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Purification and characterization of a peptide from *Bacillus licheniformis* showing dual antimicrobial and emulsifying activities

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ABSTRACT

Bacillus licheniformis strain P40 produces a bacteriocin-like substance (BLS) that inhibits important pathogenic and food spoilage bacteria such as *Listeria monocytogenes*, *Streptococcus* spp., *Bacillus cereus*, and *Erwinia carotovora*. The antimicrobial peptide produced by *B. licheniformis* P40 was purified by ammonium sulfate precipitation, gel filtration chromatography on Sephadex G-100 and reversed phase chromatography on Source-RPC. The purification was about 100-fold with a yield of 0.3%. The molecular mass of the purified BLS about 800 Da, as determined by mass spectroscopy. The BLS was resistant for up to 100 °C and pH ranging 3–10, but lost its activity when treated with proteases and trichloroacetic acid. Reaction with ninhydrin produced the yellowish color instead the characteristic purple. Data from infrared spectroscopy also indicate the peptide is cyclic, resembling the lipopeptides surfactin and lichenisin. The BLS also showed emulsifying properties with several hydrophobic compounds, and dual antimicrobial and emulsifying activity in a meat model system. This antimicrobial peptide presents potential for use in as a food biopreservative or biodetergent.

1. Introduction

Bacteriocins are antimicrobial peptides produced by bacteria, often presenting a bactericidal effect against closely related species (Riley & Wertz, 2002). Bacteriocins from lactic acid bacteria (LAB) have been largely studied with the perspective of food protection against pathogenic and spoilage bacteria (Cleveland, Montville, Nes, & Chikindas, 2001; O'Sullivan, Ross, & Hill, 2002). Despite the intensive research on LAB bacteriocins, increasing attention have been addressed to the antimicrobial peptides produced by several other classes of bacteria (Riley & Wertz, 2002).

Some species of *Bacillus* have a history of safe use in the industry, including the production of food additives (de Boer & Diderichsen, 1991). This genus presents a great variety of species that produce bacteriocins or bacteriocin-like substances (BLS), which display antimicrobial activity against food-borne pathogenic microorganism. These include *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus amyloliquefaciens*, and other (Kamoun et al., 2005; Sirtori, Cladera-Olivera, Lorenzini, Tsai, & Brandelli, 2006).

Bacillus licheniformis strain P40, isolated from the Amazon basin fish *Leporinus* sp., produces a BLS that may be used as a biopreservative for control of pathogenic bacteria such as *Listeria monocytogenes* and *Bacillus cereus* (Cladera-Olivera, Caron, & Brandelli, 2004). This BLS was also effective to combat soft rot development caused by *Erwinia carotovora* in potatoes (Cladera-Olivera, Caron, Motta, Souto, & Brandelli, 2006). In this report, the purification and chemical characterization of this BLS is described. The dual antimicrobial and emulsifying activity was tested in a meat model system.

2. Materials and methods

2.1. Microorganisms

The bacterium *B. licheniformis* P40, previously isolated and characterized (Cladera-Olivera et al., 2004) was used for production of BLS. *L. monocytogenes* ATCC 7644 was used as an indicator strain. The organisms were stored at −20 °C in Trypticase Soy Broth (TSB) medium (Acumedia, Lansing, MI, USA) containing 20% (v/v) glycerol. The bacteria were propagated twice in fresh TSB medium before use.

2.2. Assay of antimicrobial activity

Antimicrobial activity was monitored by disc diffusion assay as described elsewhere (Motta & Brandelli, 2002). Aliquots (20 μl) were applied onto cellulose discs on agar plates previously inoculated with a swab submerged in indicator strain suspension containing about 10^8 CFU ml^{-1}. Plates were incubated at 37 °C for 24 h and the clearing zones were measured around the discs. The BLS titre was determined by the serial 2-fold dilution method.

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2.3. Culture conditions and BLS production

B. licheniformis P40 was grown in TSB broth for 48 h at 30 °C and then culture supernatant was obtained by centrifugation at 10,000g for 15 min at 4 °C. The supernatant was filtered through a 0.22 μm membrane (Millipore, Billerica, MA, USA), and the filtrate was considered as crude BLS preparation.

2.4. BLS purification

The crude filtrate was submitted to ammonium sulfate precipitation at 20% saturation. After centrifugation at 10,000g for 20 min at 4 °C, the pellet was suspended in 100 mmol l^{-1} phosphate buffer pH 6.0 and applied to a column of Sephadex G-100 (Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 10 mmol l^{-1} phosphate buffer pH 6.0 as mobile phase. Fractions of 1 ml were collected. Fractions presenting antimicrobial activity were pooled and sterilized through a 0.22 μm filter membrane (Millipore, Billerica, MA, USA). These fractions were applied to a column of Source-RPC (Pharmacia Biotech), eluted with a gradient of 0–100% (v/v) acetonitrile (Merck, Darmstadt, Germany) containing 0.1% (v/v) trifluoroacetic acid (TFA) (Merck). The fractions positive for BLS activity were pooled, and stored at 4 °C until used.

2.5. Effect of enzymes, temperature, chemicals, and pH on antimicrobial activity

The effect of enzymes, chemicals, temperature, and pH on BLS activity was determined as described elsewhere (Bizani & Brandelli, 2002). Proteolytic enzymes tested were Pronase E, trypsin (Sigma, St. Louis, MO, USA), papain, and proteinase K (Merck). Organic solvents (acetone, chloroform, 1-butanol, ethanol, and toluene) were used at working concentrations of 50% (v/v). The detergents Tween 20, Tween 80, and Triton X-100 were tested in a final concentration of 10% (v/v) and sodium deoxycholate at 10 mg ml^{-1}). In the case of trichloroacetic acid (TCA), the BLS was previously incubated with the acid at 100 mg ml^{-1} for 2 h at 4 °C, centrifuged at 10,000g for 5 min and neutralized before to accomplish the test of antimicrobial activity. After the treatments, residual activity was determined against *L. monocytogenes* ATCC 7644.

2.6. Chemical characterization of BLS

Determination of free thiol groups was carried out essentially as described elsewhere (Sangali & Brandelli, 2000). To 1 ml sample were added 0.2 ml NH_4OH, 1 ml of 0.5 g l^{-1} NaCN and 1 ml water. The mixture was incubated for 20 min at 25 °C and then 0.2 ml of 0.5 g l^{-1} sodium nitroprusside was added. Absorbance at 530 nm was determined within 2 min. Free amino groups were determined by reaction with ninhydrin (Moore & Stein, 1957) before and after peptide hydrolysis with 6 mol l^{-1} HCl at 110 °C for 16 h. Samples of the peptide were also analyzed for reaction with 1-fluoro-2,4-dinitrobenzene (Goodwin, 1968). For determination of tryptophan, 1 ml sample was mixed with 2 ml $FeCl_3$–$HClO_3$ (0.01 mol l^{-1} $FeCl_3$ in 35% $HClO_3$). Absorbance at 530 nm was determined after 25 min at 25 °C (Minamisawa, Seki, Onodera, Kubota, & Asami, 1992).

2.7. Spectroscopy

The BLS was analyzed by UV–visible, infrared spectroscopy and mass spectroscopy. The FTIR spectrum was recorded as KBr pellet in a Mattson FTIR spectrometer (Thermo Fischer Scientific, Waltham, MA, USA). UV–visible spectrum was obtained using a Shimadzu UVX 1600 (Shimadzu, Columbia, MD, USA) double beam spectrophotometer. Mass spectra were recorded on a MALDI-TOF mass spectrometer (Waters-Micromass, UK).

2.8. Emulsifying activity

The capacity of BLS to emulsify several hydrophobic substances was tested. BLS was mixed with 10 ml of kerosene, soybean oil, olive oil, hexane, or toluene and allowed to stand for 24 h at room temperature (Willumsen & Karlson, 1997). After this time the emulsifying index (E_{24}) was determined by

$$E_{24} = \frac{\text{height of the emulsion layer}}{\text{height of the total layer}} \times 100 \quad (1)$$

2.9. BLS activity in meat model system

Beef (160 g) was mixed with ice water (90 g) and the emulsifier: BLS (10 ml, fraction from Sephadex G-100), isolated soy protein (1 g), or soy lecithin (1 g). The mixture was chopped for 4 min at 3000 rpm in a cutter. Approximately 10 g of each meat emulsion was placed into polypropylene tubes and centrifuged at 8000g for 15 min. The emulsion height before and after centrifugation was measured, and the emulsion stability (ES) was calculated as percentage of initial emulsion height. Other tubes were inoculated to give 10^3 CFU ml^{-1} *L. monocytogenes* and incubated for 10 days at 4 °C. Samples were homogenized in a blender for 2 min following by decimal serial dilutions before enumerating by plating in Oxford Listeria agar (Acumedia, Lansing, MI, USA). Three replicates of each sample were analyzed.

3. Results

BLS was produced by *B. licheniformis* P40 and the antimicrobial substance secreted to the culture supernatant was purified. The results of the purification are summarized in Table 1. The protocol resulted in a purification of 100-fold and a yield of 0.3%. Gel filtration chromatography resulted in an important step of purification since the activity eluted not coinciding with the main peak of protein (Fig. 1A). The BLS was further purified through the chromatographic column Source-RPC and only three column fractions showed antimicrobial activity, coinciding with a single peak of protein (Fig. 1B).

BLS activity was resistant to temperature (not shown) and pH (Fig. 2) Antimicrobial activity was quite resistant to the proteolytic enzymes trypsin, papain, and proteinase K, but not to Pronase E (Table 2). Among the chemicals tested, only butanol and trichloroacetic acid caused loss of BLS activity (data not shown).

The BLS reaction with ninhydrin resulted a pale yellow color instead the characteristic purple, which was observed after peptide hydrolysis. In addition, BLS was not reactive with FNDB, indicating the absence of free $-NH_2$. Negative reaction for –SH was also observed, as well as for tryptophan. However, the UV–visible spectrum indicates the presence of aromatic amino acids. Also, typical absorbance of peptide bonds at 220 nm was observed (data not shown).

BLS was analyzed by infrared and mass spectroscopy. The FTIR spectrum showed that the substance contains peptide bonds. Characteristic bands at 1540 and 1640 cm^{-1}, corresponding to amide II and amide I, respectively (C═O, C═N stretching), and at 3250 cm^{-1} (N–H stretching of proteins) were observed (Fig. 3A). In addition, the infrared spectrum revealed bands at 2850–2955 cm^{-1}, characteristic of aliphatic C–H stretching of fatty acids (Fig. 3A). The MALDI-TOF MS was chosen to measure the mass of the compound. The mass spectrum showed a homologous $[M+H]^+$ ion at *m/z* 803.5 (Fig. 3B).

The partial characterization of BLS P40 suggests that this substance exhibit amphiphilic properties. The putative emulsifying activity of this substance was then investigated. The BLS showed