Selected characteristics of a bacteriocin from Lactobacillus sake 449

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SUMMARY: The antagonistic activity of Lactobacillus sake 449, detected with Lactobacillus fermentum CECT285 as the indicator microorganism, was evaluated in the MRS and BHI broths and in a semisynthetic defined medium (SDM) with several supplements. The antagonistic activity was a growth-associated property, being detected and quantified when L. sake 449 was grown at either 8, 16, 25 and 32 ºC. The antagonistic effect of a concentrated culture supernatant of L. sake 449 was active against several lactobacilli, Staphylococci and listeriae, but none of the Gram-negative bacteria tested were inhibited, those included among others, S. typhimurium and Y. enterocolitica. The activity was degraded completely by treatment with papain, protease II, protease XIV, pepsin and trypsin. However, the activity of the concentrated supernatants was resistant to heat, about 70% of the original activity remained after heating for 20 min at 100 ºC. The antagonistic activity was bacteriostatic against the indicator organism rather than bacteriocidal. When the concentrated supernatant was passed through a column of Sephadex G-150 we observed the formation of aggregates.

INTRODUCTION: The lactic acid bacteria have the potential to inhibit the growth of pathogenic and spoilage bacteria and the possibility exists of using them to improve the hygienic quality and to extend the shelf-life of different meat and meat products (RACCACH et al., 1979; RODRIGUEZ et al., 1989; SCHILLINGER and LUCKE, 1989). Reduction of pH and removal of carbohydrates are the primary effects exerted by these bacteria (DAESCHEL, 1989), but they are also capable of producing other inhibitory substances such as hydrogen peroxide, diacetyl, bacteriocins and other secondary metabolites that are antagonistic toward other microorganisms (KLAENHAMMER, 1988; DAESCHEL, 1989). Bacteriocins produced by lactic acid bacteria are interesting to the meat industry for its possible uses as food preservatives, once they have been adequately characterized. We report in this communication some selected characteristics of a bacteriocin produced by L. sake 449, a lactic acid bacteria previously isolated from Spanish dry fermented sausages.

MATERIALS AND METHODS

Microorganisms, microbial growth and cell-free cultures: A Gram-positive rod previously isolated from Spanish dry fermented sausages was identified as Lactobacillus sake 449 as described by SCHILLINGER and LUCKE (1987). The microorganism was grown on MRS (De MAN et al., 1960) broth (Oxoid) or in a semisynthetic mediun (SDM) containing (l^{-1}) in distilled water: yeast extract, 5 g; dextrose, 10 g; di-ammoniumhydrogen citrate, 2 g; NaCl, 2 g; KH_2PO_4, 1 g; $MnSO_4 \cdot 1H_2O$, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 0.01 g and Tween 80, 1 ml, with the final pH adjusted to 6.1 with 1N HCl. The microorganism was also grown in a brain heart infusion broth (BHI, Oxoid) and in the SDM medium with the following supplements: tryptone, tryptose, proteose peptone, casitone and peptone from Difco and the "Lab Lemco" powder from Oxoid. Cells were removed by centrifugation at 1200 g for 10 min. This was followed by neutralization of the supernatant to pH 6.2 with 1N NaOH and filtration through a 0.22 µm pore size filter (Millipore). The culture supernatant was lyophilized and afterwards it was resuspended in 4 mM phosphate buffer, pH 7.0 to a concentration corresponding to twenty-fold the original concentration.

Measurement of the antimicrobial activity: For this assay, sterile Whatman nº 3 filter paper discs of 7 mm diameter containing 0.030 ml of concentrated supernatants were placed on prepoured agar plates overlaid with about 3×10^5 cells of the various microorganisms investigated in 6 ml of soft MRS, APT or BHI agar. Plates were incubated at 32 ºC and the antimicrobial activity of the supernatans was quantified by measuring the diameter of the clear zones of inhibition around the discs. To determine the bacteriostatic or bacteriocidal mode of action of the antagonistic activity of L. sake 449 against the sensitive organism L. fermentum CECT 285, different volumes of a twenty-fold concentrated supernatant of L. sake 449 were added to 5 ml of the indicator microorganism (approx. 1×10^5 cells $\cdot$ ml^{-1}) in MRS. At appropriate intervals, the number of viable cells in the culture tubes was determined by pour plating MRS agar plates and incubating for 2 d at 32 ºC.

In the control tubes the indicator microorganism was tested for the effect of 0.05 ml of a 20-fold concentrated supernatant of L. sake 23, a lactobacilli not displaying a detectable antimicrobial activity.

Sensitivity to heat and proteolytic enzymes: The concentrated culture supernatant from L. sake 449 was heated in glass ampoules (10 x 30 mm) at 100 ºC for 20 min and the remaining activity was determined by the disc diffusion assay. To test its sensitivity to proteases the concentrated supernatant was treated with papain, protease II, protease XIV, trypsin and pepsin, all enzymes from Sigma (U.S.A), each at a final concentration of 1 mg . ml^{-1}. Samples with and without proteases were incubated at 32 ºC for 12 h. Residual activity was determined by the agar diffusion assay. Initial studies showed that none of the enzymes themselves exerted any inhibitory effect against the primary test organism L. fermentum CECT285.

Elution of the antagonistic activity of L. sake 449 by gel filtration: A lyophilized culture supernatant from L. sake 449 was resuspended in a phosphate citrate buffer, pH 5.6 with 1M urea, to a concentration corresponding to twenty-fold of the original concentration and 20 ml of this solution was passed through a column (3.2 x 40 cm) of Sephadex G-150 fine (Pharmacia) previously equilibrated with a phosphate-citrate buffer pH 5.6 containing 0.1M urea. The eluate in fractions of 5 ml each was subjected to absorbance readings at 280 nm to determine its protein content whereas the inhibitory activity of the fractions was evaluated as indicated above using L. fermentum CECT285 as the indicator microorganism.

RESULTS AND DISCUSSION

Lactobacillus sake 449, a lactic acid bacteria previously isolated from Spanish dry fermented sausages was tested for its antagonistic activity against L. fermentum CECT285 since previous experiments indicated its sensitivity towards antimicrobial activities of lactic acid bacteria. L. sake 449 was further grown in MRS or in a semisynthetic defined medium (SDM) with several protein supplements, as well as in the brain heart infusion (BHI) broth, either supplemented or not. Results from Table I indicates that the BHI broth, either supplemented or not, result inadequate for the expression of the antagonistic activity of L. sake 449, and suggests the existance of regulatory mechanisms involved in the synthesis of antimicrobial compounds by lactic acid bacteria.

TABLE I. Antimicrobial activity of Lactobacillus sake 449 grown in different media

Culture medium	Specific inhibitory activity ($mm^2 . ml^{-1} . KU^{-1}$)
MRS	38
SDM + Tryptone	31
SDM + Tryptose	36
SDM + Proteose peptone	31
SDM + Casitone	26
SDM + "Lab Lemco" powder	30
SDM + Peptone	33
BHI	Nd
BHI + Tryptone	Nd
BHI + Tryptose	Nd
BHI + "Lab Lemco" powder	Nd
BHI + Peptone	Nd

Nd = No detectable

The MRS or the SDM broth with several supplements result adequate for the expression of the antagonistic activity of L. sake 449. Although few work have been done concerning the effect of different nutrients on the production of antagonistic compounds from lactic acid bacteria (REDDY and RANGANATHAN, 1983; BATISH et al., 1990), the data generated should be interesting to study genetic regulatory mechanisms or to predict the possible inhibition and/or repression of the antimicrobial activity of L. sake 449 in different meat and meat products.

The production of bacteriocins has been reported to occur at various stages in the cell growth cycle (DAESCHEL et al., 1990). In the present study, the antagonistic activity was a growth-associated property, being detected and quantified when L. sake 449 was grown in MRS or the SDM-Tryptose broths at either 8, 16, 25 and 32 ºC. The antagonistic activity was maximum at 32 ºC, being accumulated during the mid log phase of growth and remaining active after 48 h, apparently not being subjected to adverse effects from residual proteolytic activities or conversion to other metabolites.

The concentrated culture supernatant of L. sake 449 was tested on various Gram-positive and Gram-negative bacteria (Table II). The supernatant was active against strains of L. curvatus, Car. divergens, Leu. mesenteroides, List. monocytogenes and Staph. aureus, but none of the Gram-negative bacteria tested were inhibited; these included, among others, the foodborne pathogens S. typhimurium and Y. enterocolitica. It appears that the lactobacilli and the strains of List. monocytogenes are more sensitive than the staphylococci. The sensitivity of listeriae to an antagonistic compound produced by a Lactobacillus species is not surprising as they are closely associated with the genus Lactobacillus (RUHLAND and FIEDLER, 1987).

The addition of a concentrated culture supernatant of L. sake 449 in a freshly inoculated culture of L. fermentum CECT285 resulted in a cessation of its growth; this result indication a bacteriostatic mode of action. This result along with others (RACCACH et al., 1989; AHN and STILES, 1990; SOBRINO et al., 1991), may indicate that the presence in meat and meat products of lactic acid bacteria with a bacteriostatic mode of action may be more common than realized at present.

The antagonistic activity of L. sake 449 was destroyed by treatment with papain, protease II, protease XIV, pepsin and trypsin. However, the activity of the concentrated supernatant was resistant to heat; about 70% of the original activity remained after heating for 20 min at 100 ºC. The antagonistic activity remained during frozen storage.

A concentrated culture supernatant of L. sake 449 was also eluted through a column of Sephadex G-150. Figure 1 shows its elution pattern, protein content and antimicrobial activity, while it seems that the antagonistic compound elutes as an aggregate of a high molecular mass.

TABLE II. Inhibitory spectrum of the antibacterial compound produced by Lactobacillus sake 449

Indicator species	Strain Nº	Origin[a]	Inhibition by the culture supernatant from L. sake 449[b]
Gram-positive bacteria			
Lactobacillus curvatus	Lb726	IMTH	+++
Lactobacillus fermentum	285	CECT	+++
Carnobacterium divergens	LV13	FRIB	+++
Leuconostoc mesenteroides	394	CECT	+++
Listeria monocytogenes	5105	NCTC	+++
Listeria monocytogenes	7973	NCTC	++
Listeria monocytogenes	LI5 sv 1/2	FVM	+++
Staphylococcus aureus	Scott A	FVM	+++
Staphylococcus aureus	137	FRI	++
Staphylococcus aureus	196E	FRI	-
Staphylococcus aureus	349	FRI	+
Staphylococcus aureus	361	FRI	+
Gram-negative bacteria			
Escherichia coli enterotoxigenic	B41	IEKC	-
Salmonella typhi	409	CECT	-
Salmonella typhimurium	T91	CENAN	-
Yersinia enterocolitica	14405	IPP	-

[a] Abbreviations: CECT, Colección Española de Cultivos Tipo (Valencia, Spain), CENAN, Centro Nacional de Alimentación y Nutrición (Madrid, Spain); FRI, Food Research Institute (Madison, USA); FRIB, AFRC Insitute of Food Research (Bristol, UK); FVM, Facultad de Veterinaria (Madrid, Spain); IEKC, International Escherichia and Klebsiella Centre (Copenhagen, DK); IMTH, Institut fur Mikrobiology, Toxicology und Histologie (Kulmbach, FRG);IPP, Institut Pasteur (Paris, France).

[b] Symbols for agar difussion assay: +++, Large inhibition zone (7 mmm); ++, Medium inhibition zone (5-7 mm); +, Small inhibition zone (5 mm); -, No inhibition zone.

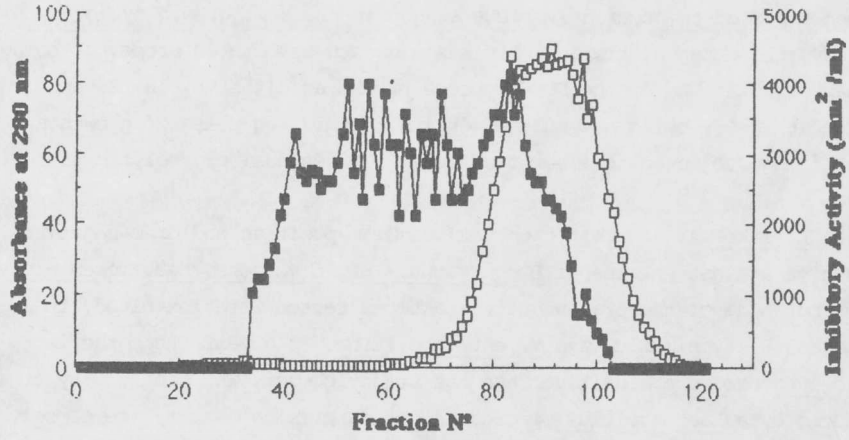

Figure 1. Elution pattern of a concentrated culture supernatant of Lactobacillus sake 449 through a column of Sephadex G-150. Absorbance at 280 nm (□). Inhibitory activity (■).

According to preliminary studies involved in this work, the antibacterial compound produced by L. sake 449 is proteinaceus in nature, heat resistant and bacteriostatic, forming aggregates when synthesized in a semisynthetic defined medium with tryptose. Due to the difficulties encountered in the biochemical purification of bacteriocins to homogeneity (BHUNIA et al., 1988; KLAENHAMMER, 1988; MURIANA and KLAENHAMMER, 1991), experiments are in progress to evaluate the possibilities of these aggregates to induce the synthesis of immunoglobulins that would be tested to purify the antibacterial activity of L. sake 449 by immunoadsorption chromatography.

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