MPE against the lethal effect of EV was exerted at a dose of 21 µg/g and was evident 24 h and 1-4 weeks after administration. To understand the chemical nature of substances responsible for the protection, an *in vivo* test was set up with two fractions obtained by Hi-prep separation of MPE, one containing proteins (P) and the other one non-proteins compounds (NP) from MPE (Figure 1). NP fraction contain small molecules like L-DOPA responsible for E_{280} absorbance, free aminoacid, ions, fatty acids as already reported (18, 19).

Figure 1
Table 1

The *in vivo* test showed that P and NP fractions exerted protection in different ways. As showed in table 1, the NP fraction conferred short-term protection (1 day) while the P fraction for a longer time (1, 2 and 3 weeks after administration). When we comparatively injected mice with MPE, P and NP fractions once a week for 3 weeks, the protective effect of the P fraction specifically increased. Some compounds in the NP fraction may be adjuvants in the long term protective effect because P fraction was less active when used alone. Only with a booster dose of P fraction the total effect was restored.

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Three well resolved protein peaks, P1, P2, P3 and one non protein fraction (NP) obtained by further purification of MPE on Sephacryl S-200 were further purified by gel filtration on Sephacryl S-200 HR, are showed in Figure 2.

Figure 2

Antibodies against EV induced in mice by extracts of Mucuna Pruriens – The protective activity of MPE, P1, P2, P3 was tested for their capacity to raise in mice antibodies against EV proteins. IgG were purified from anti-MPE, anti-P1, anti-P2, anti-P3 and preimmune mice serum with protein G affinity separation and used in western blot experiments.

When anti-MPE IgG were tested against EV proteins, two protein bands with apparent molecular weights of about 25 KDa (EV25) and 16 KDa (EV16) were detected. The signal was only visible under reducing conditions implying that the epitope on the native EV protein was in a cryptic state. When MPE proteins were incubated with the specific anti-MPE IgG, as positive control, a pattern similar to that obtained with silver staining was achieved, indicating that almost all MPE proteins were highly antigenic (Figure 3A).

When the protein fractions, obtained by partial purification of MPE on Sephacryl S-200, were injected into mice, we also obtained antibodies against EV. Antibodies raised in mice by injecting P2 fraction gave similar results to those obtained with anti-MPE IgG, while when anti-P3 IgG was used, only EV16 was detected. No signal against any EV proteins was obtained using IgG of mice treated with P1 fraction (anti-P1 IgG). Positive controls of anti-P1, P2, P3 IgG were the corresponding P1, P2, P3 fractions (Figure 3B).

Antibodies against proteins of Mucuna pruriens induced in mice by administration of EV- Under reducing conditions, some anti-EV IgG reacted with at least three bands of MPE proteins with MW in the range 22-28 Kda (MPE 22-28) as shown in Figure 3C. These proteins mainly belong to P2 fraction. EV proteins reacted strongly against the specific anti-EV IgG under non reducing conditions and this sample was used as positive control.

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Figure 3

Neutralization of lethal potency of EV – The capacity of anti-MPE, anti-P1, anti-P2, anti-P3 IgG to neutralize the toxicity *in vivo* of EV was tested after incubation of the fractions with venom and results were reported in Table 2. No neutralization was observed in control groups 1 and 2 (negative controls); lethal potency of EV was neutralized by anti-EV IgG obtained from mice treated with non lethal dose of EV (group 3, positive control). Anti-MPE (group 4) and anti-P2 (group 6) showed neutralizing effects similar to that of group 3; less neutralization was obtained with anti-P3 (group 7) and no neutralizing effect with anti-P1 (group 5). A 50% survival percentage was considered a satisfactory neutralizing effect.

Table 2

Immunodiffusion test – To confirm the results of western blot experiments and to ascertain the presence of one or more common antigenic epitopes in MPE and EV proteins, the double diffusion test was used. When anti-MPE IgG was tested against MPE and EV proteins, a pattern shown in Figure 4a was obtained indicating coalescence of antigens. When anti-EV IgG was tested against MPE, P2, P3 and EV, one precipitin line was formed in all cases (Figure 4b).

Figure 4

Protein identification by ESI-MS/MS – For the identification of proteins involved in this phenomenon, enzymatic in gel digestion and ESI-MS/MS analysis of MPE and EV immunoreactive proteins was performed. The most intense peaks in the mass spectrum were automatically selected using Sequest software for the database search. The proteins are identified by correlation of the experimentally obtained MS/MS spectra to the theoretically predicted peptide MS/MS spectra of proteins present in database. Two peptides of triptic digested mixture of EV16 protein were found with sequences NLFQFAEMIVK (Figure 5A) and DNLNTYDKK (Figure 5B) matching that of Russell's viper phospholipase A_2.

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Figure 5

DISCUSSION

The present results demonstrate that extracts of *Mucuna pruriens* seeds protects mice against the toxic effects of *EV.* It does so by an immunological mechanism based on a series of specific epitopes common to some vegetal and venom proteins.

The *in vivo* test results indicate that administration of MPE proteins raised antibodies responsible for the protection observed. The long-term protection was infact more complete when the P fraction was administered according to an immunization protocol (once a week for 3 weeks). Several proteins in the P2 fraction are involved in raising antibodies, while the most purified P3 fraction is less active. We demonstrated by western blot analysis that certain antibodies, induced in mice by injection of vegetal extracts, reacted directly with certain EV proteins, and immunodiffusion experiments pointed out the cross-reaction between MPE and EV proteins.

Western blot analysis carried out using anti-MPE and anti-P2 IgG showed that only EV proteins with molecular weight around 25 and 16 KDa were targets of the antibodies raised in mice after injection of MPE or P2; the toxic effect *in vivo* of EV was neutralized when the venom was preincubated with these IgG before injection. EV25 and EV16 may be the most toxic components of EV acting in concert and their neutralization seems to impair the toxic mixture making it non lethal. Anti-P3 IgG did not totally neutralize the venom, presumably because it only react with a single protein of EV (EV16).

Electrospray mass spectrometry combined with a peptide sequence tag search lead to the identification of the proteins with an estimated MW of 16 KDa in the EV16 band as PLA_2. The sequences of two peptides obtained by trypsin digestion matched the sequence of

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Russell's viper PLA_2 (*Daboia russellii*) while the venom we used was certified to be from *Echis carinatus sochureki*. This may be explained by the fact that only one sequence of *Echis carinatus* PLA_2 is depositated in the database whereas various isoenzymes are known in literature (13, 14, 15, 27) and EV varies with country of origin and other factors (28) as showed in our experiments and declared by our supplier. Since the genera *Echis* and *Daboia* are very similar and both belong to the subfamily *viperinae*, the *Echis carinatus* PLA_2 that we analyzed may be a genetic variant, the sequence of which is closest to that of *Russell's viper*. In our recent paper (29) we demonstrated the *in vitro* effects of anti-MPE on EV enzymes catalyzing prothrombin transformation. PLA_2 is known to be implicated in that reaction and our present results confirmed that PLA_2, the enzyme largely responsible for the lethal effects of snake venom, is the putative target of the antibodies induced by MPE.

Analysis of in gel trypsin digestion of the EV25 band, gave us no significant conclusions because no sequence of EV proteins with an estimated MW of 25 KDa have been yet reported in databases.

We also tried to identify the specific MPE protein/s responsible for the observed phenomenon. Our results suggest the presence of similar epitopes in EV and MPE proteins. This enabled us to use anti-EV IgG to detect specific proteins of MPE, limiting the numbers of animals necessary for *in vivo* testing during protein purification. We succeeded in identifying a group of MPE immunoreactives proteins (at least 3 bands) very likely due to isoforms. When tryptic digested mixture of these bands were analised by ESI-MS/MS no significant matching were obtained considering that the genome and proteome of *Mucuna pruriens* is still unexplored and no protein sequences are reported in database. A molecular biology approach is required to identify these proteins and it will be a topic for future researches.

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It seems surprising that proteins of EV and MPE could have common epitopes, however other examples of sequences shared by plant and snake venom proteins have been reported. The lectin domain, contained in all plant chitin-binding proteins, shows a sequence similarity to disintegrins of crotalid and viperid snake venoms (30). Another example is the similar overall folding pattern of the three-dimensional structures of snake venom postsynaptic neurotoxins and the domains of wheat germ agglutinin (31). All venom proteins have different functional activities but they show structural and evolutionary relationships: they are derived from a common precursor and share conserved domains (32, 33, 34), some of which are common to plant proteins. The C-type lectin domain (CTL or carbohydrate-recognition domain, CRD) has highly conserved structure (35, 36), is responsible for carbohydrate binding and has been found in all Ca-dependent type lectin-related proteins (CLPs), echicetin, ECLVIX/XBp and CA. Like many other leguminosae plants, *Mucuna pruriens* seeds contain legume lectins (37) that belong to the same family of C-type animal lectins and may contain the same CTL domain of CLPs proteins of EV.

We can conclude that when MPE proteins are injected into mice in such a way as to induce abundant antibodies production, a polyclonal serum against epitopes present on one or more EV proteins is obtained. If MPE extract and some of its proteins protect mice against EV PLA_2 or other snake venom proteins which show procoagulant and anticoagulant activities, very likely they could interfer in the coagulation process.

The present fundings open new perpectives in the field of vaccine by natural products and may be useful in the therapy of snakebite and other coagulation disorders.

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FIGURE LEGENDS

Fig. 1. **Chromatographic separation of P and NP fractions of MPE**. 5 ml MPE solution (5.4 mg/ml) was loaded on a HiPrep 26/10 column and eluted with 50 mM Tris buffer pH 7 at a flow rate of 5 ml/mi. The fractions were monitored at 280 and 254 nm and for their conductivity.

Fig. 2. **Chromatographic purification of MPE by gel filtration**. A) 5 ml MPE solution (5.4 mg/ml) was applied to a Sephacryl S-200 HR column (2.6 x 80 cm) and eluted with 50 mM Na_2HPO_4 pH 7.5 at a flow rate of 1.5 ml/min. Three protein peaks at 280 nm (P1, P2, P3) and a non protein fraction (NP) were obtained. B) SDS-PAGE of MPE and the fractions obtained in A were loaded and silver stained. Molecular weights of LMW standards are indicated on the left (St).

Fig. 3. **Immunological detection of EV and MPE proteins**. A) Western blot analisis of EV with anti-MPE IgG. MPEr: 2 µg of MPE proteins under reducing conditions (positive control); EVr: 90, 60 and 30 µg of venom proteins under reducing conditions; EVnr: 90, 60 and 30 µg of venom proteins under non-reducing conditions. Anti-MPE IgG was diluted 1:200 in blocking buffer and incubated overnight at 37°C. EV: silver staining of venom proteins under reducing conditions. Molecular weight of LMW standards are on the right. B) Western blot analysis of EV with anti-P1, anti-P2 and anti-P3 IgG. 1 ug P1, P2 and P3 were positive controls for their respective IgG. EVr: 60 µg and 120 µg of venom proteins dissolved in reducing buffer and loaded in lanes 1 and 2 respectively. Molecular weights of standard are on the left. Anti-P1, anti-P2 and anti-P3 IgG were diluted 1:200 in blocking buffer and incubated overnight at 37°C. C) Western blot analysis of MPE proteins with anti-EV IgG. EVnr: 2 µg of venom proteins under non reducing conditions (positive control); MPEnr: 3, 9 ,

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27 µg of Mucuna proteins under non reducing conditions; MPEr 3, 9, 27 µg of Mucuna proteins under reducing conditions. Anti-EV IgG was diluted 1:400 in blocking buffer and incubated overnight at 37°C. MPEr (right) shows silver stain of reduced MPE proteins.

For each experiment, binding was detected by incubating the membrane with peroxidase-conjugated goat anti-mouse IgG, diluted 1:2000 in blocking buffer. Proteins were then visualized by ECL at a short exposure time.

Fig. 4**. MPE and EV proteins tested for their capacity to precipitate mouse IgG in Ouchterlony double immunodiffusion experiments.** a) 10 µg anti-MPE IgG was deposited in well 1, 20 µg MPE proteins in well 3 and 20 µg EV proteins in well 2. *b)* 10 µg anti-EV IgG was deposited in well 1, MPE in well 2, 20 µg P2 in well 3, 20 µg P3 in well 4 and 20 µg EV in well 5.

Fig. 5. **Identification of EV16 band by mass spectrometry.** The most abundant peaks of triptic digested mixture of EV16 band were selected, tandem mass spectrum was performed and the sequence stretch, together with its starting mass, its end mass, and the molecular weight of the peptide were entered in the database search programme (Sequest) where they were converted to a peptide sequence tag. Two peptide were partially sequenced. A) NLFQFAEMIVK corresponds to the fragmentation of ion with m/z 670.5; B) DNLNTYDKK corresponds to the fragmentation of a ion with m/z 982.2.

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Table 1

In vivo protective effect of Mucuna pruriens against Echis carinatus venom

Time course of *in vivo* effect of MPE, P and NP fractions against EV. Groups of 8 mice were injected with the indicated fractions and treated with a minimum lethal dose of EV (2 mg/Kg) 24 h, 1 week and 3 weeks later. Control group was injected with saline and then EV. Survivors were counted 24 h after EV injection. All fractions were injected intraperitoneally with doses proportional to body weight (µg/g). All groups received one injection except group A in the last column which was immunized with one injection a week for 3 weeks.

Fractions	Dose (µg/g)		Survivors / total			
	dry extract	proteins	1 day	1 week	3 weeks	3 weeks (A)
MPE	21	4.2	6 / 8	5 / 8	7 / 8	8/8
P	21	4.1	2 / 8	3 / 8	5 / 8	8/8
NP	21	0	5 / 8	3 / 8	2 / 8	2/8
Control	0	0	0/8	0/8	0/8	0/8

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Table 2

Neutralization activity of IgG fractions

Minimal lethal dose of EV was incubated at 37°C for 1 h with 100 µl of the IgG fractions indicated (mixture). This was the dose for a 30 g mouse. Five groups of 12 mice were used. Groups 1 and 2 were negative controls, group 3 positive control.

group	Mixture	Survivors/total
1	EV + saline	0/12
2	EV + preimmune IgG	0/12
3	EV + anti-EV IgG	7/12
4	EV + anti-MPE IgG	6/12
5	EV + anti-P1 IgG	0/12
6	EV + anti-P2 IgG	5/12
7	EV + anti-P3 IgG	3/12

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Figure 1

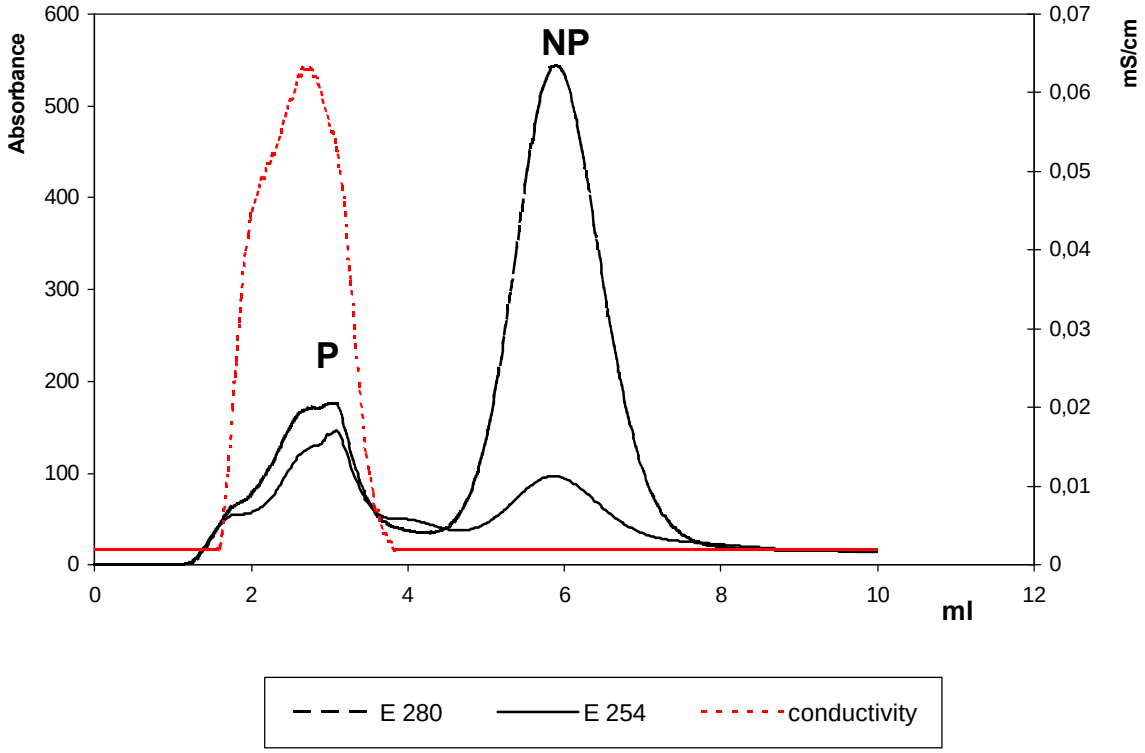

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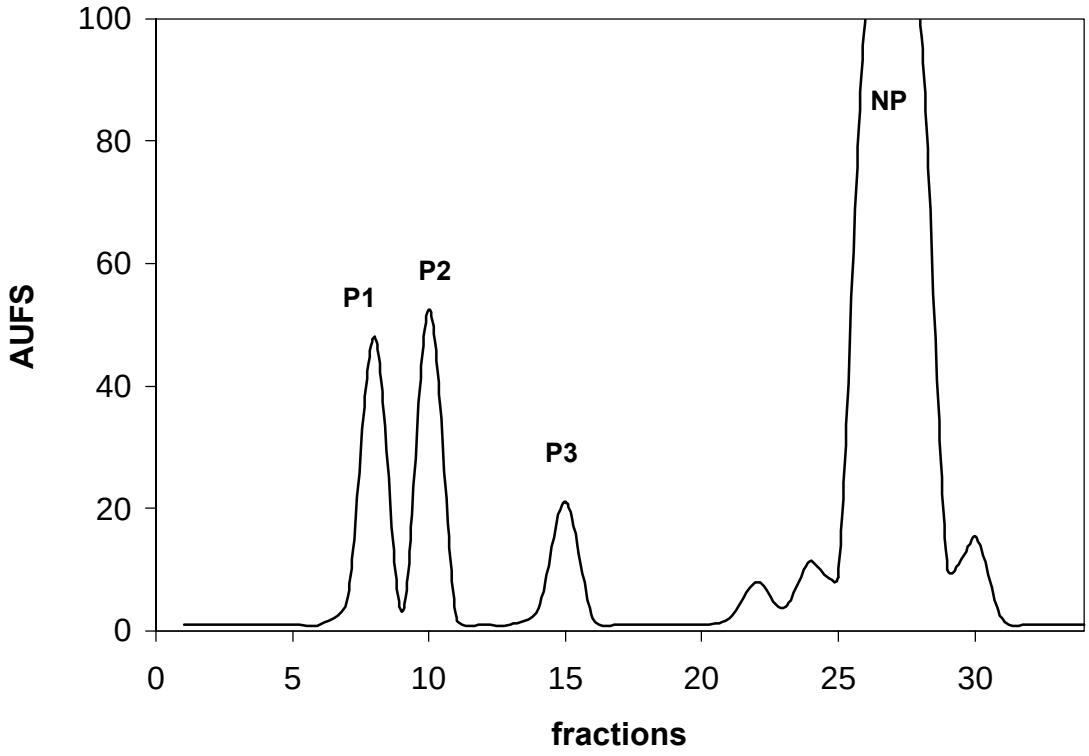
Figure 2

A.



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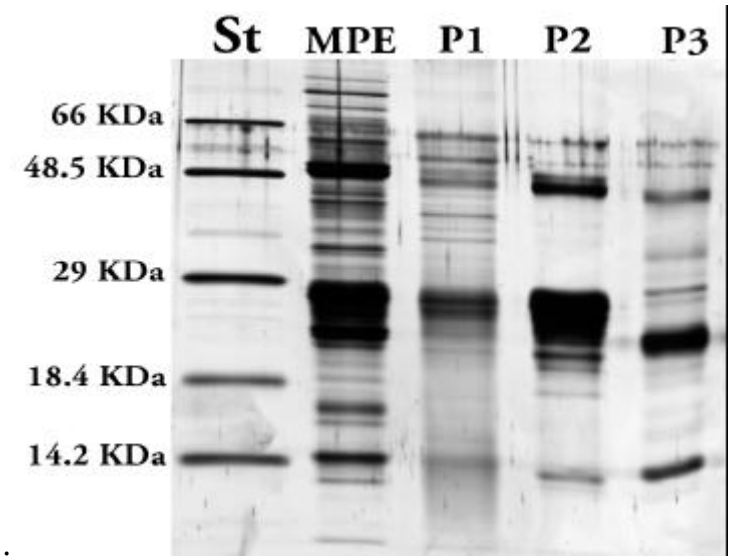

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Figure 3

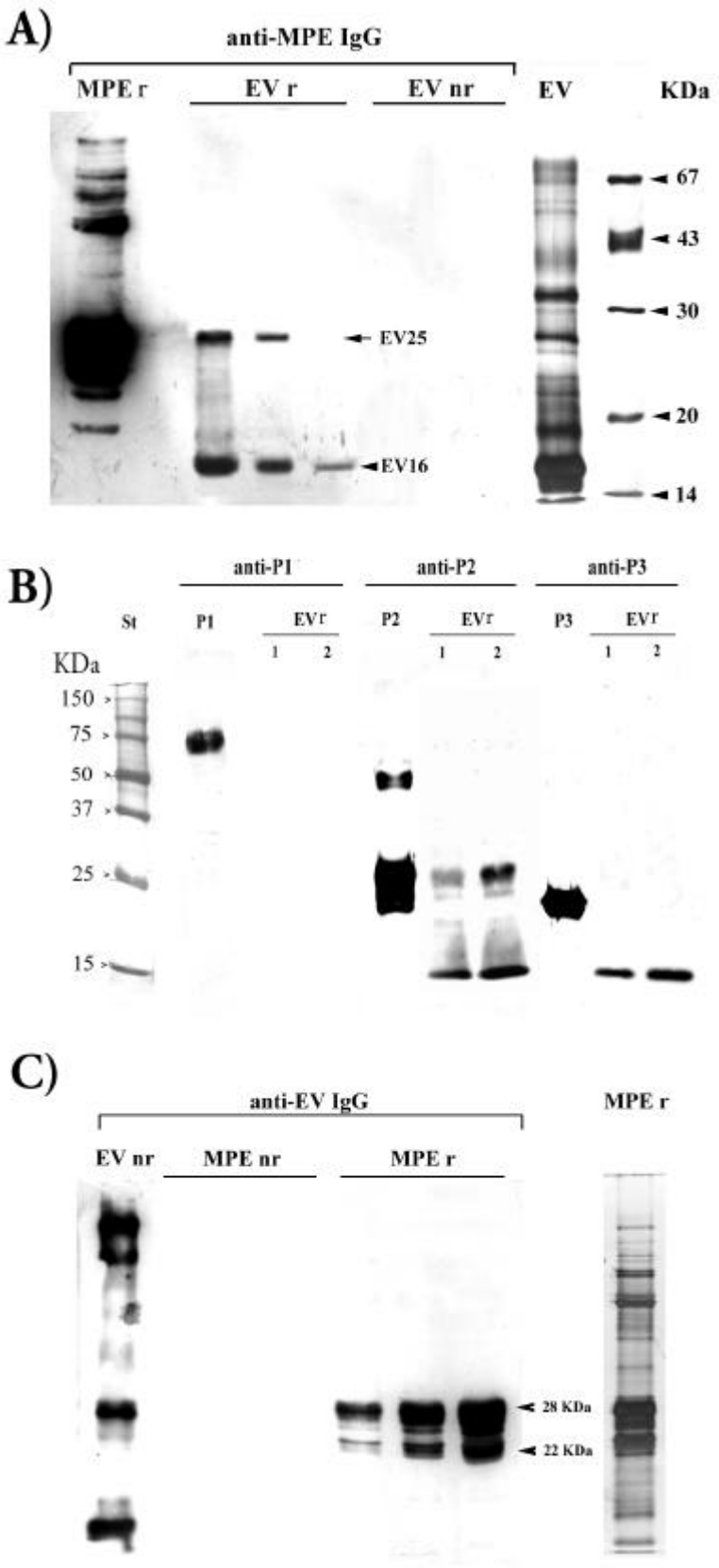

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Figure 4

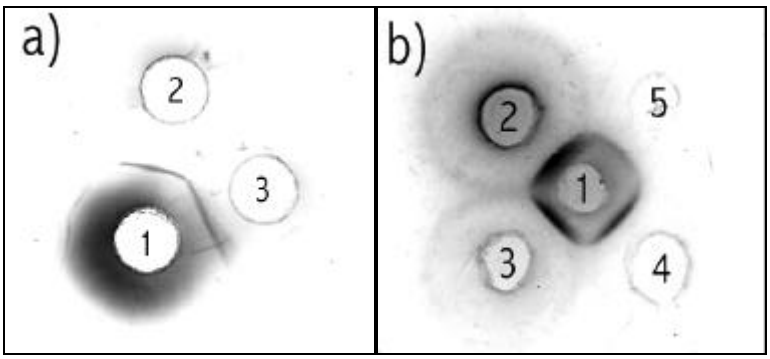

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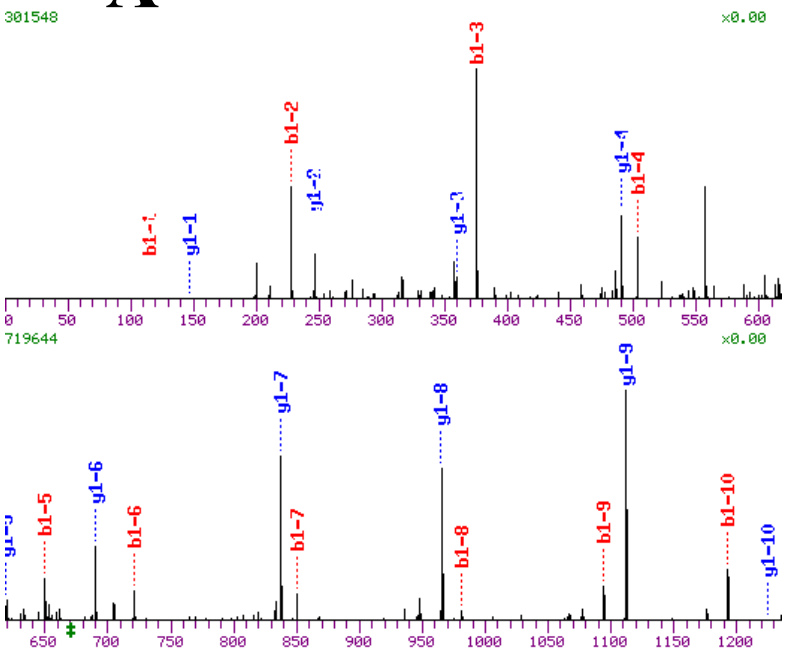
Figure 5

Seq	#	b	y	(+1)
N	1	115.1	-	11
L	2	228.3	1226.5	10
F	3	375.4	1113.3	9
Q	4	503.6	966.2	8
F	5	650.7	838.0	7
A	6	721.8	690.9	6
E	7	850.9	619.8	5
M	8	982.1	490.7	4
I	9	1095.3	359.5	3
V	10	1194.4	246.3	2
K	11	-	147.2	1

Seq	#	b	y	(+2)
N	1	58.1	-	11
L	2	114.6	613.8	10
F	3	188.2	557.2	9
Q	4	252.3	483.6	8
F	5	325.9	419.5	7
A	6	361.4	345.9	6
E	7	426.0	310.4	5
M	8	491.6	245.8	4
I	9	548.2	180.2	3
V	10	597.7	123.7	2
K	11	-	74.1	1

A



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L	3	343.4	882.0	7
N	4	457.5	768.8	6
T	5	558.6	654.7	5
Y	6	721.7	553.6	4
D	7	836.8	390.4	3
K	8	965.0	275.3	2
K	9	-	147.2	1

Seq	#	b	y	(+2)
D	1	58.5	-	9
N	2	115.6	498.5	8
L	3	172.2	441.5	7
N	4	229.2	384.9	6
T	5	279.8	327.9	5
Y	6	361.4	277.3	4
D	7	418.9	195.7	3
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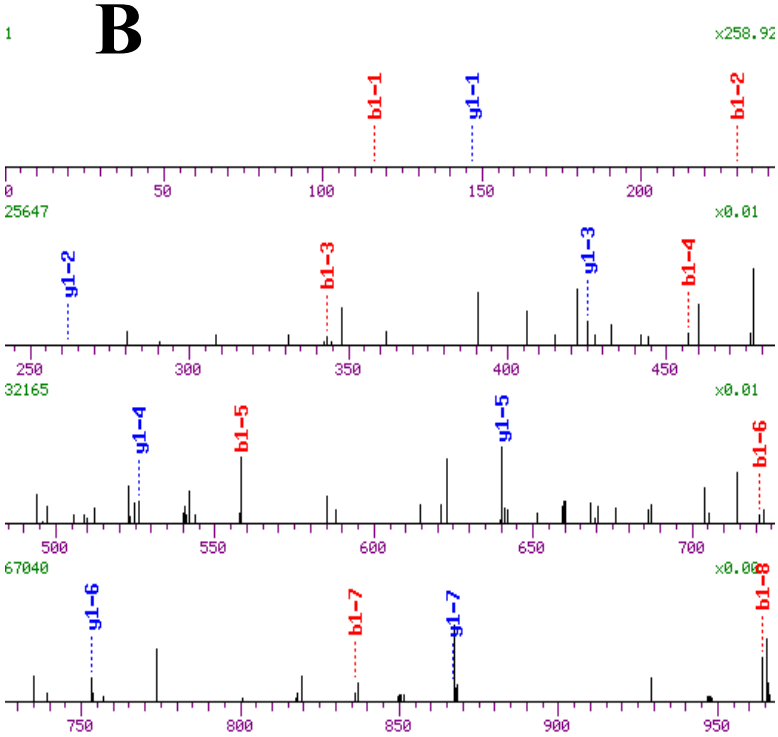
K	9	-	74.1	1

B



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Proteins from Mucuna pruriens and enzymes from Echis carinatus venom: characterization and cross-reactions

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J. Biol. Chem. published online February 26, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201387200

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