Isolation and characterization of bacteriocins produced by lactic acid bacteria

Susana Ribeiro, Márcia C. Costa, Airidas Dapkevicius, Maria de Lurdes Enes Dapkevicius, Célia C.G. Silva*

CITA-A, Universidade dos Açores, Angra do Heroísmo, Açores, Portugal
* susanaribeiro@uac.pt

Keywords: lactic acid bacteria, cheese, bacteriocins, *Listeria monocytogenes*

ABSTRACT

This study presents the characterization of eight bacteriocins produced by lactic acid bacteria (LAB) previously isolated from a traditional cheese, Pico cheese. All isolates were active against *Listeria monocytogenes* and only three against *Clostridium perfringens.* Bioactivity was studied after treatment with different enzymes, organic solvents, surfactants, temperature, pH and NaCl. The antimicrobial activity of LAB isolates was inactivated by the addition of proteases, thus confirming the proteinaceous nature of active substances. Bacteriocins were found to be heat resistant and remained active at a wide range of pH values. However, bioactivity was sensitive to some organic solvents tested. Surfactants had no influence in the antimicrobial activity with the exception of sodium dodecyl sulfate (SDS). Bacteriocin activity was stable in the presence of 2% NaCl. Bacteriocins revealed a bacteriocidal and bacteriostatic mode of action towards the indicator strain *L. monocytogenes*. Adsorption of bacteriocins to producer cells was not observed. Bacteriocins were further purified by ammonium sulfate precipitation, SDS-PAGE, anionic chromatography and RP-HPLC. Molecular weight was calculated as 3-3,5kDa by SDS-PAGE. Bacteriocins spectrums of activity and interesting properties as heat-resistant, pH tolerance and stability in presence of surfactants make them advantageous for application as biopreservatives in food industry.

1. INTRODUCTION

Consumers have been consistently concerned about possible adverse health effects arising from the presence of chemical additives in their foods. As a result, consumers are drawn to natural foods with no chemical preservatives added. This perception has stimulated research interest in finding natural but effective preservatives. The contribution of LAB to the improvement of food safety and stability of fermented foods has long been known, with their contribution to flavour and aroma development and spoilage retardation [1]. The preservative effect is not only due to acidic conditions that these bacteria create in foods, but also their capacity for producing and excreting inhibitory substances. These processes include hydrogen peroxide, ethanol, diacetyl, carbon dioxide, bacteriocin or antibiotic-like substances [2].

Bacteriocins are ribosomally synthesized proteinaceous compounds active directly against taxonomically related bacteria [3]. Intensive research into the bacteriocins produced by LAB has received considerable attention during recent years for their possible use as preservatives in food, with a resultant reduction in the use of chemical preservatives.
In this study, bacteriocins produced by eight LAB isolates obtained from Pico cheese were first characterized. In addition, bacteriocins were partially purified and estimated their molecular weight.

2. MATERIALS AND METHODS

2.1 Microorganisms

The isolates tested were selected from LAB obtained from Pico cheese. They were identified by the API50 CH system, as *Enterococcus faecalis* (L2B21K3, L3B1K3, L3A21K6, L3A21K7), *Lactobacillus paracasei* (L3A21M1, L3A21M3, L3A21M8) and *Lactococcus lactis* (L3A1M6).

2.2 Bacteriocin activity assays

The inhibitory activity against *Listeria monocytogenes* ATCC 7466, *Listeria innocua* ATCC 33090 and *Clostridium perfringens* ATCC 8357 strains was assayed by an agar well diffusion assay (WDA) with cell-free supernatants (CFS) [4]. To rule out inhibitory effect of lactic acid and/or H2O2, the pH of CFS was adjusted to 6.5 with 1N NaOH, and incubated with catalase from bovine liver (EC 1.11.1.6, 5mg/mL), at 37^{o}C for 1h.

2.2 Effect of enzymes, pH, heat treatment and chemicals on bacteriocins activity

Cell free supernatants (CFS) sensitivity to proteolytic enzymes was investigated by the addition of proteinase K, trypsine, α-chymiotrypsin and α-amylase (Sigma-Aldrich), at final concentration of 1 mg/mL. In order to determine the effect of pH on bacteriocin activity, the pH of CFS was adjusted to pH values ranging from 2-12 using 1 M NaOH or 1 M HCl and incubated for 2 hours at 25°C before neutralizing and performing the agar WDA. Sensitivity of bacteriocins to heat was checked by heating the CFS for 30 min or 60 min at 100°C and 15min at 121°C, after which activity was assessed against *L. monocytogenes*. To test the effect of NaCl concentration on bacteriocin production, LAB isolates were growth in MRS broth at different levels of NaCl and antimicrobial activity was tested by well diffusion assay. The effect of CFS treatments with SDS, Tween 80, Triton X-100, urea and organic solvents (ethanol, methanol, isopropanol, acetone, chloroform, hexane and acetonitrile) on bacteriocin activity was also assessed by performing the agar WDA.

2.3 Adsorption studies and mode of action

Adsorption of the bacteriocins to producer strain cells was studied by using the method described previously by Yang [5]. The sensitivity of *L. monocytogenes* to CFS containing bacteriocins was evaluated according to Pinto *et al* [6].

2.4 Partial purification and molecular size of bacteriocins

The cells were harvested (4500 *g*, 20 min, 4°C) and the peptides precipitated from the CFS with 80% saturated ammonium sulphate, gradually added by slow stirring at 4°C. The mixture was held overnight at 4°C without stirring and then centrifuged (4500 *g* for 30 min, 4°C). The pellet was solubilized with 1 ml of 5 mM phosphate buffer (pH 6.5).

Peptides from two isolates (L2B21K3 and L3A21K6) were purified using a Sep-Pak cartridge micro-column (AccellTM Plus QMA, Waters Millipore). Further purification was performed by reverse-phase liquid chromatography (RP-HPLC). A 20µl sample was injected into a C18 reverse-phase column. Elution was performed at a flow rate 1mL min^{-1} using a gradient from 75% solvent A (0.1% (v/v) acetic acid in MQ water) and 25% solvent B (0.1% acetic acid in 100% acetonitrile) to 5% and 95% of solvents A and B, respectively. The molecular mass of the bacteriocins was determined by tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). This was carried out in parallel using two gels, so that, after electrophoresis at 200 mV for 3 h, one of the gels was stained with EZblue Gel Staining Reagent (Sigma–Aldrich), while the other was used to detect inhibitory activity in a bioassay using *L. monocytogenes* as the indicator strain [7]. A low molecular weight marker (Sigma-Aldrich) with sizes ranging from 1.06 to 26.6 kDa was used. The molecular mass of bacteriocins was estimated from a linear plot of log (Mr) of markers *vs* migration distances.

3. RESULTS AND DISCUSSION

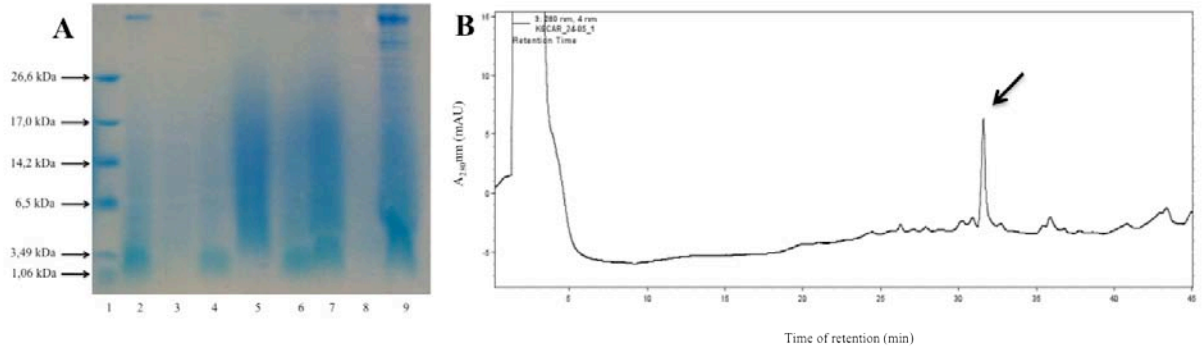
Neutralized CFS from all LAB isolates displayed activity against the indicator strain *Listeria monocytogenes* and *Listeria innocua*, while only three were active against *Clostridium perfringens.* The proteinaceous nature of active substances were confirmed by treatment with proteolytic enzymes, since CFS from all isolates lost the inhibitory activity after treatment with proteinase K, trypsin and α-chymotrypsin, whereas they were not affected by α-amylase. In addition, antimicrobial substances present in neutralized CFS were heat stable, like most bacteriocins. They were stable after treatment for 30 and 60 min at 100ºC and one of them (L3A21M1) maintained antimicrobial activity at autoclave temperature (121ºC for 15 min). CFSs produced by six of the isolates were also stable over a wide pH range, indicating that such bacteriocins may be active in acidic as well as nonacidic foods. Also, antimicrobial activity of CFS was not influence with 2% NaCl. Some isolates were sensitive to 4% NaCl, but three isolates identified as *E. faecalis* (L2B21K3, L3B1K3 and L3A21K6) continue to produce bacteriocins at 10% of NaCl. The sensitivity to detergents, denaturating agents and organic solvents was also evaluated. Most of denaturing agents, with the exception of SDS, did not affect antimicrobial activity of bacteriocins. In contrast, treatments with organic solvents result in decrease or total loss of antimicrobial activity for some isolates.

No activity was detected after treatment of isolates with 100mM at pH 2.0 suggesting that the bacteriocins did not adhere to the producer cells.

Addition of the bacteriocins to *L. monocytogenes* culture at the early logarithmic phase of growth revealed a bacteriostatic and bactericidal mode of action (data not shown).

Following precipitation with 80% ammonium sulphate, the bacteriocins were resolved by SDS-PAGE, and the peptides bands visualized after Comassie Blue staining (Figure 1A). SDS-PAGE gels were also revealed with bioindicator *L. monocytogenes*, and an inhibition zone between 3.0 and 3.5 kDa was detected. This molecular mass is similar to that found by other authors for bacteriocin like substances [1,3]. Bacteriocins produced by isolate L3A21K6 was further purified by anion-exchange chromatography and RP-HPLC. RP-HPLC chromatogram revealed a single pick exhibiting anti-listeria activity (Figure 1B). This purified peptide revealed an anionic charge uncommon to the bacteriocins described in literature [2,3].

Figure 1- A - Tricine SDS-PAGE gel of partially purified bacteriocins, stained with EZblue Reagent. Lane 1: low molecular weight marker; Lane 2: L3A21M1, Lane 3: L2B21K3, Lane 4: L3A21M3, Lane 5: L3A21M8, Lane 6: L3B1K3, Lane 7: L3A21K7, Lane 8: L3A21K6, Lane 9: L3A1M6. B - RP-HPLC chromatogram of purified bacteriocin obtained from isolate L3A21K6. Arrow indicates the major peak with 32 min retention time exhibiting anti-listeria activity.



4. CONCLUSIONS

Much of the interest in structure/function analysis of LAB produced bacteriocins is driven by their potential applications. The properties of the bacteriocins studied, like the inhibition of pathogenic strains, their stability over a wide pH range, heat resistance and high salt tolerance makes them promising agents in food preservation. Further studies on food systems and more purification steps are needed for the practical application of isolated bacteriocins.

Acknowledgements

This work is financed by National founds from FCT – “Fundaç ncia e Tecnologia”, Project PTDC/AGR-ALI/104385/2008.

References

[1] RP Ross, S Morgan, C Holl, Int J Food Microbiol, 2002, 79, 3.16.
[2] H Ghalfi, N Benkerroum, M Ongena, M Bensaid, P Thonart, Food Res Int, 2010, 43, 33-39.
[3] J Meyer, P Rogne, C Opegård, H Haugen, P Kristiansen, Curr Pharm Biotechnol, 2009, 10, 19-37.
[4] JR Tagg, AR McGiven, Appl Microbiol, 1971, 21, 943.
[5] MCJR Yang, B Ray, Appl Environ Microbiol, 1992, 58 (10), 3355-3359.
[6] A Pinto, M Fernades, C Pinto, H Albano, F Castilho, Int J Food Microbiol, 2009, 129, 50-58.
[7] H Schagger, G Von Jagow, Anal Biochem, 1987, 166, 368-379.