Korean J. Food Sci. An. Vol. 35, No. 2, pp. 211~215 (2015)
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DOI http://dx.doi.org/10.5851/kosfa.2015.35.2.211
ISSN 1225-8563 eISSN 2234-246X

ARTICLE

Antilisterial Effect of Bacteriocin SH01, Obtained from *Enterococcus faecium* SH01, in Ground Beef

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Abstract

From the previous study, *Enterococcus faecium* SH01 was isolated from *mukeunji*, an over-ripened *kimchi*, and it produced bacteriocin SH01. Bacteriocin SH01 showed an inhibitory effect against *Listeria monocytogenes* ATCC 19111, a bacterial strain causing human listeriosis. Crude bacteriocin SH01 was purified by ammonium sulfate precipitation and its inhibitory activity at two concentrations (500 and 1,000 AU/g) against *Listeria monocytogenes* ATCC 19111 was investigated in ground beef at increasing temperatures (5, 10, 15, and 20°C) for 8 d. The number of *Listeria monocytogenes* ATCC 19111 significantly decreased ($p<0.05$) as the concentration of bacteriocin increased from 500 to 1,000 AU/g. Intrinsic crude protease activities in ground beef were examined and increased as the temperature increased. Experiments varying both the concentrations of added bacteriocin SH01 and temperature demonstrated a maximum inhibition (2.33 log reduction of bacteria) in samples containing 1,000 AU/g of bacteriocin SH01 incubated at 20°C. When the crude bacteriocin SH01 solution (1,280 AU/mL) was incubated with crude protease solutions at different temperatures, its activity decreased by only half (640 AU/mL), as assessed in an agar well diffusion assay. The finding that the antilisterial activity of bacteriocin SH01 increased with temperature can be explained by the fact that higher temperatures increase bacterial membrane fluidity, thereby promoting the cellular penetration of bacteriocin SH01 into *L. monocytogenes*. Bacteriocin SH01 may be an excellent candidate as a biopreservative for controlling *L. monocytogenes* growth in ground beef.

Key words: antilisterial bacteriocin SH01, ground beef

Received November 24, 2014 / Revised February 12, 2015 / Accepted February 12, 2015

Introduction

Listeriosis is a severe foodborne infection caused by the consumption of foods that are contaminated by *Listeria monocytogenes* (Silva *et al*., 2009). Particularly, immunocompromised persons, such as pregnant women, newborn children, and the elderly are very susceptible to *L. monocytogenes* infection (Cho and Kim, 2001; Lee *et al.*, 1993). The outbreaks of listeriosis have been traced to many foods, such as fresh meats, fermented foods, vegetables, and poultry (Cho *et al.,* 2011b; Koseki and Isobe, 2005; Schlech ^{3rd} *et al*., 1983; Sommers *et al*., 2003). Because it is ubiquitous in nature, is relatively resistant to acid and salt, and has the ability to grow at refrigeration temperatures, the potential for post-processing contamination of foods with *L. monocytogenes* is high (Cho *et al*., 2011a; Nielsen *et al*., 1990; Swaminathan and Gerner-Smidt, 2007). Typically, ground beef is used for many foods, i.e., hamburger patty, and it can be easily contaminated by *L. monocytogenes* and support the growth of the pathogen, leading to serious listeriosis (Jang *et al*., 2007).

Currently, the procedures recommended for avoiding contamination of foods by *L. monocytogenes* are employing HACCP and using antimicrobial compounds, such as organic acids, fatty acids, herb essential oils, NaCl, and chemical preservatives (Neito-Lozano *et al*., 2006; Pucci *et al*., 1988; Todd *et al*., 2011). However, the antilisterial effects of these compounds are not strong, and there is an aversion towards the use of chemical preservatives in food. Many studies have suggested that some bacteriocin-producing lactic acid bacteria or purified bacteriocins may be used as natural preservatives in various foods (Cleveland *et al*., 2001; Eckner, 1992; Kim, 1993). However, since bacteriocins are proteinaceous in nature, to be effective, they must maintain their antibacterial activity in foods where the intrinsic protease activity is high, such as ground beef. In previous studies, a bacteriocin-producing strain of bacteria, *Enterococcus faecium* SH01, was isolated from *Mukeunji*, an over-ripened *kimchi*, and the bac-

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teriocin SH01 was found to have antilisterial activity (Seo *et al*., 2014).

The objectives of this study were to examine the antilisterial effect of bacteriocin SH01 in ground beef at different storage temperatures, with the purpose of evaluating its potential uses as a biopreservative.

Materials and Methods

Bacterial strains and culture conditions

L. monocytogenes ATCC 19111, a strain causing human listeriosis, was grown in 10 mL brain heart infusion (BHI) broth (Oxoid, UK) for 24 h at 37°C, with two consecutive transfers. The bacterial cells were obtained by centrifugation (1736R, LaboGene, Korea; 16,600 g for 15 min at 4°C) and washed twice in 10 mL of sterile peptone water (0.1%, w/v; Difco, USA). After a second washing, the cell suspension was diluted with sterile 0.1% (w/v) peptone water to a number of 5-6 Log CFU/mL.

Bacteriocin SH01-producing *Enterococcus faecium* SH01 were grown in 10 mL of MRS broth (Difco, USA) for 24 h at 37°C, with two consecutive transfers.

Preparation of crude bacteriocin

E. faecium SH01 was inoculated (2%, v/v) into 800 mL of MRS broth and incubated at 37°C for 24 h until the bacteria reached the late exponential phase of growth. The cells were harvested by centrifugation (16,600 g, 15 min at 4°C) and the cell-free supernatant was obtained by filtration through a 0.20 μm-pore size membrane filter (Sartorius, Germany). While stirring at 4°C, $(NH_4)_2SO_4$ (Samchun Pure Chemical Co., Ltd., Korea) was added to the filtrate to achieve 50% saturation. The saturated solution was centrifuged (16,600 g, 15 min at 4°C) and the precipitate was recovered. The precipitate was reconstituted in distilled and deionized water (ddH_2O) and dialyzed against ddH_2O using a benzoylated-cellulose dialysis sac (MWCO of 1.2 kDa; Sigma, USA) for 24 h at 4°C (Moon *et al*., 2004).

Estimation of bacteriocin titer

The bactericidal activities of the bacteriocin for the following steps were assayed by the agar well diffusion method with certain modifications (Seo *et al*., 2014). *L. monocytogenes* ATCC 19111 indicator was grown in MRS broth until reaching its mid-exponential phase of growth. The approximate number of *L. monocytogenes* ATCC 19111 on the plate was 5×10^6 CFU. The bacteriocin titer, expressed as arbitrary units (AU/mL), is defined as the reciprocal of the highest dilution that showed a clear zone of inhibition around the well of the indicator lawn.

Sample treatment

The effect of bacteriocin SH01 on the growth of *L. monocytogenes* in ground beef at different concentrations and storage temperatures was studied. Fresh (< 24 h) ground beef was purchased from a local supermarket and a *L. monocytogenes* suspension was inoculated to make initial levels of approximately 5-6 Log CFU/g. Crude bacteriocin solution (1,280 AU/mL) was added to the samples and mixed thoroughly in a stomacher bag to make final concentration of bacteriocin at 500 AU/g and 1,000 AU/g. Sterile water was used for the control. The samples were incubated at 5, 10, 15, and 20°C for 8 d.

Microbiological analysis

At day 8, samples (5 g) were aseptically taken and mixed with 45 mL of sterile 0.1% (w/v) peptone water and homogenized for 2 min in a stomacher (BagMixer 400, Interscience, France). Resulting slurries were serially diluted in 0.1% (w/v) sterile peptone water, surface plated onto PALCAM Listeria Selective Agar (Oxoid, UK) with supplement (SR0150E, Oxoid, UK) (Solomakos *et al*., 2008) and incubated at 37°C for 48±24 h. All the plating was repeated three times.

Protease activity in ground beef

In order to examine whether bacteriocin is destroyed by intrinsic proteolytic enzymes in ground beef during storage, total proteolytic activity was measured at different temperatures (5, 10, 15, and 20°C). At day 8, 10 g of ground beef were taken from each sample and mixed with 100 mL of distilled water in flasks and incubated in a shaking incubator (150 rpm) at 25°C for 4 h. The meat exudates were centrifuged (17,000 g) at 4°C for 10 min. The supernatants were filtered (0.25 μm, Sartorius, Germany) and used as crude protease solutions. Three mL of 0.6% (w/v) casein solution (pH 7.0; C4765-10ML, Sigma, USA) was mixed with 1 mL of crude protease solution and incubated at 30°C for 10 min. The reaction was stopped by the addition of 5 mL of 0.4 M trichloroacetic acid (TCA; T4885-500G, Sigma, USA) at 30°C for 30 min. The precipitate was filtered (Whatman No. 1, ADVANTEC, Japan) and 2 mL of the filtrate was neutralized with 5 mL of 0.4 M sodium carbonate and incubated with 1 mL of 1 N Folin-Ciocalteu's reagent (Sigma, USA) at 30°C for 30 min. The absorbance at 660 nm was measured using a spectrophotometer (Smart Plus SP-1900PC,

Table 1. Antilisterial activity of bacteriocin SH01 in ground beef at different concentrations and temperatures at day 8

Bacteriocin concentration	Temperature (°C)	Number of *L. monocytogenes* (Log CFU/mL)		
		Control[a]	Bacteriocin SH01[b]	a-b[2)]
500 AU/g[1)]	5	6.34±0.07	5.84±0.10	0.5
	10	6.31±0.23	5.34±0.03	0.97
	15	6.21±0.10	5.19±0.01	1.02
	20	5.94±0.07	4.87±0.17	1.07
1,000 AU/g	5	5.24±0.04	4.71±0.20	0.53
	10	5.66±0.23	4.51±0.20	1.15
	15	5.16±0.18	3.54±0.24	1.62
	20	4.31±0.44	1.98±0.23	2.33

[1)]AU/g represents the reciprocal of the highest inhibitory dilution in the two-fold dilution assay.
[2)]Log reduction of the number of *L. monocytogenes* ATCC 19111.

Woongki Science, Korea). Protease analyses were performed in triplicate. One unit of activity was defined as the amount of enzyme that liberated 1 μM of tyrosine per mL of reaction mixture per min.

Sensitivity/Endurance of bacteriocin in crude protease solution

The crude protease solution (25 μL), obtained as described above, was mixed with 25 μL of crude bacteriocin (1,280 AU/mL) and incubated at 5, 10, 15, and 20°C for 1 h. The residual bacteriocin titer was measured by a two-fold dilution method using the agar well diffusion assay, as described above.

Statistical analysis

Microbiological and protease analyses were performed in triplicate. Data were subjected to analysis for significant differences between individual treatment groups using an SPSS 20 statistical package (SPSS Ltd., UK). Microbiological data and protease activity data were analyzed by ANOVA for identification of mean differences. A probability level of $p<0.05$ was used in determining the statistical significance of all experimental data.

Results and Discussion

Antilisterial activity at different bacteriocin concentrations and temperatures

The initial numbers of *L. monocytogenes* found in all ground beef samples were not significantly different ($p>0.05$). Addition of bacteriocin SH01 at 500 and 1,000 AU/g significantly decreased ($p<0.05$) the number of *L. monocytogenes* from the samples incubated at all temperatures examined. The antilisterial activity was higher in samples containing 1,000 AU/g bacteriocin compared to the 500 AU/g samples. Several researchers also found that the degree of inhibition of *L. monocytogenes* increased with increasing concentrations of nisin (500 to 1,000 IU/g) and lactocin 705 (500 to 1,000 AU/g) (Pawar *et al*., 2000; Solomakos *et al.*, 2008; Vignolo *et al*., 1996).

Antilisterial activity increased as the incubation temperature increased. Remarkably, a maximum 2.33 log reduction of *L. monocytogenes* was obtained from samples containing 1,000 AU/g bacteriocin incubated at 20°C. Similar phenomena were reported by Abee *et al*. (1994) and Thomas and Wimpenny (1996) that the action of nisin against *L. monocytogenes* was drastically reduced at decreased temperatures. We haven't examined the fatty acid composition of indicator cell at different temperatures, but several researchers (Castellano *et al*., 2001; Mazzotta and Montville, 1997; Singh *et al*., 2001; Vignolo *et al*., 2000) also have reported that a change in membrane fatty acid composition (unsaturated versus saturated) and phospholipid content led to a decrease in membrane fluidity, as well as the inability of bacteriocin SH01 to penetrate more rigid membranes at lower temperatures.

Protease versus bacteriocin activities at different temperatures

The protease activities of samples gradually increased as the temperature increased (Table 2). The protease activity of the samples stored at refrigeration temperatures was rather low, while the protease activity of the samples stored at 15 and 20°C was relatively high. No significant differences ($p>0.05$) in the samples stored at 5 and 10°C were found, while protease activity of the samples at 15 and 20°C were significantly higher ($p<0.05$) compared to the samples stored at 5 and 10°C.

Bacteriocin SH01 is a small peptide with a molecular weight of approximately 3 kDa (Seo *et. al*., 2014). We predicted that it could be partially inactivated in the presence of intrinsic meat protease activity. After incubation

Table 2. Intrinsic protease activity of ground beef at different temperatures at day 8

Temperature (°C)	Protease Activity (units/mL)[1)]
5	13.716±1.00
10	14.088±0.45
15	19.902±2.51
20	25.855±1.70

[1)]One unit of activity was defined as the amount of enzyme that liberated 1 μM of tyrosine per mL of reaction mixture per min.

Table 3. Residual bacteriocin titer after protease treatment at different temperatures

Temperature (°C)	Bacteriocin titer (AU/mL)[1)]
5	640
10	640
15	640
20	640

[1)]Crude protease solution was incubated with 1,280 AU/mL bacteriocin solution for 1 h at different temperatures and assayed by two-fold dilution against *L. monocytogenes* ATCC 19111.

of bacteriocin SH01 with crude protease solution at different temperatures, the activities of all of the samples decreased by one half (Table 3). It appears that the antilisterial activity of bacteriocin SH01 was partially affected by intrinsic protease activity present in ground beef.

In conclusion, the antilisterial activity of bacteriocin SH01 increased in a dose-dependent manner. Intrinsic protease activity in ground beef increased as the temperature increased. Approximately 50% of the bacteriocin SH01 activity was lost by intrinsic protease activity. However, maximum antilisterial activity (2.33 log reduction) was obtained at 20°C. The temperature abuse in a refrigerator can easily increase internal temperatures up to 20°C, which could cause an outbreak of listeriosis. Based on these properties, bacteriocin SH01 can be used as an effective antilisterial food preservative in ground beef if it is safe for human consumption.

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