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Antimicrobial and Antioxidant Peptide from *Bacillus* Strain CBS73 Isolated from Korean Food

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Abstract

An antimicrobial peptides-producing *Bacillus* strain CBS73 was isolated from fermented food (kimchi) that produces low-molecular-weight proteins with broad-spectrum antimicrobial activity. Our goal was to explore the therapeutic potential of antimicrobial substances produced by *Bacillus* species. Peptide CBS73 was purified from *Bacillus subtilis subsp. subtilis* with identity of 99.79%. It was found to be stable at pH 4.0-10.0 and temp 20-60°C. A protein band around 5.2 kDa was detected in tricine-SDS-PAGE and band was confirmed by MALDI-TOF test. Peptide CBS73 showed antimicrobial activity against MDR bacteria. The minimal inhibitory concentration (MIC) of peptide CBS73 for vancomycin-resistant *S. aureus* (VRSA), vancomycin resistant *Enterococci* (VRE) and *Salmonella typhimurium* ranged from 10-40 μg/mL. The antioxidant activity of peptide CBS73 was measured by DPPH scavenging, reducing power activity and total phenolic content. Cell viability and NO production result showed less cytotoxic effect upto 12 μg/mL. Peptide CBS73 could be a promising antimicrobial agent for clinical application.

Keywords: Antimicrobial Peptide, Antimicrobial, Antioxidant, Clinical Application

1. Introduction

Peptides with biological activities belong to a large and diverse family of natural products, which include antibiotics, enzyme inhibitors, plant or animal toxins, and immunosuppressant[1]. *Bacillus* and related species of bacteria have the object of particular interest because of their safety, their extensive distribution in very diverse habitats, and their significant ability to survive adverse conditions due to the development of endospores.

Bioactive peptides are fragments of protein that exhibit an effect on body functions or conditions of living beings and may influence human health. Within bioactive peptide, Anti-microbial peptides (AMPs) are widely used as effectors of innate immunity in plants and animals. Drug-resistant infections has been increased which exhibit a serious challenge to antimicrobial therapies. A group of antimicrobials, named bacteriocins, have been studied much because they have a great potential in controlling the antibiotic-resistant pathogens. AMPs represent a new family of antibiotics that have stimulated effectiveness against drug resistant infections[2].

Antimicrobial peptides (AMPs) are a diverse class of naturally acting molecules that are produced as the first line of defense by all multicellular organisms. AMPs have salubrious character and production of antimicrobial peptides by Bacillus strains has been progressively characterized in the recent past and many peptides produced by this group of bacteria have been widely employed for fermentation technology to manufacture antibiotics. The AMPs from Bacillus spp., includes different classes of bacteriocins[3], antimicrobial surface-active biosurfactants like glycopeptides, lipopeptides, and nonribosomally synthesized cyclic peptides[4,5].

The objective of our work was to determine the activity and effect of AMPs which can act as a broad-spectrum antibiotic and achieve potent antioxidant properties. AMPs are naturally synthesized compound that exert effects against microbes. These naturally produced compounds can be alternative to the available antibiotic although most of the antibiotics have been resistant to human body. In this study, we put our determination in utilizing our novel peptide CBS73 isolated from fermented

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food kimchi. It shows stability and efficacy towards a wide range of tested conditions which may work on different diseases.

2. Materials and Methods

2.1. Materials

Sepharose CL-6B. Sephadex G-50 Sephadex G-25, and Sephadex G-10 were obtained from Pharmacia (Uppsala, Sweden). All other chemicals and reagents were of extra pure analytical grade.

2.2. Bacterial Strain and Culture Conditions

Bacillus strain CBS73 was isolated from kimchi which is capable to produce antimicrobial peptide. Identification of strain was carried out based on morphological characteristics and 16S rRNA sequence analysis according to Bergey's 'Manual of systematic bacteriology' and our previous report[6]. The effect of various carbon sources was determined using media composed of 1% yeast extract combined with 1% supplements such as Glucose, Mannitol, Starch, Lactose, Fructose, Sorbitol, Maltose or Sucrose on AMP production. Subsequently, the effect of nitrogen source was determined using medium containing 1% fructose combined with 1% supplements such as Beef extract, Malt extract, Tryptone, Yeast extract, Oat meal, Soytone, Peptone, Dried yeast on AMP production. Moreover, the effect of metal ions on AMP production was assessed in media containing the best carbon source (1% fructose), the best nitrogen source (1% beef extract) and 0.01% supplements such as Na_2HPO_4, NaH_2PO_4, $MgSO_4$, $ZnSO_4$, $MgCl_2$, KH_2PO_4, $FeSO_4$, NaCl or $CaCl_2$. Fermentation was carried out at 37°C in 250 mL Erlenmeyer flasks containing 50 mL of media with constant shaking at 170 rpm.

2.3. Production of AMP and Purification

Bacillus sp. CBS73 was cultured in 2 L baffled flask containing 400ml of mass culture medium (1% fructose and 1% beef extract) for 24 h at 37°C. The cell free supernatant was mixed with ammonium sulphate (30-80% saturation) and was kept at 4°C with overnight stirring[7]. The precipitate was recovered by centrifugation (10,000×g) for 50 min at 4°C. The precipitate was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) buffer. Ultrafiltration membrane (30, 10, and 1 kDa, Millipore Corp.) was used for molecular weight cut off (MWCO). Purification was carried with Sepharose CL-6B column (2.2 cm × 84 cm) using the same buffer. Furthermore, the active fractions were collective, concentrated and further purified with Sephadex G-50 column (1.5 cm × 89 cm) forward to Sephadex G-25 column (1 cm × 54 cm) using the same buffer system and subjected to purity analysis. The protein content was estimated using the Bradford Method[8].

2.4. Antimicrobial Activity

Minimal Inhibitory Concentration (MIC) test was carried out based on agar dilution method. It was used to determine the lowest concentration of assayed antimicrobial peptide which inhibits the growth of the bacteria. For the test, agar solutions with specified numbers of bacterial counts were allowable promptly onto nutrient agar plates containing antimicrobial peptide concentrations and reference standard antibiotic and incubated at 37°C[9,10].

Antimicrobial activity was measured by a filter paper disc (8 mm, Toyo Roshi Kaisha Ltd., Japan) saturated with antimicrobial sample (40 μL) was placed on the surface of Petri dish containing Muller Hinton Agar (MHA). Agar disk diffusion method[10] used and a clear zone of inhibition surrounding the paper disc was measured in millimeter (mm).

2.5. Polyacrylamide Gel Electrophoresis and MALDI-TOF

Tricine-SDS (tricine–sodium dodecyl sulfate) polyacrylamide gel electrophoresis was used to determine the purity and molecular weight of AMP[11]. Electrophoresed gel was stained by Coomassie Brilliant Blue R-250 solution. The band was confirmed by MALDI-TOF test.

2.6. Stability of AMP

To analyse the optimum thermal stability of CBS73, samples were incubated to 20, 40, 60, 80 and 100°C for 30 min and 121°C with 15 kPa for 15 min before examining the residual activity. Here, pH stability was determined using different buffer at a range of pH 2.0-10.0.

2.7. Effect of Chemicals on AMP Activity

The effect of various chemicals such as chelating agents (EDTA), detergents (Triton X-100, Tween 20, Tween 80), and solvents (acetone, chloroform, metha-

nol, ethanol, ethyl acetate, trichloroacetic acid) on antimicrobial activity of AMP was observed.

2.8. Effect of Proteolytic Enzymes

CSB73 was treated with 1mg/ml of each enzyme and incubated at room temperature for 1 h and then boiled for 2 min at 100ºC for enzyme inactivation. Peptide solution without any proteolytic enzymes treatment was taken as none (100%).

2.9. Anti-oxidant Activity of Peptide CBS73

2.9.1. DPPH Radical Scavenging Activity of CBS73

Free radical scavenging DPPH ((1, 1-Diphenyl-2-pycrylhydrazyl) method was used for the estimation of antioxidant activity[12]. Ascorbic acid was used as positive control and the absorption was measured on spectrophotometer at 517 nm. The following equation was applied to calculate the capability to scavenge the DPPH radical.

Percentage scavenging = (Ao – As)/ Ao * 100

Ao = absorbance of DPPH radical.

As = absorbance of test or reference sample.

Therefore, the percentage of scavenging activity was following plotted against the concentration and the regression equation was obtained to calculate IC50 (to inhibit DPPH radical formation by 50%, micro molar concentration was required)[13]. The experiment was conducted in triplicate.

2.9.2. Reducing Power of CBS73 AMP

Ferric reducing antioxidant power was measured by following the method of Wu et al.[14], with some minor modification. The control was prepared by using distilled water instead of the sample. Increasing the absorbance at 700 nm indicates increasing along with reducing power of protein hydrolysates. Ferrous ion chelating activity of the hydrolysates was measured according to the method of Klompong et al.[15]. Distilled water was used as control and the absorbance was measured at 562 nm.

2.9.3. Total Phenol Contents of Peptide CBS73

The total phenol content of the samples was measured using the Folin-Ciocalteau's reagent as described by Gulcin et al.[16]. An aliquot of the samples was mixed with Folin-Ciocalteau's reagent dissolved in ethanol, and distilled water and mixed for 1 min. Sodium carbonate (15%) was added to the mix. The solution had its volume adjusted with distilled water. After 2 h, absorbance was measured at 700 nm. A standard curve was prepared using gallic acid. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of samples.

2.10. Cell Viability and NO Production

Cell viability was measured after 24 h incubation. Survival rates were tested with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in Raw 264.7 cells. Raw 264.7 cells were incubated in the presence or absence of 1-100 μg/mL peptide for 24 h. Cells were incubated with the various concentration of peptide for 30 min, followed by treatment with 1 μg/mL of LPS and incubated for 24 h. The amounts of NO were determined using the Griess reagent in the culture medium. Each bar shows the mean ± SD of three independent experiments performed in triplicate. The process of assessment using MTT assay is described in our previous reports[17].

3. Results and Discussion

3.1. Identifcation of the Bacterial Strain

Screening and isolation of bacterial strain was carried according to our previous reports[10,18-20]. Bacterial strain CBS73 showed morphological similarities to genus *Bacillus*. The local isolate with Bacillus species was compared with multiple gene sequence alignment obtained from 16S rRNA gene sequence of various Bacillus species. The local isolate showed closest resemblance to Bacillus subtilis subsp. subtilis with identity of 99.79%. The Phylogenetic tree constructed by 16S rRNA gene sequence is shown in Fig. 1.

3.2. Optimization of the Culture Media and Production of AMP

For the high yield of any antimicrobial compound, thus need to have a suitable compound in growth media. Keeping MRS as standard, antimicrobial activity was checked on the basis of different carbon, nitrogen and metal ion source according to our previous reports[10,19] Among the carbon, nitrogen source and metal ion checked, Fructose, beef extracts and NaCl were more

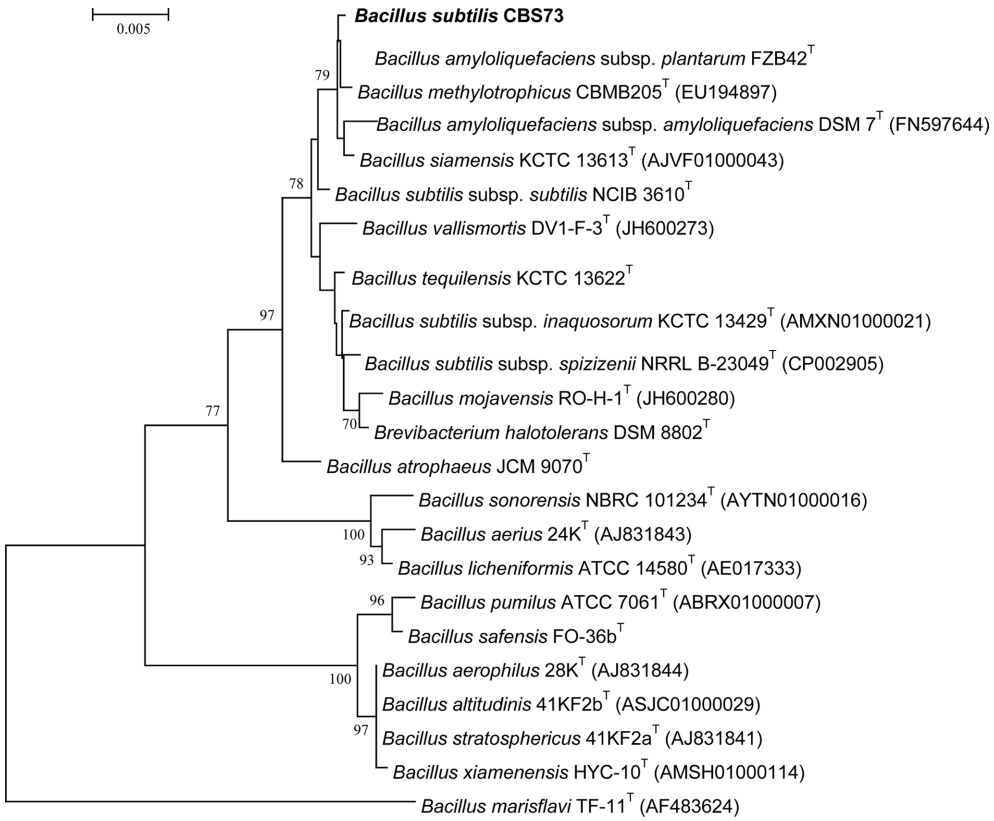

Fig. 1. Phylogenetic tree, based on the complete 16S rRNA gene sequence, showing the relationships between the strain CSB73 and closely related taxa of the genus *Bacillus*. Reference sequences were retrieved from GenBank under the accession number indicated in parentheses after the strain name. Numbers of nodes are percentage bootstrap values based on 1000 replications and bar: 0.005 substitutions per nucleotide position.

effective(Data not shown). Therefore, we used 1% fructose, 1% beef extracts and 0.01% NaCl as the optimal medium. Hence, *Mycobacterium smegmatis* ATCC 9341 was preferred as the indicator organism to check the activity. The *Bacillus* isolate CBS73 produced peptide at 37°C for 24 h.

3.3. Purification of Antimicrobial Peptide

After cultivation, the aliquot was recovered by centrifugation (10,000 × g) for 50 min. In ammonium sulfate precipitation process, 30-80% ammonium sulfate concentrate showed the highest antimicrobial activity. Gel separation chromatography was performed by Sepharose CL-6B and then active fractions were collected and loaded on Sephadex G-50, Sephadex G-25 and Sephadex G-10 (Fig. 2). Protein profiles were determined by Tricin SDS-PAGE. By the process of protein deter-

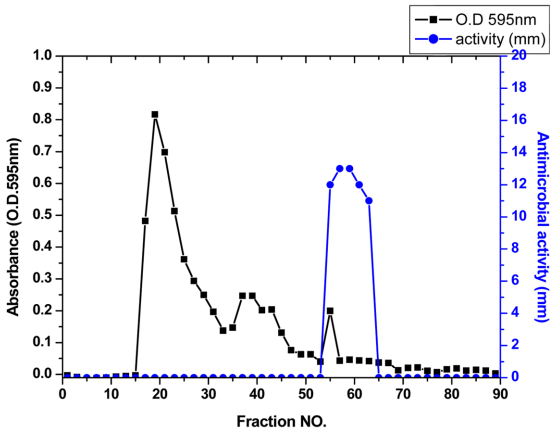

Fig. 2. Elution profile of Bacillus sp. CBS73 antimicrobial peptide. Gel filtration chromatography with Sephadex G-25 column (1.0 cm × 54 cm). The proteins were eluted at a flow rate of 1 ml/min.

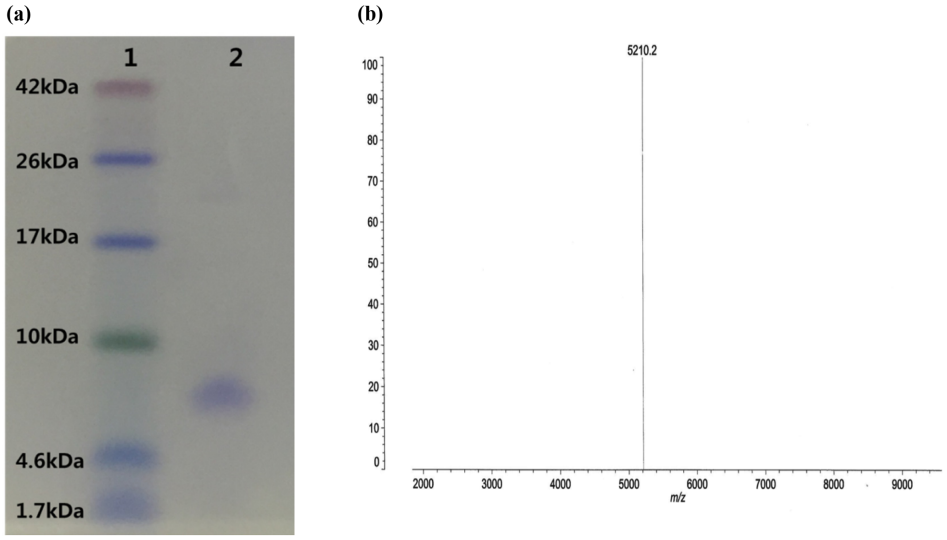

Fig. 3. Determination of the molecular weight (a) Tricine SDS-PAGE of CBS73 peptide. Lane 1; protein size marker with the corresponding value in kDa on the left; Lane 2; purified CBS73 peptide. (b) MALDI-TOF data.

mination, the molecular weight was found near 5,000 Da. For verification, MALDI-TOF process was used and molecular weight was confirmed as 5210.2 Da. (Fig. 3).

3.4. Effects of Temperature and pH on Antimicrobial Activity

Peptide CBS73 was stable around temperature 20-60°C whereas it lost about 16% of its activity at 80°C. On analyzing the residual activity, it was highly stable around pH 4-10. The autoclaved sample lost its activity completely (data not shown). It was found to be stable over wide range of temperature (20-60°C) and pH (4-10) conditions which was comparable and even wider range than other similar studies[21-25].

3.5. Effect of Chemicals on Antimicrobial Activity

The residual activity of peptide CBS73 remained stable in the presence of chelating agents such as EDTA and solvents such as acetone, chloroform, ethanol etc. Tween 20 and Tween 80 had similar effects. Triton X-100 and Trichloroacetic acid inhibited enzyme activity slightly (Data not shown).

3.6. Effect of Various Proteolytic Enzymes

The antimicrobial activity of peptide CSB73 checked against different proteolytic enzymes revealed that the enzyme activity was not lost during proteolytic degradation. Table 2 shows the effect of various proteolytic enzymes on peptide CBS73.

3.7. Antimicrobial Activity of Peptide CBS73

Peptide CSB 73 shows strong antimicrobial activity against *Salmonella typhimurium* KCTC 1925, VRE 89, and VRSA. The reference antibiotics taken were bacitracin and vancomycin. From the result against these resistant microorganisms we can use AMP CBS73 as a potent drug. Result on comparative study of our antimicrobial peptide with reference antibiotics (10 μg/mL) is shown in Table 1.

3.8. Antioxidant Activity of Peptide CBS73

DPPH scavenging activity of CBS73 was assessed by measuring changes in the absorbance at 517 nm. the concentration of 500 μg/mL and suppression rate was found 9.7±1.35. For DPPH method, Ascorbic acid was used as active control group for the measurement of antioxidant. As compared to ascorbic acid, the effect of peptide CBS73 was checked at all concentration(1-500 μg/mL). Peptide CBS73 showed a percentage inhibition 9.7±1.35% at the concentration of 500 μg/mL whereas ascorbic acid exhibited inhibition 88.26±0.26% at the same concentration.

Reducing power antioxidant activity of peptide CBS73 was checked at the concentration of 500 μg/mL that the

Table 1. Minimum inhibitory concentration of Bacillus sp. CBS73 peptide

Test organisms		MIC(μg/mL)		
		CBS73	Bacitracin	Vancomycin
Alcaligenes faecalis ATCC 1004	G(-)	80	>80	>80
Salmonella typhimurium KCTC 1925	G(-)	10	>80	>80
Escherichia coli KCTC 1923	G(-)	80	>80	>80
*ESBL B1**	G(-)	80	>80	.>80
*ESBL P3**	G(-)	80	>80	>80
*ESBL S1**	G(-)	80	>80	>80
*ESBL U4**	G(-)	80	>80	>80
*ESBL W1**	G(-)	80	>80	>80
Pseudomonas aeruginosa KCTC 1637	G(-)	80	>80	>80
*IMP 120***	G(-)	80	>80	>80
*IMP 123***	G(-)	>80	>80	>80
*IMP129***	G(-)	80	>80	>80
Bacillus subtilis ATCC 6633	G(+)	80	>80	>80
Micrococcus luteus ATCC 9341	G(+)	80	1.25	1.25
Mycobacterium smegmatis ATCC 9341	G(+)	0.15	0.15	0.15
Enterococcus faecalis ATCC 29212	G(+)	>80	20	1.25
*VRE 4****	G(+)	20	20	>80
*VRE 6****	G(+)	>80	>80	>80
*VRE 82****	G(+)	40	5	>80
*VRE89****	G(+)	40	>80	>80
*VRE98**	G(+)	>80	80	>80
*VRSA*****	G(+)	20	>80	>80
Staphylococcus aureus KCTC 1928	G(+)	80	10	1.25
*MRSA693E******	G(+)	>80	10	1.25
*MRSA 4-5******	G(+)	80	2.5	1.25
*MRSA 5-3******	G(+)	80	2.5	1.25
*MRSA S3******	G(+)	80	40	1.25
*MRSA U4******	G(+)	80	80	0.625
*MRSA S1******	G(+)	80	40	1.25
*MRSA B15******	G(+)	80	40	1.25

*ESBL, Extended spectrum beta lactamase *Escherichia coli*; **IMP, Imipenem resistant *Pseudomonase aeruginosa*; ***VRE, Vancomycin resistant *Enterococcus faecium*; ****VRSA, Vancomycin resistant *Staphylococcus aureus*; *****MRSA, Methicillin resistant *Staphylococcus aureus*.

absorbance value was indicated 0.566±0.03 as compared to control Ascorbic acid 1.384±0.026.

Total phenolic content in peptide CBS73 was calculated using a standard curve prepared for gallic acid (500 μg/mL), and result was 2.712±0.06. By using same concentration, peptide CBS73 was expressed its result 0.339±0.03.

The antioxidant properties of peptide CSB73 were checked using free radical DPPH method and it was comparable to the standard ascorbic acid, which indicates its activity in terms of hydrogen atom donating capacity/electron transfer competence.

3.9. Cell Viability and NO Production

The effect of Peptide CBS73 on cell viability in RAW264.7 cells were showed in Fig. 4. Survival rates

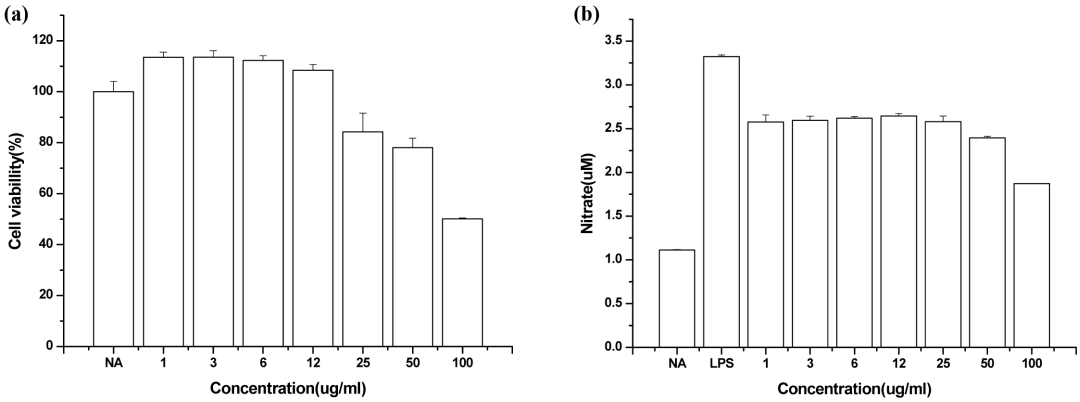

Fig. 4. Effect of the CBS73 antimicrobial peptide on cell viability and NO production (a) Cell viability was measured after 24 h incubation. Survival rates were tested with MTT assay in Raw 264.7 cells. Raw 264.7 cells were incubated in the presence or absence of 1-100 μg/ml peptide for 24 h. (b) Cells were incubated with the various concentration of peptide for 30 min, followed by treatment with 1 μg/ml of LPS and incubated for 24 h. The amounts of NO were determined using the Griess reagent in the culture medium. Each bar shows the mean ± S.D of three independent experiments performed in triplicate.

Table 2. Effect of various proteolytic enzymes

Proteolytic enzymes	Residual activity (%)
None	100
Lipase	102.0±5.8
Proteinase K	105.8±5.8
Protease	102.0±5.8
a-chymotrypsin	98.2
Trypsin	96.4±5.8
Pepsin	98.1±5.8
Papain	102.0±11.0

* Peptide was treated with 1 mg/mL of each enzyme and incubated at room temperature for 1 h and then boiled for 2 min at 100°C for enzyme inactivation. Peptide solution without any proteolytic enzymes treatment was taken as none (100%).

were tested with MTT assay. Cell viability was checked with various doses (1-100 μg/mL) of peptide CBS73. The result showed less cytotoxic effect up to 12 μg/mL. Further, examining NO generation of peptide CBS73, the antioxidant activity of peptide CSB73 was measured. Pre-treatment with peptide CBS73 decreased NO release remarkably at doses 12 μg/mL.

4. Conclusions

The result presented here determine the activity of novel AMP CBS73 purified from *Bacillus* species isolated from Korean traditional fermented food kimchi. Peptide CBS73 exhibits broad spectrum antimicrobial activity as well as antioxidant activity. Our result collectively recommended that peptide CBS73 serve as a capable candidate for developing therapeutic drug for bacterial infection and antioxidant activity.

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