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Cloning, characterization, and heterologous expression of a candidate Hirudin gene from the salivary gland transcriptome of *Hirudo nipponia*

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Hirudin is a pharmacologically active substance in leeches with potent blood anticoagulation properties. Although recombinant hirudin production isolated from *Hirudo medicinalis* Linnaeus and *Hirudinaria manillensis* Lesson is known, to our knowledge, this study is the first to report recombinant hirudin expression and production from *Hirudo nipponia* Whitman. Thus, the present study aimed to clone and characterize the full-length cDNA of a candidate hirudin gene (c16237_g1), which is localized on the salivary gland transcriptome of *H. nipponia*, and further evaluate its recombinant production using a eukaryotic expression system. The 489-bp cDNA possessed several properties of the hirudin “core” motifs associated with binding to the thrombin catalytic pocket. A fusion expression vector (pPIC9K-hirudin) was constructed and successfully transformed into *Pichia pastoris* strain GS115 via electroporation. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blot analysis confirmed hirudin expression. The recombinant protein was expressed with a yield of 6.68 mg/L culture. Mass spectrometry analysis further confirmed target protein expression. The concentration and antithrombin activity of purified hirudin were 1.67 mg/mL and 14,000 ATU/mL, respectively. These findings provide a basis for further elucidating the molecular anticoagulation mechanism of hirudin, and address China’s growing market demand for engineered *H. nipponia*-derived hirudin and hirudin-based drugs.

Hirudo nipponia is a blood-sucking leech that has been reported to possess substantial medicinal value in traditional Chinese medicine, which is first recorded in the classic book on Chinese Materia Medica, *Shen-Nong-Ben-Cao-Jing* (ca. 100 AD)^{1–3}. *H. nipponia* has been widely used to treat cardiovascular and cerebrovascular diseases, as well as cerebral thrombosis, coronary heart disease, and cerebral edema^{4–6}. It is also listed in the Pharmacopoeia of the People’s Republic of China owing to its powerful antithrombin activity⁷.

One of the main pharmacologically active substances in blood-sucking leeches is hirudin, which is a naturally occurring peptide with blood anticoagulant property, produced in the salivary glands of *H. nipponia*. It is the strongest natural thrombin-specific inhibitor identified to date^{8–11}. Hirudin and several hirudin analogues possess a specific anticoagulant effect and are widely used in clinical settings. Among these, two recombinant hirudins and a hirudin analogue have gained marketing approval from the United States Food and Drug Administration, for various applications¹². The anticoagulant activity of *H. nipponia* is significantly higher than those of the other two leech species (*Whitmania pigra* Whitman and *Whitmania acranulata* Whitman) listed in the Pharmacopoeia of the People’s Republic of China¹³.

Wild *H. nipponia* species have experienced a sharp population decline due to environmental pollution, over-harvesting, and large-scale habitat destruction, resulting in the depletion of raw material resources to meet the high demand for hirudin^{13,14}. Despite progress in artificial breeding, the commercial production of

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this medicinal leech is yet to be realized¹³. Thus, safe and effective methods to produce hirudin are needed to prevent endangering natural wild *H. nipponia*, thereby preventing the depletion of natural resources while simultaneously promoting the modernization of traditional Chinese medicine. Hence, utilizing genetically engineered microorganisms to produce recombinant hirudin is a potentially effective strategy^{15–17}.

Natural hirudin exists in three isoforms (HV1, HV2, and HV3), comprising 65 or 66 amino acid residues, with a molecular weight of approximately 7000 Da^{18–22}. Recently, genetic recombinant technology has been used to meet pharmacological and clinical demands through bacterial and eukaryotic expression systems, including *Escherichia coli* and *Pichia pastoris*, which secrete recombinant hirudin isolated from *H. medicinalis* and *Hirudinaria manillensis* Lesson^{15–18,23–25}. However, few studies have focused on expressing the hirudin gene and the production of recombinant hirudin from *H. nipponia* using molecular biotechnology techniques^{26,27}.

In a previous study²⁷, we showed that a candidate hirudin gene (c16237_g1) is localized on the salivary gland transcriptome of *H. nipponia*. Accordingly, the present study aimed to clone and characterize the full-length cDNA sequence of c16237_g1. We further aimed to evaluate the use of a eukaryotic expression system for recombinant hirudin production. To the best of our knowledge, this is the first report of expressing recombinant hirudin protein from *H. nipponia*. Therefore, this study will lay the foundation for further research in determining the molecular anticoagulation mechanism of hirudin. It will also address China's current market requirement for engineered *H. nipponia* hirudin and meet the huge demand for hirudin-based clinical drugs, including products targeting important cardiovascular and cerebrovascular diseases.

Results

Molecular cloning of the hirudin gene in *H. nipponia*. Based on the annotated hirudin transcript (c16237_g1) (sequence shown in Supplementary data) in the salivary gland transcriptome data of *H. nipponia*, one intermediate fragment of 271 bp, a 201-bp fragment amplified using 5'-RACE, and a 217-bp fragment using 3'-RACE (Fig. 1) were assembled using DNASTAR ver. 7.1 to obtain the full-length sequence of the hirudin gene in *H. nipponia*. The full-length cDNA of hirudin was 489 bp, including a 5'-terminal untranslated region (UTR) of 123 bp, a 3'-terminal UTR of 114 bp, and a 252-bp open reading frame (ORF) encoding an 83-aa protein polypeptide. The nucleotide and predicted amino acid sequences of the full-length cDNA are shown in Fig. 2. The complete hirudin cDNA sequence has been deposited in the GenBank database under accession number GenBank MN 116511.

Sequence analysis and multiple sequence alignment of hirudin. The 489-bp full-length cDNA sequence encoded an 83-aa hirudin protein, containing a 20-aa N-terminal signal peptide and 63-aa mature protein sequence (Fig. 2). After removing the signal peptide, the predicted molecular weight and the theoretical isoelectric point (pI) of the mature protein were 6.97 kDa and 6.72, respectively. The protein contained eight negatively charged residues (Asp and Glu) and eight positively charged residues (Arg and Lys). The aliphatic index was 37.14 and the grand average of hydropathicity was -1.070 .

Homologous protein search using the National Center for Biotechnology Information (NCBI)-blastp tool in GenBank revealed 62, 60, 54, 53, 53, 52, and 48% similarities to the *H. medicinalis* hirudin variant HV3 (PA) (ALA22935.1), *H. manillensis* hirudin variant HV1 (P81492.2), *H. medicinalis* hirudin variant HV1 (VV) (ALA22934.1), *H. medicinalis* hirudin variant HV2 (ALA14576.1), *H. verbana* hirudin variant HV1-VV (APA20831.1), *H. orientalis* hirudin variant HV3-PAF (APA20857.1), *H. manillensis* hirudin-HM1 (Q07558.1), and *Poecilobdella manillensis* hirudin (CAA51293.1), respectively. The multiple sequence alignment of hirudin is shown in Fig. 3, in accordance with the known structural information in the hirudin database^{26–28}. The highly conserved motif was ³⁴CLC³⁶; the GSNV region was conservatively replaced by the chemically similar ³⁸NSNL⁴¹ sequence in *H. nipponia*; and six cysteines residues, presumably involved in three disulfide bonds, were also found to be evolutionarily conserved.

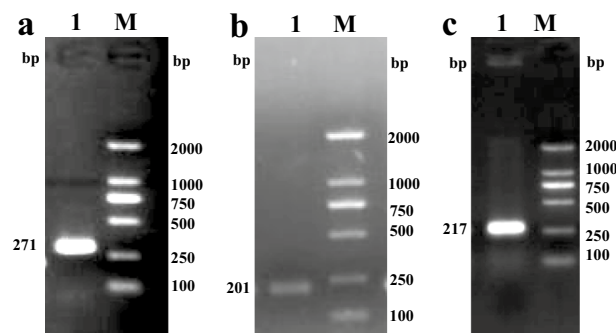


Figure 1. Amplification of a candidate hirudin gene from the salivary gland transcriptome of *Hirudo nipponia*. (a) Intermediate fragment amplification. (b) 5'-rapid amplification of cDNA ends (RACE) amplification. (c) 3'-RACE amplification (1: Target fragment; M: Marker DL2000). The polymerase chain reaction (PCR) products were analyzed on a 1% agarose gel. The original gel is presented in Fig. S1.

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TGTTCCTTTATTCGCGAGTGTAGTCTTAGCGGGGGCCGGTGCCTGTCCGAGGCGGGCTGCTT
CGCGCGCGGGTTGGGGGGAGCCTTTCAGGATCTCAATCGGATCTGAAAAAATCTCAACG
ATGTTCTCTCTGAAATGTTTCGTCGTCTGTGGCAGTTTGCATCTGCACGTCTCAAGCT
M F S L K L F V V L L A V C I C T S Q A

CAGCATTCAAAGATTGCTCAGACAGCAATCCGACTCCATGCTTGTGCGAAAAATAGTAAT
Q H F K D C S D S N P T P C L C E N S N

CTCTGTGCTTTTGGTAACACTGTGTATCTGGGCCACCAAGAAATGCATCATAAAAGTA
L C A F G N T C D L G P P K K C I I K V

TCACCACCTCCACCTCGGAGAAAAGAAAAATAACAACAAAGGAAGTAAATCTGATTAC
S P P P T S E K E K N N N K G S K S D Y

GATTATTATTAACAACCCAGAATAAGCACC AATGCAGATAAATGGTTTTAAACGTTT
D Y Y *

CCACGGTGTGATCAATAAACGACGTTTTTCAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AA
    
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Figure 2. Full-length cDNA sequence of hirudin. (The signal peptide is marked in red; the initiation codon is shown in bold; the termination codon is indicated by an asterisk; 5'-terminal untranslated region (UTR) is shown in box; 3'-terminal UTR is marked with a straight line; and the other parts represent the mature hirudin amino acid sequence).

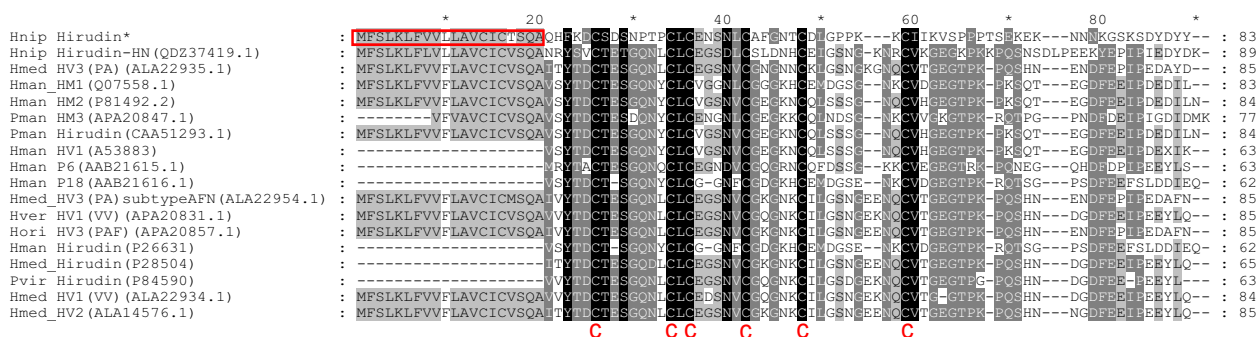


Figure 3. Alignment of hirudin protein amino acid sequences (*: candidate sequence from the present study; red box: predicted secretory signal peptides; C conserved cysteines residues, *Hnirp* *Hirudo nipponia*, *Hmed* *H. medicinalis*, *Hman* *Hirudinaria manillensis*, *Pman* *Poecilobdella manillensis*, *Hver* *H. verbena*, *Hori* *H. orientalis*, *Pvir* *Poecilobdella viridis*).

Restriction analysis of the recombinant plasmid pPIC9K-hirudin. The positive transformants were screened and digested using EcoRI and NotI. Following double enzyme digestion, two bands were observed because the recombinant plasmid was 9276 bp in length and the target gene was approximately 217 bp in length. These results indicated that the recombinant plasmid pPIC9K-hirudin had been constructed successfully (Fig. 4).

Polymerase chain reaction detection of the recombinant yeast transformant GS115/pPIC9K-hirudin. Primers targeting the yeast alcohol oxidase 1 (*AOX1*) gene were used for polymerase chain reaction (PCR) amplification. Figure 5 shows two bands corresponding to sequences that were completely amplified in *P. pastoris* GS115/pPIC9K and GS115/pPIC9K-hirudin. A band of approximately the same size (~2200 bp) was identified as the *P. pastoris* GS115 *AOX1* gene^{29,30} along with a copy of the recombinant integrated target gene of approximately 720 bp (Fig. 5b). Thus, the PCR and sequencing results (shown in Supplementary sequence S2) demonstrated successful transformation of the recombinant plasmid pPIC9K-hirudin into *P. pastoris* GS115 cells.

Target protein quantification and purification. Samples were analyzed via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting after 24, 48, and 72 h induction. SDS-PAGE analysis, revealed a product purity of 95%. As indicated in Fig. 6, specific bands were visualized at approximately 15 kDa, thereby revealing successful target protein expression in the yeast culture supernatants. After expanded culture, induction, and purification, hirudin was successfully obtained in the supernatant, as determined using 15% SDS-PAGE (Fig. 7a). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) results revealed the precise amino acid sequence for the purified protein (Fig. 7b). The spectrum showed a clearly visible peak at the position corresponding to the expected molecular mass of hirudin (Fig. 7c). After obtaining

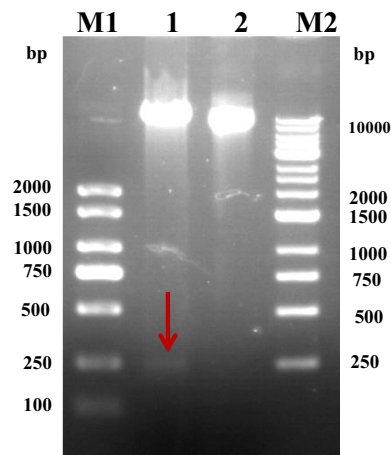


Figure 4. Analysis of the recombinant plasmid pPIC9K-hirudin through double enzyme digestion (Lane M1; Marker 2000; Lane 1: recombinant plasmid digested with EcoRI and NotI; Lane 2: recombinant plasmid; Lane M2: Marker DL 10,000). The target gene is indicated using a red arrow (217 bp). The products were analyzed on a 1% agarose gel. The original gel is presented in Fig. S2.

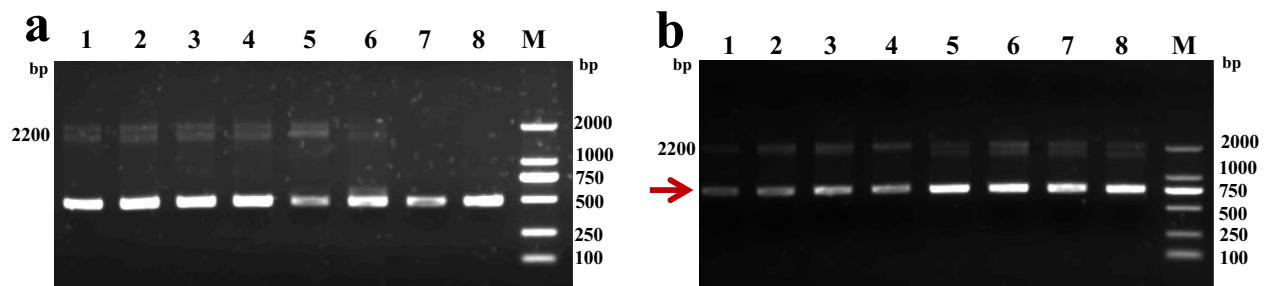


Figure 5. PCR detection of *Pichia pastoris* GS115 transformation with the recombinant plasmid pPIC9K-Hirudin. (a) *P. pastoris* GS115 cells transformed with the empty pPIC9K plasmid (GS115/pPIC9K). (b) *P. pastoris* GS115 transformants with pPIC9K-hirudin (GS115/pPIC9K-hirudin) (M: Marker DL2000). The recombinant integrated target gene is indicated using a red arrow (~720 bp). The PCR products were analyzed on a 1.5% agarose gel. The original gel is presented in Fig. S3.

an expression yield of 6.68 mg/L culture, the purified protein concentration was found to be 1.67 mg/mL, using the Bradford method³¹.

Antithrombin activity of hirudin. The antithrombin activity of purified hirudin and the commercially available product were 14,000 antithrombin units (ATU)/mL and 12,000 ATU/mL, respectively.

Discussion

The present study describes the successful expression and production of recombinant hirudin from *H. nipponia*. The hirudin expression yield was found to be considerably higher than that reports for other hirudin variants or hirudin in leeches^{26,32}. Recombinant hirudin was successfully obtained in the purified form, with the antithrombin activity comparable to commercially available hirudin.

Hirudin is currently the strongest known natural thrombin inhibitor, which interacts with thrombin to form a tight equimolar complex^{33–36}. The structure of a protein determines its physicochemical properties and biological functions, and disulfide bonds play a critical role in protein stability by cross-linking different regions of polypeptide chains³⁷. The three-dimensional structure of hirudin shows a tightly folded N-terminal globular region formed by three disulfide bonds and a flexible C-terminal tail³⁸. Our analysis revealed that the putative hirudin protein was 6.97 kDa with six cysteine residues, which presumably form three pairs of disulfide bonds, and thus, might greatly strengthen and stabilize the tertiary structure of the mature protein³⁹. In addition, multiple sequence alignment confirmed the presence of a highly conserved “core” motif (³⁴CLC³⁶) in the *H. nipponia* sequence, which is associated with hirudin’s binding to the active center of thrombin to block its hydrolytic activity⁴⁰. Natural hirudin loses its anticoagulant properties on degradation or oxidation of the disulfide bonds³⁸.

The methylotrophic yeast *P. pastoris* has been widely used, particularly after the release of its genome sequence in 2009, for producing various heterologous proteins⁴¹. More than 500 proteins have been successfully expressed

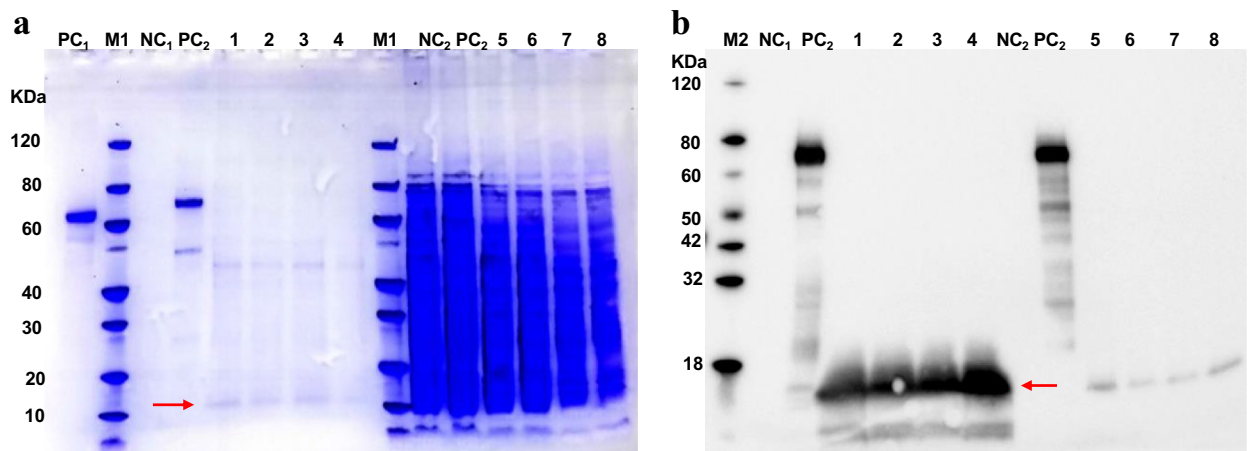


Figure 6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting results of the induced products. **(a)** SDS–PAGE analysis of pPIC9K–hirudin expression in recombinant *Pichia pastoris*. **(b)** Western blot analysis of recombinant hirudin. (Lane M1: Protein MW marker (Broad); Lane M2: protein Marker (GenScript, Cat. No. M00673); Lane PC₁: bovine serum albumin (1 µg); Lane PC₂: positive control (GenScript, Cat. No. M0101); Lane NC₁: medium without induction; Lane NC₂: cell pellet without induction. Lane 1: supernatants from GS115/pPIC9K–hirudin after 24 h induction; Lane 2: Supernatants from GS115/pPIC9K–hirudin after 48 h induction; Lanes 3, 4: Supernatants from GS115/pPIC9K–hirudin after 72 h induction; Lane 5: Precipitate from GS115/pPIC9K–hirudin after 24 h induction; Lane 6: Precipitate from GS115/pPIC9K–hirudin after 48 h induction; Lanes 7, 8: Precipitate from GS115/pPIC9K–hirudin after 72 h induction.) The target protein is indicated using a red arrow (~ 15 kDa). Recombinant hirudin was analyzed on a 15% SDS–PAGE gel. The original gel is presented in Fig. S4.

in *P. pastoris* to date, including a variety of proteins with high medicinal or economic value, such as antibodies and vaccines⁴². High secretory capacity, a strong AOX promoter, and the presence of a glycosylation pathway are three distinct advantages of yeast expression systems over other eukaryotic systems^{43–45}. In this study, the pPIC9K–hirudin recombinant plasmid was successfully constructed and effectively expressed in *P. pastoris* GS115. In addition, the target protein was secreted extracellularly, which further aided protein purification. However, the size of the target protein was not consistent with the putative molecular weight of hirudin, as determined using SDS–PAGE. The factors influencing exogenous gene expression in *P. pastoris* are complex and diverse, such as the structural, physical, and chemical characteristics of the exogenous protein; transformant phenotype; and the induction conditions³⁰. Owing to the structural characteristics of hirudin, which predominantly exists as dimers or polymers, a multimeric aggregate appears on analysis using SDS–PAGE^{46–48}. Thus, dimer formation may account for the molecular weight of hirudin protein observed in the present study, which was considerably higher than the expected molecular weight.

The thrombin titration method is commonly used to determine the specific activity of recombinant hirudin^{6,9,10}. Several complex and varied factors, including the temperature, incubation time, and sample processing methods^{6,15,29}, have been identified during the assay to determine antithrombin activity. Our results showed that the antithrombin activity of purified hirudin was 14,000 ATU/mL, which was higher than that obtained for commercially available hirudin products.

Conclusions

In conclusion, a candidate hirudin gene was amplified from the salivary gland transcriptome of *H. nipponia*, and the resulting predicted amino acid sequence was characterized. Furthermore, a eukaryotic hirudin expression plasmid, *P. pastoris* GS115/pPIC9K–hirudin, was successfully constructed and characterized using SDS–PAGE and western blotting. Mass spectrometry analysis further confirms successful protein expression. The recombinant protein was expressed with a yield of 6.68 mg/L culture. The concentration and antithrombin activity of the purified hirudin was 1.67 mg/mL and 14,000 ATU/mL, respectively. These results lay the foundation for future studies to evaluate the structure and properties of hirudin, the development of anticoagulant drugs, and the large-scale production of purified, mature hirudin protein for commercial and medicinal applications.

Methods

Enzymes, vectors, media, and strains. T4 DNA ligase, KOD-Plus-Neo polymerase, EcoRI, NotI, and SacI were purchased from Bao Bioengineering, Co., Ltd. (Dalian, China). PMD18-T vector, pPIC9K vector, and *E. coli* DH5α were purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Minimal dextrose (MD), buffered glycerol-complex (BMGY), and buffered methanol-complex (BMMY) medium were prepared according to the recipe provided in the Invitrogen *Pichia pastoris* expression kit instruction manual.

Animals and salivary tissue sample collection. All leeches were obtained from an adult *H. nipponia* colony grown in a medical leech breeding base of the Chongqing Academy of Chinese Materia Medica

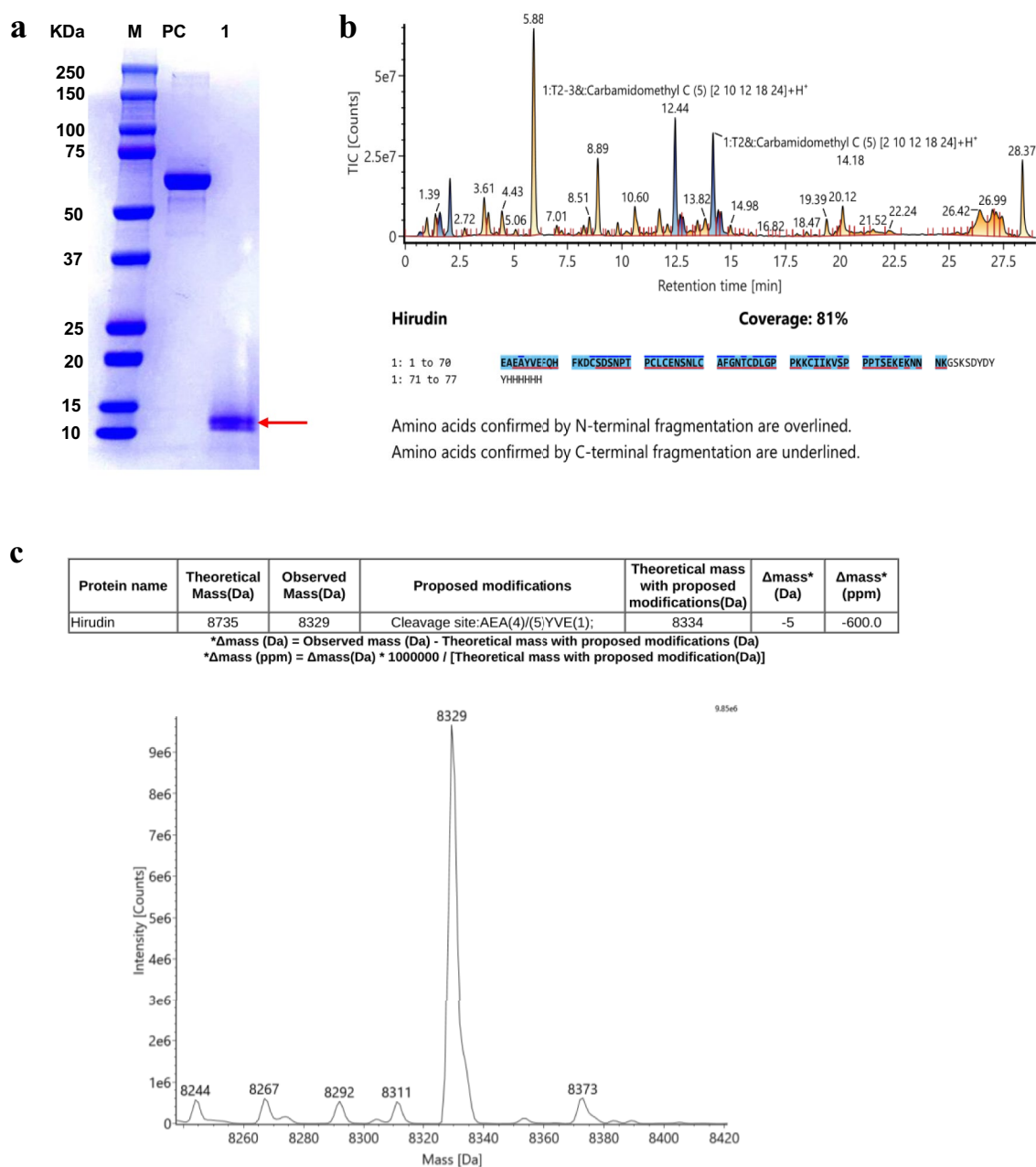


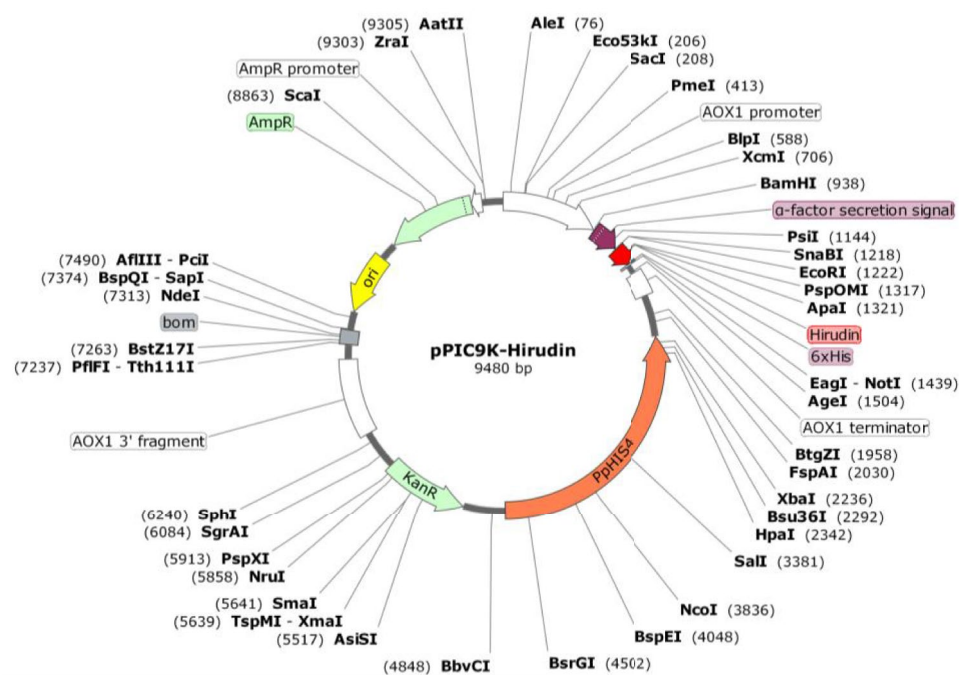
Figure 7. SDS-PAGE and mass spectrometric analysis of the purified protein. (a) SDS-PAGE detection of purified hirudin (M: Protein marker (Bio-rad, Cat. No. 1610374S). PC: BSA (2 μ g). Lane 1: Purified hirudin.) (b) Tryptic digestion peptides and sequence of hirudin. (c) Liquid chromatography-tandem mass spectrometry detection of hirudin. The peak represents a mass of 8329 Da. Purified hirudin was analyzed on a 15% SDS-PAGE gel. The original gel is presented in Fig. S5.

(Chongqing, China). Fifty healthy leeches were maintained in an aquaculture net cage filled with 15 L of dechlorinated tap water at 20–22 °C with a 12 h light/dark cycle prior to dissection. Every 5 days, half of the water was replaced with fresh water. Salivary tissue masses lying posterior to the three muscular jaws were removed aseptically using a sterilized dissecting tool; subsequently, they were rinsed in 0.5% bleach for 1 min followed by rinsing in deionized water for 1 min^{49–51}. The tissues were then stored in RNAlater at – 80 °C (Qiagen, Hilden, Germany), according to the manufacturer’s specifications.

RNA extraction and cDNA preparation. Total RNA was extracted from the aforementioned salivary tissues of *H. nipponia* using Trizol reagent (Tiangen, Beijing, China), according to the manufacturer’s instructions. RNA quality was assessed by electrophoresing on a 1.0% agarose gel, and the RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Species	Protein	Protein accession number	Identity (%)*	E-value	References**
<i>Hirudo nipponia</i>	Hirudin-HN	QDZ37419.1	57	6e-21	²⁶
<i>Hirudo medicinalis</i>	HV2	ALA14576.1	53	2e-22	⁵²
<i>Hirudo medicinalis</i>	HV3-PA	ALA22935.1	52	2e-23	⁵²
<i>Hirudo orientalis</i>	HV3-PAF	APA20857.1	50	1e-22	⁵³
<i>Hirudo medicinalis</i>	HV3-PA Subtype AFN	ALA22954.1	50	2e-22	⁵²
<i>Hirudo medicinalis</i>	HV1(VV)	ALA22934.1	50	4e-24	⁵²
<i>Hirudo verbana</i>	HV1-VV	APA20831.1	49	2e-22	⁵³
<i>Hirudinaria manillensis</i>	HM1	Q07558.1	49	2e-22	²⁴
<i>Hirudinaria manillensis</i>	HM2	P81492.2	48	9e-21	²⁴
<i>Poecilobdella manillensis</i>	Hirudin	CAA51293.1	48	9e-21	²⁴
<i>Poecilobdella manillensis</i>	HM3	APA20847.1	46	5e-16	⁵³
<i>Poecilobdella viridis</i>	Hirudin	P84590	36	1e-09	⁵⁰
<i>Hirudo medicinalis</i>	Hirudin	P28504	36	2e-09	⁵⁴
<i>Hirudinaria manillensis</i>	Hirudin	P26631	33	1e-04	⁵⁵
<i>Hirudinaria manillensis</i>	Hirudin P18	AAB21616.1	33	1e-04	²³
<i>Hirudinaria manillensis</i>	Hirudin HV1	A53883	33	1e-07	⁵⁶
<i>Hirudinaria manillensis</i>	Hirudin P6	AAB21615.1	30	3e-06	²³

Table 2. Relevant species and their hirudin proteins. *Identity with hirudin was calculated using protein BLAST (protein vs. protein). **The initial report of the hirudin sequence of these organisms.



> recombinant Hirudin

ATG-- α -factor signal peptide--EcoRI--**Hirudin**--His tag--Stop codon--NotI

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLLEGDFDVAVLPFSNSTNNGLLFINTTIIASIAAKEEG
VSLEKREAEAYVEFQHFQKDCSDSNPTPLCENSNLCAFGNTCDLGPCKCIKVSPPPTSEKEKNNKGSKSDY
DYYHHHHHH..

Figure 8. A schematic map of the recombinant plasmid pPIC9K-hirudin. (The target gene is marked in red).

designated as GS115/pPIC9K-hirudin, and *P. pastoris* cells transformed with the empty pPIC9K plasmid were used as the control and named GS115/pPIC9K.

Induction and expression of recombinant yeast transformants. The yeast clones expressing pPIC9K-hirudin were inoculated into a 250-mL bottle containing 50 mL BMGY medium overnight, after identification using PCR. The yeast was cultured at 28 °C with shaking at 250 rpm until the optical density at 600 nm (A_{600}) reached 2–4. The yeast was then collected and induced in 80 mL of BMMY medium until the A_{600} reached 1.5 and cultured continuously at 28 °C for 3 days. During induction, the samples were collected every 24 h and methanol was added to obtain a final concentration of 1% (v/v). Samples were centrifuged at 12,000×g for 2 min at 4 °C. The supernatant and precipitate were collected and analyzed using a 15% SDS-PAGE, as described previously⁵⁷.

Western blot analysis. Supernatants and precipitates from GS115/pPIC9K-hirudin were loaded on a 150 g/L SDS-PAGE for protein separation and transferred to a polyvinylidene difluoride membrane through electrotransfer (54 mA, 80 min). After blocking with 1.5% bovine serum albumin (BSA) in blocking buffer at 4 °C overnight, the membrane was incubated with 1000× dilutions of mouse-anti-His mAb (GenScript, Nanjing, China) at room temperature for 1 h, washed thrice with tris-buffered saline, and mixed with 1000× dilutions of rabbit anti-mouse IgG antibody coupled with horseradish peroxidase (Invitrogen) at room temperature for 1 h. After washing, the membrane was treated with TrueBlue™ Peroxidase Substrate (Takara, Dalian, China) for 1 min.

Purification of recombinant hirudin. The yeast clone expressing pPIC9K-hirudin was amplified, cultivated, and induced as described, and the culture supernatant was harvested for purification at 72 h post induction. The supernatant was carefully resuspended in a binding buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0), loaded onto Ni-nitrilotriacetic acid resin (GenScript), washed, and eluted as recommended by the manufacturer. The protein purification system used an imidazole gradient (0–500 mM) as the target protein eluent. The samples were then analyzed using 15% SDS-PAGE and LC-MS/MS. The Bradford method³¹ was used to determine the concentration of the purified protein.

Mass spectrometric analyses of purified hirudin. The purified protein was filtered through a 0.5-mL Ultracel-10K filter, and 5 µg protein was loaded on the BioAccord LC-MS System. The purified protein was first separated on an ACQUITY UPLC protein BEH C4 column and eluted from the column using gradient elution from 95% water with 0.1% formic acid to 95% acetonitrile with 0.1% formic acid. Intact protein was analyzed using a time-of-flight mass spectrometer with an electrospray ion source. Protein mass was deconvoluted using the UNIFI software suite.

Antithrombin activity analysis. The antithrombin activity of purified recombinant hirudin was evaluated according to the Pharmacopoeia of the People's Republic of China (2020 edition)⁷ and a previously reported method^{8,15}. Commercially available hirudin (MedChem Express, Monmouth-Junction, USA) was used as the control. Briefly, antithrombin activity was quantitatively measured via titrating a solution of thrombin and expressed in ATU: one unit is defined as the neutralization of one NIH unit of thrombin (Sigma, Germany) at 37 °C. For the activity assay, 200 µL of 0.05% bovine fibrinogen solution prepared with 50 mM Tris-HCl buffer (pH 7.4) was thoroughly mixed with 100 µL of the sample, and then 5 µL of 40 NIH/mL thrombin solution (0.2 NIH unit) was added progressively and mixed gently. The above reaction mixture was incubated at 37 ± 0.5 °C for 1 min. The formation of a fibrin clot within 1 min was considered to be the endpoint of the titration. Otherwise, another 5 µL of thrombin solution was added continuously until a fibrin clot was observed.

Ethical approval. We declare that the experiments described in this paper comply with the current laws in China. This article does not contain any studies with human participants performed by any of the authors.

Data availability

The datasets generated and/or analyzed during the current study are available in the “[NCBI] repository (<https://www.ncbi.nlm.nih.gov/nucleotide/MN116511>)”.

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Author contributions

P.S. and J.W. designed the study and prepared the manuscript. P.S. and H.J.Y. conducted the experiments. S.J.C. and F.Y.T. analyzed the data. Z.H.L. supervised the practical and theoretical works. All authors approved the final version before submission.

Competing interests

The authors declare no competing interests.

Additional information

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