Characterization of lactosporin, a novel antimicrobial protein produced by Bacillus coagulans ATCC 7050

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Abstract

Aims: To characterize the antimicrobial protein produced by Bacillus coagulans used in the probiotic dietary supplement (Lactospore® Probiotic, Sabinsa Corp., Piscataway, NJ, USA).

Methods and Results: Bacillus coagulans ATCC 7050 was grown at 37°C for 18 h. The cell free supernatant was concentrated 10-fold (lactosporin preparation, LP). The antimicrobial activity of LP was confirmed against Micrococcus luteus ATCC 10420 in a well diffusion assay. The proteinaceous nature of LP was determined following exposure to different enzymes. The activity of LP was pH-dependent but stable to heat. The isoelectric point of LP was determined to be 3.5–4.0. PCR analyses showed no similarity between lactosporin and known antimicrobial proteins produced by the Bacillus spp.

Conclusions: Lactosporin is a novel antimicrobial protein. Initial characterization indicates that it may fall outside of the conventional classification of class I and II bacteriocins. Loss of activity after exposure to a number of proteolytic enzymes and lipase suggest that lactosporin may posses a lipid moiety which contributes to its inhibitory activity.

Significance and Impact of the Study: The unique characteristics of lactosporin, including its antimicrobial activity against pathogenic micro-organisms, indicate that it may have potential for application in foods and personal care products.
ribosomally-synthesized and post-translationally modified antimicrobial peptide produced by B. subtilis. This cyclic bacteriocin is active against a variety of Gram-positive bacteria including the foodborne pathogen L. monocytogenes (Shelburne et al. 2007). Hyronimus et al. (1998) reported a heat-stable BLIS produced by a strain of Bacillus coagulans. The amino acid sequencing of this antimicrobial peptide revealed its similarity to pediocin, a bacteriocin produced by Pediococcus acidilactici.

In this study we describe a new antimicrobial compound named lactosporin produced by a strain of B. coagulans isolated from a probiotic dietary supplement, Lactospore® Probiotic (Sabinsa Corp., Piscataway, NJ, USA). Lactosporin-producer strain was first isolated in 1933 and described as Lactobacillus sporogenes. It was later classified as B. coagulans since this strain possesses key features that are identical to the aforementioned spore-former. It should be mentioned that in many cases, commercial products containing B. coagulans use the name Lact. sporogenes on the labels for marketing purposes, although it is not an accepted name for this species according to the Bergey’s manual of determinative bacteriology (Cheng 1974). Lactosporin exhibits some characteristics similar to bacteriocins; however, it differs from the previously-reported bacteriocins from Bacillus spp., such as coagulin produced by B. coagulans, and subtilosin A produced by B. subtilis.

Materials and methods

Bacterial strains and growth condition

Producer strain B. coagulans ATCC 7050 was grown aerobically at 37°C in Difco™ Lactobacilli MRS broth (Becton, Dickinson,) with aeration for 24 h. The indicator strains Micrococcus luteus ATCC 10420, L. monocytogenes Scott A, Escherichia coli O157:H7 ATCC 43859 and Salmonella enterica serovar Enteritidis were cultured aerobically in tryptic soy broth (Becton, Dickinson) (enriched with 6 g l⁻¹ yeast extract and 2·5 g l⁻¹ glucose) at 37°C except for M. luteus ATCC 10420, which was grown at 30°C. Micrococcus luteus is widely used as an indicator micro-organism for bacteriocin detection assays (Li et al. 2005; Wirawan et al. 2006). In addition, nine vaginal Lactobacillus strains isolated from the healthy human subjects were tested in the course of this study along with the following pathogens: (i) Gardnerella vaginalis ATCC 14018; (ii) vancomycin-resistant Enterococcus faecium; (iii) methicillin-resistant Staphylococcus aureus and (iv) Streptococcus agalactiae (GBS) strains 35 and 749, and E. coli (urogenital strain). All of the above-mentioned micro-organisms were grown on tryptic soy agar (TSA; Becton-Dickinson) supplemented with 5% volume per volume (v/v) sheep blood. Gardnerella vaginalis strain was grown on a selective human blood tween bilayer agar medium (Becton-Dickinson). The Lactobacillus strains were propagated on MRS agar plates and the vaginal pathogens were grown anaerobically in a glove box (Plas-Labs, Lansing, MI, USA) at 37°C overnight. The cultures used in this study were maintained as frozen stocks at −80°C in a bio-freezer.

Production of lactosporin

The production of lactosporin was carried out in a fermenter (ABEC, USA) with a total volume of 150 L. Bacillus coagulans inoculum in MRS (5% v/v) was added to the production medium (MRS broth). After 24 h, the culture was centrifuged (10 000 g) to remove the cells and the supernatant was collected, concentrated 10-fold by lyophilization and filter-sterilized through a 0·45 µm filter (Nalgene, Rochester, NY, USA). The cell-free supernatant was named lactosporin preparation (LP). For some experiments LP was partially purified by dialysis through a 1 kDa-cutoff dialysis membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA).

Inhibitory activity of lactosporin

The antimicrobial activity of LP was determined by a well diffusion assay as described by Cintas et al. (1995) with minor modifications. A 4-ml lawn of soft (0·7% agar) TSA enriched with 6 g l⁻¹ yeast extract and 2·5 g l⁻¹ glucose, containing 10⁶ CFU ml⁻¹ of the indicator strain M. luteus ATCC 10420 was poured on top of an enriched hard (1·5% agar) TSA layer. LP sample (200 µl) was added to 16-mm wells punched in the solidified bi-layer agar. Plates were kept in the refrigerator overnight to allow the sample to diffuse into the agar and subsequently incubated at 37°C for 18 h. The inhibitory activity of LP was also tested against L. monocytogenes Scott A, E. coli O157:H7 (ATCC 43859) and Salm. Enteritidis as described above. In this and all other experiments nisin A was used as a positive control. The commercial preparation (Sigma, St Louis, MO, USA) contains 2·5% nisin A in denatured milk solids and was solubilized using nisin diluent (HCl solution at pH 2·0). This preparation of nisin A was chosen since it is not different from commercially available generally recognized as safe (GRAS) preparation of nisin that is used for food preservation.

Effect of pH and temperature on activity of lactosporin

The inhibitory activity of LP was estimated at different pH values, by adjusting its pH (approx. 5·2) using 3N HCl and 3N NaOH followed by incubation at room
temperature for 2 h. Eight aliquots of the LP, representing pH values 3–10 (without adjusting the pH back to the original pH of lactosporin) respectively, were tested for inhibitory activity against the indicator micro-organism by the well diffusion assay. The thermostability of LP was determined by incubating the LP samples at 30, 37, 50, 60, 80 and 100°C for 30 min. Aliquots (1 ml) of LP were dispensed in microcentrifuge tubes and heated to the desired temperatures in a heating block (dry bath incubator, Fisher scientific). The temperature was monitored using a thermometer that was installed in the heating block. Heat-treated samples were cooled in the air to room temperature and subsequently tested for activity against *M. luteus* ATCC 10420 in a well diffusion assay.

**Sensitivity of lactosporin to enzymes**

The sensitivity of lactosporin to enzymes was tested by incubating LP samples in the presence of 10 mg ml⁻¹ of each enzyme for 2 h at their optimal temperature, having adjusted the LP and enzyme mixture to the optimal pH for activity of that specific enzyme according to the manufacturer’s protocol (Sigma, St. Louis, MO, USA). The enzymes tested and their respective conditions are as follows: (i) proteinase K (37°C, pH 7.5); (ii) pepsin (37°C, pH 2.0); (iii) z-chymotrypsin (25°C, pH 7.8); (iv) protease (37°C, pH 7.5); (v) trypsin (25°C, pH 7.6); (vi) catalase (25°C, pH 7.0) and (vii) lipase class I, II and VII (37°C, pH 7.2) (Sigma). After incubation, the pH of the mixture was adjusted to the original pH of the LP (approx. 5.0) with 3 N HCl or 3 N NaOH and the samples were analysed for antimicrobial activity in the well diffusion assay with *M. luteus* as an indicator. Controls used for this experiment included LP samples treated with specific solvents used for preparation of each enzyme stock solution (LP + enzyme solvents) (Sigma) and MRS medium treated with 10 mg ml⁻¹ of each enzyme solution (MRS + enzymes). Similarly the pH of controls was first adjusted to the optimum pH for activity of enzymes and after the incubation was adjusted back to the optimal pH for activity of lactosporin. A 10 mg ml⁻¹ nisin stock solution was exposed to 10 mg ml⁻¹ z-chymotrypsin and was used as a control for this experiment.

**Detection of antimicrobial activity on SDS-PAGE and native-PAGE**

Both 5× and 10× concentrated LP samples were used for visualization of the antimicrobial activity using native PAGE ready gels (10–20% Tris-HCl; Bio-Rad, Hercules, CA, USA). Two duplicate samples were separated on the same gel (100 V). At the end of electrophoresis the gel was cut into two halves, vertically. One part was stained with silver stain (Bio-Rad, Hercules, CA, USA) and the other part was tested for antimicrobial activity in overlay test as described previously (Bhunia *et al.* 1988) with *M. luteus* as the reference micro-organism.

**Determination of the antimicrobial activity of lactosporin as arbitrary units ml⁻¹**

Arbitrary unit (AU) ml⁻¹ is defined as a reciprocal of the highest dilution of an antimicrobial compound which gives a visible zone of inhibition in the overlay assay against the indicator micro-organism (Callewaert and De Vuyst 2000). Eight serial twofold dilutions of a partially purified and 20× concentrated and dialysed LP were prepared. The antimicrobial activity of each dilution was determined in a well diffusion assay against sensitive strain *M. luteus*. Nisin A (10 mg ml⁻¹ stock solution) was used as a positive control for this experiment. Twofold dilutions of nisin A stock solution were similarly prepared to a dilution of 2⁻⁸ and tested for inhibitory activity in the well diffusion assay.

**Determination of the isoelectric point of lactosporin**

The isoelectric point (pI) of lactosporin was determined by using Rotofor® (Bio-Rad, Hercules, CA, USA), an isoelectric focusing apparatus. A 50-ml sample of partially-purified 2× concentrated LP was applied to the Rotofor® unit in an ampholyte preparation with a pH range of 3–10 (BioLyte, Bio-Rad). The separation procedure was performed as described by the supplier. Following separation, 20 fractions were collected, and the pH of all fractions was adjusted to approx. 4.5–5.0 with 3 N HCl or 3 N NaOH. The activity of each fraction was determined by well diffusion assay against *M. luteus*.

**PCR testing**

PCR was used to investigate the possible relatedness of the antimicrobial protein produced by *B. coagulans* ATCC 7050 with the known bacteriocins produced by *Bacillus* spp. including coagulin produced by *B. coagulans* I₄ (Hyronimus *et al.* 1998), and subtilin and subtilosin, which are produced by *B. subtilis* (Klein *et al.* 1992; Stein *et al.* 2004). Genomic DNA was extracted from overnight cultures of *B. coagulans* and *B. subtilis* ATCC 6633. Briefly, the cells were harvested by centrifugation at 13 000 g for 3 min at room temperature and resuspended in 0.5 mol l⁻¹ EDTA pH 8.0. The cell suspension was treated with 100 µl of 20 mg ml⁻¹ lysozyme, 10 µl of 20 mg ml⁻¹ proteinase K and 8 µl of 2·5 U µl⁻¹ mutanolysin (all from Sigma, St Louis, MO, USA) at 37 °C for 60 min. The purification of genomic DNA from the cell
Lysate was conducted using the Wizard® SV genomic DNA purification system (Promega Corp., Madison, WI, USA) according to the manufacturer’s protocol. *B. coagulans* I4 plasmid pUC19 containing a 4.9 kb fragment encoding coagulin operon, and genomic DNA were a kind gift from Dr Maria Urdaci (Enita de Bordeaux, Gradignan Laboratoire de microbiologie, France). Primers specific for the known *Bacillus* bacteriocin structural genes coaA, spaS, and sboA were designed, and synthesized by Sigma Genosys (Germany) (Table 1). PCR-based detection of these genes was accomplished using a master mix containing the appropriate primer pair, dNTPs (10 mmol l−1), buffer, and 0.2 U HotMaster Taq polymerase (all PCR reagents were supplied by Eppendorf, Hamburg, Germany) according to the manufacturer’s specifications. PCR was performed using an Applied Biosystems GeneAmp PCR System 2400 apparatus (Applied Biosystems, Foster City, CA, USA) and products were separated on a 1% agarose gel.

### Results

#### Spectrum of antimicrobial activity of lactosporin

Lactosporin was active against the Gram-positive microorganisms *M. luteus* and *L. monocytogenes* when tested in the well diffusion assay, but not against Gram-negative bacteria. LP also did not show inhibitory activity against most of the vaginal lactobacilli tested (Table 2), in contrast to nisin which inhibited all the vaginal lactobacilli tested. The activity of LP was 16 AU ml−1 when tested against the indicator strain *M. luteus*.

#### Sensitivity of lactosporin to different pH, temperature and enzymes

Testing of LP at a wide range of pH values showed it to be more active at lower pH values with total loss of activity occurring at pH values over 7.0 (Fig. 1). Upon heat-testing, the antimicrobial compound was stable at a temperature range of 30–60°C after 30 min of incubation, and only a 1 mm decrease in the size of inhibition zone occurred when lactosporin was heated at 100 and 80°C. (Fig. 2). There was no detectable antimicrobial activity following exposure of LP to: (i) the proteolytic enzymes proteinase K, trypsin, *α*-chymotrypsin and protease, and (ii) lipase classes II and VII (data not shown).

#### Determination of isoelectric point of lactosporin

Isoelectric focusing of partially-purified LP with a Rotofor® cell yielded 4 active fractions. The zones of inhibition of tested samples from fractions 18, 19 and 20 were large (5, 7 and 12 mm respectively), without a clearly-defined edge; in contrast, the zone of inhibition of fraction 1 was small with a well-defined edge. Further experiments showed that the antimicrobial activity seen in fractions 18–20 was due to the ampholyte used in the Rotofor system (Riazi et al. 2007), and that fraction 1 is the active fraction of the LP. The pI of lactosporin was estimated to be approx. 3.5–4.0 (Fig. 3).

### Table 1 Primers specific for the structural genes of coagulin, subtilin and subtilosin used in this study

<table>
<thead>
<tr>
<th>Bacteriocin name</th>
<th>Producing organism</th>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulin*</td>
<td><em>Bacillus coagulans</em></td>
<td>CoaAFwd</td>
<td>5′-GGTGTTAACAGGATTGGAGGTT-3′</td>
<td>(Le Marrec et al. 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CoaDRev</td>
<td>5′-GGTGCTAAAACTGATGGTCGAT-3′</td>
<td></td>
</tr>
<tr>
<td>Subtilin†</td>
<td><em>Bacillus subtilis</em></td>
<td>SpaFwd</td>
<td>5′-CAAAGTCGATTTGGTTGATGAGT-3′</td>
<td>(Klein et al. 1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SpaRev</td>
<td>5′-GGCAGTTGGTGGTTGATGGTGAAGA-3′</td>
<td></td>
</tr>
<tr>
<td>Subtilosin†</td>
<td><em>B. subtilis</em></td>
<td>SboAFwd</td>
<td>5′-GCAGCAAGTGGCATATTCTTAACA-3′</td>
<td>(Stein et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SboARev</td>
<td>5′-GCAGCAAGTGGCATATTCTTAACA-3′</td>
<td></td>
</tr>
</tbody>
</table>

*Primer sequence based on GenBank accession number AF300457.
†Primer sequence based on GenBank accession number AJ430547.

### Table 2 Inhibitory activity of lactosporin preparation against tested bacteria

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 710</td>
<td>0</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 711</td>
<td>0</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 748</td>
<td>0</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 735</td>
<td>0</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 701</td>
<td>0</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 807</td>
<td>0</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 618</td>
<td>0</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 757</td>
<td>3</td>
</tr>
<tr>
<td><em>Lactobacillus</em> 758</td>
<td>4</td>
</tr>
<tr>
<td><em>Gardnerella vaginalis</em></td>
<td>8</td>
</tr>
<tr>
<td>GBS 35</td>
<td>0</td>
</tr>
<tr>
<td>GBS 749</td>
<td>0</td>
</tr>
<tr>
<td>VRE</td>
<td>0</td>
</tr>
<tr>
<td>MRSA</td>
<td>0</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (Urogenital)</td>
<td>0</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>0</td>
</tr>
<tr>
<td><em>Salm. Enteritidis</em></td>
<td>0</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>4</td>
</tr>
</tbody>
</table>
Lactosporin activity was detected following analysis by native-PAGE. This was represented by zones of inhibition for both the five and 10-fold concentrated LP on the half of the gel overlaid with *M. luteus*, corresponding to the two bands observed on the silver-stained gel portion (Fig. 4). Following SDS-PAGE, a band was visualized with a molecular mass of 25–30 kDa, however no zone of inhibition was observed on the gel portion overlaid with *M. luteus* (data not shown).

**PCR analysis**

PCR using primers specific for the known *Bacillus* bacteriocin structural genes coaA, spaS, and sboA yielded the expected-sized product from *B. coagulans* I4 (coaA) and *B. subtilis* ATCC6633 (spaS and sboA), but not from the lactosporin-producer *B. coagulans* ATCC 7050.

**Discussion**

In this study, lactosporin, an antimicrobial compound produced by the probiotic-derived *B. coagulans* ATCC 7050 strain, has been isolated and partially characterized. Its spectrum of activity included the food-pathogen *L. monocytogenes*, but like most other bacteriocins derived from Gram-positive bacteria (Abee et al. 1995), it did not inhibit any of the Gram-negative bacteria tested. The activity of this antimicrobial substance is remarkably
pH-dependent, but is not affected by a wide range of temperature. Furthermore, the activity of this antimicrobial compound was no longer detected after exposure to a number of proteolytic enzymes such as proteinase K and protease, thus indicating the proteinaceous nature of this inhibitory substance. Loss of activity following exposure to lipase suggests that this antimicrobial compound may be a protein with an associated lipid moiety which appears to be required for activity similar to class IV bacteriocins (Klaenhammer 1993). The most recent classifications of bacteriocins exclude class IV, which leaves no room for our new BLIS to be placed in. Of the remaining three classes, only class III gathers large-size molecules such as zooncin A from Strepococcus equi ssp. zooepidemicus, millericin B from Streptococcus milleri, streptococcin A-M57 from Strepococcus pyogenes and dysgalacticin from Strepococcus dysgalactiae (Simmonds et al. 1996; Beukes et al. 2000; Heng et al. 2004, 2006). However, these bacteriocins are heat-labile substances from various species of Strepococcus whereas lactosporin is produced by the Bacillus spp. and is heat stable. Therefore, it is highly possible that the newly isolated lactosporin belongs to antimicrobial lipopeptides or surfactin-like molecules (Yu et al. 2002; Stein 2005).

A protein band with a molecular weight of about 25–30 kDa was visualized on the stained portion of a gel following SDS-PAGE. However, no corresponding antimicrobial activity of this protein was detected on the portion of the gel overlaid with the indicator micro-organism. The loss activity might be due to the irreversible denaturation of this protein by SDS or reduction of disulfide bridges that might be present in the structure of this antimicrobial protein with 2-fl-mercaptoethanol. On the other hand, antimicrobial activity of lactosporin was detected on an overlaid portion of a gel following native-PAGE. Although the band with molecular weight of approx. 37 kDa corresponding to this zone of inhibition was visualized on the stained portion of the gel, conclusions regarding the exact molecular weight of this protein cannot be made due to the fact that proteins in native electrophoresis systems migrate according to their charge, and not their weight. Hyronimus et al. (1998) and Le Marrec et al. (2000) have conducted comprehensive studies on coagulin, the BLIS produced by B. coagulans I4. Unlike lactosporin, the activity of which is highly pH-dependent, the activity of coagulin was retained at a wide range of pH (3–8). It has been reported that coagulin belongs to the group of pediocin-like bacteriocins (class IIa), with slight differences in structure when compared to pediocin PA-1 and AcH. In general, class IIa bacteriocins are characterized as small (<10-kDa) molecules with a net positive charge and basic pI values (8–10) (Drider et al. 2006). The results of this study indicate that lactosporin is an anionic protein with a pI value of 3.5–4 which is in contrast to the characteristics of class IIa bacteriocins. Genetic analysis through PCR-screening also demonstrated no relatedness between the antimicrobial protein produced by B. coagulans ATCC 7050, and coagulin produced by B. coagulans I4, or to subtilin and subtilosin produced by B. subtilis. These data collectively suggest that lactosporin is a novel antimicrobial protein with characteristics different to these previously-described bacteriocins produced by Bacillus spp.

Antimicrobial proteins that are naturally produced by LAB are currently receiving well-deserved attention due to the potential health problems associated with the use of chemical antimicrobial agents in foods and personal care products, and because of the rapidly growing microbial resistance to conventional antibiotics (Abee et al. 1995; Cleveland et al. 2001). These issues have created a demand for the use of safe and natural antimicrobial compounds in foods and personal care products, for preservation and medical applications. Previous studies have demonstrated that a large number of antimicrobial substances produced by bacteria, such as bacteriocins and BLIS, are inhibitory to some pathogenic micro-organisms such as L. monocytocegetes. Nisin is the most studied bacteriocin with FDA-approved GRAS status for application in certain food products (US Food and Drug Adminstration 1988). Produced by Lactococcus lactis ssp. lactis, it belongs to the antibiotic class of bacteriocins and shows activity against a large number of Gram-positive bacteria (Cleveland et al. 2001). The heat stability, increased antimicrobial activity at low pH values, along with its activity against foodborne pathogens and micro-organisms associated with bacterial vaginosis, are the very important factors that make lactosporin, too, a great potential candidate for food and medical/personal care applications.

Acknowledgements

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References

Lactosporin characterization

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