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Purification of leucocin A for use on wieners to inhibit *Listeria monocytogenes* in the presence of spoilage organisms

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ABSTRACT

The aims of this study were to improve the method for purification of leucocin A to increase yield of peptide and to evaluate the efficacy of leucocin A and an analogue of leucocin A (leucocin N17L) to inhibit the growth of *Listeria monocytogenes* on wieners in the presence of spoilage organisms. Leucocin A was produced by *Leuconostoc gelidum* UAL187 and purified with a five-fold increase in yield; leucocin N17L was synthesized replacing asparagine at residue 17 with leucine. Five strains of *L. monocytogenes* associated with foodborne illness were used to assess bacteriocin efficacy *in vitro* and *in situ*. Minimum inhibitory concentrations could not be determined in broth; however, on agar the minimum inhibitory concentrations ranged from 11.7–62.5 μM and 62.5– > 500 μM for leucocin A and leucocin N17L, respectively. Leucocin N17L was less effective than the native bacteriocin at controlling the growth of *L. monocytogenes*. The inactivation profiles of *L. monocytogenes* in broth in the presence of leucocin A suggested each isolate had different levels of resistance to the bacteriocin as determined by the initial bactericidal effect. The formation of spontaneously resistance subpopulations were also observed for each strain of *L. monocytogenes*. *In situ*, wieners were inoculated with the spoilage organisms, *Carnobacterium divergens* and *Brochothrix thermosphacta*, followed by surface application of purified leucocin A, and inoculated with a cocktail of *L. monocytogenes*. Wieners were vacuum packaged and stored at 7 °C for 16 d. Leucocin A reduced the counts *L. monocytogenes* on wieners during storage, regardless of the presence of *C. divergens*. *B. thermosphacta* was unaffected by the presence of leucocin A on wieners over the duration of storage. This study suggests that leucocin A may be beneficial to industry as a surface application on wieners to help reduce *L. monocytogenes* counts due to post-processing contamination even in the presence of spoilage organisms. However, further investigation on the ability of *L. monocytogenes* to form spontaneous resistance to class II bacteriocins on food matrices during prolonged storage is warranted.

1. Introduction

Listeria monocytogenes is associated with ready-to-eat (RTE) products due to post-processing contamination (Flint et al., 2005). *L. monocytogenes* typically affects immunocompromised, elderly or pregnant individuals (Health Canada, 2011) and has a fatality rate of 20–40% (Drevets and Bronze, 2008; Farber and Peterkin, 1991; Gálvez et al., 2008). *L. monocytogenes* can grow over a wide range of temperatures, including refrigeration temperatures (Health Canada, 2011). This poses a concern for the safety of RTE meat products, as they typically are not subjected to an additional heating step prior to consumption (Zhu et al., 2005). Strict guidelines have been developed to control *L. monocytogenes* in RTE products (Health Canada, 2011). With consumer demands for the food industry to increase availability of fresh, RTE and minimally processed products, research on novel methods of biopreservation is needed. The potential of biopreservatives to meet consumer demands and maintain product integrity have been demonstrated against a diverse group of foodborne pathogens, including *L. monocytogenes* (Gálvez et al., 2010).

Biopreservatives are antimicrobial products of living organisms (Gálvez et al., 2010) and include bacteriocins, which are ribosomally synthesized either with or without posttranslational modification, are excreted out of the cell and have a narrow-spectrum of activity (Cotter et al., 2005). Nisin has been approved for use in foods in over 50 countries (Gálvez et al., 2008). However, nisin cannot be used with raw meat as it reacts with glutathione, which inactivates the peptide (Rose et al., 1999). Class IIa bacteriocins have antilisterial activity on meats and can be applied to RTE meat products (Zhu et al., 2005).

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Leucocin A is a plasmid mediated, 37 amino acid residue Class IIa bacteriocin with a molecular mass of 3930 Da that is produced by *Leuconostoc gelidum* UAL187 (Hastings and Stiles, 1991). *Leuc. gelidum* UAL187 was isolated from chilled, processed meat stored in an atmosphere containing CO_2 (Hastings and Stiles, 1991) making it an ideal candidate for application on RTE meat products. Leucocin A is a good candidate for use on RTE meat products due to its stability under extreme conditions (100 °C for 20 min at pH 2–3); however, it is degraded by various proteases (Hastings and Stiles, 1991). Class IIa peptides have a conserved, charged, hydrophilic N-terminus motif of $YGNGV(X)C(X)_4C(X)V(X)_4A$ (X denotes any amino acid residue) that forms a β-sheet structure (Bodapati et al., 2013; Fregeau Gallagher et al., 1997), which allows for electrostatic interactions association between the target cell surface and the bacteriocin (Fimland et al., 2005). The cysteine residues (located at positions 9 and 14 in leucocin A) form a disulphide bridge that is important for proper conformation and antibacterial activity (Fregeau Gallagher et al., 1997; Sit et al., 2011). The more hydrophobic C-terminus residues are suspected to play a role in receptor protein interactions allowing for receptor mediated recognition mechanisms to elucidate antibacterial activity (Kaur et al., 2004). The mode of action is based on pore formation and ion disruption of the target cell (Kaur et al., 2004). The antilisterial activity and inhibitory action against a range of other lactic acid bacteria, including *Enterococcus faecalis* (Hastings and Stiles, 1991) make leucocin A a candidate for further investigation for preservation use in RTE meat products. However, proteolytic cleavage of leucocin A on wieners at residue 17 between asparagine and tryptophan has been observed and neither portion of the peptide had activity against *L. monocytogenes* (unpublished data, Kaur). To prevent fragmentation, a synthetic analogue (leucocin N17L) was synthesized where the asparagine was replaced by a leucine.

The objectives of this research were to purify sufficient quantities of leucocin A and to use the purified leucocin A in experiments to determine the efficacy of leucocin A to inhibit the growth of *L. monocytogenes* in the presence of natural spoilage microbiota on vacuum packaged RTE meat. The spoilage organisms of interest were *Brochothrix thermosphacta* and *Carnobacterium divergens*. A variant of leucocin A was synthesized to determine if activity against *L. monocytogenes* could be increased.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Strains of *Listeria monocytogenes* (Table 1) developed as a cocktail for challenge tests were obtained from Cornell University (Fugett et al., 2006). *Carnobacterium divergens* UAL9, a sensitive indicator for leucocin A, was obtained from the University of Alberta Lactic Acid Bacteria culture collection. *Brochothrix thermosphacta* strains A401, B401, P104, P107 were previously isolated from RTE meat products (Miller et al., 2014). All culture strains were stored in 30% glycerol in media at − 80 °C until needed.

Prior to use in experiments, cultures were streaked onto appropriate culture media, and a single colony was inoculated into broth media. *Leuc. gelidum* UAL187 was grown anaerobically at 25 °C in semi-defined casamino acids (CAA) media (Hastings et al., 1991). Strains of *L. monocytogenes* were grown aerobically at 37 °C in tryptic soy broth (TSB; Becton, Dickinson and Company, New Jersey, USA). Strains of *C. divergens* and *B. thermosphacta* were grown anaerobically and aerobically, respectively, at 25 °C in All Purpose Tween (APT; Becton, Dickinson and Company).

Table 1
Strains of *L. monocytogenes* (Fugett et al., 2006).

L. monocytogenes	Lineage	Serotype	Origin
FSL C1-056	II	1/2a	Isolated from human sporadic case '98
FSL J1-177	I	1/2b	Isolated from human sporadic case '97
FSL N3-013	I	4b	Isolated human listeriosis epidemic '88–'90
FSL R2-499	II	1/2a	Human isolate associated with US outbreak '00
FSL N1-227	I	4b	Food isolate associated with US outbreak (Bilmar, wiener outbreak '98–'99)

2.2. Production and purification of leucocin A

Leucocin A was purified using a modified protocol of Hastings et al. (1991). To produce leucocin A, *Leuc. gelidum* UAL187 was grown in CAA media without Tween® 80 (initial pH of 6.5; 2% v/v inoculum), at 25 °C for 24 h under anaerobic conditions. For purification, the spent culture broth was not centrifuged after fermentation and the entire cell suspension was loaded onto a column packed with 60 g/L Amberlite® XAD®-2 (Supelco, Bellefonte, PA, USA) resin equilibrated with trifluoroacetic acid (TFA; 0.1% v/v) at 5 mL/min. The column was washed sequentially with 500 mL each of sterile distilled water (dH_2O) 10 mL/min, ethanol (EtOH) (20% v/v) 10 mL/min and EtOH (40% v/v) 10 mL/min. Leucocin A was eluted with 800 mL EtOH (75% v/v) with TFA (0.1% v/v) at 5 mL/min. The eluent was concentrated on a Buchi® rotary evaporator (Brinkman Instruments, Westbury, NY, USA) at 30 °C under vacuum and lyophilized (Labconco® FreezeZone, Kansas City, MO, USA). Lyophilized samples were washed with 1 mL of 100% acetonitrile (ACN) two times to remove residual colour compounds. Washed samples were solubilized in TFA (0.1% v/v) for further purification using reversed-phase high performance liquid chromatography (RP-HPLC; Prostar 210, Varian Inc., Palo Alto, CA, USA). Samples were injected onto a semi-preparative C18 column (Grace™ Vydac™, Fisher Scientific, Edmonton; 10 mm × 250 mm, particle size 5 μm) and eluted with a gradient of 15% to 85% ACN-TFA 0.1% (v/v) dH20 with a flow rate of 1 mL/min, leucocin A was eluted at ~28.5 min. Leucocin A identity was confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF). Pure leucocin A was lyophilized and stored at − 20 °C until needed for applied experiments on meat, at which point it was suspended in TFA (0.1% v/v) to the desired concentration.

Peptides were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a UHPLC system (Waters nanoAquity Waters, Milford, MA) coupled to a QTOF analyser (Water Premier, Waters). The sample (5 μL) was loaded onto a nano trap cartridge (PepMap C18, 300 μm × 1 mm, Thermo Scientific, Sunnyvale, CA) coupled to a nano analytical column (75 μm × 150 mm, 100 Å, PepMap C18 column, Thermo Scientific). Desalting on the peptide trap was achieved by flushing the trap with 99% eluent A (0.1% formic acid in water) and 1% eluent B (0.1% formic acid in acetonitrile) at a flow rate of 10 μL/min for 3 min. Peptides were separated at a flow rate of 350 nL/min with 2% eluent B for 3 min, a gradient of 2 to 10% eluent B over 3 min, a gradient of 10 to 98% eluent B over 42 min, 98% eluent B for 10 min and 2% eluent B for 5 min. Analysis was done manually using the MassLynx software supplied by Waters. Pure leucocin A was lyophilized and stored at − 20 °C until needed, at which point it was suspended in TFA (0.1% v/v) to the desired concentration.

2.3. Synthesis of leucocin N17L

Leucocin N17L (Fig. 1) was synthesized as a 37 amino acid peptide using previously standardized solid phase method (Bodapati et al., 2013) with 0.2 mM equivalent of Wang resin. During synthesis, a leucine was substituted for an asparagine at residue 17. The synthesis was carried out using an automatic peptide synthesizer following double coupling procedures. The pure peptide was subjected to oxidative folding (disulphide formation) by air oxidation in freshly degassed Tris buffer (50 mM, pH 8.3) solution, with gentle stirring in an