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Purification, amino acid sequence and characterization of the class IIa bacteriocin weissellin A, produced by *Weissella paramesenteroides* DX

Maria Papagianni [a,*], Emmanuel M. Papamichael [b]

[a] Department of Hygiene and Technology of Food of Animal Origin, School of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece
[b] Department of Chemistry, University of Ioannina, Ioannina 45-110, Greece

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ABSTRACT

Weissella paramesenteroides DX has been shown to produce a 4450-Da class IIa bacteriocin, weissellin A, composed of 43 amino acids with the sequence KNYGNGVYCNKHKCSVDWATFSANIANNSVAMAGLTGGNAGN. The bacteriocin shares 68% similarity with leucocin C from Leuconostoc mesenteroides. Computational analyses predict that the bacteriocin is a hydrophobic molecule with a beta-sheet type conformation. Weissellin A exhibited various levels of activity against all gram-positive bacteria tested, but was not active against Salmonella enterica Enteritidis. The antimicrobial activity was not associated with target-cell lysis. The bacteriocin retained activity after exposure to 121 °C for 60 min or to −20 °C for 6 months, and to pH 2.0–10.0. It was not sensitive to trypsin, α-chymotrypsin, pepsin and papain, but was inactivated by proteinase K. At a dissolved oxygen concentration of 50%, weissellin A was produced with growth-associated kinetics. The properties of weissellin A make this bacteriocin a potentially suitable agent for food and feed preservation.

1. Introduction

Many bacteria produce ribosomally synthesized antimicrobial peptides (bacteriocins) with diverse structures, molecular masses, biochemical characteristics, spectra of activities and sequence homologies (Papagianni, 2003). These are usually membrane-permeabilizing cationic peptides with less than 50 amino acid residues (Papagianni, 2003). Depending on the presence of modified amino acid residues, the bacteriocins are classified as class I (modification) or class II (no modifications). One group of class II bacteriocins, the class IIa of "pediocin-like" bacteriocins, are gaining increasing interest because of their strong antilisterial activity, and heat and pH stability (Fimland et al., 2005). A considerable number of pediocin-like bacteriocins have been reported in the literature but only a few of them have been isolated and thoroughly characterized (Hastings et al., 1991; Héchard et al., 1992; Henderson et al., 1992; Holck et al., 1992; Metivier et al., 1998; Tichaczek et al., 1992). Elucidation of their amino acid sequences and the genetic determinants have provided valuable insights into the common characteristics of the subclass, e.g. the -YGNGV- motif in the N-terminus, the structure/function relationships and the modes of action of these compounds.

In this study we report the complete amino acid sequence and describe the characteristics of a class IIa bacteriocin, designated as weissellin A, which is produced by *Weissella paramesenteroides* DX isolated from a traditional Greek sausage.

2. Methods

2.1. Bacterial strains and growth conditions

The sausage isolate DX used in this study, was identified as *W. paramesenteroides* phylogenetically by comparison of a 708-base pair 16SrDNA sequence using BLAST (results not shown here). *Listeria inocua* ATCC BAA-680D was used as indicator microorganism for bacteriocin activity, and the strains listed in Table 1 were used to delineate the antimicrobial spectrum of the bacteriocin.

2.2. Bacteriocin production in batch fermentation

The isolated strain of *W. paramesenteroides* DX was maintained in M17 + 2% glucose agar (prepared with addition of 1.5% granulated agar to broth media) and grown statically in M17 + 2% glucose broth at 30 °C for 60 h (M17 was from Sharlau Microbiology, Spain). Mid-logarithmic phase cultures (OD_{600} = 1.2) were used as inoculums (2% v/v) for cultivation in a BIOFLO 110 New Brunswick Scientific stirred tank reactor with a working volume of 2L. The agitation system consisted of two 6-bladed Rushton-type impellers (52 mm), operated at the stirrer speed of 150 rpm. The temperature was maintained at 30 °C. The culture pH at inoculation time was 6.0 and no pH control was applied during cultivation. The

* Corresponding author. Tel.: +30 2310 999804; fax: +30 2310 999829.
E-mail address: mp2000@vet.auth.gr (M. Papagianni).

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Table 1
Inhibition spectrum of culture supernatant of *W. paramesenteroides* DX containing weissellin A.

Indicator organism	Medium	Incubation temperature	Aeration	Sensitivity to weissellin A[a]
Bacillus cereus LMG13569	BHI	37	Aerobic	85
Clostridium sporogenes NCTC533	RCM	37	Anaerobic	100
Clostridium thiaminolyticum ATCC15579	RCM	37	Anaerobic	78
Enterococcus faecalis NCTC8176	MRS	37	Microaerophilic	75
Lactobacillus brevis ATCC8287	MRS	37	Microaerophilic	65
Lactobacillus bulgaricus LMG13551	MRS	37	Microaerophilic	65
Lactobacillus casei ATCC344	MRS	37	Microaerophilic	75
Lactobacillus curvatus ATCC51436	MRS	30	Microaerophilic	10
Lactobacillus jensenii ATCC25258	MRS	37	Microaerophilic	70
Lactobacillus plantarum CECT220	MRS	30	Microaerophilic	70
Lactobacillus sakei CECT906T	MRS	30	Microaerophilic	20
Lactococcus lactis LM0230	MRS	30	Microaerophilic	75
L. lactis ATCC11454	MRS	30	Microaerophilic	20
L. lactis IL1403	MRS	30	Microaerophilic	72
L. lactis subsp. *cremoris* MC1363	MRS	30	Microaerophilic	15
Leuconostoc mesenteroides ATCC19254	MRS	25	Microaerophilic	20
Listeria inocua ATCC BAA-680D	BHI	30	Microaerophilic	92
Listeria monocytogenes ATCC19111	BHI	30	Microaerophilic	100
Micrococcus luteus CECT241	NB	30	Aerobic	100
Pediococcus acidilactici ATCC25740	MRS	30	Microaerophilic	55
Pediococcus pentosaceus ATCC 33316	MRS	30	Microaerophilic	50
P. pentosaceus LMG13560	MRS	30	Microaerophilic	50
Salmonella enteritidis ATCC13076	SS	25	Microaerophilic	0
Staphylococcus carnosus LMG13564	BHI	37	Microaerophilic	92

[a] Mean values of at least three experiments, expressed as a percentage of the inhibition zone diameter of the standard test strain *Micrococcus flavus* ATTC 400.

dissolved oxygen tension (DOT) was maintained at 50% by sparging the reactor with a mixture of N_2 and atmospheric air, adjusted by using two mass flow controllers, and the DOT was kept constant by feedback regulation. Samples were taken every 2 h. Runs were carried out in triplicate and repeated if experimental variation exceeded 10%. Fermentation kinetic parameters were calculated by numerical differentiation in MS Excel.

2.3. Determination of biomass, lactate, and glucose concentrations

Cell dry weight was determined by filtering 5 ml of broth through nitrocellulose filters (pore size, 0.45 μm, dried in a microwave oven at 150 W for 15 min), washing twice with 10 ml of distilled water and dried in a microwave oven (150 W, 15 min). Lactic acid concentration was determined with the EnzyPlus D/L Lactic Acid kit by Diffchamb AB (Diffchamb, Sweden). Glucose was determined using a glucose oxidase/peroxidase assay kit by Sigma Aldrich (Sigma Aldrich Co., UK).

2.4. Determination of bacteriocin activity

Antimicrobial compound attached to cell walls was removed by using the cell adsorption–desorption method for bacteriocin extraction as was described by Yang et al. (1992) with minor modifications. The pH of samples of *W. paramesenteroides* DX cultures was adjusted to 6.5 by addition of 1 N NaOH. The cultures were stirred at 150 rpm for 3 h at room temperature, the cells were collected by centrifugation at 10,000×*g* (4 °C) for 30 min, washed twice with 100 ml of 5 mM sodium phosphate buffer (pH 6.5), collected again by centrifugation at 10,000×*g* (4 °C) for 30 min and finally resuspended in 50 ml of 100 mM NaCl and HCl solution (pH 2.0). The cell suspension was stirred for 12 h at 4 °C, then subjected to centrifugation at 10,000×*g* (4 °C) for 15 min. The supernatant was filtered through 0.22 μm pore-size filters, concentrated to 0.1 volume by polyethylene glycol dialysis (PEG, Sigma, M_r 20,000) and again filter sterilized. This material was designated as "crude bacteriocin preparation" and was frozen at −20 °C when not used immediately. For activity determinations, serial twofold dilutions of the crude bacteriocin extract were made in sterile distilled water, the pH of samples was adjusted to 6.5 with 1 N NaOH and 30 μl were delivered into wells in M17 + 2% glucose agar plates previously seeded with 10 μl of a fresh overnight culture of *Micrococcus flavus* ATCC 400 (10^6 cells/ml) and incubated at 30 °C. The sample titer was defined as the reciprocal of the highest dilution at which activity was still obtained and was expressed in activity units (AU)/ml (Papagianni et al., 2006). Bacterial strains and growth conditions for the activity spectrum determination of the crude bacteriocin preparation are listed in Table 1.

For each indicator organism the appropriate solid medium was used. Anaerobic and microaerophilic growth was insured by growing the cultures in an incubator with controlled CO_2 partial pressure. 30 μl aliquots of cell-free culture supernatant fluid (pH adjusted at 6.5) were spotted on the appropriate solid media (1.5% w/v agar) seeded with a fresh culture of test cells, at logarithmic phase of growth. Plates were incubated at the optimum conditions for each test microorganism (Table 1) and examined for the presence of clear zones of inhibition. Strain sensitivities were expressed as a percentage (based on zone diameter measurements) of *M. flavus* ATCC 400 sensitivity to 200 AU of bacteriocin.

2.5. Crude bacteriocin preparation

Following extraction from producer cells, the bacteriocin-containing solution was concentrated using a vacuum concentrator centrifuge and subjected to a series of blue native polyacrylamide gel electrophoresis (BN PAGE) and native PAGE runs according to Wittig et al. (2006). Isolation of the protein of interest in a single band was achieved by carrying out native electrophoresis at pH 6.1 in the absence of urea, using gels prepared with a mixture of 15% (w/v) acrylamide and 0.4% (w/v) bisacrylamide. The exact position of the antimicrobial protein was determined after covering the native electrophoresis gel with solid medium (nutrient agar) containing cells of the indicator microorganism (*M. flavus*). The Rainbow protein molecular weight marker (2.35–46 kDa) (Amersham International, Amersham UK) was used for molecular weight comparisons.