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# Evaluation of antibacterial peptide synthetized by *Lactobacillus paracasei* (1-g) isolated from Mongolian airag

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## Abstract
In this study, we aimed to purify antibacterial peptides like bacteriocin from the cell-free supernatant of L.paracasei 1-g isolated from traditional Mongolian Airag. The antibacterial peptide was purified by a sequential purification method via ammonium sulfate precipitation, gel filtration, and ion exchange chromatography on SP-Sepharose and Reversed phase chromatography. The purification exhibited a 25-fold increase compared to the cell-free supernatant. The purified peptide displayed antibacterial activity of 6400 AU/ml at neutral pH against indicator strains. The peptide portrayed high thermal stability sensitivity to proteolytic enzymes. The molecular weight of the peptide was 2056.424 Da as measured by MALDI TOF MS analysis.

**Keywords:** Airag, antibacterial peptide, bioactive peptides, *Lactobacillus paracasei*

## Introduction
Antibacterial peptides (ABP) are small parts of proteins secreted from living organisms and are synthesized as defense molecules against pathogenic bacteria. It is considered the first line of self-defense in occupied eukaryotic and prokaryotic cells [1]. The gram-negative Escherichia coli produced an antibacterial peptide known as colicin, which was noted as the first antibacterial peptide in 1925 [2]. Three years later, in England, the initial discovery of Nisin was made; Nisin is an antibacterial peptide synthesized by lactic acid bacteria formerly known as Lactococcus lactis [3-5]. Many kinds of microorganisms produce antibacterial peptides. In particular, Lactic acid bacteria (LAB) antimicrobial peptides have exceptional characterizations for their applications that are inherently stable to high temperatures, have a wide pH range, and are sensitive to proteolytic enzymes [5]. In recent years, many scientists have studied the production of antibacterial peptides from LAB and their applications. For instance, applications include bio preservatives for the food industry in order to assist in extending the shelf life of food products [6.7] and also in human therapeutics as an antibiotic [8]. The Mongolian Airag is one of the famous traditional fermented beverages made by wild-type starters and non-pasteurized fresh mare's milk [9]. Since ancient times, traditional Airag has been accepted with numerous health benefits by: providing nourishment, promoting digestion and the immune system, improving physical strength, controlling blood cholesterol level, and preventing infections, especially tuberculosis [10], hypertension [11], and gastroenteritis [12-14]. Moreover, Abdel-Salam et al. (2010) reported that high-fiber content in airag reduced mercury toxicity in rats [15]. Prior studies have thoroughly analyzed Airag's microbial diversity (lactic acid bacteria and yeast), biochemical characterization, and technological conditions [9, 13, 16-21]. Only a few studies have described the production of antibacterial peptides synthesized by LAB isolated from Airag, but their exact substance compositions were unclear.

These findings are directly related to studies on culture broth, which includes an analysis of many kinds of polysaccharides, peptides, trace elements, vitamins and organic acids, etc [23]. For that reason, researchers have been focusing on the purification and identification of antibacterial peptides to further study antibacterial effects, antibacterial mechanisms, and their applications [24-26]. Miao et al. (2014) reported a small peptide known as bacteriocin with 2113.842 Da purified from Lactobacillus paracasei subsp strain isolated from Tibetan kefir. This bacteriocin exhibited a broad potential for antimicrobial activity, including against?? Similar to?? gram +/- bacteria and some fungi such as Aspergillus, Rhizopus and Penicillium [23]. This paper aims to isolate and evaluate antibacterial peptides from the cell-free supernatant of Lactobacillus paracasei (1-g) derived from Mongolian traditional Airag in order to determine their antibacterial activity against indicator strains E.coli and B.subtilis.

**Material and methods**

In this study we used Lactobacillus paracasei (1-g) isolated from fermented horse milk airag. Bacterial samples (200µl) were kept at -80°C in 20% glycerol. Escherichia coli (E. coli) (ATCC25922) and Bacillus subtilis (NBRC 13722) were obtained from Japan's National Institute of Technology and Evolution (NITE). MRS Broth and Nutrient Broth (Oxoid Limited, England). Antibacterial activity of cell free supernatant (CFS) against indicator strains at neutral pH

Antibacterial activity against indicator strains (E. coli and B.subtilis) was performed using Fleming's well diffusion method [5]. L. paracasei (1-g) (2×105 cell/mL) was incubated in 3 ml MRS broth at 37°C for 24 h. The number of L. paracasei (1-g) was counted using a 100 ml cell counter, and L. paracasei (1-g) with to 4×108 cell/ml was observed. The cell suspension was centrifuged for 5 min at 8000G to remove cell pellets from L. paracasei (1-g). 0.1 N NaOH adjusted the pH of the CFS to 6.5. The CFS was passed through a sterile filter (pore size of 0.22 mm), then 50ml CFS was loaded into a 5 mm diameter hole on the LB agar plate previously cultivated with E. coli and B.subtilis, respectively, by 2-fold serial dilution, and the plate was incubated overnight at 30°C. The inhibition zone around the well was measured and calculated using the highest 2-fold serial dilution reciprocal.

***Protease treatment of CFS***

The effect of different enzymes on antibacterial substance activity was determined as described by Batdorj et al. (2006) [9]. 200 µl of filter-sterilized CFS was incubated with 20 µl of proteinase K (pH 7.5, 37°C), trypsin (pH 8.0, 25°C), a-chymotrypsin (pH 7, 37°C) and catalase (pH 7.5, 37°C), respectively. Two hours later, all treatments were heated at 100°C for 5 minutes for enzyme inactivation. The negative controls were used as unheated samples. The antibacterial activity of the protease-treated supernatant was measured against E. coli and B. subtilis. The thermal stability of the active substances was measured on neutralized cell-free supernatant (2 ml) and incubated on a water bath at 100°C for 15, 30 and 45 min. The residual activity was evaluated against the E. coli and B.subtilis, respectively.

***Production of Antibacterial peptides on Cell cultivation***

The first fermentation of L.paracasei (1-g) was carried out in MRS Broth at 37°C for 24 h. Then, the second fermentation was carried out in a 1-liter flask containing 750ml MRS broth and incubated at 37°C for 48 hours. During fermentation, samples were taken at 6-hour intervals, centrifuged at 8000 g for 20 minutes, then filtered through a 0.22 µm membrane filter. CFS was heated at 80 °C for 20 min for protease inactivation [25] and concentrated to 1/5th volume by dialysis.

***Isolation and Purification of Antibacterial peptides from Lactobacillus paracasei (1-g) strain***

We isolated antibacterial peptides by sequential purification methods such as ammonium sulfate precipitation (ASP), Amicon Ultra centrifugal filter units, gel filtration and ion exchange chromatographical techniques.

For ASP, cell-free supernatant was placed in solute to ascending ammonium sulfate saturation at 70%, and the pellets were centrifuged at 8000g for 15 min. It was resuspended in 100 ml pH 7 PBS. The concentrated crude peptide was subjected to an Amicon Ultra-15 centrifugal filter unit using 10 kDa molecular weight cut-offs (Merck Ltd, Germany). One fraction was derived and screened against indicator strains. The active fraction was purified as described in previous work using same column and techniques (5).

***Determination of protein concentration***

Protein concentration was measured by the Protein assay kit based on the Bradford method (Quick Start kit for Protein Assay, Bio RAD, U.S.). Adding an acidic dye to the protein solution and subsequent measurement at 595 nm with a standard curve provides a relative measurement of protein concentration [15].

**Results**

*Production of Antibacterial peptides*

The relationship between the cell growth of L.paracasei (1-g) and the production of antibacterial peptides in MRS broth are summarized in Figure 1. The results show that antibacterial substance production started in the early to mid-stage of the exponential phase, reaching 1600 AU/ml during the higher exponential phase. Notably, there is a significant increase in antibacterial peptide production during 12 to 16 h.

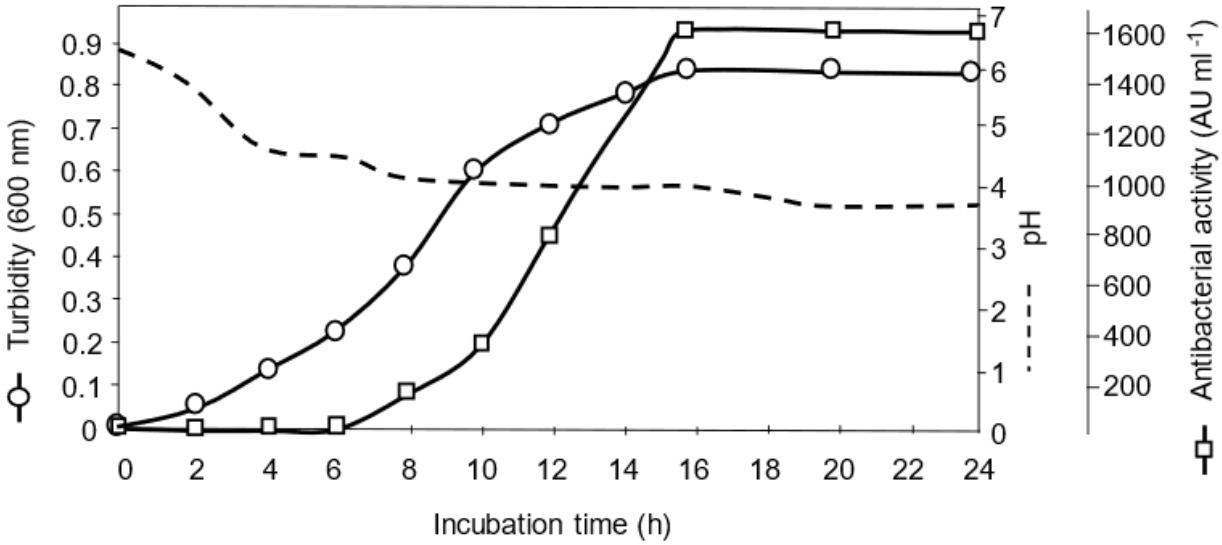


Figure 1. The production of antibacterial peptide and growth of Lactobacillus paracasei (1-g) in MRS at 37°C in 2 h intervals, absorbance at 600 nm. Antibacterial peptide concentration is expressed as arbitrary units per milliliter (AU/ml) against E. coli

**Effect of enzymes and high temperature treatment of CFS**

LAB generated ABPs have exceptional characterizations for their applications that are inherently stable to high temperatures, have a wide pH range, and are sensitive to proteolytic enzymes [5,9]. Table 1 shows CFS with antibacterial activity following the effect of enzymes and heat treatments at a neutral pH. Antibacterial activity was wholy exposed after treatment with three kinds of protease and catalase for negative control and treated at optimal pH and temperatures. The inhibitory activity of CFS was not reduced when treated with hydrogen peroxide-inhibiting catalase. Next, we determined the high-temperature stability of CFS. Table 1 shows that the inhibitory activity did not decrease when heated at 100 ºC for 15 and 30 min. These results are similar to some studies about bacteriocin, such as ST28MS and ST26MS, produced by Lactobacillus plantarum isolated from molasses. It was observed that

after protease and heat treatment, there was no decrease in antibacterial activity [28].



High-temperature stability is a significant feature of using bacteriocin to extend the shelf life, and food processing procedures usually include heating steps [9].

Table 1.

Effect of high temperature and 3 different protease treatments of CFS by L.paracasei (1-G) against indicator strains at a neutral pH

| Treatment | Antimicrobial activity, AU/mL | |
|---|---|---|
| | *E.coli* | *B.subtilis* |
| Protease treatment | | |
| Before treatment | 1600 | 1600 |
| Proteinase K | - | - |
| Trypsin | - | - |
| α-chymotrypsin | - | - |
| Heat treatment | | |
| Before treatment | 1600 | 1600 |
| 100°C | | |
| 15 min | 1600 | 1600 |
| 30 min | 1600 | 1600 |
| 45 min | 800 | 800 |

*The cell free supernatant with antimicrobial activity was measured by the agar diffusion assay. The activity exhibited AU/mL. AU-arbitrary unit.*
*(-) no inhibition.*

**Production and purification of Antibacterial peptides**

Lactobacillus paracasei (1-g) was grown in MRS broth (pH 6.8) for 24h at 37 ºC. The CFS was dissolved to ascending 70% ammonium sulfate precipitation and fractioned using Amicon Ultra centrifugal filter units. The crude peptides below 5 kDa were the only active fraction against E.coli and B.subtilis. A step column chromatography system, such as SP Sepharose Fast flow and RP-HPLC, further purified the active fraction. In ion exchange chromatography, out of the 50 fractions, the 21st to 34th fractions displayed antibacterial activity. The antibacterial activity of these fractions was 44800 AU/mg of protein, which was a 25-fold increase compared to cell-free supernatant, the results of which are shown in Table 2. In the next purification step, the latest active fraction was loaded to RP-HPLC equipped with an Agilent Bio SEC-3 column and eluted with Water:ACN containing 0.1% TFA gradient elution solvent system as resulted in Figure 3.

Then, absorption peaks were re-fractionated, after which a single fraction was used for antibacterial activity against indicator strains. A single peak (4) was observed at a retention time of 24.1 min.

These results indicate that the purification steps were successful. The active fraction was acquired around 2000 Da band in Tricine SDS PAGE with antibacterial activity against B.subtilis, shown in Figure 2. Further, the purified active peptide was analyzed using MALDI TOF MS that demonstrated the presence of a peptide 2,056.424 Da in size. These results indicated that L.paracasei (1-g) produced potent antibacterial peptides. Similarly, Jingping et al. (2016) reported that antibacterial peptides produced by L.paracasei HD1-7 isolated from Chinese Sauerkraut juice were purified using three-step purification. From the results, one kind of bacteriocin, also named paracin 1.7, has a 10 kDa molecular weight. It was sensitive to proteases and was heat resistant.

According to other results, a bacteriocin-like antibacterial peptide with low molecular mass was isolated and purified from cell-free supernatant of Lactobacillus species L.paracasei, L.rhamnosus, and L.plantarum [29].



Kanockporn et al. purified and identified one kind of bacteriocin from the cell-free supernatant of L.paracasei HL32 against Porphymoranus gingivalis. This strain has potent antibacterial activities against food-borne pathogens [30]. The primary structure of peptide is analyzed by LC-MS/MS, and peptide sequential analyses and the antimicrobial mechanism will be elucidated

Table 2.

Purification of antibacterial peptide produced by L.paracasei 1-g

| Purification step | Volume (ml) | Activity unit (AU/ml) | Total activity | Total protein (mg) | Purification fold |
|---|---|---|---|---|---|
| Cell free supernantant | 750 | 1600 | $120x10^4$ | 675 | 1 |
| ASP | 100 | 3200 | $32x10^4$ | 47 | 7.6 |
| SP Sepharose | 7 | 6400 | $4.48x10^4$ | 1 | 25 |

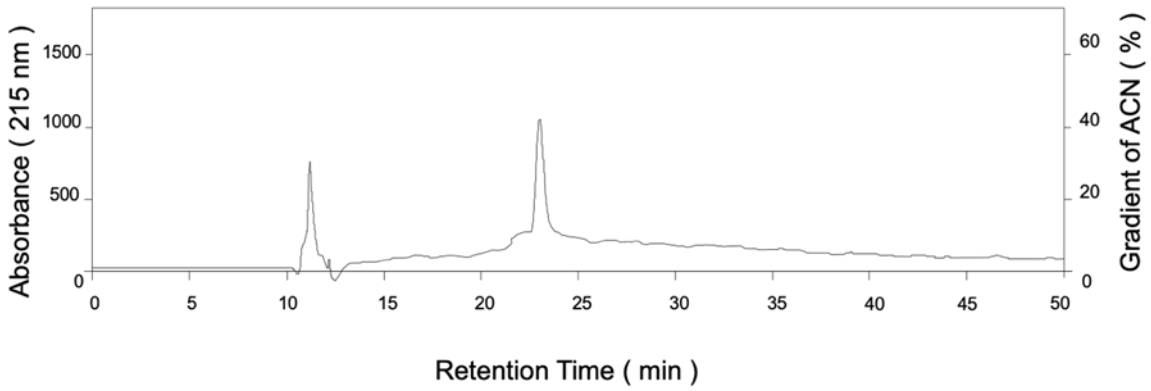


Figure.3. HPLC profiles of antibacterial peptide.
Column: Agilent Bio SEC-3 column (3µm, 150A, 4.6 x 300 mm).
Solvent system: Water (100%): ACN (100%), gradient: 0 to 50 min.

In this study, we have purified antibacterial peptides synthesized by L. paracasei 1-g that was isolated from Mongolian traditional Airag.

It was found to have potent antibacterial activity at a neutral pH against gram-positive and negative pathogenic bacteria, high thermal stability, and sensitivity to proteolytic enzymes. Similar results were reported by Tariful et al. with Lactobacillus paracasei ssp paracasei-1, which was isolated from traditional yogurts from Kulna region [31].

Proteolytic enzyme-mediated inactivation indicates the identity and inhibitory substance as bacteriocin. Previously, Uugantsetseg et al. reported that Lactobacillus paracasei DU-8 is a studied probiotic bacterium that exhibits antioxidant activity with strains isolated from Mongolian airag [32]. However, research on the purification of antibacterial peptides by Lactobacillus paracasei isolated from airag still need to be done.

The antibacterial peptides produced by wild type LABs from locally fermented foods and beverages may serve as candidates for a new food preservative and an alternative to antibiotics. Further works are in progress to better define this purified peptide, and the relationship between protein primary structure and antibacterial mode action against gram-positive and -negative pathogenic bacteria will be examined.

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