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Suspected mode of antimycotic action of brevicin SG1 against *Candida albicans* and *Penicillium citrinum*☆

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ABSTRACT

Brevicin SG1, a novel, broad spectrum bacteriocin produced by *Lactobacillus brevis* SG1 isolated from 'ogi', a traditional Nigerian fermented cereal, was studied for its effects on fungal hyphal growth and morphology. The bacteriocin caused some morphological changes and a decrease in the total mass of *Candida albicans* and, cellular damage and an inhibition of pre-formed germ tubes in *Penicillium citrinum*. The inhibitory action was concentration- dependent, confirming that antibiosis was the mode of antagonism.

Transmission scanning electron microscopy (TSEM) study revealed that the bacteriocin caused hyphal destruction and lysing of the cell wall in *P. citrinum* and distortion of shape in *C. albicans*. TSEM result suggested that the fungal cell wall and the cell membrane were the main target of this antifungal compound and the suspected mode of action was fungiolytic. Disintegration of fungal hyphae was probably due to a membrane disorder causing increased membrane permeability and the consequent osmotic effect.

The bacteriocin was stable over a wide range of pH (1–10) especially between pH 5–7 while its production was maximal at pH 6.5. The production and stability of the bacteriocin at a broad pH makes it a suitable agent for controlling spoilage organisms of most foods and for using it as starter cultures of fermented foods. It also has the potential for probiotic usage.

1. Introduction

Fungal contamination of food causes economic losses and health problems due to the decomposition of food products, deterioration of organoleptic properties and the potential production of mycotoxins or allergenic conidia (Hussein & Brasel, 2001). There is therefore the need to prevent their growth in food products. Chemical preservatives (fungicides) that are commonly used to control the growth of fungi are not appropriate for food because of their side effects. Developing natural alternatives with no side effect are therefore imperative. Researches in biopreservation have led to the use of metabolites from lactic acid bacteria in food preservation (Papagianni, 2003).

Lactic acid bacteria (LAB) have been used for centuries in the fermentation of a variety of fermented foods; this is because they improve the organoleptic properties and preservation of such foods (Soomro, Masud, & Anwar, 2002). The preservative ability of LAB in foods is attributed to the production of antimicrobial metabolites like bacteriocin, diacetyl, hydrogen-peroxide, low-molecular weight substances and organic acid, which contribute both to the safety and flavour of many food products (Adebayo & Aderiye, 2007). They are natural candidate for use in biopreservation because they are naturally safe and have been consumed by man for centuries without any known side effect.

Bacteriocins, the most potent of the antimicrobial substances produced by LAB are ribosomally-synthesized proteins with antimicrobial activity (Savadogo, Quattara, Basole, & Traore, 2006). Bacteriocins generally exert their antimicrobial action by interfering with the cell wall or the membrane of target organisms, either by inhibiting cell wall biosynthesis or causing pore formation, subsequently resulting in death (O' Sullivan, Ross, & Hill, 2002). It was hypothesized that the action of bacteriocins of LAB occurs either by adsorbing to receptor cells and eventually causing its death, inhibition of synthesis of proteins or nucleic acid, or altering the membrane permeability (O' Sullivan et al., 2002; Tagg, Dajani, & Wannamaker, 1976). The effectiveness of bacteriocin is judged by such properties like activity level, activity spectrum and biostability.

☆ The experiment was performed in the Microbiology Unit of the Central Research Laboratory of the University of Ado-Ekiti, Nigeria.

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In a recent study, we isolated lactic acid bacteria from indigenous foods that produce bacteriocins that have broad spectra and exhibit a high level of activity (Adebayo & Aderiye, 2009). The bacteriocins were heat and enzyme stable, storable for several days at room temperature and active against a host of spoilage and pathogenic microorganisms. After an extensive screening of the bacteriocin for antifungal activity we have already reported that the bacteriocins were effective against many moulds and yeasts and brevicin SG1 from *Lactobacillus brevis* SG1 was the most potent (Adebayo & Aderiye, 2010). However for any practical application of the LAB isolates in biocontrol, there is a need to study their mode of action. The suspected mechanism of antagonism of brevicin SG1 on fungal cells is reported in this study.

2. Materials and methods

2.1. Microbiological cultures and media

The bacteriocin, *brevicin* SG1 was obtained from *L. brevis* SG1, isolated from sorghum 'ogi' in our previous work (Adebayo & Aderiye, 2009). The fungal strains are *Penicillium citrinum*, obtained from stored fermented foods and *Candida albicans* UCH 2007, yeast isolate from clinical cultures of University College Hospital (UCH), Ibadan, Nigeria. The *L. brevis* SG1 was cultured in de Mann, Rogosa and Sharpe (MRS) broth at 30 °C and the test fungi in Potato Dextrose Broth (PDB) or agar at ambient temperature. All the experiments in this study were performed in triplicate using dual cultures.

2.2. Preparation and purification of culture supernatant

This was performed as described previously (Adebayo & Aderiye, 2009). The *L. brevis* SG1 cells were cultivated anaerobically in MRS broth at 30 °C for 48 h and removed by centrifugation (4000 rpm, at 40 °C for 10 min). The cell free cultural supernatant (CFCS) was neutralized to pH 7 with 0.1 mM NaOH and treated with catalase to eliminate the effect of lactic acid and hydrogen peroxide. Purification was by ammonium sulphate precipitation and dialysis. The bacteriocin was filter-sterilized and stored at −22 °C when required.

2.3. Determination of bacteriocin titre

The titre of bacteriocin produced was quantified by serial two fold dilutions of bacteriocin in phosphate buffer (0.5 mM, pH 7.0). One arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was multiplied by a factor of 100 to obtain the activity unit per millilitre (AU ml^{-1}) of the original sample (Graciella et al., 1995).

2.4. Determination of pH stability and pH optima

The pH stability of the bacteriocin was determined by assaying over the pH range 1–10 using either 1 mol l^{-1} NaOH or HCl. After incubation for 1 h, it was readjusted to pH 7 and then assayed for the residual activity. For the determination of pH optima, *L. brevis* SG1 was grown at various initial pH (4–7). The growth and activity were assessed by the spectrometric (600 nm) and agar well diffusion assay respectively, as previously described (Bonade, Dagnan, & Garver, 2001).

2.5. Inhibition of fungal growth

The spectrophotometric method was used to determine the inhibitory effect of *brevicin* SG1 on mycelia growth of the test yeast while the method of Strom, Sjogrem, Broberg, and Schnurer (2002) was employed for fungal spore/cell determination. For the spectrophotometric assay, 10 ml of sterile PDB containing 10^5 cells of *C. albicans* and 2 ml each of different concentrations of brevicin SG1 were added into 100 ml Erlenmeyer flasks. A mixture of 2 ml of sterile MRS broth and 10 ml of PDB containing 10^5 cells of the test yeast served as control. The flasks were incubated at 25 °C and the absorbance was measured after 24 h at 490 nm to evaluate the growth of the test yeast in the presence of the bacteriocin. The percentage inhibition was determined by comparing the growth in the control with the treated yeast.

2.6. Determination of the mode of action

The mixture was prepared as described above but with the following modifications. The yeast cell was grown in corn meal broth and incubated for only 6 h. After which the effects of brevicin SG1 on yeast cells were assayed by scanning electron microscopy of samples. Also, the mixtures of *P. citrinum* and the bacteriocin were incubated for 48 h after which the effects on hyphal cells were observed with the transmission scanning electron microscope (TSEM).

2.7. Inhibition of germ-tube elongation

The modified method of Walker, Emslie, and Allan (1996) was employed. A total of 250 μl of various concentrations of the bacteriocin was added to 250 μl of PDB culture containing 4×10^5 spores of test fungi per ml that was previously incubated for 24 h. The control experiment involved the addition of sterile MRS broth only to the cultured fungal spore suspension. The vials were mixed for 10 s and incubated at 25 °C and 100% relative humidity (R.H.) for 6 h in sterile Eppendorf tubes. After which the contents of each Eppendorf tube were carefully mixed and 100 μl from each treatment was pipetted onto sterile glass microscope slide. The slide was observed under the microscope and the length of the germ tubes was measured using the micrometre attached to the ocular lens of the microscope. At least 50 germ tubes per slide were considered. The % inhibition of germ tube elongation was determined by comparing the control with the treated.

2.8. Statistical analysis

The data obtained were analyzed by ANOVA and the tests of significance were determined by Duncan's multiple range test (DMRT) (Fallant, 2000).

3. Results

3.1. Influence of pH on bacteriocin stability and production

The inhibitory activity was stable over a wide pH range (1–10) (Fig. 1). Full biological activity was retained at the pH values ranging from pH 5.0 to 7.0, whereas the activity was reduced by 25% at extremes of pH (pH 1.0, 9.0 and 10.0). Table 1 shows the growth and production of the inhibitory substance by *L. brevis* SG1 in the MRS medium adjusted to pH values between 4.0 and 7.0 and incubated at 30 °C. Bacteriocin production was observed in all the test pH. The optimum growth was recorded at initial pH 6.5 while the highest activity unit (AU) was at final pH value not below 4.0. The critical limit for growth and inhibitor production was between final pH values 4.0 and 5.5. There was practically no difference between the AU recorded at initial pH 6.0 and 6.5. Coincidentally, the initial pH of 6.5 was the optimum for both growth and bacteriocin production indicating a direct relationship between growth and antifungal production.