PURIFICATION AND PARTIAL CHARACTERISATION OF AN ANTIFUNGAL BACTERIOCIN FROM Bacillus sp. Sh10 ASSOCIATED WITH MARINE CARPET CLAM (Paphia textile)

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ABSTRACT

This study was conducted to purify and characterize an anti- C. albicans bacteriocin produced by Bacillus sp. Sh10 isolated from marine carpet clam, Paphia textile. Extracellularly produced bacteriocin was purified by 80 % ammonium sulfate precipitation and size exclusive gel chromatography using superdex-200 column chromatography. The purification steps resulted in 3.68-fold increase in specific activity and recovery of 20.66 % of total activity. The purity rate and molecular mass of 11 KDa of this compound were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Profile analysis of this bacteriocin by electrospray mass spectrometry (LC/MS/MS) showed 43 % protein sequence coverage with a putative uncharacterized protein from Bacillus cereus strain B4264.

Keywords: Marine bacteria, antifungal bacteriocin, Candida albicans, Bacillus sp., purification

INTRODUCTION

Bacteriocins are generally defined as antimicrobial peptides produced by bacteria that inhibit or kill microorganisms closely-related or non-related to produced bacteria (Leroy & De Vuyst, 2004; Cotter et al., 2005). The members of Bacillus genus are known to produce a variety of bacteriocins consisting of a hydrophobic fatty acid linked to a hydrophilic peptide chain (Roongsawang et al., 2011). This complex structural diversity warrants the broad spectra of inhibition of some Bacillus strains, which includes not only Gram-positive and Gram-negative bacteria, but fungi and amoeba as well (Benitez et al., 2011; Shelburne et al., 2007; Cladera-Ólvera et al., 2004). The pathogenicity of C. albicans makes it particularly troublesome for patients with immunosuppression conditions. Indeed increasing numbers of diabetics, HIV type-C patients, post-surgical patients, premature infants, and trauma patients are progressively vulnerable to C. albicans infections. These infections can be localized to the gastrointestinal, oral, or vaginal tracts, or be disseminated across multiple sites and organs, which can be fatal. The mortality rate in patients infected with C. albicans, exceeds 40% (Godlundsson et al., 2005). During the past five decades, systemic antifungal treatments have been devised (Ostrozyk-Zeiherner et al., 2010); yet, new antifungals are urgently needed to offset the rising incidence of invasive fungal infections arising from C. albicans and other pathogens becoming drug-resistant (Shahin et al., 2015; Amel et al., 2015; Adebayo & Aderiye, 2011). To be effective, these new treatments will need to go beyond conventional drug targets, to focus instead upon new targets such as novel metabolic pathways or virulence factors.

This report describes the partial purification and identification of anti-C. albicans bacteriocin, which is produced by Bacillus sp. sh10 isolated from Paphia textile.

MATERIAL AND METHODS

Isolation, detection and partial purification of bacteriocin

In the present study, a bacteriocin produced from Bacillus sp. sh10 was used. This microorganism was isolated previously from carpet clam (P. textile) (Shayesteh et al., 2014, 2015). Inorganic salt medium (Pridham & Gottlieb, 1948) containing 2% tryptone and 1% glucose as nitrogen and carbon source, respectively followed by 2% sodium chloride was obtained as an optimized medium for bacteriocin production by isolate sh10 (Shayesteh et al., 2014). In addition, the optimized culture media was adjusted to pH 8.0 and bacteria culture was incubated at 30 °C under the aerobic condition for 30 h. Afterwards, the supernatant was achieved by centrifuging bacteria culture at 3000 × g for 30 min at 4 °C. The supernatant which was obtained after centrifugation was then filtered by 0.22 μm membrane filter followed by ammonium sulfate precipitation at a different percentage of saturation (40 % - 60 % and 80 %) at 4 °C. After moderate agitation, the precipitated protein obtained by centrifugation (3000 g, 30 min, 4 °C), was dissolved in 0.1 M phosphate buffer (pH 7.0) followed by dialysing via dialysis membrane (2 KDa cut-off, Sigma) against the same buffer at 4 °C for 24 h (Rajaram et al., 2010) and applied as ammonium sulfate fraction.

The antimicrobial activity of bacteriocin against C. albicans ATCC 10231 was determined using the spot-on-lawn method (Seuk-Hyun & Ched, 2000). In brief, in each step of purification, 10 μL of bacteriocin was spotted onto the surface of tryptone soy agar overlaid by 1.5 × 105 CFU/mL cells of the C. albicans followed by incubation at 30 °C for 48 h. A bacteriocin titer was determined as the reciprocal of the highest twofold dilution indicating the complete inhibition of the indicator lawn and was presented in activity units (AU) per ml of culture media. The titer of the bacteriocin solution, in AU/mL was calculated as (10000/D), where D is the dilution factor and is the dose (Hoover & Harlander, 1993).

Gel filtration chromatography

The dialyzed protein was placed in a Superdex-200 column (AKTA purifier UPC-900) at room temperature. The protein was eluted with phosphate buffer (0.1 M, pH 7.0) at a flow rate of 0.5 mL/min and screened at 280 nm. Fractions (1 mL) were collected and evaluated in terms of antimicrobial activity by spotting aliquote (10 μL) on a lawn of C. albicans, as described previously. The molecular weight of bacteriocin was calculated through gel filtration with apotinin (6.5 kDa), ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), canolalbumin (75 kDa), aldolase (158 kDa), and ferritin (440 kDa) as references.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The active fractions acquired from gel filtration chromatography were analyzed by 15% SDS-PAGE and Tris-Glycine buffer system (Laemmli, 1970) to verify the purity. A pre-stained broad-range molecular mass marker (11-245 KDa, GeneDirex) was utilized to assess the bacteriocin molecular weight. In order to enter the samples into the separating gel, the electrophoresis was performed at 50 V and was followed by a constant power of 100 V until samples achieved the endpoint of the separating gel. Fractions containing a single band were pooled and designed as gel filtrate fractions.

Direct detection of the bacteriocin activity on SDS-PAGE

The protein band activity in SDS-PAGE was assayed as described by Cherif et al. (2001). After the samples were run, the gel was vertically cut into two halves. The first half was stained by Coomassie blue R-250 to observe the protein bands; the other half was rinsed with deionized water for 2 h with intermediate steps of changing the water every 20 min. Afterward, the gel was placed in a sterile Petri dish and subjected to UV for 20 min for sterilization. The gel was then overlaid with Mueller Hinton soft agar seeded with 1.5 × 10^8 CFU/mL cells of C. albicans followed by incubation at 30 °C. After 24-48 h of incubation, the gel was examined to determine the growth inhibition zone caused by bacteriocin.

Determination of protein concentration

The protein concentrations of the samples obtained from culture supernatant, ammonium sulfate precipitation, and gel filtration were determined in accordance with Bradford assay (Bradford, 1976). Bradford solution was added to 100 μL aliquots containing different bovine serum albumin (BSA) concentrations and bacteriocin samples. These samples were incubated at room temperature for 10 min. The absorbance at 595 nm was also determined using a spectrophotometer. BSA standards were used to create a standard curve, and the protein concentration of the samples was calculated on the basis of this standard curve.

Identification of bacteriocin using mass spectrometry (LCMS/MS)

Active bands on SDS-PAGE corresponding to a molecular weight of approximately 11 kDa was excised from the gel. The process involved in-gel de-staining, reduction with dithiothreitol, alklylation of the thiols with iodoacetamide, and in-gel tryptic digestion and extraction of the resulting peptides (Proteomics International Facility, Australia). The extracted peptides were analyzed through electrospray ionization mass spectrometry by Agilent 1260 Infinity HPLC System coupled with an Agilent 6540 mass spectrometer. Tryptic peptides were loaded onto a 5 μm 300 SB C18 column (Agilent) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v). The spectra were analyzed to recognize the target proteins by Mascot sequence matching software [Matrix Science] with Ludwig NR database.

RESULTS

Extraction, purification, and molecular weight determination of bacteriocin produced by Bacillus sp. Sh10

The highest bacteriocin activity was found at 80% of ammonium sulfate precipitation, which resulted in a 3.68-fold increase in the specific activity. This purification step resulted in 494.42 AU/mg and 20.66 % of specific activity and total activity recovery, respectively (Table 1). The separation profile of gel filtration chromatography (superdex-200) achieved from crude ammonium sulfate precipitate, resulted in two major peaks (Figure 1). Among 36 fractions collected, antimicrobial activity was trailed in fractions 23-27 which were located in the second peak (Figure 1). When active fractions obtained from gel chromatography were compared to the standard curve, the molecular weight of eluted peptides in these fractions corresponded to ~13-6 kDa. The present purification step resulted in 546.22 AU/mg and 6.5% of specific activity and total activity recovery, respectively (Table 1). The ammonium sulfate precipitate and active fractions of bacteriocin collected from size exclusion gel chromatography were examined by SDS-PAGE (Figure 2). The accurate molecular weight of antimicrobial peptide was measured by SDS-PAGE. In the case of ammonium sulfate precipitation, seven major and other bands were observed with different molecular weights compared with a broad range molecular marker (Figure 2A). The bacteriocin activity against C. albicans ATCC 10231 corresponded to a low-molecular-weight band with an estimated molecular mass of 11 KDa (Figure 2B). Based on the active fractions from gel chromatography, fractions 23 and 24 contained more than one band, indicating the impurity of these fractions. However, fractions 25, 26, and 27 showed single sharp bands as indicated by SDS-PAGE (Figure 2). The bacteriocin activity of fractions 23-27 on SDS-PAGE against C. albicans ATCC 10231 (Figure 2B) was exhibited as a clear zone of inhibition on the corresponding band (~11 KDa). Therefore, fractions 25, 26, and 27 with the single band were pooled and designed as gel filtrate fraction of bacteriocin.

![Figure 1](image-url)  
Elution profile of bacteriocin from isolate Sh10 on superdex-200 column chromatography

| Purification steps | Volume (mL) | Activity unit (AU/mL) | Protein (mg/mL) | Total protein (mg) | Total activity (AU) | Specific activity (AU/mg) | Purification fold | Recovery (%) |
|-------------------|-------------|----------------------|----------------|-------------------|-------------------|--------------------------|----------------|-------------|
| Culture supernatant | 1000        | 1200                 | 8.93           | 8930              | 1200000          | 134.38                   | 1              | 100         |
| Ammonium sulfate precipitation (80%) | 80          | 3100                 | 6.27           | 501.6             | 248000          | 494.42                  | 3.68             | 20.66       |
| Gel filtration | 30          | 2600                 | 4.76           | 142.8             | 78000            | 546.22                  | 4.06             | 6.5         |

^1Activity unit (AU/mL) = Reciprocal of the highest dilution × 1000/volume of bacteriocins added; ^2Protein concentration measured by Bradford’s method; ^3Total protein = protein concentration × total volume; ^4Total activity = Activity (AU/mL) × Total volume; ^5Specific activity (AU/mg) = Total activity of the subsequent purification step/Total protein of the same step; ^6Fold purification = Specific activity of subsequent step/ Specific activity of crude preparation; ^7Recovery (%) = Total activity of subsequent step × 100/Total activity of crude preparation.
Figure 2 SDS-PAGE profile of bacteriocin produced by Bacillus sp. Sh10. A, Gel stained with coomassie blue R250. Lane 1, broad range molecular weight marker (Gen Direx); lane 2, bacteriocin precipitated with 80% ammonium sulfate; lanes 3,4,5,6,7, gel exclusive chromatography fractions 23-27 respectively; B, SDS-Polyacrylamide gel overlaid with C. albicans ATCC 10231 showing the inhibitory effect (arrow) of bacteriocin. Lane 1, bacteriocin precipitated with 80% ammonium sulfate; lanes 2,3,4,5,6, gel exclusive chromatography fractions 23-27, respectively.

Identification of bacteriocin by LC-MS/MS

The gel-filtration purified band that showed anti-Candida activity in SDS-PAGE gel overlay assay was exposed to LC-MS/MS analysis. The result from Mascot Sequence Matching Software [Matrix Science] with Ludwig NR database showed 43% identity of bacteriocin produced by isolate Sh10 with uncharacterized protein Tax_id=405532 from B. cereus [strain B4264]. Out of 105 amino acids of the uncharacterized protein Tax_id=405532, 46 amino acids were detected by LC-MS/MS, and the subsequent data analysis showed a 43% sequence similarity with the score of 175 with the uncharacterized protein Tax_id=405532 from B. cereus [strain B4264] (Figures 3 and 4). Based on the LC-MS/MS, the peaks corresponding to NFNTIDVTMNLNELYVLSGR (score: 66, expected value: 0.0085), SIDILSGDK (score: 18, expected value: 1.3e+03), SIDILSGDK (score: 33, expected value: 41), SIDILSGDKEAIQLNK (score: 70, expected value: 0.0043), and EAIQLNKYTISFNKPGK (score: 5, expected value: 1.3e+04) were detected in the mass spectra (Figure 3A-E).

In addition, the detected amino acids by LC-MS/MS did not show identity or homology to the antimicrobial peptides available at the antimicrobial peptide database. The overall view of the matched protein is summarized below.

Protein view:
MASCOT search result: Protein trJB7HDM0j/BCB4264_A3844 B7HDM0_BACC4 Uncharacterized protein n=3 Tax_id=405532 [Bacillus cereus (strain B4264)] Database: Ludwig NR Score: 175 Nominal mass (M): 11558 Da Calculated PI: 7.77 Protein sequence coverage: 43%

Figure 3 MALDI-TOF spectra of peptide: (A) peptide NFNTIDVTMNLNELYVLSGR, (B) peptide SIDILSGDK, (C) peptide SIDILSGDK, (D) peptide SIDILSGDKEAIQLNK and (E) peptide EAQLNKYTISFNKPGK.

Figure 4 Amino acid sequence of uncharacterized protein n=3 Tax_id=405532 [Bacillus cereus (strain B4264)]. The underline sequences reveal those that matched with that of experimental sample from SDS-PAGE active band. The sequence coverage was 43 % with a score of 175 [Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 59 indicate identity or extensive homology (p<0.05)].
Fractional protein purification is usually performed through ammonium sulfate precipitation as it facilitates concentration of dilute protein solutions (Whitford et al., 2003). This procedure prevents protein denaturation and makes it possible to restore activity after re-dissociation. Proteolysis and contamination by bacteria of proteins are hindered by ammonium sulfate as well (Harris & Angal, 1989). Enhanced purification is generally achieved by taking an ammonium sulfate cut. Bioactivity assays of the ammonium sulfate precipitate fraction and active fraction were performed in vitro, lysozyme, α-amylase, lipase, and proteasome K did not alter bacteriocin activity, but complete inactivation was observed after treatment with trypsin. The inhibitory compound was heat resistant and the antimicrobial activity of the present bacteriocin did not alter after autoclaving (Shayesteh et al., 2015). Although the present bacteriocin possessed the same range of molecular weight as other bacteriocins from *B. cereus* group members but differed in physico-chemical characteristics. For example, crenin GN105 (9 KDa) produced by *B. cereus* GN105 (*Naclerio et al., 1993*) differed from the present bacteriocin in terms of heat stability (stable until 75 °C), Tocchin (10.5 KDa) and thuricin (11.6 KDa) produced by *B. thuringiensis* spp. *tochigensis* HD868 (Paik et al., 1997) and *B. thuringiensis* BMGI.7 (Cherif et al., 2001), respectively, both differed from the present bacteriocin in terms of proteinase K susceptibility and heat stability (complete inactivation by heating at 121 °C for 20 min). However, SDS-PAGE identifies as BLIS of approximately 10 kDa compounds synthesised by *Mexican B. thuringiensis*, including Morpin 269 from *B. thuringiensis* spp. *morrisoni* (LBIT 269), kurtaspin 287 from *B. thuringiensis* spp. *kurstaki* (LBIT 287), kenyacin 404 from *B. thuringiensis* spp. *kenyae* (LBIT 404), entomocin 420 from *B. thuringiensis* spp. *entomocidus* (LBIT 420), and tolworthcin 524 from *B. thuringiensis* spp. *tolworthi* (LBIT 524) (Barboza-Corona et al., 2007; De la Fuente-Salcedo et al., 2008). Depending on heat stability, two uniform groups of such BLIS was distinguished via partial purification. The first group comprises Morpin 269 and kurtaspin 287, which show tolerance to heat and retain their stability up to 80°C. Meanwhile, the second group consists of the rest of the BLIS, which exhibit resistance to heat and retain their stability up to 121°C. The characteristic which differentiates BLIS from second group from the present bacteriocin is their complete loss of activity after exposure to proteinase K. The heat resistance, protease sensitivity, and low molecular weight of the present bacteriocin indicate that it belongs to group II bacteriocin (Klaenhammer, 1993).

The gel-purified band corresponding to approximately 11 KDa was analyzed by LC–MS/MS. Mascot software showed 43% similarity to an uncharacterized protein from *B. licheniformis* B4264 (46 out of 105 amino acids showed homology). We compared the present bacteriocin’s amino acid sequence with those of other bacteriocins in the NCBI and Antimicrobial Peptide Database. Sequence comparison showed no matches to the partial sequence obtained from LC–MS/MS and to the uncharacterized protein from *B. cereus* B4264. The homology between the present bacteriocin and previously isolated bacteriocins could not be defined. Partial purification of bacteriocin produced by isolate Sh10 is a novel bacteriocin. The present bacteriocin has a high amount of cationic amino acids, a net charge of +1, and a high percentage of hydrophobicity (42%). Given these characteristics, bacteriocin produced by *Bacillus* sp. Sh10 may exert its action by interacting with cell-wall-associated or membrane-associated binding sites in sensitive *C. albicans* cells. These findings indicated that the net positive charge of present bacteriocin would interact with the negative charges of phospholipids in the cell walls of sensitive cells by a strong electrostatic interaction, resulting in pore formation, which allowed for the efflux of intracellular materials, ultimately leading to cell death. According to Akkam (2016), antifungal peptides are cationic and possess an inhibitory effect on two pathogenic fungi, *A. fumigatus* and *C. albicans*. In *A. fumigatus*, antifungal peptides are cationic and possess membrane permeabilization. Peptide hydrophobicity is also controlled by the peptide synthesis and antimicrobial activity for food. Nature Reviews Microbiology, 3 (10), 777-788. http://doi:10.1038/nrmicro1273

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