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Purification and pore forming activity of two hydrophobic polypeptides from the secretion of the Red Sea Moses sole (Pardachirus marmoratus)*

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Running title

Pore Forming Activity of Pardaxins.

The abbreviations used are: PMC, Pardachirus marmoratus Moses sole secretion after desalting on Sephadex G-25; PX, pardaxin derived from Sephadex G-100 chromatography; PXI, II, pardaxin I, II, derived from HPLC; PTH: phenylthiohydantoin derivative; SDS, sodium dodecyl sulphate; DSS, disuccinimidyl suberate: kD, kilodaltons; CU_{50}, cytotoxic unit 50%.

Summary

A new column chromatography procedure, based on ion exchange, chromatofocusing and reverse phase HPLC was employed to isolate the two main proteaceous, toxic, cytolytic, pore forming factors from the secretion of the Red Sea Moses sole Pardachirus marmoratus. Pardaxin I, comprising 10% of the gland secretion proteins, was shown to be 5-10 times more toxic, cytolytic and active in membrane pore formation that pardaxin II (8% of gland secretion proteins). Gel-electrophoresis, amino acid analysis and N-terminal amino acid sequence reveals a high degree of homogeneity and resemblance between the two toxins. They are rich in aspartic, serine, glycine, alanine, and devoid of arginine, tyrosine, and tryptophan. Their N-terminal was found to be NH_2^{Γ}-Gly-Phe-Phe. Their hydrophobicity is evident from the chromatographic behavior on a hydrophobic resin, presence of nine successive hydrophobic residues on the NH_2^{Γ}-terminal and a decrease in drop size during elution of active fractions during chromatographic purification. The minimal molecular weight of Pardaxin I is about 3500 as determined by SDS gel electrophoresis and amino acid analyses. It is composed of 35 amino acids and is free of carbohydrate and sialic acid residues. Mass spectrometry of the ethyl acetate extract of the gland secretion and purified toxin reveals the presence of sterols in the secretion but their absence in the purified toxins.

Pardaxin I was iodinated without affecting its chemical and pore forming properties. It binds to liposomes of different phospholipid compositions. In hyperpolarized unilamellar liposomes pardaxin I produced a fast, non-specific permeabilization and in multilamellar liposomes, a slow, cation specific pore. It is suggested that pardaxins exert their effects due to their hydrophobic and pore formation properties.

By means of some 200 glands located along its dorsal and anal fins, the Red Sea Moses sole exudes a secretion into its environment. This secretion is toxic to fish and repels sharks (1,2). The secretion contains several high and low molecular weight components of biological interest. Using gel permeation column chromatography, the higher molecular weight proteins were fractionated into several fractions, the main toxic component was named pardaxin (3,4). This toxin was characterized as a single, helical, monomeric, acidic protein with four disulfide bridges and a molecular weight of about 13,000 daltons (4,5). Diverse pathological and pharmacological effects, induced by the fish toxic secretion or pardaxin were in a range of 10^{-9} M - 10^{-5} M (as reviewed in Table I). These effects seem to be associated with a perturbation of membrane function and a change in permeability. Using planar artificial membranes (6) and liposomes (7) formation of pores by the Moses sole secretion was reported. We have been interested in the mechanism of pardaxin induced pores in the hope that the toxin could provide a well characterized model for the interaction of cytotoxic polypeptides with biological and artificial membranes. Recently, it was shown that the secretion of the Pardachirus sole in addition to toxic proteins (45) contains also toxic and hemolytic steroid aminoglycosides with shark repellent activity (8), thus requiring a reevaluation of the structure-function relationships of the fish secretion components and of the homogeneity of pardaxin isolated by gel permeation (3,4). In the present study we present a new procedure for purification of the proteous, cytotoxic, pore inducing factors, from the Moses sole secretion, free of sterol components. It was shown that the hydrophobic proteous toxin binds to and permeabilizes liposomes. These properties of pardaxins suggest they may be of potential value as probes in membrane research.

Experimental procedures

Materials

Gels for column chromatography, Sephadex G-10, G-25, G-75 and G-100, QAE-Sephadex A-25, Phenyl Sepharose CL 4B, Sepharose CL-4B, PBE-94, polybuffer 74, and low molecular weight calibration kit were purchased from Pharmacia, Uppsala, Sweden. Ovalbumin, bovine albumin fraction V, soybean lecithin, sodium cholate, gramicidin D and Dowex 50 x 8 (50-100 mesh) were obtained from Sigma Chemical Co., St. Louis, MO, USA. 3,3'-diethylthiodicarbocyanine iodide (dis-C_2-(5)) was purchased from Molecular Probes, Inc., Junction City, OR, USA and valinomycin from Calbiochem, San Diego, CA. ^{86}Rb and Bolton Hunter reagent were purchased from Amersham Radiochemicals. Phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and cholesterol, analytically pure were purchased from Sigma, St. Louis. Melittin, electrophoretically pure was purchased from Makor Chemicals, Jerusalem, Israel. Polyoxyethylene lauryl ether (BRiJ35) and disuccinimidyl suberimidate were obtained from Pierce Chemical Co., Rockford, IL. Cytotoxins devoid of phospholipases A_2 activity were purified from cobra venom and scorpion venom (18). All other chemicals were of analytical grade.

Toxic secretion. The toxic secretion of the Red Sea Moses sole Pardachirus marmoratus (Pisces, Soleidae) was obtained according to the method described by Clark and Chao (1) and stored in a lyophilized form.

Purification of Pardaxins

Gel filtration. 0.4 gr of water extract of the lyophilized secretion was applied on a column of 3 x 50 cm filled with Sephadex G-25 superfine, eluted by

0.1 M ammonium acetate pH 5.0 buffer at a flow rate of 400 ml/hr and fractions of 150 drops/tube were collected. 200 mg of the toxic fraction obtained was applied on a column of 2.5 x 100 cm filled with Sephadex G-100, equilibrated and eluted with 0.1 M ammonium acetate buffer, pH 5.0 at a flow rate of 30 ml/hr and fractions of 150 drops per tube were collected. Estimation of the molecular weight of pardaxins, derived from QAE-Sephadex, was performed on Sephadex G-75 (V_t=130 ml; V_o=45 ml) using 1.0 M ammonium acetate buffer, pH 6.5 at a flow rate of 50 ml/hr and fractions of 6.5 ml/tube were collected. The peak elution volume of each marker and toxin was measured according to the absorbance at 280 nm. The column was calibrated with Pharmacia low molecular weight, gel filtration calibration kit. The molecular weight were determined by interpolation of the linear plot of log M_w versus R_f. All purification procedures were carried out at room temperature.

Ion exchange chromatography. Pardaxin obtained from Sephadex G-100 chromatography was further purified on a column of QAE-Sephadex (1.5 x 20 cm) equilibrated with 0.05 M ammonium acetate, pH 6.7. Two active fractions were obtained by step-wise increases of elution buffer molarity up to 2.0 M. After removal of the salts by dialysis, the toxic fractions were lyophilized.

Chromatofocusing. The two pardaxins isolated by the QAE-Sephadex were purified by two steps of column chromatofocusing. Columns (1.5 x 20 cm) charged with PBE-94 gel were equilibrated with 0.025 M immidazole buffer, pH 7.3 and eluted with polybuffer 74 (0.0093 mmole/pH unit/ml) pH 3.3. The gels were subsequently eluted by 1 M ammonium acetate, pH 6.8 and 6 N guanidine-HCl, pH 7.5. Samples of each second tube were tested for fish lethality, cytotoxicity and pore formation activity, using the respective buffers as controls. The toxic peaks were pooled, dialyzed from the ampholytes extensively and desalted on a column (3 cx 50 cm) of Sephadex G-10 or hydroxylapatite. In

the present study, for sequence analysis we have used pardaxin I (PXI) and pardaxin II (PXII) at this stage of purification.

Reverse-phase HPLC. Pardaxins I and II derived from chromatofocusing were eluted from LKB ultropac column (Spherisorb ODS 2, 3 µm) with a linear gradient from 25% acetonitrile to 85% acetonitrile in 1% trifluoroacetic acid, for 30 min. Peaks were monitored by 226 nm absorption. The collected polypeptide peaks were lyophilized and submitted for biological assays, gel electrophoresis and amino acid analysis.

Hydrophobic chromatography. Was carried out as previously described (9). The pardaxins were loaded on a Phenyl-Sepharose CL-4B column, (1.5 x 12 cm) pre-equilibrated with 2.0 M ammonium acetate buffer, pH 6.7. The column was eluted with a step-wise decrease of ammonium acetate molarity to 0.05 M, followed by a step-wise increase to 6 N guanidine HCl in the 0.05 M ammonium acetate elution buffer. A flow rate of 50 ml/hr was employed and fractions of 70 drops/tube were collected. After removal of salts by dialysis the fractions were tested for biological activities.

Amino acid analysis

50 µg duplicate samples of PXI and PXII, from reverse phase HPLC, were dissolved in 0.4 ml of 6 N HCl and hydrolyzed for 24 hr and 48 hr at 110°C in vacuum sealed ampules. The amino acid composition was determined on an automatic Dionex 500 or Beckman Model 121M amino acid analyzer. Corrections for losses of labile amino acids were routinely made.

Sequence determination

The chromatofocusing derived pardaxins were analyzed by automated Edman degradation in a Beckman sequencer 890 C. The degradation was essentially that described by Hunkapiller and Hood (10) using 0.25 M Quadrol, 5% phenylisothiocyanate with single cleavage. Conversion of the phenylthiazo derivatives

was achieved with 1 N HCl for 10 min at 80°C. The phenylthiohydantoin (PTH) derivatives were identified by high pressure liquid chromatography on a Spectra Physics 8000 instrument equipped with Zorbax ODS 5 micron (250 x 4.6 mm) column; a flow rate of 1.2 ml/min was used for the mobile phase which consisted of an increasing gradient of methanol from 15% to 43% in 0.1 M sodium acetate buffer, pH 4.5, followed by an isocratic elution step (10 min) of 43% methanol and finally a step of 90% methanol (11). Finally, the PTH-amino derivatives were back hydrolized with hydriodic acid 47% at 130°C for 19 hr in vacuum sealed tubes (12) followed by amino acid analysis on a Dionex 500 instrument.

Sialic acid analysis

Sialic acid was determined by a slight modification of the procedure of Hahn, et al. (13).

Carbohydrate analysis

The carbohydrate content of secretion and toxins was determined using the phenol sulfuric acid reaction described by Dubois, et al. (14).

Sterol isolation and derivatization

Four hundred mg of P. marmoratus secretion and samples of 30 mg of PXI and PXII after chromatofocusing were suspended in 0.1 M ammonia and diluted with 10 volumes of acetone. The precipitate was removed by centrifugation (10,000 g). The acetone supernatant was partitioned between ethyl acetate and water as recently described (8). After evaporation of the ethyl acetate the residue was examined for toxicity and pore formation activity. Ethyl acetate extracts were submitted for methanolysis in 5% HCl in methanol, for 6 hours at 65°C.

Mass spectrometry

Samples of ethyl acetate extract, obtained from 30 mg protein of the toxic secretion and the purified toxins, after acid methanolysis were subjected to mass spectral analysis. Electron impact mass spectra (70 eV) were recorded

with a Varian MAT 731 mass spectrometer. Source temperature was in the range of 250 to 300°C and probe temperature was 50-250°C according to the volatility of the sample.

Polyacrylamide gel electrophoresis

Slab, SDS polyacrylamide gel electrophoresis was carried out according to Laemmli (15). Lyophilized pardaxins samples were dissolved in sample buffer (10% glycerol, 5% SDS, 1 mM dithiothreitol 5% β-mercaptoethancl in 0.025 M Tris-HCl, pH 6.8) and immediately boiled for 30-60 minutes. Discontinuous SDS gel electrophoresis in the presence of urea was performed using the experimental conditions described by Anderson et al. (16). Proteins were fixed for 4 hr in 40% methanol, 10% acetic acid, 1% trichloroacetic acid and stained with silver stain using the Bio Rad kit.

Crosslinking of Pardaxin I

Chemical crosslinking of pardaxin I dissolved in water, butanol or different detergents was carried out with disuccinimidyl suberimidate (DSS) according to Pilch and Czech (46). 50 µg of Pardaxin I were incubated with 1 mg/ml DSS (diluted in dimethyl sulfoxide) for 10 hr at room temperature. The reaction was stopped by adding 1/2 volume of twice concentrated sample buffer and heating the samples for 30 min at 100°C. 50 µl aliquots of each sample were analyzed by SDS-gel electrophoresis.

Protein determination

The protein content was determined according to its absorbance at 280 nm and according to Lowry, et al. (17) using bovine serum albumin as standard.

Assay of fish toxicity

Was determined as previously described (3) using Gambusia affinis 1-2 gr body weight.

Hemolytic activity

The hemolytic activity was determined in a suspension of washed human erythrocytes according to Primor and Zlotkin (3). One unit of hemolytic activity (HU_{50}) corresponds to the amount of compound (µg Lowry protein) producing 50% of the hemolysis induced by distilled water towards 5×10^6 cells/ml.

Cytotoxic activity

Exclusion method. The cytotoxicity was determined on a suspension of lymphoma malignant cells by the trypan blue exclusion method as described (18). One cytotoxic unit corresponded to the amount of test substance (µg Lowry protein/2×10^6 cell suspension/ml) which is cytotoxic to 50% of the cells following 4 hr incubation.

^{51}Cr release assay. Human erythroblastoid cells were preloaded with $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.). All assays were carried out in triplicate in conical wells of microtiter plates in a total volume of 0.2 ml of 0.25 M sucrose in 0.02 M Tris containing 10% fetal bovine serum, 1 mM $MgCl_2$, pH 7.4. Each well received 1×10^5 cells and the toxins were added at the appropriate concentrations. The microtiter plates were incubated for 1 hr at 37° in a humidified, 5% Co_2 incubator. To terminate the assay, plates were centrifuged for 5 min at 500 g and 100 µl of supernatant was removed from each well for gamma counting. Lysis in all experiments was calculated according to the following formula:

$$\frac{\text{experimental cpm} - \text{control cpm}}{\text{maximum cpm} - \text{control cpm}} \times 100$$

The maximum release is calculated by adding 0.5% Triton X-100 to an aliquot of cells. Control release is defined as the spontaneous cpm released from the cells incubated with medium alone. This value usually doesn't exceed 5-10% of maximum release.

Liposomes. Unilamellar vesicles were prepared by the octyl glucoside dialysis method of Mimms, et al. (22) and further detailed in Loew, et al. (7). This results in a stock suspension of large unilamellar vesicles in a buffer containing 50 mM K_2SO_4, 0.5 mM EDTA and 10 mM HEPES titrated to pH 7.2. Na^+ and choline buffers are identical except that Na_2SO_4 or choline sulfate replaced K_2SO_4. These liposomes are subjected to phosphate analysis, internal volume determination, and electron microscopy in order to derive the concentration of liposomes and their size distribution. For isotope uptake experiments, multilamellar liposomes were prepared as described by Garty, et al. (21). 40 mg acetone extracted soybean lecithin were suspended in 1 ml buffer containing 25 mM immidazole pH 7.0, 5 mM EDTA and 150 mM KCl. The suspension was vortexed for 10 min and sonicated in a bath sonicator until it became translucent.

Pore formation assays

Diffusion potential. A valinomycin mediated diffusion potential was produced across the membrane of K^+ containing liposomes which have been diluted into K^+ free buffers and monitored by the fluorescence of the voltage sensitive dye 3-3-diethylthiodicarbocyanine iodide. The fluorometric detection of the pore mediated collapse of the liposome diffusion potential was measured as previously described (7,19).

Rubidium assay. The assay was performed essentially as described (21) with slight modifications. 200 µl of multilamellar liposome mixture were passed through a 1 ml Dowex-50 column, eluted with 150 mM Tris Cl, 25 mM immidazole (pH 7.0) and 5 mM EDTA. This step exchanges the external KCl with Tris-HCl without changing the total volume. The eluted vesicles were diluted two-fold in the same elution buffer to which gramicidin or pardaxin had been added. The assay was initiated later at room temperature by adding 5 uCi ^{86}Rb at different times in order to separate the liposomes from the medium, 100 µl

aliquotes were applied on Dowex (0.8 x 5 cm volumns) eluted with 1.5 ml of 175 mM ice-cold sucrose (21). The amount of ^{86}Rb trapped within the vesicles was measured by a scintillation counter. The ^{86}Rb content is expressed as a fraction of initial total radioactivity in the vesicle reaction medium (mean of two duplicate experiments).

Radiolabelling of pardaxin I

Pardaxin I, obtained from chromatofocusing, was iodinated by the Bolton-Hunter method (23) at pH 8.0 using 0.1 M phosphate-saline buffer, for 1 hr at 4°C. The reaction was stopped with 0.3 M glycine buffer, pH 8.0. Iodinated toxin was separated from unreacted reagents by gel filtration on 15 ml columns of Sephadex G-25 and eluted with 0.1 M phosphate-saline buffer containing 0.3% gelatin or ovalbumin. At the above pH, a specific activity of about 0.5 mCi/mg toxin was obtained. The eluted toxin was preserved at -20°C in phosphate-saline buffer, pH 7.4 and used in assays within 14 days following iodination. Every iodinated batch of toxin was tested for pore formation activity, and analyzed by SDS-polyacrylamide gel electrophoresis and chromatofocusing.

Binding assay

Liposomes made from different phospholipid compositions were prepared from soybean and pure synthetic phospholipids mixed at different ratios, using the sonicate-freeze thaw-sonicate method (20). The liposomes were isolated by gel filtration through a Sepharose CL-4B column (50 ml) eluted with saline phosphate buffer. 5 ul liposomes (0.05 mg lipid) were incubated for 30 min at room temperature with 0.7 x 10^6 cpm ^{125}I-labeled pardaxin I in 0.2 ml saline phosphate buffer, pH 7.4 containing 0.1% ovalbumin. The incubation was terminated by addition of 0.8 ml cold buffer, the mixtures applied to separate Sepharose CL-4B columns (10 ml gel) at a flow rate of 8 ml/hr and fractions of 0.3 ml/tube were collected. The radioactivity of each elution tube was counted.

Total radioactivity associated with the liposomal peak was measured and calculated as a percentage of total radioactive toxin submitted for the assay.

Electron micrographs of liposomes

A diluted sample of unilamellar liposomes was deposited on electron microscope grids coated with polyvinyl formal solution, and negatively stained with 1% uranyl acetate in water for 1 min. After drawing off the staining solution, the grid was allowed to dry at room temperature. The grids were examined with a Philips EM-410 electron microscope at different magnifications (30-150 K) and typical liposomal fields were photographed.

RESULTS

Purification of two pardaxins

Separation of fish secretion on Sephadex G-100 gel chromatography (Fig. 1B) preceeded by a desalting step by chromatography on Sephadex G-25 (Fig. 1A) resulted in fractionation of several components of different molecular weight. Fraction II (PX represents 80% of secretion proteins) is the main toxic and hemolytic fraction (about 80% of the original activies). During these chromatographic procedures the collection of fractions was set at a fixed number of drops per tube. We have consistently noticed a significant decrease in the volume of the collected active fractions. This effect was observed during all chromatographic steps on the different resins (Fig. 1-4). The active fraction PX obtained from Sephadex G-100 (Fig. 1B) was separated by anion exchange chromatography into two main toxic and hemolytic components which we assign pardaxin I (PXI) and pardaxin II (PXII) (Fig. 2). Each of the PXs obtained from QAE-Sephadex was further purified by two steps of chromatofocusing. A decreasing pH gradient from 6.5 to 5.0 resulted in elution from PXI of three

non-active components (80% of PX1, QAE-Sephadex) (Fig. 3A). PXI at this stage of purification is still partially contaminated by sterols. However, a re-chromatography of this fraction (PXI), after desalting, using a new chromato-focusing column and a modification of the pH gradient (from 6.4 to 3.0) re-sulted in almost final purification of the PXI. The toxin is eluted at pH 4.0 (Fig. 3B), a pH which is near to its isoelectric point (data not given). At this stage of purification the toxin is completely free of sterols (see next paragraph). An identical preparative chromatofocusing method has been used for purification of PXII derived from QAE-Sephadex chromatography (Fig. 4). A decreasing pH gradient of polybuffer from 7.0 to 3.2 resulted in elution of two non-active components (60% of PXII, QAE-Sephadex), PXII eluted at pH 3.7 (Fig. 4A). This toxin was also further purified by rechromatography under the same conditions of pH gradient (Fig. 4B). Final separation of chromatofocused PXI and PXII from contaminating peptides and residual ampholytes (Fig. 5, first peaks) has been achieved by reverse phase HPLC, resulting with a single, active component eluted with 45% acetonitrile. (PX-second peaks, Fig. 5). PXI and PXII represent 10% and 8% of fish secretion proteins, possessing 50-60% and 5% of fish secretion cytotoxicity and pore formation activity, respectively.

Chemical characteristics of PXI and PXII

Pardachirus secretion and the derived pardaxins, PXI and PXII, were tested for the presence of carbohydrates, sialic acids, lipids, and sterols after the appropriate extraction procedure. Lipids, carbohydrates and sialic acid could not be detected in 10 mg of PXI and II and sterols not in 30 mg. The mass spectroscopy of the extract of active fraction (PXC obtained from Sephadex G-25 (Fig. 1A) indicates presence of several sterols (Fig. 5, PXC) to the extent of about 2-3% of the protein content. A typical steroid indicative, metastable peak, for the transition 398 m/e - 18 (H_2O) → 380 m/e was detected. Steroid

aminoglycosides were found and characterized in the secretion of P. pavonicus and P. marmoratus (8,25). However, mass fragmentation spectra of extracts of pardaxins I and II do not show the presence of such sterols in 30 mg samples (Fig. 6, PXI and PXII). The secretion was found to contain about 0.08 mg galactose equivalents and 0.02 mg sialic acid, but no lipids, per 10 mg of secretion protein. Sialic acid could not be detected in 10 mg samples of PXI or PXII. Mass spectra of PXII do indicate the presence of some carbohydrate components (Fig. 6).

According to the amino acid composition (Table II), pardaxins I and II are composed of 35 and 31 amino acids, respectively. Both toxins are devoid of tyrosine, arginine and tryptophan, and rich in aspartic serine, glycine, glutamic and alanine. A clear similarity in amino acid composition is observed between pardaxins I and II and the proteous P1-3 toxins (Table II) recently isolated from another species of Pardachirus sole (45). The amino terminal sequence of pardaxins is presented in Table III. The major method of identification of the PTH-derivatives was high pressure liquid chromatography and it was confirmed by back hydrolysis and amino acid analysis. Both toxins have identical N-termini: NH_2-Gly-Phe-Phe (Table III), comprising mainly hydrophobic residues. The minor heterogeneity of pardaxins N-terminal (Table III) could reflect either a variability of the amino terminal sequence or most probably, the presence of minor peptides that co-purify with the toxins during the process of isolation from the fish secretion (Fig. 5, first peak).

Molecular weight and homogeneity of pardaxins

Using a discontinuous SDS polyacrylamide slab gel electrophoresis system (16) molecular weight values of 2.9-3.5 kD were obtained for PXI (Fig. 7A) and PXII (data not shown). However using Laemmli method (15) higher molecular

weight values of 3.5-4.3 kD were obtained for both toxins (Fig. 5 and 7B) most probably reflecting some abnormal SDS-pardaxins interactions (16). Difficulties in assigning precise molecular weight by electrophoresis of pardaxins after chromatofocusing or HPLC separations were found to be due to a process of aggregation. Pardaxins aggregates were not separated by 10 min boiling and probably represent an intrinsic, salient property of water soluble pardaxins, e.g., storage at 4°C longer than 2 hr led to aggregates (data not shown). However when pardaxin I and II were transfered directly from the lyophilized powder to the sample buffer and boiled for 30 min the above 3.5 kD molecular weight values were measured (Fig. 7). Crosslinking of pardaxin I in the presence of sodium cholate was carried out with the bifunctional reagent disuccinimidyl suberate. Upon SDS gel electrophoresis, pardaxins olygomers were observed (Fig. 7-B6), representing multiples of 3.0-3.5 kD molecular weight unit. The minimal molecular weight of pardaxins I and II given by amino acid analyses (Table II) are 3529 and 3488 for PXI and PXII, respectively.

The chromatographic mobilities on Sephadex G-75 gel filtration of PXI and PXII fractions from QAE-Sephadex (Fig. 2) indicate a molecular weight of about 15 kD (Fig. 8). Analytical ultracentrifugation in the same buffer (1 M ammonium acetate) resulted with maximal molecular weight of 15 kD and 16 kD for PXI and PXII, respectively (data not shown). Most probably these values represent a tetramer aggregate. These findings suggest that pardaxin monomers (~3.5 kD) self aggregates in aqueous buffers and interact with detergent micelles, probably to yield stable, compact configurations. This possibility deserves further investigation.

Pardaxins homogeneity is indicated by: 1) the symmetry of the HPLC peaks (Fig. 5); b) single bands upon SDS gel electrophoresis (Fig. 5 and 7: A-1; B-5,7); c) high homogeneity of the N-terminal (data not shown); d) the symmetry of the diffusion peaks upon analytical ultracentrifugation (data not shown).

Hydrophobic characteristics of pardaxins

The high hydrophobicity of these toxins is indicated by several independent methods: (a) Presence of pardaxins in aqueous solution resulted in a significant decrease of the solution drop volume (Fig. 1-4,9); (b) Behavior of pardaxins on Phenyl-Sepharose hydrophobic chromatography (Fig. 9). Only 6M guanidine HCl could elute the toxins from the resin. In addition, the larger elution volume required for PXI and its lower recovery from the column, suggest that PXI may be more hydrophobic than PXII. (c) As calculated from the amino acid analyses (Table II), PXI and PXII contains an average hydrophobicity of 51.5% and 54.8%, respectively indicating a higher proportion of hydrophobic amino acid residues over hydrophilic in these toxins. (d) The NH_2-terminal of pardaxins (Table III) represent a compact hydrophobic segment composed of nine hydrophobic amino acid residues. Comparing the N-terminal amino acid sequence of PXI derived from chromatofocusing with the sequence of pardaxin derived from Sephadex G-100 previously reported (Table III), substantial agreement is noted but a discrepancy exist at residue 8. The partial blockage of PXII terminal starting at step 3 (Table III) and the presence of carbohydrate residues in the extract of this toxin as indicated by mass fragmentation spectra (Fig. 6) is an indication of some type of association between this proteous toxin and carbohydrate-like components, reminiscent of the purification process.

Radioactive labeling of PXI and binding to liposomes

The Bolton-Hunter reagent (23) was employed for iodination of PXI. In order to determine optimal conditions, the toxin was labeled at different pHs. The maximum specific radioactivity was obtained at pH 8.0 resulting in 0.5 mCi/mg protein. The radiolabeled toxin was checked for pore formation activity in liposomes and was found to preserve all of its activity compared to the native PXI (Fig. 11). Iodinated toxin shows a single protein peak on Sepharose

CL-4B (Fig. 10) gel chromatography. Chromatofocusing of iodinated toxin under identical conditions described for the native toxin resulted in a main peak (90% of radioactivity) eluted at pH 3.8. Figure 10 shows a typical elution profile from Sepharose CL-4B column of ^{125}I-labeled PXI and liposomes. The degree of toxin binding was not significantly dependent on the lipid composition; the following lipids were surveyed: (a) phosphatidylcholine; (b) phosphatidylcholine-phosphatidylinositol (10:1); (c) phosphatidylcholine-phosphatidylserine (10:1); (d) phosphatidylcholine-cholesterol (4:1); (f) soybean phospholipid. For all of them a binding ratio of about 3×10^4 moles lipids per 1 mole PXI, was estimated.

Fluorometic analysis of pore formation in unilamellar liposomes by pardaxins

A valinomycin mediated diffusion potential is produced across the membrane of K^+ containing liposomes (which have been diluted into K^+ free buffer) and monitored by the fluorescence of a voltage-sensitive dye (7,19). As indicated in Fig. 11, addition of 7.5×10^{-12} M gramicidin into the cuvete, causes an immediate collapse of the membrane potential on a fraction of the liposome population as expressed by the recorded recovery in fluorescence. (A subsequent slow reduction in fluorescence is often observed and reflects, we believe, reabsorption of released dye into the large pool of vesicles which are still polarized.) PXI at a concentration 5×10^{-10} M produced a similar fluorescence recovery. PXII elicited a similar effect at ten-fold higher concentrations (Fig. 11). These effects are comparable with those of gramicidin with respect to the rate of fluorescence recovery, as well. Pardaxins show an effect of partial recovery indicating that their action is not mediated by detergent lysis of the liposomes but through a pore. The same behavior was observed in sodium, choline and sucrose solutions, indicating a non-selective pore. The pore forming activity as a function of toxin concentration was

measured for several cytolysins. The molar concentration of pore can be estimated by using gramicidin as a standard or directly from the product of the fractional fluorescence recovery and the liposome concentration (7). Both methods give comparable results and allow the estimation of pore forming activity listed in Table V. For PXI it has been shown (7) that the concentration dependence of the pore forming activity can be treated via Poisson statistics and fits a model in which the pore is composed of between 4 and 12 monomers. Comparing these effective doses it is evident that PXI posses the highest specific pore formation activity. Electron microscope examination of unilamellar liposomes incubated for 30 min with PXI or PXII at 10^{-9} - 10^{-10} M do not reveal any significant lysis, swelling or other morphological changes. However, at about 10^{-7} M an extensive lysis of the large and disruption or aggregation of the small liposomes were observed (Fig. 12).

^{86}Rb flux through gramicidin and PXI induced pores in multilamellar liposomes

The experiments were performed using the method of Garty, et al. (21), which allows detection of ion selective pores in liposomes by measuring uptake of the radioactive ion against a large chemical gradient of the same unlabeled ion. Under our experimental conditions, as a consequence of K gradient an electrical diffusion potential is spontaneously formed, the magnitude being determined by the relative permeabilities of K, Cl and Tris through the liposomal membrane. Only in those vesicles containing pardaxin pores, is the K^+ permeability greater than Cl and Tris permeabilities, thus resulting in the formation of a K^+ diffusion potential. ^{86}Rb added will tend to equilibrate with the membrane potential, therefore accumulating selectively into the vesicles containing the pardaxin pores. Table IV shows the time course of ^{86}Rb uptake into soybean phospholipid multilamellar liposomes containing 150 mM KCl in the presence and absence of gramicidin and PXI and a large potassium gradient. Gramicidin at 10^{-7} M within 1 min of incubation produced 15.4% of ^{86}Rb

uptake. PXI (10^{-7} M) however at 1 min had no measurable effect. Nevertheless, it produced 5.5% of ^{86}Rb uptake after 30 min incubation (Table IV). Comparing to gramicidin, under identical conditions, PXI show a slow time course of ^{86}Rb uptake. It is possible that this slow uptake is a reflection of the organization process of pardaxin I molecules to form an active channel or is the result of some complex behaviour of the channel resulting with both conductance of ^{86}Rb and dissipation of the gradient. However, in the absence of a K gradient or toxins the uptake of ^{86}Rb was found insignificant.

The cytotoxic effect of pardaxins

The dose response curves of cytotoxic effects of PXI and PXII on mouse lymphoma cells, as determined by the trypan blue exclusion method are presented in Fig. 13. PXI (CU_{50}: 1.3×10^{-8} M) was found to be about five times more cytotoxic than PXII (CU_{50}: 6.5×10^{-8} M). A similar ratio was found on human erythroblastoid cells, using the ^{51}Cr release assay (PXI-CU_{50}: 0.7×10^{-9} M); PXII-CU_{50}: 0.5×10^{-8} M). The cytotoxic and pore forming effect of PXs and other venom cytolysins were compared on human red blood cells and mouse lymphoma (Table V). Melittin displays the highest cytotoxic activity but produces 500 times less pores than PXI (Table V). These data suggest that cytotoxicity is not necessarily directly associated with membrane pore formation.

Discussion

In the present study we developed a new chromatographic procedure for the purification of the proteaceous, cytolytic and pore forming factors named pardaxin I and pardaxin II from the gland secretion of the Moses sole *P. marmoratus*. Pardaxin I was found to be completely free of steroid aminoglycosides present in the Moses sole secretion and which coelutes with the

proteous toxins during the different chromatographic steps. The chromatofocusing procedure employed for toxin purification resulted in the complete resolution of these two types of components. The reverse phase HPLC purification step efficiently separate the tight interaction between pardaxins and residual ampholytes and peptide contaminants. Our results offer conclusive evidence that both PXI and PXII retain significant biological activity in the absence of the non-proteous components of the secretion. Pardaxins are acidic amphipathic polypeptides composed of 31-35 amino acids. Their amino acid composition indicates about 1:1 ratio between apolar-hydrophobic and polar-hydrophilic residues (Table II) and their N-terminal sequence show the presence of an exceptionally high hydrophobic segment, properties found in membrane and surface proteins (37).

Pardaxins I and II spontaneously aggregate in aqueous buffers and upon crosslinking in water or detergents. Different types of oligomeric forms were formed (Fig. 7 and unpublished experiments) resembling bee venom melittin (47). Although we don't know what causes the appearances of these aggregates it may be possible that its related to pardaxins structure or to the removal of the above sterol components during chromatography. It may be that the above toxic proteins and sterols are associated in the Moses sole secretion in a surfactant complex promoting its stability in an aqueous medium in analogy to the structural organization of serum lipoprotein complexes (38) or lung surfactant lipid-protein complexes (39). This hypotheses deserve an examination using reconstitution experiments with the purified pardaxins and the isolated or synthetic sterols.

Previous studies have shown that the Pardachirus secretion contains factor(s) capable of increasing the electrical conductance in lipid bilayers and a mechanism of channel formation was suggested (6). We prepared radiolabeled PXI

to assess its binding to liposomes. We also utilized two liposomal systems to detect the pore activity of PXI and PXII. The recovery of the valinomycin-induced decrease of fluorescence produced by these toxins provides a sensitive assay (10^{-10} M - 10^{-9} M) which was also used to assess the pore activity of the radiolabeled PXI. The pore activity of PXI and PXII was expressed within 1 min. This effect, however, was observed in the presence and the absence of Na^+, SO_4^{2-} and sucrose indicating lack of specificity in transporting these solutes (Fig. 10). In contrast, using the pore activity assay based on the differential transport to Cl^- and Rb^+, pore activity of a substance could only be detected if it is characterized by a non-equal permeability to anions and cations (21). The accumulation of ^{86}Rb in liposomes treated with PXI could be achieved only if more K^+ than Cl^- ions were transported from the liposomes to the medium. Here, PXI activity is characterized by a long time-course of about 10-15 min. We do not know the biological significance of a fast-nonspecific and slow-cation specific pores. But it may be a result of the much greater sensitivity of the fluorescence assay. In a planar bilayer, it was shown previously that the pore activity of the compound(s) present in the secretion occurred only when the potential is made positive with respect to the trans side of the membrane (6). It is, therefore, also tempting to attribute the fast formation of pore activity to the potential difference induced by valinomycin. It is possible that potential effect is to align pardaxin I monomers in the membrane so that aggregation is facilitated or alternatively to promote their insertion into the membrane which most probably occurs spontaneously due to the interaction with hydrophobic sequence segments, like the NH_2-terminal. In both tested liposomal systems, PXI and PXII differ from gramicidin which clearly transports more cations over anions and reaches maximum effect within 1 min (Fig. 11 and Table IV). Contrary to lipid perturbation, pardaxin I pore formation in both liposomes and bilayers (6) fulfills

several criteria: a)the depolarization or the conductance (6) reached steady state upon each addition of toxin (Fig. 14); b) both unilamellar liposome depolarization (Figure 14 and Ref. 7) and black lipid membrane conductance (6) show concentration dependencies which indicate aggregation numbers of 4-6 monomers/pore, probably forming a conductivity pathway of a barrel stave type (40). Although the structural details of the pardaxin pore require further elucidation, it is tempting to correlate this aggregation number with one of the oligomeric forms of pardaxin I detected in aqueous solutions. The observation that small liposomes aggregate after pardaxin I treatment (Fig. 12) also suggests some pardaxin mediated vesicular fusion most probably by promoting membrane contact as expected from a phospholipid binding protein (41).

It appears that pardaxin I exert its cytotoxic effects (Table 1) by at least two mechanisms depending on concentration and time of exposure. At low concentrations ($<10^{-7}$M) most probably it permeabilize cell membrane to cations, triggering cell death by a pathway shared in common with a variety of other cytolysins (42,48). At higher concentration (10^{-7} - 10^{-4}) most probably pardaxins act like other typical surface proteins (43) or detergents, causing cell destruction by rapid dissolution of cell membranes into mixed micelles. Although, it is quite conceivable that the same mechanism of pore formation underlies pardaxin toxicity or repellancy to fish, we cannot exclude the existance of specific receptors on the luminal (external) side of the gills, which have been found to be the target organ of Moses sole secretion (44). The molecular mechanisms of pardaxin interaction with artificial and biological membranes is under investigation. Nevertheless, the present study shows that the polypeptide toxic principle component of the flat fish secretion(pardaxin I) was obtained free of steroid aminoglycosides, display unique hydrophobic and amphipathic properties and has the capability of binding to liposomes with the formation of ion permeable pores.

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Legends to Figures

Fig. 1. Gel filtration column chromatography of P. marmoratus secretion on Sephadex G-25 (A) and Sephadex G-100 (B). A: 0.4 g of lyophilized secretion was applied on 3 x 50 cm column. B: 0.2 g of the active material obtained from Sephadex G-25 was applied on 2.5 x 100 cm column. Elution buffer consists of ammonium acetate 0.1 M, pH 5.0. The absorbance was monitored at 280 nm (---). The elution was carried out at a constant rate of 400 ml/h for Sephadex G-25 and 30 ml/h for Sephadex G-100 and the collection of the eluant was set for 150 drops per tube. The broken line represents the volume of the collected elution buffer per tube. The double headed arrow indicates the location of toxic activity.

Fig. 2. Column chromatography of the active fraction on QAE-Sephadex. 0.04 g of the active fraction obtained from Sephadex G-100 (double arrow area in Fig. 1A) were applied on 1.5 x 60 cm column equilibrated with ammonium acetate 0.05 M pH 6.7. Elution was carried out with increased concentrations of equilibration buffer at 50 ml/h and 150 drops per tube. The absorbance was monitored at 280 nm (●-●). The broken line represents the volume of the collected elution buffer per tube. The double arrow indicates the location of activity of toxicity in fish and cytotoxicity.

Fig. 3. Purification of PXI on chromatofocusing chromatography. A: 0.150 g of the first active fraction (PXI) obtained from QAE-Sephadex (Fig. 2) were applied on a column (1.5 x 15 cm) of PBE-94 chromatofocusing gel equilibrated with immidazole buffer 0.025 M, pH 6.4. B: 0.05 g of the PXI obtained from Fig. 3A were further rechromatographed. The elution was first carried out using polybuffer 74 (0.0093) mmole/pH unit per ml) pH 5.0 (for A) and pH 3.3 (for B), then the elution continued in the presence of ammonium acetate (1 M, pH 6.8) and guanidine (6N, pH 7.5), at 40 ml/h. The collection of the eluant was set for 130 drops per tube and 110 drops per tube for A and B,

respectively. The absorbance was monitored at 280 nM (●-●), the pH (Δ--Δ) and the volume (O-O) was measured in each tube. The double arrow indicates the collected active fraction.

Fig. 4. Purification of PXII by chromatofocusing chromatography. A: 0.015 g of the second active fraction (PXII) obtained from QAE-Sephadex (Fig. 2) was applied on a column (1.5 x 15 cm) of PBE-94 chromatofocusing gel equilibrated with immidazole buffer 0.025 M, pH 7.3. B: 0.05 g of the PXII obtained from Fig. 4A were further rechromatographed. The elution was first carried out using polybuffer 74 (0.0093) mmole/pH unit per ml) pH 3.3, then the elution continued in the presence of ammonium acetate (1 M, pH 6.8) and guanidine (6N, pH 7.5), at 40 ml/h. Additional details as presented in the legends for Fig. 3.

Fig. 5. Reverse phase HPLC fractionation and SDS polyacrilamide gel electrophoresis of pardaxins. Pardaxin I (A, 200 µg) and Pardaxin II (B, 300 µg) derived from chromatofocusing (Fig. 3B and 4B) were eluted on Spherisorb ODS 2 (3 um) with two linear gradients of acetonitrile in 1% trifluoroacetic acid: a) from 15% (at zero time) to 25% (at 20 min); b) from 25% (at 20 min) to 85% (at 50 min). Flow rate was 0.9 ml/min; temperature was ambient. Compositions for PXI and PXII peaks are given in Table II. Right panels, SDS-gel electrophoresis in 15% polyacrilamide of HPLC fractions (10 µg) (from left to right): first line: first peak eluted at 2 min; second line: shoulders of pardaxins, eluted at 29 min; third line: pardaxins eluted at 32 min; fourth line: aprotinin-marker (arrow, 6.5 kD).

Fig. 6. Mass spectra of ethyl acetate extractable compounds of P. marmoratus secretion (PMC), PXI, and PXII. 0.03 g of each substance were submitted for methanolysis procedure. The star (*) indicates that the peak is out of scale. Note that PXI and PXII are devoid of peaks in the range of m/e 300-410.

Fig. 7. Molecular weight determination of native and crosslinked pardaxin I by different conditions of sodium dodecyl sulfate polyacrilamide gel electrophoresis. A- Discontinuous SDS polyacrylamide gel electrophoresis with 8 M urea of pardaxin I (Fig. 5A) (20 µg, line 1) and markers: aprotinin (6.5 kD, line 2), lysozyme (14.3 kD, line 3) and Bio-Rad low molecular weight standards (line 4). B- Crosslinking of pardaxin I with disuccinimidyl suberate was performed as described under Experimental Procedures: native PXI in sodium sodium cholate (line 5); crosslinked PXI in sodium cholate; native PXI in 10% dimethyl sulfoxide (line 7); aprotinin (line 8); lysozyme (line 9) and Bio-Rad low molecular weight standards (line 10). Arrows indicate the mobility of markers and numbers indicate molecular weight (kD).

Fig. 8. Estimation of molecular weight of PXI and PXII be gel filtration on Sephadex G-75. For details see Experimental Procedures.

Fig. 9. Hydrophobic chromatography of PXI and PXII on Phenyl-Sepharose. A: 0.03 g of the first active fraction (PXI) B: 0.025 of the second active fraction (PXII) obtained from QAE-Sephadex (Fig. 2) were applied on a column (1.5 x 15 cm) of Phenyl-Sepharose equilibrated with ammonium acetate (2 M; pH 6.7). The elution was initiated by dilution of the equilibration buffer to 1.0 M and 0.05 M and by adding 6 M guanidine and was carried out at 50 ml/h and the eluant was collected at 70 drops per tube. The absorbance was monitored at 280 nm (●-●) and the volume was measured in each tube (---). Note that the pardaxins were eluted only in the presence of 6 M guanidine and that PXII was eluted before PXI.

Fig. 10. Chromatography on Sepharose CL-4B of iodinated PXI, large unilamellar liposomes and the incubation mixture of iodinated PXI with liposomes. Iodinated PXI (●-●), liposomes (○-○); 0.05 mg of liposomal lipids were incubated with 0.3 µg of ^{125}I-PXI (0.7 x 10^6 cpm) for 30 min at 20°C and then submitted for chromatography (▲--▲).

Fig. 11. Pore activity of PXI and PXII and gramicidin in large unilamellar liposomes. PXI and PXII were obtained from rechromatofocusing chromatography (Fig. 3C and Fig. 4B, respectively). The 4 traces are records from 4 separate experiments. The first trace shows a complete experiment and displays the partial recovery of fluorescence due to incorporation of gramicidin into a small fraction of the vesicle population. The next three traces were recorded after the diffusion potential had already been established at a gain identical to that established before adding gramicidin in the first trace. Toxins are introduced to the cuvette at the indicated arrows.

Fig. 12. Electron micrographs of negative stained liposomes treated with P. marmoratus secretion (PMC) and PXI. 1: A typical view of liposomal preparation, consists of large (b) and small (a) vesicles. 2: Liposomes (0.1 mg lipid) were incubated with PXI (1 µg) and with 5 µg PMC (insert). Note that PMC and PXI produced complete disappearance of large vesicles and aggregation of small particles (stars).

Fig. 13. The cytotoxic activity of P. marmoratus secretion (PMC), PXI and PXII on mouse lymphoma cells. The reaction mixture contained 3×10^6 cells and different doses of toxins. Following incubation, the % of survival cells was determined by the trypan blue exclution method. Insert: A-control cells, B-cells incubated with pardaxin I, 25 µg/ml.

TABLE I

Activities of Pardachirus marmoratus secretion and its derived toxin

Effects	Tested system	Dose range	Reference
Lethality	Fish	10^{-6} -10^{-8}	Primor et al (5,33)
	Mice and rats	10 mg/Kg body weight	Primor & Lazarovici (26)
Pharmacological	Inhibition or activation of short circuit current in fish epithelia	10^{-5} - 10^{-6}	Primor (27)
	Smooth muscle contraction in guinea pig ileum	3×10^{7}	Primor (28)
	Leakage of urea and sodium from gills	1.5×10^{-6}	Primor et al (29,35,44)
Enzymatic	Activation of acethylcholine esterase	7×10^{-5}	Primor & Lazarovici (26)
	Inhibition of $Na-K^{+}$ ATPase	10^{-8}	Primor et al (30)
Histopathology	Red blood cells (human)	2×10^{-6}	Primor & Zlotkin (3)
	Red blood cells (dog)	2×10^{-7}	Primor & Lazarovici (26)
	Virion distruction	$1.5\ 10^{-5}$	Pal et al (31,32)
	Frog neuromuscular junction	5×10^{-6}	Spira et al (34)
	Fish gill	6×10^{-8}	Primor et al (30)
	Shark rectal gland	6×10^{-5}	Primor et al (29)
Permeabilization of artificial lipid membranes	Release of 6-carboxy fluorescein	2×10^{-8}	Zlotkin & Barenholtz (24)
	Electrical conductance	10^{-8} - 10^{-10}	Moran et al (6)
	Depolarization of liposomes	7×10^{-10}	Loew et al (7)
Reduction of surface tension	Drops of saline	10^{-4} - 10^{-6}	Moran et al (6)

TABLE II
Amino acid composition of pardaxins I and II purified from

P. marmoratus secretion compared to P-toxins isolated from P. pavonicus

Amino acid	P. marmoratus (a		P. pavonicus (b		
	PXI	PXII	P1	P2	P3
Asp	4.22 (4)	3.79 (4)	0	0	0
Thr	1.23 (1)	0.92 (1)	1	1	1
Ser	3.78 (4)	3.75 (4)	7	7	7
Glu	2.98 (3)	2.71 (3)	3	2	3
Pro	0.76 (1)	1.05 (1)	2	2	2
Gly	6.33 (6)	4.23 (4)	3	4	3
Ala	2.82 (3)	2.83 (3)	3	3	3
Val	1.77 (2)	1.13 (1)	1	1	1
Ile	1.93 (2)	1.85 (2)	3	4	3
Leu	2.25 (2)	0.62 (1)	4	3	3
Tyr	0	0	0	0	0
Phe	2.27 (2)	1.80 (2)	3	3	4
His	0.95 (1)	0.94 (1)	0	0	0
Lys	1.91 (2)	1.23 (1)	2	2	2
Arg	0.33 (0)	0.23 (0)	0	0	0
Trp	0	0	0	0	0
Cys	1.32 (2)	1.51 (2)	0	0	0
Met	0	1.19 (1)	0	0	0
Total Residues	35	31	32	32	32
Molecular weight	3529	3188	3284	3212	3318
Total hydrophylic (3	48.5%	45.2%		44%	
Total hydrophobic (4	51.5%	54.8%		53-56%	

a. PXI and II derived from HPLC (Fig. 5).
b. Calculated from Thompson et al. (45).
c. The sum of Asp, Glu, Lys, Pro, Ser, Thr (36).
d. The sum of Ala, Cys, Gly, His, Ile, Leu, Met, Phe, Val (36).

TABLE III

Determination of the amino acid sequence of the NH_2-terminal of pardaxin I and pardaxin II

	Pardaxin I				Pardaxin II[c]	Proposed sequence of Pardaxin[d]
Cycle No.	Amino acid	Amount (nmol)	% Yield[a]	id[b]	Amino acid	Amino acid
1	Gly	18.7	---	A,B	Gly	Gly
2	Phe	13.5	100	A,B	Phe	Phe
3	Phe	10.7	79	A,B	Phe (Ile,Ala)	Phe
4	Ala	11.5	92	A,B	Phe	Ala
5	Leu	8.6	86	A,B	Pro	Leu
6	Ile	5.1	79	B	---	Ile
7	Pro	4.3	80	B	---	Pro
8	Gly	13.7	99	B	---	Lys
9	Ile	NQ	---	B	---	Ile
10	Glu	NQ	---	B	---	Ile

a) Percent repetitive yield based on 50 nmol of starting material, calculated from cycle 2. Most probably, the amount of Gly in cycle 1 and 8 is overestimated due to its presence in the sequenation buffers. NQ-Not quantitated.

b) i.d.-Methods of identification: A-High pressure liquid chromatography; B-Back hydrolysis.

c) Residues were determined qualitatively, mainly by back hydrolysis. Residues in brackets correspond to the minor components; A non proteous compound was released from step 3 which is interfering with the sequence determination producing a yellow derivative after the conversion step, soluble in ethyl acetate and methanol.

d) Taken from Zlotkin and Barenholz (24).

TABLE IV

^{86}Rb uptake in liposomes in the presence of gramicidin and pardaxin I[a]

Substance (M)	Time (Min) 1	5	15	20
Gramicidin (10^{-7})	15.40	13.30	9.50	6.10
Pardaxin I (10^{-9})	0.02	0.40	0.80	1.40
(10^{-7})	0.02	2.00	3.00	5.50
Control	0.02	0.05	0.20	0.40

a) The data (% of total radioactivity) represent mean of triplicate experiments in which liposomes were diluted in Tris-EDTA-immidazole buffer, pH 7.2 containing the different compounds. The uptake of control liposomes and the standard errors don't exceed 0.5% of total radioactivity.

TABLE V

Cell toxicity and pore formation specific activities of paradaxins, mellitin, cobra and scorpion cytotoxin[a]

Cytotoxin	Cytotoxicity erythrocytes[b]	Cytotoxicity lymphoma cells[c]	Pore forming[d] relative activity
Pardaxin I	40	50	5.00
Pardaxin II	2-8	5-10	0.60
Melittin	455	2000	0.01
Cobra	15	500	0.03
Scorpion	30	5-10	0.07

a) For definitions of cytotoxic and pore formation units see "Experimental Procedures."

b) Number of hemolytic units per mg protein, tested on washed human erythrocytes.

c) Number of cytotoxic units per mg protein obtained with mouse lymphoma cells.

d) Number of moles of pores multiplied by 10^{-10}, per mg protein.

Fig 1A

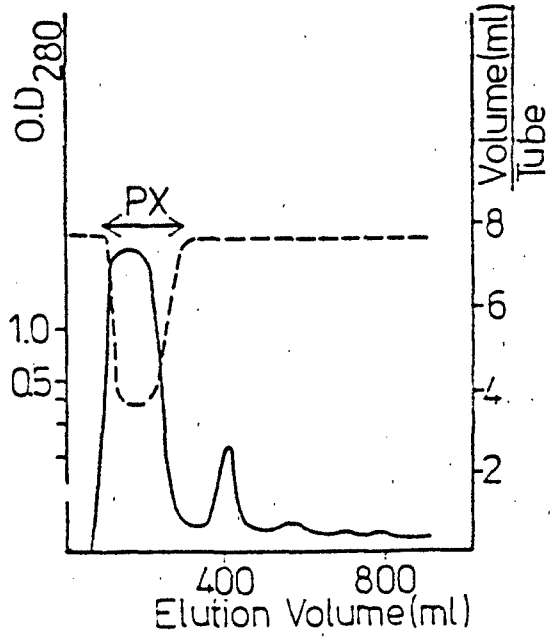


Fig 1B

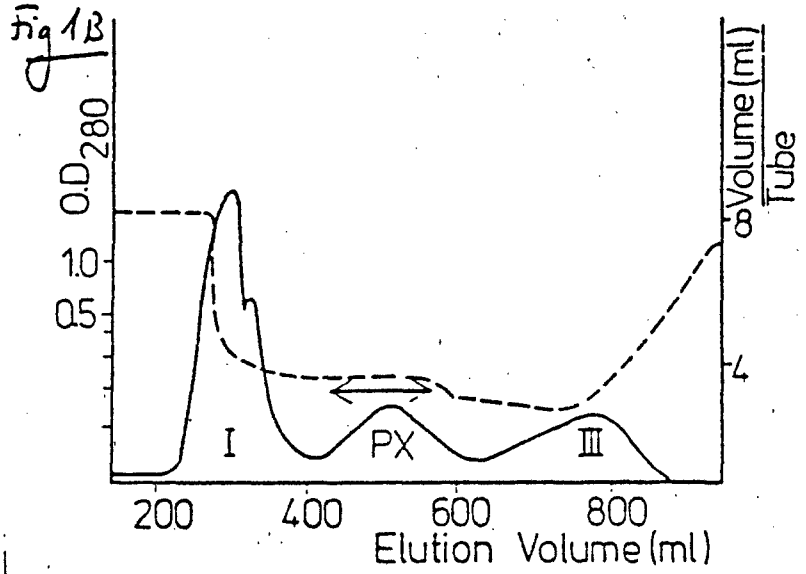


Fig 2

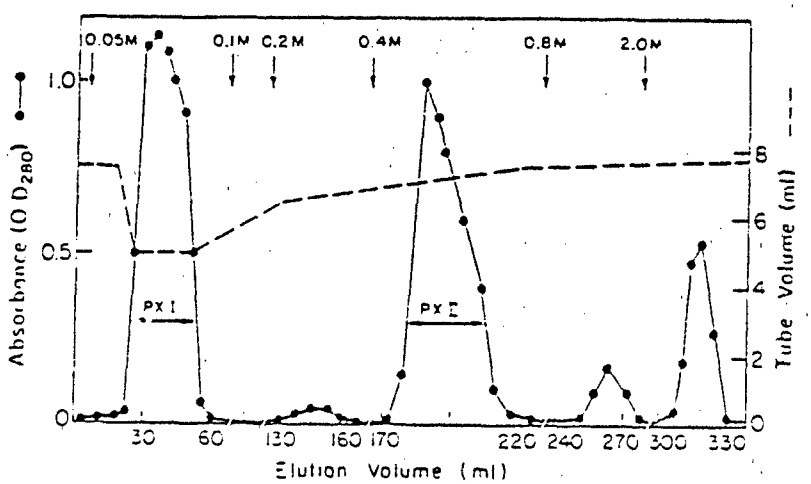


Fig 3

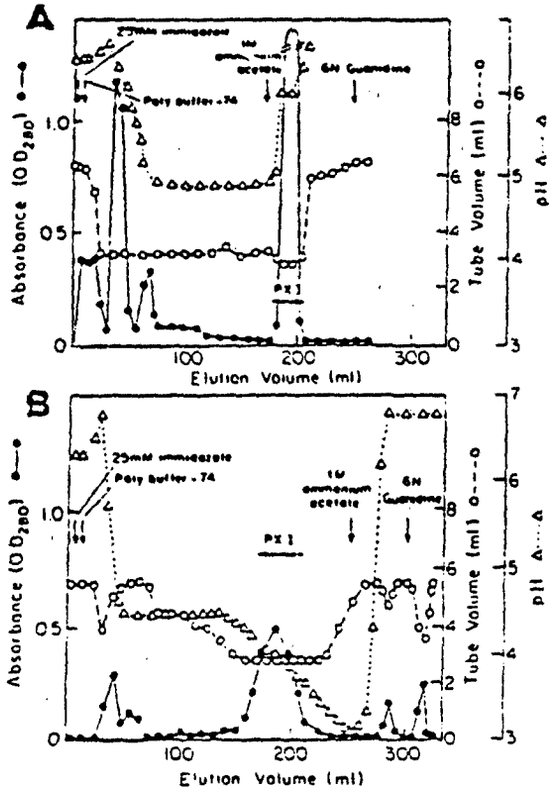


Fig 4

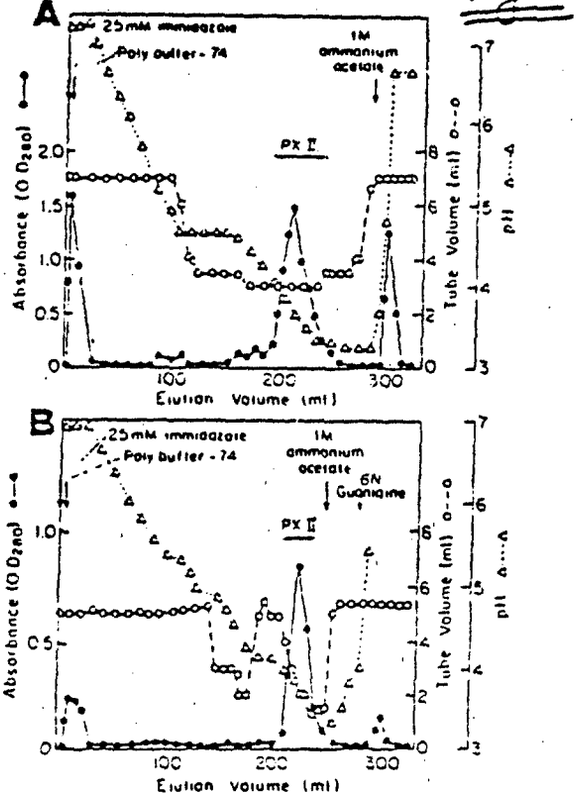


Fig 5

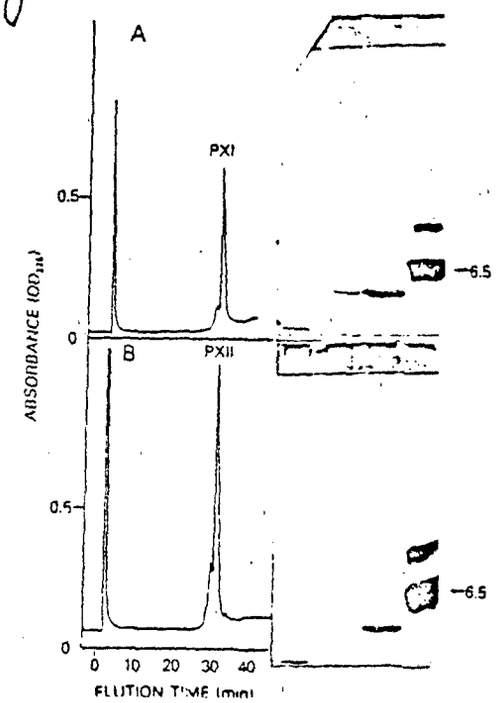


Fig 6

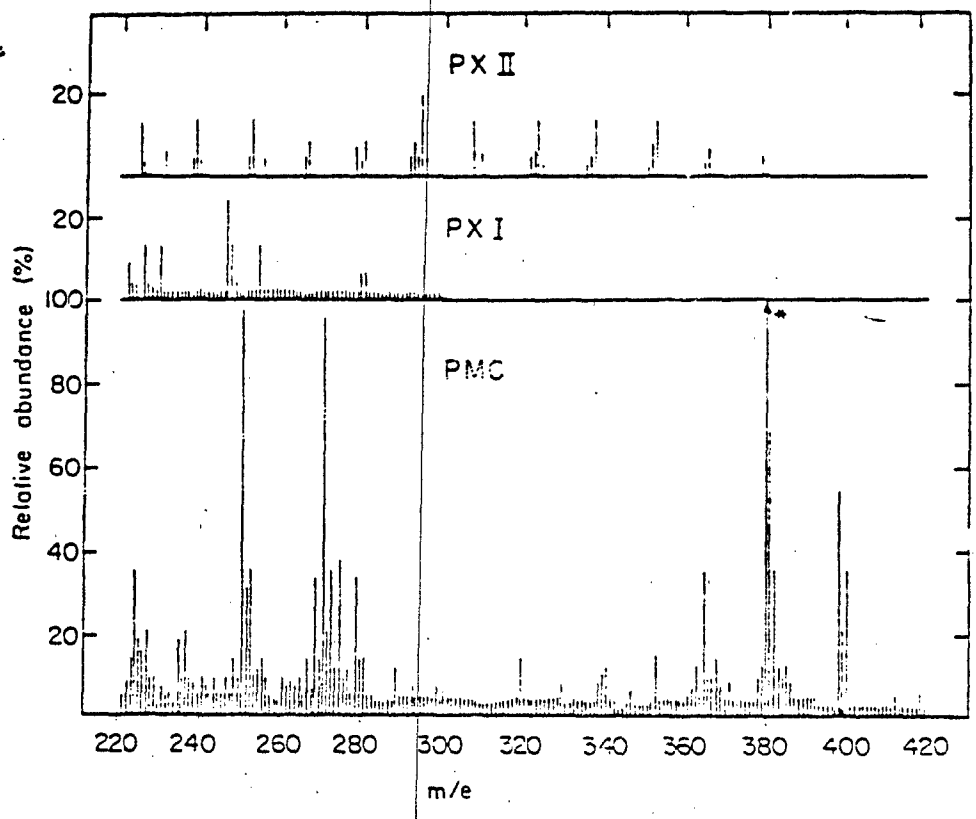

PX II
PX I
PMC
Relative abundance (%)
20
20
100
80
60
40
20
220 240 260 280 300 320 340 360 380 400 420
m/e

Fig 7

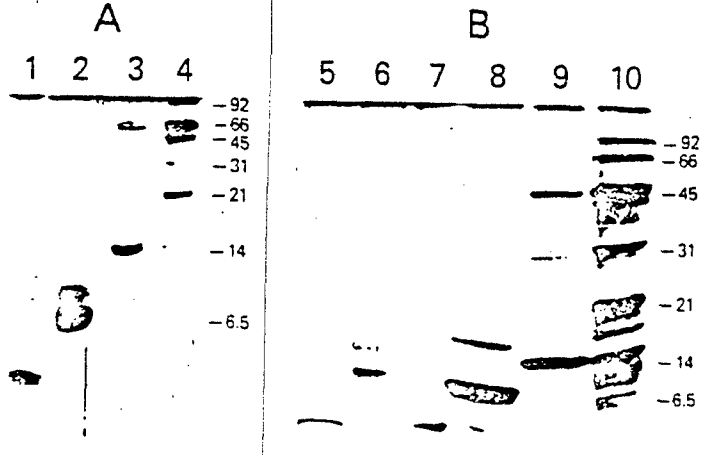

A
1 2 3 4
92
66
45
31
21
14
6.5
B
5 6 7 8 9 10
92
66
45
31
21
14
6.5

Fig 8

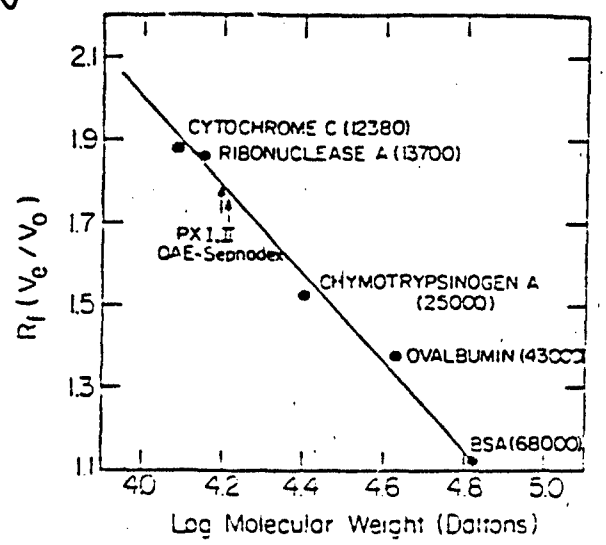


Fig 9

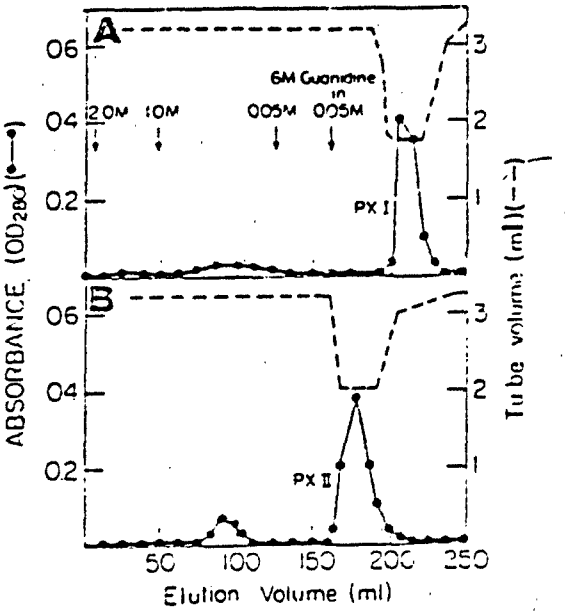


Fig 10

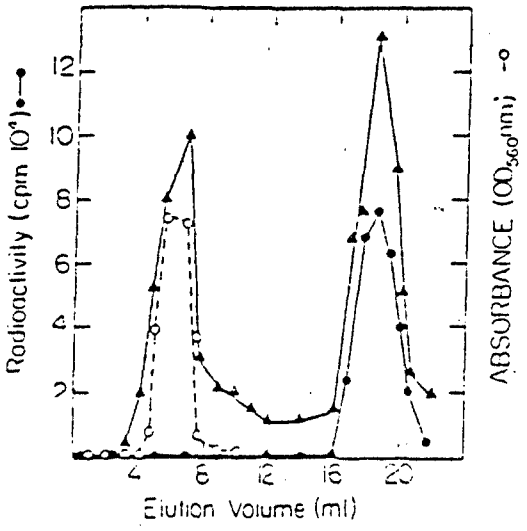


Fig 11

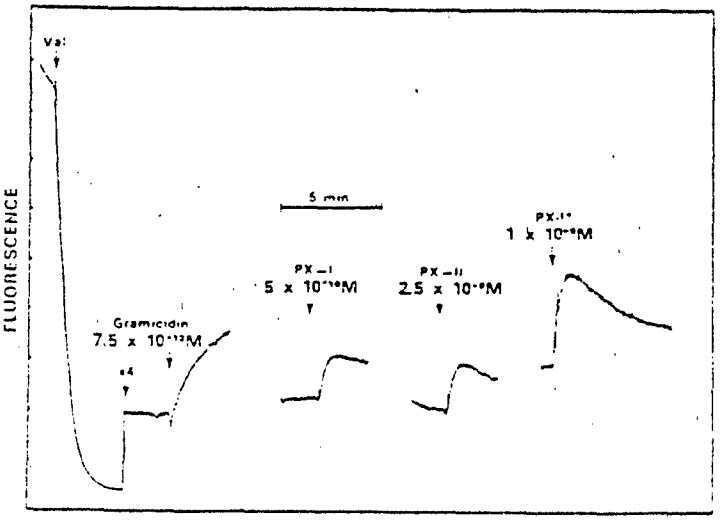


Fig 12

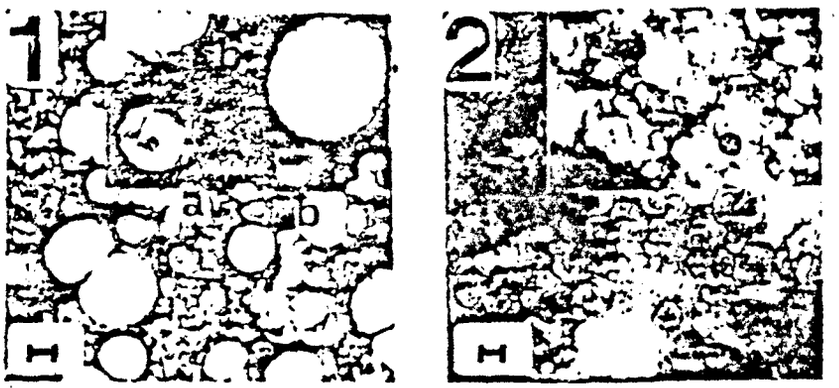
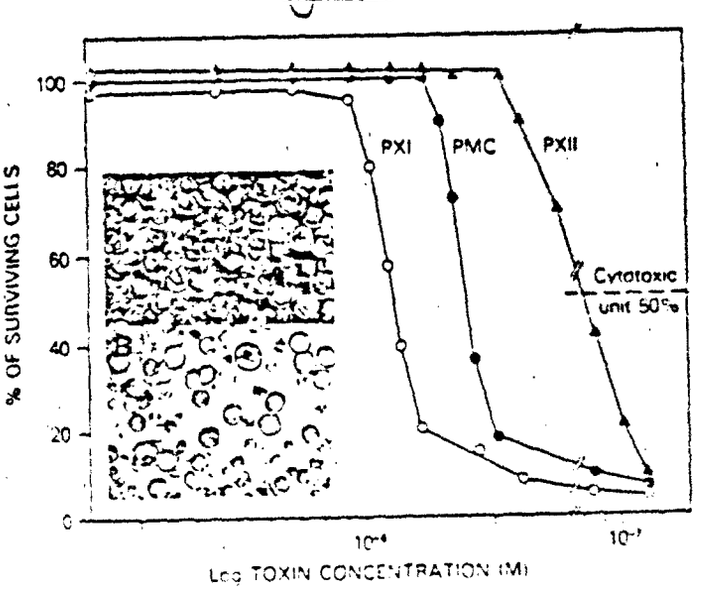

1
a
b
a
b
2

Fig 13


100
80
60
40
20
0
% OF SURVIVING CELLS
PXI
PMC
PXII
Cytotoxic unit 50%
Log TOXIN CONCENTRATION (M)