Current Microbiology Vol. 54 (2007), pp. 282–286
DOI: 10.1007/s00284-006-0414-x

Current Microbiology
An International Journal

Purification and Partial Characterization of an Antimicrobial Peptide Produced by a Novel *Bacillus* sp. Isolated from the Amazon Basin

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Received: 11 August 2006 / Accepted: 20 November 2006

Abstract. An antimicrobial peptide produced by a new *Bacillus* species isolated from the Amazon Basin was purified and characterized. The antimicrobial peptide was purified by ammonium sulfate precipitation, gel filtration, and ion exchange chromatography, and after the final purification step, one active fraction was obtained, designated BLS P34. Direct activity on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was observed. A single band on SDS-PAGE suggested that the peptide was purified to homogeneity and had a molecular mass of about 5 kDa. The molecular weight (MW) was accurately determined by mass spectroscopy as 1456 Da. The purified BLS P34 remained active over a wide temperature range and was susceptible to all proteases tested.

Bacteriocins are ribosomally synthesized antimicrobial peptides that are usually inhibitory to strains that are closely related to the producing bacteria. These antimicrobial compounds are thought to provide the producer strain with the selective advantage over others strains. Bacteriocins produced by gram-positive bacteria are often membrane-permeabilizing cationic peptides with fewer than 60 amino acid residues [12].

The classification of bacteriocins takes into account the chemical structure, heat stability, molecular mass, enzymatic sensitivity, presence of modified amino acids, and mode of action of these chemicals [7, 14]. Three classes of bacteriocins can be distinguished. (1) Lanthibiotics containing the modified amino acids lanthionine; the well-studied bacteriocins nisin [11] and epidermin [1] belong to this group. (2) Low-MW bacteriocins (smaller than 10 kDa) formed exclusively by unmodified amino acids. Within this group, specific antilisterial compounds, bacteriocins formed by two peptides acting synergistically and thiol-activated peptides, can be found. (3) High-MW bacteriocins: heat labile proteins larger than 30 kDa.

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The genus *Bacillus* includes a variety of species with a history of safe use in industry. Commercial products that are currently obtained from *Bacillus* spp. include enzymes, antibiotics, amino acids, and insecticides. The potential of *Bacillus* species to produce antibiotics has been recognized for more than 50 years, and peptides' antibiotics represent the predominant class. Many bacteriocins or bacteriocin-like substances (BLS) in the genus *Bacillus* have been reported, such as thuricin, cerein 7, cerein 8A, subtilosin A, and surfactin [3, 12, 12, 21].

We have screened a number of bacterial cultures obtained from the Brazilian Amazon Basin for the production of antimicrobial substances. The microorganism *Bacillus* sp. P34 was isolated from the teleost fish Piau-com-pinta (*Leporinus* sp.) and produces an antimicrobial peptide, which inhibited the food-borne pathogen *Listeria monocytogenes* [18]. This paper describes the purification and some physicochemical properties of the antimicrobial peptide BLS P34.

Materials and Methods

Bacterial Strains and Media. The produced strain was identified as *Bacillus* sp. P34 and characterized as described elsewhere [18]. The indicator strain was *Listeria monocytogenes* ATCC 7644. Brain heart

Table 1. Purification of BLS P34 produced by *Bacillus* sp. P34

Purification step	Total protein (mg)	Total activity (AU)	Specific activity (AU mg^{-1})	Purification fold	Yield (%)
Crude supernatant	1906.38	144,000	76	1.0	100
Precipitation	21.38	92,160	4384	57.7	64.0
Sephadex G-100	8.4	51,200	6095	80.2	35.56
DEAE Sepharose	0.9	12,000	13,333	175.43	8.33
DEAE Sepharose	0.27	5400	20,000	263.15	3.75

infusion (BHI) medium (Oxoid, Basingstoke) was used for maintenance of strains with 20% (v/v) glycerol at –20°C. The cultivation of strains was performed aerobically.

Antimicrobial Activity. Antimicrobial activity was determined by the agar-disk diffusion assay [17]. An aliquot of 20 µl antimicrobial substance was applied to disks (6 mm) placed on agar plates previously inoculated with a suspension of the indicator strain. Plates were incubated at 37°C for 24 h. The antimicrobial activity titre was determined by the serial twofold dilution method as described elsewhere [17].

Hemolytic Activity. The hemolytic activity was determined on sheep blood agar plates [2]. An isolate of *Staphylococcus aureus* with known hemolytic activity was used as a positive control.

Purification Protocol. *Bacillus* sp. P34 was cultivated aerobically in 500-ml Erlenmeyer flasks containing 200 ml of TSB broth at 30°C, 180 cycles min^{-1} for 24 h. Cells were harvested by centrifugation at 10,000*g* for 15 min at 12°C, and the resulting supernatant was filtered through 0.22-µm membranes (Millipore, Bedford, MA). The cell-free culture filtrate was submitted to precipitation with ammonium sulfate to 20% saturation. The resulting pellet was re-suspended in 10 mM sodium phosphate buffer, pH 7.0, and applied to a gel filtration column (Sephadex G-100, Pharmacia Biotech, Uppsala, Sweden) and eluted with 10 mM sodium phosphate buffer, pH 7.0. Fractions positive for antimicrobial activity were pooled and applied to a column of DEAE-Sepharose (Pharmacia Biotech, Uppsala, Sweden), eluted with this same buffer followed by a gradient from 0 to 1.5 M NaCl. The active peaks were dialyzed and rechromatographed according to the same process. Fractions were monitored for A280 nm using an EM-1 EconoUV monitor (Bio-Rad Laboratories, Hercules, CA).

The determination of soluble protein was carried out by the Folin phenol reagent method [16] with bovine serum albumin as standard.

Direct Detection on Polyacrylamide Gels. Antimicrobial activity was detected on polyacrylamide gels as described previously [4]. Briefly, the samples were applied to 14% polyacrylamide gels and electrophoresed at 20 mA per gel. The gels were then washed with sterile distilled water to remove SDS and antimicrobial activity was tested against *L. monocytogenes*. Other gels were stained with Commassie blue to observe peptide bands. MW standards were from Sigma (St. Louis, MO).

Effects of Enzymes and Heat on Antimicrobial Activity. Samples of purified bacteriocin were treated at 37°C for 1 h with 2 mg ml^{-1} final concentration of the following enzymes: trypsin, papain, pronase E, and proteinase K. Samples were then boiled for 2 min to inactivate the enzyme. To analyze thermal stability, samples of bacteriocin were exposed to temperatures ranging from 40° to 90°C for 30 min, 100°C for 10, 20, 30, 40, 50, and 60 min, and 121°C/105 kPa for 15 min. After the treatments, the samples were tested for antimicrobial activity against *L. monocytogenes*.

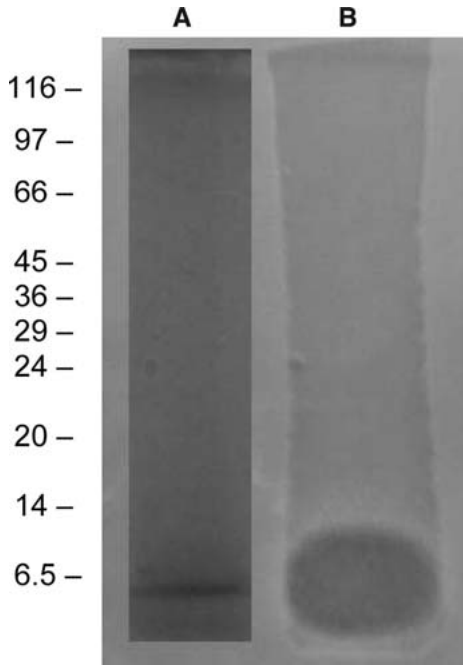

Fig. 1. Gel electrophoresis analysis of BLS P34. Samples of the purified bacteriocin submitted to SDS-PAGE and stained for proteins with Coomassie blue (**A**) and tested for antimicrobial activity against *Listeria monocytogenes* (**B**).

Mass Spectroscopy. A sample of purified BLS P34 was dialyzed against MilliQ water and freeze-dried. This material was dissolved in 0.046% trifluoroacetic acid and applied to a C18 chromatographic resin (Vydac). The column was eluted with 80% acetonitrile 0.046% TFA and concentrated in a vacuum centrifuge (SpeedVac SC100, Savant). The sample was analyzed in a MALDI-TOF mass spectrometer (Ettan MALDI-TOF ProSystem, Amersham Biosciences, Sweden) operating in reflecton mode and using a matrix of α-ciano-4-hydroxycinnamic acid.

Infrared Spectroscopy. The infrared spectrum was measured as a potassium bromide pellet. Four scans of the sample were taken using a Mattson 3020 Fourier transform infrared (FTIR) spectrophotometer (Madison, WI).

Results

The antimicrobial peptide produced by *Bacillus* sp. P34 was purified from the culture supernatant by combination of ammonium sulfate precipitation, gel filtration,

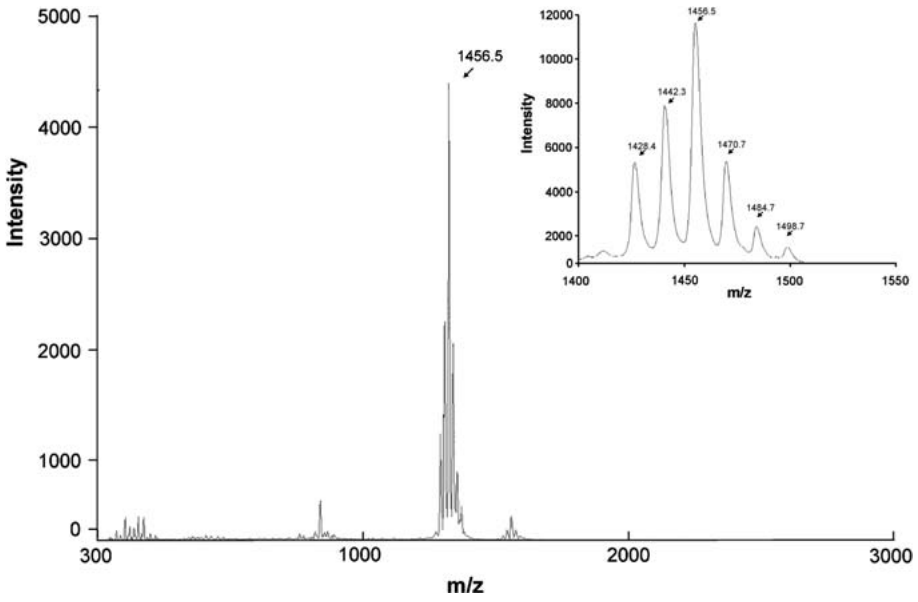

Fig. 2. Mass spectrum of purified BLS P34. Inset, detailed view of the region m/z 1440–1550.

and ion exchange chromatography. The results of BLS P34 purification are summarized in Table 1. The final specific activity of the purified peptide was approximately 263-fold greater than that in the culture supernatant and the final recovery was 3.75%.

The purified BLS P34 was analyzed by SDS-PAGE, revealing a single band of about 5 kDa (Fig. 1). The antibacterial activity could be demonstrated by overlaying the other part of the gel, containing the same purified peptide, with media containing the indicator strain *L. monocytogenes*. An inhibitory zone was observed at the same R_f that was visualized in the stained gel (Fig. 1).

The hemolytic activity was assayed on sheep blood agar plates and negative reactions were observed with fresh or freeze-dried preparations of BLS P34 (not shown).

Aliquots of BLS P34 were treated with trypsin, papain, pronase E, and proteinase K and the antimicrobial activity was lost with all proteolytic enzymes tested. The purified BLS showed residual activity after heat treatments, its initial activity remaining at 70% after 60 min at 100°C. Total loss of activity was only observed after autoclaving (121°C, 105 kPa) for 15 min.

In order to prove the purity and to determine accurately the molecular mass of the BLS P34, mass spectroscopy analysis was carried out revealing a molecular mass of 1456 Da (Fig. 2). The mass spectroscopy analysis showed a cluster of 6 peaks that were observed at m/z 1498, 1484, 1470, 1456, 1442, and 1428, differing from each other by 14 Da (Fig. 2, inset).

The infrared spectrum of BLS P34 showed characteristic absorption valleys at 3478 and 1651, which

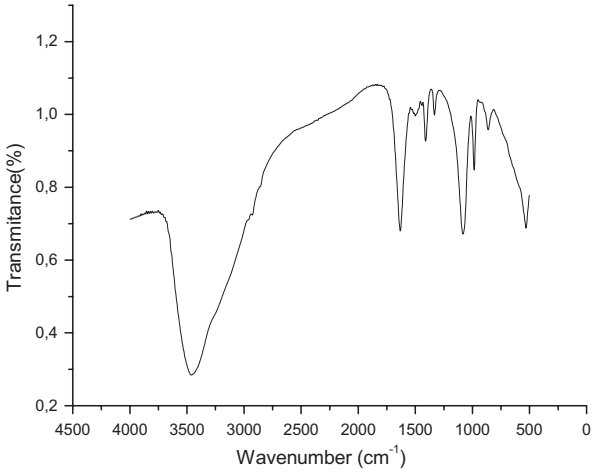

Fig. 3. Fourier transform infrared (FTIR) spectrum of BLS P34.

indicate the substance contains peptide bonds (Fig. 3). Valleys that results from C-H stretching (1434 and 1101 cm^{-1}) indicate the presence of aliphatic chains.

Discussion

In this work, a bacteriocin-like substance produced by *Bacillus* sp. P34 was purified and characterized. The antimicrobial substance was purified by sequential precipitation, gel filtration, and ion-exchange chromatography process. A single band of about 5 kDa was observed when estimated by SDS-PAGE, suggesting that the BLS P34 had been purified to homogeneity. The molecular mass was determined by mass spectroscopy as 1456 Da. This discrepancy can be explained on the basis of the abnormal behavior of some highly hydro-

phobic proteins in SDS-PAGE [13]. This property has been associated with bacteriocins and BLS presenting a strong hydrophobic nature [4, 5, 22].

The FITR and mass spectra of the BLS P34 offer additional information about the hydrophobic nature of the peptide. Analysis of the FTIR spectrum show typical absorption bands corresponding to N-H stretching of proteins and peptide bonds, solid evidence that the substance contained a peptide in its structure. In addition, absorption bands indicating aliphatic chains may be related to the predominance of the hydrophobic amino acids or the presence of a fatty acid in the structure. The mass spectroscopy analysis showed a cluster of 6 peaks that were observed at m/z 1498, 1484, 1470, 1458, 1442, and 1428. These peaks differ by 14 Da, suggesting a series of homologous molecules or fragments having different lengths of fatty acid chain (CH_2 = 14 Da). This substance may be a surfactin-like compound, belonging to a family of lipopeptide antibiotics often termed biosurfactants [9].

These data may suggest that aggregates of BLS P34 can be formed in aqueous solution, which could be maintained by hydrophobic interactions. Thus, formation of BLS P34 aggregates can very likely occur in natural conditions in which a large number of bacteria simultaneously produce antibiotics as the nutrients become limited. These aggregates can prevent diffusion and loss of the antibacterial activity, maintaining its concentration at high levels in the surrounding bacterial population.

The antimicrobial activity was sensitive to all proteases tested, additional evidence that a peptide moiety is associated with its activity. The heat stability resembled some BLS produced by *Bacillus amyloliquefaciens* [15] and *Bacillus liqueniformis* [6], while other BLS from *Bacillus* are often less resistant to thermal treatments [3]. According to its properties of size and protein stability data, BLS P34 could be associated with the group of *Listeria*-active class Ib bacteriocins [7, 14].

Antimicrobial peptides have been associated with molecules such as hemolysins. Indeed, some bacteriocins have hemolytic activity [5]. However, this activity was not associated with the BLS P34 as evaluated by the lack of hemolysis on blood agar plates.

The role of antimicrobial production for the *Bacillus* sp. P34 is still under speculation. The best-accepted theory is that peptide antibiotics may play a role in competition with other microorganisms during spore germination [8, 19]. These antimicrobial substances are found not only among bacteria, but also as part of a defense system in higher organisms [10, 19]. It has been proposed that antagonism mediated by cationic peptides may represent the conservation through the course of evolution of a general mechanism of antibiosis [20]. The detection of novel antibiotics produced by *Bacillus* species would, therefore, be helpful in providing an understanding of the intrinsic (if any) role of antimicrobial activity in the life cycle of those organisms.

ACKNOWLEDGMENTS

This work received financial support from CNPq, Brazil.

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