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Inhibition of toxicogenic *Bacillus cereus* in rice-based foods by enterocin AS-48

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Abstract

The antimicrobial effect of the broad-spectrum bacteriocin enterocin AS-48 against the toxicogenic psychrotrophic strain *Bacillus cereus* LWL1 has been investigated in a model food system consisting of boiled rice and in a commercial infant rice-based gruel dissolved in whole milk stored at temperatures of 37 °C, 15 °C and 6 °C. In food samples supplemented with enterocin AS-48 (in a concentration range of 20–35 μg/ml), viable cell counts decreased rapidly over incubation time, depending on the bacteriocin concentration, the temperature of incubation and the food sample. Enterotoxin production at 37 °C was also inhibited. Heat sensitivity of endospores increased markedly in food samples supplemented with enterocin AS-48: inactivation of endospores was achieved by heating for 1 min at 90 °C in boiled rice or at 95 °C in rice-based gruel. Activity of enterocin AS-48 in rice gruel was potentiated by sodium lactate in a concentration-dependent way.

Keywords: Bacteriocin; Enterocin AS-48; *Bacillus cereus*; Rice-based foods; Biopreservation

1. Introduction

Bacillus cereus is a food-poisoning bacterium that may cause two types of gastrointestinal disorders: the emetic syndrome, caused by ingestion of a preformed toxin in the food, and the diarrhoeal syndrome, caused by a different toxin that can be formed in the food but also in the small intestine (Granum and Lund, 1997; Granum, 2001). Due to its ubiquitous distribution in nature, *B. cereus* occurs frequently in a wide range of food raw materials. Rice-based products and farinaceous foods such as pasta and noodles are frequently contaminated and involved in *B. cereus* poisoning (Kramer and Gilbert, 1989). Levels of *B. cereus* greater than 10^3 cfu/g have been found in both cooked and uncooked rice and in cereal products all over the world (Gilbert et al., 1974; Mortimer and McCann, 1974; Raevuorie et al., 1976; Schiemann, 1978; Shinagwa et al., 1979; Bryan et al., 1981; Holmes et al., 1981; Lee et al., 1995; Rusul and Yaacob, 1995; te Giffel et al., 1997; Nichols et al., 1999; Little et al., 2002; Sarrías et al., 2002). Other foods including meats, milk, sauces and desserts are also frequently implicated in *B. cereus* food poisoning (Notermans et al., 1997). Dried milk products are frequently contaminated with *B. cereus* spores. In foods containing both dairy and cereal ingredients, such as cereal-based infant foods, the risk of *B. cereus* poisoning may even be higher (Becker et al., 1994), and contaminated weaning foods are frequently responsible for episodes of diarrhoea in children (Motarjemi et al., 1993; Motarjemi and Nout, 1996). *B. cereus* spores survive pasteurization processes with decimal reduction times at 100 °C of 2.2–5.4 min and, in addition, vegetative cells from some strains can grow down to 4–5 °C (Dufrenne et al., 1995; Choma et al., 2000). Under specific conditions, mild heat treatments might even activate rather than inactivate dormant spores, thereby increasing the risk of

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pathogen outgrowth and food poisoning (Kim and Foegeding, 1990).

The demands of consumers for mildly processed foods with a limited refrigerated shelf-life have promoted research to improve processing technologies that may lower the risk of *B. cereus* poisoning. Natural antimicrobial substances, such as bacteriocins, are being investigated for food preservation and to replace chemical preservatives (Cleveland et al., 2001; Devlieghere et al., 2004), although nisin is currently the only bacteriocin widely used (Thomas et al., 2000). Some bacteriocins, such as nisin or lacticin 3147, prevent spore outgrowth and enterotoxin production by *B. cereus* (Jaquette and Beuchat, 1998; Morgan et al., 2001). Enterocin AS-48 is a broad-spectrum cyclic peptide produced by *Enterococcus faecalis* (Gálvez et al., 1986, 1989a; Martínez-Bueno et al., 1998; González et al., 2000), with a bactericidal mode of action (Gálvez et al., 1991) against several foodborne pathogenic bacteria (Gálvez et al., 1989b; Abriouel et al., 1998; Mendoza et al., 1999). The antimicrobial activity of enterocin AS-48 against *B. cereus* has been established in conventional culture media under laboratory conditions (Abriouel et al., 2002) as well as in cheese inoculated with a bacteriocinogenic strain (Muñoz et al., 2004). In this work, we describe the antimicrobial activity of enterocin AS-48 against a toxicogenic psychrotrophic strain of *B. cereus* in a model food system consisting of boiled rice and in a rice-based infant formula dissolved in whole milk.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

The psychrotrophic and enterotoxigenic strain *B. cereus* LWL1 (Dufrenne et al., 1995) was kindly supplied by Dr. F.M. van Leusden (Microbiological Lab. for Health Protection, Natl. Inst. Publ. Health and Environ., The Netherlands). The strain *E. faecalis* A-48-32 (a cured mutant from strain *E. faecalis* S-48 producing solely enterocin AS-48; Gálvez et al., 1985; Martínez-Bueno et al., 1990) was used to produce enterocin AS-48. The cured mutant strain *E. faecalis* B-48-47 (Martínez-Bueno et al., 1990) was used as negative control. *E. faecalis* S-47 (Gálvez et al., 1985) was used as test strain to determine bacteriocin activity. Bacilli and enterococci were cultivated in Brain Heart Infusion broth (BHI; Scharlab, Barcelona, Spain), at 37 °C. Tryptic Soy Agar (TSA, Scharlab) was used as plating medium for viable cell count. Enterococci and *B. cereus* were routinely stored at 4 °C in BHI-agar slants. Strains were maintained as frozen stocks at −80 °C in 40% glycerol.

2.2. Preparation of endospore suspensions

Spore crops were obtained according to Beuchat et al. (1997). Cultures grown in BHI broth for 24 h were surface spread on a solid sporulation medium consisting of nutrient agar (NA, Oxoid, Madrid) supplemented with 0.05 g/l of $MnSO_4$ (NAMS agar) and incubated for 4 days at 37 °C to obtain at least 90–95% spores. Spores were collected with a sterile cotton swab and resuspended in sterile distilled water (3 ml per plate). The pool of spores collected from the different plates was centrifuged at 5000×*g* for 15 min at 4 °C, washed two times with sterile distilled water by repeated centrifugation, and finally resuspended in sterile distilled water (6–7 log units/ml, as determined by plating on TSA) and stored in Eppendorf tubes at −20 °C until use. Spores were activated to germinate by heat (80 °C for 10 min), followed by 1-h incubation on ice. The number of spores was determined by serially diluting the heat-shocked spore suspensions in sterile saline solution and plating by triplicate on TSA. Plates were incubated for 48 h at 37 °C and the grown colonies were counted.

2.3. Preparation of bacteriocin extracts and determination of bacteriocin activity

Enterocin AS-48 was obtained from cultured broths of the producer strain *E. faecalis* A-48-32 after concentration by cation exchange chromatography as described by Abriouel et al. (2003). The partially purified bacteriocin samples obtained by this process contained enterocin AS-48 as the sole antibacterial substance, with an estimated purity of 48% (Abriouel et al., 2003). A cultured broth of the non-bacteriocinogenic mutant strain *E. faecalis* B-48-47 concentrated by an identical procedure was added (8.0%, vol/vol) as negative control. Bacteriocin concentrates and negative control concentrates were filtrated through 0.22-μm pore size low protein-binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. Samples were serially diluted and tested (100 μl) for bacteriocin activity against the indicator strain *E. faecalis* S-47 by the agar well diffusion method using stainless steel cylinders of 8 mm (outer) diameter (Gálvez et al., 1986). One arbitrary unit (AU) was defined as the highest dilution producing a visible (9 mm diameter) zone of inhibition. A specific activity value of 3.5 AU/μg protein was previously determined for purified enterocin AS-48 (Abriouel et al., 2003).

2.4. Effect of enterocin AS-48 on vegetative cells and endospores of B. cereus in boiled rice and in rice gruel

Vegetative cells from exponential-phase cultures of *B. cereus* LWL1 (incubated for 8 h at 37 °C in BHI broth) as well as endospore suspensions prepared as described above were inoculated into the desired rice-based preparation. Boiled rice was prepared from round grain white rice (SOS Cuétara S.A., Madrid). Rice (200 g) was boiled in water (250 ml) for 30 min, and the resulting paste was diluted 1:5 with distilled water under stirring for 10 min to obtain a homogeneous slurry (pH 8.79). Rice gruel was prepared from a commercial rice-based infant formula (rice cream; Nestlé España, S.A., Esplugas del Llobregat, Barcelona) by dissolving 25 g of the commercial powder in 200 ml of whole milk (Puleva SA., Granada, Spain) at 70 °C under stirring (pH 6.71). Food samples (10 ml each, in duplicate) were pre-cooled at desired incubation temperatures for 1 h before inoculation with *B. cereus* and addition of bacteriocin. Enterocin AS-48 was