Salusin-β, an Antimicrobially Active Peptide against Gram-Positive Bacteria

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Salusin-β has been detected in numerous mammalian tissues and has been shown to have various effects on the cardiovascular system. In this study, we showed that salusin-β exhibited potent antibacterial activity against Gram-positive microorganisms such as Bacillus subtilis NBRC 3513, Bacillus megaterium ATCC 19213, Staphylococcus aureus NBRC 12732, and Staphylococcus epidermidis NBRC 12933. A cytoplasmic membrane-depolarizing assay using the DiSC3(5) dye revealed that the addition of 4 nmol/mL of salusin-β caused the leakage of fluorescence dye from Staphylococcus aureus NBRC 12732. The antimicrobial potency and circular dichroism (CD) spectroscopy of five analogs related to salusin-β were examined to determine structure–function relationships in its N- and C-terminal regions. The results obtained suggest that the N-terminal sequences of the salusin-β molecule are important for the expression of the potent antimicrobial activity of this peptide. A profile corresponding to that of the α-helix conformation was observed in the salusin-β solution.

Key words salusin-β; antibiotic activity; antibacterial action mechanism; structure–function relationship; circular dichroism (CD) spectroscopy

Salusin-α and salusin-β are endogenous bioactive peptides that have been identified in a human full-length enriched cDNA library using bioinformatics analyses by Shichiri et al.1) Salusin-α is composed of 28 amino acids (H-Ser-Gly-Ala-Leu-Pro5-Pro-Ala-Pro-Ala-Ala10-Pro-Arg-Pro-Ala-Leu15-Arg-Ala-Gln-Arg-Ala20-Gly-Pro-Ala-Gly-Pro25-Gly-Ala-Lys-NH2) and salusin-β of 20 amino acids (H-Ala-Ile-Phe-Ile-Phe2-Ile-Arg-Trp-Leu-Leu16-Lys-Leu-Gly-His-His15-Gly-Arg-Ala-Pro-Pro30-Off). Salusins have been detected in various human tissues such as the nervous system, cardiovascular system, kidneys, monocytes, and macrophages, as well as human body fluids such as plasma and urine.1–3) Many studies have investigated the biological activities of these peptides demonstrated that they acted on the cardiovascular system. Rapid and temporary hypotension and bradycardia were observed following the intravenous administration of these peptides to rats.1) Watanabe et al. reported that the development of atherosclerosis may have been accelerated by salusin-β and suppressed by salusin-α via the regulation of acyl-CoA cholesterol acyltransferase-1 in acute coronary syndrome.5) They also showed that salusin-α was a useful biomarker, with better sensitivity and specificity for detecting coronary artery disease.5

Analytical examinations of biological fluids such as human milk, cow milk, cheese whey, and blood have recently revealed that there are many bioactive peptides including salusin peptides in these liquids.6,7) This study showed that the peptides in cow milk may be an important and nutritious food for (neonatal) calves and humans due to their biological and physiological properties. Some peptides such as hepcidin, lactoferricin, casecidin, and isracidin, which have been detected in human, cow and goat milk, have been shown to exhibit antibacterial activities against both Gram-positive and Gram-negative bacteria.8–11) In the present study, we determined the antibacterial activity and action mechanism of synthetic salusin-β. We synthesized salusin-β-related peptides and subjected them to biological assays in order to determine the function of the salusin-β molecule itself. Furthermore, the conformations of these peptides were studied using circular dichroism (CD) spectroscopy to obtain a better understanding of the structure–function relationship of synthetic peptides.

Experimental

General

HPLC was performed on an apparatus equipped with two 510 pumps (Waters Corp., MA, U.S.A.), a U6K injector (Waters), Lambda-Max Model 481 LC Spectrophotometer (Waters), 680 Automated Gradient Controller (Waters), and Chromatocorder 21 (System Instruments Co., Ltd., Tokyo, Japan). Gel filtration column chromatography was performed using Toyopearl HW-40-S (Tosoh Corp., Tokyo, Japan). Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was conducted on a Model Voyager RP BioSpectrometry Workstation (PerSeptive Biosystems, Inc., MA, U.S.A.). 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids and all reagents for peptide synthesis were purchased from Watanabe Chem., Ind., Ltd. (Hiroshima, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. The protective groups of the amino acid side chains used here were 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf), trityl (Trt) and tert-butyloxy carbonyl (Boc) for Arg, His, and Lys, respectively. Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification.

Preparation of Synthetic Peptides

Salusin-β and its related peptides were synthesized using a continuous flow solid phase method with a Fmoc-strategy by an automated peptide synthesizer (Model Pioneer; Life Technologies, CA, U.S.A.). The peptides prepared included salusin-β, salusin-β (1–11),...
H-Ala-Ile-Phe-Ile-Phe<sub>5</sub>-Ile-Arg-Trp-Leu-Leu<sup>10</sup>-Lys-Leu-Gly-His-His<sup>15</sup>-Gly-Arg-Ala-Pro-Pro<sup>36</sup>-OH

**[1] Salusin-β**(1-11)
H-Ala-Ile-Phe-Ile-Phe<sub>5</sub>-Ile-Arg-Trp-Leu-Leu<sup>10</sup>-Lys-OH

**[2] Salusin-β**(1-17)
H-Ala-Ile-Phe-Ile-Phe<sub>5</sub>-Ile-Arg-Trp-Leu-Leu<sup>10</sup>-Lys-OH

**[3] Salusin-β**(7-17)
H-Arg-Trp-Leu-Leu<sup>10</sup>-Lys-Leu-Gly-His-His<sup>15</sup>-Gly-Arg-OH

**[4] Salusin-β**(7-20)
H-Arg-Trp-Leu-Leu<sup>10</sup>-Lys-Leu-Gly-His-His<sup>15</sup>-Gly-Arg-Ala-Pro-Pro<sup>36</sup>-OH

**[5] Salusin-β**(11-20)
H-Lys-Leu-Gly-His-His<sup>15</sup>-Gly-Arg-Ala-Pro-Pro<sup>36</sup>-OH

Fig. 1. Structures of Salusin-β and Synthetic Peptides [1]–[5]

(1–17), (7–17), (7–20), and (11–20) (Fig. 1). As an example, the synthesis of human salusin-β was described below.

The peptide chain was elongated on Fmoc-Pro-tritylcarboxamidomethyl polyethylene glycol resin (0.2 mmol, 100–200 mesh, Watanabe Chemical Ind., Hiroshima, Japan) with the Fmoc derivatives of amino acids such as Ala, Arg(Pbf), Gly, His(Trt), Ile, Leu, Lys(Boc), Phe, and Pro. The Fmoc-amino acids (4-fold excess amount of each) were successively introduced to the resin according to the peptide sequence using 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo(4,5-b) pyridinium 3-oxide hexafluorophosphate (HATU) as a coupling reagent. After the construction of the desired sequence, the protected peptide-resin (1.25 g) was treated with trifluoroacetic acid (TFA) (16.5 mL) in the presence of thioanisole (1.0 mL), phenol (1.0 g), H<sub>2</sub>O (1.0 mL), ethanedithiol (0.5 mL), and trisopropylsilane (0.2 mL) for 120 min at room temperature to give a crude preparation of salusin-β (190 mg). The crude salusin-β (90 mg) obtained was purified by reverse phase HPLC on a Develosil ODS-HG-5 column (2 × 25 cm, Nomura Chemical Co., Seto, Japan) with a solvent system of 0.01 mol/L hydrogen chloride/acetonitrile (70/30, v/v%) over 30 min at a flow rate of 10 mL/min to give the desired salusin-β (10.7 mg).

**Determination of the Antibacterial Activities of the Synthetic Peptides** Bacillus subtilis NBRC 3513, Bacillus megaterium ATCC 19213, Staphylococcus aureus NBRC 12732, Staphylococcus epidermidis NBRC 12933, Enterococcus faecalis NBRC 3989, Escherichia coli NBRC 12734, Pseudomonas aeruginosa NBRC 3080, Salmonella Typhimurium NBRC 12529, Klebsiella pneumoniae NBRC 3317, and Serratia marcescens NBRC 3046 were grown overnight at 37°C on nutrient agar medium and harvested in sterile saline. The densities of the bacterial suspensions were determined at 600 nm, using a standard curve relating absorbance to the number of colony forming units (CFU).

Minimum inhibitory concentrations (MICs) of the synthetic peptides against several bacterial strains were assayed using the microplate dilution method as follows: 100 µL of a serial dilution of the synthetic peptide in 10% dimethyl sulfoxide (DMSO)-sterile saline solution was added to a mixture of 10 µL of the bacterial suspension (approximately 10<sup>8</sup> CFU/mL) and 90 µL of Mueller–Hinton broth (Difco Laboratories, NJ, U.S.A.) in each well of a flat-bottomed microplate (Corning Laboratory Sciences Company, NY, U.S.A.). The highest peptide concentration tested was 128 nmol/mL. The plates were then incubated overnight at 37°C for the MIC evaluation. The replacement of the peptide solution with sterile saline alone was used as the control. MIC was expressed as the lowest final concentration (nmol/mL) at which no growth was observed. Antibiotic activity tests were performed 7–10 times for each peptide and we obtained the same values for each peptide within the limits of error.

**Cytoplasmic Membrane Depolarization Assay** The interaction between salusin-β and the bacterial cytoplasmic membrane was determined using the membrane potential sensitive cyanine dye; 3,3′-dipropylthiadicarbocyanine iodide (DiSC3(5)) (Sigma-Aldrich Co., LLC, MO, U.S.A.).

Staphylococcus aureus NBRC 12732 was grown to the mid-log phase at 37°C in trypt-soya broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The bacterial suspension was centrifuged (2000×g for 5 min at room temperature), washed twice with wash buffer (5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mmol/L glucose, pH 7.4) and resuspended in assay buffer (the wash buffer containing 0.1 mol/L KCl) to an OD<sub>600</sub> of 0.5.

A 100 µL aliquot of the bacterial suspension was added to 889 µL of assay buffer, followed by 1 µL of 1 mmol/L DiSC3(5) in DMSO. The bacterial suspension was then incubated at 20°C until the stable reduction of fluorescence due to dye uptake and quenching in bacteria in response to an intact membrane potential. Salusin-β or gramicidin S (GS) was then added at final concentrations of 0 to 4 µmol/L and 2 µmol/L, respectively. Changes in fluorescence in response to membrane depolarization were recorded with a Hitachi F-2700 Fluorescence Spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at an excitation wavelength of 622 nm and emission wavelength of 670 nm at 20°C.

**CD Spectra of the Synthetic Peptides** CD spectra were obtained using a model J-725 spectropolarimeter (JASCO Ltd., Tokyo, Japan), with a 1.0-mm quartz cell at room temperature. CD spectroscopy of salusin-β and its analogs [1]–[5] was performed with methanol (MeOH) solution at a concentration of 1.0×10<sup>−4</sup> mol/L.

**Results and Discussion**
**Synthesis of Peptides** The synthesis of salusin-β and its analogs [1]–[5] (Fig. 1) was performed as reported in Experimental. The product was extensively purified by HPLC and gel-filtration prior to the examination of its biological activity.
The homogeneity of the purified peptide was confirmed by analytical HPLC and MALDI-TOF mass spectrometry (Table 1). The purity of the synthetic peptides was >98%.

**Antimicrobial Activity of Salusin-β and Its Fragments**

The MIC values of salusin-β, i.e., the minimum amounts of the peptides necessary for the complete inhibition of growth of ten kinds of bacteria, were shown in Table 2. The bacterial strains used in this study were five kinds each for Gram-positive (A–E in Table 2) and Gram-negative (F–J in Table 2) bacteria, respectively. The synthetic salusin-β tested here exhibited high antimicrobial activity against Gram-positive bacteria such as *S. aureus* NBRC 12732, *B. megaterium* ATCC 19213, and *B. subtilis* NBRC 3513, but slightly lower activity against *S. epidermidis* NBRC 12933, and no activity against *Enterococcus faecalis* NBRC 3989. On the other hand, the peptide exhibited no antibacterial potency against Gram-negative bacteria such as *E. coli* NBRC 12734 and *S. Typhimurium* NBRC 12529. The result that salusin-β has potent antibacterial activity, especially against Gram-positive bacteria such as *S. aureus* NBRC 12732 and *B. megaterium* ATCC 19213, has not been reported previously. We also assessed the antibacterial activity of synthetic salusin-α using the same protocol, but did not find any potency for either Gram-positive or Gram-negative bacteria (data not shown). The potent antibacterial characteristics toward Gram-positive microbes were only found in salusin-β. Aydin recently reported that biological fluids such as human milk, cow milk, cheese whey, and blood contained many bioactive peptides such as salusin peptides. These findings suggest that the salusin-β peptide in milk may act as an antibacterial agent against contaminating microorganisms in the fluid.

The structure–function relationship of salusin-β as an antibiotic has not yet been the subject of any study. We synthesized five analogs of salusin-β, and subjected them to antibacterial assays in order to clarify the biological effects of the peptide chain length of salusin-β. We designed analogs [1], [2], [3], [4] and [5] which might be released by activities of endogenous peptidases such as trypsin from salusin-β in vivo. Analogs [1] and [2], which lack 9 and 3 amino acid residues at the C-terminal region relative to the salusin-β peptide molecule, exhibited activity against all the Gram-positive microorganisms tested here. Although the antibacterial potency of [2] was higher than that of [1], the activity of [2] was lower than that of salusin-β itself. However, the MIC values of [2] and salusin-β against *S. epidermidis* NBRC 12933 were 8 and 16 nmol/L, respectively. These results indicated that the antibiotic activity of [2] toward this bacterium was two times higher than that of salusin-β. The removal of 6 and 3 amino acid moieties from the N-terminal and C-terminal regions of salusin-β led to a further decrease in antibacterial activity. Analog [3] did not exhibit any activity against *S. epidermidis* NBRC 12933. The activities of analog [3] toward *B. subtilis* NBRC 3513, *B. megaterium* ATCC 19213, and *S. aureus* NBRC 12732 were lower than those of analog [1]. On the other hand, the antimicrobial activities of analog [4] and [5], in which 6 and 10 amino acid residues were eliminated in the N-terminal of salusin-β, were reduced against all the Gram-positive microorganisms tested here. Analog [4] showed very low activities against three kinds of bacteria, and no potency toward *S. epidermidis* NBRC 12933. Furthermore, analog [5] exhibited no bacteriostatic effects against all the Gram-positive microorganisms tested here. The order of the antimicrobial potencies of the synthetic peptides examined in this study was salusin-β > [2] > [1] > [3] > [4] > [5]. These results suggest that the N-terminal region of salusin-β is more necessary than the C-terminal region for the expression of antimicrobial activity.

**Cytoplasmic Membrane Depolarization**

Inhibiting the biological synthesis of bacterial nucleic acids, proteins, and the cell wall is the action mechanism of antibiotics. The principal mode of antibiotic action has been proposed to result from an interaction between antibacterial peptides and the cell membrane. The antibacterial potencies of the synthetic peptides examined in this study were salusin-β > [2] > [1] > [3] > [4] > [5]. These results suggest that the N-terminal region of salusin-β is more necessary than the C-terminal region for the expression of antimicrobial activity.

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**Table 1. MALDI-TOF Mass Data of Salusin-β and the Synthetic Peptides [1]–[5]**

| Peptide   | Formula                  | [M+H]+, m/z Calculated | Found |
|-----------|--------------------------|------------------------|-------|
| Salusin-β | C113H170N82O24           | 2342                   | 2342  |
| [1]       | C63H92N13O12             | 1419                   | 1419  |
| [2]       | C102H157N29O18           | 2077                   | 2077  |
| [3]       | C63H101N23O12            | 1372                   | 1372  |
| [4]       | C74H114N16O12            | 1419                   | 1419  |
| [5]       | C82H128N24O18            | 1637                   | 1637  |

**Table 2. Antibacterial Activities of Salusin-β and the Synthetic Peptides [1]–[5]**

| Peptide   | MIC Values (nmol/mL)  |
|-----------|-----------------------|
| Salusin-β | 8                     |
| [1]       | 32                    |
| [2]       | 16                    |
| [3]       | 64                    |
| [4]       | 128                   |
| [5]       | 128                   |

**Table 3. Antibacterial Activities of Salusin-β and the Synthetic Peptides [1]–[5]**

| Peptide   | MIC Values (nmol/mL)  |
|-----------|-----------------------|
| Salusin-β | 8                     |
| [1]       | 32                    |
| [2]       | 16                    |
| [3]       | 64                    |
| [4]       | 128                   |
| [5]       | 128                   |

**Note:** Minimum inhibitory concentration (MIC) values were determined by a microplate dilution method with 10⁵ organisms per mL medium. Antibacterial activity tests were performed 7–10 times for each peptide. *A. B. subtilis* NBRC 3513, *B. megaterium* ATCC 19213, *C. Staphylococcus aureus* NBRC 12732, *D. Staphylococcus epidermidis* NBRC 12933, *E. Enterococcus faecalis* NBRC 3989, *F. Escherichia coli* NBRC 12734, *G. Pseudomonas aeruginosa* NBRC 3080, *H. Salmonella Typhimurium* NBRC 12529, *I. Klebsiella pneumoniae* NBRC 3317, *J. Serratia marcescens* NBRC 3046.
membrane of target microorganisms. In this study, we investigated the mode of the antibacterial action of salusin-β using *S. aureus* NBRC 12732, which was the most sensitive toward this peptide, and compared it with GS, which disrupted the bacterial cell membrane.\(^{14}\)

The results of the cytoplasmic membrane-depolarizing assay measured using DiSC\(_3\)(5) dye are shown by increases in fluorescence units in Fig. 2. The addition of 2 nmol/mL of GS to *Staphylococcus aureus* caused the leakage of dye from this bacterium. Activity was observed as a value of approximately 43 fluorescence units 150 s after the addition of this peptide. On the other hand, salusin-β caused the slow leakage of dye from the microorganism tested here, and the fluorescence unit value was 50 in 300 s after the peptide was added. The intensity of leaked fluorescence depended on the concentration of salusin-β. These results suggested that salusin-β also had the ability to depolarize the bacterial cytoplasmic membrane, and this may be linked to the antibacterial activity of this agent.

**CD Spectra of the Synthetic Peptides** The CD spectra of salusin-β and synthetic peptides analogs [1]–[5] in methanol are shown in Fig. 3. The conformation of salusin-β exhibited a strong negative band near 207 nm, and a shoulder at the 215–225 nm region. These CD patterns of salusin-β resembled a peptide having an α-helix conformation. The CD spectrum of analog [2] was found to have a curve similar to that of salusin-β, whereas the trough (207 nm) was slightly shallow. Furthermore, the CD spectra of analog [1] and [3]–[5] were very different from that of salusin-β. Although the antibacterial activity of analog [2] was slightly less than that of salusin-β, low bacteriostatic activity was observed in analogs [1] and [3]–[5]. These results suggest that contribution of the α-helix conformation to the expression of the potent antimicrobial activity of salusin-β peptide is not clear in this CD spectrum study.

In conclusion, we demonstrated that salusin-β exhibited potent antibacterial activity against Gram-positive microorganisms. Salusin-β had the ability to depolarize the cytoplasmic membrane, and this may be linked to the antibacterial activity of this agent. The partial deletion of the N-terminal region of salusin-β decreased the antimicrobial activity of the peptide more than the deletion of the C-terminal region. A profile corresponding to that of the α-helix conformation was observed in the salusin-β solution.

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