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Two antimicrobial and nematicidal peptides derived from sequences encoded *Picea sitchensis*

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Two antimicrobial peptides (piceain 1 and 2) derived from sequences encoded *Picea sitchensis* are identified. Their amino acid sequences are KSLRPRCWIKIKFRCKSLKF and RPRCWIKIKFRCKSLKF, respectively. One intra-molecular disulfide bridge is formed by these two half-cysteines in both piceain 1 and 2. Antimicrobial activities of synthesized piceains against several kinds of microorganisms were tested. They showed antimicrobial activities against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and fungus *Candida albicans* but little antimicrobial activity against *Bacillus subtilis*. The results of nematicidal test showed they exerted strong nematicidal activities against *Caenorhabditis elegans*, following exposure for 5 h at concentrations as low as 10 μg/ml. They had weak hemolytic abilities against human and rabbit red cells. At the concentration of 250 μg/ml, they induced red cell hemolysis of less than 5%. Circular dichroism spectra of the two antimicrobial peptides were investigated in several solutions. Their main secondary structure components are β-sheet and random. The current work provides a novel family of antimicrobial and nematicidal peptides with unique disulfided loop containing nine amino acid residues. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *Picea sitchensis*; antimicrobial and nematicidal peptides; innate immunity

Introduction

Antimicrobial peptides are key effectors in the innate immunity. Antimicrobial peptides act as the first defensive line against microorganism infection because of their rapid and direct microorganism-killing abilities. Antimicrobial peptides may be more than 100 times faster than other proteins such as IgM on killing microbes [1]. In addition, some antimicrobial peptides also have cytotoxicity against tumor cells [2], hemolytic activity [3], spermicidal activity [4–7] and abilities to induce mast cell degranulation and histamine release [8,9]. A large amount of antimicrobial peptides have been found in animals, plants, and microorganisms. Especially, many antimicrobial peptides have been identified from animals, such as amphibians and insects. Amphibian skins are pools to isolate and identify antimicrobial peptides. More than 50 families of antimicrobial peptides have been identified from skins of Pipidae, Hylidae, Hyperoliidae, and Ranidae amphibians [10–13]. Insects represent the largest class within the animal kingdom in terms of species number. The best characterized aspect of the insect immunity is the synthesis by the fat body and certain blood cells of antimicrobial peptides/polypeptides and their rapid release into the hemolymph [14]. Since the original report of cecropin from Boman [15], more than 170 antimicrobial peptides/polypeptides have been found in insects [14].

There are 271 plant antimicrobial peptides in a database recently updated [16]. These plant antimicrobial peptides are classified as cyclotides, defensins, hevein-like, impatiens, knottins, lipid-transfer proteins, shepherins, snakins, thionins or vicilin-like, Maize Basic Peptide-1 (MBP-1)-1 and β-barellin [16]. They are from families of Amaranthaceae, Andropogoneae, Brassicaceae, Oryzeae, Santalaceae, Spermacoceae, Triticeae, Vicieae, and Violaceae [16]. No antimicrobial peptide has been found from Pinaceae. The current work identified two antimicrobial peptides from *Picea sitchensis*.

Materials and Methods

Peptide Synthesis

According to available *P. sitchensis* genome information, a protein precursor (EF087003.1 MIKRLNYLRDNSVIFFFSSYKKKSLR-PRCWIKIKFRCKSLKF) was found to contain possible antimicrobial sequences. According to the sequences, four short peptides (KSLRPRCWIKIKFRCKSLKF and RPRCWIKIKFRCKSLKF with or without intra-molecular disulfided bridge) were synthesized by GL

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Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by HPLC and mass spectrometry to confirm purity higher than 98%.

Antimicrobial Test

Microorganisms including Gram-positive bacterium *Staphylococcus aureus* (ATCC 25 923), *S. aureus* (ATCC 43 300, methicillin resistance), *Bacillus subtilis* (ATCC 6633), Gram-negative bacteria *Escherichia coli* (ATCC 25 922), *E. coli* ML-35P (penicillin resistance), *E. coli* 08 040 722 (clinically isolated strain), *E. coli* 08A866 (clinically isolated strain), *Pseudomonas aeruginosa* PA01 (streptomycin resistance), *P. aeruginosa* (ATCC 27 853), *P. aeruginosa* 08 031 014 (clinically isolated strain), and fungus *Candida albicans* (ATCC 20 032) were obtained from Kunming Medical College. They were first grown in Luria–Bertani broth or yeast extract–peptone–dextrose broth as our previous methods [10]. MICs of tested samples against these microorganisms were determined as previous reports [10]. Briefly, serial dilutions of each sample were made in 50 µl liquid broth in 96-well plates, then each well was inoculated with 50 µl of the test organism to a final concentration of approximately 10^5 Colony-Forming Units (CFU)/ml. The MIC was defined as the lowest concentration of test peptides to inhibit microorganism growth after overnight incubation at 37 °C.

Nematicidal Test

Caenorhabditis elegans was obtained from Yunnan University and maintained on oatmeal medium (oatmeal 20 g, water 80 ml) at 25 ± 2 °C. For the nematicidal assay, 5 µl aliquots of samples at various concentrations were introduced into 96-well MultiDish wells containing 95 µ 1 aqueous suspensions (distilled water) of approximately 50 adults of *C. elegans* and incubated at 25 ± 2 °C for 5 or 15 h. To prevent bacterial growth, the nematode suspensions also contained streptomycin (30 µg/ml) and chloramphenicol (30 µg/ml). Nematode mortality was expressed as described by Leyns *et al*. [17]. *C. elegans* thoroughly exposed to 100 µ 1 distilled water served as controls. The experiment was performed three times, with five replicates per treatment.

Hemolytic Assays

Human and rabbit red blood cells were used for hemolytic assay. Human was bled by vein puncture and rabbit was bled by cardiac puncture. Tested samples were incubated with human or rabbit red blood cells in Alsever's solution (in g/l: NaCl, 4.2; citric acid· 3Na· $2H_2O$, 8.0; citric acid·H_2O, 0.55; D-glucose, 20.5) according to the methods reported by Bignami [18]. Samples were serially diluted and incubated with rabbit red blood cells at 37 °C for 30 min. After centrifugation, the supernatant absorbance at 540 nm was measured. Maximum hemolysis was determined by adding 1% Triton X-100 to the red cells.

Transmission Electron Microscopy

The effects of antimicrobial peptides on Gram-positive *S. aureus* ATCC 25 923 were observed by transmission electron microscopy according to the methods described by Friedrich *et al*. [19]. Exponential-phase bacteria were treated with the peptides (100 µg/ml) for 30 min at 37 °C. After treatment, the bacteria were centrifuged at 300 × *g* for 10 min, and the pellets were fixed with 2.5% buffered glutaraldehyde for 1 h. The cells were then postfixed in 1% buffered osmium tetroxide for 1 h, stained en bloc with 1% uranyl acetate, dehydrated in a graded series of ethanol washes, and embedded in white resin. The buffer used was 0.1 M sodium cacodylate, pH 7.4. Thin sections were prepared on copper grids using an LKB-V (Sweden) and stained with 1% uranyl acetate and lead citrate. The resin and grids were purchased from Marivac (Halifax, Nova Scotia, Canada). Microscopy was performed with a JEM1011 microscope under standard operating conditions.

Table 1. Antimicrobial activity of antimicrobial peptides from *P. sitchensis*

Microorganisms	MIC[a] (µg/ml) P1	P2
S. aureus ATCC 25923	9.38	9.38
S. aureus ATCC 43300	9.38	9.38
B. subtilis ATCC 6633	>100	>100
C. albicans ATCC 20032	9.38	18.75
E. coli ATCC 25922	18.75	37.5
E. coli ML-35P	18.75	37.5
E. coli 08040722 (CI)	75	75
E. coli 08A866 (CI)	37.5	37.5
P. aeruginosa PA01	9.38	9.38
P. aeruginosa ATCC 27853	9.38	18.75
P. aeruginosa 08031014 (CI)	37.5	37.5

[a] Minimal peptide concentration required for total inhibition of cell growth in liquid medium. These concentrations represent mean values (±20%) of three replicates.
P: picein, CI: clinically isolated strain.

CD Spectroscopy

CD data were obtained with a Jasco J-810 CD spectrophotometer using a 0.2 mm path length cylindrical cuvette. The response was measured using wavelengths from 200 to 250 nm with a 0.2 nm step resolution and a 1 nm bandwidth. The rate of 100 nm/min and a response time of 0.25 s were used, and the spectra were averaged over eight scans as our previous method [20]. Samples were prepared by dissolving the peptide powder in TFE/H_2O mixtures or in SDS micelles of different concentrations. The experimental temperature was 25 °C. In each case, the CD spectrum of the solvent was subtracted from the spectrum of the peptide. The secondary structure elements of the peptides were estimated according to the Chang formula [21].

Results

Antimicrobial Activities

Picein 1 and 2 contain 20 and 17 amino acid residues with a predicted isoelectric point of 11.13 and 11.08, respectively. There are eight and seven basic amino acid residues (Arg or Lys) in picein 1 and 2, respectively, and no acidic residues. Both of them contain a intra-molecular disulfided bridge. Both picein 1 and 2 were synthesized and their antimicrobial abilities were tested. They exerted antimicrobial activities against the tested microorganisms including Gram-positive and Gram-negative bacteria and fungi as listed in Table 1 but they had no antimicrobial ability against *B. subtilis*. They have the same antimicrobial effects on antibiotics-resistant and common strains. The antibiotic activity was proven to be lethal for the susceptible strain. Among the

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Table 2. Nematicidal activity of antimicrobial peptides from *P. sitchensis* against *C. elegans*

Antimicrobial peptides (μg/ml)	Nematode mortality (%)[a]			
	P1		P2	
	5 h	15 h	5 h	15 h
10	55.9	50.2	50.7	41.8
25	71.6	68.1	53.6	50.2
50	72.7	71.7	55.8	57.3
100	75.8	72.0	55.7	64.0
200	76.7	70.9	57.3	66.1

[a] These percentages represent mean values (±20%) of three replicates. P: piceain.

Table 3. Hemolytic activity of antimicrobial peptides from *P. sitchensis*

Antimicrobial peptides (mg/ml)	Hemolysis (%)[a]			
	P1		P2	
	HRC	RRC	HRC	RRC
0.0625	0.7	1.5	0.6	2.2
0.125	0.8	7.2	0.8	4.7
0.25	5.9	7.8	5.2	5.2
0.5	7.6	9.5	5.3	12.4
1	7.8	57.1	11.3	66.3

[a] These percentages represent mean values (±20%) of three replicates. P: piceain; HRC: human red cell; RRC: rabbit red cell.

tested microorganisms, they showed the strongest antimicrobial activities against *S. aureus* and *P. aeruginosa*. The MICs against *S. aureus* and *P. aeruginosa* are about 9.38 μg/ml.

Nematicidal Activities

Both piceain 1 and 2 at serial concentrations (10, 25, 50, 100, and 200 μg/ml) exerted strong nematicidal activities against the *C. elegans* as listed in Table 2. At the same incubated time point, piceain 1 induced higher mortality of the adults of *C. elegans* than piceain 2. After exposure to piceains at 10 μg/ml for 5 and 15 h, piceain 1 induced 55.9 and 50.2% mortality, respectively, piceain 2 induced 50.7 and 41.8% mortality, respectively. When the concentration reaches up to 25 μg/ml, the mortality of piceain 1 suddenly increased to 71.6 (5 h) and 68.1% (15 h), respectively, while piceain 2 increased less obviously; From 50 μg/ml onwards, no more significant increases in mortality of both piceains were observed.

Hemolytic Activities

As listed in Table 3, the hemolytic activities of both piceain 1 and 2 with five different concentrations (0.0625, 0.125, 0.25, 0.5, and 1 mg/ml) against human and rabbit red cells were tested. In most of the tested concentrations, they showed weak hemolytic abilities against both human and rabbit red cells. The only exception is the concentration at 1 mg/ml. About 1 mg/ml of piceain 1 and 2 induced 57.1 and 66.3% rabbit red cell hemolysis, respectively while the hemolysis rate against human red cell is 7.8 and 11.3%,

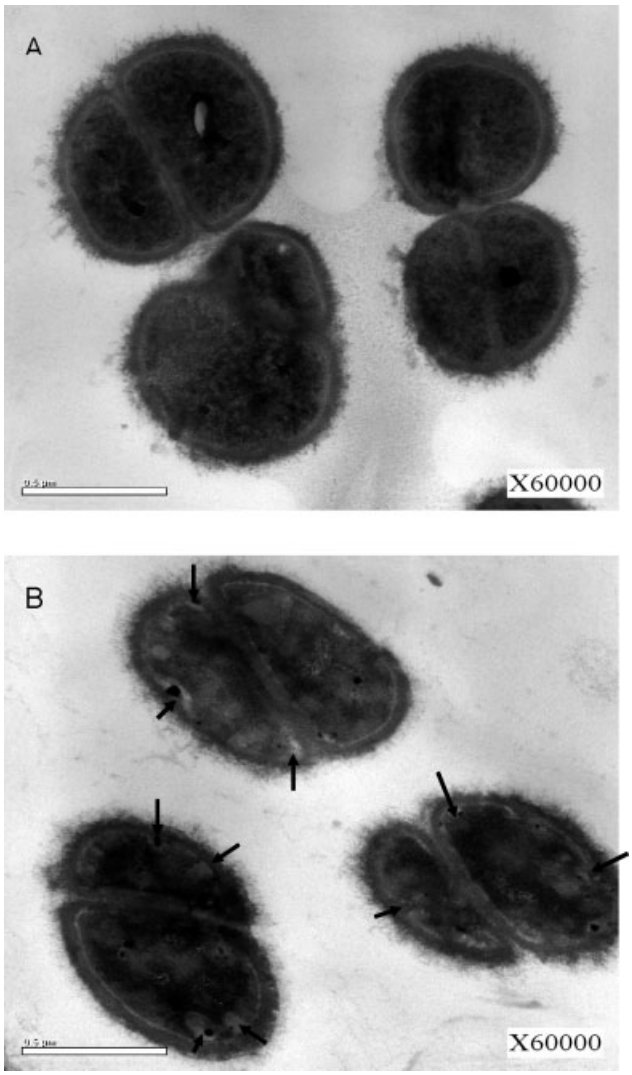

Figure 1. Ultrastructure of *S. aureus* treated by piceain 1 (100 μg/ml). (A) Control; (B) treated by piceain 1. The mesosome structures are indicated by arrows.

respectively. Their hemolytic ability to rabbit red cells is stronger than human red cells.

Transmission Electron Microscopic Analysis

It was found that piceain 1 killed bacteria by directly affecting its cell wall and membrane (Fig. 1). These cell walls and membranes are significantly destroyed. Large laminar mesosomes were seen to arise from the septa and cell wall of piceain 1-treated bacteria, as indicated by the arrows in Fig. 1 while no detectable mesosome structures were found in the control group of untreated bacteria (Fig. 1A). These mesosomes may be indicative of cytoplasmic membrane alteration induced by the cationic peptides [19].

Secondary Structure Analysis

The conformation distribution of both piceain 1 and 2 was analyzed. CD spectroscopy was used to study secondary structure of piceains (Table 4). Their main patterns of CD in all the tested solutions are β-sheet and random. They have no characteristic negative absorption at the 210 and 225 nm, suggesting that these

Table 4. Secondary structural components of piceains in different solutions

Antimicrobial peptides	Helix (%)[a]		Beta (%)[a]		Turn (%)[a]		Random (%)[a]	
	P1	P2	P1	P2	P1	P2	P1	P2
0.9% NaCl (w/v)	0.0	0.0	28.6	50.7	13.0	0.0	58.4	49.3
20 mM Tris-HCl, pH 7.0	0.0	0.0	4.4	35.7	8.9	11.4	55.4	84.2
SDS (mM)								
0	0.0	0.0	35.7	4.4	8.9	11.4	55.4	84.2
30	0.0	2.4	53.0	30.1	4.5	0.0	42.5	67.5
60	0.0	2.5	64.7	43.1	0.0	0.0	35.3	54.4
90	6.4	0.0	15.8	39.5	22.5	2.7	55.3	57.8
120	0.0	1.6	41.2	50.4	0.0	0.0	58.8	48.0
150	0.0	0.0	63.5	63.9	0.0	0.0	36.5	36.1
TFE (v/v)								
0 : 10	0.0	0.0	35.7	4.4	8.9	11.4	55.4	84.2
1 : 9	0.0	0.0	35.1	46.0	10.5	0.0	54.4	54.0
3 : 7	6.5	0.0	42.7	56.8	0.0	0.0	50.8	43.2
5 : 5	3.7	9.1	58.5	42.3	0.0	5.0	37.8	43.6
7 : 3	2.9	2.3	58.3	50.0	0.0	3.3	38.8	44.4
9 : 1	9.2	7.5	52.3	52.7	1.6	0.0	36.9	39.8

[a] These percentages represent mean values (±20%) of three replicates.
P: piceain.

peptides have no α-helix in these solutions. Tris-HCl (20 mM, pH 7.0) has obvious effect on the CD components of piceain 2. Only 4.4% β-sheet is in 20 mM Tris-HCl, compared with at least 30% β-sheet in other solutions. It appears that 20 mM Tris-HCl destroyed β-sheet component of piceain 2 and increased its random component. In addition, 90 mM SDS also destroyed β-sheet component of piceain 1 and increased its random component (Table 4).

Discussion

Many antimicrobial peptides contain single intra-molecular disulfide bond. The disulfide bond motifs differ in sizes of disulfide-bridged segments. Most of those segments are composed of 7, 8, and 10–13 amino acid residues, no report of disulfide-bridged segment composed of 9 residues. The current piceains contain unique disulfided loop composed of nine amino acid residues and provide a novel family of antimicrobial peptides.

Most of plant antimicrobial peptides are from herbaceous plants although there are 271 plant antimicrobial peptides in recent database [16]. Only a few antimicrobial peptides are found from tree plants belonging to Santalaceae or Violaceae [16]. No antimicrobial peptide has been found from Pinaceae. *P. sitchensis* is belonging to *Picea* of Pinaceae. Its full genome information is available now. In this report, piceain antimicrobial peptides are identified from the genome sequences of *P. sitchensis*. Piceains do not show similarity to other known plant antimicrobial peptides. Both piceain 1 and 2 showed antimicrobial abilities against wide spectrum of microorganisms including Gram-positive and Gram-negative bacteria and fungus, either common or drug-resistant strains (Table 1). In addition, they contain little hemolytic activities against human red cells although high concentration (1 mg/ml) of piceains induced 50–60% rabbit red cell hemolysis. Piceains proved novel candidates for development of anti-infective agents.

Transmission electron microscopy was used to study the possible antimicrobial mechanism against *S. aureus* (Fig. 1). After treatment by piceain 1, the interface between the cell wall and membrane is not clear; in some regions, the interface even disappeared because of lysis of both or their separation. Moreover, some fibers extend from the cell surface in the piceain 1-treated bacteria, which together with the observations above suggests that piceain 1 acts on and disrupts the cytoplasmic membrane, leading to its dissolution and finally death of the cell itself. As the cytoplasmic membrane serves an important role in cell wall synthesis and turnover, perturbing it possibly also affect cell wall functions and autolysin regulation [22]. This process observed in the interaction between piceain 1 and *S. aureus* is similar to the putative mode of cationic antimicrobial peptides acting on Gram-positive bacterium [21]. In addition, a condensed substance was found in the cytoplasm, which implies that an intracellular or alternative target probably exists in addition to interactions with the cell walls. It could presumably be condensation of the DNA in this bacterium.

Our data also confirm the existence of piceains with nematicidal activity against adult nematodes. Nematodes as plant parasite have inflicted serious damage, their invasion resulted in the retarded growth and development of the agricultural crops and trees. For controlling nematodes, chemical fumigants have been widely used for the present although most of them are highly toxic to human and animals, so non-fumigant nematicides become more and more important [23]. The present results indicate that both piceain 1 and 2 show strong nematicidal activities against the *C. elegans* at low concentration in short time. Piceains were identified as dual acting nematicidal and antimicrobial peptides which may provide great potential alternative to currently used nematicides and may act as the first defensive line against microorganism infection or nematodes invasion for the plants.

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