A Research Note

Bacteriocin Production by Lactic Acid Bacteria Isolated from Regional Cheeses: Inhibition of Foodborne Pathogens

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ABSTRACT

Four strains of enterococci isolated from Argentina regional cheeses were found to produce bacteriocins that were active against several lactic acid bacteria. Among them, enterocin CRL35 produced by Enterococcus faecium CRL35 was also inhibitory to foodborne pathogens like Listeria monocytogenes and Staphylococcus aureus. These antimicrobial compounds were sensitive to proteases and heat stable; inhibitory activity of enterocin CRL35 showed also to be stable at extreme pHs, heat treatment, and storage in different conditions.

Key Words: Bacteriocin, enterococci, lactic acid bacteria.

Lactic acid bacteria (LAB) play a fundamental role in microbial ecology synthesizing a variety of antimicrobial factors such as organic acids, H$_2$O$_2$, diacetyl, and bacteriocins (8).

Several bacteriocins produced by lactic acid bacteria have been described (11). While most of them have narrow inhibitory spectra, some, like nisin (7), pediocin PA-1 (12) and sakacin A (14), are also active against foodborne pathogens like L. monocytogenes and S. aureus and, thus, have a potential as natural food preservatives.

With the aim to finding antimicrobial substances, a screening of 350 strains isolated from Argentinian regional cheeses was performed. This paper describes the preliminary characterization of four bacteriocins and, especially, an antimicrobial compound produced by E. faecium CRL35 that is inhibitory to foodborne pathogens.

MATERIALS AND METHODS

Strains, media and detection of bacteriocin activity.

The 350 analyzed (CRL strains) strains, originally isolated in CERELA from regional cheeses (Tafi cheeses), belong to the genera Lactococcus, Lactobacillus and Enterococcus (6). All the strains were maintained freeze-dried and stored at 4°C. Before use, cells were subcultured three times in Laptg (13), M17glu (16) and methicillin-resistant Staphylococcus (MRS) broth (3), for enterococci, lactococci and lactobacilli, respectively. Listeria monocytogenes CRL1111 was cultured in Laptg broth (13).

In the search for an indicator and a bacteriocin producer strain, the 350 strains were challenged between themselves in groups of 10. Antagonistic activity was assayed by the well diffusion method (4). Soft agar medium was prepared with reduced concentration of glucose (0.2%).

The activity of the bacteriocin preparation expressed in arbitrary units per milliliter (AU/ml) was calculated with the formula $(1,000/30) 	imes (1/D)$, where 30 is the volume of the sample utilized in microliters, and D is the highest dilution that inhibited the growth of the indicator strain at 18 h of incubation (2).

Crude extract preparations of bacteriocins.

Crude extracts were attained precipitating the producer strains supernatant with different concentrations of $(\text{NH}_4)_2\text{SO}_4$; resuspending the pellet in $1/100$ original volume with buffer phosphate, 50 mM; pH 7; and dialyzing overnight in the same buffer in benzoylated tubing (Sigma Chemical Co., St. Louis, MO).

Effect of enzymes on the antimicrobial activity.

The enzymes trypsin, protease type IV, alpha-chemotrypsin, pronase E and catalase were obtained from Sigma and used as described by Parente and Hill (9).

Effect of pH and temperature on the antimicrobial activity.

The heat resistance of the different bacteriocins was tested by heating the supernatants at 80, 90 and 100°C for 20 min. After cooling to room temperature (c.a. 22°C), the extracts were tested for antimicrobial activity.

The activity at different pHs was assayed using the filtered supernatants through a 0.45 μm filter. These were adjusted to pH 2, with hydrochloric acid (HCl), and to pH 7 and 10, with sodium hydroxide (NaOH), incubated for 4 h at room temperature, and then tested for antimicrobial activity.

Mode of action and production of enterocin CRL35.

The mode of action of enterocin CRL35 was determined as described by Parente and Hill (9).

To study bacteriocin production as a function of time, an overnight culture of E. faecium CRL 35 was washed in buffer phosphate, 50 mM; pH 7 and 0.1 ml was inoculated in 5 ml of Laptg broth and incubated at 37°C. At 1-h intervals, optical density was measured (560 nm) and cell-free supernatant fluids were used to determine their inhibitory activity against Lactobacillus plantarum CRL 98.
RESULTS AND DISCUSSION

From a screening of 350 strains isolated from regional cheeses (Tafí cheeses), 25 strains produced antimicrobial activities. However, only four strains were chosen for further studies since their antimicrobial activity showed to be insensitive to catalase, remain active after neutralization at pH 7, sensitive to protease, nondialyzable and inhibitory to several LAB. The selected strains were *E. faecium* CRL35, *Enterococcus faecalis* CRL268 and CRL291 and *Enterococcus* sp. CRL504. The antimicrobial substances were sensitive to alpha-chymotrypsin, pronase E and protease type IV and resistant to trypsin, with the sole exception of the CRL504 supernatant that was sensitive to all of them. These results indicate that the inhibitory substances produced by CRL35, CRL291, CRL268 and CRL504 are proteinaceous and could be considered bacteriocins. Therefore, the corresponding bacteriocins were called enterocins CRL35, CRL268, CRL291 and CRL504, respectively.

The effect of pH and different temperatures on the stability of the enterocins was tested. All the bacteriocins were stable for 20 min at 80°C. Enterocins CRL268, 291 and 35 were stable for 10 min at 100°C, and only enterocins CRL 291 and 35 were stable for 10 min at 120°C. Changes in pH had no effect on bacteriocin stability, since they were not inactivated after 4 h at pH 2, 7 and 10.

Inhibition of foodborne pathogens by LAB bacteriocins has been already reported (5). In our studies, only enterocin CRL35 was found to be active against the foodborne pathogens *S. aureus* and *L. monocytogenes*. Enterocin CRL35 has a comparable profile of sensitivity to proteolytic enzymes to the one described for nisin. However, *E. faecium* CRL35 was sensitive to nisin, and *L. lactis* American Type Culture Collection (ATCC) 11454, the nisin producer strain, was resistant to enterocin CRL35.

To determine whether enterocin CRL35 had either a bactericidal or a bacteriostatic mode of action, the viability of *L. monocytogenes* CRL1111 was monitored in the presence of the CRL35 crude extract containing 800 AU/ml. Even though the turbidity was maintained unchanged, the number of viable cells (CFU) declined after incubation for 5 h indicating that enterocin CRL35 has a bactericidal mode of action against *L. monocytogenes* (Fig. 1, 2).

Besides the characteristics mentioned above, it was found that enterocin CRL35 precipitates with 60% (NH₄)₂SO₄ and that it remains active after 60 days at 20°C, 30 days at 4°C and 7 days at room temperature (c.a. 22°C). On the other hand, it is produced at the late period of the log phase of growth (Fig. 3).

Although enterococci have been used as fecal indicator in microbiological food analysis, they have been isolated in environments far removed from fecal contamination. They can be used, indeed, in artisanal cheeses and their use as starter cultures has been proposed for cheddar cheese (15), fontina cheese (1) and water-buffalo Mozzarella cheese (10).

Since the bacteriocins characterized in this paper, and especially enterocin CRL35, may be of technological value, further biochemical and genetic studies are being carried out to further analyze these antimicrobial compounds.

Figure 1. Effect of enterocin CRL35 on the growth of *L. monocytogenes* CRL1111. (∙) indicate control growth without bacteriocin, and (△) express growth with enterocin CRL35 added in the point indicated by the arrow.

Figure 2. Kinetics of death of resting cells of *L. monocytogenes* exposed to enterocin CRL35 in 50 mM potassium phosphate buffer, pH 7. (○): control, no bacteriocin added, (*): 800 AU/ml of enterocin CRL35 added.

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