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Isolation, Purification, and Characterization of Antimicrobial Peptide Produced by *Saccharomyces boulardii* №6569

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Abstract

Object: To identify microorganisms that are newly isolated biologically and microbiologically and determine their characteristics by purifying AMP produced by yeast. ***Methods:*** We measured the antibacterial activity of AMP using microliquid dilution and turbidity methods and purified them using membrane filtration and gel filtration methods. The molecular weight of AMP produced by *Saccharomyces boulardii* was measured using Acquity UPLC SQD-2 and MS. ***Results:*** We confirmed that yeast-№18, identified biologically and molecularly, isolated from apple peel, which was grown in our country, was *S. boulardii.* After ultrafiltered *S. boulardii* (Sb) culture, the total antibacterial activity of antimicrobial peptide (AMP) was 12825 AU, and the yield was 95.7%. It was then filtered with DEAE-Sephadex A25, CM-Sepharose, and Sephadex-G15, with a total antibacterial activity of 1192AU and a yield of 8.9%. The molecular weight (MW) of AMP was 600–930 Da in the Acquity UPLC SQD-2 analysis. This AMP had no hemolytic activity at 1.56~50 µg/ml and was heat stable and sensitive to trypsin and pepsin. It also has potent antibacterial activity against Gram-positive and Gram-negative pathogens and a broad spectrum of activity against methicillin-resistant *Staphylococcus aureus*) and *Clostridioides difficile*, classified as bacteria that cause especially dangerous infections by the WHO. **Conclusion:** The yeast that is isolated from apple peel of our country is *S. boulardii* and MW of AMP from it is 930 Da.

Keywords: Antimicrobial peptide, *Saccharomyces boulardii,* probiotics, yeast, purification, antibacterial activity

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INTRODUCTION

With the rapid increase in pathogens that are resistant to antibacterial agents, it has become one of the most serious public health problems [1, 2], and a new strategy is required to conquer it [3, 4]. In the last 20 years, antimicrobial peptides, which are highly sensitive to persister cells and do not acquire resistance, have been widely used as antimicrobial alternatives [5].

Antimicrobial peptides have been isolated from many living forms, including microbes, plants, invertebrates, and vertebrates, and they have been gaining attention as they particularly affect several pathogens, including bacteria, fungi, and viruses, and do not affect eukaryotes [6, 7, 8, 9, 10]. Researchers have produced antimicrobial peptides from microbes or by expressing recombinant genes in microbes [11, 12]. AMP has several advantages over antibiotics: first, it limits resistance induction

by characterization of its antimicrobial mechanism; second, it is possible for topical indication of the drug; third, it has a broad antimicrobial spectrum; fourth, it has activity at a concentration of nanomole; fifth, it has no toxicity to eukaryotes; and sixth, it is possible to chemically synthesize AMP analogs, which have modifiable biological activity [5].

Therefore, researchers have recently focused on obtaining antimicrobial peptides produced by microbes, which are cheap and capable of mass production [13–15, 2, 16, 17]. They have purified acidocin LCHV, a small AMP with a molecular weight of 1.1 kDa produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine and was extremely heat stable (90 min at 130°C), was active over a wide pH range, and was found to be sensitive to proteolytic enzymes (trypsin, pepsin, and proteinase K) [18]. Acidocin LCHV has a broad spectrum of activity against both Gram-positive and Gram-negative pathogens, including several that are classified as especially dangerous infections by the WHO, as well as MRSA and *Clostridioides difficile* [15, 18]. Researchers have suggested that AMP produced by probiotic strains will be stable for use in the treatment of patients with infectious diseases [18].

Saccharomyces boulardii is a non-pathogenic and non-toxic yeast, a probiotic yeast that is only admitted by WHO [19, 20]. In 1923 *S. boulardii* was isolated from lychee peel by Boulard for the first time, who discovered the traditional secret, using the peel to treat cholera in Indochina. *S. boulardii* is a second to *S. cerevisiae* yeast strains, known as *S. cerevisiae* Hansen CBS 5926, which is different from *S. cerevisiae* and has special advantages. It is comparatively acidic and bile stable and has natural resistance to antibiotics [19, 20]. It also has a serine protease of 54 kDa which resolves A toxin produced by *Clostridioides difficile* [13, 21] and is efficient in treating and preventing diarrhea associated with recurrent *Clostridioides difficile* and antibiotics [22–24]. Sougioultzis et al. reported that researchers purified *S. boulardii* anti-inflammatory factor (SAIF) from *S. boulardii*, which has a molecular weight of 1.1 kDa, is heat stable and water-soluble, and blocks NF-kB activation and NF-κB-mediated IL-8 gene expression in intestinal epithelial cells and monocytes [25–27].

Therefore, our study was conducted to purify antimicrobial peptides from newly isolated *S. boulardii* cultures and to verify their characterization.

MATERIALS AND METHODS

Strains and Culture Medium

Bioidentical used two yeast strains (No. 18, No. 9) isolated from apple (Hwangju) peel and *S. cerevisiae* IF 995 as control yeast [28]. Our study for molecular biological identification of isolated yeast was conducted with a specific primer design for 5.8s rDNA and internal transcribed spacer (ITS) sequence to classify and identify yeast of *Saccharomyces genus* at the interspecific level, establish conditions for optimum PCR reaction, analyze gene sequence of PCR product, BLAST with data of sequence analysis, discriminate species using it, and confirm its identity. Other standard strains used in this study were kept at -50°C after freeze-drying in the Food and Medical Supplies Certification Department of the Public Health Ministry, and antibiotic-resistant strains were isolated and identified at Pyongyang University of Medical Science and Central District Hospital. Before use, they were cultured according to the idiographic productive condition after moving two times in a suitable culture medium.

We poured 100 ml of peptone-yeast-glucose culture fluid (peptone 10 g, yeast extract 3 g, glucose 20 g, water 1 L, pH 6.0~6.5) into a culture flask and sterilized at 115°C for 40 min. Fresh incubate of *S. boulardii,* shake-cultured in this culture flask for 20 h, seeded at 3%(v/v) in peptone-yeast-glucose culture medium, and shake-cultured under aerobic conditions at 37°C for 60 h (180~200 r/min).

Antimicrobial Test Using Microliquid Dilution

We determined the antibacterial activity of the AMP solution against Gram-positive and Gram-negative pathogens by microliquid dilution [29, 21]. Independent colonies cultured in agar plate

culture medium were seeded in broth (selected proper ones for bacteria to examine), cultured at 37°C for 18 h, gained cells were obtained, the number of living bacteria was measured, and used for testing. In each well of 96 microplates, we poured 100 µl of culture fluid, poured 100 µl of AMP solution, and 30 µg/ml of protein concentration on the first well, and diluted it two times. In each well, we poured and mixed 10 µl of suspension for testing, which was $6.8\sim8.2\times10^5$ cfu (colony forming units)/ml and measured the absorbance at 540 nm using an ELISA plate reading device (Bio-Rad) after culturing at 37°C for 18 h. We determined the minimal concentration of antimicrobial peptide as the MIC for which bacterial growth was completely inhibited. The MIC is the average value measured four times independently.

Antimicrobial Activity by Turbidity Method

Eight milliliters of M-H (M-H) culture fluid and suspension ($5.4\times10^4\sim5.4\times10^5$ cfu/ml) were poured and mixed into a large test tube. Then, this solution was divided into the same four test tubes, 2 mL of AMP solution was poured 2 mL into two test tubes, 0.85% of physiological salt solution was poured into 2 mL in other test tubes as a control group, separately measured absorbance of one test tube at 540 nm and shake-cultured at 37°C for 4~5 h. In the control and experimental groups, the absorbance increment before culturing against that after culturing was obtained. (We defined it as 1 AU of antimicrobial activity when the decreasing rate of absorbance of the test group was 1% per 1 ml of sample, compared with the control group.)

It is calculated as follows.

$$\text{Antimicrobial activity (AU/ml)} = (A_{540cont.} - A_{540test})/A_{540cont.} \times (100/V_{test})$$

Measure of Protein Concentration

Protein determination was conducted by the Bradford method [30].

Test of Hemolysis Activity

The hemolytic activity of antimicrobial peptide was determined using white mice and human erythrocytes isolated from 3.8% sodium citrate blood [31]. (White mouse erythrocyte; exsanguinated from orbital, human erythrocyte; conservative blood B.) 4 mL of erythrocytes were prepared after centrifugation at 1500 r/min at 4°C for 10 min. Erythrocytes were washed 3 times with 0.01 mol PBS (phosphoric acid buffer: 0.8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 1.15 g/L Na_2HPO_4, pH 7.2) and suspended 10% (v/v) in physiological salt solution. Then they were affected with a variety of concentrations of antimicrobial peptides dissolved in PBS (protein concentration; 70, 50, 25, 12.5, 6.25, 3.15, 1.56 µg/ml) at 37°C for 1 h and centrifugalized at 1500 r/min for 10 min. Superliquor was measured at 414 nm using an ELISA plate reading device (Bio-Rad). Hemolysis (0%) and hemolysis (100%) were measured using a reference point made of erythrocytes in erythrocyte suspension in PBS and processed with 0.1% Triton X-100[6, 32].

Hemolysis (%) =(sampling H-negative control group H)/(positive control group H-negative control group H)×100

Sensitivity Test of Antimicrobial Peptide Against Physicochemical Factor

To determine the sensitivity of antimicrobial peptide against proteinase, 0.5 mol AMP specimens (protein concentration 30 µg/ml) were separately poured into four small test tubes, affected with 0.5 ml trypsin (Sigma Chemical Co., St. Louis, MO) dissolved in 0.05 mol Tris-HCl buffer (pH 8.0) and 0.5 ml pepsin (Sigma Chemical Co., St. Louis, MO) dissolved in 0.2 mol sodium citrate buffer (pH 6.0), and antimicrobial activity was measured by microliquid dilution (strains for measurement of antimicrobial activity; *Staphylococcus aureus* ATCC 25923). The control group was not affected by proteinase [18, 16].

To determine the effect of temperature on the antimicrobial peptide, AMP solution was added at various temperatures of 60, 70, 80, 90, 121, and 132°C for 30 min, and the pH was regulated to 3–9 with HCl and NaOH to determine the effect of pH and measure its antimicrobial activity by microliquid dilution [18, 33]. To detect amino acids, 1 ml AMP solution was heated with 0.1 ml ninhydrin in a water bath and considered positive if it was dyed purple. To detect the peptide, 1 ml antimicrobial peptide solution was mixed with 1 ml biuret, and if it was dyed blue-purple, it was estimated as positive.

Purification and Characterization of Antimicrobial Peptide in *S. boulardii* Culture Fluid

1. *Purification of antimicrobial peptide produced from S. boulardii' culture fluid:* Cultured *S. boulardii* was centrifugalized at 3000 r/min for 30 min to eliminate cells and ultrafiltered at 5000 Da. After the filtrate was heated and concentrated to 1/2, it was passed through a DEAE-Sephadex A25 column (1.6×18 cm), already balanced with 10 mmol Tris-HCl buffer (pH 8.0) [34]. Compartments with antimicrobial activity among passed specimens each 5 ml were collected and they were heated, concentrated into 1/5, passed through CM-Sepharose column (3.5×15 cm), already balanced with 0.01 mol acetic acid buffer (pH 4.4), (velocity 0.8 ml/min) and demarcated each 5 ml by eluting gradually with 0.5 mol NaCl [35, 27]. 4.5 ml compartments with antimicrobial activity, collected and concentrated to 1/10, were passed through a Sephadex G-15 column (1.8×25 cm), already balanced with 10 mmol phosphoric acid buffer (pH 7.2) and demarcated each 5 ml by eluting with the same buffer (0.4 ml/min).
2. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE):* SDS-PAGE determined molecular weight, according to Schägger H, and von Jagow [36]. The MW of purified antimicrobial peptide was obtained by SDS-PAGE with 16.5% displacing gel (16.5% acrylamide and 0.5% bisacrylamide) and 4% fixing gel (4% and 0.5% bisacrylamide). Each specimen was mixed with 4%(w/v) SDS, 12% (w/v) glycerol, 50 mmol Tris-HCl buffer, 2% (v/v) mercaptoethanol, and 0.01%(w/v) bromophenol blue and regulated pH 6.8 with concentrated hydrochloric acid.

   This mixture was boiled for 3 min, poured on a well, and electrophoresed at 30 V of initiating voltage for approximately 1 h till the band was completely separated into an isolated gel and sequentially at 150 V. The gel was then fixed with 40% (v/v) methanol and 10% (v/v) acetic acid for 30 min and dyed using a silver impregnation kit (Bio-Rad), as described in the standard maneuver [37]. Insulin (5807 Da, made in Germany), lysozyme (14.4 kDa, Serva), bovine serum albumin (BSA, 66.2 kDa, Serva), and vitamin K_{12} (1355 Da, made in Germany) were used as MW markers.
3. Analysis of super-performance liquid chromatography-mass spectrometry: The MW of the purified antimicrobial peptide was obtained by Acquity UPLC SQD-2 [33, 38]. Purification of the high-activity compartment passed through the Sephadex G-15, was determined by Acquity UPLC SQD-2 (Acquity UPLC® BEH 130 C18 1.7 µm, 2.1×150 mm). 10 µl activity compartment was eluted at a velocity of 0.2 ml/min at 30°C for 10 min using a 0.1% formic acid solution-acetonitrile solution (70:30). Then mass analysis of MS was scanned 100~3000 by electro spray ionization at 3.5 V of capillary voltage, 0 V cone voltage, 125°C of origin temperature, 0 L/hr. of gas flux, 650 L/hr. of desolvation gas flux and 350°C of desolvation temperature.

RESULT

Isolation and Identification of Yeast

This study was conducted using molecular biology to identify two yeast strains (No. 18, No. 19) considered as *S. boulardii,* biologically identified, and grown at 37°C among 14 yeast strains isolated from a variety of peels, earth, yogurt, and cereal. To identify the isolated yeast, specific primers were designed to discriminate *S. boulardii* from *S. cerevisiae* using PCR.

5.8s rDNA and ITS, well-reflected interspecific or intraspecific variation of Saccharomyces genus yeast, were collected from the GenBank database, multiple-lined up by cluster V using the MegAlign program of DNASTAR, and primers were designed to discriminate between *S. boulardii* and *S. cerevisiae.* The characteristics of primer design are shown in Table 1.

As shown in Table 1, using designed specific primers, isolated yeasts distinguished *Saccharomyces boulardii* from *Saccharomyces cerevisiae* by the glass bulb method in PCR.

Table 1. Primer's array and its characteristic values are designed in 5.8s rDNA and ITS array.

Primer	Primary structure	Length	GC content (%)	Tm (ºC)
Sac-R	TCCGTAGGTGAACCTGCGG	19	63.2	55.7
Sac-F-b	TTTACTGGGCAAGAAGACAG	20	45.0	47.1
Sac-F-c	TTTACTGGGCAAGAAGACAA	20	40.0	47.6
Sac-R	TCCTCCGCTTATTGATATGC	20	45.0	49.6

As a result, PCR was performed when No. 18 yeast used Sac-F-b/Sac-R, a specific primer of *S. boulardii,* and No. 9 yeast used Sac-F-c/Sac-R, a specific primer of *S. cerevisiae* (Figure 1).

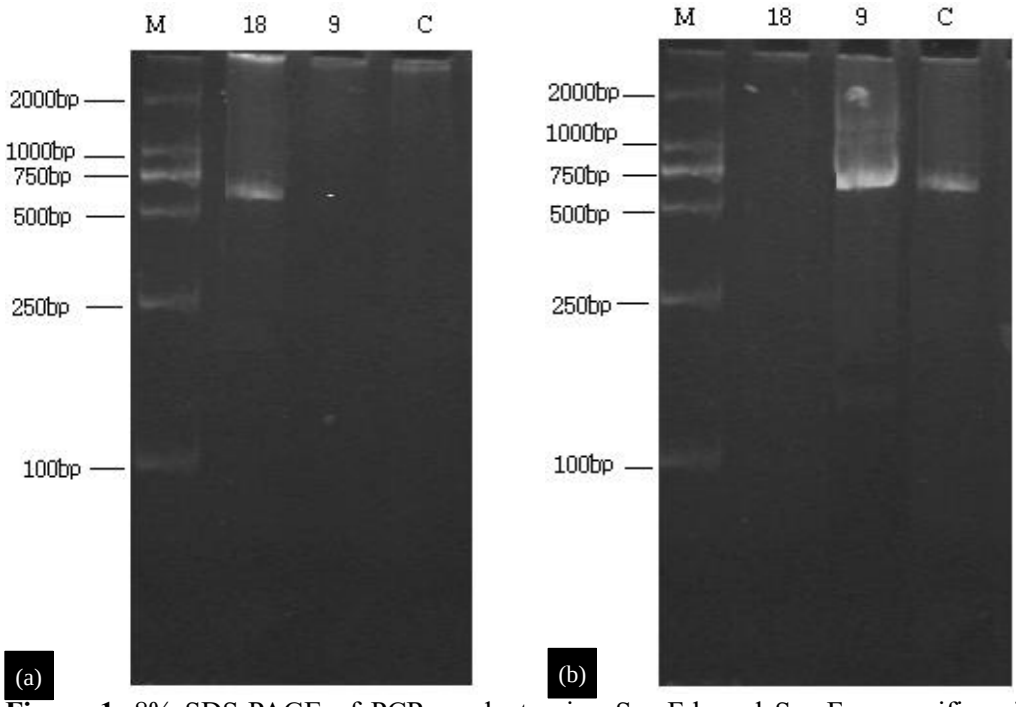

Figure 1. 8% SDS-PAGE of PCR product using Sac-F-b and Sac-F-c, specific primer, (a) Primer Sac-F-b/ Sac-R, (b) primer Sac-F-c/Sac-R). 18-yeast № 18, 9-yeast № 9, c-reference *Saccharomyces cerevisiae* IF 995.

To confirm the PCR results, the product obtained under optimum PCR conditions was repurified, and the first-degree structure of the PCR product was analyzed by dideoxy procedure using an ABI PROSM-310 type DNA sequence analysis device. Homologous species were explored using the NCBI GenBank database and BLAST retrieval and analyzed phylogenetically. As a result, it was identified as 100% with *Saccharomyces boulardii* in the sequence analysis data and NCBI GenBank database and phylogenetically confirmed as *Saccharomyces boulardii.*

Purification and Characterization of Antimicrobial Peptide in *S. boulardii* Culture Fluid

Purification's degree of antimicrobial peptide following the purification stage

Table 2. Purification's degree of antimicrobial peptide following the purification stage.

Purification stage	Total protein (mg)	Total activity (AU)*	Inactivity (AU/ml)	Refining degree	Yield (%)
Supernatant	324.5	13400	41.3	1.0	100
ultra-filter	45.6	12825	281.3	6.8	95.7
DEAE-Sephadex A25	5.9	3010	510.2	12.4	22.4
CM-Sepharose	1.2	1712	1426.7	34.5	12.8
Sephadex-G15	0.7	1192	2128.6	51.5	8.9

AU (Arbitrary Unit)

After *S. boulardii* was ultrafiltered, the total activity was 12825AU the yield was 95.7%, and it was then filtered with DEAE-Sephadex A25, CM-Sepharose, and Sephadex-G15. The total antibacterial activity was 1192AU and the yield was 8.9% (Table 2).

MW Determination by SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The MW of purified antimicrobial peptides was determined by SDS-polyacrylamide gel electrophoresis (PAGE).

Brands of standard materials including insulin (5807 Da), lysozyme(14.4 kDa), and BSA (66.2 kDa) appeared, but brands did not appear in the well-poured vitamin K12 (1355 Da) and purified antimicrobial peptide. This showed that the MW of the purified antimicrobial peptide was less than 5807 Da and that the specimen did not have any other proteins whose MW were greater [39, 38].

MW Decision by Mass Spectrometer

The MW of the purified antimicrobial peptide was obtained using Acquity UPLCSQD-2. As shown in Figure 2(a) and (b), the purified AMP specimen peaked at 2.44 min at Acquity UPLCSQD-2, and the MW of the purified antimicrobial peptide was 930 Da in the MS analysis.

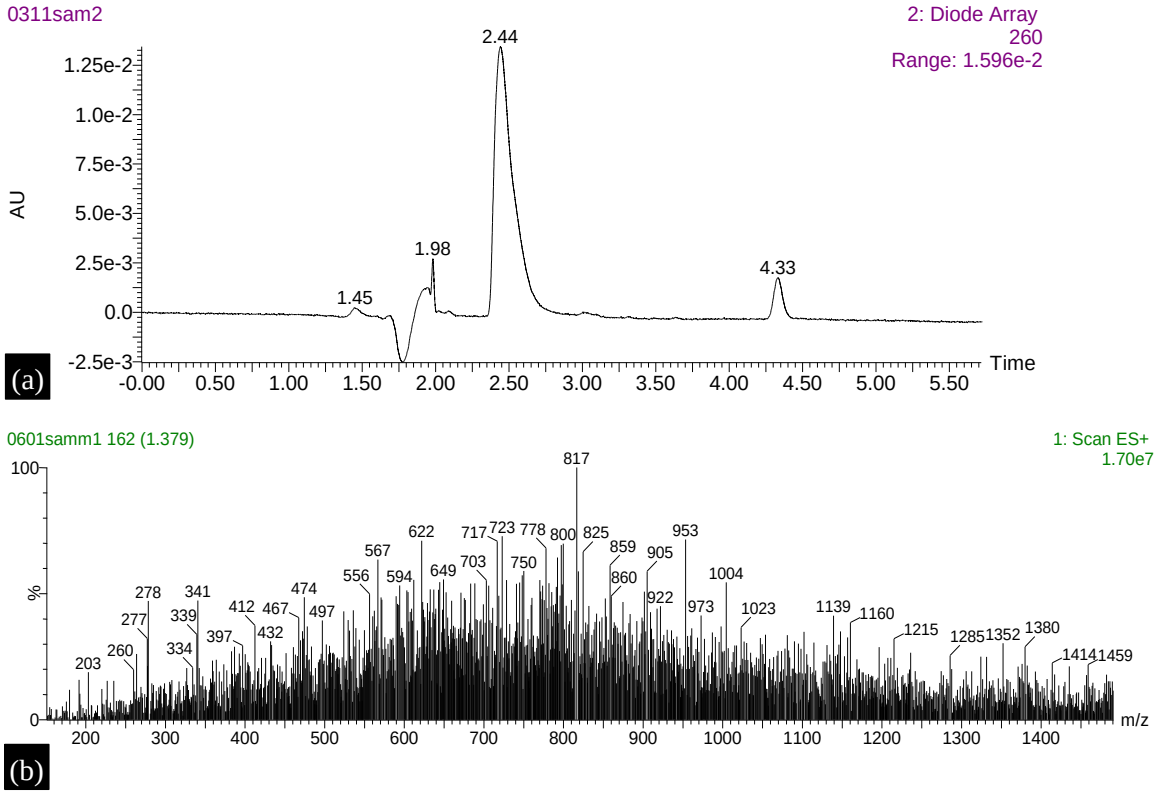

Figure 2. Acquity UPLC SQD-2 and MS of purified antimicrobial peptide

As shown in Figure 2(a), the AMP sample had a high peak at 2.44 min in Acquity UPLC SQD-2, the MW of AMP was 600–100 Da in MS analysis, and at 817 Da, it had one higher peak (Figure 2(b)).

Antibacterial of Purified Antimicrobial Peptide

The MIC of the purified AMP solution (protein concentration 30 μg/ml) was measured using a microliquid dilution assay.

Table 3. MIC of purified AMP against reference and isolated strains

Strains	MIC(μg/ml)
S. aureus ATCC 25923	0.41±0.06
MRSA (isolated strain)	0.47±0.00
S. aureus-37(isolated strain)	0.41±0.06
Streptococcus (isolated strain)	0.35±0.07
Clostridioides difficile ATCC 9689	0.86±0.18
E. coli O_{111}	1.40±0.27
E. coli O_{157}:H_7	1.63±0.23
Sh. flexneri ATCC 11836	1.63±0.23
P. aeruginosa ATCC 10145	2.79±0.54
Salmonella enterica serovar Typhimurium ATCC 14028	2.47±0.49

n=4

As shown in Table 3, MIC against gram-positive bacteria was 0.35~0.86 μg/ml, and that against gram-negative bacteria was 1.40~2.79 μg/ml. Significant differences between the MIC of S. aureus ATCC 25923, antibiotic-sensitive bacteria, and MRSA, antibiotic-resistant bacteria, were not observed ($p>0.05$).

Sensitivity Test of Antimicrobial Peptide Against Physicochemical Factor

- *Test score of hemolysis activity:* We tested white mice and human erythrocytes for the hemolysis activity of the purified antimicrobial peptides. Hemolysis activity was not observed when a variety of 1.56~70 μg/ml antimicrobial peptides were mixed with white mice and human erythrocytes [32, 40].
- *Sensitivity test of antimicrobial peptide against physicochemical factor:* **(**1) Antibacterial activity of antimicrobial peptide according to enzyme concentration.

The antibacterial activity of the AMP solution was affected by various factors, and its sensitivity was estimated.

Table 4. Sensitivity test of antimicrobial peptide against physicochemical factor.

Index		Antibacterial activity	Reaction
Enzyme	Pepsin	−	
	Trypsin	−	
Heat	70~132℃	+	
pH	3~9	+	
solvent	Ethyl alcohol	+	
	Methyl alcohol	+	
	Acetic acid	+	
Confirmatory reaction	Ninhydrin		Positivity
	Burette		Positivity

−: No antibacterial activity, +: Antibacterial activity, n=4

As shown in Table 4, the purified antimicrobial peptide had no antibacterial activity after being affected by trypsin and pepsin; its antibacterial activity remained after heating at 70–120°C was active in the pH range of 3–9 and was not affected by ethyl alcohol, methyl alcohol, or acetic acid. Ninhydrin and burette reactions to the purified antimicrobial peptide solution were positive.

DISCUSSION

The rapid increase in antibiotic resistance has resulted in obstacles to clinical treatment in the last 20 years. In particular, the emergence of multidrug-resistant (MDR) bacteria demands new antibacterial and antibacterial factors. The study for antimicrobial peptides, which did not acquire resistance and had a broad antimicrobial spectrum, had widely been conducted to use them as antimicrobial alternatives.

Therefore, we aimed to purify microbes that could produce many antibacterials from a variety of peels, earth, yogurt, and cereal.

- *Isolation and identification of yeast:* This study was conducted using molecular biology to identify two yeast strains (No. 18, No. 19) considered as *S. boulardii*, biologically identified, and grown at 37°C among 14 yeast strains isolated from a variety of peels, earth, yogurt, and cereal. To identify the isolated yeast, specific primers were designed to discriminate *S. boulardii* from *S. cerevisiae* using PCR.

  5.8s rDNA and ITS, well-reflected interspecific or intraspecific variation of Saccharomyces genus yeast, were collected from the GenBank database, multiple-lined up by cluster V using the MegAlign program of DNASTAR, and primers were designed to discriminate between *S. boulardii* and *S. cerevisiae*.

  The results showed that the sequence of Sac-F-b, a positive primer of *S. boulardii,* was TTTACTGGGCAAGAAGACAG, Sac-R, the reverse primer, was TCCGTAGGTGAA-CCTGCGG, the sequence of Sac-F-c, the positive primer of S. cerevisiae IF 995 was TTTACTGGGCAAGAAGACAA and Sac-R, the reverse primer, was TCCTCCGCTTATTGATATGC.

  PCR was performed using genomic DNA isolated by a glass bulb method with a designed specific primer, at various aniline temperatures, and a proper method to isolate genomic DNA and optimum PCR conditions were established. An experiment to distinguish *Saccharomyces boulardii* from *S. cerevisiae* by PCR was conducted; as a result, yeast No.18 was *Saccharomyces boulardii* and yeast No. 19 was *S. cerevisiae* on 8% PAGE. To confirm the PCR results, the product obtained under optimum PCR conditions was repurified, and the first-degree structure of the PCR product was analyzed by a dideoxy procedure using an ABI PROSM-310 DNA sequence analysis device. Homologous species were explored using the NCBI GenBank database and BLAST retrieval and analyzed phylogenetically. As a result, it was completely equal to *Saccharomyces boulardii* in sequence analysis data and the NCBI GenBank database and phylogenetically identified as *Saccharomyces boulardii.* These yeast strains were registered as *Saccharomyces boulardii* 6569-2018 at the National Strain Conservation Institute.

  S. boulardii is a non-pathogenic and non-toxic yeast isolated from lychee peel for the first time in Indochina in 1923. It is a probiotic yeast that was only admitted by the WHO Health Organization.

  Probiotics are live microbial food supplements that beneficially affect the host animal by improving the intestinal microbial balance." The benefits of many probiotics in humans have been reported, and Eli Metchnikoff demonstrated the role of LAB for the first time. In the past, we thought that probiotic microorganisms, including LAB symbiosis in the intestine, counteracted disturbances in the intestinal flora, reduced the risk of colonization by pathogenic bacteria, had immunomodulatory effects and antagonism to pathogens by producing organic acids, and restored the intestinal balance disorder by producing antibacterial substances such as bacteriocins. However, acidocin LCHV, a small AMP with a molecular weight of 1.1 kDa

produced by *Lactobacillus acidophilus* n.v. Er 317/402 strain Narine, has been recently reported, which broadens the antibacterial mechanism of probiotic microorganisms and has a wider range.

These data demonstrate how probiotic microorganisms, including LAB, restore intestinal balance and kill pathogenic bacteria. The same is true for S. boulardii, which is a probiotic.

We supposed that the yeast we isolated would produce AMP because it was a probiotic and conducted experiments to purify it.

- *Purification and characterization of an antimicrobial peptide in S. boulardii's culture fluid:* After *S. boulardii* was ultrafiltered, the total activity was 12825AU and the yield was 95.7%. It was then filtered with DEAE-Sephadex A25, CM-Sepharose, and Sephadex-G15. The total antibacterial activity was 1192AU and the yield was 8.9%. The MW of purified antimicrobial peptides was determined by SDS-polyacrylamide gel electrophoresis (PAGE). Protein bands were not detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This result was thought to have erupted early because the MW of AMP was too small, and there were no other proteins whose MW was greater than 5807 Da.

  The MW of the purified antimicrobial peptide was obtained using ACQUITY UPLCSQD-2. The purified AMP specimen peaked at 2.44 min at ACQUITY UPLCSQD-2, many peaks appeared between 600 and 1000 Da in MS analysis, and a higher peak appeared at 817 Da. As a result, we assumed that the MW of the purified antimicrobial peptide was 817 Da. The literature [25] only mentioned that SAIF was isolated and purified from *S. boulardii* with a molecular weight of 1.1 kDa, which is heat stable and water-soluble, and blocks NF-kB activation and NF-κB-mediated IL-8 gene expression in intestinal epithelial cells and monocytes but did not offer antimicrobial activity. Therefore, this study was conducted to test the antimicrobial activity of AMP.
- *Antibacterial of purified antimicrobial peptide:* MIC of purified AMP solution (protein concentration 30 μg/ml) was measured by microliquid dilution. As shown in Table 3, MIC against gram-positive bacteria was 0.35~0.86 μg/ml, and that against gram-negative bacteria was 1.40~2.79 μg/ml. There were no significant differences between the MIC of *S. aureus* ATCC 25923, the antibiotic-sensitive bacteria, and MRSA, the antibiotic-resistant bacteria($p>0.05$). These results were similar to those reported in the literature [18, 2], which indicated that AMP is sensitive to antibiotic-resistant bacteria and has a broad spectrum of activity against both Gram-negative and gram-positive pathogens. It also was sensitive to several bacteria, classified as causing Especially Dangerous Infections by WHO, and had a wide range of pathogens including MDR. These results show that AMP, which is sensitive to both antibiotic-resistant and antibiotic-sensitive bacteria, has a beneficial curative effect in clinical treatment.
- *Sensitivity test of antimicrobial peptide against physicochemical factor:* Until now, more than 5000 types of AMP have been purified, but their introduction to clinical treatment has been limited. This is because AMP has both hemolytic and proteolytic activities. We tested white mice and human erythrocytes for the hemolytic activity of the purified antimicrobial peptide. Hemolytic activity was not observed when a variety of 1.56~70 μg/ml antimicrobial peptides were mixed with white mice and human erythrocytes.

  The purified antimicrobial peptide had no antibacterial activity when measured after treatment with trypsin and pepsin. This indicated that the peptide substance was dissolved by these enzymes. The antibacterial activity of the AMP solution was affected by various factors, and its sensitivity was estimated.

  The activity of the purified antimicrobial peptide remained after heating to 132°C, was active over pH 3-9, and was not affected by ethyl alcohol, methyl alcohol, or acetic acid. These results were completely similar to the references [18], which mentioned that AMP was extremely heat stable, active over a wide pH range, sensitive to proteolytic enzymes, and not affected by ethyl alcohol, methyl alcohol, and acetic acid. We confirmed that the purified substance was

composed of amino acids and peptides by performing experiments for amino acids and peptides. AMP, isolated and purified from *S. boulardii*, is thought to be extremely stable and effective in clinical treatment.

CONCLUSION

Antimicrobial peptide from this yeast, whose molecular weight is between 600~1000 Da, had high sensitivity to antibiotics as MIC of AMP against gram-positive, negative strain is 0.35~0.86 μg/ml, 1.40~2.79 μg/ml, high antibacterial activity against resistant bacteria, thermal resistance, no hemolysis in the extent of 1.56~70 μg/ml of protein concentration and was stable for the animal organism.

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Disclosure of Any Conflict of Interest

None.

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