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Bacteriocin-like inhibitory substances (BLIS) produced by *Bacillus cereus*: Preliminary characterization and application of partially purified extract containing BLIS for inhibiting *Listeria monocytogenes* in pineapple pulp

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ABSTRACT

The application of conventional preservatives in food has been reassessed due to the toxicity of some of classical additives, and it is important to search for new food preservation strategies. Thus, this study aimed to characterize an antilisterial bacteriocin-like inhibitory substances (BLIS) produced by *Bacillus cereus* LFB-FIOCRUZ 1640 and to evaluate the potential of the BLIS in pineapple pulp biopreservation. The proteinaceous nature of BLIS was confirmed by susceptibility to the enzyme α-chymotrypsin and it showed thermal stability for up to 30 min at 80 °C and in a wide pH range. After partial purification of the BLIS with Amberllite XAD-16 resin and cation-exchange SP-Sepharose "Fast Flow", it was obtained a purification factor of 4.1 times and 30% of yield, and SDS-PAGE analyses revealed that the peptide has approximately 24.8 kDa. The Minimum Bactericidal Concentration (MBC) of 2 mg mL^{-1} was determined with the biological indicator *Listeria monocytogenes* and this concentration of BLIS was applied in a model system containing pineapple pulp. The BLIS was bactericidal towards *L. monocytogenes* after 24 h at 37 °C, and these results indicate the application of the BLIS produced by *B. cereus* LFB-FIOCRUZ 1640 in food, especially for fruit pulp biopreservation.

1. Introduction

Nowadays, pulps of tropical fruits are products with considerable commercial potential, since they keep many nutritional properties of the fruits and they are important food sources (Fernandes, 2007). To increase shelf life and to ensure the preservation of pulps and fruit juices, industries use processes such as pasteurization, dehydration, high pressure treatments, freezing and refrigeration (Awuah, Ramaswamy, & Economides, 2007). According to Mattietto and Matta (2011), pasteurization is widely used in the processing of fruit pulp and it is usually combined with chill storage, which allows the safety for marketing this product. Nevertheless, the occurrence of pathogens such as *Listeria monocytogenes* has been reported in juices and fruit pulps with low acidity (Chan & Wiedmann, 2009; Penteado & Leitão, 2004). *L. monocytogenes* is a pathogenic Gram-positive bacterium, facultative anaerobic, psychrotrophic, transmitted mainly through food and affects specific risk groups such as pregnant women, newborns, the elderly and immunocompromised people (Montville, Matthews, & Kniel, 2012).

To ensure food safety, there is a great interest in biopreservation procedures involving the use of saprophytic microorganisms and/or their metabolites, either to inhibit pathogens or to extend shelf-life (Stiles & Hastings, 1991). The main bacterial metabolites with potential for use as biopreservatives are the antimicrobial peptides, which are described as bacteriocins and bacteriocin-like inhibitory

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substances (BLIS), and may be produced by many bacteria species. Bacteriocins compose a heterogeneous group of peptides that are synthesized by the ribosomes, with *ca.* 30 to 60 amino acids. They may also vary in activity, mechanism of action, molecular mass, biochemical properties and genetic origin (Abee, Krockel, & Hill, 1995; Cleveland, Montville, Nes, & Chikindas, 2001; Gálvez, López, Abriouel, Valdivia, & Omar, 2008). BLIS are defined as antimicrobial peptides that were not fully characterized with regard the amino acid sequences and biochemical properties (Settanni & Corsetti, 2008). The BLIS act as antagonistic substances, with bactericidal or bacteriostatic potential against Gram-positive and/or Gram-negative bacteria, and they are innocuous for the producer strain (Allison & Klaenhammer, 1998; Cleveland et al., 2001; Oscáriz, Lasa, & Pisabarro, 1999).

Bacillus cereus is a Gram-positive bacterium widely spread in several environments (*e.g.* soil and plants), thereby contaminating many food products (Montville et al., 2012). However, despite the fact that *B. cereus* may cause food spoilage and food poisoning, the metabolites from some strains can be used for food biopreservation. In addition, the production of bacteriocins and BLIS have been reported in many *B. cereus* strains (Abriouel, Franz, Omar, & Gálvez, 2011; Oscáriz et al., 1999; Senbagam, Gurusamy, & Senthilkumar, 2013; Sevim, Karaoglu, Sevim, & Canakci, 2013; Zhong et al., 2013). Furthermore, *Bacillus* spp. can produce other compounds related to defense mechanisms, survival, sporulation, antimicrobial peptides, antibiotics and lipopeptides (Abriouel et al., 2011). Bioactive metabolites produced by *Bacillus* species also have attracted the interest of pharmaceutical industries due to large structural diversity, production and stability in different conditions of pH and temperature, in addition to broad antimicrobial spectrum of activity (Abriouel et al., 2011; Sansinenea & Ortiz, 2011).

Bacteriocins and BLIS from *Bacillus* species have potential for antibacterial therapy and food biopreservation as they have low allergenic potential, activity at low concentrations and they are easily degraded in the gastrointestinal tract. Moreover, these substances do not increase the selection of resistant microorganisms and they can be active against pathogens and spoilage bacteria, such as *B. cereus*, *L. monocytogenes* and *Staphylococcus* spp (Cleveland et al., 2001; Lohans & Vederas, 2012; Riley & Wertz, 2002).

The studies on BLIS produced by *Bacillus* species are still scarce in the literature, which suggest that the prospect of bacteria of this genus with antagonist activity may contribute to the discovery of new classes of antimicrobial compounds (Abriouel et al., 2011). Thus, the aim of this work was to partially purify and characterize the BLIS produced by *B. cereus* LFB - FIOCRUZ 1640, isolated from pineapple pulp, and to evaluate the potential of partially purified extract of BLIS for biopreservation by the inhibition of *L. monocytogenes* in pineapple pulp.

2. Materials and methods

2.1. Bacterial cultures

B. cereus LFB-FIOCRUZ 1640 was isolated from pineapple pulp (*Ananas sativus*), identified by phenotypic and molecular tests. The strain was selected for this study due to inhibitory activity against *L. monocytogenes* in preliminary tests (data not shown). *B. cereus* was cultured in MRS (De Man, Rogosa and Shape) broth (Oxoid, UK) for 24 h at 30 °C, and stored in the same broth with 20% glycerol (Synth, Brazil) at −80 °C. Other bacterial strains used in this study are described in Table 1.

2.2. Antimicrobial spectrum and protease susceptibility of the antagonistic substance

The spectrum of inhibitory activity was evaluated according to Lewus, Kaiser, and Montville (1991), using the indicator strains described in Table 1. The protease susceptibility of the antagonistic substance produced by *B. cereus* LFB-FIOCRUZ 1640 was tested by the method described by Lewus et al. (1991) and modified by De Martinis and Franco (1998). Two microliters of culture were inoculated on TSA-YE agar (Trypticase soy agar with 0.6% of yeast extract, Oxoid) and incubated anaerobically at 30 °C for 24 h. Following, 2 mm wells were made in the agar, near to the bacterial spot, and filled with 20 μL of protease solutions (20 mg mL^{-1} of protease type XIV from *Streptomyces griseus* and 20 mg mL^{-1} α-chymotrypsin, all purchased from Sigma–Aldrich, USA). Sterilized purified water was used as negative control, and inoculated plates were incubated at 30 °C for 2 h. Next, each plate was overlaid with soft BHI agar (0.8% agar) inoculated with *ca.* 10^6 CFU mL^{-1} of *B. cereus* (non bacteriocinogenic strain, Table 1), followed by incubation at 30 °C for 24 h. The absence of inhibition halo in the presence of proteolytic enzymes was indicative of the production of antagonistic peptides.

2.3. Quantification of BLIS activity and total proteins

BLIS activity was quantified by agar antagonism assays (critical dilution method), as described by Mayr-Harting, Hedges, and Berkeley (1972), using *L. monocytogenes* ATCC 19115 and *B. cereus* (non bacteriocinogenic) as indicator strains. The results were expressed in arbitrary units per mL (AU mL^{-1}). Quantification of total protein was performed as described by Bradford (1976).

2.4. Evaluation of BLIS stability under different pH and temperatures

B. cereus LFB-FIOCRUZ 1640 was cultured in MRS broth for 24 h at 30 °C, followed by centrifugation at 14,000 *g* for 10 min at 4 °C (Sorvall Legend Mach 1.6R Sorvall, USA). The culture supernatant was filter sterilized (0.22 μm GVWP membrane, Millipore, USA) to obtain a cell-free supernatant (CFS), which was submitted to different temperatures (60, 70, 80 and 90 °C) for 30 min and at 121 °C for 15 min. The evaluation of BLIS stability at different pH was performed according to Bizani and Brandelli (2002) with modifications. Aliquots of CFS were adjusted to pH 2, 4, 6, 8 and 10 with NaOH 4 mol L^{-1} or HCl 4 mol L^{-1} and kept at 10 °C for 24 h. The remaining antimicrobial activity was quantified as described previously (item 2.3) and compared with a non-treated CFS (control).

2.5. BLIS purification using XAD-16 resin and SP-Sepharose "Fast Flow"

B. cereus LFB - FIOCRUZ 1640 was cultured in MRS broth without Tween and used to obtain the CSF, as described previously. The purification was conducted as proposed by Sebei, Zendo, Boudabous, Nakayama, and Sonomoto (2007) and Martin-Visscher et al. (2008). A glass column (2.5 cm × 50 cm, Bio-Rad Laboratories, Hercules, CA, USA) was filled with 40 g of Ambelite XAD-16 resin (Sigma–Aldrich), previously conditioned with isopropyl alcohol (JT Baker, CA, USA) and washed with 4 L of purified water. The CFS was applied in the column, which was further washed with 300 mL of water and 300 mL of 20% ethanol aqueous solution (Synth). The BLIS was eluted with 300 mL of 70% isopropyl alcohol aqueous solution acidified to pH 3.0. After removing the organic solvent, the remaining solution was applied in a SP-Sepharose "Fast Flow" resin packed into a C10 column (GE