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Isolation and biochemical characterisation of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* An6

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ABSTRACT

This study focuses on the isolation and characterisation of a peptide with bacteriocin-like properties from *Bacillus amyloliquefaciens* An6. Incubation conditions were optimised, and the effects of the incubation period and of carbon and nitrogen sources were investigated. The produced bacteriocin was partially purified with ammonium sulphate precipitation, dialysis and ultrafiltration and was then biochemically characterised. Maximum bacteriocin production was achieved after 48 h of incubation in a culture medium containing 20 g/L starch and 10 g/L yeast extract, with an initial pH 8.0 at 30 °C under continuous agitation at 200 rpm. The bacteriocin was sequentially purified and its molecular weight was determined to be 11 kDa by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The bacteriocin was relatively heat-resistant and was not sensitive to acid and alkaline conditions (pH 4.0–10.0). Its inhibitory activity was sensitive to proteinase K but was resistant to the proteolytic action of alcalase, trypsin, chymotrypsin and pepsin. In conclusion, bacteriocin An6, owing its wide spectrum of activity as well as its high tolerance to acidic and alkaline pH values, temperature and proteases shows great potential for use as a food biopreservative.

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1. Introduction

Bacteriocins are bacterial ribosomally synthesised antimicrobial peptides that are lethal to bacteria other than the producing strain [1]. Owing to their potential use as natural preservatives, bacteriocins produced by lactic acid bacteria have been the subject of intensive investigation in recent years [2]. In contrast, bacteriocins from the genus *Bacillus* have attracted little attention even though some *Bacillus* spp., such as *Bacillus subtilis* and *Bacillus licheniformis*, are 'generally recognised as safe' bacteria [3]. *Bacillus* is an interesting genus to search for inhibitory substances [4], and *Bacillus amyloliquefaciens* is one of the major producers of these substances, including several bacteriocins. Unlike bacteriocins produced by lactic acid bacteria, which have a narrow antimicrobial spectrum [5], bacteriocins from *Bacillus* exhibit distinct diversity in their inhibitory activities [6].

We have screened a number of *Bacillus* strains isolated from various sources for the production of inhibitory substances against domestic animal pathogens. The selected isolate *B. amyloliquefaciens* An6 exhibits a potential antimicrobial effect against some important domestic animal pathogens, including Gram-positive and Gram-negative bacteria. In the present study, we report the production, inhibitory spectrum and properties of this antimicrobial compound.

2. Materials and methods

2.1. Antagonistic strain

In a previous work, during a screening programme on protease-producing strains that have potential industrial applications, *B. amyloliquefaciens* An6 was isolated from a soil sample collected from an industrial complex/plant producing detergent. It was identified according to the methods described in *Bergey's manual of determinative bacteriology* [7] and on the basis of 16S rDNA sequence analysis. It was assigned the GenBank accession no. FJ517583. This strain was found to produce multiple proteases, and fibrinolytic BAF1 enzyme was partially purified and characterised [8]. This strain was also found to produce high amounts of biosurfactants (based on their ability to reduce surface tension from 71 mN/m to 30 mN/m). In the present study, An6 strain was also tested for its ability to produce bacteriocin.

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2.2. Antimicrobial activity assay

2.2.1. Agar diffusion method

Antibacterial activity was tested against Gram-positive and Gram-negative bacterial strains, including *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 4698, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 19430, *Klebsiella pneumoniae* ATCC 13883 and *Escherichia coli* ATCC 25922. Antifungal activity was tested against *Aspergillus niger* I1, *Mucor rouxii* DSM 1191 and *Botrytis cinerea*. Antimicrobial activity was initially assessed by the agar well diffusion method described by Millette et al. [9]. Culture suspensions of the tested micro-organisms were prepared and were serially diluted to 10^6 CFU/mL for bacterial strains and to 5×10^4 spores/mL for fungal strains. Luria–Bertani (LB) agar (Sigma Chemical Co., St Louis, MO) and Sabouraud dextrose agar (Sigma Chemical Co.) were used, respectively, for bacterial and fungal strains. Briefly, 100 μL of each suspension was spread over the surface of the LB/Sabouraud plate and was allowed to dry. Wells (7 mm depth, 6 mm diameter) were then cut in the agar and a 60 μL sample (2 mg/L) of the crude bacteriocin-like substance was delivered into them. The plates were then incubated for 24 h at 37 °C for bacteria and for 72 h at 30 °C for fungal strains. The diameter of the inhibition zone was measured and the results are reported in millimetres. The experiment was conducted in triplicate.

2.2.2. MTT test and determination of the minimum inhibitory concentration (MIC)

The MIC of bacteriocin An6 against various micro-organisms was determined by a broth microdilution assay. The inoculum of each bacterium was prepared in LB medium and suspensions were adjusted to 10^6 CFU/mL of bacteria cells estimated by absorbance at 600 nm. Serial dilutions of bacteriocin ranging from 5 mg/mL to 156 mg/mL were prepared in a 96-well plate (MidSci, St Louis, MO). Each well of the sterile microplate included 100 μL of the diluted bacteriocin, 100 μL of the LB growth medium and 10 μL of inoculum. The bacitracin zinc (Bio Basic, Markham, Ontario, Canada) and LB medium were used as positive and negative controls, respectively. Following overnight incubation at 37 °C, growth of the tested micro-organism was monitored at 600 nm by a microtitre plate enzyme-linked immunosorbent assay (ELISA). The MIC was defined as the lowest concentration of sample required for complete inhibition of bacterial growth.

2.3. Bacteriocin production under different conditions

Production of antimicrobial activity was first determined in four different media at 30 °C for 48 h: M1 (LB); M2 (yeast extract 10 g/L, glucose 20 g/L); M3 (yeast extract 10 g/L, peptone 10 g/L, casein 20 g/L); and M4 (glucose 20 g/L; $(NH_4)_2SO_4$ 2.3 g/L; K_2HPO_4 1 g/L; $MgSO_4$ 0.5 g/L; KCl 0.5 g/L; glutamic acid 2 g/L, $CuSO_4$ 1.6 mg/L; $Fe_2(SO_4)_3$ 1.2 mg/L; $MnSO_4$ 0.4 mg/L). At the end of each incubation period, antimicrobial activity was detected by the agar disk diffusion assay.

2.3.1. Optimisation of the culture medium and growth conditions

Various carbon sources such as lactose, galactose, sucrose, inulin, starch, maltose and glucose were evaluated for their effect on bacteriocin production by *B. amyloliquefaciens* An6 strain at a concentration of 2%. The best carbon source was further optimised in the range of 2–30 g/L. The basal medium contained starch as carbon source supplemented with 1% of different organic (casein, yeast extract, soya peptone and pastone) and inorganic nitrogen sources (ammonium sulphate, ammonium chloride and sodium nitrate). The best nitrogen source was further optimised in the range of 2–20 g/L. The period of incubation (0–72 h), temperature (30 °C and 37 °C) and pH (range 4.0–10.0) were examined for their effect on antagonistic activity production by the An6 strain.

2.3.2. Kinetics of bacteriocin production

Flasks containing 25 mL of nutrient broth were inoculated with *B. amyloliquefaciens* An6 cells from glycerol stock and were incubated at 37 °C in an incubator shaker for 18 h. This was used as a pre-culture inoculum. The kinetics of bacteriocin production was conducted at 30 °C for 72 h in optimised medium (starch 20 g/L and yeast extract 10 g/L). Growth was followed by measuring the optical density at 600 nm (OD_{600}). Samples were withdrawn at desired time intervals by centrifugation at $10\,000 \times g$ for 15 min and the supernatants were tested for bacteriocin activity against *S. aureus*. Antagonistic activity was expressed in terms of arbitrary units per millilitre (AU/mL). One arbitrary unit (AU) against an individual indicator strain was defined as the reciprocal of the highest dilution that still produced a minimum detectable zone of inhibition and expressed as AU/mL.

2.4. Partial purification of the bioactive substance

Strain An6 was grown in 500 mL of medium containing starch 20 g/L and yeast extract 10 g/L at 30 °C in a rotary shaker at 200 cycles per min for 48 h. Cells were removed by centrifugation at $10\,000 \times g$ for 15 min. The supernatant was precipitated with ammonium sulphate at 80% saturation under chilled conditions for 18–24 h. The precipitated proteins collected by centrifugation ($10\,000 \times g$, 30 min), was suspended in 5 mL of 20 mM phosphate buffer solution (pH 6.8) and was dialysed using a 1 kDa cut-off membrane (Sigma Chemical Co.) against the same buffer at 4 °C overnight. The dialysate was then applied to a stirred ultrafiltration cell (Millipore 8400; Merck Millipore, Darmstadt, Germany) using a 10 kDa cut-off membrane (PBGC membrane; Merck Millipore). The fraction with antimicrobial activity obtained was designated as the crude bacteriocin-like substance (CBLS).

2.5. Direct detection of the antimicrobial activity on Tricine–SDS-PAGE gels

Briefly, 20 μL of the CBLS and low-molecular-mass standards ranging from 3 to 45 kDa were subjected to Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis (Tricine–SDS-PAGE) carried out on 15% polyacrylamide gels using Tricine as trailing ion [10]. Following electrophoresis conducted at 100 V for 90 min, the gel was cut vertically. The first part, containing the sample and protein standards, was stained with staining solution (0.25% Coomassie brilliant blue R-250, 25% isopropanol, 8% acetic acid) to determine the molecular weights of separated protein bands. The other part of the gel was assayed for direct detection of inhibitory activity according to the method described by Barboza-Corona et al. [11] with slight modification. Briefly, this part was washed with phosphate buffer for 3 h. The gel was aseptically placed in a sterile Petri dish with 5 mL of soft brain–heart infusion medium (0.75% agar, w/v) containing ca. 0.1 mL of overnight culture of *S. aureus*. The Petri dish was incubated at 37 °C for 24 h and was observed for the presence of an inhibition zone.

2.6. Effects of enzymes, heat and pH on antimicrobial activity

CBLS was assessed for its sensitivity to proteases and other enzymes. Enzymes (obtained from Sigma Chemical Co.) and their respective buffers were: trypsin, chymotrypsin and alcalase (0.05 M Tris hydrochloride, pH 8.0); proteinase K (1 N NaOH, pH 6.5); pepsin (0.05 M glycine HCl, pH 2.0); and catalase as non-

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