

Study on a Novel Analgesic Peptide from the Digestive Juice of Land Leeches

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Abstract: *Haemadipsa*, also known as land leeches, is a genus of annelids feeding on blood. Although the biology and reproductive ecology of the species have been studied, no previous research has shown the analgesic active ingredients in their bodies. In this work, the digestive tract secretions of *Haemadipsa guangchuanensis* have been investigated using chromatography, RP-HPLC (reversed-phase high performance liquid chromatography), and an *in vivo* study on mice. The result of the study proved the existence of an analgesic peptide, HGSN1, in the body of the leech. Furthermore, the result suggests that the active peptide HGSN1 is a key ingredient secreted by *Haemadipsa guangchuanensis* to avoid being noticed while sucking blood. As HGSN1 can potentially be adapted as a precursor compound to develop novel analgesic drugs, it would be of great importance to carry out further research on the structural analysis of the peptide.

1 Introduction

1.1 Motivation The motivation to perform this study arose during a field trip to Lanshan County, Yongzhou, Hunan province. Land leeches are common in such subtropical areas, and were found sucking blood from hosts on several unnoticeable occasions⁽¹⁾. This phenomenon was of great interest to conduct research on.

According to former studies⁽²⁻³⁾, *Haemadipsa* is a genus of annelids commonly known as land leeches. At present, there are nearly 20 species of *Haemadipsa* found in China. This raises the question how land leeches have developed their blood-sucking ability without causing any pain to the host. Is there any certain substance(s) in the digestive juice that can help suppress the transmission of pain signals and paralyze their hosts? The question evoked a strong desire to study the active analgesic and paralytic ingredients of land leeches, a common animal in this province.

1.2 Previous studies The existing studies on *Haemadipsa* are mainly focused on three areas: 1. research on its biological characteristics including morphology, classification, reproduction, ecology, etc. ⁽¹⁻⁶⁾; 2. genomic DNA research, where PCR technology is used to isolate the Haemadin gene (an anticoagulant protein) from *Haemadipsa hainana* and obtain its complete genome sequence ⁽⁷⁾; 3. the medicinal value of *Haemadipsa*. However, few studies have been carried out to identify the active analgesic peptides in the digestive juice of land leeches.

2 Methods and Materials

2.1 Extraction and Preparation of the Digestive Tract Secretions of Land Leeches

2.1.1 Stimulus Method with a Pulse Electronic Stimulator A pulse electronic stimulator was used to reduce the harm of land leeches. The voltage and stimulation frequency were adjusted before the experiment. Ultrapure water was used to clean the living *Haemadipsa guangchuanensis*. Rubber gloves were used to prevent leakage current when collecting the secretion. 100 leeches were placed in a beaker containing 50mL of 0.9% NaCl solution. The poles of the stimulator were held in each hand and kept in contact with the solution in the beaker for 3 to 5 seconds. The process was repeated 4 to 5 times, causing leeches to secrete white foam-like secretions. Thereafter the secretion was washed with 0.9% NaCl solution and stored at -80 ° C.

2.2 Gel Filtration Chromatography of the Crude Extract 2 mL of *Haemadipsa guangchuanensis* secretions obtained through the stimulus method were loaded onto a Dextran G-50 gel column (26 mm×100 cm) equilibrated with Tris-HCl buffer (0.02 mol/L, pH 7.8). The secretion was then eluted with the equilibrium buffer solution of the same concentration using a flow rate of 0.3 mL/min. The automated fraction collector was set at a rate of 3.0 mL/tube and the wavelength of the ultraviolet detector was set at 280nm and 215nm. The elution peaks were collected and refrigerated at -20 ° C. Millipore devices were used to obtain 3K and 10K Centrifugal Filter Device were further used to obtain substances that have a relative molecular mass between 3K and 10K, which is roughly the mass range for proteins. The concentrate was retained for use.

2.3 RP-HPLC Purification of the Crude Extract The purification of the active protein was performed with a Reverse Phase- High Performance Liquid Chromatography (RP-HPLC) system. The elution peaks were collected at 280 nm. The crude extract sample was obtained after freeze-drying and stored at -80 ° C. The wavelength for the second HPLC reaction was set at 215nm and 280nm with the column temperature at 37 ° C to better detect the existence of proteins. The elution gradient is shown in Table 2-2.

Table 2-1 RP-HPLC Gradient Elution Table for the Crude Extract of *Haemadipsa Guangchuanensis* Secretions

| Time (min) | Flow rate(ml/min) | Solution A (%) | Solution B (%) | Curve |
|------------|-------------------|----------------|----------------|-------|
| 0.0 | 3.0 | 100.0 | 0.0 | 6.0 |
| 10.0 | 3.0 | 95.0 | 5.0 | 6.0 |
| 11.0 | 3.0 | 95.0 | 5.0 | 6.0 |
| 40.0 | 3.0 | 67.0 | 33.0 | 6.0 |
| 50.0 | 3.0 | 62.0 | 38.0 | 6.0 |
| 60.0 | 3.0 | 30.0 | 70.0 | 6.0 |
| 70.0 | 3.0 | 0.00 | 100.0 | 6.0 |

Table 2-2 Gradient Elution Table for the Second RP-HPLC Purification of the Target Peptide

| Time (min) | Flow rate(ml/min) | Solution A (%) | Solution B (%) | Curve |
|------------|-------------------|----------------|----------------|-------|
| 0.0 | 1.0 | 90.0 | 10.0 | 6.0 |
| 5.0 | 1.0 | 77.0 | 23.0 | 6.0 |
| 25.0 | 1.0 | 67.0 | 33.0 | 6.0 |
| 28.0 | 1.0 | 60.0 | 40.0 | 6.0 |
| 33.0 | 1.0 | 10.0 | 90.0 | 6.0 |
| 45.0 | 1.0 | 10.0 | 90.0 | 6.0 |

3 Further results and Analysis

3.1 Extraction and Preparation of the Digestive Tract Secretions of Land Leeches The stimulus method with a pulse electronic stimulator was proved to be a simple and effective approach to obtain relatively pure secretion samples. The stimulus method is an ideal approach for the extraction and preparation of the digestive tract secretions of land leeches. Through repeated experiments, it was found that the optimal stimulation parameters were as follows: voltage: 4-5V, stimulation duration: 3-5 seconds, interval: 2 minutes and frequency: 4-5 times till white liquid or foam appears around the anterior sucker of the leeches.

3.2 Gel Filtration Chromatography of the Crude Extract and Centrifugal Ultrafiltration Gel filtration chromatography was used to separate and purify the crude extract. Four elution peaks (Figure 3-1) were obtained through gel filtration chromatography and elution peak IV was selected for centrifugal ultrafiltration. After two times, 80 minutes of centrifuge, 1.8 mL of ultra-filtered solution with a molecular mass between 3,000 and 10,000 was obtained.

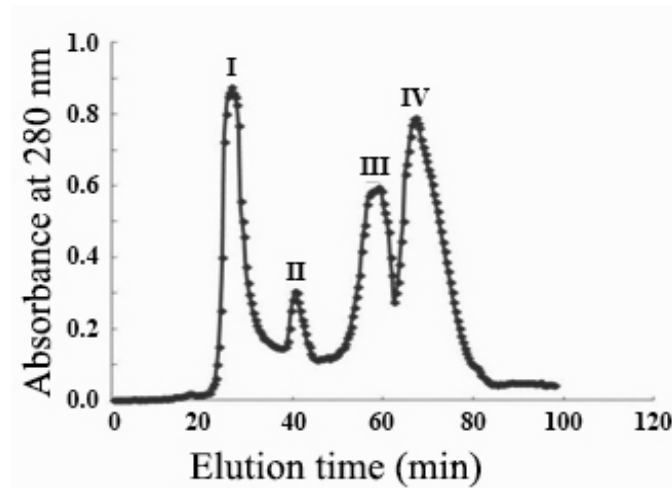


Figure 3-1 Gel Filtration Chromatogram of the Crude Extract Sample

3.3 RP-HPLC Purification and Sequencing of the Sample Components The active polypeptide sample was subjected to the (RP-HPLC) for purification. The retention time of the component elution peaks is shown in Figure 3-2 (the first and second HPLC elution peak). After this, the sample of HGSN1 was sent to a biotechnology company and sequenced. The sequence is: CPQVCPAIYQPVFDEFGRMYSNSCEMQRARCLRG. The molar mass was further calculated and measured through mass spectrum.

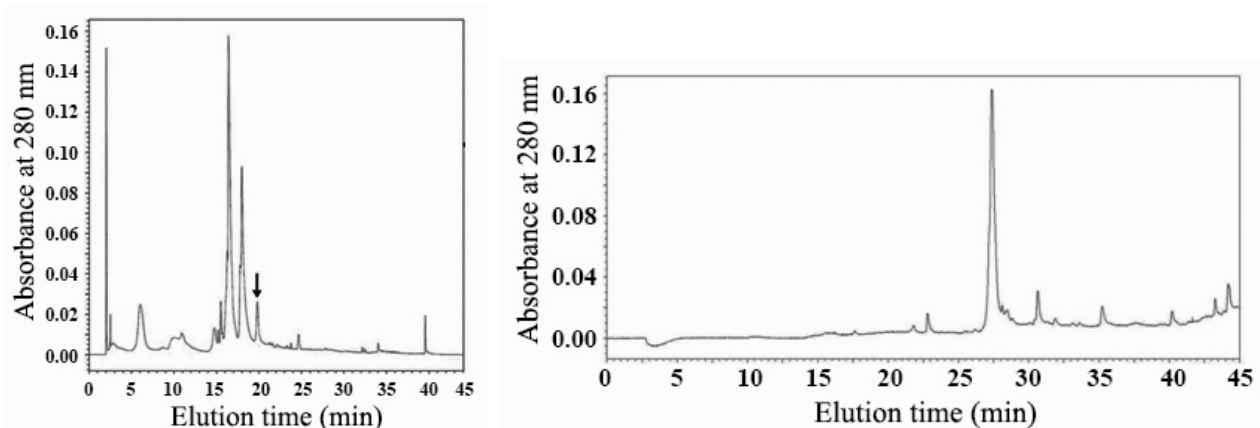


Figure 3-2 The first and second HPLC elution peak

3.4 The Analgesic Activity Test of Target Peptide A mice experiment was performed using formalin, a widely used inflammatory chemical in the study of acute and chronic pain mechanisms. After subcutaneous injection of 5.0% formalin solution in the hind paws of the mice, the mice immediately showed signs of agitation. A typical biphasic behavioral response to pain was observed, and could be divided into phase 1 (0-5 min, acute pain) and phase 2 (15-30 min, chronic pain). At a dosage of 1.0 $\mu\text{mol/kg}$, no significant difference ($p > 0.05$) on reaction was observed among different groups on phase 1 pain. When giving doses of either 2.0 $\mu\text{mol/kg}$ or 4.0 $\mu\text{mol/kg}$, a statistically significant difference was observed in the morphine group and the target peptide group (HGSN1) on the first phase pain response ($p < 0.05$), and morphine was found to be more effective. Compared with saline, the second phase nociceptive response of the morphine group and the target peptide group (HGSN1) was significantly reduced ($p < 0.05$). Furthermore, there was no significant difference between the

morphine group and the target peptide group (see Figure 3-3). Through separation and purification, a new active polypeptide with relative molecular mass of 3951.8 was identified and named as HGSN1. Animal experiments showed that at a dosage of 2 $\mu\text{mol/kg}$, HGSN1 had a significant inhibitory effect on formalin-induced inflammatory pain in mice.

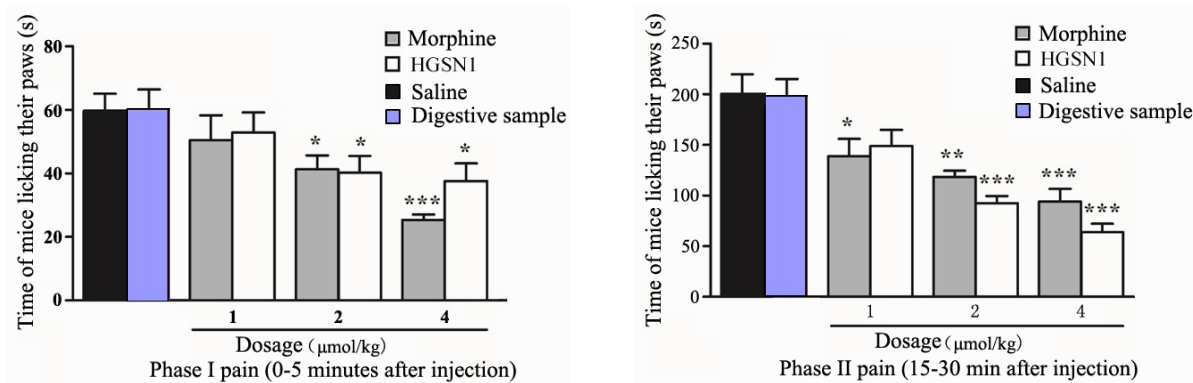


Figure 3-3 The result of different groups

4 Conclusion and Innovative Findings

As a genus of annelids that feed on blood, land leeches have evolved their blood-sucking ability to overcome a series of barriers, such as piercing the skin, preventing the host's blood from coagulating, and quickly completing the blood-sucking process without causing the alert and inflammation symptoms of hosts.

This study adopted a stimulus method to collect the digestive tract secretions from *Haemadipsa guangchuanensis*. Through separation and purification, a new active polypeptide with a relative molecular mass of 3951.8 was identified and named as HGSN1. Animal experiments showed that at a dosage of 2 $\mu\text{mol/kg}$, HGSN1 had a significant inhibitory effect on formalin-induced inflammatory pain in mice. It can serve as a precursor molecule for novel analgesic drugs and is of great significance for the research of protection from land leeches and drug development.

References

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