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Phospholipid nanovesicles containing a bacteriocin-like substance for control of *Listeria monocytogenes*

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Abstract

Bacillus licheniformis strain P40 produces a bacteriocin-like substance (BLS) that has potential to be used as a natural biopreservative for control of pathogenic and food microorganisms. The objective of this study was the encapsulation of BLS in phosphatidylcholine vesicles, evaluating its antimicrobial activity against *Listeria monocytogenes*. The size of the nanovesicles with the BLS was around 570 nm and of the nanovesicles without BLS was of approximately 484.8 nm, as determined by light scattering with a He–Ne laser (λ=632.8 nm) as light source. The encapsulated BLS showed inhibitory activity against *L. monocytogenes* as observed by agar diffusion assay. Complete inhibition of *L. monocytogenes* growth was observed with the addition of 100 and 50 AU mL^{-1} of encapsulated and free BLS, respectively. A reduction in the number of viable cells to zero was observed after 10 min incubation with 400 AU mL^{-1} of either encapsulated or free BLS. The encapsulated BLS was stable for up to 30 days at 4 °C. These results indicate that nanovesicles containing BLS may have potential for use as food preservative.

Keywords: Bacteriocin; Nanovesicles; Encapsulation; *B. licheniformis*; *L. monocytogenes*

Industrial relevance: The increased concern on minimally processed food and natural additives has been stimulated many studies on the utilization of antimicrobial peptides as biopreservatives. The incorporation of bacteriocins into nanovesicles may represents an interesting alternative for controlled release and increased stability of bacteriocins.

1. Introduction

Nanovesicles represent promising systems for drug delivery and storage. These systems have acquired increasing attention as a tool to target drugs to its site of action or to optimize drug circulation *in vivo*, allowing reduction of side effects as well as making treatments easier. In the same way, nanovesicles can be used to protect a series of chemical compounds, being more commonly used for drugs. They can be also used to encapsulate molecules of larger sizes, like the polymer chitosan (Anderson, Burdick, & Langer, 2004; Drummond et al., 2005; Hashida, Kawakami, & Yamashita, 2005; Pavelic, Skalko-Basnet, Filipovic-Grcic, Martinac, & Jalsenjak, 2005). Recently, a number of potential applications of nanoparticles in food and related industries have been described (Sanguansri & Augustin, 2006).

Bacteriocins are proteinaceous compounds produced by bacteria, often presenting a bactericidal effect against closely related species (Riley & Wertz, 2002). Lactic acid bacteria have been largely studied with the perspective of food protection against pathogenic and spoilage microorganisms (Cleveland, Montville, Nes, & Chikindas, 2001; O'Sullivan, Ross, & Hill, 2002). Also, bacteriocins are 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; Pedersen, Bjornvad, Rasmussen, & Petersen, 2002). This genus presents a great variety of species that produce bacteriocins or bacteriocin-like substances (BLS),

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which display antimicrobial activity against food-borne pathogenic microorganism. These include *B. subtilis* (Zheng, Yan, Vederas, & Suber, 1999), *B. thuringiensis* (Kamoun et al., 2005), *B. amyloliquefaciens* (Lisboa, Bonato, Bizani, Henriques, & Brandelli, 2006) and *B. cereus* (Bizani & Brandelli, 2002).

B. licheniformis P40, isolated from the Amazon basin fish *Leporinus* sp., produces a BLS that may be used as a biopreservative for control of pathogenic and spoilage microorganisms such as *L. monocytogenes* and *Bacillus cereus* (Cladera-Olivera, Caron, & Brandelli, 2004). This BLS was effective to combat soft rot development caused by *Erwinia carotovora* in potatoes (Cladera-Olivera, Caron, Motta, Souto, & Brandelli, 2006). The aim of this work was to develop phosphatidylcholine (PC) nanovesicles containing the BLS produced by *B. licheniformis* P40 and to characterize its antimicrobial activity against *L. monocytogenes*.

2. Materials and methods

2.1. Production of antimicrobial substance

B. licheniformis strain P40 was grown in Trypticase Soy Broth (TSB) medium (Acumedia Manufacturers, Lansing, MI, USA) at 30 °C in a rotary shaker at 125 cycles min^{-1} for 48 h. Cells were harvested by centrifugation at 10,000 ×*g* for 15 min at 4 °C. Ammonium sulfate was added to the supernatant to achieve 20% (w/v) saturation and the mixture was kept at 4 °C for 18 h. After centrifugation at 10,000 ×*g* for 15 min at 4 °C, the pellet was suspended in 10 mM phosphate buffer pH 6.0 and immediately submitted to a gel filtration chromatography using a Sephadex G-100 matrix (Pharmacia Biotech, Uppsala, Sweden). The column was eluted with 10 mM phosphate buffer pH 6.0 as the mobile phase. Fractions of 1 mL were collected. Fractions presenting antimicrobial activity were pooled and sterilized through a 0.45 μm filter membrane. This fraction was used for encapsulation in phospholipid nanovesicles.

2.2. Production of nanovesicles

The nanovesicles were prepared by the reverse phase evaporation method as follows (Mertins, Cardoso, Pohlmann, & Silveira, 2006): 60 mg of phosphatidylcholine (95%; Solae, Esteio, Brazil) were dissolved in 10 mL of ethyl acetate and 200 μL of MilliQ water were dropped into the solution to form a water in oil (W/O) emulsion, which was sonicated at 400 Hz for 2–3 min at 30 °C, using a USC 700 ultrasonic processor (Unique, São Paulo, Brazil). This procedure yielded a homogeneous opalescent dispersion of reverse micelles. The organic solvent was taken out using a rotatory evaporator (Fisatom, São Paulo, Brazil) at 30–35 °C under vacuum, resulting in a high viscous organogel. The organogel was reverted to nanovesicles with the addition of 5 mL of MilliQ water under shaking. Samples were filtered through 0.45 μm pore membranes and placed into dust free cells for light scattering measurements. The nanovesicles containing bacteriocin (NCB) were prepared by the same procedure, adding 50, 100 or 200 μL of the BLS solution (0.98 mg protein mL^{-1}) plus MilliQ water to a final volume of 200 μL. The concentration of BLS entrapped in the nanovesicles was determined using the Folin-phenol reagent method (Lowry, Rosebrough, Farr, & Randall, 1951), using a calibration curve developed with bovine serum albumin as protein standard.

2.3. Light scattering

Light scattering was performed on a Brookhaven BI-200M goniometer, BI-9000AT digital correlator (Brookhaven Instruments, Holtsville, NY, USA) with a He–Ne laser ($\lambda = 632.8$ nm) as light source. An interference filter was used before detecting the signal on the photomultiplier. The sample cell was placed in the index-matching liquid decahydronaphthalene (Sigma-Aldrich, Saint Louis, MO, USA).

The apparent values of hydrodynamic radius R_h (related to the diffusional dynamics of a vesicle) were obtained in this work by Dynamic Light Scattering (DLS) at 90°. The time correlation functions were measured in the multi-τ mode using 224 channels and data treatment was made by means of the Provencher Software CONTIN as described elsewhere (Mertins et al., 2006). The R_h determination was made by means of the Stokes–Einstein relation using the diffusion coefficient D_{app} determined by Eq. (1):

$$D_{app} = \frac{k_B T}{6\pi\eta_0 R_h} \quad (1)$$

where k_B the Boltzmann constant, η_0 the viscosity of the solvent, T the absolute temperature and R_h the hydrodynamic radius.

2.4. Antimicrobial activity assay

The antimicrobial activity was detected by agar diffusion assay. An aliquot of 10 μL of the purified free BLS, NCB and nanovesicles free of bacteriocin were applied on agar plates previously inoculated with a swab submerged in indicator strain (*L. monocytogenes* ATCC 7644) suspension, which corresponded to a 0.5 McFarland turbidity standard solution, (approximately 10^7 CFU mL^{-1}). Plates were incubated at 37 °C for 24 h. The reciprocal value of the highest dilution that produced an inhibition zone was taken as the activity unit (AU) per mL (Motta & Brandelli, 2002).

To verify the antimicrobial activity of the NCB, an aliquot of 10 μL was applied in the plate previously inoculated with *L. monocytogenes*. The NCB were also submitted to heating for 100 °C for 3 min before being tested for antimicrobial activity. The purified free BLS was used as positive control, and nanovesicles free of bacteriocin as negative control.

2.5. Mode of action on L. monocytogenes ATCC 7644

Sterile 96-well microplates (Corning, NY, USA) were filled with 0.1 mL of serial dilutions of the NCB (between 6.25 and 400 AU mL^{-1}). The NCB were diluted in 10 mM phosphate buffer pH 6.0 from a 3200 AU mL^{-1} stock solution. A