Japan Journal of Food Engineering, Vol. 10, No. 1, pp. 45 – 53, Mar. 2009

◇◇◇ Original Paper ◇◇◇

Growth-Inhibition of *Lactobacillus hilgardii*, a Bacterium Related to Hiochi, in the Mizu-Koji Process by Bacteriocins from Lactic Acid Bacteria

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The bacteriocins produced by *Lactococcus lactis* subsp. *lactis* C101910 (C101910) and NBRC 12007 (NBRC 12007) were used to prevent the growth of *Lactobacillus hilgardii*, a hiochi bacterium, at the mizu-koji stage in the sake brewing process. The bacteriocins produced by C101910 and NBRC 12007 were slowly inactivated in the koji extract by proteases, depending on the pH and temperature of the koji extract. However, the activities of bacteriocins from C101910 and NBRC 12007 remained about 50% and 70% of the initial values, respectively, even after incubation at pH 3 and 10℃ for 12 h. By adding the bacteriocin solutions, prepared according to the method reported previously, from C101910 and NBRC 12007 to koji extract (pH 3 and 10℃) at a volume ratio of 5% (v/v) and 1% (v/v), respectively, the number of viable *L. hilgardii* cells decreased to below the detection limit (1.0×10^2 cells/ml) from the initial concentration (3.2×10^5 cells/ml) within 12 h. In mizu-koji consisting of rice koji and lactic acid solution at pH 3 and 10℃, the growth-inhibitory effect of bacteriocins from C101910 and NBRC 12007 on *L. hilgardii* decreased as compared with that in koji extract. However, the number of viable *L. hilgardii* cells decreased by more than two orders of magnitude as compared with the initial value (1.0×10^5 cells/ml) by adding the bacteriocin solution at a volume ratio of 10% (v/v).
Key words: bacteriocin, hiochi, *Lactobacillus hilgardii*, koji extract, mizu-koji

1. Introduction

Spoilage of sake, called hiochi in Japanese, is caused by several *Lactobacillus* species (hiochi bacteria). Because hiochi bacteria are alcoholophilic and alcohol-tolerant, they can grow in sake with a high concentration of ethanol. The growth of hiochi bacteria seriously deteriorates the quality of sake by lowering the pH, inducing the occurrence of turbidity, and by the production of off-flavors [1, 2]. Koji is frequently contaminated with hiochi bacteria. To prevent the growth of hiochi bacteria in the sake making process, lactic acid is initially added to seed mash (moto) and fermentation is carried out at a low temperature [3]. The endogenous acidity and the low temperature are effective in preventing outbreaks of hiochi bacteria, but it is impossible to completely suppress the growth of hiochi bacteria. To avoid the spoilage of sake caused by hiochi bacteria during storage and distribution, pasteurization is carried out at about 65℃ for a short time (2–3 min) or a microfiltration device is used to remove hiochi bacteria. However, due to incomplete inactivation and insufficient removal of hiochi bacteria, they frequently deteriorate the quality of sake, especially in non-pasteurized (fresh) sake.

In the previous study, by determining the partial 16S rRNA gene sequences of the predominant spoilage bacteria isolated from deteriorated sake using SI medium ([4], commercial standard medium for detecting hiochi bacteria, Brewing Society of Japan, Tokyo) with 10% ethanol, it was possible to identify the major species of *Lactobacillus* that cause hiochi in Niigata Prefecture as *Lactobacillus fructivorans*, *Lactobacillus paracasei*, and *Lactobacillus hilgardii* [5]. To prevent hiochi from occurring in sake brewing, the exploitation of bacteriocins with antimicrobial activity against the strains of *Lactobacillus* described above was considered [6]. Recently, it was reported that some lactic acid bacteria produce bacteriocin in koji

(Received 15 Nov. 2008: accepted 22 Dec. 2008)
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extract medium supplemented with rice protein hydrolyzate (RPH), which can be legally used in the sake brewing process [7], and that the culture supernatant containing the bacteriocin was found to be effective in inhibiting the growth of *L. hilgardii* in MRS medium [5].

This study examined the application of bacteriocins in the sake brewing process, and investigated not only the influence of proteases from koji on the antimicrobial activity of bacteriocins but also the stability of growth-inhibitory activity against *L. hilgardii* in koji extract under different incubation conditions. To apply bacteriocins to the stage for making seed mash (moto) in the sake brewing process, the growth-inhibitory activity of bacteriocins against the hiochi bacterium in mizu-koji was also examined.

2. Materials and Methods

2.1 Microorganisms

Lactococcus lactis subsp. *lactis* C101910 (C101910), a nisin Z producer, and *Lactococcus lactis* subsp. *lactis* NBRC 12007 (NBRC 12007), a nisin A producer, were used as bacteriocin-producing bacteria. C101910 was isolated from lake water by the authors [8]. *L. hilgardii* NBRC 15886^{T} was used as an indicator microorganism. *L. lactis* subsp. *lactis* NBRC 12007 and *L. hilgardii* NBRC 15886^{T} were purchased from NITE (National Institute of Technology and Evaluation) Biological Research Center. The bacterial strains were stored at −80℃ in 25% glycerol.

2.2 Bacteriocin production

Bacteriocin production was carried out in a medium bottle using 10% (v/v) koji extract medium supplemented with RPH. The koji extract solution used as the medium for bacteriocin production and RPH (Shimada Kagaku, Nagaoka) were prepared as described previously [7]. Bacteriocin-producing lactic acid bacteria were cultured statically at 30℃ for 24 h at an initial turbidity of 0.1–0.2 at 660 nm. The supernatant was prepared by centrifugation (21,000×*g* for 10 min) of the culture broth obtained after cultivation of bacteriocin-producing lactic acid bacteria and used as a bacteriocin solution as described previously [5]. The activities of the bacteriocin solutions were 3.5×10^{3} U/ml for C101910 and 2.8×10^{3} U/ml for NBRC 12007. The bacteriocin solutions contained less than 10 g/l lactic acid, 10–14 g/l total sugar, and 3–4 g/l glucose, and the pH of bacteriocin solutions was 4–5.

2.3 Determination of antimicrobial activity

The antimicrobial activity of bacteriocin was determined by the agar diffusion method using a stainless cup according to the procedure reported previously [7]. The agar concentration of each medium was adjusted to 1%. Commercial nisin A (Sigma Chemical Co., St. Louis, USA) was used as a standard. The antimicrobial activity was determined by measuring the diameter of the clear growth-inhibitory zone around the cup. One unit of antimicrobial activity was defined as the amount of bacteriocin showing a growth-inhibitory zone diameter equal to that obtained using 1 ng of pure commercial nisin A standard solution as reported previously [7].

2.4 Inactivation of bacteriocin in koji extract

Koji extract was prepared according to the official method of the National Tax Agency Japan [i] as described below. Fifty mM acetate buffer solution (110 ml, pH 5.0) was added to 30 g of rice koji and then the suspension was allowed to stand at 4℃ overnight. After removing the precipitate by centrifugation (21,000×*g* for 10 min), the resultant supernatant was sterilized by filtration using a membrane with a pore size of 0.20 μm (Dismic 25CS020AS, Advantec Toyo Co., Tokyo). The sterile filtrate obtained was used as koji extract. The koji extract was analyzed for activity of acid protease as described below. The pH and temperature of the koji extract were adjusted to pH 3, 4, and 5 by adding 4 N HCl solution, and 10℃, 20℃, and 30℃, respectively. The bacteriocin solution was added to the koji extract under the different conditions and incubated for 48 h. Aliquots of the mixture were withdrawn at intervals and then heated at 80℃ for 20 min. After the heat treatment, the residual activities of enzymes in koji extract were inactivated, but the bacteriocin activities did not decrease due to their high thermal stability [9–13]. The residual activity of the bacteriocins was determined by the agar diffusion method described above.

2.5 Growth-inhibitory activity of bacteriocin

To examine the influence of pH and temperature on the growth-inhibitory activity of bacteriocin, viable cells of *L. hilgardii* NBRC 15886^{T} (2.5–3.8×10^{5} cells/ml) were incubated for 6 h in koji extract containing a bacteriocin solution at a volume ratio of 1% (v/v). Koji extract was prepared as described above and used without further treatment . The pH and temperature of the koji extract

were adjusted to pH 3, 4, and 5 by adding 4 N HCl solution, and 10℃, 20℃, and 30℃, respectively. To evaluate the effect of bacteriocin concentration on the growth-inhibitory activity, viable cells of *L. hilgardii* NBRC 15886^T (1.0–3.2×10^5 cells/ml) were incubated for 48 h in koji extract containing a bacteriocin solution or mizu-koji containing a bacteriocin solution at a volume ratio of 0.5% to 10% (v/v). Mizu-koji consisted of 3 g of rice koji, 77 μl of lactic acid, and 11 ml of distilled water [14]. The bacteriocin solution was prepared and sterilized by filtration as described above prior to addition to the koji extract and mizu-koji. The inhibitory effect of each bacteriocin solution on the cell growth of the indicator microorganism was evaluated by measuring the concentration of viable cells as described below.

2.6 Adsorption of bacteriocin and *L. hilgardii* cells to koji

In the adsorption experiment, both intact koji and koji without enzymatic activities were used. The latter was prepared by heating at 90℃ for 3 h. The bacteriocin solution was added to mizu-koji at a volume ratio of 1% (v/v) and the mixture was allowed to stand at pH 3 and 10℃ for 1 h. After the incubation, the residual koji was separated as a precipitate by centrifugation (21,000×*g* for 10 min), and the resultant supernatant was analyzed for bacteriocin activity by the agar diffusion assay. In the same manner, the degree of adsorption of *L. hilgardii* cells (initial viable cell number: 1.5–2.0×10^5 cells/ml) to koji was estimated by measuring the viable cells in the supernatant as described below.

2.7 Other analytical methods

The activity of acid protease in koji extract was measured by the official method of the National Tax Agency Japan using casein as a substrate [i]. Protein concentration was determined by the method of Lowry *et al.* [15] using bovine serum albumin as a standard. The number of viable cells was counted by the plate culture method using SI or MRS [16] agar medium containing 20 g/l glucose. When *L. hilgardii* was inoculated into mizu-koji, to completely recover the cells the suspended sample for measuring the number of viable cells was prepared by thoroughly crushing koji particles in a sterile pouch bag using a wood hammer. The viable cell concentration was expressed as colony forming units per milliliter (CFU/ml).

3. Results

3.1 Inactivation of bacteriocin in koji extract

It is well known that bacteriocin is a proteinaceous substance that is hydrolyzed by proteases [9–11]. Figure 1 shows the inactivation of bacteriocins produced by C101910 and NBRC 12007 in intact koji extract. The activity of acid protease in koji extract was confirmed by measuring at pH 3 and 40℃ in accordance with the official method. The enzymatic activity was 1.45×10^3 U/g-koji, which almost corresponds to that of koji used in the sake making process [i]. The residual activity of bacteriocin after incubation of the mixture under different conditions was determined by the agar diffusion method. Both kinds of bacteriocin were very stable in buffer solution without koji extract containing proteases under every condition tested (data not shown). As shown in Figs. 1A and 1C, the influence of temperature was examined at 10℃, 20℃, and 30℃ when the pH was maintained at 3. As shown in Figs. 1B and 1D, the influence of pH was examined at pH 3, 4, and 5 when the temperature was kept at 10℃. The activities of bacteriocins from C101910 and NBRC 12007 significantly depended upon both the temperature and the pH of koji extract. Little or no activity of bacteriocins from C101910 and NBRC 12007 was observed within 12 h at pH 3 and 30℃. As shown in Figs. 1A and 1C, the inactivation rate slowed with lower incubation temperature. The inactivation rate gradually increased with elevating the pH value at 10℃. The conditions at pH 3 and 10℃ are usually used for preparation of seed mash [14]. When the mixture was incubated at pH 3 and 10℃ for 12 h, the activities of bacteriocins from C101910 and NBRC 12007 remained about 50% and 70% of the initial values, respectively. Under these conditions, the activities of both bacteriocins were detected at more than 10% of the initial value even after an incubation time of 48 h.

3.2 Growth-inhibitory activity of bacteriocin in koji extract under different conditions

Table 1 shows the growth-inhibitory activity of bacteriocin solution under different conditions of pH and temperature. When *L. hilgardii* was inoculated into the koji extract without bacteriocin solution, the number of viable cells was maintained at 2.5–3.8×10^5 cells/ml for 6 h, which almost corresponded to the initial value. However, by adding bacteriocin solution to the koji extract at a vol-

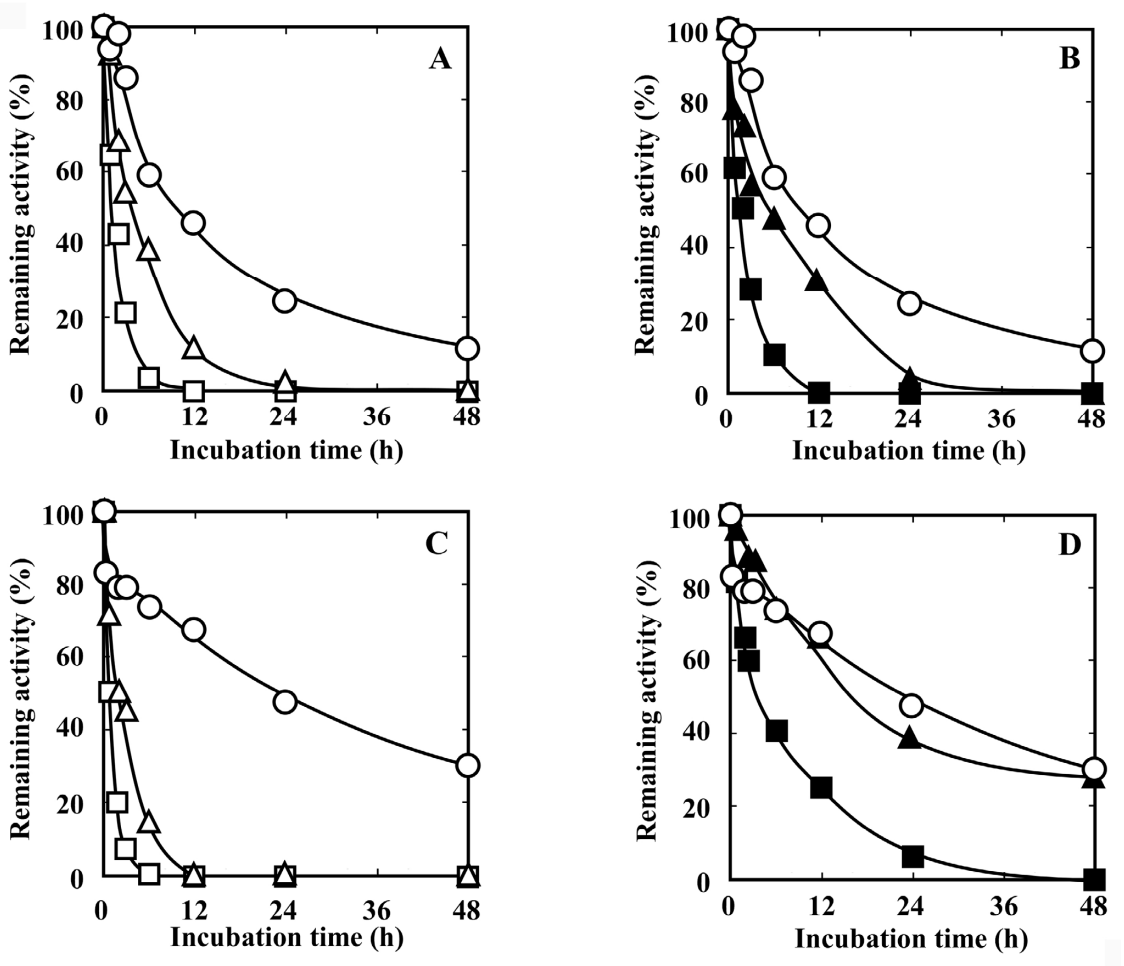

Fig. 1 Time courses of inactivation of bacteriocins produced by *L. lactis* subsp. *lactis* C101910 (A, B) and *L. lactis* subsp. *lactis* NBRC 12007 (C, D) in koji extract. Influences of temperature (A, C) and pH (B, D) of koji extract are shown. Symbols: open circles, pH 3.0 at 10℃; open triangles, pH 3.0 at 20℃; open squares, pH 3.0 at 30℃; closed triangles, pH 4.0 at 10℃; closed squares, pH 5.0 at 10℃.

Table 1 Influence of pH and temperature on antimicrobial activity of bacteriocin solution.

pH	Temperature (℃)	Viable cells (CFU/ml)[1]		
		Control[2]	C101910	NBRC 12007
5	10	$3.4\pm0.52\times10^5$ (100 ± 15)[3]	$1.2\pm0.12\times10^5$ (34 ± 3.4)[3]	$4.4\pm3.3\times10^4$ (13 ± 9.6)[3]
4	10	$3.2\pm0.31\times10^5$ (100 ± 9.7)	$4.3\pm0.88\times10^4$ (13 ± 2.7)	$2.9\pm2.5\times10^3$ (0.91 ± 0.78)
3	10	$3.3\pm0.39\times10^5$ (100 ± 12)	$1.3\pm0.33\times10^4$ (4.0 ± 1.0)	$<10^2$ (<0.030)
3	20	$3.1\pm0.73\times10^5$ (100 ± 24)	$9.0\pm2.9\times10^2$ (0.29 ± 0.10)	$<10^2$ (<0.032)
3	30	$2.8\pm0.70\times10^5$ (100 ± 25)	$<10^2$ (<0.036)	$<10^2$ (<0.036)

[1] The data indicate the number of viable cells at 6 h after the addition of bacteriocin solution from C101910 or NBRC 12007 was added to the koji extract medium under different conditions. The values (±standard deviations) present averages of triplicate determinations.

[2] In the control experiment, no bacteriocin was added to the medium.

[3] The values in parentheses are the percentages of the control experiment for each condition.

ume ratio of 1% (v/v), the concentration of viable cells decreased depending upon the pH and temperature of the koji extract. At 10℃, the concentration of surviving cells increased as the pH value increased. On the other hand, increasing incubation temperature resulted in a significant increase in growth-inhibitory activity at pH 3 against the hiochi bacterium. Consequently, by adding the bacteriocins from C101910 and NBRC 12007 to koji extract at pH 3 and 30℃, the number of viable cells decreased below the detection limit (1.0×10^2 cells/ml)

on agar medium. Under all conditions tested, the activity of bacteriocin (nisin A) from NBRC 12007 was higher than that of bacteriocin (nisin Z) from C101910. In the koji extract with nisin A at pH 3, no or very few viable cells were detected, irrespective of the temperature. The results suggest that the bacteriocins exhibited a rapid sterilizing action against the cells of *L. hilgardii* prior to complete inactivation by proteases in koji extract.

3.3 Growth-inhibitory activity of bacteriocin in koji extract

Figure 2 shows the effect of bacteriocin concentration in koji extract on the growth-inhibitory activity against *L. hilgardii*. In the koji extract without bacteriocin, the number of viable *L. hilgardii* cells was maintained at a constant level for 48 h, suggesting that *L. hilgardii* could neither grow nor was killed at pH 3 and 10℃. Although the bacteriocin might be hydrolyzed and partially inactivated by proteases extracted from koji, as shown in Fig. 1, the viable cell number of the hiochi bacterium reduced with the increasing concentration of bacteriocin. When the ratio of bacteriocin solution from C101910 was 1% (v/v), the viable cell number at 48 h decreased by a factor of about 150 as compared with the initial cell concentration. Furthermore, when the ratio was elevated to 5% (v/v), the viable cell number reduced to below the detection limit (1.0×10^2 cells/ml) within 12 h. The activity of bacteriocin produced by NBRC 12007 was higher than from C101910. The number of viable *L. hilgardii* cells decreased rapidly by more than three orders of magnitude within 1 h as compared with the initial value. The results showed that the bacteriocins possessed sufficient growth-inhibitory activity to sterilize the hiochi bacterium even in koji extract containing proteases at pH 3 and 10℃.

3.4 Growth-inhibitory activity of bacteriocin in mizu-koji

In practical sake brewing processes, koji is mixed with water, and the mixture is allowed to stand at 10-12℃ for 1-2 h to fully extract enzymes from koji prior to the addition of steamed rice and yeast cells. It was postulated that hiochi bacteria may be killed by the addition of bacteriocin at the stage for making mizu-koji; therefore, the growth-inhibitory activity of bacteriocins against *L. hilgardii* was evaluated in preparation of mizu-koji. Figure 3 shows the growth-inhibitory activity of bacteriocin against *L. hilgardii* in mizu-koji. In the preliminary experiment, it was confirmed that almost all of the viable cells added to mizu-koji were recovered by the method described in the Materials and Methods. Judging from the negligible change in the viable cell number, *L. hilgardii* could not grow in mizu-koji even for an incubation of 48 h, in a similar manner to the experiment using koji extract. When the growth-inhibitory activity of the bacteriocins from C101910 and NBRC 12007 was compared at the same concentration, the growth-inhibitory activity observed in koji extract (Fig. 2) was higher than that

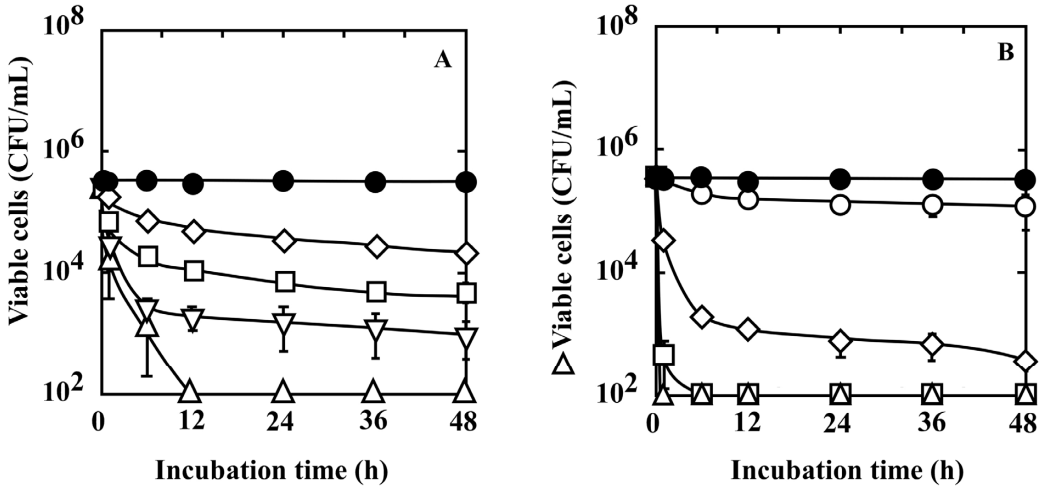

Fig. 2 Effect of bacteriocin concentration on growth-inhibition of *L. hilgardii* NBRC 15886^T in koji extract. The bacteriocin solution from *L. lactis* subsp. *lactis* C101910 (A) or *L. lactis* subsp. *lactis* NBRC 12007 (B) was added to koji extract at pH 3.0 and 10℃. Symbols: closed circles, 0% (control); open circles, 0.1% (v/v); open diamonds, 0.5% (v/v); open squares, 1% (v/v); open reversed triangles, 2% (v/v); open triangles, 5% (v/v). The date present the mean values (n=3). The error bars indicate the standard deviations but are hidden behind the symbols when the standard deviations are very small.

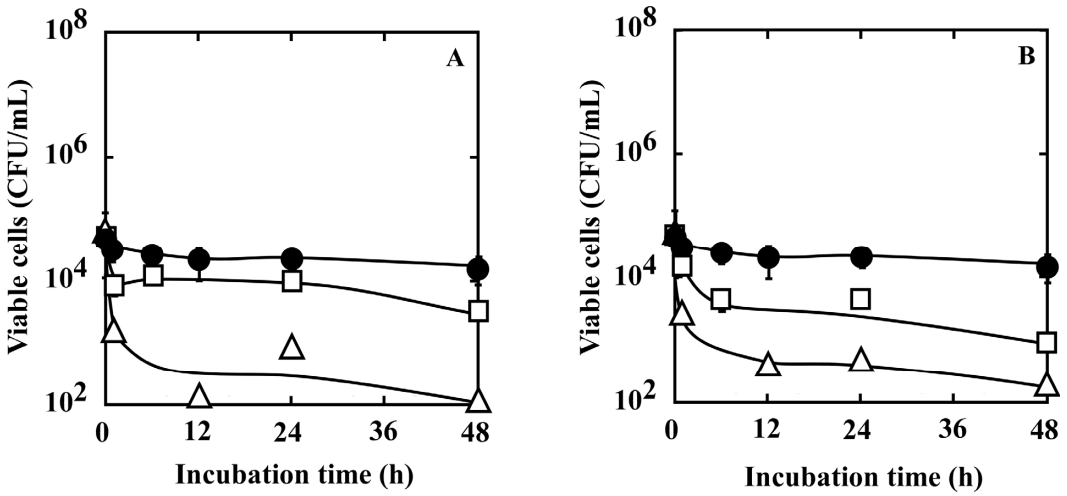

Fig. 3 Effect of bacteriocin concentration on growth-inhibition of *L. hilgardii* NBRC 15886^T in mizu-koji. The bacteriocin solution from *L. lactis* subsp. *lactis* C101910 (A) or *L. lactis* subsp. *lactis* NBRC 12007 (B) was added to mizu-koji at pH 3.0 and 10℃. Symbols: closed circles, 0% (control); open squares, 1% (v/v); open triangles, 10% (v/v). The data represent mean values (n=2). The error bars indicate the standard deviations but are hidden behind the symbols when the standard deviations are very small.

obtained in mizu-koji. When the ratio of bacteriocin solution from C101910 was 1% (v/v), the viable cell number at 48 h decreased to one-half of the initial cell concentration. Furthermore, in the case of elevating the concentration to 10% (v/v), the viable cell number decreased to near the detection limit (1.0×10^2 cells/ml). In the experiment using bacteriocin solution from NBRC 12007 at a concentration of 1% (v/v), the number of viable cells decreased by approximately two orders of magnitude as compared with the initial value. Moreover, the viable cell number reduced to near the detection limit (1.0×10^2 cells/ml) when the bacteriocin solution was added at a volume ratio of 10% (v/v).

3.5 Adsorption of bacteriocins and *L. hilgardii* cells to rice koji

To clarify the reason why the growth-inhibitory effect of bacteriocins on *L. hilgardii* decreased in mizu-koji as compared with koji extract, the degree of adsorption of *L. hilgardii* cells, as well as bacteriocin, to intact and heat-treated koji particles was investigated at pH 3.0 and 10℃. Figure 4 shows the amounts of adsorption of bacteriocins and *L. hilgardii* cells to two kinds of rice koji. A fraction (35-40%) of the total bacteriocin activity was adsorbed to intact koji after 1 h. The residual activity of bacteriocins in the supernatant obtained after mixing with intact koji was slightly lower than that after mixing with heat-treated koji. This result seems to be mainly because of the slight inactivation of bacteriocin by proteases in intact koji as shown in Fig. 1. On the other hand, about 77% and 75% of *L. hilgardii* cells were adsorbed to intact koji and heat-treated koji, respectively.

4. Discussion

Many studies have reported that bacteriocins are readily hydrolyzed by several proteases such as trypsin, pancreatin, and α-chymotrypsin, under the optimum conditions for each protease [9-12]. In this study, the stability of bacteriocins produced by C101910 and NBRC 12007 in koji extract was investigated, because rice koji is known to include highly active proteases. Although the activity of acid protease in koji extract was high at pH 3 and 40℃, the enzymatic activity was significantly affected by the pH and temperature of the reaction mixture. Under standard conditions for the preparation of mizu-koji, namely pH 3.0 and 10℃, the protease activity was depressed to some extent and consequently the residual activities of bacteriocins from C101910 and NBRC 12007 were found to be more than 60% of the initial value after incubation in koji extract for 6 h (Fig. 1). By adding the bacteriocin solutions to the koji extract at pH 3.0 and 10℃, the concentration of viable cells of *L. hilgardii* decreased as shown in Table 1. The growth-inhibitory activity significantly depended upon the pH and temperature of the koji extract in a similar manner to the antimicrobial activity of bacteriocins as determined by the agar diffusion assay (Fig. 1). When the temperature was kept at 10℃, the

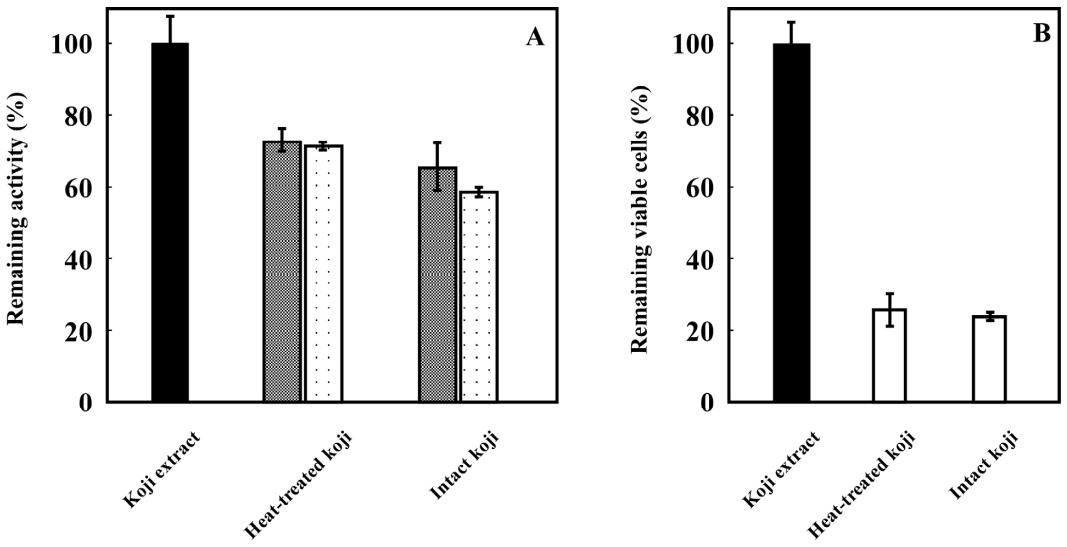

Fig. 4 Adsorption of bacteriocin (A) and *L. hilgardii* cells (B) to koji. The bacteriocin solution from *L. lactis* subsp. *lactis* C101910 (hatched bars) or *L. lactis* subsp. *lactis* NBRC 12007 (dotted bars) and *L. hilgardii* cells (open bars) were added and incubated at pH 3.0 and 10℃ for 1 h. The remaining activity of bacteriocin and the remaining number of viable cells in the supernatants after the incubation are shown. The values when using koji extract are expressed as 100% (solid bars). The data represent mean values (n=3). The error bars indicate the standard deviations.

growth-inhibitory activities of bacteriocins from C101910 and NBRC 12007 at pH 5.0 were much lower than those at pH 3.0. The decrease in the growth-inhibitory activity at pH 5.0 was probably due to the instability of bacteriocins at pH 5 [9, 13, 17, 18] as well as the rapid inactivation of bacteriocins by proteases in koji extract as shown in Figs. 1B and 1D. When the pH was maintained at 3.0, the bacteriocin from NBRC 12007 showed growth-inhibitory activity against *L. hilgardii* at the temperature tested. On the other hand, the growth-inhibitory activity of the bacteriocin from C101910 against *L. hilgardii* was enhanced with increasing temperature. The increase in the growth-inhibitory activity was probably attributable to the activation of bacteriocin by the elevation of temperature [19, 20]. The positive effect of temperature on the sterilizing action of bacteriocin was considered to be larger than the negative effect of inactivation in koji extract at 30℃ by proteases. The positive effect of temperature seems to be caused by not only the increase in the sterilization rate of the bacteriocin but also by changes in the fluidity of bacterial cell membranes [19, 20], although the detailed mechanism remains unclear.

As shown in Table 1, the bacteriocins exhibited a growth-inhibitory effect on *L. hilgardii* in koji extract at pH 3.0 and 10℃, although they were partially inactivated by hydrolysis by proteases from the koji (Fig. 1). As the concentration of bacteriocins increased, the number of viable *L. hilgardii* cells decreased more rapidly. When the volume ratio of the bacteriocin solution added was 5% (v/v) for C101910 and 1% (v/v) for NBRC 12007, the viable cell number decreased below the detection limit (1.0×10^2 cells/ml) within 12 h and 1 h, respectively (Fig. 2). At pH 3.0 and 10℃, the bactericidal action of bacteriocins against the hiochi bacterium seems to be very rapid as compared with the inactivation rate by hydrolysis by proteases from the koji [9, 13, 19]. The growth-inhibitory activity of bacteriocins against *L. hilgardii* decreased in mizu-koji (Fig. 3) as compared with that in koji extract (Fig. 2). The decrease in the growth-inhibitory effect of bacteriocins on *L. hilgardii* in mizu-koji as compared with that in koji extract seemed to be because of the adsorption of *L. hilgardii* cells as well as bacteriocin to koji particles as shown in Fig. 4. That is, the decrease in contact frequency caused by the adsorption of both *L. hilgardii* cells and bacteriocin to koji is probably responsible for the decrease in the growth-inhibitory activity of bacteriocin in mizu-koji. However, the number of viable *L. hilgardii* cells in mizu-koji decreased by more than two orders of magnitude as compared with the initial value by adding the bacteriocin solutions at a higher volume ratio of 10% (v/v).

As described above, the susceptibility to proteolytic degradation and the interaction with components in foods are considered as the important factors in the effi-

ciency of bacteriocins and the required dosage in foods [12]. For example, Aasen *et al.* [21] showed that 40–50% of the total antimicrobial activity of nisin A was lost even after 5 h because of endogenous proteases in salmon that had not been heat-treated. The comparison of antimicrobial efficacy of nisin between in the liquid medium and in a model food system with rice was reported by Jamuna *et al.* [22]. They showed that the growth-inhibitory activity of nisin A against *Listeria monocytogenes* and *Staphylococcus aureus* was lower in a food system than that in liquid medium. Grande *et al.* [23] reported that the bacteriocin concentration required to exhibit the same bactericidal effect against *Bacillus cereus* as that in culture broth was about 5-fold higher in a model food system consisting of boiled rice slurry. In this study, it was found that the addition of bacteriocin solution was effective in reducing the viable cell number of *L. hilgardii* in mizu-koji as well as in koji extract, which both contain proteases. Spoilage of sake is presumed to be frequently caused by contamination of koji with hiochi bacteria. Therefore, the addition of bacteriocin solution at the initial stage for preparing mizu-koji is considered to be potentially important for preventing outbreaks of hiochi bacteria in the sake brewing process because the initial cell concentration of *L. hilgardii* could be reduced to a very low value (nearly 1.0×10^2 cells/ml). However, the present results suggested that the bacteriocin solution necessary to reduce the viable cell number of hiochi bacteria in mizu-koji below the detection limit (1.0×10^2 cells/ml) was more than 10% (v/v) of the total volume of mizu-koji suspension. To alleviate the influence of hiochi bacteria on the quality of sake, further study is necessary to reduce the volume of bacteriocin solution required by producing the higher activities of bacteriocin.

Acknowledgements

We wish to thank Shimada Chemical Industries, Nagaoka, for the kind gift of rice protein preparation. We are grateful to Mr. Mamoru Narita of Taiyo Sake Brewing, Murakami, Japan, for helpful discussions. The study was partially supported by Grant-in-Aids for the Research Development Project from Niigata Industrial Creation Organization (NICO), Feasibility Study Program from the Japan Science and Technology Agency, and a Grant for the Promotion of Niigata University Research Projects.

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「日本食品工学会誌」, Vol. 10, No. 1, p. 54, Mar. 2009

◇◇◇ **和文要約** ◇◇◇

乳酸菌由来バクテリオシンを用いた水麹工程における火落ち関連細菌 *Lactobacillus hilgardii* の増殖阻害

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Lactococcus lactis subsp. *lactis* C101910（C101910）および *Lactococcus lactis* subsp. *lactis* NBRC 12007（NBRC 12007）が生産するバクテリオシンを用いて、清酒製造プロセスの中の水麹工程において、火落菌である *Lactobacillus hilgardii* の増殖を阻害することを検討した。C101910 および NBRC 12007 が生産するバクテリオシンは、pH と温度に依存して、プロテアーゼ活性を有する麹抽出液中でゆっくりと失活した。しかし、C101910 および NBRC 12007 が生産するバクテリオシンの活性は、pH 3、10℃の条件で 12 時間処理した後でも、それぞれ初期の活性の約50%と約70%が残存していた。既に報告した方法に従って調製した C101910 および NBRC 12007 由来のバクテリオシン溶液を、麹抽出液（pH 3、10℃）にそれぞれ 5% (v/v) および 1% (v/v) の割合で添加することによって、*L. hilgardii* の生細胞数は 12 時間以内に、初期に添加した濃度（2.5–3.2×10^5 cells/ml）に比べて検出限界（1.0×10^2 cells/ml）以下まで減少した。米麹と乳酸溶液を含む水麹（pH 3、10℃）中では、C101910 および NBRC 12007 由来のバクテリオシンの *L. hilgardii* に対する増殖阻害活性は、麹抽出液中に比べて低下した。しかし、バクテリオシン溶液の添加割合を 10% にすることによって、*L. hilgardii* の生細胞数は、初期に添加した値（1.0×10^5 cells/ml）に比べて 2 オーダ以上減少した。

（受付 2008 年 11 月 15 日，受理 2008 年 12 月 22 日）

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