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Inhibition of multidrug resistant *Listeria monocytogenes* by peptides isolated from combinatorial phage display libraries

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ABSTRACT

The aim of the study was to isolate and characterize novel antimicrobial peptides from peptide phage library with antimicrobial activity against multidrug resistant *Listeria monocytogenes*. Combinatorial phage-display library was used to affinity select peptides binding to the cell surface of multidrug resistant *L. monocytogenes*. After several rounds of affinity selection followed by sequencing, three peptides were revealed as the most promising candidates. Peptide L2 exhibited features common to antimicrobial peptides (AMPs), and was rich in Asp, His and Lys residues. Peptide L3 (NSWIQAPDTKSI), like peptide L2, inhibited bacterial growth *in vitro*, without any hemolytic or cytotoxic effects on eukaryotic cells. L1 peptide showed no inhibitory effect on *Listeria*. Structurally, peptides L2 and L3 formed random coils composed of α-helix and β-sheet units. Peptides L2 and L3 exhibited antimicrobial activity against multidrug resistant isolates of *L. monocytogenes* with no haemolytic or toxic effects. Both peptides identified in this study have the potential to be beneficial in human and veterinary medicine.

1. Introduction

Listeria monocytogenes is a widely distributed pathogen causing arthritis and severe central nervous system infection collectively referred as listeriosis. Listeriosis represents a public health problem as it is fatal in up to 30% of all reported cases, and it has been widely associated with food-borne epidemics during the last decade (Farber and Peterkin, 1991; Ramaswamy et al., 2007). *Listeria* is a facultative intracellular pathogen that replicates in the cytosol of eukaryotic non-phagocytic cells (Cossart and Kocks, 1994; Ramaswamy et al., 2007). *Listeria* are tolerant to extreme conditions such as low pH, low temperature and high salt concentrations, and can be found in a variety of environments (Van Renterghem et al., 1991; MacGowan et al., 1994; Sleator et al., 2003). In veterinary medicine, *L. monocytogenes* is an unwelcome pathogen responsible for significant economic losses at the farm level due to the high morbidity and mortality of animals (Oevermann et al., 2010). In ruminants (cattle, sheep and goats) *L. monocytogenes* is mostly acquired through the consumption of contaminated feed and food; and manifests itself as central nervous system (CNS) disease and uterine infections (Low and Donachie, 1997; Kathariou, 2002). Conversely, contaminated animal-derived food products which are not processed before consumption (*e.g.*, raw food) represent a direct link between human infections, and *L. monocytogenes* in farm animals and the environment (Nightingale et al., 2004). In humans, *Listeria* affects preferentially individuals with impaired cell-mediated immunity, pregnant women and the elderly (Wilson and Drevets, 1998). Experimental data show that *L. monocytogenes* is able to invade and infect the CNS through several different mechanisms and can result in meningitis, meningoencephalitis, rhombencephalitis, cerebritis and brain abscess (Wilson and Drevets, 1998).

In general, listeriosis is treated with ampicillin and Bactrim (trimethoprim-sulfamethoxazole), which are considered as first-line drugs for invasive diseases, while trimethoprim-sulfamethoxazole is recommended in the case of penicillin allergy (Gyurko et al., 2000). Shortly after the initial detection of the first multidrug resistant clinical strain of *L. monocytogenes*, other strains of *Listeria* spp. resistant to one or more antibiotics were reported (Poyart-Salmeron et al., 1990; Charpentier et al.,

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1995; Aureli et al., 2003; Li et al., 2007). Currently, food-borne and clinical isolates of *Listeria* have shown resistance to multiple antibiotics including trimethoprim, gentamicin, streptomycin, erythromycin, kanamycin, sulfamethoxazole and rifampin (Roberts et al., 1996; Charpentier and Courvalin, 1997; Purwati et al., 2001; Christensen et al., 2011). Multidrug resistance of *L. monocytogenes* has been linked to the presence of a self-transferable plasmid or by conjugative transposons both originating in *Enterococcus* and *Streptococcus* (Poyart-Salmeron et al., 1990; Doucet-Populaire et al., 1991).

Short cationic peptides with antimicrobial and/or immunomodulatory activity represent a promising alternative to traditional antibiotics. Antimicrobial peptides have been found in multicellular organisms where they constitute part of the innate immunity (Boman, 1991; Gudmundsson et al., 1996; Zasloff, 2002; Radek and Gallo, 2007; Schauber and Gallo, 2008). The mode of antimicrobial action of these defence peptides is related to the disruption of cell membrane or interaction with intracellular components (Hale and Hancock, 2007; Mihajlovic and Lazaridis, 2010), resulting in inhibition of cell-wall, nucleic-acid and protein synthesis or inhibition of enzymatic activity (Cudic and Otvos, 2002; Nicolas, 2009). The advantage of antimicrobial peptides as defence weapons consists in their non-specific mechanism of action, which makes it difficult for microbial pathogens to develop resistant mutants to overcome peptide intervention.

Previously, it was shown that *L. monocytogenes* was strongly inhibited by human defensins, protamine and magainin, as well as plant-derived peptides such as thionin and snaking (Lopez-Solanilla et al., 2003). Listeriocidal activity has also demonstrated for the novel antibiotic Khal. Khal was derived from the AMP halocidin, and is capable penetrating the host cell without causing membrane damage, and attack intracellular resident *Listeria*. *In vivo* experiments clearly demonstrated that intravenous administration of Khal has significant therapeutic effect on the survival of infected animals (Jang et al., 2007). The high susceptibility of *Listeria* to antimicrobial peptides opens the possibility of using such molecules in therapeutic strategies, and in food preservation (Lopez-Solanilla et al., 2003).

Combinatorial phage-display is a powerful tool for the selection of peptides binding to a cell surface, and has been used to generate therapeutic peptides (Kay and Castagnoli, 2003; Bishop-Hurley et al., 2005; Fang et al., 2006; Bishop-Hurley et al., 2010). In this contribution, combinatorial phage display was used to isolate peptides binding to the outer surface of *L. monocytogenes*. Bound phage clones were sequenced and based on their percent occurrence three peptides were selected and tested for their antimicrobial activities *in vitro*. In further analyses, the listeriocidal properties of the L2 and L3 peptides were confirmed. The L2 and L3 peptides share a number of features common to AMPs, and have the potential to play a significant role in the development of therapeutic strategies against multidrug resistant *Listeria*.

2. Material and methods

2.1. *Cultivation of* L. monocytogenes

Multidrug resistant *L. monocytogenes* isolated from the cerebrospinal fluid was obtained from Palacky University in Olomouc, Czech Republic. *Listeria* was grown on blood agar overnight at 37 °C and then in the enrichment broth (Oxoid, Slovakia). The culture centrifuged and bacterial cells were counted using flow cytometer (FACS single-laser flow cytometer Accuri™ C6, Becton-Dickinson, Heidelberg, Germany). The suspension was then diluted according to experimental procedure.

2.2. *Selection of phages with affinity to* L. monocytogenes

Phage display library Ph.D-12 (New England Biolabs, Frankfurt am Main, Germany) (2×10^{10} PFU) was incubated with *L. monocytogenes* (4×10^5 cells) in 100 μl LB medium at 37 °C for 1 h with gentle shaking. After incubation, the culture was centrifuged at 2500*g* for 15 min at room temperature, and unbound phages were washed with PBS-T (PBS containing 0.1% Tween 20) for at least three times. The bound phages were eluted with 0.2 M Glycin-HCl (pH 2.2). Eluate containing phages with affinity to the bacteria was neutralised with 1 M Tris–HCl (pH 9.0) and the phages were separated from bacterial cells by centrifugation at 2500*g* for 15 min at room temperature. Supernatant containing phages was recovered, and phages were amplified in *Escherichia coli* ER 2738 (New England Biolabs, Frankfurt am Main, Germany) for 4.5 h at 37 °C by shaking. Amplified phages were precipitated from cleared *E. coli* culture with polyethylene-glycol precipitation (20% PEG8000 in 2.5 M NaCl) as per manufacturer's instructions (New England Biolabs, Frankfurt am Main, Germany). Four rounds of the biopanning were performed. In each round of biopanning the titer of the phages was determined, and the efficiency was evaluated.

2.3. *Isolation of phage DNA and sequencing*

Phages from the fourth round of bioapanning were used to prepare phage stocks to isolate genomic DNA and subsequent nucleotide sequencing. The procedure is described in details in manufacturer's instructions (Ph.D-12, New England Biolabs, Frankfurt am Main, Germany). DNA sequencing of isolated phage clones was performed using an Applied BiosystemsAvant 3010 automated sequencing system with the ABI BigDye™ Terminator sequencing kit (Life Technologies, Bratislava, Slovakia). The DNA sequences were translated into amino acid sequences (Biomatters, San Francisco, USA).

2.4. *Peptide sequence analysis using bioinformatic tools*

Physicochemical properties of the peptides (Molecular weight, pI) were predicted using Geneious software. Hydrophobicity, net charge and sequence similarity with existing peptides deposited in the repository was performed using Antimicrobial Peptide Database server (http://aps.unmc.edu/AP/main.php). Homology modelling was done using the package MODELLER (https://salilab.org/modeller/) (Modeller, San Francisco, USA). Biotinylated form of peptides was synthesized commercially (Shafer-N, Copenhagen. Denmark).

2.5. *Conformation analysis of peptides by CD spectrometry*

The secondary structure of peptides was assessed by CD spectrometry using JASCO J-810 (JASCO, Easont, USA). Peptides were diluted in 50 mM phosphate buffer (50 mM Na_2HPO_4 + 50 mM NaH_2PO_4) pH 7.4 to a final concentration of 300 μM. The measurements were recorder using JASCO J-810CD spectrometer (JASCO, Easont, USA) under nitrogen gas flow of 8 l/h at a temperature of 25 °C. Spectra were recorded between 190 and 270 nm, using a 1 mm cuvette at a scan speed of 50 nm/min. We performed an average of five measurements for each sample. The average absorption was corrected by buffer and then baseline to zero using the average of readings between 190 and 270 nm.

2.6. *ATR (attenuated total reflectance) Fourier transform infrared spectroscopy of peptides*

Synthesized peptides were purchased from Schafer-N (Denmark). Peptides were dissolved in ultra-pure water to