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Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*

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Abstract In response to epidermal injury, *Parasilurus asotus*, a catfish, secreted a strong antimicrobial peptide into the epithelial mucosal layer. The molecular mass of the antimicrobial peptide, named parasin I, was 2000.4 Da, as determined by matrix-associated laser desorption ionization mass spectrometry. The complete amino acid sequence of parasin I, which was determined by automated Edman degradation, was Lys-Gly-Arg-Gly-Lys-Gln-Gly-Gly-Lys-Val-Arg-Ala-Lys-Ala-Lys-Thr-Arg-Ser-Ser. Eighteen of the 19 residues in parasin I were identical to the N-terminal of buforin I, a 39-residue antimicrobial peptide derived from the N-terminal of toad histone H2A [Kim et al. (1996) Biochem. Biophys. Res. Commun. 229, 381–387], which implies that parasin I was cleaved off from the N-terminal of catfish histone H2A. Parasin I showed strong antimicrobial activity, about 12–100 times more potent than magainin 2, against a wide spectrum of microorganisms, without any hemolytic activity. Circular dichroism spectra of parasin I indicated a structural content of 11% α-helix, 33% β-sheet, and 56% random coils. The β-sheet axial projection diagram of parasin I showed an amphipathic structure. Our results indicate that the catfish may produce parasin I from its histone H2A by a specific protease upon injury to protect against invasion by microorganisms.

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Key words: Antimicrobial peptide; Histone H2A; Epidermal injury; Catfish; *Parasilurus asotus*

1. Introduction

Antimicrobial peptides, which are widespread in nature and are among the earliest developed elements of innate immunity, are important components of the natural defenses of living organisms against invading microorganisms [1,2]. Many different kinds of antimicrobial peptides have been found from amphibians [3], insects [4], mammals [5], plants [6], microorganisms [7,8], and fishes [9,10]. Most of the antimicrobial peptides kill target cells rapidly and specifically, and have unusually broad activity spectra [9,11]. In addition to the microbicidal activity, antimicrobial peptides may also have other functions such as promotion of wound healing [12], stimulation of monocyte chemotaxis [13], and inhibition of cytokine response [14].

Mucous surfaces of living organisms are under constant attack from microorganisms. However, invasive infections are rare, remain localized, and heal rapidly. Recent reports have established antimicrobial peptides as the host-defense effector molecules which protect the mucous epithelia from invading microbes [15]. Some of these agents of mucosal immunity include andropin from the ductal epithelial cells of the reproductive tract of *Drosophila* [16], magainins from the mucosal skin surface and the gastrointestinal tract of *Xenopus laevis* [17], buforin I from *Bufo bufo gagarizans* [11], tracheal antimicrobial peptide (TAP) from the bovine tracheal mucosa [18], and pleurocidin from *Pleuronectes americanus* [19].

Even though many antimicrobial peptides have been reported in the mucous layer of amphibians, insects, and mammals [11,16,18], only a few have been found in the mucous layer of aquatic organisms [9,10,22]. In this study, we report the discovery of a strong and inducible antimicrobial peptide from the epithelial mucosal layer of the catfish, *Parasilurus asotus*.

2. Materials and methods

2.1. Microorganisms

All of the microorganisms used in this study were obtained from the American Type Culture Collection (ATCC). The following microorganisms were used: *Bacillus subtilis* ATCC 62037, *Staphylococcus aureus* ATCC 15752, *Streptococcus mutans* ATCC 25175, *Pseudomonas putida* ATCC 17426, *Escherichia coli* ATCC 27325, *Salmonella typhimurium* ATCC 15277, *Serratia* sp. ATCC 21074, *Cryptococcus neoformans* ATCC 34881, *Saccharomyces cerevisiae* ATCC 44774, and *Candida albicans* ATCC 10231.

2.2. Peptide purification

Catfishes were injured by scratching the skin (16 cm^2) with a sandpaper and 5 h after the wounding, the catfishes were stunned by electro-shock. The proteinaceous epithelial mucosal layer was scraped off from both the unwounded and wounded catfishes. The mucus (20 g) collected from the catfish skin was then homogenized using a Waring blender (Waring, New Hartford, CT, USA) in 200 ml of extraction medium (0.2 M sodium acetate, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 20 000×*g* for 30 min in a Himac SCR20BR (Hitachi, Tokyo, Japan) and the supernatant was collected. The peptides in the supernatant were subsequently subjected to reverse-phase concentration using a Sep-Pak C18 cartridge (Millipore, Milford, MA, USA) which was activated with 80% acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) (buffer A) and flushed with 0.1% (v/v) TFA (buffer B) to remove the excess acetonitrile. After being loaded with the supernatant, the Sep-Pak C18 cartridge was washed with 20 ml of buffer B and the peptides trapped in the cartridge were eluted with 6 ml of buffer A. The eluate was then lyophilized and subsequently resuspended in buffer B. The resuspended sample was applied to a C18 reverse-phase high-performance liquid chromatography (HPLC) column (3.9×300 mm, Delta Pak, Millipore) and elution was achieved with a linear gradient of 0–80% acetonitrile in 0.1% TFA for 2 h at a flow rate of 1 ml/min. Each fraction was lyophilized, resuspended in water, and was assayed for antimicrobial activity against *B. subtilis* ATCC 62037. The peaks with antimicrobial activity were identified and pooled. The purity of the isolated peptide was assessed by reverse-phase HPLC and matrix-associated laser desorption ionization mass spectroscopy (MALDI-MS) (Kratos Kompact

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MALDI, Manchester, UK). The amount of the isolated peptide was determined by amino acid analysis as described by Park et al. [9].

2.3. Antimicrobial and hemolytic activity assay

Antimicrobial activity of the samples was determined during each purification step by the radial diffusion assay using *B. subtilis* ATCC 62037 as described by Lehrer et al. [20]. A 20 ml culture of *B. subtilis* ATCC 62037 cells in mid-logarithmic phase was washed with cold 10 mM sodium phosphate buffer (NaPB), pH 7.4, and resuspended in 10 ml of cold NaPB. A cell suspension containing 1×10^6 bacterial colony forming units (CFUs) was added to 6 ml of underlayer agar (10 mM sodium phosphate, 1% (v/v) trypticase soy broth (TSB), 1% (w/v) agarose, pH 6.5) and the mixture was poured into a Petri dish. 5 μl samples were added directly to the 3-mm wells which were made on the solidified underlayer agar. After incubation for 3 h at 37°C, the underlayer agar was covered with a nutrient-rich top agar overlay (6% (w/v) TSB, 1% (w/v) agarose) and incubated overnight at 37°C. Antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 3-mm wells. The minimal inhibitory concentrations (MICs) of the isolated peptide against the microorganisms were determined as described by Moore et al. [21]. 100 μl microorganism suspensions (10^5 CFU/ml) in 3% (w/v) TSB was mixed with serial two-fold dilutions of the isolated peptide in a sterilized 96-well microtiter plate (Nunc F96 microtiter plates, Denmark). The final concentration of the peptide ranged over 0.5–200 μg/ml. The 96-well plate was incubated overnight at 37°C and the inhibition of growth was determined by measuring the absorbance at 620 nm on a Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). MIC was defined as the lowest concentration of peptide that inhibited growth. Hemolytic activity of the antimicrobial peptide was assayed as described by Park et al. [9].

2.4. Molecular mass determination and amino acid sequence analysis

Molecular mass of the antimicrobial peptide was determined by MALDI-MS. Approximately 20 nmol of the lyophilized peptide was dissolved in 50% acetonitrile containing 7% (w/v) sinapinic acid and mixed with a Pt probe. After removing the solvent in warm air, the peptide, adsorbed to the Pt probe, was applied to a vacuum chamber and analyzed. Amino acid sequencing was performed by the automated Edman degradation method on an Applied Biosystems gas phase sequencer, Model 477A (Foster City, CA, USA). Amino acid sequence homology searches were performed by computerized query of the GenBank/EMBL Data Bank. The antimicrobial peptide, whose complete amino acid sequence was confirmed by amino acid analysis, was synthesized at the Korea Basic Research Institute (Seoul, Korea) to compare the antimicrobial activity between the isolated and synthetic peptide.

2.5. Circular dichroism

For circular dichroism (CD) analysis, peptide samples were dissolved in either 50 mM NaPB or 50% (v/v) trifluoroethanol (TFE) in 50 mM NaPB. The CD spectra were recorded at room temperature on a Jasco model J-715 spectropolarimeter (Jasco, Tokyo, Japan) with a cell path length of 1 mm. Five scans per sample were performed and averaged over the wavelength range 200–250 nm [22]. Ellipticity was reported as mean residue ellipticity [θ] (degrees cm^2 $dmol^{-1}$). The contents of α-helix, β-sheet and random coils were estimated as described by Greenfield and Fasman [23].

3. Results

3.1. Peptide purification

The mucosal layer was scraped off from the wounded and unwounded catfishes as described in Section 2 and immediately homogenized in the extraction media. The crude extracts were centrifuged and the supernatants were subsequently concentrated by solid phase extraction on Sep-Pak C18 cartridges. The reverse-phase HPLC analysis of the wounded catfish extract showed the presence of several peaks which were not present in that of the unwounded catfish (Fig. 1A,B). This experiment was repeated several times with the same results. Peaks a, b, and c in Fig. 1B were individually collected and assayed for antimicrobial activity. Of the three induced peaks, only peaks a and c showed antimicrobial activity. Antimicrobial activity assay revealed that peak a showed over 200 times stronger antimicrobial activity than peak c. Therefore, only peak a, which showed the strongest antimicrobial activity, was purified to homogeneity using C18 reverse-phase HPLC and fully characterized. Peaks b and c, which did not show significant antimicrobial activity, were not further characterized except for the molecular mass. The purity of the antimicrobial peptide in peak a was confirmed to be over 95% homogeneous by reverse-phase HPLC (Fig. 1B, inset) and MALDI-MS (data not shown). The total amount of the purified antimicrobial peptide recovered was about 0.1 μg/g mucus. The purified antimicrobial peptide, named parasin I (derived from the genus name of the catfish, *Parasilurus*), was used for further chemical and biological analyses.

3.2. Antimicrobial and hemolytic activity

The antimicrobial activity of parasin I was tested against 10 different microorganisms, including Gram-negative and Gram-positive bacteria and fungi. The MICs (Table 1) indicate that parasin I was approximately 12–100 times more potent than magainin 2, which was purified from *Xenopus laevis* [17], and up to 8 times more potent than buforin I, a potent antimicrobial peptide which was isolated from *Bufo bufo gagarizans* [11]. The antimicrobial activity of the synthetic parasin I, which was synthesized by the solid-phase synthesis method and quantitated by amino acid analysis,

Table 1
Minimal inhibitory concentrations of parasin I, buforin I and magainin 2

Microorganism	Minimal inhibitory concentration (μg/ml)		
	Parasin I	Buforin I	Magainin 2
Gram-positive			
Bacillus subtilis ATCC 62037	1	4	50
Staphylococcus aureus ATCC 15752	2	4	50
Streptococcus mutans ATCC 25175	1	8	100
Pseudomonas putida ATCC 17426	2	4	50
Gram-negative			
Escherichia coli ATCC 27325	1	8	100
Salmonella typhimurium ATCC 15277	2	4	25
Serratia sp. ATCC 21074	4	8	50
Fungi			
Cryptococcus neoformans ATCC 34881	2	4	12
Saccharomyces cerevisiae ATCC 44774	2	4	25
Candida albicans ATCC 10231	1	4	25

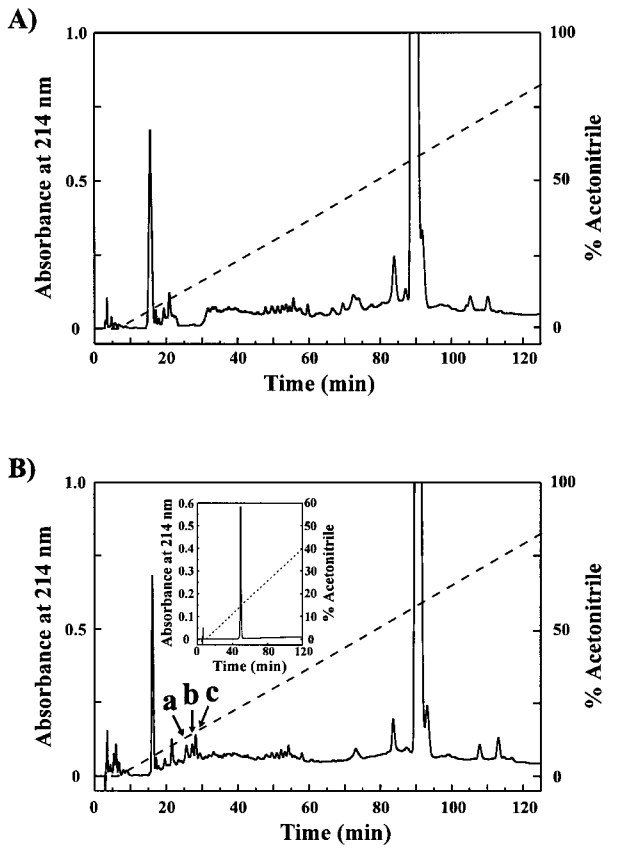


Fig. 1. Reverse-phase HPLC analyses of extracts of unwounded (A) and wounded *Parasilurus asotus* (B). The samples were loaded on a 3.9×300 mm Delta Pak C18 column and elution was achieved with a linear gradient (dotted line) of acetonitrile in aqueous trifluoroacetic acid (80% acetonitrile/0.1% TFA). Absorbance was monitored at 214 nm (solid line). Peaks a, b, and c indicate the absorption peaks that were detectable only in the extract of injured catfish and the elution position of the isolated antimicrobial peptide is indicated by peak a. The inset in B represents the HPLC profile of the purified peptide with a slow gradient.

was found to be identical to that of natural parasin I. Furthermore, when tested against human erythrocytes, parasin I (200 μg/ml) caused only 0.2% hemolysis whereas melittin, a hemolytic peptide [24], effected 99.2% hemolysis at the same concentration.

3.3. Molecular mass and amino acid sequence analysis

The molecular mass of parasin I was 2000.4 Da as determined by MALDI-MS (data not shown). Amino acid sequence analysis revealed that parasin I consisted of 19 residues, including three arginines and five lysines, which contributed to the net charge of +8 (Fig. 2). The complete amino acid sequence of parasin I was Lys-Gly-Arg-Gly-Lys-Gln-Gly-Gly-Lys-Val-Arg-Ala-Lys-Ala-Lys-Thr-Arg-Ser-Ser. The molecular mass of parasin I obtained by MALDI-MS was in good agreement with the mass calculated from the amino acid sequence. This result indicates that post-translational modifications were not present in parasin I. Amino acid sequence homology search showed that parasin I was highly homologous to the N-terminal region of histone H2A, a replication-dependent protein (Fig. 2). Parasin I was identical to the N-terminal of buforin I [11] in 18 of the 19 residues, which corresponds to 95% homology. Parasin I also shared 89% homology with the N-terminal of human histone H2A.5 [25]. The molecular mass of the two peptides in peaks b and c (Fig. 1B) was 4203 and 3937 Da, respectively (data not shown). However, these two peptides were not further studied because of their poor antimicrobial activity.

3.4. Circular dichroism

The secondary structure of parasin I was estimated using a spectropolarimeter in the absence and presence of TFE (Fig. 3A). The CD spectra were baseline-corrected and smoothed by the algorithm provided by the manufacturer. When analyzed by the method of Greenfield and Fasman [23], the CD spectrum of parasin I in 50 mM NaPB indicated that the contents of α-helix, β-sheet, and random coils were 11%, 33%, and 56%, respectively. Under the hydrophobic condition of 50% TFE solution, the contents of α-helix, β-sheet, and random coils changed to 0%, 37%, and 63%, respectively.

4. Discussion

The present study describes the isolation and characterization of a potent antimicrobial peptide, parasin I, from the mucous layer of the wounded catfish skin, which is one of the first reports of an inducible antimicrobial peptide in fish. Parasin I has a molecular mass of 2000.4 Da and consists of 19 amino acids, including three arginines and five lysines, which contribute to the net charge of +8. Parasin I was found only in the skin mucous extracts of the injured catfish and not in the uninjured catfish (Fig. 1). The detection of parasin I only in the wounded catfish skin indicated that injuring the catfish skin stimulated the production or secretion of parasin I into the mucous layer. When threatened or injured, catfish secretes a thick gel-like layer of proteinaceous materials, which includes antibodies and lysozyme [26,27], to its skin

Peptide	Amino acid sequence
	1 5 10 15 20 25
Parasin I	K G R G K Q G G K V R A K A K T R S S
Buforin I	A G R G K Q G G K V R A K A K T R S S R A G L Q F ...
Human histone H2A.5	S G R G K Q G G K A R A K A K T R S S R A G L Q F ...

Fig. 2. Amino acid sequence of parasin I. The sequence of parasin I, obtained by automated Edman degradation, is aligned with those of buforin I and human histone H2A.5. The conserved residues are boxed.

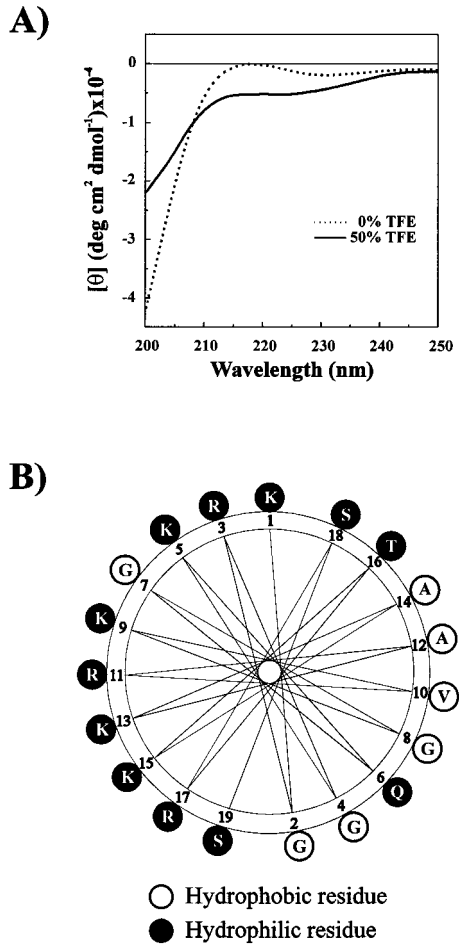


Fig. 3. CD spectra (A) and axial projection diagram (B) of parasin I. The CD spectrum of parasin I was measured in 50 mM NaPB (dashed line) and 50% TFE in 50 mM NaPB (solid line). The axial projection diagram of parasin I was drawn with 170° rotation per residue.

surface mainly from the unicellular glands of the epidermis [28]. It is also known that amphibians, such as *X. laevis*, *Bombina* sp., and *Phyllomedusa* sp., produce and store antimicrobial peptides in the granular glands and release their contents onto the epithelia upon adrenergic stimulation or injury [29]. A similar secretory immune system may also be responsible for the release of the antimicrobial peptide parasin I in the catfish skin. The other peptides (Fig. 1B, peaks b and c), which did not show significant antimicrobial activity, may have other functions related to epidermal injury.

The very high homology between parasin I and the N-terminal region of histone H2A, the replication dependent protein, suggests that parasin I might be cleaved off from the N-terminal of catfish histone H2A by a specific protease cleavage. Production of antimicrobial peptides from the larger peptides or proteins with other known functions by processing or alternative splicing has been reported [1,11,30–32]. This group of antimicrobial peptides include buforin I [11], GIP(7–42), DBI(32–86) [30], XPF, CPF [31], and lactoferricin [32]. Buforin I is a 39-amino acid antimicrobial peptide produced from the N-terminal of histone H2A in the toad stomach by a specific proteolytic cleavage [33]. XPF is believed to be produced by an initial cleavage of the xenopsin precursor by a 'trypsin-like' enzyme and the subsequent removal of the C-terminal arginine residue by a 'carboxypeptidase B-like' enzyme [31]. Recently, Fiorucci et al. [34] reported that tryptase, an inducible trypsin-like serine proteinase from the mast cell granules, has a preferential cleavage site in the histone H2A molecule at a monobasic site, 20 residues downstream of the N-terminus. Interestingly, the N-terminal fragment that is produced from this hydrolysis corresponds to the parasin I molecule with an additional arginine residue at the C-terminus. It remains to be studied whether a series of enzymatic cleavages is involved in the production of parasin I from histone H2A.

Parasin I showed a strong antimicrobial activity towards Gram-negative bacteria, Gram-positive bacteria, and fungi without any hemolytic activity (Table 1). The MICs of parasin I were in the range of 1–4 μg/ml. The most potent antimicrobial peptides have been reported to kill susceptible bacteria in vitro at concentrations in the range of 0.25–4 μg/ml [35], which indicates that parasin I is one of the most potent antimicrobial peptides found so far. The antimicrobial property of most antimicrobial peptides is generally attributed to their amphipathic secondary structures with a net positive charge [36]. The secondary structure of parasin I was investigated to see if it formed, like many antimicrobial peptides, an amphipathic structure. The CD spectra of parasin I in 50 mM NaPB revealed a structural content of 11% α-helix, 33% β-sheet, and 56% random coils. The addition of TFE, an α-helix inducing solvent, slightly increased the β-sheet content (37%) in parasin I (Fig. 3A). This result suggests that parasin I formed a β-strand, in both hydrophilic and hydrophobic environments, instead of the linear amphipathic α-helix which is common for many other antimicrobial peptides. An axial projection diagram of parasin I was drawn with 170° rotation per residue to determine whether it was possible for parasin I to form an amphipathic β-strand (Fig. 3B). The axial projection of parasin I formed a nearly perfect amphipathic β-strand with seven of the eight cationic residues on the hydrophilic side of the β-sheet plane. Our results in this study indicate that catfish produce a strong antimicrobial peptide from histone H2A in the epithelial mucosal layer upon epidermal injury to protect against the invasion of microorganisms. Further works on the identification of the protease responsible for cleaving parasin I off from histone H2A and the determination of the molecular mechanism of antimicrobial action of parasin I are in progress.

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