Recombinant expression and predicted structure of parborlysin, a cytolytic protein from the Antarctic heteronemertine *Parborlasia corrugatus*

Matej Butalaa, Daniel Šega a, Blaž Tomca a, Zdravko Podlesek a, William R. Kem b, Frithjof C. Küpper c, Tom Turk a, *a*

**a** Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia  
**b** Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, FL, 32610, USA  
**c** Oceanlab, University of Aberdeen, Main Street, Newburgh, AB41 6AA, Scotland, UK

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The heteronemertine *Parborlasia corrugatus* contains a cytolytic protein, parborlysin, which after extensive purification was found by Edman sequencing to be a mixture of several homologues. To investigate this microheterogeneity and enable the analysis of single toxins, we have obtained seven parborlysin isoform genes from *P. corrugatus* collected in Antarctica. Total RNA was isolated from the homogenized head region and parborlysin genes were identified from a cDNA library using degenerate primers. The translated sequences reveal that the isoforms are ~10 kDa basic (pI~10) proteins of which all but one harbour six cysteine residues. We generated a model of the three dimensional structure of parborlysins, which suggests that they are composed of five alpha-helical segments that include large, exposed hydrophobic surfaces. Finally, we constructed plasmids and inserted them into *Escherichia coli* to obtain overexpressed amino- or carboxy-terminal polyhistidine-tagged parborlysin isoforms fused to the third domain of the *E. coli* periplasmic-protein TolA to facilitate toxin isolation. One of the isoforms adversely affected growth in the *E. coli* expressing it. Although we succeeded in isolating one of the recombinant parborlysin constructs, it lacked haemolytic activity.

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1. Introduction

Nemertines (also called nemerteans and ribbon worms) are a phylum of predatory, unsegmented worms which are generally toxic (Bacq, 1936; Kem, 1971). While the evolutionarily more ancestral heteronemertines apparently release defensive toxins directly from integumentary glands, hoplonemertines possess alkaloid offensive and defensive toxins which can also be injected into their prey by means of a mineralized stylet bearing venom apparatus in their proboscis (Kvist et al., 2014). Almost eighty years ago Bacq (1936) demonstrated that nemertines are toxic (Bacq, 1936), but it was not until the early 1970s that toxins were first isolated from a few relatively common species, including the hoplonemertines *Paranemertes peregrina* (Kem, 1971) and *Amphiporus angulatus* (Kem et al., 1976; Kem and Blumenthal, 1978), and the heteronemertine *Cerebratulus lacteus* (Kem, 1976; Kem and Blumenthal, 1978). Nemertines are now known to produce a variety of alkaloid and peptide toxins. Anabaseine, the first alkaloid to be isolated, stimulates a variety of nicotinic receptors and currently serves as a lead compound for the design of drug candidates targeting neurodegenerative, neurodevelopmental and anti-inflammatory diseases (Kem et al., 1997; Kem, 2000).

So far the peptide toxins of only two large heteronemertines, northern hemisphere *Cerebratulus lacteus* and southern hemisphere *Parborlasia corrugatus*, have been investigated. Two types of *Cerebratulus lacteus* toxins were isolated, sequenced and pharmacologically characterized. The B toxins, named because they elute from Sephadex columns after the larger A toxins, are approximately 6 kDa in molecular size (Kem, 1976). The B toxins are neurotoxins that potently paralyse and kill crustaceans, but are not toxic when injected into mice. These highly basic peptides possess eight cysteinyl residues and the four disulfide bonds of the most abundant variant Cl-BIV have been determined (Blumenthal et al., 1981). By CD and laser Raman spectroscopic methods Cl-B-IV was estimated...
to contain a high proportion (>70%) of α-helical secondary structure but no B-pleated sheet (Kem et al., 1990). This was consistent with the solution structure of CI-B-IV determined by NMR (Hansen et al., 1992). The A toxins are of approximately 10 kDa size and are cytolytic to mammalian erythrocytes and Ehrlich ascites tumor cells (Kem and Blumenthal, 1978). Like the B toxins they are localized within the skin and proboscis and are most readily isolated from body mucus secretions. The most abundant and well-studied A toxin, CI-AII, is composed of 95 amino acids. It has hydrophobic domains near the N- and C-termini, and both domains can interact with and penetrate into phospholipid bilayer membranes (Blumenthal, 1982). The C-terminal part of the toxin, which seems particularly important for cytolytic activity, is postulated to contain a relatively hydrophobic helical hairpin structure; the B-turn region separating the two putative membrane spanning helices of this segment can apparently penetrate to the other side of the membrane (Dumont and Blumenthal, 1985). A recent transcriptomic study found that some other closely related heteronemertines that also belong to family Lineidae, namely C. lacteus, Cerebratulus marginatus, Ramphogordius lacteus, Lineus ruber and Lineus longissimus, also express CI-AII homologues (Whelan et al., 2014).

A very similar cytolytic toxin named parborlysin has been isolated from Parborlassia corrugatus, a heteronemertine (Berne et al., 2003) common to near shore marine benthic communities in the cold waters of South America and Antarctica, where it is an important predator and scavenger (Heine et al., 1991). It is the most abundant and well-studied A toxin, CI-AII, is composed of 95 amino acids. It has hydrophobic domains near the N- and C-termini, and both domains can interact with and penetrate into phospholipid bilayer membranes (Blumenthal, 1982). The C-terminal part of the toxin, which seems particularly important for cytolytic activity, is postulated to contain a relatively hydrophobic helical hairpin structure; the B-turn region separating the two putative membrane spanning helices of this segment can apparently penetrate to the other side of the membrane (Dumont and Blumenthal, 1985). A recent transcriptomic study found that some other closely related heteronemertines that also belong to family Lineidae, namely C. lacteus, Cerebratulus marginatus, Ramphogordius lacteus, Lineus ruber and Lineus longissimus, also express CI-AII homologues (Whelan et al., 2014).

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2. Materials and Methods

2.1. Collection and preparation of P. corrugatus tissue

Specimens of P. corrugatus were collected by SCUBA diving in the area of British Antarctic Survey Rothera Research Station, Adelaide Island, Antarctica by one of the coauthors (FCK), at Hangar Cove on 31 Dec 2010 and at Honey Bucket on 6 Jan 2011. Collections were typically made between 10 and 20 m depth where the species is very common. After collection, the animals were placed temporarily in a circulating local sea water aquarium (0 °C) and dissected a few hours later. Approximately 10 cm³ of tissue of the heteronemertine head region was immersed in RNAlater (Quiagen), an RNA stabilization reagent and shipped on ice to the University of Ljubljana (Slovenia), where it was stored at −80 °C. For tissue RNA extractions, RNAzap was used to prepare RNAse free equipment. Tissue was thawed and 1 cm² area excised with a scalpel, cut into small pieces and homogenized with an Elvejem-Potter grinder in 10 cycles at 4 °C for 30 min after addition of the homogenized tissue or the purified parborlysin.

2.3. Preparation of the cDNA library

The P. corrugatus RNA was isolated from the homogenized tissue with the SV Total RNA Isolation System (Promega). Isolated RNA was checked on a 1.5% ethidium bromide-stained agarose gel and concentration determined spectrophotometrically by Nanodrop ND-1000 (Thermo Scientific). RNA was stored at −80 °C until use. Synthesis of the double-stranded cDNA from P. corrugatus poly(A⁺) RNA (4 µg) was performed with Just cDNA Double-Stranded cDNA Synthesis Kit™ (Agilent Technologies) according to the manufacturer’s instructions. To amplify the parborlysin coding region we engineered the degenerate primer 5′-GGNTGCGCCNGNTAVCCNGG-3′ corresponding to the amino acid N-terminal sequence obtained by Edman degradation of native parborlysin (Berne et al., 2003). Touchdown™ PCR was performed with Pfu DNA polymerase (Thermo Scientific) using the degenerate primer and oligo(dT)20 (5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′) primers in an UnoII PCR machine (Bioterrra). PCR products were separated and approximately 400 bp and the 300 bp fragments excised from the 1% agarose gel and purified with GeneJET Gel Extraction Kit (Thermo scientific). Rest of the bands observed on the gel were not included in the study as just the three other bands of a size (<150 bp), which is smaller than the expected length of the parborlysin gene, were detected in addition to the DNA/RNA background smear. DNA fragments were cloned into the blunt cloning vector pJET according to the CloneJET™ PCR cloning kit manual (Thermo Scientific), using the chemically competent DH5α E. coli strain. Plasmid DNA from the transformants grown on selective Luria–Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml) was isolated with GeneJET Plasmid Miniprep Kit™ (Thermo Scientific). Positive plasmid clones were determined by restriction analysis and 12 of the plasmid constructs were sequenced by Macrogen.

2.4. Sequence analysis and parborlysin structure prediction

In order to identify the putative parborlysin coding regions, the identified nucleotide sequences were translated to amino acid sequences using ExPaSy/Translate tool. By using the ExPaSy/Protein tool (Gasteiger et al., 2003) we predicted the isoelectric points and molecular weights of the parborlysin isoforms. Blastp (Altschul et al., 1990) search was carried out to query identified sequences against the non-redundant protein sequences database (Altschul et al., 1990) and to determine similarity to the C. lacteus cytotoxin A-III (UniProt ID: P01527). ClustalW (Larkin et al., 2007) was used to align multiple protein sequences and the BoxShade server (Gasteiger et al., 2003) was used to shade the alignments. Prediction of the parborlysin clone P1 three-dimensional structure was made using the I-Tasser server (Roy et al., 2010) and contact restraints were assigned for the cystein residues 16 and 22, 33 and 37, 47 and 60. Visualization of the parborlysin tertiary structure was performed using Visual molecular dynamics™ (Humphrey et al., 1996).

2.5. Cloning of the recombinant parborlysin

In this study generated oligonucleotide primers P1UP (5′-GGCATATTGCTGGCAGATTG-3′) and P2DO (5′-GGCTGGACAGCTTGGCAGGCTATTGAG-3′) or P1UP and P2UP (5′-GGGCTACGGAGGCTGAGAATTGGAC-3′) or P3UP (5′-GGCCATAATEGGTCGCCGCTATTGAC-3′) and P3DO (5′-GGCCATATTGGCTGCCTCGG-3′) were used to PCR amplify parborlysin isoform P1 or P2 or P3 coding sequences, respectively, by using Pfu DNA polymerase (Thermo Scientific).
under the following conditions: 3 min at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C, and a final step of 7 min at 72 °C. Ndel, Xhol cut and gel purified DNA fragments were ligated into the expression vector pET21c harbouring under a T7 promoter the sequence for the C-terminal His6-tag positioned downstream of the thrombin cleavage site to obtain the pPAR1H, pPAR2H and pPAR3H for the parborlysin isoforms 1–3, respectively. Subsequently these plasmid constructs were cut by Xhol and ligated with Xhol cut PCR amplified tolAII region from pTolAII (Andersluh et al., 2003) to obtain plasmids pTolUP (5'-CAACTC-GAGCTGTTCCGGCCCGATCCAACATTGGCGCATGGG-3') and pTolDO (5'-CAACTGAGCGTGGTAACTGATTCCGATGGG-3') were used under the conditions as described above. P1_U (5'-GGGGATCCGGGTGGCCGGCGTATCCGGG-3') and P1_D (5'-GGGACGCTTCCTTGTGCCAGCCTTATTGAT-3') primers were used to amplify parborlysin isoform P1 coding sequence by using Pfu DNA polymerase (Thermo Scientific), under the stated conditions. The BamHI/Mul-cut and gel purified DNA fragment was ligated into the expression vector pET21c (Andersluh et al., 2003) to prepare pHPART1 plasmid, harbouring under a T7 promoter the sequence for the N-terminal His6-tag positioned upstream of the tolAII sequence, a thrombin cleavage site and a parborlysin 1 isoform gene. Following transformation into E. coli DH5x, several clones were checked by sequencing (Macrogen) and the appropriate plasmid constructs isolated.

2.6. Expression and purification of the recombinant parborlysin

To overexpress the parborlysin isoforms cloned into pET21c expression vector and the parborlysin effect on the growth of the producing strain, the E. coli rosetta-gami(DE3) pLysS (Novagen) strain harbouring one of the plasmid constructs was transferred into LB medium with 100 µM ampicillin (Ap) and 25 µM chloramphenicol, and cultivated overnight with shaking. Overnight culture was inoculated (dilution 1:50) in LB broth supplemented with Ap (100 µg/ml) with aeration at 37 °C to an OD600 of 0.6 when samples were collected and 0.8 mM isopropyl thio-β-D - galactoside (IPTG) added to the culture which was further grown at 37 °C. The samples were collected before and 3 h after IPTG induction and equilibrated to an OD600 of 0.6 to detect protein levels at equal cell densities during bacterial growth. Cell pellets were resuspended in 10 µl NuPAGE LDS sample buffer, 10 µl of DTT and 20 µl of ddH2O and heated (95 °C, 5 min) before loading equal amounts of the samples on a 12% SDS-PAGE gel (Invitrogen).

To express the His6-TolAIII-parborlysin 1 protein, the pHPART1 plasmid was transformed into the E. coli minc (Baba et al., 2006) strain harbouring a ΔDE3, which was inserted into the strain by using a ΔDE3 Lysogenization Kit (Novagen). Overnight culture was inoculated (dilution 1:50) into 500 ml of LB Ap media at 37 °C at 180 rpm until the broth OD600 reached 0.6 when the IPTG was added to a final concentration of 0.8 mM and bacteria were left growing for additional 2 h. Cells were then pelleted, lysed and inclusion bodies washed in 20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 2% Triton X-100 (v/v). Inclusion bodies were lysed in 20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 50 mM β-mercaptoethanol and 6 M guanidinium chloride. Subsequently Ni-NTA chromatography under denaturing conditions was performed and the eluted protein in 20 mM Tris–HCl (pH 8.0), 0.3 M NaCl, 10% glycerol (v/v) and 1 mM imidazole was refolded by dialysis against 20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole and 1 mM β-mercaptoethanol in a spectrum/por dialysis membrane (MWCO 6–8,000, Spectrum labs). Protein was stored at 4 °C within the dialysis membrane tubes. Thrombin kit (Novagen) was used to cleave the His6-TolAII region from parborlysin 1 and the proteins, mixed with NuPAGE LDS sample buffer (Invitrogen) and reduced by 10 mM DTT and heated at 95 °C for 5 min before loading, were resolved by SDS–PAGE in 12% gradient gels (Invitrogen) in MES running buffer and visualized by Coomassie blue staining with PageRuler™ prestained protein ladder plus (Thermo Scientific) used as a standard.

3. Results and discussion

Sequence microheterogeneity of parborlysin initially isolated from P. corrugatus indicated that this species harbours several genes for this cytolytic toxin (Berne et al., 2003). We initially assayed whether the tissue of P. corrugatus specimens collected in the area of Rothera Research Station (Adelaide Island, maritime Antarctica) contained haemolytic activity. This was confirmed (Fig. S1) and was consistent with the earlier paper showing that parborlysin is haemolytic (Berne et al., 2003). In order to obtain nucleotide sequences of genes related to parborlysin we isolated total P. corrugatus RNA from approximately 1 cm² of tissue of a single individual. Subsequently, degenerate primers were designed according to a previously identified N-terminal amino acid sequence (Berne et al., 2003), which allowed us to construct the parborlysin plasmid library. By nucleotide sequencing and a BlastP search with the translated amino acid sequences of the seven nucleotide sequences with very close similarity (p-distance 0.174) to the C. lacteus cytotoxin A-III amino acid sequence (Table S1 with GenBank accession numbers). The translated sequences of the seven parborlysin isoforms, approximately 90 amino acids long, imply that the proteins have a high isoelectric point and harbour six (six isoforms) or 5 (one isoform) cysteine residues (Table 1, Fig. 1). The presence of 6 cysteine residues implies that three disulphide bonds occur in almost all parborlysin isoforms. This is also the characteristic of other polypeptide lysins, for instance C. lacteus toxin B-IV (Blumenthal and Kem, 1977) and the saposin-like polypeptides (Liepinsh et al., 1997), which likely increase the resistance of these molecules to a variety of physical and chemical stresses.

In order to obtain tentative (prediction-based) insight into the structural characteristics of the parborlysin we generated a structural model of the 93 amino acid parborlysin isoform 1 by using the I-Tasser server (Yang et al., 2015). The ab initio modelling suggests that the toxin's secondary structure is exclusively composed of α-helices (Fig. 2A). Some unpublished data on Cl-AIII are at least qualitatively consistent with this secondary structure prediction; using CD and Raman spectroscopic methods approximately 60% α-helix and only 11% β-pleated sheet were estimated for this homologous toxin (Kem and Williams, in preparation).

The predicted disulphide bond between cysteine residues 47 and 60 and the proximities of the other four cysteines in isoform 1 to each other, as shown in Fig. 2A, suggests disulphide pairings (16–37, 22 to 33 and 47 to 60) that are consistent with what was found for CIA-III (Blumenthal et al., 1981). This provides further support for the predicted structure shown in Fig. 2A.

Several different approaches were attempted to obtain a properly folded recombinant protein. Parborlysin seems to harbour substantial surface-exposed hydrophobic areas (Fig. 2B and C), which implies that the protein is poorly soluble in polar solvents. Thus, to enhance the solubility, we separately fused three parborlysin isoforms to the third domain of the periplasmic protein TolA (TolAII) from E. coli that has previously been used to facilitate higher yields and easier purification of several proteins (Andersluh et al., 2003). The parborlysin (isoform 1, 2 or 3)–TolAII–His6-TolAIII proteins were IPTG induced in the E. coli rosetta-gami(DE3) pLysS strain. The results obtained imply that the overexpressed parborlysin isoform 1, but neither of the two other tested protein constructs, had a bacteriostatic effect on the growth of the producing bacteria (Fig. 3A and B). Purification of these protein
Constructs under native or denaturing conditions by using a Ni-NTA Fast Start Kit (Quiagen) was unsuccessful. In addition, we prepared a pHTPAR1 plasmid to overexpress and isolate the N-terminally his-tagged TolAIII-parborlysin isoform 1 and test whether this affected the isolation of the protein. In the protein construct the parborlysin isoform 1 and the TolAIII domain are separated by a thrombin cleavage site. As parborlysin appeared to be toxic to the producing bacteria, we used an E. coli strain that has a capability of enhanced

Table 1
Characteristics of the identified parborlysin isoforms (P1–P7) and of cytotoxin A-III. The protein sequences are shown with the number of amino acid (AA) residues and with the underlined cysteine residues. Molecular weights (Mw) and theoretical isoelectric points (pi) of the proteins are presented.

<table>
<thead>
<tr>
<th>Protein sequence</th>
<th>Mw (kDa)</th>
<th>pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (93 AA)</td>
<td>10.075</td>
<td>10.32</td>
</tr>
<tr>
<td>P2 (93 AA)</td>
<td>9.712</td>
<td>10.25</td>
</tr>
<tr>
<td>P3 (93 AA)</td>
<td>9.638</td>
<td>10.15</td>
</tr>
<tr>
<td>P4 (93 AA)</td>
<td>9.752</td>
<td>10.19</td>
</tr>
<tr>
<td>P5 (91 AA)</td>
<td>9.699</td>
<td>10.15</td>
</tr>
<tr>
<td>P6 (91 AA)</td>
<td>9.470</td>
<td>10.12</td>
</tr>
<tr>
<td>P7 (92 AA)</td>
<td>9.655</td>
<td>10.13</td>
</tr>
<tr>
<td>Cytotoxin A-III (95 AA)</td>
<td>9.809</td>
<td>10.00</td>
</tr>
</tbody>
</table>

Fig. 1. Amino acid sequence alignment of the parborlysin isoforms (P1–P7) and of C. lacteus cytotoxin A-III. Identical amino acids are shaded in black and residues with similar physical and chemical characteristics shaded in gray.

Fig. 2. Three-dimensional model of the parborlysin 1 isoform. (A) Predicted structure of the parborlysin isoform 1 shown in white, with N-terminal region in ribbon representation in blue and cysteine residues (presented as stick model) in red. (B) The same orientation of parborlysin model as shown in panel A. (C) An orientation obtained by rotation of orientation B through 90°. In both B and C, hydrophobic surfaces are orange and charged residues are yellow.
suggesting that the protein was not properly folded or that it requires a free N-terminal end for its activity.

In conclusion, we present the first report of the nucleotide sequences of seven parborlysin isoforms. The translated sequences indicate that the isoforms are small, basic proteins of around 90 amino acids, neutrally charged at about pH 10. Furthermore, all but one of the isoforms seems to contain 6 cysteine residues, the modelling of which (Fig. 2A) suggests that potential cysteinyll residues forming pairs in parborlysin lie in close proximity and are likely to form disulphide bonds conferring stability to the protein structure. Presumably, due to the hydrophilic nature of the parborlysin, the isolation of properly folded recombinant parborlysins proved challenging. To enable further characterisation of the biochemical and cytotoxic characteristics of this protein our study suggests that other expression approaches including eukaryotic expression should be attempted.

**Ethical statement**

No experiments on animal or human were done in the study encompassed.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2015.09.044.

**Transparency document**

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**References**


