Protoplasma
https://doi.org/10.1007/s00709-026-02243-5

RESEARCH

Hirudins and ornatins in five species of the genus *Placobdella* (Annelida: Hirudinea: Glossiphoniidae)

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Received: 6 February 2026 / Accepted: 26 June 2026

Abstract
Leeches of the genus *Placobdella* Blanchard, 1893 (Annelida: Rhynchobdellida: Glossiphoniidae) are mainly distributed across North and Central America, but at least two species are also present in the palearctic region: the European turtle leech *Placobdella costata* Müller, 1846, and the North African turtle leech *Placobdella nabeulensis* Ben Ahmed et al., 2023. All species of the genus are hematophagous and feed on vertebrates, but are known to preferentially target aquatic and semi-aquatic reptiles like turtles and snakes. *Placobdella ornata* Verrill, 1872, is the original source of ornatins, a class of potent platelet aggregation inhibitors. However, the whole repertoire of bioactive peptides, including anticoagulation factors remains largely underexplored for *Placobdella*, and functional characterizations of putative antithrombotics are scarce. Here we describe the genes and cDNAs that encode putative hirudins and ornatins in both of the palearctic and three American species of the genus *Placobdella*. A selection of putative hirudins and ornatins was recombinantly expressed and functionally characterized using appropriate coagulation and platelet aggregation assays, and our data confirm the expression of functional hirudins and ornatins in the studied leeches. In addition, our genetic analyses strongly support the hypothesis that blood feeding evolved only once in the evolutionary history of leeches.

Keywords *Placobdella* · Hirudin · Ornatin · Blood coagulation · Platelet aggregation · Hematophagous leeches

Handling Editor: Martina Sombetzki.

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Published online: 06 July 2026

Introduction

Leeches are segmented worms that form the subclass Hirudinea within the phylum Annelida (Phillips et al. 2019). Crudely, leeches are traditionally divided into two major clades based on the morphology of their feeding apparatus: Rhynchobdellida Blanchard, 1894, whose members possess a proboscis, and Arhynchobdellida Blanchard, 1894, without a proboscis. The former group includes the Glossiphoniidae Vaillant, 1890, jawless dorsoventrally flattened leeches that inhabit freshwater ecosystems. The latter group includes the Hirudiniformes Caballero, 1952, in which the feeding apparatus is usually characterized by the presence of jaws (Kuo and Lai 2019). Many leeches of both groups are hematophagous, whereas others are macrophagous or liquidosomatophagous (Lynggaard et al. 2022). The medicinal use of hematophagous leeches, consequently also termed medicinal leeches, traces far back in human history and was common in many civilizations throughout time (Elliott and Kutschera 2011; Abdualkader et al. 2013; Marchiori et al. 2024). However, mainly representatives of jawed leeches were and are regularly used for medical purposes, including the European medicinal leech *Hirudo medicinalis* Linnaeus, 1758, the Mediterranean medicinal leech *Hirudo verbana* Carena, 1820, the African medicinal leech *Hirudo troctina* Johnson, 1816, the Asian medicinal leeches *Hirudo nipponia* Whitman, 1886, and *Hirudinaria manillensis* Lesson, 1842, the North-American medicinal leech *Macrobdella decora* Say, 1824, or the Australian medicinal leech *Richardsonianus australis* Bosisto, 1859 (Kvist et al. 2013). By contrast, proboscis-bearing leeches are much less frequently considered for the direct use in medical practice, yet members of the Central and South American genus *Haementeria* de Filippi, 1849, may represent an exception (Oceguera-Figueroa 2012). As a consequence, only few studies have specifically addressed the antithrombotic repertoire of rhynchobdellid leeches. Again, the genus *Haementeria* represents a remarkable exception (Sawyer et al. 1991; Seale et al. 1997; Faria et al. 1999; Oliveira et al. 2012; Amorim et al. 2015; Pfordt et al. 2022). Antistasin, a factor Xa inhibitor that was isolated from the Mexican leech *Haementeria officinalis* de Filippi, 1849 (Tuszynski et al. 1987), is probably the most well-known *Haementeria*-derived anticoagulant (Iwama et al. 2021). Factor Xa is a crucial component of the blood coagulation-cascade and facilitates the cleavage of prothrombin to thrombin (Núñez-Navarro et al. 2019). The activity of thrombin in turn is inhibited by direct thrombin-inhibitors like hirudin (Markwardt 1956). Both antistasin and hirudin are hence inhibitors of the secondary hemostasis. In contrast, the primary hemostasis (the process of platelet aggregation) is inhibited by leech-derived factors like decorsin and ornatin. Whereas decorsin was first described from *M. decora* (Seymour et al. 1990), ornatin originates from *Placobdella ornata* Verrill, 1872 (Mazur et al. 1991). Both factors are potent glycoprotein IIb/IIIa antagonists and contain a highly conserved RGD motif (Lazarus and McDowell 1993).

Like the genus *Haementeria*, the genus *Placobdella* Blanchard, 1893, also belongs to the order of *Rhynchobdellida* Blanchard, 1894, and the family of *Glossiphoniidae* Vaillant, 1890. The genus *Placobdella* currently comprises about 25 different species that inhabit large areas of North America (de Carle et al. 2017). Members of the genus are hematophagous and primarily ectoparasitic on turtles (Bielecki et al. 2012), but occasionally also feed on amphibians, birds and mammals including humans (Moser et al. 2014; Cichocka et al. 2021). *Placobdella costata* Müller, 1846, represents the only European member of the genus. The species is widely distributed across Europe, and the distribution range largely overlaps with the distribution range of its natural host, the European mud turtle *Emys orbicularis* Linnaeus, 1758 (Bielecki et al. 2012). A contemporary study showed that the genetic diversity between the various populations of *P. costata* may suggest up to seven separately evolving European lineages throughout its range, five of which exhibit degrees of separation at the molecular level to warrant species level status (Kvist et al. 2022). One of these lineages was recently confirmed as a second Palaearctic member of the genus *Placobdella*, namely *Placobdella nabeulensis* (Ben Ahmed et al. 2023). This species occurs in distinct regions of Tunisia and Algeria in northern Africa.

So far, investigations into members of the genus *Placobdella* have mainly focused on the description and analysis of morphological (Richardson et al. 2017; Sawyer 2022) and genetic (de Carle et al. 2017; Ben Ahmed et al. 2023) markers or on the composition of their bacterial symbionts (Manglicmot et al. 2020). Besides the historical identification of ornatins, investigations into the presence and diversity of anticoagulants in the salivary glands of these animals are rare. In 2016, Mark Siddall et al. conducted a comparative transcriptomic analysis of three members of the genus *Placobdella*, namely *Placobdella ali* Hughes & Siddall, 2007, *Placobdella kwetlumye* Oceguera-Figueroa, Kvist, Watson, Sankar, Overstreet & Siddall, 2010 and *Placobdella parasitica* Say, 1824, and the authors identified a broad variety of putative anticoagulants including hirudins and ornatins (Siddall et al. 2016). Functional investigations of the respective factors, however, are still missing. In 2024, the complete genome sequence data of *P. costata* were deposited in the nucleotide database of GenBank by the Wellcome Sanger Darwin Tree of Life Programme including the sequences of 18 chromosomes (accession numbers OZ194472.1 - OZ194489.1) and the mitochondrial genome (accession number OZ194490.1). Strikingly,

the information on the genome was neither published nor is it available on the website of the Darwin Tree of Life Programme.

Over the last years, we have successfully applied the approach to conduct in-depth analyses and re-analyses of genome and transcriptome sequence data of several leech species in order to further explore the diversity of putative anticoagulants (Khan et al. 2025). Whenever feasible, factors of interest were expressed as recombinant proteins and subsequently functionally characterized (Pfordt et al. 2022; Müller et al. 2024; Schulz et al. 2025; Tolksdorf et al. 2025). The availability of both transcriptome and genome sequence data has enabled such analyses also for *P. costata*. The primary aim of the present study was hence to identify, and functionally characterize putative anticoagulants in five species of *Placobdella*, with a special focus on hirudins and ornatins. The second aim, however, was to eventually answer the question whether or not the hirudins and ornatins of glossiphoniid leeches also belong to the hirudin superfamily whose members are characterized not only by similar structural features of the respective molecules, but also by a common gene structure (Müller et al. 2019).

Materials and methods

GenBank transcriptome and genome data

Transcriptome data for members of the genus *Placobdella* were obtained from the following GenBank sequence read archives (SRAs): SRX3734179 (*P. ali*), SRX3734173 (*P. kwetlumye*) and SRX1335044 (*P. parasitica*), respectively. Chromosome-level genome data of *P. costata* were obtained from GenBank accession numbers OZ194472.1 - OZ194489.1.

Animal collection, tissue preparation, genomic DNA isolation and sequencing

Individuals of *Placobdella costata* were collected in November 2018 from the Kapengraben in Saxony-Anhalt, Germany (51.81488 N, 12.40539 E) (collection codes 695 and 696, Kvist et al. 2022), whereas individuals of *Placobdella nabeulensis* were collected in June 2021 from El Malaabi Dam (36.81666 N, 10.98333 E) in Menzel Temime located in the North-East of Tunisia (Ben Ahmed et al. 2023). The caudal suckers of two individuals of *P. costata* and one individual of *P. nabeulensis* were dissected and placed in 2 ml microcentrifuge tubes each. Prior to extraction of genomic DNA, 350 µl of the lysis buffer ML 1 and 25 µl of proteinase K solution (E.Z.N.A. Mollusc DNA kit, Omega Bio-tek Inc., Norcross, GA) were added to the samples and the tissues were manually homogenized using scissors. DNA extractions were performed according to the manufacturer's protocol. Purified genomic DNA was eluted with sterile double distilled water and stored at −20 °C. For whole genome sequencing, the samples were sent to the Competence Centre for Genomic Analysis (Kiel, Germany). The genomic DNA was quality-controlled and quantified via fluorometric measurement. Library preparation and sequencing were carried out at the Competence Centre for Genomic Analysis (Kiel, Germany). Library preparations were done using the Illumina DNA Prep Kit (Illumina, Berlin, Germany) according to the manufacturer's protocol. Sequencing of the libraries was performed on an Illumina NovaSeq 6000 S4 device, using 2 × 150 bp reads on v1.5 Flowcells. The raw genome sequence data of both the two individuals of *P. costata* and the single individual of *P. nabeulensis* were deposited in BioStudies (The European Bioinformatics Institute, EMBL-EBI, Sarkans et al. 2018) and received the following accession number: S-BSST2339 (DOI: https://doi.org/10.6019/S-BSST2339). The data can be freely accessed and used. During the course of the present study a high-quality, chromosome-level reference genome of *P. costata* was generated by the Wellcome Sanger Institute and deposited in GenBank (GCA_964276725.1). All *P. costata*-related sequence information in the manuscript hence refer to the reference genome.

Bioinformatics and graphical tools

Raw genome sequence data were imported into Geneious v9.1.8 (Biomatters Ltd., Auckland, New Zealand; Kearse et al. 2012) for further analyses. Basic Local Alignment Search Tool (BLAST) searches were performed using the NCBI web portal (https://blast.ncbi.nlm.nih.gov/Blast.cgi), BioEdit v7.2.5 (Hall 1999) or Geneious v9.1.8 with adjusted parameter settings for both word size and the expected threshold values. Multiple sequence alignments (MSA) were generated using ClustalX 2.1 (Larkin et al. 2007) or the CLC Sequence Viewer software package v8.0 (QIAGEN, Aarhus, Denmark) using default settings. Particular attention was paid to the conservation of disulfide bond-forming cysteines. Alignments were exported as msf-files and further processed using Gene-Doc v2.7 (Nicholas and Nicholas 1997). Signal peptide sequences were predicted using the SignalP6.0 server (Teufel et al. 2022) and the Phobius web portal (Käll et al. 2007). Graphs were generated and analyzed using GraphPad Prism V5.01 (GraphPad Software, Boston, MA, USA).

Gene synthesis

The cDNA fragments of putative hirudins, hirudin-like factors (HLFs) and ornatins of *P. costata* were synthesized using the GeneArt gene synthesis service of Thermo Fisher Scientific (Darmstadt, Germany).

Amplification and cloning of putative hirudin and decorsin cDNAs

For the amplification of putative hirudin and decorsin cDNAs, primers were derived from the respective transcriptome or genome sequences. A list of all primers that were used in the study is provided in Supplementary Information File Table S1. PCR reactions were performed using Q5 high-fidelity DNA polymerase (New England Biolabs, Frankfurt a. M., Germany), fragments of relevant sizes were purified and cloned into the expression vector pQE30Xa (QIAGEN, Hilden, Germany). Successfully cloned cDNAs were sequenced for control purposes by Biosearch Technologies (LGC, Berlin, Germany) or by Eurofins Genomics (Cologne, Germany).

Expression, purification, processing, and quantification of putative hirudins and decorsins

The detailed procedure to express, purify, process and quantify the respective recombinant hirudins and decorsins of *P. costata* was described in numerous recent publications (e.g. Müller et al. 2016; Wang et al. 2025; Tolksdorf et al. 2025). Briefly, all factors were either expressed in *Escherichia coli* laboratory strains DH5α at a cultivation temperature of 37 °C (Hanahan 1985) or SHuffle® T7 at a cultivation temperature of 30 °C (New England Biolabs, Frankfurt a. M., Germany; Lobstein et al. 2012). To obtain the recombinant proteins, we applied an expression and purification system that was developed by QIAGEN (Hilden, Germany). The pQE30Xa vector encodes a factor Xa protease recognition site that is located between the His-tag coding region at the 5′ side and the multiple cloning site at the 3′ side. A subsequent factor Xa protease-treatment cleaves off the His-tag and results in a recombinant protein that is devoid of any vector-derived amino acid residues at the N-terminus. The successful expression, purification and factor Xa treatment of all factors was controlled by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses. Molar concentrations of final protein solutions were calculated by dividing the absorbance at 280 nm by the molar absorption coefficient according to the equation $\varepsilon = (nW \times 5{,}500) + (nY \times 1{,}490) + (nC \times 125)$ (Gill and von Hippel 1989; Pace et al. 1995).

Blood coagulation assays

To verify the thrombin-inhibitory potencies of putative hirudins of *P. costata*, we performed a thrombin time test (TTT; reference range 16.8–21.4 s) using a BFT II analyzer (Siemens Healthcare, Erlangen, Germany). All steps were carried out according to the manufacturer's instructions. Protein samples were diluted with buffer to reach final concentrations in the reaction assays of 3.2 µmol/l or 0.32 µmol/l, respectively. The desired amount of substrate was directly transferred into the test cuvette immediately before the plasma was added. Dade® Ci-Trol® 1 (Siemens Healthcare, Erlangen, Germany) was used as standardized human plasma. The incubation of reaction mixtures was carried out at 37.4 °C. Measurements that reached 300 s before any coagulation was detected were stopped and considered as complete inhibition of coagulation. Blood coagulation tests were performed in three to four technical replicates.

Platelet aggregation assays

Platelet aggregation was analyzed by light transmission aggregometry (LTA) with human blood samples that were obtained from healthy human volunteers after written informed consent and approval from the institutional ethics committee. Blood collection, sample preparation, and the subsequent experimental procedures were performed as described in Müller et al. (2024) and Tolksdorf et al. (2025). Briefly, 10 ml of venous blood was taken from the antecubital vein using an S-Monovette® (Sarstedt, Nürnbrecht, Germany) prefilled with citrate buffer. The first centrifugation step of the blood collection tube was performed at 200 g for 20 min at room temperature. After centrifugation, the supernatant (platelet-rich plasma, PRP) was transferred, and the remaining blood was centrifuged again for 10 min at 2000 g at room temperature. The supernatant was dedicated as platelet-poor plasma (PPP), transferred, and used as a reference value for maximal platelet aggregation. Measurements were performed using either an APACT-4004 aggregometer (LABiTec, Ahrensburg, Germany) or a TA-8 V aggregometer (Diagnostica Stago S.A.S., Asnièressur-Seine, France). The snake venom-derived platelet aggregation inhibitors eptifibatide and tirofiban (Sigma-Aldrich, Taufkirchen, Germany) were used as positive controls for complete inhibition of platelet aggregation. PRP was pre-incubated with the respective test and control compounds (final concentration 3.2 µmol/l) or buffer for 1 min at 37 °C. For the measurement, PRP was subsequently transferred into the test cuvettes and stimulated with ADP (200 µmol/l; Hart Biologicals, Hartlepool, UK; final concentration 5 µmol/l) after 1 min of runtime. The final volume in each test cuvette was

250 µL of diluted PRP. All experiments were performed at 37 °C over a time period of 400 s. Maximal aggregation in percentage and the area under the curve were calculated as quantitative output parameters (Zhou and Schmaier 2005). Platelet aggregation tests were performed in two to four technical replicates.

Results

Identification of putative hirudin and ornatin cDNAs in *P. ali*, *P. kwetlumye* and *P. parasitica*

Whereas ornatins have already been described from *P. ornata* many years ago (Mazur et al. 1991), the investigations of Siddall et al. (2016) for the first time revealed the presence of putative hirudins in members of the genus *Placobdella*. Altogether, the authors identified putative cDNAs of hirudin in *P. kwetlumye* and *P. parasitica* and putative cDNAs of ornatin in *P. ali* and *P. parasitica*, respectively. However, none of the putative hirudins or ornatins was functionally verified so far. Our in-depth re-analysis of the transcriptomic data confirmed the results of Siddall et al. (2016), but we were able to identify additional putative hirudin and ornatin-encoding cDNAs. In total, we identified two putative hirudins in *P. ali* (Pali_HV1 and Pali_HV2), three putative hirudins in *P. kwetlumye* (Pkwe_HV1, Pkwe_HV2 and Pkwe_HV3) and one putative hirudin in *P. parasitica* (Ppar_HV1). Pkwe_HV1 and Ppar_HV1 were already described by Siddall et al. (2016). The corresponding contigs of all putative hirudins-encoding cDNAs are provided in Supplementary Information Files S1-S3.

The identification of putative ornatin-encoding contigs turned out to be a challenging task and required some manual sequence editing including the deletion or integration of single nucleotides to obtain meaningful open reading frames (ORFs) and/or the deduction of consensus sequences from multiple sequence alignments. However, we eventually identified three putative ornatin-encoding cDNAs in *P. ali* (Pali_OV1-3) and one cDNA in both *P. kwetlumye* (Pkwe_OV1) and *P. parasitica* (Ppar_OV1), respectively. The corresponding contigs with all marked manual sequence modifications are provided in Supplementary Information Files S1-S3.

Identification of putative hirudin and ornatin genes in *P. costata* and *P. nabeulensis*

Based on the cDNA sequences of putative hirudins and ornatins of *P. ali*, *P. kwetlumye* and *P. parasitica* we subsequently screened the genome of *P. costata* to identify the respective gene sequences. Based on our analyses, the genome of *P. costata* contains three genes that encode putative hirudin variants (HV), namely Pcos_HV1-3, and three genes that encode putative ornatin variants (OV), namely Pcos_OV1a, _OV1b and _OV2. All genes are located on chromosome 18, the exact position and orientation of each gene are provided in Table 1.

Table 1 Position and orientation of putative hirudin (HV) and ornatin (OV) genes on chromosome 18 in *P. costata*. rev+comp indicates reverse and complementary orientation

gene	position
Pcos_HV3	3,772,705–3,773,531 rev+comp
Pcos_HV2	5,750,243–5,752,539 rev+comp
Pcos_HV1	5,755,993–5,756,621 rev+comp
Pcos_OV1a	9,984,659–9,985,686 rev+comp
Pcos_OV1b	9,989,819–9,991,063 rev+comp
Pcos_OV2	10,001,584–10,002,179 rev+comp

All hirudin genes are composed of four exons and three introns, whereas the ornatin genes are composed of three exons and two introns and apparently lack the third intron and the fourth exon of the hirudin genes which encode the elongated acidic C-terminal tail of hirudin (see Fig. 1). For each gene, the exon/intron composition, the location of the introns and the characteristics of the exon boundaries (boundary-overlapping versus non-overlapping codons) exactly match the structures of all hirudin and decorsin/ornatin genes analyzed so far. A schematic representation of the gene structures of Pcos_HV1 and Pcos_OV2 is provided in Fig. 1. Table 2 lists the exon and intron lengths, and the annotated sequences for each of the genes are provided in Supplementary Information Files S4 (hirudin genes) and S5 (ornatin genes).

To verify the data for *P. costata* we generated and analyzed raw genome sequence data of *P. nabeulensis*, the second Palearctic representative of the genus *Placobdella*. The raw Illumina reads were manually assembled, and all putative hirudin and ornatin genes of *P. costata* could successfully be identified and reconstructed in *P. nabeulensis* with the exception of intron1 of both the Pnab_HV2 and Pnab_OV1 genes and intron 3 of the Pnab_HV2 gene. Exon and intron lengths of all putative hirudin and ornatin genes in *P. nabeulensis* are in close proximity to the values of the respective genes of *P. costata* (see Table 2 for details). The annotated sequences for all genes are provided in Supplementary Information Files S6 (hirudin genes) and S7 (ornatin genes).

Structural and functional characterization of putative hirudins

The amino acid sequences of all putative hirudins of both the three North American and the two Palearctic *Placobdella* species were derived from the respective cDNA or

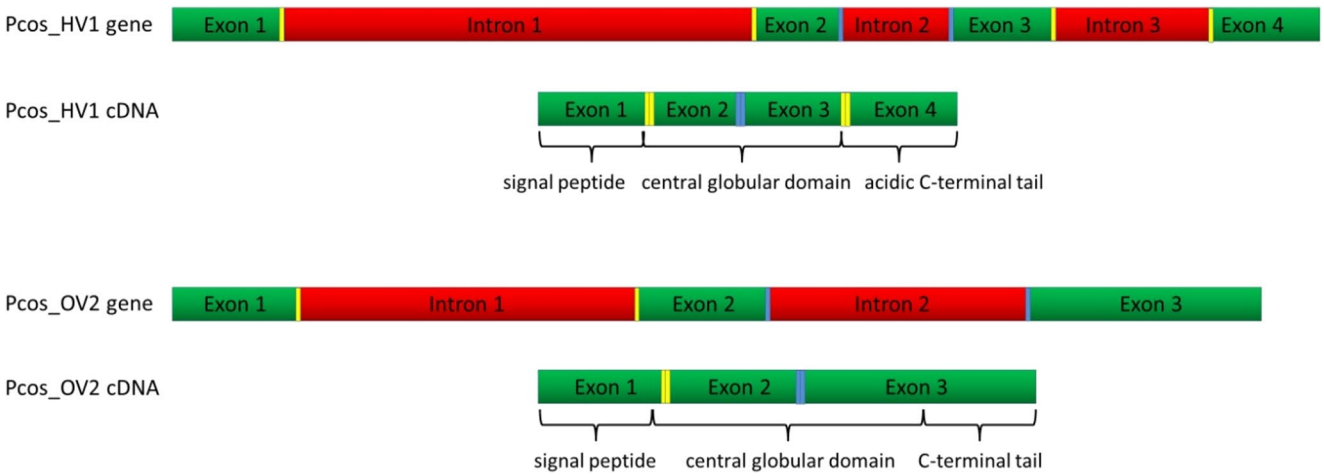

Fig. 1 Schematic representation of hirudin Pcos_HV1 and ornatin Pcos_OV2 gene and cDNA structures in *P. costata*. Exons are labeled in green, introns are labeled in red. Exon boundary-overlapping codons are labeled in yellow, non-overlapping codons are labeled in blue. Sizes are adjusted relative to the size of exon 1 of the Pcos_HV1 gene (see Table 2 for details)

Table 2 Comparison of putative hirudin (HV) and ornatin (OV) gene structures in *P. costata* (Pcos) and *P. nabeulensis* (Pnab)

Gene	Exon1	Intron1	Exon2	Intron2	Exon3	Intron3	Exon4
Pcos_HV1	61 bp	256 bp	50 bp	58 bp	58 bp	84 bp	62 bp
Pcos_HV2	61 bp	1540 bp	50 bp	85 bp	58 bp	429 bp	80 bp
Pcos_HV3	55 bp	344 bp	62 bp	71 bp	73 bp	151 bp	35 bp
Pnab_HV1	61 bp	255 bp	50 bp	58 bp	58 bp	84 bp	62 bp
Pnab_HV2	61 bp		50 bp	85 bp	58 bp		80 bp
Pnab_HV3	55 bp	335 bp	62 bp	75 bp	73 bp	151 bp	77 bp
Pcos_OV1a	70 bp	663 bp	65 bp	116 bp	114 bp		
Pcos_OV1b	70 bp	880 bp	65 bp	116 bp	114 bp		
Pcos_OV2	70 bp	183 bp	74 bp	140 bp	129 bp		
Pnab_OV1	70 bp		65 bp	116 bp	105 bp		
Pnab_OV2	70 bp	194 bp	74 bp	140 bp	138 bp		

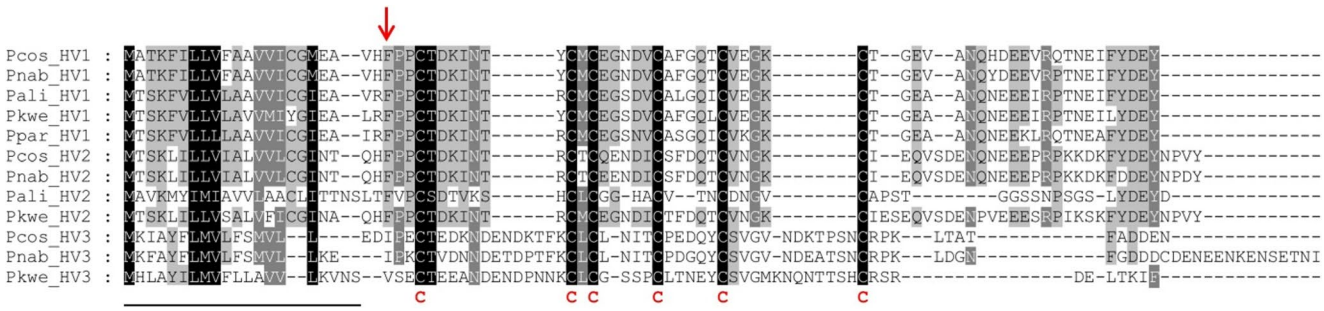

Fig. 2 Multiple amino acid sequence alignments of putative hirudin variants HV1-3 of *P. costata* (Pcos_HV1-3), HV1-3 of *P. nabeulensis* (Pnab_HV1-3), HV1 and HV2 of *P. ali* (Pali_HV1 and 2), HV1-3 of *P. kwetlumye* (Pkwe_HV1-3) and HV1 of *P. parasitica* (Ppar_HV1). A black background indicates fully conserved residues; a gray background indicates partially conserved residues. The six conserved cysteine residues giving rise to the three-dimensional structure of the central globular domain are marked in bold and red. The essential phenylalanine residue at position 3 is marked with an arrow. The signal peptide sequence is underlined. Abbreviations are used according to the IUPAC code

gene sequences. A multiple sequence alignment (MSA) of all putative hirudins is provided in Fig. 2, their biochemical features are summarized in Table 3.

All putative hirudins contain the six highly conserved cysteine residues, and both the lengths (49–72 amino acid residues without the signal peptides) and the acidic pI values (3.94–5.23) are in the range of functional hirudins. However, all HV3 variants apparently lack the essential phenylalanine (or tyrosin) residue at position 3 of the mature protein (see Fig. 2; Lazar et al. 1991).

Table 3 Structural and biochemical features of putative hirudin variants HV1-3 of *P. costata* (Pcos_HV1-3), HV1-3 of *P. nabeulensis* (Pnab_HV1-3), HV1 and HV2 of *P. ali* (Pali_HV1-2), HV1-3 of *P. kwetlumye* (Pkwe_HV1-3) and HV1 of *P. parasitica* (Ppar_HV1). The length is without the signal peptide sequence. *aa* amino acids, *MW* molecular weight, *kDa* kilodalton, *pI* isoelectric point

Factor	Length in aa	MW in kDa	pI value
Pcos_HV1	56	6382.99	4.25
Pcos_HV2	60	7149.79	4.49
Pcos_HV3	58	6532.12	4.12
Pnab_HV1	56	6378.01	4.09
Pnab_HV2	62	7304.85	4.27
Pnab_HV3	72	7979.53	3.94
Pali_HV1	56	6292.01	4.29
Pali_HV2	49	4970.41	4.34
Pkwe_HV1	56	6313.03	4.13
Pkwe_HV2	64	7426.18	4.38
Pkwe_HV3	54	6017.56	4.64
Ppar_HV1	56	6266.02	5.23

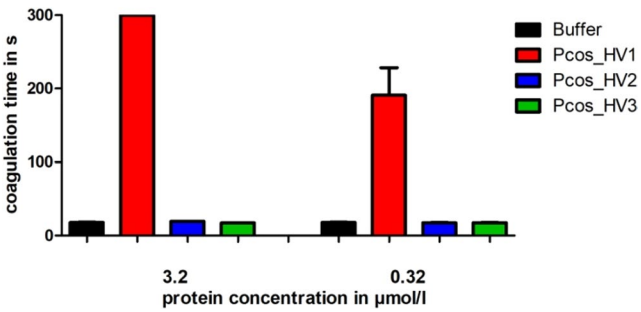

Fig. 3 Standard blood coagulation assays of putative hirudins Pcos_HV1-3 of *P. costata* using the thrombin time test (TT). All test compounds were used at final concentrations of 3.2 or 0.32 µmol/l, respectively. Results are the mean of three to four independent measurements, bars indicate standard deviation (SD)

For the functional characterization, we focused on the putative hirudins of *P. costata*. All three hirudin variants, namely Pcos_HV1-3, were expressed as recombinant proteins in *E. coli* strain DH5α and further processed as described in the Materials and Methods section. The thrombin time tests revealed that Pcos_HV1 displayed a high thrombin-inhibitory potency, but neither Pcos_HV2 nor Pcos_HV3 did negatively influence the activity of thrombin (Fig. 3). Pcos_HV1 can hence be termed a true hirudin, whereas Pcos_HV2 and Pcos_HV3 are rather HLFs.

Gain-of-function analyses of Pcos_HV2 and HV3

The N-terminus of hirudin is of special importance for its interaction with hirudin, and a replacement of the first hydrophobic valyl residue in hirudin variant HV1 of *H. medicinals* with polar amino acids results in a dramatic increase in the inhibition constant K_i (Wallace et al. 1989). Whereas Pcos_HV1 contains a valyl residue at position 1,

Table 4 Nomenclature and N-terminal amino acid sequences of wild-type and mutant variants of Pcos_HV2 and HV3

Factor	*N*-terminal sequence
Pcos_HV1	VHFPPC
Pcos_HV2	QHFPPC
Pcos_HV2V	VHFPPC
Pcos_HV2L	LHFPPC
Pcos_HV3	EDIPEC
Pcos_HV3Q	QHFPKC
Pcos_HV3V	VHFPPC
Pcos_HV3L	LHFPPC

both Pcos_HV2 and HV3 contain polar amino acid residues at the respective position: a glutamine residue (Q1) in Pcos_HV2 and a glutamyl residue (E1) in Pcos_HV3. Strikingly, within the N-terminal five amino acid residues of Pcos_HV2 only the Q1 residue differs from the N-terminus of Pcos_HV1 (see Fig. 2). A closer look at the conjunction of exons 1 and 2 of the Pcos_HV2 and Pnab_HV2 genes (see Fig. 1 and Supplementary Material Files S4 and S6) revealed that with only one nucleotide substitution the codons that encodes the polar glutamine residue Q1 (CAA) can be changed to codons that encode a hydrophobic leucine residue (CTA) and with two substitutions to codons that encode a hydrophobic valyl residue (GTA). To evaluate the effects of variations of the N-terminal amino acid sequences on the thrombin-inhibitory potency of Pcos_HV2 we hence constructed two variants that contain either a valyl residue (Pcos_HV2V) or a leucine residue (Pcos_HV2L) at position 1. In addition we constructed respective variants of Pcos_HV3 that contain either the N-terminal five amino acid residues of Pcos_HV2 (Pcos_HV3Q), Pcos_HV2V (Pcos_HV3V) or Pcos_HV2L (Pcos_HV3L), respectively. Table 4 lists all Pcos_HV2 and HV3 variants and their N-terminal amino acid sequences.

All variants of Pcos_HV2 and HV3 were expressed as recombinant proteins in *E. coli* strains DH5α and SHuffle T7® and further processed as described in the Materials and Methods section. The thrombin time tests revealed that both Pcos_HV2V and HV2L displayed moderate, but clearly detectable thrombin-inhibitory potencies, whereas none of the Pcos_HV3 variants negatively influenced the activity of thrombin. The inhibitory potencies of recombinant Pcos_HV2V and HV2L did not differ between the two *E. coli* strains (see Fig. 4 for the recombinant factors expressed in *E. coli* strain DH5α and Supplementary Information File Figure S1 for the recombinant factors expressed in *E. coli* strain SHuffle® T7). Interestingly, in both bacterial expression systems the leucine residue at position 1 conferred higher thrombin-inhibitory potencies of the respective recombinant Pcos_HV2 variants compared to the variants containing a valyl residue at position 1. It is worth to note in that context that lepirudin, a commercially available

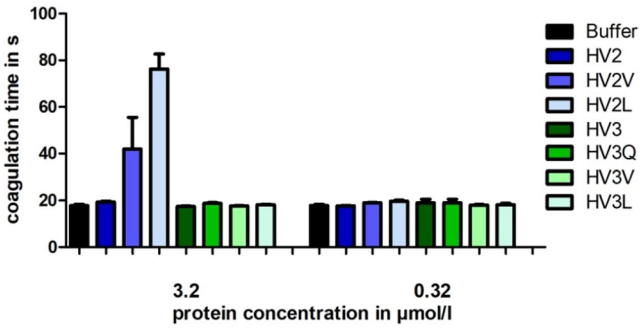

Fig. 4 Standard blood coagulation assays of Pcos_HV2 and HV3 variants of *P. costata* using the thrombin time test (TT). All factors were expressed in *E. coli* strain DH5α. All test compounds were used at final concentrations of 3.2 or 0.32 µmol/l, respectively. Results are the mean of three independent measurements, bars indicate standard deviation (SD)

Table 5 Structural and biochemical features of pitative ornatin variants OV1a, 1b and 2 of *P. costata* (Pcos_OV1a, 1b and 2), OV1 and 2 of *P. nabeulensis* (Pnab_OV1 and 2), OV1-3 of *P. ali* (Pali_OV1-3), OV1 of *P. kwetlumye* (Pkwe_OV1) and OV1 of *P. parasitica* (Ppar_OV1). The length is without the signal peptide sequence. *aa* amino acids, *MW* molecular weight, *kDa* kilodalton, *pI* isoelectric point

Factor	Length in aa	MW in kDa	pI value
Pcos_OV1a	62	7073.28	9.44
Pcos_OV1b	62	7104.33	9.41
Pcos_OV2	70	8358.83	9.50
Pnab_OV1	59	6733.86	9.25
Pnab_OV2	73	8560.07	9.15
Pali_OV1	50	5738.61	9.04
Pali_OV2	57	6404.56	9.54
Pali_OV3	56	6052.96	8.64
Pkwe_OV1	68	7634.84	9.80
Ppar_OV1	52	5722.23	4.90

recombinant hirudin (rec-Hirudin, HBW 023, Refludan®), is a variant of hirudin HV2 (hirudin-IT) of *H. medicinalis* (Harvey et al. 1986) and contains a leucine residue at position 1 instead the wildtype isoleucine residue (Markwardt 1994).

Structural and functional characterization of putative ornatins

The amino acid sequences of the putative ornatins of all five *Placobdella* species were derived from the respective cDNA or gene sequences. A multiple sequence alignment (MSA) of the putative ornatins is provided in Fig. 5, their biochemical features are summarized in Table 5.

All putative ornatins except Pnab_OV1 contain the six highly conserved cysteine residues, however, the essential RGD/KGD motif (Lazarus and McDowell 1993) is absent in Pnab_OV1, Pali_OV3 and Ppar_OV1 (see Fig. 5). The basic pI value of most putative ornatins except for Ppar_OV1 (see Table 5) is often observed in functional ornatins (Mazur et al. 1991). However, a basic pI does not seem to be an essential biochemical feature (Pfordt et al. 2022).

For the functional characterization we focused on the putative ornatins of *P. costata*. Again, Pcos_OV1a and OV2 were expressed as recombinant proteins in *E. coli* strain DH5α and further processed as described in the Materials and Methods section. Platelet function analyzed by light transmission aggregometry, however, revealed that none of the factors exhibited an inhibitory effect on platelet aggregation (Fig. 6A und C). To evaluate whether or not the genetic background of the bacterial host strain may have negatively influenced the correct folding and hence the activity of the recombinant ornatins Pcos_OV1a and 2, we repeated their expression and purification, but used the *E. coli* strain SHuffle® T7 as an alternative host. The strain was specifically developed to improve the correct formation of disulfide bonds in recombinant proteins (Lobstein et al. 2012). Indeed, the recombinant ornatins Pcos_OV1a and 2 that were produced by *E. coli* strain SHuffle® T7 negatively affected ADP-induced platelet aggregation, both in terms of shape of the curve and the maximal aggregation value (Fig. 6B). A statistical analyses revealed that the area under the curve (AUC) values of both putative ornatins Pcos_OV1a and Pcos_OV2 significantly differed from the negative control

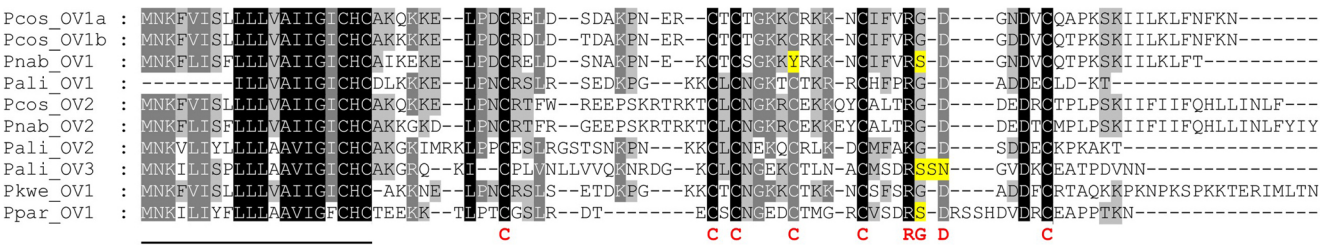

Fig. 5 Multiple amino acid sequence alignments of putative ornatin variants OV1a, 1b and 2 of *P. costata* (Pcos_OV1a, 1b and 2), OV1 and 2 of *P. nabeulensis* (Pnab_OV1 and 2), OV1-3 of *P. ali* (Pali_OV1-3), OV1 of *P. kwetlumye* (Pkwe_OV1) and OV1 of *P. parasitica* (Ppar_OV1). A black background indicates fully conserved residues; a gray background indicates partially conserved residues. The six conserved cysteine residues giving rise to the three-dimensional structure of the central globular domain and the RGD motif are marked in bold and red, divergent residues are labeled in yellow. The signal peptide sequence is underlined. Abbreviations are used according to the IUPAC code

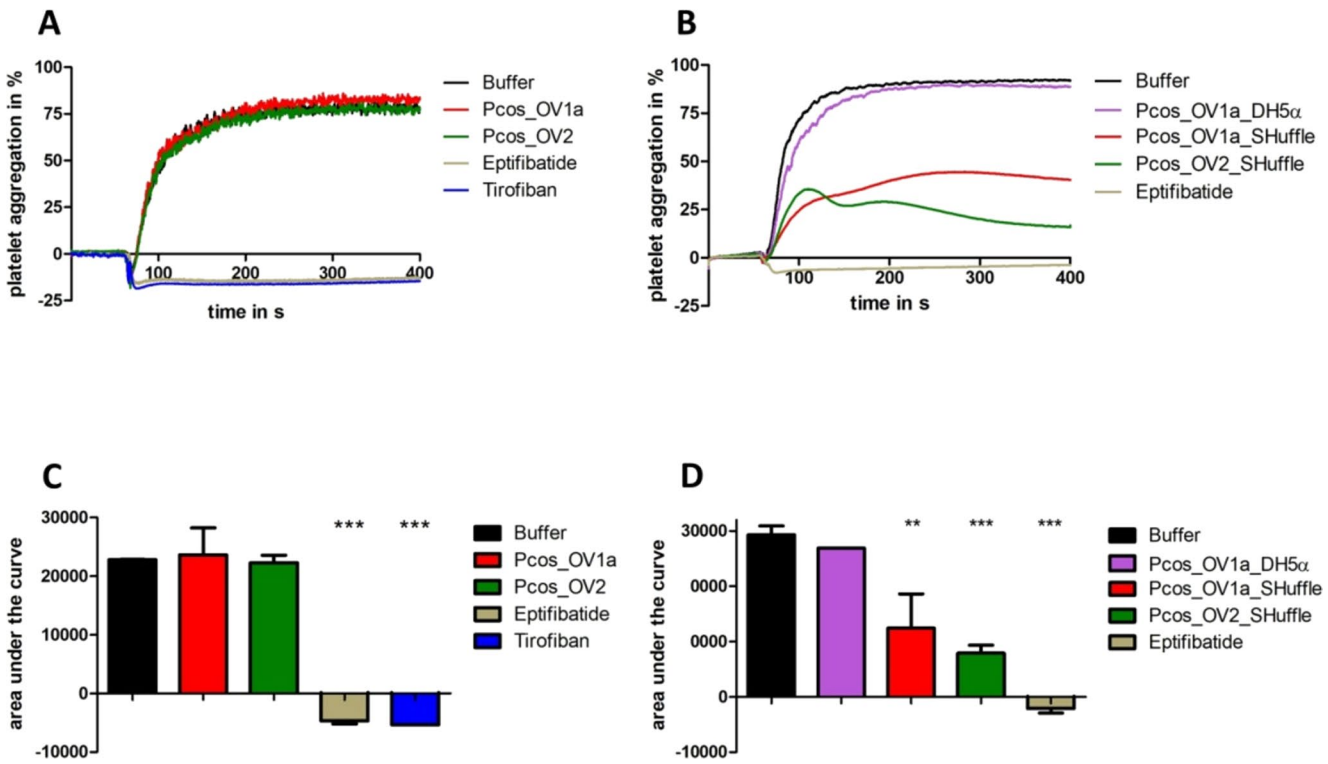

Fig. 6 Standard platelet aggregation assays of putative decorsin variants Pcos_OV1a and Pcos_OV2 of *P. costata* expressed in *E. coli* strains DH5α (A+C) or SHuffle® T7 (B+D). A and B display the aggregation curves, B and D the derived AUC values. Tirofiban and eptifibatide were used as positive control substances for the complete inhibition of aggregation; buffer was used as a negative control. All test compounds and the control substances were used at a final concentration of 3.2 µmol/l. Platelet aggregation was induced by the addition of adenosine diphosphate (ADP) in a final concentration of 5 µmol/l. Results are the mean of two to four independent measurements, bars indicate SD. ** indicates a p-value≤0.01, *** indicates p-values≤0.001

samples (buffer) with p-values≤0.01 (Pcos_OV1a) and ≤0.001 (Pcos_OV2), respectively (Fig. 6D). Both factors can hence be considered as active ornatins.

Discussion

Leeches are frequently used for medical purposes, but the term "medicinal leeches" does not refer to a specific taxon of leeches and is rather a description of their anthropogenic use. So far, almost exclusively jawed (or arhynchobdellid) leeches (Hirudiniformes) have been used by humans to treat various maladies including cardiovascular disorders, wound healing and problems associated with plastic reconstructive surgery (Singh 2010; Hackenberger and Janis 2019; Nair et al. 2022). Consequently, in-depth investigations of the therapeutic potential of proboscis-bearing leeches (especially Glossiphoniidae) are rare. Notably, leeches of the glossiphoniid genus *Placobdella* comprise a broad variety of anticoagulants including putative hirudins and ornatins (Siddall et al. 2016), yet detailed analyses of their biochemical properties and functional characterizations are still pending. In a series of previous studies we have already determined the structures of hirudin, hirudin-like factor and decorsin/ornatin genes in representatives of various arhynchobdellid leech species including the European medicinal leeches *H. medicinalis*, *H. verbana*, *H. orientalis* and *H. troctina* (Müller et al. 2016; Ben Ahmed et al. 2024), the Asian medicinal leeches *Hirudinaria manillensis* Lesson, 1842, *Whitmania pigra* Whitman, 1884, and *Hirudo nipponia* (Müller et al. 2017, 2022; Tolksdorf et al. 2025; Kalatehjari et al. 2026) and the North American medicinal leech *Macrobdella decora* (Müller et al. 2019). The generation of genome sequence data of two Palearctic species of the genus *Placobdella* enabled us to conduct respective analyses in representatives of glossiphoniid leeches. The primary aim of the present study was to take a step forward on the track to close the "analytical gap" between glossiphoniid and arhynchobdellid leech species. Transcriptomic sequence data of three North American species and genome sequence data of the two Palearctic representatives of the genus *Placobdella* were analyzed for the presence of putative hirudin and ornatin cDNAs or genes. In all five leech species, respective coding sequences for both factors could be identified (see Figs. 2 and 5). Whereas hirudins are present in all hematophagous leech species analyzed

so far and may even occur in predatory leeches (Müller et al. 2022), decorsins/ornatins are apparently absent in European members of the genus *Hirudo* (Babenko et al. 2020; Kvist et al. 2020) and in the African medicinal leech *Asiaticobdella fenestrata* (Schulz et al. 2025) and may hence have been lost during evolution. However, the verification of hirudin and ornatin genes in representatives of the glossiphoniid leech genus *Placobdella* indicates that independent genes of both types of anticoagulants were probably present already in the last common ancestor (LCA) of Hirudinida (or true leeches) (Tessler et al. 2018; Kuo and Lai 2019; de Carle et al. 2025). Strong support for this assumption comes from the observation that the gene structures of both the hirudin and the decorsin/ornatin genes are highly conserved in glossiphoniid and arhynchobdellid leeches including the exact position of introns and the presence of exon-boundary overlapping and non-overlapping codons (see Fig. 1; Müller et al. 2016; Müller et al. 2019; Lukas et al. 2022). Whether or not the LCA of the hirudin and ornatin genes dates back even further in time and is already present in Hirudinea Lamarck, 1818, a group that also includes the family Piscicolidae Johnston, 1865, and the two orders Acanthobdellida Livanow, 1905, and Branchiobdellida Holt, 1965 (de Carle et al. 2025), still remains to be defined.

A recurring feature in hematophagous leeches is the presence of multiple copies of anticoagulant genes including genes that encode hirudins and ornatins/decorsins (Liu et al. 2023; Zhao et al. 2024; Khan et al. 2025; Kalatehjari et al. 2026), very likely to prevent deleterious effects of occasional gene loss events. The genome sequence data of *P. costata* only partially confirm this observation with the presence of just a single gene that encodes a functional hirudin (Pcos_HV1)and three genes that encode functional ornatins (Pcos_OV1a/b and OV2) (see Fig. 2+3 and 5+6). However, with only a single amino acid substitution (Q1 to either V1 or L1) Pcos_HV2 can gain a moderate thrombin-inhibitory potency (see Fig. 4). We can only speculate whether the current situation of Pcos_HV2 is the consequence of a past loss-of-function event (GTA ◊ CTA ◊ CAA) or primes the leech for a future gain-of-function event (CAA ◊ CTA ◊ GTA), but each scenario is not mutually exclusive. Unfortunately, the biological targets of both Pcos_HV2 and HV3 remain obscure and are difficult to identify.

In *P. nabeulensis* the Pnab_OV1 gene encodes a factor that lacks the fourth conserved cysteine residue and may hence be inaccurately folded (see Fig. 5). With that. *P. nabeulensis* may contain only single genes that encode a functional hirudin (Pnab_HV1) and a functional ornatin (Pnab_OV2). Interestingly, both Pnab_OV1 and Ppar_OV1 of *P. parasitica* comprise RSD motifs instead of the canonical RGD/KGD motif that facilitates integrin binding (Lazarus and McDowell 1993; Reiss et al. 2006). An RSD motif may be functionally equivalent to an RGD motif (Van de Walle et al. 2008), but this remains to be tested for ornatins. Based on the current status of investigations, neither *P. costata* nor *P. nabeulensis* contain genes that encode putative multimeric hirudins/HLFs or decorsins.

The recombinant expression of putatve hirudins and decorsins/ornatins in bacterial hosts for subsequent functional analyses is well established in our lab. In a recent publication we compared two *E. coli* strains with different characteristics, namely DH5α (Hanahan 1985) and SHuffle® T7 (Lobstein et al. 2012). The strain SHuffle® T7 supports the formation of disulfide bonds, generally a crucial step in correct folding of cystein-containing proteins (Feige et al. 2018) and in correct folding of members of the hirudin superfamily in particular (Dodt et al. 1985; Mazur et al. 1993; Micheletti et al. 2003; Wu et al. 2013). We deciphered that a preparation of recombinant hirudin HV1 of *H. medicinalis* (Hmed_HV1) that was expressed in SHuffle® T7 at its optimal cultivation temperature of 30 °C revealed a higher thrombin inhibitory potency compared to a preparation of Hmed_HV1 that was expressed in DH5α at its optimal cultivation temperature of 37 °C (Wang et al. 2025). The current study partially supports this observation: the putative ornatins Pcos_OV1a and OV2 did not exhibit platelet aggregation inhibitory potencies when both factors were expressed as recombinant proteins in *E. coli* strain DH5α at 37 °C. However, platelet aggregation was negatively affected when both factors were expressed in *E. coli* strain SHuffle® T7 at 30 °C (see Fig. 6). In contrast, the thrombin-inhibitory potencies of the recombinant Pcos_HV2V and HV2L variants did not differ between the two bacterial expression systems (see Fig. 4 and Supplementary Information Figure S1). Nevertheless, it might be a reasonable option to consequently use the strain SHuffle® T7 for the expression of recombinant putative hirudins, ornatins and other cysteine-rich proteins.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00709-026-02243-5.

Acknowledgements The authors are thankful to the colleagues who generated and provided the genome and transcriptome datasets explored in the present study. In addition, we would like to thank all members of the Animal Physiology Working Group at the University of Greifswald for their help and support. Special thanks go to Jan-Peter Hildebrandt and Undine Lauf for their critical reading of the manuscript.

Author contributions C.M. conceived the ideas, designed the methodology and performed the sequence-based investigations; R.B.A. and C.G. collected the animals; R.B.A., C.M., C.T. and R.W. performed the experiments; C.M.,R.B.A. and S.K. analyzed the data and drafted the manuscript; C.M., B.H.R. and G.J. supervised the experimental work.

Funding Open Access funding enabled and organized by Projekt DEAL.

Data availability Reference genome chromosome-level sequence data of P. costata are deposited in GenBank under the accession numbers OZ194472.1 - OZ194489.1. Information on specific gene locations are provided either within the manuscript or as Supplementary Information Files. The raw genome sequence data of P. costata and P. nabeulensis generated in the present study were deposited in BioStudies under the following accession number: S-BSST2339 (DOI: 10.6019/S-BSST2339). Images that illustrate the expression, purification and processing of all recombinant factors are available on request from the corresponding author.

Declarations

Ethical approval The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the University Medicine Greifswald (protocol code BB 20/12, approval date 25 July 2018) and by the Institutional Review Board (or Ethics Committee) of the University Medicine Oldenburg, Carl von Ossietzky University Oldenburg (ethics vote 2024-034, approval date 27 March 2024).

Consent to participate and consent for publication Written informed consent was obtained from all subjects involved in the study.

Competing interests The authors declare no competing interests.

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