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Effect of acidic pH on antibacterial action of peptide isolated from Korean pen shell (*Atrina pectinata*)‡

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Recently, the rapid emergence of microbial pathogens which are resistant to currently available antibiotics has triggered considerable interest searching for naturally occuring antimicrobial peptides (AMPs). Because AMPs from food organisms are comparatively nontoxic, a number of them are used as sources, purified in new antibiotics. Herein, an antibacterial peptide (heat-stable KPS-1) was isolated from Korean pen shell (*Atrina pectinata*) by the following procedures: solvent-extraction, heating, ultrafiltration, and RP-HPLC. The molecular weight of KPS-1 (4549.1 Da) was revealed by MALDI-TOF/MS analysis. Interestingly, KPS-1 inhibited *in vitro* growth of Gram-negative bacteria, including *Escherichia coli, E. coli* O157, *Pseudomonas aeruginosa, Enterobacter sakazakii*, and *Salmonella typhimurium,* at pH 5.2, rather than at pH 7.2. Its minimal inhibitory concentrations (MICs) were ranged from 20 to 80 μg/ml; however, it was not effective against human red blood cells at a concentration of 500 μg/ml. This suggests that this peptide is useful as a clinical agent for some human organs in an acidic environment. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Korean pen shell; antibacterial peptide; heat-stable; minimal inhibitory concentration

Introduction

Antimicrobial peptides (AMPs) play an important role in innate defense system and display broad-spectrum activity against invasion from pathogenic bacteria, fungi, and enveloped viruses [1]. AMPs have been isolated from diverse organisms, including animals, bacteria, insect, and plants [2–5]. The oceans, covering 71% of the surface of the earth, contain approximately half of the total global biodiversity, ranging between 3 and 500 $\times$ 10^6 different species [6]. It is noteworthy that AMPs from marine organisms have good potential when they are developed as a therapeutic agent, because AMPs originated from these organisms were generally salt-resistant [7] and *in vivo* condition acquires a high salinity (~150 mM). Although they have no acquired immunity [6], because marine invertebrates have developed an effective innate immunity, a number of AMPs were discovered and characterized from marine invertebrates including marine sponge [8], annelida [9], mollusks [10], tunicates [11], and crustaceans [12,13].

Among various species, most AMPs in crustaceans such as arasin-1 [14], callinectin [15], crustins [16], and penaeidin [17] were isolated from crabs and shrimp. The penaeidins are prominent AMPs from crustaceans [17], which are specific against Gram-positive bacteria via a strain-specific inhibition mechanism that includes rapid killing activity or bacteriostatic properties [18]. Although pen shells contain many proteins, proteins or peptides with antimicrobial activity had not yet been studied.

Here, we isolated an antibacterial peptide from Korean pen shell (*Atrina pectinata*). The heat-stable peptide exhibits inhibitory activity against Gram-negative bacteria.

Materials and Methods

Biological Materials

Korean pen shell (*A. pectinata*) was obtained from the Village Information Center in Jangheung, Korea. For antibacterial activity, *Escherichia coli* (ATCC 25 922), *E. coli* O157 (ATCC 43 895), *Staphylococcus aureus* (ATCC 25 923), and *Pseudomonas aeruginosa* (ATCC 15 692) were obtained from American Type Culture Collection. *Listeria monocytogenes* (KCTC 3710), *Enterobacter*

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Abbreviations used: *CFU, colony forming unit.*

sakazakii (KCTC 2949), and *Salmonella typhimurium* (KCTC 1926) were obtained from Korean Collection for Type Cultures.

Extraction and Isolation Using Ultrafiltration

The powder of pen shell meats were obtained by grinding in liquid nitrogen, using a mortar and pestle, and then solvent-soluble proteins were extracted with organic solvent (50% methanol, v/v) at 4 °C for 3 h. To remove solvent-insoluble materials, the extracts were centrifuged at 13.700 × *g* for 20 min and the supernatants were then freeze-dried, after which the samples were heated to obtain heat-stable peptides at 70 °C for 20 min. Heat-denatured precipitants were removed by centrifugation for 20 min at 13.700 × *g*. The heat-stable supernatants were subjected to ultrafiltration with a membrane of 10 000 molecular weight.

Purification of antibacterial peptide

Aliquots of the ultrafiltrates were injected to a reverse phase C_{18} column (5 μm, 300 Å, 4.6 × 250 mm; Vydac, Hesperia, CA, USA) on HPLC system (Shimadzu, Kyoto, Japan). Peptides dissolved in 0.1% (v/v) TFA in HPLC grade water (solvent A) and were loaded on a C18 RP-HPLC column in equilibrium with 0.1% TFA. Peptides were separated with a gradient of 10–60% acetonitrile for 50 min at a flow rate of 1 ml/min. The elutes were monitored by measurement of the absorbance at 214 nm. Each fraction was pooled and dried in the freeze-dryer. Each of the peak fractions were collected and assayed for antibacterial activity. The purified peptides were confirmed as one molecule on tricine SDS-PAGE [19].

Tricine SDS-PAGE

The 16.5% tricine acrylamide gel, based on the formulation by Schagger and von Jagow [20], were used to separate the peptides with a low molecular weight. Ten micrograms of each sample were loaded onto each well. Gels were fixed in 5% glutaraldehyde, followed by staining with 0.025% (w/v) Coomassie Blue G-250 in 10% acetic acid, and then destaining with 10% (v/v) acetic acid.

Protein Quantification

Determination of protein concentration was performed according to the method of bicinchoninic acid (BCA; Pierce, Rockford, IL, USA), using BSA as the calibration standard [21].

Antibacterial Activity

The antibacterial activity of each fraction was assessed using microdilution assay. Bacteria were grown in appropriate culture media at 37 °C for overnight and collected in mid-log phase. Twofold serial dilutions of each sample in 10 mM sodium phosphate, pH 5.2 or 7.2 containing 10% culture media were placed in 96-well microtiter plates (Nunc, Roskilde, Denmark) and then an aliquot of cell suspension (5×10^5 CFU/ml) was added to each well. The samples were incubated at 37 °C for 24 h. At the end of the incubation, the turbidity of each well was measured by absorbance at 600 nm using a microtiter reader (Molecular Devices Emax, Sunnyvale, CA, USA). The lowest concentration of peptide that completely inhibited bacterial growth was defined as the minimal inhibitory concentration (MIC). The MIC values were calculated as an average of several independent experiments, conducted in triplicate [22]. For radial diffusion assay, pre-grown bacteria were washed once with 10 mM sodium phosphate, pH 5.2, and 15 ml of underlay agarose gel containing 0.03% (w/v) tryptic soy broth, and 1% (w/v) low electroendosmosis type agarose, with 5×10^5 CFU/ml, was poured into a perti dish and the gel was solidified. The indicated concentration of peptide (8 μl) solution was added into 3-mm diameter wells that have been punched in underlay gels and the plate was incubated at 37 °C for 3 h. The underlay gel was then covered with overlay gel solution containing 6% culture media and 1% agarose and the plate was further incubated at 37 °C for 24 h to visualize a clearance zone.

Hemolysis

Fresh human red blood cells (hRBCs) from a healthy donor were centrifuged at 800 *g* and washed with phosphate buffered saline (PBS) until the supernatant was clear. The hRBCs [8% (v/v) of final concentration] were added in twofold serially diluted peptide in PBS. After incubation with mild agitation for 1 h at 37 °C, the samples were then centrifuged at 800 *g* for 10 min and absorbance of the supernatant was then measured at 414 nm. Hundred per cent hemolysis is defined as the absorbance of hRBCs containing 1% Triton X-100 and zero hemolysis consisted of hRBCs suspended in PBS. Each measurement was conducted in triplicate and the percentage of hemolysis was calculated using the following equation:

$$\%\text{hemolysis} = \frac{(\text{Abs}_{\text{peptide}} - \text{Abs}_{\text{PBS}})}{\text{Abs}_{\text{Triton-X100}} - \text{Abs}_{\text{PBS}}} \times 100 \qquad (1)$$

Results and Discussion

Antibacterial peptide from Korean pen shell (*A. pectinata*) was purified by the following four steps: extraction, heating, ultrafiltration, and C_{18} RP-HPLC. The collected pen shells were washed twice with distilled water, quickly frozen in LN_2, and then ground to soft powder. Fifty percent of methanol (v/v) was used to extract the solvent-soluble proteins and peptides from the pen shell powder (Figure 1(A)). Almost all proteins with high molecular weight were removed after centrifugation because they denatured and aggregated in the high percentage of solvent. The extract (100 μg/ml) showed an antibacterial activity against *E. coli* (Figure 1(B)). In the next procedure, in order to obtain the heat-stable peptide, the extracted substances were heated to 70 °C for 20 min (Figure 2(A)). As shown in lane 1 of Figure 2(A), high molecular weight proteins, over 26.6 kDa, were successfully eliminated in this step, compared with Figure 1(A), and these substances had an antibacterial activity (number 3 in pH 5.2 of Figure 2(B)). Peptide-antibiotic is easily destabilized or denatured at high temperatures during long-term preservation, because it is composed of amino acids. This heat-stable character is one important factor for pharmaceutical application. Although a pure peptide was not isolated in this step, solvent- and heat stabilities suggested that stable structure of peptide was probably required for antibacterial activity.

The ultrafiltration which followed was performed by using membrane with molecular weight limit of 10 kDa (Figure 2(A)). The cut-off fractions showed major bands under 10 kDa on tricine SDS-PAGE gel (Figure 2(A), lane 3). In order to investigate pH-dependent or independent action, the above fractions were assessed for antibacterial activity against *E. coli* under neutral (pH 7.2) or acidic (pH 5.2) conditions. Figure 2(B) shows that almost all fractions displayed the better antibacterial activity in pH 5.2 than

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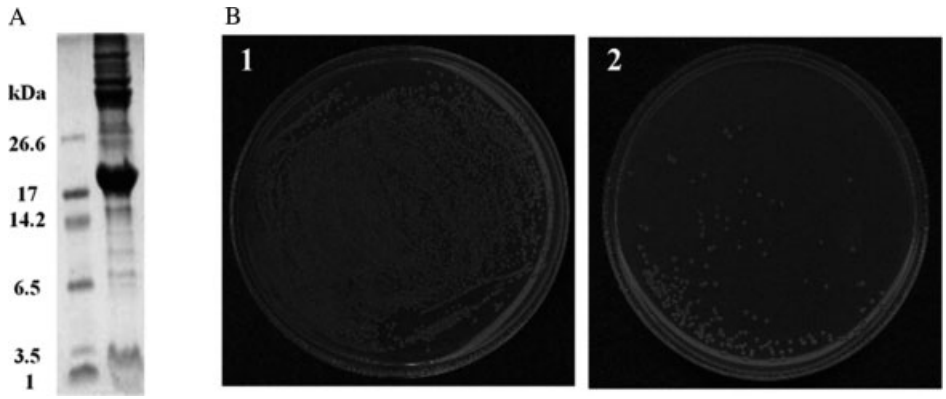

Figure 1. Tricine SDS-PAGE of total proteins from pen shells (A). The total proteins extracted with 50% (v/v) methanol. Lane 1, molecular size marker; lane 2, extracted proteins (10 μg). Antibacterial activity of extracted total proteins from pen shells against *E. coli* (B). 1, untreated cell; 2, treated cell (100 μg).

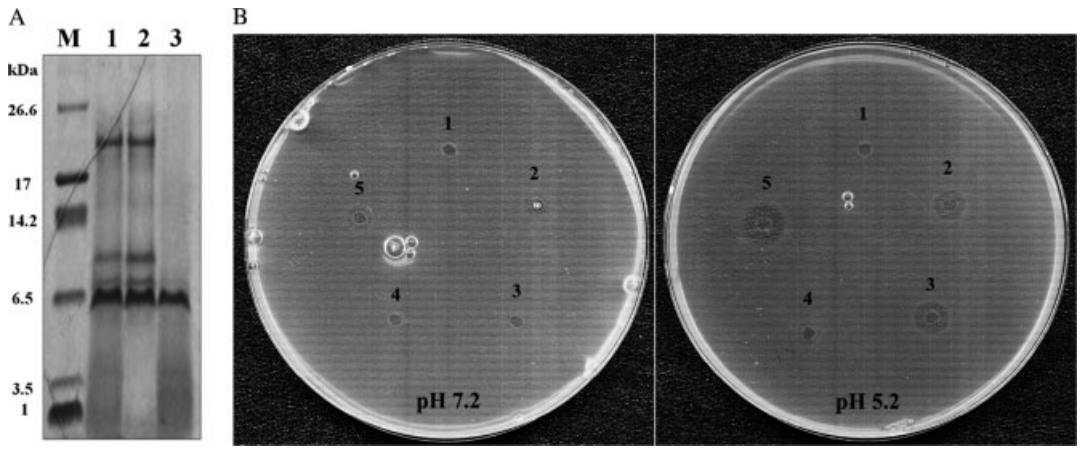

Figure 2. Tricine SDS-PAGE of heat-stable and ultrafiltrated proteins (A). The total proteins were heated at 70 °C for 20 min and the supernatants were ultrafiltrated. M, molecular size marker; 1, heat-stable proteins; 2, fraction over 10 kDa; 3, fraction under 10 kDa. The pH-dependent antibacterial activity of heat-stable and ultrafiltrated proteins against *E. coli* (B). 1, untreated; 2, extracted fraction (50 μg); 3, heat-stable fraction (25 μg); 4, fraction over 10 kDa (25 μg); 5, fraction under 10 kDa (25 μg).

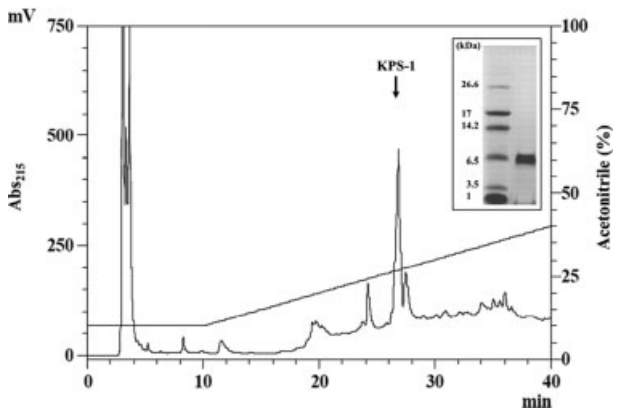

Figure 3. RP-HPLC profile and Tricine SDS-PAGE analysis of KPS-1 peptide. The sample was injected to an HPLC system with a vydac C_{18} column. The elution was achieved with a linear gradient of acetonitrile in 0.1% TFA. The indicated peak fraction (as arrow) was run on Tricine SDS-PAGE.

in pH 7.2, and moreover, the high molecular proteins over 10 kDa did not have activity against *E. coli*.

The low molecular fractions with the strongest antibacterial activity were applied to a C_{18} RP-HPLC (Figure 3). After examination of the antibacterial assay for all peak fractions, we collected a peak fraction which is arrowed in Figure 3. The single peptide was detected near a molecular weight of 6.5 kDa on 16.5% tricine SDS-PAGE gel (inset in Figure 3). An accurate molecular mass was analyzed to be 4549.1 Da by using MALDI-TOF/MS (Figure 4(A)). The purified peptide showed a potent antibacterial activity against Gram-negative bacteria (*E. coli, E. coli* O157 and *P. aeruginosa*) in radial diffusion assay; however, the growth of *S. aureus* was not inhibited (Figure 4(B)). These findings indicated that the antibacterial activity of purified peptide was successfully isolated and it was named as KPS-1. As shown in Table 1, the yield of purified KPS-1 from *A. pectinata* was 73 μg/200 g of meat powder.

Table 1. Summary for purification of KPS-1 from *Atrina pectinata*

Step	Amount of proteins (mg/200 g powder)	Recovery (%)
Extraction with 50% (v/v) MetOH	28.72	100
Heat	9.04	31.47
Ultrafiltration	2.21	7.69
RP-HPLC	0.073	0.25

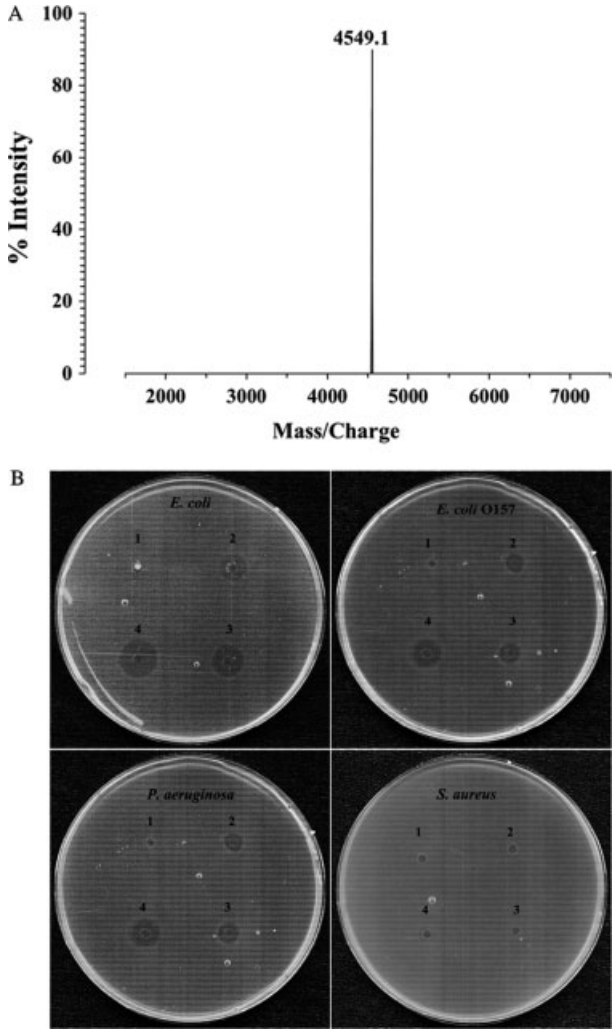

Figure 4. Mass spectrum of KPS-1 (A). Purified peptide was run on MALDI-TOF/MS. Radial diffusion assay of KPS-1 against *E. coli*, *E. coli* O157, *P. aeruginosa*, and *S. aureus* in pH 5.2 (B). 1, untreated; 2, 1 μg of KPS-1; 3, 2 μg of KPS-1; 4, 4 μg of KPS-1.

In order to determine MICs of KPS-1 against the growth of several bacteria, we performed antibacterial assay with microdilution method in both pH 7.2 and 5.2 conditions. Table 2 shows that the inhibition spectrum of KPS-1 in bacterial growth was limited to both Gram-negative bacteria and pH 5.2, having MICs ranged from 20 to 80 μg/ml, but melittin used as a positive control, displayed a potent inhibition activity against bacterial growth in both pH. The better activity of KPS-1 in acidic condition suggested that it may have histidine residues in amino acid sequence or disulfide bonds by cystein residues. Antibacterial activity in acidic conditions may be therapeutically applied in several fields, such as in cosmetics or food safety. Furthermore, antibacterial peptides acting in the acidic environment are expected to protect from infection against pathogenic bacteria, because some human tissues, namely the skin, vagina, urinary tract, and oral cavity, are physiologically acidic pH [23,24].

The hemolytic effect of KPS-1 was assessed against hRBCs to determine the toxicity toward eukaryotic cells. The KPS-1 peptide showed nonhemolytic activity against hRBCs at a concentration of 500 μg/ml, whereas melittin induced 100% hemolysis at 15.6 μg/ml (Figure 5).

Table 2. MICs of KPS-1 in pH 5.2 and 7.2 against bacterial strains

	MIC (μg/ml)			
	KPS-1		Melittin	
Bacteria	pH 5.2	pH 7.2	pH 5.2	pH 7.2
Gram (−)				
E. coli	20	160	6	6
E. coli O157	20	160	6	6
E. sakazakii	40	160	6	6
P. aeruginosa	40	>160	12	6
S. typhimurium	80	>160	12	12
Gram (+)				
S. aureus	>160	>160	6	6
L. monocytogenes	>160	>160	12	12

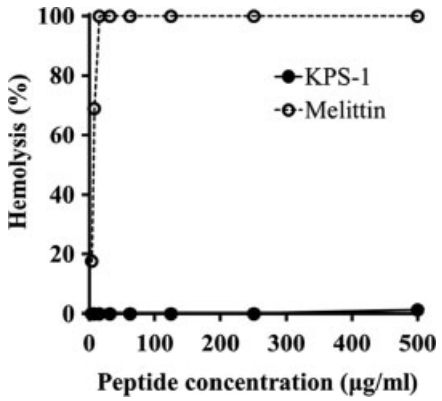

Figure 5. Hemolytic effects of KPS-1 and melittin against hRBCs.

Currently, Gram-negative bacteria such as *E. coli* and *P. aeruginosa* are causing much concern because of the rapid spread of multi- or extremely resistant strains to traditional antibiotics and there is a lack of a promising candidate which is active against Gram-negative pathogens, due to the lipopolysaccharidic component of their cell wall. In this view point, to overcome serious health care problems by highly resistant Gram-negative bacteria, a KPS-1 peptide from pen shell with antibacterial activity against Gram-negative bacteria is interesting as an alternative antibiotic.

Although pen shell is already known to have a high amount of protein, to be a low calorie food containing all the essential amino acids plus iron, and to effectively prevent both hardening of arteries and anemia, the studies so far of the attributes of pen shell are too few. Although further study is needed to characterize the purified peptide, e.g. analysis of amino acid sequence and a study of the mechanism of the purified peptide, we have demonstrated the antibacterial activity of the purified peptide from the pen shell. We expect that this peptide will be used for development as antibacterial agent.

Conclusions

In conclusion, an antibacterial peptide was purified from comb pen shell in Korea. Compared to those of nonnatural origins, AMPs from endogenous origins have an advantage for *in vivo* application, due to their being non-cytotoxic. The purified peptide, which exerted an antibacterial action against Gram-negative bacteria in an acidic

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pH condition, was thermostable and had a molecular weight of 4549.1 Da on MALDI-TOF/MS analysis. Thus we think that this peptide could be useful in the field of new antibiotic drugs development, for microbial infections occurring in body areas with physiologic acidic pH.

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