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Interactions between two carnobacteriocins Cbn BM1 and Cbn B2 from *Carnobacterium maltaromaticum* CP5 on target bacteria and Caco-2 cells

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ABSTRACT

Two purified class IIa carnobacteriocins Cbn BM1 and Cbn B2, from *Carnobacterium maltaromaticum* CP5, were evaluated for antimicrobial activity against pathogenic, spoilage and lactic acid bacteria. Then, the presence of a synergistic mode of action of these two carnobacteriocins on *Listeria* sp., *Enterococcus* sp. and *Carnobacterium* sp. was investigated. A synergistic mode of action between Cbn BM1 and Cbn B2 on sensitive target bacteria was demonstrated using the FIC index method. Combinations of carnobacteriocins enhanced their antibacterial activities and MICs were significantly reduced, between 2- and 15-fold, by the addition of the second bacteriocin. To improve the safety of the bacteriocins as biopreservative agents, the cytotoxicity of the combination of theses two bacteriocins was determined on Caco-2 cell line. However, these two peptides used alone or in combination, at concentration 100-fold higher than those required for antimicrobial activity, were not cytotoxic. This suggests that the two carnobacteriocins produced by *C. maltaromaticum* CP5 could be potential natural agents for food preservation.

1. Introduction

Antimicrobial peptides (AMP) are frequently found in all vegetal and animal kingdoms, from the bacteria to the mammals. These peptides can prevent infections and/or eliminate the competitive bacterium living in the same environment than the AMPs producer (Reddy et al., 2004). Consequently, they are potential therapeutic and food preservative agents (O'Sullivan et al., 2002). The AMPs produced by the lactic acid bacteria (LAB), called bacteriocins, are ribosomally synthetized without post-traductional modification and are generally active against related phylogenetically bacteria (Tagg et al., 1976). They were classified into four classes, with the class II the most represented (Klaenhammer, 1993). This class displays an anti-*Listeria* activity and shares high similarities in their primary sequence with an N-terminal consensus sequence (YGNGV) and presents two cysteine residues involved in a disulfide bridge (Fimland et al., 2005). Among the class IIa bacteriocins synthesized by LAB strains, *Carnobacterium* is an important producer secreting around 12 distinct bacteriocins (Leisner et al., 2007). *Carnobacterium maltaromaticum* CP5 isolated from a French mold ripened cheese produces two carnobacteriocins, CP51 and CP52 (Herbin et al., 1997). CP51 is similar to carnobacteriocin BM1 (Cbn BM1) from *C. piscicola* LV17, isolated from vacuum-packed meat (Ahn and Stiles, 1990) and its derivative *C. piscicola* LV17B (Quadri et al., 1994) and to piscicocin V1b synthesized by *C. piscicola* V1 (Bhugaloo-Vial et al., 1996). Other strains of *C. maltaromaticum* produced Cbn BM1 like *C. maltaromaticum* UAL26 (Gursky et al., 2006). CP52 is similar to carnobacteriocins B2 (Cbn B2) (Quadri et al., 1994). These bacteriocins, Cbn BM1 (43 AA) and Cbn B2 (48 AA), are active against Gram-positive bacteria, including *Carnobacterium* sp., *Enterococcus* sp. and *Listeria* sp. strains (Mathieu et al., 1993; Quadri et al., 1994).

Among the potential strategies to enhance the LAB bacteriocin activity, the use of combinations of them was proposed. Synergistic action between two bacteriocins produced by the same strain has been already reported for the two bacteriocins (mesenterocins 52A and 52B) produced by *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 (Limonet et al., 2004; Revol-Junelles et al., 1996) and for enterocins A and B produced by *Enterococcus faecium* T136 (Casaus et al., 1997).

In this paper, the spectra of carnobacteriocins Cbn BM1 and Cbn B2 were determined to confirm the natural antimicrobial activity of the two purified peptides. The presence of a synergistic mode of action of these two carnobacteriocins on *Listeria* sp., *Enterococcus* sp. and *Carnobacterium* sp. was investigated. To ensure their safety as biopreservative agents, the cytotoxicity of the combination of theses two peptides was determined on Caco-2 cell line.

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2. Materials and methods

2.1. Bacterial strains and culture conditions

Carnobacterium, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria*, *Staphylococcus*, *Streptococcus* and *Weissella* strains were purchased from different public collections (Table 1). The strains from *Carnobacterium* and *Listeria* genus were cultivated in trypticase soy broth (Biokar Diagnostics, Beauvais, France) supplemented with 6 g L^{-1} of bacto-yeast extract (Biokar) (TSB-YE); *Enterococcus*, *Staphylococcus* and *Streptococcus* strains were cultivated in Elliker (Biokar) broth. *Lactobacillus*, *Leuconostoc* and *Weissella* strains were cultivated in MRS (Biokar). Incubation was performed either at 25, 30 or 37 °C. All strains were stored in appropriate culture medium supplemented with glycerol (10%) at −30 °C and propagated twice before use. Agar medium was prepared by addition of 15 g L^{-1} of bacteriological agar. Enumerations were determinated by decimal dilutions of the cultures and plated on appropriate agar medium using a spiral plating (Whitley Automatic Spiral Plater, WASP 2, AES Laboratoire).

2.2. Bacteriocins

Carnobacteriocins Cbn BM1 and Cbn B2 from *C. maltaromaticum* CP5 were produced and purified by heterologous expression in a previous work (Jasniewski et al., 2008a).

2.3. Determination of antibacterial activity

Antimicrobial activity was determined by the agar well diffusion method (Mathieu et al., 1993). Bacterial strains were inoculated in the appropriate solid medium (1.2% bacteriological agar, Biokar) supplemented with 1% Tween 80 (Merck, Darmstadt, Germany) and poured in Petri dishes. Cbn BM1 and Cbn B2 bacteriocins were prepared in phosphate buffer (5 mM, pH 6.5) to a final concentration of 250 mg L^{-1}. A combination of these two carnobacteriocins was prepared in the same buffer to a concentration of 125 mg L^{-1} for each bacteriocin. The supernatant of a 16 h culture of *C. maltaromaticum* CP5 was harmed at 80 °C during 20 min and filtered through a 0.22 μm pore diameter membrane.

A 25 μL volume of Cbn BM1, Cbn B2, combination of Cbn BM1/Cbn B2 or of the treated supernatant of *C. maltaromaticum* CP5 was placed into a 5-mm diameter agar well. Agar plates were allowed to diffuse overnight at 4 °C and then were incubated at the optimal temperature of each target strain for at least 10 h and diameters of inhibition zones were measured. Assays were performed in triplicate.

2.4. Determination of the minimal inhibitory concentration (MIC) and the fractional inhibitory concentration (FIC)

For strains susceptible to both carnobacteriocins, the minimal inhibitory concentrations (MIC) of the Cbn BM1 and Cbn B2 were determined in 96-wells plates (Nunc, Roskilde, Denmark) containing 100 μL of the tested peptides. Phosphate buffer solutions (5 mM, pH 6.5) containing the nine combinations 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0 mg L^{-1} of Cbn BM1 and of CbnB2 were prepared. For each combination, 100 μL of the appropriate 2X culture media inoculated with the susceptible strains with an initial optical density value at 660 nm of 0.02 were added. Inhibitory activities of these 81 bacteriocin(s) suspensions were determined by measuring $O.D_{660}$ values after 18 h of incubation at the optimal temperature of each tested culture (Table 1).

Inhibition data were expressed in fractional inhibitory concentrations (FIC) (Hall et al., 1983). $FIC_{Cbn\ BM1}$ = (MIC of Cbn BM1 with MIC of Cbn B2)/MIC of Cbn BM1. The FIC index was calculated with FICs for individual carnobacteriocin: $FIC_{index} = FIC_{Cbn\ BM1} + FIC_{Cbn\ B2}$. A FIC_{index} near 1 indicates additivity, <1 synergy, and >1 antagonism of the bacteriocins combination.

For Caco-2 cells, the followed combinations have been tested: 100 mg L^{-1} of Cbn BM1 without Cbn B2, 100 mg L^{-1} of Cbn B2 without Cbn BM1, 75 mg L^{-1} of Cbn BM1 with 25 mg L^{-1} of Cbn B2, 75 mg L^{-1} of Cbn B2 with 25 mg L^{-1} of Cbn BM1, 65 mg L^{-1} of Cbn BM1 with 35 mg L^{-1} of Cbn B2, 65 mg L^{-1} of Cbn B2 with 35 mg L^{-1} of Cbn BM1, 50 mg L^{-1} of Cbn BM1 with 50 mg L^{-1} of Cbn B2 and 25 mg L^{-1} of Cbn BM1 with 25 mg L^{-1} of Cbn B2.

Table 1
Activity spectra of carnobacteriocins Cbn BM1, Cbn B2, used in association and of a culture supernatant of the productive strain *C. maltaromaticum* CP5. ATCC, American Type Culture Collection, Mannassas, USA; CIP, Collection de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikro-Organismen and Zellkulturen, Göttingen, Germany; SLCC, Special *Listeria* Culture Collection, University of Wurzburg, Germany; LMA and CP, Private collection, Laboratoire de Microbiologie Alimentaire, ENSAIA-INPL Nancy, France.

Target strains		Incubation temperature (°C)	Inhibition zone diameter (mm)			
Species	Strain designation		BM1	B2	BM1 + B2	CP5
Carnobacterium divergens	DSM 20623^T	30	+++[a]	++	+++[a]	+++[a]
C. gallinarum	DSM 4847	25	+++	++	++	+++
C. mobile	DSM 4848^T	30	+++[a]	++	++	++
C. maltaromaticum	LMA 28	30	0	0	0	0
	DSM 20730^T	30	+++[a]	++	+++[a]	+++
	CP 5	30	0	0	0	0
C. viridans	CIP 107728^T	25	0	0	0	0
Enterococcus durans	CIP 55125^T	37	+++[a]	++	+++	++[a]
E. faecalis	CIP 76117	37	++[a]	++	++	+++
E. faecium	LMA 63	37	0	++[a]	++[a]	++
	DSM 20477^T	37	0	++	++	++
Lactobacillus casei subsp. casei	DSM 20011^T	30	0	0	0	0
Lb. fermentum	DSM 20052^T	37	0	0	0	0
Lb. helveticus	DSM 20075^T	37	0	0	0	0
Lb. plantarum	DSM 20174	30	0	0	0	0
Lactococcus lactis subsp. *lactis*	CIP 7056T	30	0	0	0	0
Leuconostoc citreum	CIP 103405	30	0	0	0	0
Ln. mesenteroides subsp. *dextranicum*	LMA 131	30	0	0	0	0
	DSM 20484	30	0	0	0	+[a]
Ln. mesenteroides subsp. *lactis*	DSM 20202	30	0	0	0	+[a]
Ln. mesenteroides subsp. *mesenteroides*	LMA 7	30	0	0	0	0
	LMA 7AR	30	0	+[a]	0	+[a]
	CIP 54170	30	0	0	0	0
Ln. pseudomesenteroides	CIP 103316^T	30	0	0	0	0
L. grayi	CIP 6818	30	++[a]	++[a]	+	++[a]
L. innocua	CIP 12511	30	++[a]	++[a]	++	++
L. ivanovii	CIP 12510	30	+++[a]	++[a]	+++[a]	+++[a]
L. monocytogenes	CIP 7831	37	++[a]	++[a]	++[a]	+[a]
	ATCC 15313	37	+	++[a]	++[a]	++[a]
Listeria seeligeri	SLCC 3954	37	++[a]	++[a]	++[a]	+++
Staphylococcus aureus	CIP 7625^T	37	0	0	0	0
Streptococcus thermophilus	DSM 20617	37	0	0	0	0
Weissella paramesenteroides	DSM 20288^T	30	0	0	0	0

0, no inhibition; +, ⩽ 2 mm; ++, between 2 and 8 mm; +++, > 8 mm.
[a] Regrowth in the inhibition area.