A novel antimicrobial peptide from the loach, *Misgurnus anguillicaudatus*

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Abstract A novel antimicrobial peptide, named misgurin, was isolated and characterized from the loach (mudfish), *Misgurnus anguillicaudatus*. The 21-amino-acid peptide with a molecular mass of 2502 Da was purified to homogeneity using a heparin-affinity column and C18 reverse-phase and gel-permeation high-performance liquid chromatography. The complete amino acid sequence of misgurin, which was determined by an automated amino acid sequencer, was Arg–Gln–Arg–Val–Glu–Glu–Leu–Ser–Lys–Phe–Ser–Lys–Lys–Gly–Ala–Ala–Ala–Arg–Arg–Arg–Lys. Misgurin is a strongly basic peptide which has 5 arginine and 4 lysine residues. Comparison of the amino acid sequence with those of other known antimicrobial peptides revealed that misgurin was a novel antimicrobial peptide. Misgurin showed a strong antimicrobial activity in vitro against a broad spectrum of microorganisms without significant hemolytic activity and was about 6 times more potent than magainin 2. Scanning electron microscopy confirmed that the peptide caused damage to the cell membrane by a pore-forming mechanism similar to that of magainin 2. This damage occurred at the minimal inhibition concentration (MIC), but at higher concentration than MIC it lysed the cell.

Key words: Antimicrobial; Peptide; Mudfish; Loach; (*Misgurnus anguillicaudatus*)

1. Introduction

Bacteria and fungi coexist with all living organisms. However, considering the number and diversity of microorganisms with which they are in constant contact, microbial invasion to the hosts is rare, likely because of their unique defense systems. One such system is that of non-specific immunity which comprises a wide variety of peptides with potent antimicrobial activities [1]. More than 2000 antimicrobial peptides, which possess antimicrobial activities against bacteria, fungi and enveloped viruses with little or no cytolytic activity [2,3], have been isolated from diverse biological sources, including amphibians [4], insects [5], mammals [6], plants [7] and prokaryotes [8]. Cecropins were the first well-characterized family of structurally related antimicrobial peptides that were found in a wide distribution of insects [5]. In amphibians, which are rich in antimicrobial peptides, many amphipathic α-helical antimicrobial peptides such as magainins [4], bombinins [9], buforins [10] and dermaseptin [11], as well as carboxy-terminal disulfide bridged peptides like the esculentins and brevinins [12], have been isolated from the glands of the skin and gastrointestinal tract. Defensins were found in phagocytic cells isolated from several mammalian species, including human [6]. Most of these antimicrobial peptides, although a few non-high-cationic antimicrobial peptides have been recently characterized [13], share the properties of being cationic but otherwise differ considerably in such basic features as their size, the presence of disulphide bonds and structural motifs [14].

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. Pardaxins, mytilins and tachyplesins, which were found in Moses sole fish [15], mussels [16] and horseshoe crab [17], respectively, were the only antimicrobial peptides so far isolated from marine organisms. Pardaxins, however, showed strong hemolytic activity. So far, natural fish antimicrobial peptides without hemolytic activity have not been reported. In this study, we report the purification and characterization of an antimicrobial peptide from the loach (or mudfish), *Misgurnus anguillicaudatus*, which exhibited potent antimicrobial activities against Gram-negative and Gram-positive bacteria and fungi without significant hemolytic activity.

2. Materials and methods

2.1. Peptide purification

An antimicrobial peptide was purified from the loach (*Misgurnus anguillicaudatus*) as described previously by Park et al. [10]. Loaches (200 g) were homogenized using a Waring blender (Waring, New Hartford, CT) in 400 ml of acidic medium containing 1% (v/v) trifluoroacetic acid (TFA), 1 M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl and pepstatin A at 1 μg/ml. 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 then subjected to reverse-phase concentration using a Sep-Pak C18 cartridge (Millipore, Milford, MA) which was activated with 80% acetonitrile containing 0.1% (v/v) TFA and flushed with 0.1% (v/v) TFA to remove the excess acetonitrile. After being loaded with the supernatant, the cartridge was washed with 20 ml of 0.1% (v/v) TFA and the peptides trapped in the Sep-Pak C18 cartridge were eluted with 6 ml of 80% (v/v) acetonitrile containing 0.1% (v/v) TFA. The eluate was then lyophilized and subsequently resuspended in 10 ml of 0.01 M Tris-HCl (pH 7.5) containing 0.01 M NaCl. The resuspended eluate was loaded onto a 1.0×10 cm Heparin Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M Tris-HCl (pH 7.5) containing 0.01 M NaCl [18]. The bound peptides were eluted by 0.1 M, 0.5 M and 1 M NaCl, respectively, and concentrated in 2 ml of 0.1% (v/v) TFA using Sep-Pak C18 cartridges. Five microliters of each sample was then assayed for antimicrobial activity against *Bacillus subtilis*. The active eluate was run on a C18 reverse-phase high-performance liquid chromatography (HPLC) column (3.9×300 mm, Delta Pak, Millipore). The peptides loaded into the column were eluted with a linear gradient of 0–50% in 1 h at a flow rate of 1 ml/min with buffer A (0.1% TFA in H_2O) and buffer B (0.1% TFA in acetonitrile). Each fraction was dried under vacuum and resuspended in water. Five microliters of the resuspended peptides was assayed for antimicrobial activity as before. The active fraction was further purified on an 8×300 mm Protein KW-802.5 gel-permeation HPLC column (Millipore). The purity of the isolated peptide was assessed by tricine SDS-PAGE and a matrix-associated laser

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desorption ionization (MALDI) mass spectroscopy (Kratos Kompact MALDI, Manchester, UK).

2.2. Antimicrobial assays

The antimicrobial activity was examined during each purification step by the radial diffusion assay on *B. subtilis* lawn as described by Lehrer et al. [19]. A 20 ml culture of *B. subtilis* 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 volume containing 1×10^6 bacterial CFU was added to 6 ml of underlayer agar (10 mM sodium phosphate, 1% (v/v) trypticase soy broth, 1% agarose, pH 6.5) and the mixture was poured into a Petri dish. Samples were added directly to the 3-mm wells 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 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 (MIC) of the isolated peptide against several Gram-positive and Gram-negative bacteria and fungi were determined as described by Park et al. [10]. The lowest concentration of antimicrobial peptide which showed visible suppression of growth was defined as the MIC.

2.3. Hemolysis assay

Hemolytic activity was assayed as described by Aboudy et al. [20] with a slight modification. Freshly packed sheep erythrocytes (3 ml) [9] was washed with isotonic phosphate-buffered saline (PBS), pH 7.4, until the color of the supernatant turned clear. The washed erythrocytes were then diluted to a final volume of 20 ml with the same buffer. To 190 μl of the cell suspension in microfuge tubes, peptide samples (10 μl), serially diluted in PBS, were added. Following gentle mixing, the tubes were incubated for 30 min at 37°C and then centrifuged at 4000 rpm for 5 min. One hundred microliters of supernatant was taken, diluted to 1 ml with PBS, and OD_{567} was determined. The relative optical density, as compared with that of the cell suspension treated with 0.2% Triton X-100, was defined as percent hemolysis.

2.4. Molecular mass determination and sequence analysis of the antimicrobial peptide

The molecular mass of the antimicrobial peptide was determined by MALDI mass spectroscopy. Approximately 20 nmol of 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).

2.5. Quantification of peptide

The amount of peptide was determined by amino acid analysis. The lyophilized peptide was hydrolyzed in 6 N HCl for 24 h at 110°C and converted to its phenylthiocarbamyl derivative. Samples were then analyzed using a Pico-tag analysis system on a Beckman 121 MB amino acid analyzer (Fullerton, CA).

2.6. Peptide synthesis

The isolated antimicrobial peptide was synthesized by the solid-phase synthesis method on a Milligen 9050 Pepsynthesizer according to fluoren-9-methyloxycarbony (Fmoc)-polypeptide active ester chemistry. The synthesized peptide was purified by reverse-phase HPLC and the purity was confirmed by amino acid analysis and MALDI mass spectroscopy.

2.7. Scanning electron microscopy

Scanning electron microscopy (SEM) was conducted as described previously by Shi et al. [21].

2.8. Circular dichroism

Circular dichroism (CD) spectra were measured in either 50 mM NAPB, or 50% (v/v) trifluoroethanol in 50 mM NAPB, on a Jasco model J-715 spectropolarimeter with a cell path length of 1 mm. Five scans per sample were performed over the wavelength range 200–250 nm [22]. The contents of α-helix, β-sheet and unordered structures were estimated as described by Yang et al. [23].

3. Results

3.1. Purification of the antimicrobial peptide

Loaches 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. The active eluate, after heparin-affinity chromatography, was further fractionated by reverse-phase HPLC into one single, active peak (Fig. 1A). However, the active peak was found to contain three peptides on tricine–SDS–PAGE (Fig. 1D, lane 3). Therefore, gel-permeation HPLC was used to isolate the antimicrobial peptide from the active, single peak of reverse-phase HPLC (Fig. 1B). The antimicrobial peptide purified by gel-permeation HPLC was confirmed to be about 95% homogeneous by reverse-phase HPLC (Fig. 1C), tricine–SDS–PAGE (Fig. 1D, lane 4) and MALDI mass spectroscopy (Fig. 2), and named misgurin (derived from the genus name of the loach '*Misgurnus*'). The total amount of purified misgurin recovered was 0.1 μg/g loach. The purified misgurin was used for further chemical and biological analyses.

3.2. Primary structure determination

The molecular mass of misgurin was determined to be 2502 Da by MALDI mass spectroscopy, along with a small

Table 1
Antimicrobial activities of misgurin and magainin 2

Microorganism	Minimal inhibitory concentrations (μg/ml)[a]		
	misgurin	synthetic misgurin	magainin 2
Gram positive			
Bacillus subtilis	8	8	50
Staphylococcus aureus	8	8	50
Streptococcus mutans	16	16	100
Streptococcus pneumoniae	8	8	50
Pseudomonas putida	8	8	50
Gram negative			
Escherichia coli	8	8	100
Salmonella typhimurium	8	8	25
Serratia sp.	16	16	50
Fungi			
Candida albicans	16	16	25
Cryptococcus neoformans	8	8	12
Saccharomyces cerevisiae	4	4	25

[a]Minimal inhibitory concentrations were determined by observing the zone of inhibition of growth of microorganisms around 3-mm wells containing serial dilutions of antimicrobial peptides. See Section 2 for details.

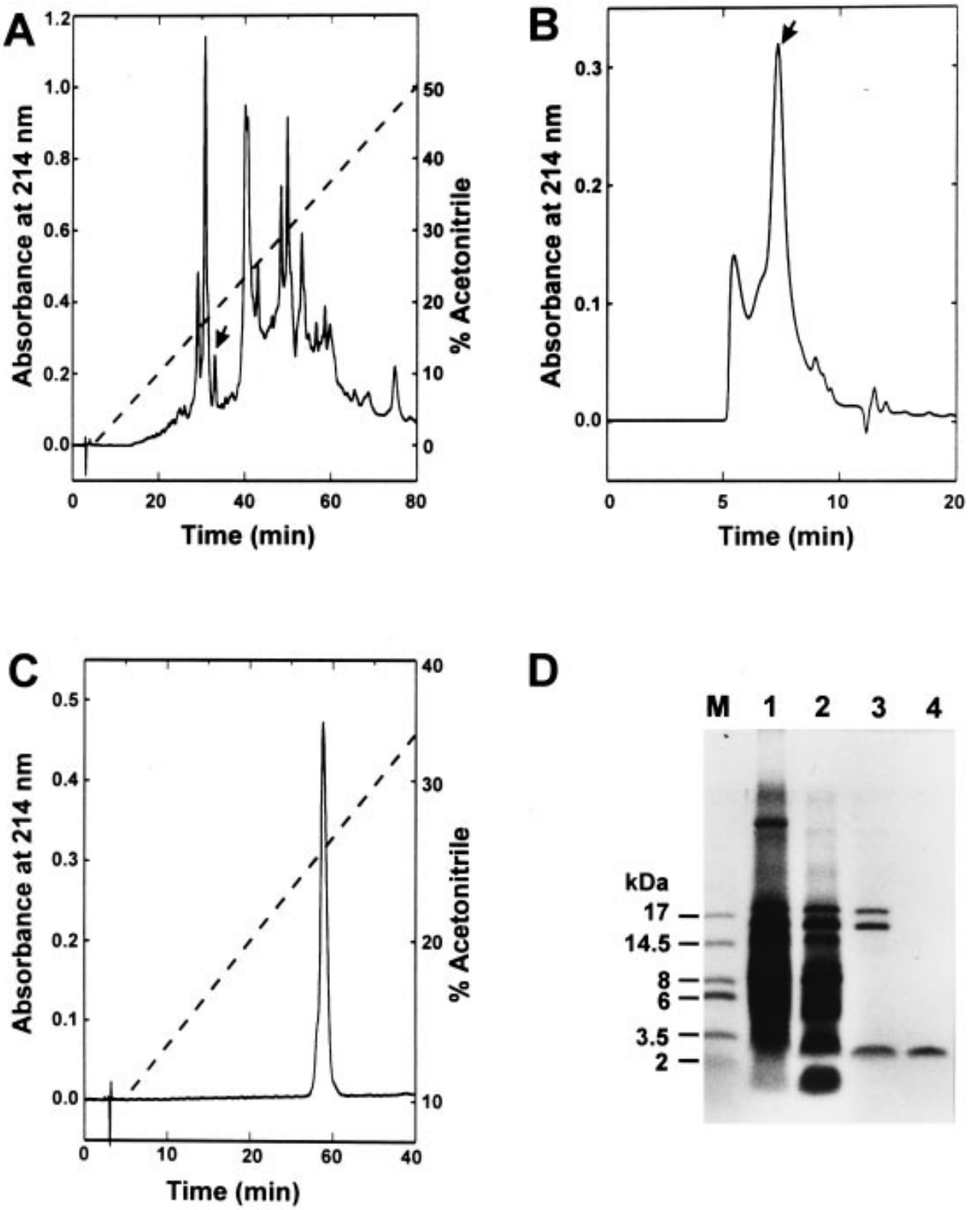

Fig. 1. Purification of misgurin. (A) Reverse-phase HPLC chromatogram of the active fraction after heparin chromatography. The active fraction was loaded on a 3.9×300 mm Delta-pak C18 column and elution was achieved with a linear gradient of acetonitrile in aqueous TFA (80% acetonitrile/0.1% TFA). The elution position of the active peak is indicated by the arrow. (B) Gel-permeation HPLC chromatogram of the active peak after reverse-phase HPLC. The active peak was loaded on a 8×300 mm Protein KW-802.5 gel-filtration HPLC column. The elution position of misgurin is indicated by the arrow. (C) Reverse-phase HPLC profile of misgurin after gel-permeation HPLC with a slow gradient. (D) Tricine–SDS–PAGE of loach peptides. Lane 1, loach crude extracts; lane 2, active fraction after heparin chromatography; lane 3, active peak after reverse-phase HPLC; lane 4, pure misgurin after gel-permeation HPLC. The molecular mass size markers (lane M) have the following sizes: 17, 14.5, 8, 6, 3.5 and 2 kDa.

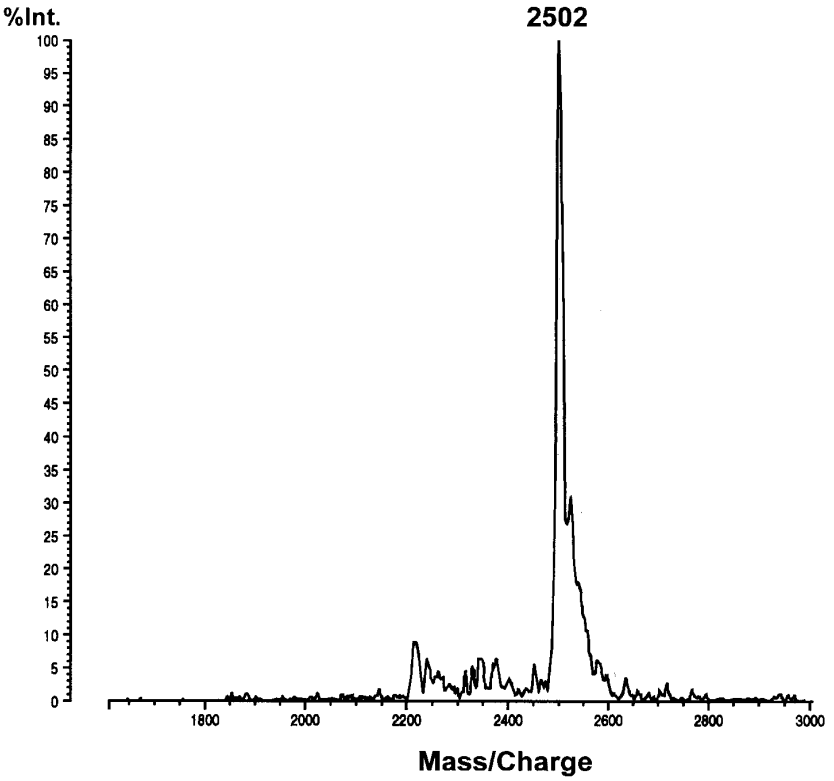

Fig. 2. Mass spectroscopic analysis of misgurin. Mass for misgurin ($MH^+ = 2502$) was determined by MALDI mass spectrometry.

minor peak of molecular mass 2537 Da representing the misgurin containing one Cl^- ion (Fig. 2). The complete sequence of misgurin was Arg–Gln–Arg–Val–Glu–Glu–Leu–Ser–Lys–Phe–Ser–Lys–Lys–Gly–Ala–Ala–Ala–Arg–Arg–Arg–Lys. The amino acid composition of misgurin, analyzed by the acid hydrolysis method (data not shown), and its molecular mass determined by MALDI mass spectroscopy were in good agreement with those obtained from the amino acid sequence. These results indicated that additional post-translational modifications were not present in misgurin.

A computer search comparing this amino acid sequence with all those published in the GenBank™/EMBL Data Bank file revealed that misgurin was a novel antimicrobial peptide.

3.3. Antimicrobial activity of the isolated peptides and synthetic peptide

The antimicrobial activity of misgurin was tested on 11 different microorganisms, including Gram-negative and Gram-positive bacteria and fungi. As shown in Table 1, misgurin displayed a strong antimicrobial activity against a broad spectrum of bacteria, including *B. subtilis*, *Staphylococcus aureus, Streptococcus mutans, Streptococcus pneumoniae, Escherichia coli*, *Serratia* sp., *Pseudomonas putida* and *Salmonella typhimurium*. Furthermore, *Candida albicans, Saccharomyces cerevisiae* and *Cryptococcus neoformance* were also killed. In addition, as compared to magainin 2 purified from *Xenopus laevis*, misgurin was approximately 6 times more potent against a broad range of microorganisms. Synthetic misgurin was also as active as the natural one. However, no appreciable hemolytic activity was observed for misgurin (up to 100 μg/ml) when tested against sheep red blood cells (Table 2).

Table 2
Hemolytic activities of misgurin and magainin 2

Concentrations	% Hemolysis of sheep red blood cells	
(μg/ml)	misgurin	magainin 2
5	0.00	0.19
10	0.00	0.19
25	0.23	0.57
50	0.23	0.95
100	0.46	1.14

3.4. SEM

The effect of misgurin and magainin 2 on the cell membrane of *B. subtilis* was examined under SEM. Peptides were added to bacteria and incubated for 1 h under the same conditions as in the antimicrobial assay. At the MIC, grooves developed on the surface of *B. subtilis*, indicating the disintegration of the cell membrane (Fig. 3C). At the concentration 5 times higher than the MIC, the cells ruptured and shrank as shown in Fig. 3D. It was evident from the electron micrographs that the inhibition of bacterial growth was associated with the destruction of the bacterial membrane (Fig. 3D).

3.5. Circular dichroism

The secondary structure of synthetic misgurin was estimated using a spectropolarimeter in the absence and presence of trifluoroethanol (Fig. 4). The contents of α-helix, β-sheet and unordered structure was calculated according to Yang et al. [23]. The CD spectrum of misgurin in 50 mM NAPB was characteristic of an unordered structure with 0% helical content. In the presence of 50% trifluoroethanol, the peptide became structured, exhibiting a high level, i.e. 63%, of α-helical folding, 4% β-sheet and 33% unordered structure.

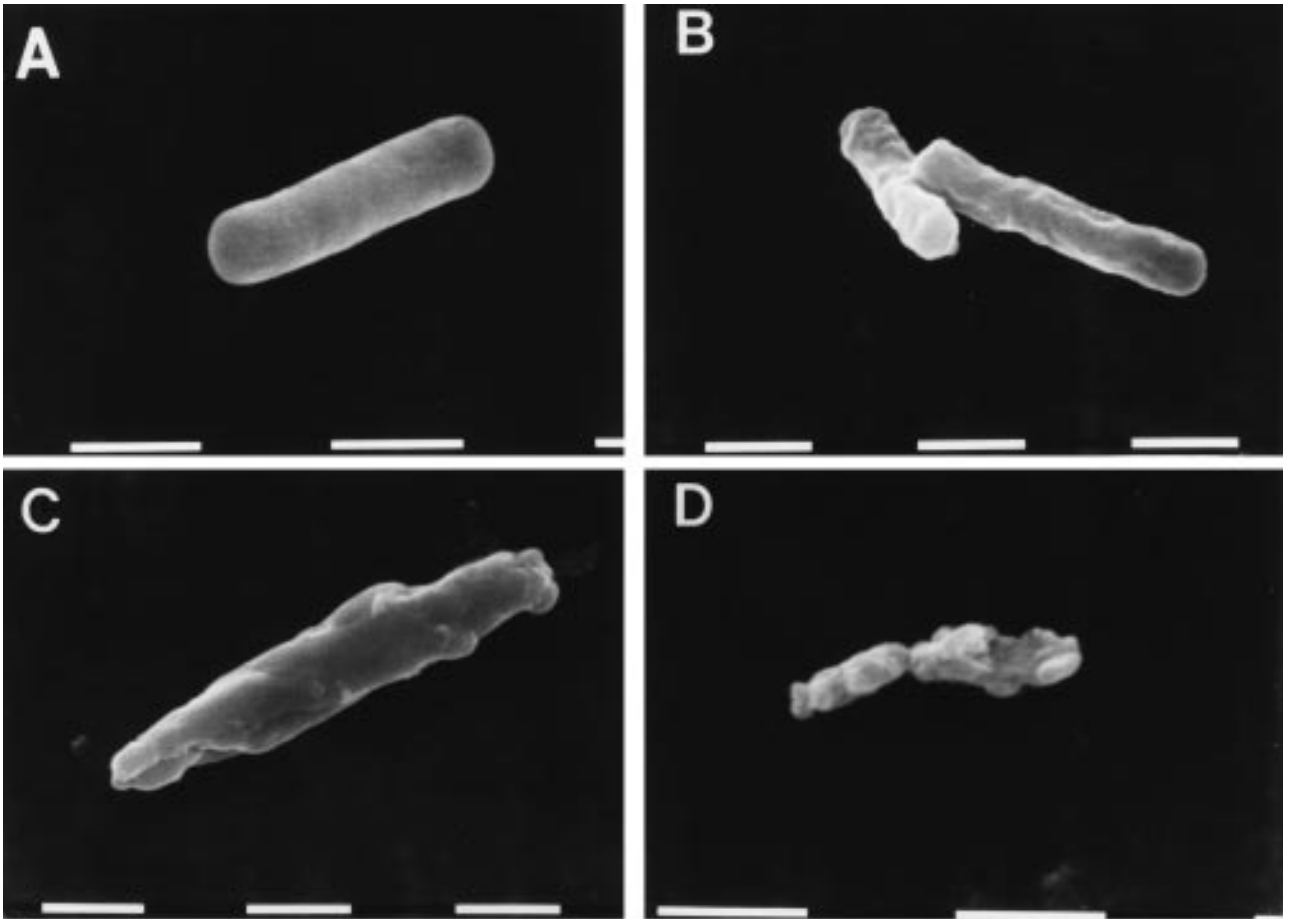

Fig. 3. SEMs of *B. subtilis* treated with magainin 2 or misgurin. *B. subtilis*, in mid-logarithmic growth at 10^7 cells/ml, was incubated with the antimicrobial peptides for 1 h. (A) Medium only; (B) magainin, 50 μg/ml (MIC); (C) misgurin, 8 μg/ml (MIC); (D) misgurin, 40 μg/ml. White bars represent 0.1 μm.

4. Discussion

The present study describes the purification and characterization of a novel antimicrobial peptide from the loach, which showed potent antimicrobial activities against a broad range of microorganisms, but without significant hemolytic activity. Antimicrobial peptides have been discovered from various life forms, from insect to human, and are now recognized as a type of innate immunity across life forms [14]. However, few fish antimicrobial peptides have been reported. To our knowledge, our peptide represents one of the first antimicrobial peptides isolated from fish which dose not possess significant hemolytic activity. Misgurin was purified to homogeneity by a three-step protocol using heparin-affinity chromatography, reverse-phase HPLC and gel-permeation HPLC. The homogeneity of the final preparation was assessed by amino acid analysis (data not shown), automated amino acid sequence determination, mass spectrometry analysis and solid-phase synthesis. MALDI mass spectrometry identified a molecular ion with a molecular mass of 2502 Da. This value was in good agreement with the calculated mass obtained from the amino acid sequence of misgurin, which indicated the absence of any secondary modifications (carboxamidation, O- and/or N-glycosidation, phosphorylation) of the constitutive amino acid side chains. Definitive confirmation of the proposed structure was achieved through solid-phase synthesis of misgurin. Synthetic misgurin showed chemical and physical properties that were indistinguishable from those of the natural compound.

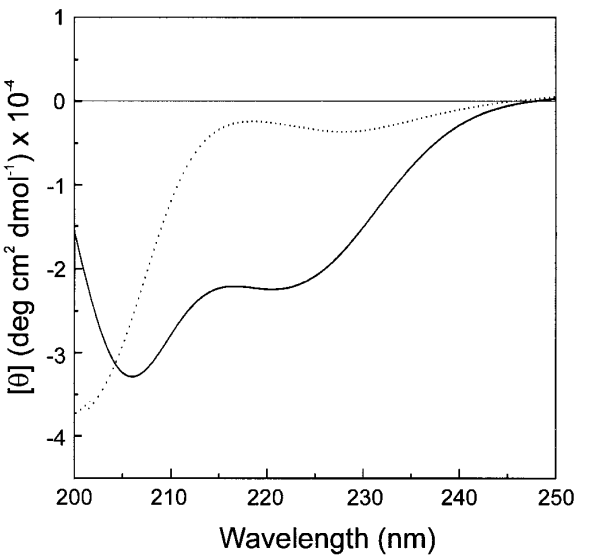

Fig. 4. CD spectra of misgurin in 50 mM NAPB in the absence or the presence of trifluoroethanol. The concentration of misgurin was 12 μM. Measurements were taken in 50 mM NAPB in the absence (dashed line) or in the presence (solid line) of 50% trifluoroethanol.

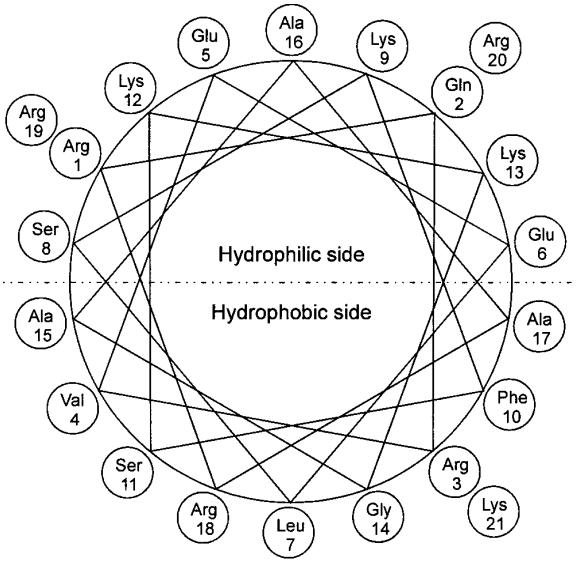

Fig. 5. Edmundson wheel representation of misgurin.

Comparison of the misgurin amino acid sequence with those in the GenBank™/EMBL Data Bank file confirmed that misgurin was a novel molecule. The primary amino acid sequence of misgurin can be fitted to an α-helical structure using an Edmundson wheel projection as shown in Fig. 5. The predicted amphipathic α-helical structure is strongly supported by the delineation of a hydrophobic and hydrophilic face on the Edmundson wheel projection. Further evidence for the amphipathic α-helical structure was obtained through CD measurement of misgurin in NAPB buffer, or in 50% trifluoroethanol. The CD spectra of misgurin in aqueous buffer were characteristic of a non-structured conformation. In the presence of a helix promoting solvent, however, misgurin became structured with a rather high level, i.e. 63%, of α-helix conformation. Misgurin did not show any homology with other known antimicrobial peptides, but structurally it belongs to one major group of antimicrobial peptides: the amphipathic α-helical peptides without cysteine (Fig. 5). The members of this group include cecropins [5], magainins [4], buforins [10] and dermaseptin [24]. Recent studies with several antimicrobial peptides from this family suggested that the peptides kill microorganisms either by forming pores and increasing the permeability [25], or by disintegration of cell membrane [26]. Misgurin may kill microorganisms in a way similar to that of the amphipathic α-helical antimicrobial peptide, because grooves were evident under SEM on the outer membrane of *B. subtilis* treated with misgurin at its MIC (Fig. 3C). The same result was also observed in bacteria treated with magainin 2, a well-known pore-forming peptide (Fig. 3B). Misgurin is highly basic (net +7) due to the presence of multiple arginine and lysine residues. This gives it an advantage over other antimicrobial peptides, including magainin 2 (net +4), in the initial contact with the target organisms, resulting in an increased antimicrobial activity. According to the suggested action mode, misgurin binds preferentially to the negatively charged phospholipids, and lies on the surface of membrane such that the positive charges of the basic amino acids interact with negatively charged phospholipid head groups or water molecules. It probably then rotates, leading to reorientation of the hydrophobic residues toward the hydrophobic core of the membrane. Finally, disintegration of the membrane occurs when the lipid packing in the bilayer structure is disrupted [26]. The cDNA cloning of misgurin and its gene expression mechanism in the loach are now under study.

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