

IMPROVED ADSORPTION-DESORPTION EXTRACTION APPLIED TO THE PARTIAL CHARACTERIZATION OF THE ANTILISTERIAL BACTERIOCIN PRODUCED BY *CARNOBACTERIUM MALTAROMATICUM* C2

Tulini, F. L.; De Martinis, E.C.P.*

Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Brasil.

Submitted: November 19, 2008; Returned to authors for corrections: February 10, 2009; Approved: November 07, 2009.

ABSTRACT

Bacteriocins are ribosomally produced peptides useful for food biopreservation. An improved adsorption-desorption process is proposed for the partial purification of the bacteriocin produced by the fish isolate *Carnobacterium maltaromaticum* C2. Analysis of extract by SDS-PAGE indicated this method may offer an alternative to improve the yield of purification of bacteriocins.

Key words: Bacteriocins, LAB, *Carnobacterium*, SDS-PAGE.

Consumers demand safe, convenient, nutritious and healthy foods, stimulating researches on new antimicrobial agents for food biopreservation. Lactic acid bacteria (LAB) are present as autochthonous microbiota in many fermented products with long history of safe consumption and this group of bacteria can ribosomally synthesize antimicrobial peptides named bacteriocins, which present potential use as biopreservatives (7, 12). Bacteriocins of LAB are cationic, hydrophobic and present high isoelectric point that facilitates the extraction from the producers cells, depending on pH (9). Bacterial cell surface presents phospholipids with anionic characteristics at neutral and acid pH and can be used as a matrix for the initial purification of LAB bacteriocins, which can be further purified using chromatographic processes based on hydrophobic interaction, cationic exchange and reverse-phase chromatography (4, 12). Yang *et al.* (16) described a method for purification of bacteriocins based on the adjustment of the pH of broth cultures and used the own bacterial cells as adsorbent material. Besides, Todorov *et al.* (15) and Khouiti *et al.* (8) observed that the adsorption of bacteriocins to the producer cells and other surfaces may be influenced by compounds such as surfactants and salts (6, 15).

The common features of the bacteriocins produced by the genus *Carnobacterium* are low molecular weight (4.2 to 5kDa) and high isoelectric point, between 9 and 10 (4). The strain *Carnobacterium piscicola* C2, isolated by from cold-smoked, vacuum-packed surubim fish, produces a bacteriocin that inhibits the growth of *L. monocytogenes* in smoked fish juices and in surubim homogenate at 10°C (1). Mora *et al.* (11) have proposed the re-classification of *Carnobacterium piscicola* as *Carnobacterium maltaromaticum* due to DNA-DNA relatedness. In the present work, the bacteriocin of *Carnobacterium maltaromaticum* C2 (previously referred as *Carnobacterium piscicola* C2) was partially purified and characterized and a two-steps adsorption-desorption extraction was proposed, which may contribute for obtaining improved bacteriocin preparation for food applications.

C. maltaromaticum C2 and *L. monocytogenes* IAL 633 were used in this study and maintained in Brain Heart Infusion broth (BHI, Oxoid, UK), cryoprotected with 20% glycerol (Synth, Brazil) at -70°C. Working cultures were prepared in BHI broth incubated at 20°C for *C. maltaromaticum* C2 and at 37°C for *L. monocytogenes* IAL633.

Bacteriocin extraction was done in a two-steps approach,

*Corresponding Author. Mailing address: Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, 14040-903, Ribeirão Preto, São Paulo, Brasil.; Tel.: 55 16 36024267 Fax: 55 16 36024725.; E-mail: edemarti@usp.br

using a modification to the method proposed by D'Angelis *et al.* (5), using *L. monocytogenes* as indicator strain for monitoring antimicrobial activity. Two liters of culture of *C. maltaromaticum* C2 was obtained in BHI broth at 20°C/18h and the pH was adjusted to 6.0. Cells were harvested at 6,048xg/20 minutes/4°C (Sorvall RC 5C Plus, USA) and the cell pellet was washed twice with phosphate buffer 5mM pH 6.0. The cell pellet was resuspended in 50ml of 0.1M NaCl solution pH 1.5 and kept at 4°C/1h under agitation with a magnetic stirrer. The suspension was centrifuged at 10,080xg/15 minutes/4°C (Sorvall RC 5C Plus) and the supernatant was collected. The remaining cell pellet was added again to the initial supernatant of culture broth, and the extraction was repeated. The cell-free supernatants obtained after the extractions were combined, dialyzed against distilled water in 1KDa exclusion membrane (Spectra/Por® Biotech Cellulose Ester Dialysis Membrane, Spectrum, USA) and freeze-dried (VirTis Lyophilizer, USA). Aliquots of the supernatants were taken after each step for quantification of bacteriocin activity, according to Mayr-Harting *et al.* (10). An extraction as described by D'Angelis *et al.* (5) was carried out for comparison purposes. Protein contents of dried powders were determined by the Bradford method (2) adapted for microassay.

Bacteriocin extracts were analyzed by SDS-PAGE, with stacking gel of 4% and resolving gel of 16%, using all chemicals from Bio-Agency (São Paulo, Brazil). Electrophoresis was done at 10mA/4h (Mini-Protean Cell 3, BioRad, USA), with trisglycin buffer solution and pre-stained low molecular weight markers (2.5 and 45kDa, Amersham Biosciences, U.K.).

Half of the gel was silver stained (Protein Silver Staining Kit, Amersham Biosciences, Sweden) and half was fixed with a solution of ethanol (40%) and acetic acid (10%) for 30 minutes. The fixed gel was washed under running distilled water for 2 hours and placed on a BHI agar plate. The gel was overlaid with 7ml of soft BHI agar (0.8%) seeded with *L. monocytogenes* (ca. 10⁶CFU/ml). After diffusion for 18 h at 4°C it was incubated for 8-12h at 37°C (5) and the resulting gel

was documented with a digital camera (Sony Cyber-Shot W35).

Bacteriocin activity obtained from *C. maltaromaticum* C2 during the purification steps are shown in Table 1. The extraction procedure used in this work resulted in 7.5% yield, compared to 2.5% of a previous reported extraction (5), with 28.0mg of dry powder containing 1.03% of proteins, and a specific activity of 622,477AU/mg (Table 1). The use of the two-steps approach resulted in more effective bacteriocin purification than other protocols based on adsorption-desorption of bacteriocins to producers cells (5, 16). This fact is related to the reversible capacity of bacteriocins to adsorb and desorb from the producers cells. Results indicate that the bacterial cells may be used to extract bacteriocins from the culture broth at least twice. Table 1 shows that not all the bacteriocin present in the initial broth can be adsorbed by the producers cells, likely due to saturation of the binding sites of the cell membrane. It also shows that not all the bacteriocin molecules desorbed at the end of extraction, indicating that some adjustments can still increase the final yield. Yang *et al.* (16) obtained good results for the acid extraction applied for purification of pediocin AcH (106.7%), nisin (93.3%), sakacin A (44.3%) and leuconocin Lcm1 (96.2%), but in this study with *C. maltaromaticum* C2 that method resulted in low yield. Even considering that it is difficult to compare interlaboratory results of bacteriocin purification due to differences in definitions of arbitrary units, levels of bacteriocin produced in the initial broth, variation in the number of cells and intrinsic cell capacity of adsorption, the present results indicate that the two-steps extraction is advantageous as larger amounts of bacteriocins can be obtained.

SDS-PAGE of the extract obtained with the double acid extraction revealed with the bioindicator *L. monocytogenes* (Figure 1B) presented a band with antimicrobial activity, revealed by silver staining (Figure 1A). This band corresponded to a compound molecular weight between 3.5 and 6.5KDa. Contaminant proteins were also detected in the silver stained gel (Figure 1A), indicating that further purification is required for the complete characterization of the

antimicrobial peptide. Other studies on the purification of antimicrobials produced by other *Carnobacterium* species revealed peptides of molecular weight similar to the bacteriocin from *C. maltaromaticum* C2, with 4,635 Da for carnocin UI49 to 6 KDa for BLIS 213 (8, 14).

In conclusion, the proposed two-steps extraction procedure offers a good alternative for improved recovery of *C. maltaromaticum* C2 bacteriocin from culture broth and can contribute for successful application of this isolate in food biopreservation.

Table 1. Bacteriocin activity and final yield during the traditional and improved acid extraction proposed for the partial purification of the bacteriocin produced by *C. maltaromaticum* C2. The two extraction of the improved method were made from the same culture broth, in sequence.

TRADITIONAL EXTRACTION		
Material	Average activity (AU/mL)	Total activity (AU)
Initial broth (2000mL)	320	640000
Bacteriocin not adsorbed (2000mL)	160	320000
Phosphate buffer pH 6.0 (50mL)	0	0
Dialysed final supernatant (200mL)	80	16000
	Yield	2.5%
IMPROVED ACID EXTRACTION		
Material	Average activity (AU/mL)	Total activity (AU)
Initial broth (2000mL)	200	400000
Bacteriocin not adsorbed (2000mL)	200	400000
1 st Phosphate buffer pH 6.0 (50mL)	0	0
Final supernatant (50mL)	200	10000
Bacteriocin not adsorbed (2000mL)	100	200000
2 nd Phosphate buffer pH 6.0 (50mL)	0	0
Final supernatant (50mL)	400	20000
Dialysed supernatant (200mL)	150	30000
	Yield	7.5%

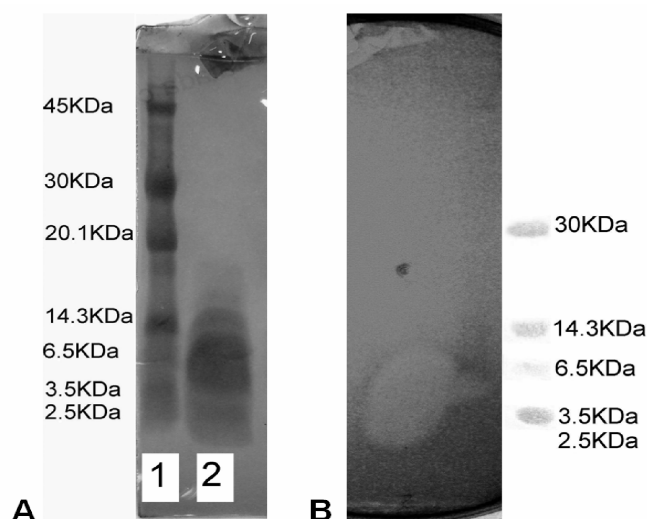


Figure 1. SDS-PAGE analysis of bacteriocin extracts obtained with the two-steps adsorption-desorption method. A) Silver stained gel. Lane 1: pre-stained low-range molecular marker; lane 2: bacteriocin extract. B) Gel prepared with the bioindicator *L. monocytogenes*. The pre-stained molecular marker indicates the molecular weight range.

ACKNOWLEDGEMENTS

F. L. T. thanks The State of São Paulo Research Foundation (FAPESP) for a scientific initiation fellowship (process # 07/50236-0). Authors thank Ana Cristina Morseli Polizello (FCFRP – USP) for excellent technical support and Prof. Maria Cristina Nonato, Ph. D. (FCFRP – USP) for helpful discussions.

REFERENCES

- Alves, V.F.; De Martinis, E.C.P.; Destro, M.T.; Vogel, B.F.; Gram, L. (2005). Antilisteral activity of a *Carnobacterium piscicola* isolated from brazilian smoked fish (Surubim [*Pseudoplatystoma sp.*]) and its activity against a persistent strain of *Listeria monocytogenes* isolated from surubim. *J. Food Prot.*, 11, 2068-2077.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72, 248-254.
- Chen, Y.S.; Yanagida, F.; Sriannual, S. (2007). Characteristics of bacteriocin-like inhibitory substances from *dochi*-isolated *Enterococcus faecium* D081821 and D081833. *Lett. Appl. Microbiol.*, 44, 320-325.
- Cintas, L.M.; Casaus, M.P.; Herranz, C.; Nes, I.F.; Hernández, P.E. (2001). Review: bacteriocins of lactic acid bacteria. *Food Science Technology International*, 7(4), 281-305.
- D'Angelis, C.E.M.; Polizello, C.; Nonato, M.C.; Spadaro, A.C.C.; De Martinis, E.C.P. (2008). Purification, characterization and N-terminal amino acid sequencing of sakacin 1, a bacteriocin produced by *Lactobacillus sakei* 1. *J Food Saf, in press*.
- Elegado, F.B.; Kim, W.J.; Kwon, D.Y. (1997). Rapid purification, partial characterization, and antimicrobial spectrum of the bacteriocin, Pediocin AcM, from *Pediococcus acidilactici* M. *Int. J. Food Microbiol.*, 37, 1-11.
- Guinane, C.M.; Cotter, P.D.; Hill, C.; Ross, R.P. (2005). Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. *J. Appl. Microbiol.*, 98, 1316-1325.
- Khouiti, Z.; Simon, J.P. (1997). Detection and partial characterization of a bacteriocin produced by *Carnobacterium piscicola* 213. *J. Ind. Microbiol. Biotechnol.*, 19(1), 28-33.
- Li, C.; Bai, J.; Li, W.; Cai, Z.; Ouyang, F. (2001). Optimization of conditions for bacteriocin extraction in PEG/salt aqueous two-phase systems using statistical experimental designs. *Biotechnol. Prog.*, 17, 366-368.
- Mayr-Harting, A.; Hedges, A.J.; Berkeley, R.C.W. (1972). Methods for studying bacteriocins. *Methods in Microbiology*, 7, 313-342.
- Mora, D.; Scarpellini, M.; Franzetti, L.; Colombo, S.; Galli, A. (2003). Reclassification of *Lactobacillus maltaromicus* (Miller *et al.* 1974) DSM 20342T and DSM 20344 and *Carnobacterium piscicola* (Collins *et al.* 1987) DSM 20730T and DSM 20722 as *Carnobacterium maltaromaticum* comb. nov. *Int. J. Syst. Evol. Microbiol.*, 53, 675-678.
- Oscariz, J.C.; Pisabarro, A.G. (2001). Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. *Int. Microbiol.*, 4, 13-19.
- Schöbitz, R.; Suazo, V.; Costa, M.; Ciampi, L. (2003). Effects of a bacteriocin-like inhibitory substance from *Carnobacterium piscicola* against human and salmon isolates of *Listeria monocytogenes*. *Int. J. Food Microbiol.*, 84, 237-244.
- Stoffles, G.; Nissen-Meyer, J.; Gudmundsdóttir, S.; Sletten, K.; Holo, H.; Nes, I.F. (1992). Purification and characterization of a new bacteriocin isolated from a *Carnobacterium sp.* *Appl. Environ. Microbiol.*, 58, 1417 – 1422.
- Todorov, S.D.; Dicks, L.M. (2006). Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. *Biotechnol J*, 1, 405-409.
- Yang, R.; Johnson, M.C.; Ray, B. (1992). Novel method to extract large amount of bacteriocins from lactic acid bacteria. *Appl. Environ. Microbiol.*, 58(10), 3335-3359.