Isolation, characterization and synthesis of a novel pardaxin isoform

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Abstract We report the isolation of a novel pardaxin isoform from the toxic secretion of the Red Sea Moses sole (*Pardachirus marmoratus*). Mass spectrometrical analysis of the newly purified peptide revealed a different primary structure compared to the previously known pardaxin isoforms. Sequence analysis disclosed an aspartic acid residue instead of glycine at position 31 of the new isoform. According to the novel sequence, a synthetic Asp-31-peptide was compared with the native compound as well as with synthetic Gly-31-pardaxin. The isolated Asp-31-pardaxin isoform and its synthetic analog exhibited identical elution properties during reverse-phase HPLC, as well as similar dose-dependent lytic effects on human erythrocytes at a concentration of 10^{-6} to 10^{-5} M. The hemolytic activity of Asp-31-pardaxins was lower than that of Gly-31-pardaxin and no synergistic effect between these peptides was found. The additional negative charge introduced by Asp-31 is likely to affect the selectivity of pardaxin pores towards a variety of ions.

Key words: Cytolytic toxin; Pardaxin; *Pardachirus marmoratus*

1. Introduction

Sole fish of the genus *Pardachirus* produce an exocrine secretion from epithelial glands located at the base of the dorsal and anal fins. This secretion contains toxic components that play an important role in the defense of predatory fish [1]. Beside aminoglycosteroids [2], peptides, known as pardaxins [3], represent the major components of the secretion [4–6]. Pardaxins have been shown to form voltage-dependent ion channels [7–9] at low concentrations [5–14] in a variety of artificial membranes and cytolysis at high concentrations [5,11]. The membranal activity of pardaxins, binding, insertion and rearrangement for pore formation, is dependent on the α-helix content as well as the intramolecular interaction between the amino- and carboxy-terminal domains [10,15]. Thus, this peptide family is structurally and functionally related to other membrane-associated peptides, e.g. cecropins [16], magainins [17] and melittin [18]. To date, pardaxins have been isolated and characterized from the western Pacific Peacock sole, *P. pavoninus* (P1, P2 and P3) [4], and the Red Sea Moses sole, *P. marmoratus* [5,6]. We refer to the *P. marmoratus* pardaxin described by Shai et al. [6] as pardaxin P4. All known pardaxins contain one single peptide chain composed of 33 amino acids and show a high tendency of aggregation in aqueous solution [5,9]. Their sequences are very homologous, differing only at positions 5, 14 or 31 (Fig. 1). The membranal effects of pardaxins are rather sensitive to structural variations such as the net charge, attached side chains, the stereochemistry of α-carbons and chain truncation [10,11,19].

In the context of our work on the internalization of immunoglobulin conjugates [20] for the treatment of experimental lymphomas [21–23], we became interested in the interaction of pardaxin conjugates with plasma membranes. In this study, we report the isolation of a novel pardaxin isoform (P5) from *P. marmoratus*. The primary structure of the native peptide has been determined by amino acid sequencing, endoproteinase cleavage and mass spectrometric techniques. To verify the identity of the novel peptide, it was compared with the corresponding synthetic peptides by reverse-phase HPLC and a hemolysis assay.

2. Material and methods

2.1. Purification of pardaxin isoform

Twelve specimens of *P. marmoratus*, caught in the Gulf of Eilat, were supplied by the Interuniversity Marine Biology Institute of Eilat, Israel. Their secretions were collected manually by gentle hand pressure of the glands. The secretions of four fish were combined and the ensuing three batches lyophilized. The lyophilisates were extracted with water and the aqueous extracts desalted on Sephadex G25 (Pharmacia) and separated by gel filtration on Sephadex G100 as described [5] and finally lyophilized. The yield of dry material was about 20 mg per fish. For analytical purposes, further purification of 100 μg samples was carried out by reverse-phase HPLC on a Nucleosil C18 PPN column (Macherey and Nagel, 5 μm, 100 Å, 2.1×250 mm) using a linear gradient from 10–80% solvent B in 45 min (solvent A, 0.5% TFA; solvent B, 0.1% TFA in acetonitrile/water 4:1; flow rate, 0.2 ml; UV detection at 215 nm). Larger amounts of pardaxin fractions (20–40 mg) were purified using a Vydac C18 column (the Separations Group, 10 μm, 300 Å, 20×250 mm; flow rate, 8 ml/min; UV detection at 230 nm).

2.2. Mass spectrometry of pardaxin

Electrospray mass spectrometry (ES-MS) was performed on an API III triple stage quadrupole mass spectrometer (SCIEX, Perkin-Elmer) equipped with the high pressure collision cell. The articulated ion spray was operated at 5 kV. Mass spectra of pardaxins, which were dissolved in 50% acetonitrile containing 0.5% formic acid, were obtained by flow infusion at 5 μl/min and by scanning the first quadrupole from m/z 400 to 2400 at a sampling rate of 3 s/spectrum with one data point per 0.5 mass units. For sequence analysis by collision-induced dissociation (CID) [24], the first quadrupole was set to the

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Abbreviations: CID, collision-induced dissociation; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; RP, reverse-phase; TFA, trifluoroacetic acid

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m/z value of the single-charged peptide as determined before. Argon was used as collision gas at a collision energy of 20 eV. The second mass spectrometer was scanned from 10 m/z to the m/z value determined for the single-charged ion. At least 20 spectra were sampled. The peptide map obtained by endoproteinase treatment of pardaxin P5 was analyzed by coupling the liquid chromatography separation to the mass spectrometer (LC/MS) using a double syringe pump (140B, Perkin Elmer/ABI) operated at a flow rate of 20 μl/min. A variable UV detector (765, Perkin Elmer/ABI) equipped with a capillary U-flow cell (LC Packings) was used to detect UV absorbance at 220 nm. The eluent was infused into the ion spray without splitting. The C18 column used for LC/MS analysis was from YMC (ODS-AQ, 1×10 mm, 120 Å, 3 μm). A gradient with solvent A, 0.8% TFA in water, and B, 0.06% TFA in 80% acetonitrile, from 10% B in A to 70% B in A over 60 min was used for the separation.

2.3. Enzymatic cleavage and peptide sequencing of pardaxin isoform P5

Enzymatic degradation of pardaxin was performed by standard treatment with endoproteinase Lys-C (Boehringer Mannheim) at an enzyme to peptide ratio of 1:50 in 250 μl 0.1 M NH_4HCO_3 for 24 h at 37°C (pH 8.2). The solution was freeze-dried and dissolved in 50 μl 0.1% TFA. The resulting peptide mixture was separated by C18 LC/MS analysis (0.5×100 mm) using a double syringe pump at a flow rate of 5 μl/min. UV absorbance was determined at 215 nm. Fractions were collected automatically every minute. Peptides were directly applied to polybrene-coated glass fiber filters for amino-terminal sequencing which was performed on a 494 Procise sequencer (Perkin Elmer/ABI) using standard conditions as recommended by the manufacturer.

2.4. Synthesis of pardaxin peptides

Pardaxins P4 and P5 were synthesized on a 433A peptide synthesizer (Perkin Elmer/ABI) using standard Fmoc solid phase chemistry [25] on a preloaded Fmoc-Glu(OtBu)-TentaGel S PHB resin (Rapp Polymere). Fmoc amino acids were coupled in the presence of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU) and diisopropylethylamine (DIEA) in *N*-methylpyrrolidinone (NMP) for 30 min. Fmoc groups were cleaved with 20% piperidine in NMP. For deprotection and resin cleavage, the peptidyl resin was treated with a fresh mixture of TFA/ethanedithiol/water (94:3:3) for 2.5 h. After filtration, the peptides were precipitated by addition of cold tert-butylmethylether and lyophilized from 10% acetic acid. Thereafter, the crude peptides were purified by reverse-phase HPLC (Vydac C18, 10 μm, 300 Å, linear gradient from 10–100% solvent B in 60 min; solvent B, 0.1% TFA/water; solvent B, 0.1% TFA in acetonitrile/water 4:1; flow rate, 8 ml/min; UV detection at 230 nm). The purity and identity of pardaxins P4 and P5 were checked by analytical HPLC and sequence analysis. The molecular masses were determined by ES-MS and calculated from $[M+2H]^{2+}$: P4, 3323.9 (calc., 3323.9); P5, 3381.2 (calc., 3381.9).

2.5. Hemolysis assay

Venous blood (10 ml) was drawn from a healthy male adult person, mixed with 1 ml 10% sodium citrate buffer, and then centrifuged. The pellet obtained was washed three times with cold isotonic NaCl. For calibration, various numbers of erythrocytes (between 10^6 and 10^8) were suspended in 1 ml of 0.2% SDS. For the hemolysis assay of the pardaxins, 10^8 erythrocytes were incubated in 1 ml peptide solution at concentrations between 10^{-7} and 10^{-5} M at 25 or 37°C for 30 min. The erythrocyte suspensions were centrifuged, and the hemoglobin absorption of the supernatants was measured at 550 nm. All data points were obtained in quadruplicate; relative standard errors were maximum 15%. The concentration of peptides used for this assay was calculated according to their absolute peptide content as determined by amino acid analysis. Pardaxin mixtures were tested in a molar ratio.

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      1         10        20        30
P1  GFFALIPKIISSPLFKTLLSAVGSALSSSGEQE
P2  GFFALIPKIISSPIFKTLLSAVGSALSSSGGQE
P3  GFFAFIPKIISSPLFKTLLSAVGSALSSSGEQE
P4  GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE
P5  GFFALIPKIISSPLFKTLLSAVGSALSSSGDQE
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Fig. 1. Amino acid alignment of pardaxins. P1, P2 and P3 are from *P. pavoninus* [4], P4 [6] and P5 are from *P. marmoratus*. Variable residues are bold-faced.

3. Results and discussion

Two major compounds were separated by reverse-phase HPLC purification of the secretion extract of *P. marmoratus* (Fig. 2). Neither HPLC nor electrospray mass spectrometry (ES-MS) revealed any significant difference between the pardaxins purified from the three batches, each containing the pardaxin of four fish. Furthermore, ES-MS analysis revealed molecular weights of 4362.5 Da and 3382.6 Da for peaks 1 and 2, respectively, in contrast to the molecular weight of *P. marmoratus* pardaxin P4 (3323.9 Da, Fig. 1) [6]. The estimated yield per fish was about 5 mg of peak 1 and 10 mg of peak 2. Edman degradation of peak 1 did not generate any amino acid, suggesting that this compound is a peptide with a protected N-terminal amino function. Since no leucine, but methionine, histidine, tyrosine and aspartic acid/asparagine are contained in this peptide, as detected by amino acid analysis, its primary structure differs considerably from that of pardaxins. Sequence analysis of peak 2 resulted in a partial amino acid sequence which was in complete agreement with positions 1 to 25 of pardaxin P4. Interestingly, peak 2 contained a shoulder with a slightly shorter retention time during HPLC analysis (Fig. 2), but identical molecular weight as the main part of peak 2. This effect may be assigned to an oligomerization tendency in aqueous solution which was reported earlier for pardaxins [10,15]. Further sequence analysis of the isolated compound corresponding to peak 2 was carried out after cleavage of the isolated peptide by endoproteinase Lys-C. Three fragments were obtained and their molecular weights and amino acid sequences determined by Edman degradation and collision-induced dissociation (CID, Table 1, Fig. 2). The molecular weights of two out of the three fragments were in agreement with the sequence positions 1–16 of pardaxin P4 from *P. marmoratus*. The carboxy-terminal fragment with a molecular weight of 1621 Da showed a difference of +58 Da compared with the corresponding fragment which should be obtained from Lys-C digestion of pardaxin P4. This fragment was further analyzed by CID [24], and a carboxy-terminal sequence of ALSSSGDQE could be unambiguously assigned. Fig. 3 shows the CID daughter spectrum of the ion $[M+H]^+$ with indicated fragment ions of the y-series. This carboxy-terminal sequence of P5 was also confirmed by Edman degradation of the separated fragment 3. In addition, amino acid analysis showed that one Asp residue is contained in the iso-

Table 1
Analysis of peptide fragments obtained by endoproteinase Lys-C cleavage of *P. marmoratus* pardaxin P5

Fragment	Sequence position	Sequence detected	MW calculated	MW experimental[c]
1	1–8	GFFALIPK[a]	892.1	893.0
2	9–16	IISSPLFK[a]	904.1	904.5
3	17–33	TLLSAVGSALSSSGDQE[a,b]	1621.7	1621.0

[a]Edman degradation, [b]collision-induced dissociation (CID), [c]electrospray mass spectrometry (ES-MS).

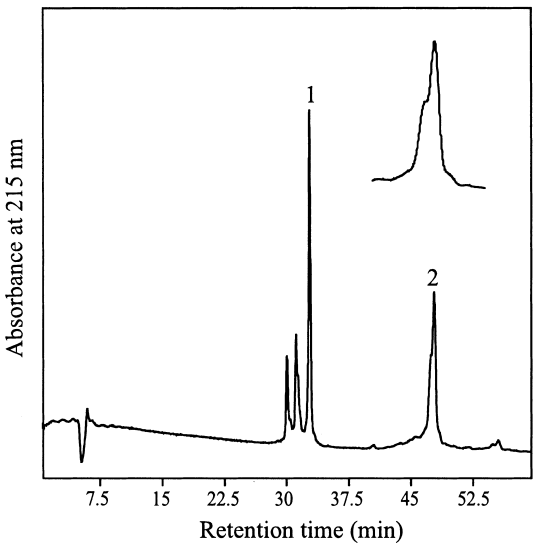

Fig. 2. Analytical reverse-phase HPLC of the toxic secretion of *P. marmoratus*. Chromatography was carried out as described in Section 2. Peaks 1 and 2 were collected and further characterized. The inset shows a stretching of peak 2 which corresponds to pardaxin P5.

lated pardaxin. The exchange of Gly-31 of pardaxin P4 by Asp is in exact accordance with the molecular weight difference between P4 and P5. Thus, the amino acid sequence of pardaxin P5 identified and isolated from *P. marmoratus* is different from pardaxin P4 [6] and represents a novel pardaxin isoform.

To verify the sequence of the isolated pardaxin P5, we synthesized a peptide according to the determined primary structure using Fmoc chemistry and compared this peptide chromatographically with the native compound. Fig. 4 reveals that pardaxin P5 isolated from *P. marmoratus* eluted with a retention time identical to that of the synthetic peptide. In contrast to the native P5, the synthetic pardaxin P5 gave a sharp peak on HPLC analysis, indicating a different oligomerization tendency. The sequence of the novel pardaxin P5 differs by one point mutation from the pardaxin P1 isolated from *P. pavoninus*. The change Asp-31→Glu-31 would require a mutation

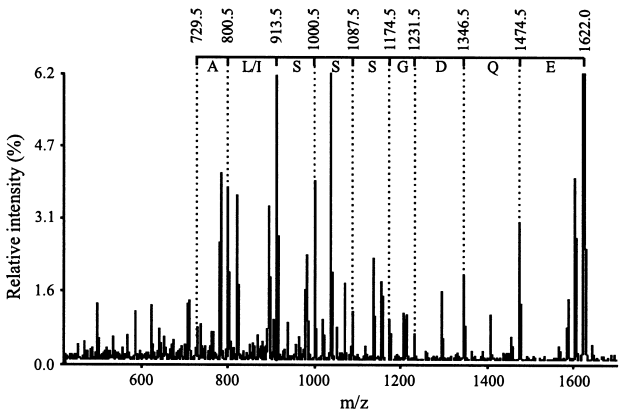

Fig. 3. Collision-induced dissociation (CID) mass spectrum of the single-charged ion $[M+H]^+$ of fragment 3 (Table 1) obtained by endoproteinase Lys-C cleavage of peak 2. Successive peptide bond cleavage of amino acid residues starting at the carboxy terminus according to the y-series is indicated.

of the third base of the Asp-31 codon. In addition, a mutation C→U, the first base of the Leu-5 codon of P1, would lead to the sequence of P3, another pardaxin found in *P. pavonius*. In contrast, no single point mutation of either P1, P3 or P5 would lead to P2. The pardaxin P2 is genetically closest to P5, differing in position 31 (Asp→Gly: C→A exchange of the first base of the Leu-14 codon). The earlier published report on the isolation of *P. marmoratus* pardaxin P4 contained a sequence analysis by Edman degradation, but no mass spectral analysis was provided [6]. It seems likely that either a misinterpretation of Edman sequencing resulted in an incorrect determination of the residue at position 31 or the toxin purified at that time was of a different composition. We suggest that the pardaxin isoform P4 may provide the mutation gap between P5 and P2. It would be one point mutation away from P5 (Asp-31→Gly-31) and another point mutation away from P2 (Leu-14→Ile-14). However, the investigation of this evolutionary structure-function relationship requires further evaluation using other *Pardachirus* species.

To assess the biological activity of the native and synthetic pardaxins, their hemolytic potency was quantified. At concentrations between 10^{-6} and 10^{-5} M, the isolated pardaxin P5 evoked a dose-dependent hemolysis (Fig. 5A). A similar dose-response curve was obtained with synthetic P5, thus providing indirect evidence for their structural identity. In contrast, pardaxin P4 synthesized according to the published sequence [6] had a four- to five-fold higher hemolytic activity. Corresponding to the hemolytic potency, P5 was found to activate phospholipase A2 [26] and to induce release of dopamine (data not shown) [27] with a lower potency compared to P4. Peak 1

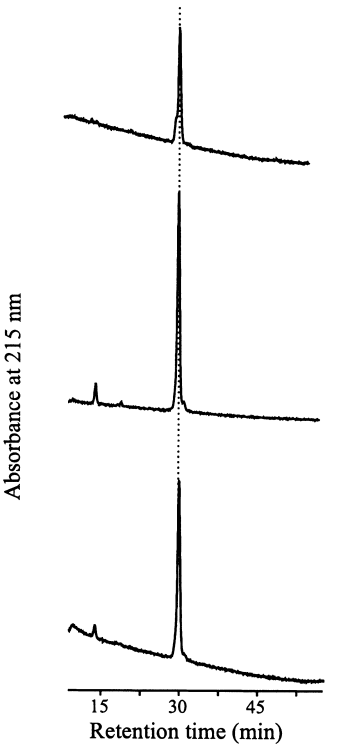

Fig. 4. HPLC comparison of native pardaxin P5 and the corresponding synthetic peptide. Upper trace: native peptide; middle trace: synthetic P5; bottom trace: coelution of equivalent amounts of native and synthetic P5. HPLC was carried out using a Nucleosil C18 PPN column with a linear gradient of 50–100% buffer B in 60 min.

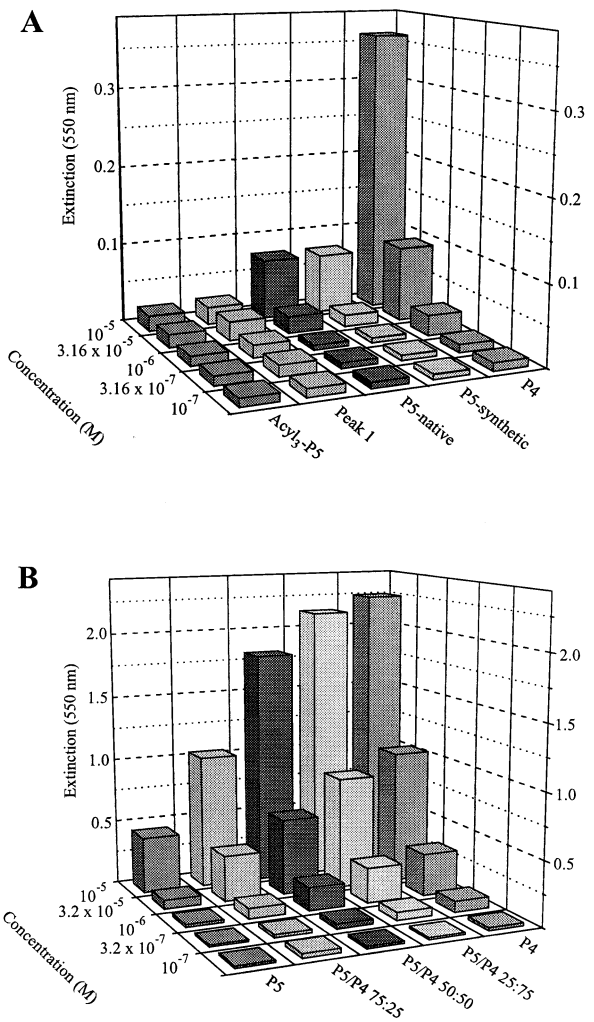

Fig. 5. Cytolytic effect of pardaxins on human erythrocytes. A: Pure pardaxins. Peak 1: by-product from the purification (Fig. 2); $Acyl_3$-P5: {Gly-1,Lys-8,Lys-16-[*N*-(2-(4′-hydroxyphenyl)acetyl)]$_3$}pardaxin-P5. B: Mixtures of pardaxins P4 and P5. As a control, incubation buffer without peptides was used, resulting in an average extinction of 0.008.

obtained from pardaxin purification (Fig. 2) as well as a derivative of native P5 isoform obtained by peracylation of primary amino groups with 2-(4′-hydroxyphenyl)acetic acid were inactive. The lack of hemolytic activity of $Acyl_3$-P5 can be explained by the complete loss of positive charges thereby impairing the amphiphatic character that enables pardaxins to aggregate and to form pores in cell membranes [14]. The occurrence of structurally closely related pardaxins in the same species may cause a synergistic physiological effect between these peptides. To examine this hypothesis, we tested the hemolytic activity of mixtures of synthetic pardaxins P4 and P5 in different molar ratios (Fig. 5B). The results of this assay demonstrate that the hemolytic effect of pardaxin mixtures is unambiguously additive, but not synergistic. Likewise, peptide mixtures containing different molar amounts of native P4 and synthetic P5 caused additively a leakage of cytosolic lactate dehydrogenase (LDH) [28], thus confirming the results obtained from the hemolysis assay (data not shown). In summary, the novel pardaxin P5 has a similar efficacy compared to P4, but a significant lower potency. There are no indications of a synergistic effect between these peptides. The different hemolytic activities of P4 and P5 may be due to differences in either their charge or their secondary structure. We predicted that the carboxy terminus is the filter of the pardaxin channel [14]. This filter is responsible for the relative selective permeability for cations. Therefore, an additional negative charge at position 31 might increase the permeability ratio for ions of pardaxin pores. Considering the presence of a variety of different pardaxins in the same species, one may speculate that this reflects a molecular resource which allows the fish to adjust to environmental changes affecting food supply or the appearance of new predators. Other animals such as snails of the species *Conus* are also provided with different structurally closely related toxic peptides causing an enhanced effectiveness for a defensive or offensive purpose.

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