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# Partial Characterization and Purification of Enterocin K25 Linked to the Plasmid in *Enterococcus* sp. K25

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**Abstract** The antimicrobial activity of partially purified enterocin K25, produced by *Enterococcus* sp. K25, was abolished by proteases such as pepsin and proteinase K. The bacteriocin was resistant to heat treatment at 75°C for 15 min and lost 75% of its activity at 100°C for 30 min. Enterocin K25 showed bactericidal mode of action against an indicator strain, *Lactobacillus plantarum* NCDO 955. Enterocin K25 was purified to 112.6-fold purity via conventional steps of ammonium sulfate precipitation, ion exchange chromatography, and reversed phase high performance liquid chromatography (RP-HPLC). The molecular mass of the purified enterocin K25 was estimated as 4.3 kDa on an electrophoresis gel. Plasmid (~6.5 kb) linkage of production of enterocin K25 was confirmed by plasmid curing.

**Keywords:** enterocin K25, bacteriocin purification, *Enterococcus* sp.

## Introduction

Bacteriocins from lactic acid bacteria (LAB) have been widely studied. To date, many LAB producing bacteriocin have been isolated from various sources and their bacteriocins were characterized (1-6). Nevertheless, LAB researchers continue to search for novel LAB bacteriocins having strong bactericidal activity against food-borne pathogens or food-spoilage bacteria (7-9). LAB bacteriocins are classified into three groups: class I, lantibiotics (nisin A); class II, small heat-stable peptides (pediocin PA-1); class III, large heat-labile proteins (helveticin J) (2). Among these bacteriocins, the class II a variants (pediocin-like bacteriocins) have been intensively studied because of their commercial availability (1). Bacteriocins belonging to class IIa have a consensus motif -YGNGV- and strong antilisterial activity and many of them have been characterized biochemically and genetically (1, 10-15). In our previous study, we isolated bacteriocinogenic *Enterococcus* sp. K25 from kimchi, a fermented vegetable, investigated its antimicrobial activities against Gram-positive and Gram-negative bacteria, and yeasts, and applied it to kimchi fermentation (16). To continue this line of research activity, we investigated the physico-chemical properties of partially purified enterocin K25, purified it completely, and confirmed the locus of the genes related its production.

## Materials and Methods

**Bacterial strains and culture conditions** *Enterococcus* sp. K25 and *Lb. plantarum* NCDO 955 were grown in Brain Heart Infusion broth (Merck, Darmstadt, Germany) and MRS broth (Merck, Darmstadt, Germany), respectively, at 37°C without agitation.

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**Preparation of partially purified enterocin K25** Supernatant of overnight-cultured *Enterococcus* sp. K25 was obtained by centrifugation at 8,000 rpm for 30 min and filtration through 0.2 μm-pore-sized membrane filters. An aliquot of culture supernatant held at 4°C was brought to 50% saturation rate by adding solid ammonium sulfate with stirring. The mixture was stirred at 4°C over 4 hr and centrifuged at 12,000 rpm for 1 hr. The resultant pellet was dissolved with distilled and deionized water, dialyzed with Spectra/Por® DispoDialyzer® (MWCO. 1,000; Spectrum, Rancho Dominguez, CA, U.S.A.) and freeze dried. The powder was stored at -80°C until use.

**Stabilities of partially purified enterocin K25 against heat and enzyme treatments** Partially purified enterocin K25 was resuspended with 50 mM of sodium phosphate buffer (pH 7.5). Aliquots of the suspension were heat treated at 75 and 100°C and the residual activities were measured. The bacteriocin was also treated with various enzymes, including proteases, according to the manufacturer's instruction. It was treated with each enzyme (1 mg/mL) for 1 hr at 37°C, the reactant was boiled for 5 min to inactivate the enzyme and the residual activity was measured.

**Mode of action of enterocin K25** Exponential-phased cells of *Lb. plantarum* NCDO 955 were washed and suspended in 20 mL of 50 mM sodium phosphate buffer (pH 7.5) to a final concentration of $10^7$ CFU/mL. Partially purified enterocin K25 [500 arbitrary units (AU)/mL] was added to the suspension with or without proteinase K (200 units/mL) and the mixture was incubated for 3 hr at room temperature. Viable cell counts of *Lb. plantarum* NCDO 955 were measured at every 30 min on MRS agar plate.

**Purification of enterocin K25** Partially purified enterocin K25 was prepared from 2 L of overnight-cultured *Enterococcus* sp. K25 as described above. Crude enterocin K25 was resuspended in 20 mM of sodium

phosphate buffer (pH 6.0) and loaded onto a CM sepharose (Sigma, St. Louis, MO, U.S.A.) column (2.5 × 14 cm) equilibrated with the same buffer. The column was washed with 20 mM of sodium phosphate buffer (pH 6.0) and a linear NaCl gradient (0-1.0 M) was applied to elute the bound proteins. The protein concentration of each fraction (5 ml) was monitored by measuring the absorbance at 280 nm. Bacteriocin activities of the fractions were measured with the serial dilution method (17). Fractions having bacteriocin activity were pooled and subjected to RP-HPLC. The HPLC column (Delta Pak, C18 5 μm, Waters, Japan) was equilibrated with 0.1% trifluoroacetic acid solution and a linear acetonitrile gradient (0-100%) was applied to elute enterocin K25. Protein concentration ($OD_{280}$) and bacteriocin activity (AU/mL) of each fraction (1 mL) were monitored and fractions having the activity were pooled and subjected to second HPLC run.

**Bacteriocin activity and protein concentration** As mentioned above, the bacteriocin activity, expressed as AU/mL, was defined as the reciprocal of the highest two-fold dilution showing inhibitory action against an indicator strain (17). The protein concentration of each purification step was measured by using an RC DC™ protein assay kit (Bio-Rad, Hercules, CA, U.S.A.).

**Tricine SDS-PAGE and bioassay** To investigate the molecular mass of enterocin K25, tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed (18). Criterion™ 10-20% gradient tris-tricine gel (Bio-Rad, Hercules, CA, U.S.A.) was assembled in a Mini-PROTEAN® 3 electrophoresis unit (Bio-Rad, Hercules, CA, U.S.A.), running buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.3) was poured into the buffer tank, and the sample was loaded into a well. The unit was run at 150 V for 60 min and the gel was fixed in a fixation solution [40% (v/v) MeOH, 10% (v/v) acetic acid] for 30 min. The fixed gel was stained by Bio-Safe™ Coomassie (Bio-Rad, Hercules, CA, U.S.A.) and washed with distilled and deionized water and the protein band was examined. To confirm whether the protein band corresponds to enterocin K25, bioassay was performed at the same time. The identical gel run in the same unit was washed with distilled and deionized water for 5 hr and the gel was loaded on an MRS agar plate. Soft agar (0.7%, w/v) seeded with an indicator strain, *Lb. plantarum* NCDO 955, was poured on the plate and incubated at 37°C for 18 hr and the inhibition zone was examined.

**Plasmid preparation and curing** Plasmid DNA from *Enterococcus* sp. K25 was prepared according to the method of O'Sullivan and Klaenhammer (19). To obtain a plasmid-cured mutant, *Enterococcus* sp. K25 was continuously cultured in MRS broth with novobiocin (3 μg/mL), curing agent, and spread on MRS agar plate periodically. The mutant was isolated via plasmid profiling of colonies randomly selected on the MRS agar plates.

## Results and Discussion

**Stabilities of enterocin K25 against heat and enzyme treatments** Prior to complete purification, physico-chemical tests using partially purified enterocin K25 were performed because the crude bacteriocin is well suited to distinguish treated samples from the control due to the presence of plenty of bacteriocin. Enterocin K25 was resistant to heat treatment at 75°C for 15 min and its activity was sustained at 50% of the original activity after heat treatment at 100°C for 15 min (Table 1). The bacteriocin activity of enterocin K25 was abolished by treatments of proteases such as proteinase K and pepsin; however, the activity was unaffected by other enzymes (Table 1). These results indicate that enterocin K25 is a proteinaceous, heat-stable compound that is a potential candidate for food biopreservative. To date, various bacteriocins from *Enterococcus* spp. have been revealed and characterized (20-23). Particularly, peptide AS-48, a cyclic bacteriocin from *Enterococcus faecalis* subsp. *liquefaciens*, has attracted research attention due to its structural stability and broad range antimicrobial activity (23, 24).

**Mode of action of enterocin K25** The viable cell count of *Lb. plantarum* NCDO 955 was significantly reduced after treatment of enterocin K25 compared with the control (no treatment), but was slightly recovered when the bacteria was treated with the bacteriocin plus proteinase K (Fig. 1). These results indicate that enterocin K25 shows a bactericidal mode of action against *Lb. plantarum* NCDO 955 and that the bacteriocin is inactivated by proteinase K. In general, bacteriocins are bactericidal peptides which act primarily by permeabilizing the membranes of susceptible microorganisms, probably through the formation of pore complexes, causing an ionic imbalance and leakage of inorganic phosphate (1).

**Table 1. Stability of enterocin K25 against heat and enzyme treatments**

| Treatment | Residual activity |
|---|---|
| Control | 100[a] |
| Pronase E | 100 |
| Proteinase K | 0 |
| Trypsin | 100 |
| α-Chymotrypsin | 100 |
| Pepsin | 0 |
| Lipase | 100 |
| α-Amylase | 100 |
| Lysozyme | 100 |
| Catalase | 100 |
| Ribonuclease A | 100 |
| 75°C, 10 min | 100 |
| 75°C, 15 min | 100 |
| 75°C, 30 min | 50 |
| 100°C, 10 min | 50 |
| 100°C, 15 min | 50 |
| 100°C, 30 min | 25 |

[a]The number shows the relative activity of partially purified enterocin K25.
Bacteriocin activities were measured by the serial dilution method as described in materials and methods (indicator strain: *Lb. plantarum* NCDO 955). All enzymes were purchased from Sigma Co. (St. Louis, MO, U.S.A.).

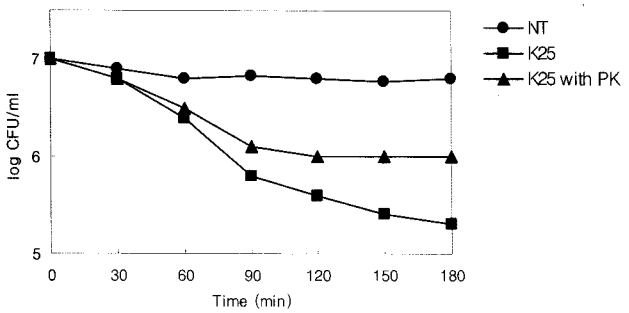


**Fig. 1. Changes of viable cell count of *Lb. plantarum* NCDO 955 as an indicator strain treated with enterocin K25.** NT, no treatment; K25, partially purified enterocin K25; PK, proteinase K. Partially purified enterocin K25 (500 AU/mL) was added to the cell suspension of *Lb. plantarum* NCDO 955 with or without proteinase K (200 units/mL) and the mixture was incubated for 3 hr at room temperature.

**Purification of enterocin K25** Purification of enterocin K25 produced by *Enterococcus* sp. K25 is summarized in Table 2. Enterocin K25 was purified from the culture supernatant and then underwent ammonium sulfate precipitation, ion exchange chromatography, and RP-HPLC to obtain a 112.6-fold increase in purity. A single peak corresponding to enterocin K25 was detected after the second HPLC run (Fig. 2). The molecular mass of the purified enterocin K25 was estimated as 4.3 kDa on a tricine SDS-PAGE gel (Fig. 3). Although this purification strategy was straightforward and successful, new strategies such as heterologous production via genetic characterization should be considered to improve the production yield and purification quality.

**Selection of plasmid-cured *Enterococcus* sp. K25 mutant** *Enterococcus* sp. K25 harbored a 6.5 kb-sized plasmid and the plasmid-cured K25 mutant was obtained (Fig. 4A). This mutant did not exhibit antimicrobial activity against *Lb. plantarum* NCDO 955, which was itself inhibited by K25 wild type (Fig. 4B). These results indicate that genes for the production of enterocin K25 are present in the plasmid. Many studies on the plasmid linkage of bacteriocin production have been reported (25-28). The structural gene for bacteriocin and its surrounding region showed an operon-like structure in which the production and exportation of, immunity to the bacteriocin, and in several cases the regulation of the bacteriocin synthesis, were involved (1).

Recently, many bacteriocinogenic LAB were isolated from kimchi and several bacteriocins from LAB were characterized biochemically and genetically (16, 25, 29-32). In our previous study, bacteriocinogenic *Enterococcus* sp. K25 strongly inhibited *Lb. plantarum*, which is recognized as an acidifying species in *Kimchi* fermenta-

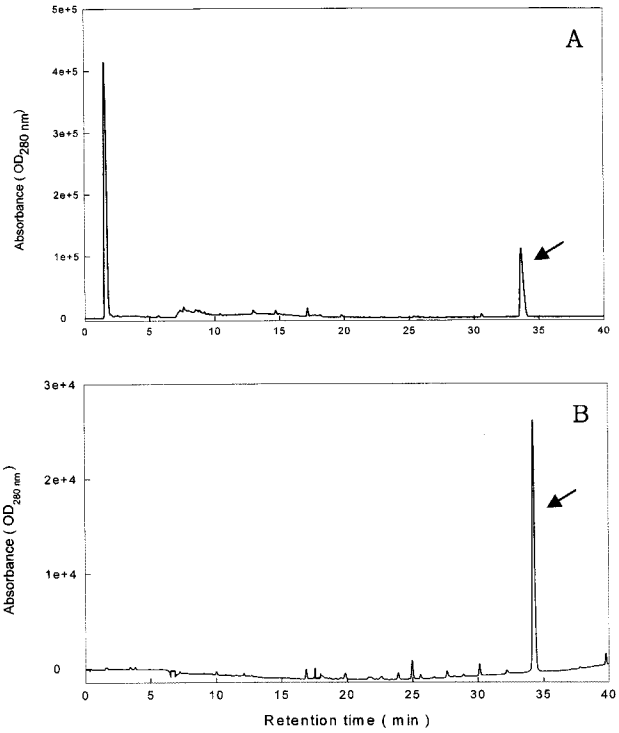


**Fig. 2. Chromatograms of partially purified enterocin K25 by RP-HPLC.** A, first run RP-HPLC; B, second run RP-HPLC. Partially purified enterocin K25 was prepared by means of ammonium sulfate precipitation and ion exchange chromatography. The arrows indicate peaks of enterocin K25.

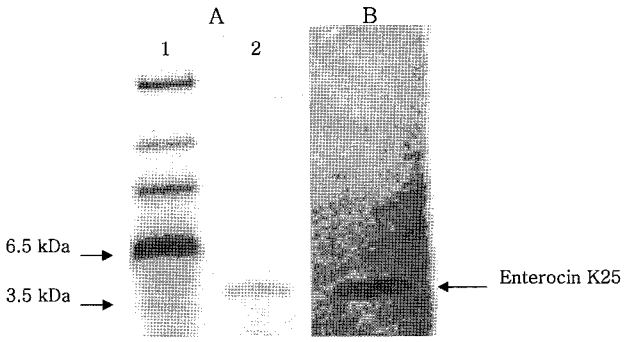


**Fig. 3. Tricine SDS-PAGE of purified enterocin K25.** A: Bio-Safe™ Coomassie (Bio-Rad, Hercules, CA, U.S.A.) staining; lane 1, polypeptide standards (Bio-Rad, Hercules, CA, U.S.A.); lane 2, purified enterocin K25. B: activity staining (indicator strain: *Lb. plantarum* NCDO 955).

**Table 2. Purification of enterocin K25 produced by *Enterococcus* sp. K25**

| Fraction | Total activity (AU) | Total protein (mg) | Specific activity (AU/mg) | Yield (%) | Fold in specific activity |
|---|---|---|---|---|---|
| Culture supernatant | 900,000 | 47.5 | 18,947 | 100 | 1 |
| Ammonium sulfate precipitation | 480,000 | 5.2 | 92,308 | 53.3 | 4.9 |
| Ion exchange chromatography | 210,000 | 1.4 | 150,000 | 23.3 | 7.9 |
| First run RP-HPLC | 96,000 | 0.09 | 1,066,666 | 10.7 | 56.3 |
| Second run RP-HPLC | 64,000 | 0.03 | 2,133,333 | 7.1 | 112.6 |

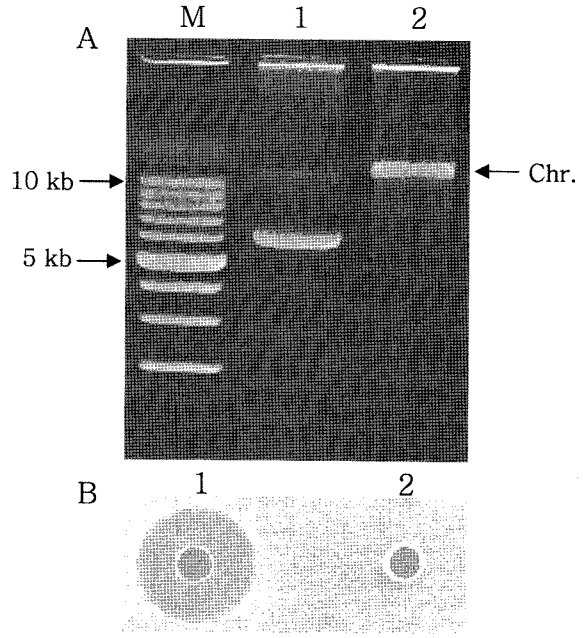


**Fig. 4. Plasmid profiles and antimicrobial activities of *Enterococcus* sp. K25 wild type and its mutant.** A: plasmid profiles; Chr., chromosomal DNA; M, supercoiled DNA ladder (Promega, Madison, WI, U.S.A.); lane 1, K25 wild type; lane 2, K25 mutant. B: antimicrobial activities of culture supernatant (well diffusion assay); 1, K25 wild type; 2, K25 mutant.

tion, and prolonged the *Kimchi* shelf life (16), indicating that *Enterococcus* sp. K25 producing enterocin K25 could be used as a biocontroller in the fermentation. To realize this potential, fundamental studies on enterocin K25 need to be performed. In this respect, it is considered that the results of the present study with regard to the physicochemical properties, purification, and confirmation of gene locus of enterocin K25 will provide a foundation for further research.

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