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Purification and Characterization of a Novel Antimicrobial Peptide from the Skin of the Hagfish, *Eptatretus burgeri*

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Abstract

A novel antimicrobial peptide, named HFS-I, was isolated and characterized from the skin of the hagfish, *Eptatretus burgeri*. The decapeptide with a molecular mass of 1279.5 Da was purified to homogeneity using a gel-filtration column, ion-exchange and C_{18} reverse-phase high performance liquid chromatography. The complete amino acid sequence of HFS-I, which was determined by a combination of an automated amino acid sequencing and FAB-MS, was F-P-W-W-L-S-G-K-Y-P-NH_2. Comparison of the amino acid sequence with those of other known antimicrobial peptides revealed that HFS-I was a novel antimicrobial peptide. HFS-I showed a weak antimicrobial activity *in vitro* against a broad spectrum of microorganisms without hemolytic activity.

Key words: antimicrobial peptide, hagfish, *Eptatretus burgeri*, hemolytic activity

INTRODUCTION

Proteins and polypeptides perform a multitude of offensive and defensive tasks in several organisms. They provide the defense systems by killing invaders as in the case of antimicrobial peptides and proteins (1). One of these systems is that of non-specific immunity which comprises diverse peptides with potent antimicrobial properties (2). In recent years, a number of peptides, which possess antimicrobial activities against bacteria, fungi and enveloped viruses with little or noncytolytic activity (3,4) have been isolated from various biological sources, including mammals (5), amphibians (6), insects (7-9), plants (10) and prokaryotes (11). Some of the most studied members include the defensins from human neutrophils (5), the magainins from the South African clawed toad, *Xenopus laevis* (6), the cecropins from Cecropia moth (7), the mastoparan B and the apidaecins from bee venoms (8,9). In amphibians, which are rich in antimicrobial peptides, many of amphipathic α-helical antimicrobial peptides such as bombinins (12), buforins (13), and dermaseptin (14), as well as carboxy-terminal disulfide bond peptides like the esculentins and brevinins (15), have been isolated from the glands of the skin, venom and the gastrointestinal tract.

In general, all these antimicrobial peptides are cationic but otherwise differ considerably in such basic features as their molecular size, presence of disulfide bonds, and structural motifs. To date, the majority of structural and biological studies on antimicrobial peptides have been limited to those from terrestrial life. Few studies have been performed on antimicrobial peptides from underwater organisms. Pleurocidin, misgurin, pardaxins, clavanins and tachyplesins, which were found in flounder (16), loach (17), Moses sole fish (18), tunicates (19) and horseshoe crab (20), respectively, are some of the antimicrobial peptides isolated from marine organisms. Pardaxins, however, showed strong hemolytic activity. So far, antimicrobial peptides from hagfish have not been reported. The objectives of this study was to purify and characterize an antimicrobial peptide from the hagfish, *Eptatretus burgeri*, which could exhibit antimicrobial activities against Gram-negative and Gram-positive bacteria without significant hemolytic activity.

MATERIALS AND METHODS

Materials

Live hagfish (*Eptatretus burgeri*) was purchased from a local market in Pusan, Korea. Fmoc-NHSAL Resin (0.43 mmol/g), Fmoc-amino acid, Diisopropylcarbodiimode (DIPCI), 1-hydroxybenzotriazole (HOBT), N, N-dimethylformamide (DMF) and piperidine were purchased from WATANABE Chemical Co. (Hiroshima, Japan). Chemicals for peptide synthesis (trifluoroacetic acid, thioanisole, 1,2-ethanedithiol, and *m*-cresol), and acetonitrile) were obtained from commercial sources and were of the highest purity available.

Peptide purification

Obtained hagfish skin from 350 *Eptatretus burgeri*, were frozen in liquid nitrogen. The procedure of the 60%-methanol (RM 60) extraction was performed using a minor modified experiment (21). The frozen material was extracted with ethanol-acetic acid (94 : 4). The extract was condensed and subsequently allowed to pass through C18 cartridges (Sep-pak, Millipore,

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Miliford, MA, USA). The retained material was eluted with force through 60% methanol.

The 60%-methanol eluate (RM 60) was concentrated under vacuum (Speed Vac concentrator) and then lyophilized. The resulting powder was dissolved in 15 ml of 5% acetic acid and fractionated on a calibrated gel filtration column (Sephadex G-25 fine, 1.6×110 cm). Fractions (15 ml) were collected at a flow rate of 90 ml/h from which aliquots (150 μl) were lyophilized and assayed for antimicrobial activity against *Bacillus subtilis*. The active fractions of gel filtration (fractions 46-54, Fig. 1) were pooled and evaporated under vacuum. This material was subjected to reversed-phase HPLC chromatography on a μ-Bondapak C_{18} (3.9×300 mm, Millipore) loaded with the extracts in water containing 0.1% trifluoroacetic acid. The elution was achieved at a flow rate of 1.0 ml/min with a 5~55% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. Two milliliter fractions were collected from which aliquots (20 μl) were assayed for antimicrobial activity.

The active fractions of reverse phase HPLC (fractions 16-17, Fig. 2) were further fractionated on a TSKgel SP-5PW cation-exchange HPLC column (7.5×75 mm, Tosoh, Japan) with a 200 min linear gradient of 0~1 M NaCl in 10 mM phosphate buffer (pH 7.2). Each aliquot (10 μl) were lyophilized and assayed for antimicrobial activity. The active peaks were subjected to reversed-phase HPLC chromatography on a μ-Bondapak C_{18} (3.9×300 mm) loaded with the extracts in water containing 0.1% trifluoroacetic acid. The elution was achieved at a flow rate of 1.0 ml/min with a 25~45% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid. Each of peaks manually was collected and assayed with an aliquot (10 μl) for antimicrobial activity. The active peak was subjected to a final HPLC purification via the ODS-2 column (4.7×150 mm, Hewlett Packard, Phase Separations Ltd., Germany). The column was eluted isocratically with 32% acetonitrile in 0.1% trifluoroacetic acid.

Molecular mass determination and sequence analysis of the antimicrobial peptide

The molecular mass of the antimicrobial activity was determined by fast atom bombardment (FAB) mass spectra using

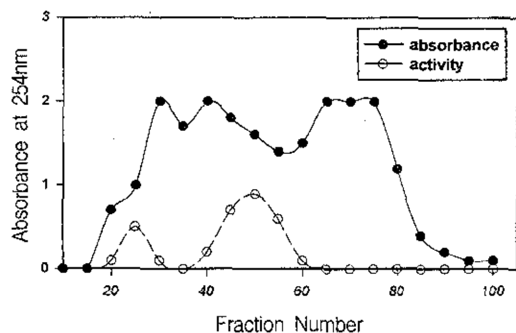

Fig. 1. Size fractionation profile of the hagfish skin extract on a Sephadex G-25 column (1.6×110 cm) using 5% acetic acid as eluant. Fifteen-milliter fractions was collected at a flow rate of 90 ml/h. Fraction 46-54 were collected for further purification.

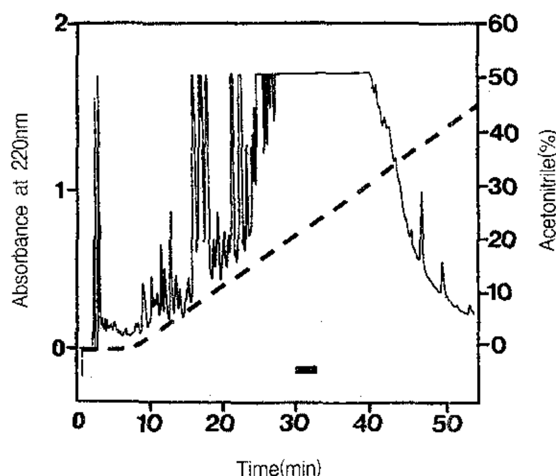

Fig. 2. Reversed-phase HPLC chromatogram of the active fraction after Sephadex G-25 chromatography. The active fractions were loaded on a μ-Bondapak C_{18} (3.9×300 mm) column and elution was achieved with a linear gradient of acetonitrile in aqueous TFA (100% Acetonitrile/0.1% TFA). The elution position of the active fraction is indicated by the black bar.

a JEOL SX-102A. The purified peptide was subjected to amino acid sequence analysis by the automated Edman degradation method on an Applied Biosystem gas-phase sequencer, Model Procise 491 (Foster City, CA, USA). This peptide was synthesized by a solid-phase peptide synthesizer (Milligen 9050) using the Fomc method and purified by HPLC. The synthetic peptide was compared with that of native peptide using reversed-phase HPLC.

Peptide synthesis

HFS-I, mastoparan B (MPB) and Apidaecin Ib were synthesized and purified as described previously (22).

Antimicrobial assay

The antimicrobial activity of the different fractions obtained during the pufification procedure was measured as follows. Each aliquot was incubated in a sterilized 96-well plate with 100 μl of a midlogarithmic phase culture of bacteria (*Bacillus subtillus*) and 0.001 of absorbance at 630 nm. Inhibition of growth was determined by measuring absorbance at 630 nm with a Microplate Autoreader E1309 (Bio-Tek instruments, Winooski, VT, USA) after incubation (18 h, 37°C). The minimal inhibitory concentrations (MIC) of the isolated peptide against several Gram-positive and -negative bacteria were determined as described previously (23).

Hemolytic assay

Hemolytic activity of erythrocytes was determined as described previously (22). The buffy coat was removed by centrifugation of freshly collected human blood, and the obtained erythrocytes were washed three times with isotonic saline and stored at 4°C. They were incubated with peptides at 37°C in 10 mM Phosphate buffer containing 150 mM NaCl buffered saline, and then centrifuged. The absorbance in the supernatant

was measure at 542 nm. The absorbance of the supernatant obtained by treatment of erythrocytes with 1% Triton X-100 was taken as 100%.

RESULTS AND DISCUSSION

Purification of antimicrobial peptide

This report describes the isolation of a novel antimicrobial peptide from the skin of the hagfish. To our knowledge, this is the first report on antimicrobial peptide production by the hagfish. Hagfish skins were homogenized in an acidic medium in order to maximize solubilization of peptides. As a result, the extracts were mostly composed of low-molecular-mass peptides. Antimicrobial peptide was purified to homogeneity by sequential elution through a series of 3 columns having different separation characteristics (Sephadex G-25, μ-Bondapak C_{18} and TSKgel SP-5PW column). The active eluate of Sephadex G-25 chromatography (Fig. 1) was further fractionated by reversed-phase HPLC (Fig. 2). The fraction with antimicrobial activity was subjected to cation-exchange HPLC (On a TSKgel SP-5PW column). The active substance was eluted at 0.75 M NaCl (Fig. 3) and this fraction was further purified on a reversed-phase HPLC (Fig. 4). Final purification was effected on a ODS-2 column (Fig. 5) and a pure peptide, designated as HFS-I (acronym name of hagfish skin).

Primary structure of HFS-I

The determined sequences and detected amounts (picomoles) of each amino acid in the amino acid sequence analysis was as follow: Phe_{250}-Pro_{99}-Trp_{106}-Trp_{148}-Leu_{82}-Ser_{15}- Gly_{42}-Lys_{36}-Tyr_{41}-Pro_{5}. Molecular ion peaks in the FAB-MS spectrum of the native peptide was 1279.5 m/z $(M+H)^+$, which is virtually identical to the mass of 1279.7 predicted for the C-terminal amidated peptide. Based on these results, the accuracy of proposed structure for HFS-I was F-P-W-W-L-S-G-K-Y- P-NH_2. To confirm the chemical identity of HFS-I and to provide sufficient quantities for futher biological experiments, the peptide was prepared synthetically. Co-injection of native HFS-I and synthetic HFS-I gave a single peak on reversed-

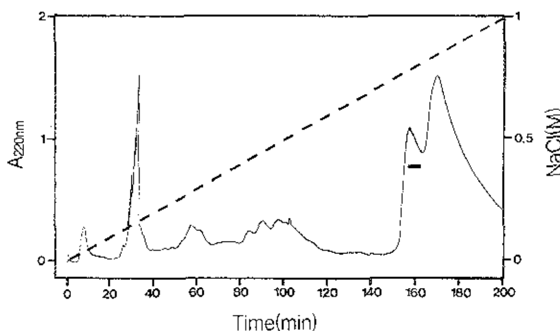

Fig. 3. Cation-exchange HPLC chromatogram of the active fraction after reversed-phase HPLC. The active fractions were loaded on a TSK-gel SP-5PW (7.5×75 mm) column and elution was achieved with a linear gradient of NaCl in phosphate buffer (pH 7.2). The elution position of the active fraction is a indicated by the black bar.

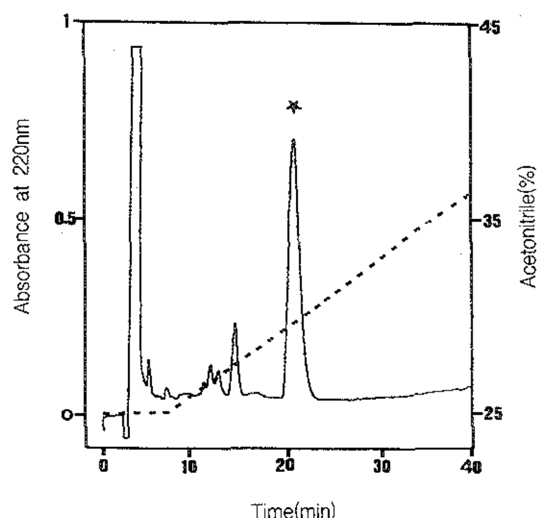

Fig. 4. Reversed-phase HPLC chromatogram of the active fraction after cation-exchange HPLC. The active fraction was loaded on a μ-Bondapak C_{18} (3.9×300 mm) column and elution was achieved with a linear gradient of 25~45% acetonitrile in 0.1% TFA. The elution position of the HFS-I is indicated by the asterisk mark.

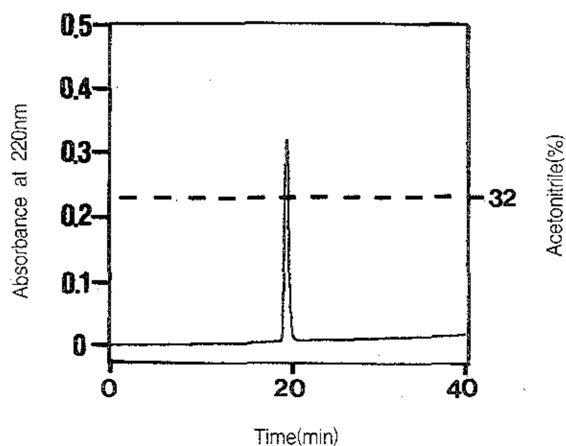

Fig. 5. Final purification step of HFS-I, performed an analytical Hewlett Packard ODS-2 column (4.7×150 mm). Elution was achieved with a isocratic condition of 32% acetonitrile in 0.1% TFA/water at a flow rate of 1 ml/min.

phase liquid chromatography (Fig. 6). Therefore, the synthetic HFS-I was used for further biological analyses.

A computer search comparing this peptide sequence to all published protein sequences in the GenBank™/EMBL Data Bank file revealed that HFS-I was a novel antimicrobial peptide. Application of the empirical rules of Chou and Fasman (24) for prediction of peptide conformations indicates that HFS-I has a high probability of forming a β-sheet structure containing one turn (23). Studies on the secondary structure of HFS-I is now in progress at our laboratories.

Antimicrobial and hemolytic activities

The antimicrobial activity of HFS-I was assayed on 10 different microorganisms, including Gram-positive and Gram-negative bacteria. Mastoparan B (MPB) and apidaecin Ib are

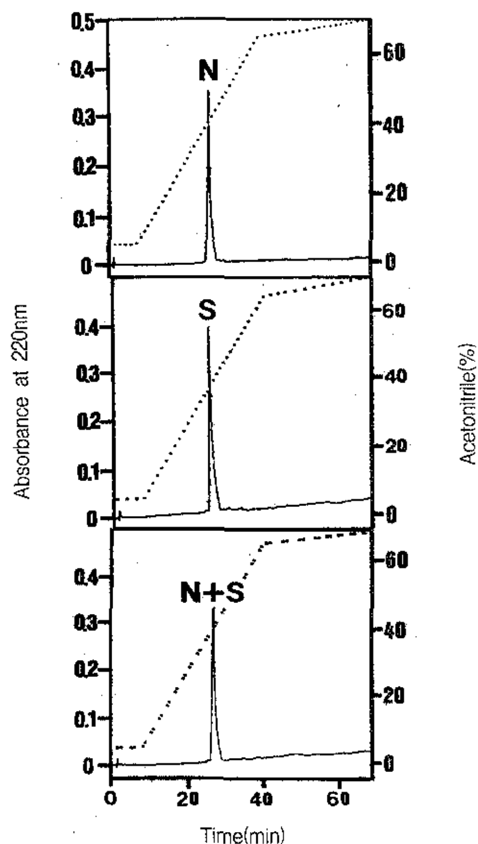

Fig. 6. Comparison between HPLC profile of the native (N) and synthetic (S) HFS-I. HFS-I were loaded onto a Hewlett Packard ODS-2 column (4.7×150 mm) and eluted with a linear gradient of 5~65% acetonitrile for 40 min in 0.1% TFA at a flow rate of 1 ml/min. N+S, represents a mixture of native and synthetic HFS-I.

antimicrobial peptide isolated from the natural source (8,9). MPB and apidaecin Ib were employed as a reference for the antimicrobial activity. The minimum inhibitory concentration of the peptides is shown in Table 1. HFS-I had weak activity against both Gram-positive and Gram-negative bacteria. On the other hand, MPB had strong activity against Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus epidermidis* but weak activity against Gram-negatives except for *Escherichis coli* and *Shigella flexneri*. The antimicrobial activity of HFS-I is less effective than those of MPB and Apidaecin Ib.

Human erythrocyte hemolytic activity was measured for HFS-I, mastoparan B and apidaecin Ib as shown in Table 2. Neither HFS-I nor apidaecin Ib showed hemolytic activity at 100 μg/ml of heparinized blood. However, mastoparan B caused considerable lysis at 50 μg/ml and about 60% lysis is observed at 100 μg/ml. These results indicate that the hemolytic activity of peptides generally increased in parallel to the increase of growth inhibitory activity.

Table 1. Antimicrobial activities of HFS-I, MPB[1] and Apidaecin Ib[2]

Organism	MIC (μg/ml)[3]		
	HFS-I	MPB	Apidaecin Ib
Staphylococcus aureus FDA 209P	>50	25	>25
S. aureus 1840	>50	>50	>25
S. epidermidis IFO 12228	>50	12.5	>25
Bacillus subtilis PCI 219	>50	3.13	>25
Escherichis coil NIHJ JC-2	>50	12.5	25
Shigella flexneri EW-10	>50	6.25	12.5
Klebsiella pneumoniae DT-S	>50	50	>25
Enterobacter cloacae IFO 12937	>50	>50	>25
Acinetobacter calcoaceticus ATCC 13008	>50	25	>25
Pseudomonas aeruginosa U-31	>50	>50	>50
Vibrio arguillarum PT-213	>100	>100	>100
Edward tarda 219	>100	>100	>100

[1]Mastoparan B: Leu-Lys-Leu-Lys-Ser-Ile-Val-Ser-Trp-Ala-Lys-Lys-Val-Leu-NH2.
[2]Apidaecin Ib: Gly-Asn-Asn-Arg-Pro-Val-Tyr-Ile-Pro-Gln-Pro-Arg-Pro-Pro-His-Pro-Arg-Leu- OH.
[3]Method: Agar dilution method. Medium: Mueller Hinton medium (Difco). Inoculum size: 10^6 cell ml^{-1}

Table 2. Hemolytic activities of HFS-I, mastoparan B and apidaecin Ib

Concentration (μg/ml)	% Hemolysis of human red blood cells		
	HFS-I	MPB	Apidaecin Ib
3.13	0.6	0.6	0.3
6.25	1.1	1.6	0.4
12.5	1.4	5.4	1.0
25	2	12	1.0
50	2	39	1.3
100	2	57	4.3

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