Food Microbiology 31 (2012) 133–136

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Short communication

Isolation and lytic activity of the *Listeria* bacteriophage endolysin LysZ5 against *Listeria monocytogenes* in soya milk

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ARTICLE INFO

Article history:
Received 15 June 2011
Received in revised form
18 January 2012
Accepted 23 January 2012
Available online 30 January 2012

Keywords:
Listeria phage
Endolysin
Biocontrol

ABSTRACT

The endolysin gene (*lysZ5*) from the genome of the *Listeria monocytogenes* phage FWLLm3 was cloned in *Escherichia coli* and characterized. Comparative sequence analysis revealed that *lysZ5* resembled the murein hydrolase *ply511* encoded by *L. monocytogenes* phage A511. The encoded protein LysZ5 had a predicted molecular mass of 35.8 kDa and was expressed in *E. coli* as an N-terminal fusion protein of 41.5 kDa. Addition of purified fusion protein to lawns of indicator bacteria showed that LysZ5 could lyse *L. monocytogenes*, *Listeria innocua* and *Listeria welshimeri,* but not *Staphylococcus aureus* or *Enterococcus faecalis*. The purified protein was able to kill *L. monocytogenes* growing in soya milk, with the pathogen concentration reduced by more than 4 $\log_{10}$ CFU ml^{-1} after 3 h incubation at 4 °C. As far as we know, this is the first report of a *Listeria* phage endolysin to control pathogens in soya milk and to demonstrate endolysin activity in foods at refrigeration temperatures. Moreover, LysZ5 may also be useful for biocontrol in other ready-to-eat foods.

1. Introduction

Listeria monocytogenes, a pathogen of humans and animals, has been implicated in several outbreaks and sporadic cases of listeriosis, resulting in numerous food product recalls. For example in 2008, New York food company Gourmet Boutique recalled 286,320 pounds of beef, pork, turkey and chicken products due to possible *Listeria* contamination (Peggy, 2008). In 2010, the Louisiana Department of Agriculture and Forestry coordinated a voluntary recall of approximately 500,000 pounds of hogs-head cheese and sausage because of possible contamination with *L. monocytogenes* (Centers for Disease Control and Prevention, 2010). Canadian company Maple Leaf Foods was associated with an outbreak of listeriosis that eventually led to 22 deaths and country-wide cases of illness in 2008 (Mason, 2009).

In China, there have not been any reported recalls of foods due to *L. monocytogenes* contamination. However, a survey of foods in China reported the prevalence of *L. monocytogenes* to be 7.1% in raw meats and 11.7% in cooked meats (Chao et al., 2007). Feng et al firstly detected *L. monocytogenes* from bean food (Feng et al., 2007), then Liu et al isolated 2 *L. monocytogenes* from 20 bean food samples in FuZhou (Liu et al., 2007). The contamination situation of *L. monocytogenes* on bean food is rather severe. The surveillance on food should be strengthened. However, soya milk, which is a popular breakfast food in China and usually stored in the refrigerator was one of the main food in bean products .The foods at highest risk of *L. monocytogenes* contamination are cold ready-to-eat foods that are not heated before consumption. This is due to the organisms' ability to grow at refrigeration temperatures (as low as −1.5 °C) (Hudson et al., 1994) and to tolerate low pH and high salt concentrations. *Listeria* is therefore well equipped to survive food-processing technologies and storage conditions, and can become endemic in food processing and storage environments.

The use of naturally occurring, virulent phages to reduce contamination of foods has advantages over the use of chemical preservatives (Greer, 2005; Hudson et al., 2005). Phage endolysins are enzymes that cleave bonds in the peptidoglycan of the bacterial cell wall. They are encoded by the phage genome and synthesized at the end of the phage lytic life cycle to release the newly produced virions from the host cell. Besides this "lysis from within", endolysins from phages of Gram-positive hosts are also able to rapidly lyse bacterial cells when they are applied exogenously (Loessner, 2005). It is thought that there is a low probability of developing resistance against the activity of endolysins as they target unique

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doi:10.1016/j.fm.2012.01.005

and highly conserved bonds in the peptidoglycan (Loeffler et al., 2003). Recombinant phage endolysins have been reported to inhibit a variety of pathogens and are under development as alternative antimicrobials for treatment of bacterial infections caused by Gram-positive bacteria (Gu et al., 2011). For biocontrol of phage or endolysin, Soni et al illustrated that the GRAS phage LISTEX P100 was useful in quantitatively reducing *L. monocytogenes* on raw salmon fillets (Soni and Nannapaneni, 2010a) and fresh channel catfish fillets (Soni et al., 2010b). Obeso et al demonstrated that purified LysH5 endolysin could kill *Staphylococcus aureus* growing in pasteurized milk and the pathogen was not detected after 4 h of incubation at 37 °C (Obeso et al., 2008); García et al reported the synergy between LysH5 and nisin to kill *S. aureus* in pasteurized milk also (García et al., 2010).

So far, a few *Listeria* phage endolysins have been characterized, such as those of phages A500, A511 (Loessner et al., 1996). However, none of them has been tested as biopreservative in soya milk; which is a popular breakfast food in China and usually stored in the refrigerator. In this work, we have cloned and overexpressed a *Listeria* phage endolysin, LysZ5, in *Escherichia coli* and its antimicrobial activity against *L. monocytogenes* evaluated in soya milk.

2. Materials and methods

2.1. Bacteria, phage and growth conditions

Bacteria used in this study are shown in Table 1. *L. monocytogenes*, *Listeria innocua* and *Listeria welshimeri* were provided by the Guangdong Culture Collection Center (GCCC). *L. monocytogenes* isolates *Lm*002, *Lm*003, *Lm*004, *Lm*005, *Lm*006, *Lm*007, *Lm*008, NJ 2–4, NJ 05, NJ 2009020, NJ 09–14, XZ 58 and XZ 056 were kindly provided by Dr. Chao (Yangzhou Center for Disease Control and Prevention (YCDC), Yangzhou, China). *L. monocytogenes* isolate 2000/47 was from a listeriosis outbreak associated with ready-to-eat meats in New Zealand (Sim et al., 2002) and *Listeria* phage FWLLm3 was previously isolated from ruminant feces in Christchurch, New Zealand (Lee, 2008). *E. coli* BL21 (DE3) and DH5α was purchased from Stratagene (La Jolla, CA, USA) and used to express the endolysin gene, transformants were selected with 100 μg ml^{-1} Kanamycin.

Table 1
Bacterial strains.

Strain	Serogroup	Source (Supplied)
L. monocytogenes GIM1.228	1/2a	Unknown (GCCC)
Lm R-2	1/2a	Raw meat isolate (YCDC)
Lm R-6	1/2b	Raw meat isolate (YCDC)
Lm002	Not tested	Raw meat isolate (YCDC)
Lm003	Not tested	Raw meat isolate (YCDC)
Lm004	Not tested	Raw meat isolate (YCDC)
Lm005	Not tested	Raw meat isolate (YCDC)
Lm006	Not tested	Raw meat isolate (YCDC)
Lm007	Not tested	Raw meat isolate (YCDC)
Lm008	Not tested	Raw meat isolate (YCDC)
NJ 2–4	Not tested	RTE[a] food isolate (YCDC)
NJ 05	Not tested	RTE food isolate (YCDC)
NJ 2009020	Not tested	RTE food isolate (YCDC)
NJ 09–14	Not tested	RTE food isolate (YCDC)
XZ 58	Not tested	RTE food isolate (YCDC)
XZ 056	Not tested	RTE food isolate (YCDC)
L. monocytogenes 19114	4a	Ruminant (ATCC)
L. monocytogenes 2000/47	1/2	Clinical (NZERL)
L. innocua GIM 1.230	6a	Unknown (GCCC)
L. welshimeri GIM 1.232	6a	Unknown (GCCC)
Staphylococcus aureus 25923	Unknown	Clinical (ATCC)
Enterococcus faecalis 29213	Unknown	Clinical (ATCC)

GCCC: Guangdong Culture Collection Center. YCDC: Yangzhou Center for Disease Control and Prevention. ATCC: American Type Culture Collection. NZERL: New Zealand Enteric Reference Laboratory.
[a] Ready-To-Eat.

2.2. DNA manipulations

Phage DNA was extracted and purified using the lambda phage genome DNA extraction kit (Biomed, Beijing, China). The DNA was digested with *Nco*I (Thermo Fisher Scientific, Waltham, MA, USA) and random fragments were cloned in pUM19-T (Biouniquer Technology Co. Ltd, Nanjing, China) in *E. coli* DH5α. Sequence data obtained was BLAST (Altschul et al., 1990) searched against the NCBI nucleotide database to reveal similarity matches. Phylogenetic analysis of *lysZ5* was conducted in MEGA4 (Tamura et al., 2007) using the *Listeria* phage sequences AJ312240 (PSA) (Zimmer et al., 2003), DQ003637 (A500) (Dorscht et al., 2009), DQ003638 (A511)(Klumpp et al., 2008), DQ003639 (B025)(Dorscht et al., 2009), DQ777769 (phiLm4) (Gasson and Payne, 2006) and EU855793 (P40) (Dorscht et al., 2009). The phylogeny was constructed by maximum parsimony with 1000 replicates and evolutionary distances computed using the Poisson correction method. All positions containing gaps and missing data were eliminated from the dataset.

2.3. Cloning and overexpression of the recombinant endolysin gene *lysZ5*

A 1005-bp DNA segment containing the predicted *lysZ5* open reading frame was amplified with the primers M5-1: 5′-A TAT GGA TCC ATG GTA AAA TAT ACC-3′ and M5-2: 5′-A AGT AAG CTT TTA TTT CTT GAT AAC TGC TCC-3′ and the product digested with the restriction enzymes *Eco*RI and *Hind*III (Thermo Fisher Scientific). The amplification product was cloned in pUM19-T vector to confirm the sequence and in vector pET 28a (+) (Novagen, Madison, WI, USA) for expression of the recombinant protein in *E. coli* BL21 (DE3). Exponentially growing BL21 (DE3)-plysZ5 (OD_{600} 0.6–0.8) were induced with 1 mM IPTG (Biomed, Beijing, China), followed by 18 h shaking at 28 °C. Cells were pelleted at 8000 *g* for 15 min at 4 °C, washed with PBS, and sonicated (Sonics & Materials Inc. Newtown, CT, USA; VCX 130PB) five times (15 × 5 s pulses, with 15 s recovery on ice). The lysates were centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatant was added to 1 ml Ni-Sepharose High Performance slurry and eluted according to the manufacturer's instructions (HisTrap HP 1 ml column, GE Healthcare, Buckinghamshire, UK). Fractions (10 μl) of purified LysZ5 were subjected to 12% (w/v) SDS-PAGE using the manufacturers (Bio-Rad Laboratories, Hercules, USA) recommended electrophoresis conditions. A 14.4–94.4 kDa molecular weight marker (Biouniquer Technology Co. Ltd) was used as standard. Protein was visualized by Coomassie staining (0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) isopropanol and 10% (v/v) acetic acid), and destained with a solution of 5% (v/v) ethanol and 10% (v/v) acetic acid. Protein was quantified by the Quick Start Bradford Protein Assay (Bio-Rad).

2.4. Identification of lytic activity and lytic spectrum

L. monocytogenes ATCC 19114 was grown to optical density (OD_{600}) = 1, centrifuged, and suspended in 50 mM phosphate buffer (pH 7.0), to OD_{600} = 1. Bacterial suspensions (0.1 ml) were added to serial dilutions of purified LysZ5 (0.1 ml) in sterile, uncoated polystyrene 96-well plates, and the decrease in OD_{600} was monitored every 2 min for 20 min, at 25 °C, in a PowerWave XS Microplate Spectrophotometer (BioTek, Winooski, VT, USA). The activity of LysZ5, expressed as units per milliliter (U ml^{-1}), was defined as the reciprocal of the highest dilution that decreased the OD by 50% at 15 min. The lytic spectrum of LysZ5 was determined by adding 100 μl endolysin (50 μg) to lawns of all 18 *L. monocytogenes* isolates,