Research Article

Bacteriocins-Like Substances Produced by Enterococcus sanguinicola Isolated from Traditional Egyptian Food Sires (Chichorium pumilum)

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Abstract

The isolated organism was screened for their antimicrobial activities against different microbial indicators bacteria. Antimicrobial activities expressed in inhibition zones against all tested indicator organisms. The antimicrobial-producing isolate was identified as Gram-positive cocci, absence of catalase, hemolytic and mannitol salt agar. PCR products used to assess the DNA similarities and multiple sequences showed 94% identity to Enterococcus sanguinicola (accession no.Gu460396). Further identification to species level, E. sanguinicola was based on pigment formation and carbohydrate fermentation reactions recorded with and API 20 Strep. The SDS-PAGE analysis was indicated that bacteriocin of E. sanguinicola is a peptide of approximately 42 kDa. No antimicrobial activity was recorded after treatment of the cells with NaCl at pH 2.0. The leakage of K+ ion increased as the concentration of bacteriocin increased. The bacteriocins had a negative effect on the respiration, where the amount of consumed oxygen decreased by increasing the bacteriocins. No toxic effect against Artemia salina larva was recorded. Complete inactivation of antimicrobial activity was observed with Proteinase K and trypsin, respectively, after incubation for 2h at pH settings between 2.0 and 12.0. No antimicrobial activity was recorded at pH values from 5 to 10. On the other hand, high antimicrobial activity was obtained at pH 3. Heat treatment on bacteriocins showed that non-significant results at 70°C or 100°C while complete loss after incubation at 70°C, 100°C and by autoclaving. Bacteriocin remained stable when treated with Tween 20 and urea where the activity reduced to zero after treatment for 5 h at 37°C but highest activities was obtained at pH 3 and detergent SDS.

INTRODUCTION

The production of bacteriocins is in general, closely associated with growth of the producing organism. Bacteriocins activities decrease more or less sharply at the end of the growth phases as a result of degradation by proteases [1]. Bacteriocins are ribosomally synthesized antimicrobial materials protein in nature produced by eubacteria [2]. Bacteriocin production is also affected by the medium composition and culture conditions, such as pH, temperature and agitation [3]. Therefore, the optimization of environmental conditions is very important for the enhancement of bacteriocin production. Some bacteriocins inhibit food spoilage and pathogenic microorganisms. Bacteriocins are inhibitory towards sensitive strains and are produced by both Gram positive and Gram negative bacteria [4,5]. Bacteriocins are inhibitory towards sensitive strains [4]. Bacteriocinogenic LAB strains may play in the food industry as starter cultures, co-cultures, or bio-protective cultures, to improve food quality and safety [6].

The Enterococcus genus, a member of the lactic acid bacteria (LAB) is found in various environments, but more particularly in the intestines of humans and other animals, also found in vegetables like, plant materials and fermented foods products [7,8]. Bacteriocins are extremely heterogeneous group of antibacterial substances. Although chemically diverse, the one unifying property is the presence of an essential protein component. Some bacteriocins appear to be either simple proteins,
glycoproteins [9] or lipo-carbohydrate protein complexes [10]. In this study, we used different vegetables samples include Sires (Chichorium pumilum). Sires are plant cultivated in Egyptian land which eats as fresh or by cooking. The isolation, identification and characterization of Enterococcus sanguinicola from different traditional Egyptian Food and bacteriocin producing ability of LAB and its inhibitory effect against food-borne pathogenic bacteria (Gram positive and Gram negative bacteria plus fungus Candida albicans) were studied.

MATERIALS AND METHODS

Source of bacterial isolates

Samples of different traditional Egyptian food were used to isolate different bacterial isolates. The bacteriocin producer Enterococcus sanguinicola was isolated from Sires (Chichorium pumilum) and Koskos. Samples of the cooked source (Koskos) was left several days to be fermented and Sires (Chichorium pumilum) was left to be un-fresh and then used for isolation. Five gram of the cooked fermented samples were homogenized in sterile physiological water, serially diluted and 100 µl was spread and plated onto MRS agar [11]. Plates were incubated overnight at 30°C ± 2°C for 24 h. Colonies were randomly selected and screened for production of antimicrobial compounds by using the Indicator organisms.

Indicator organisms

Different Gram-positive and Gram-negative bacteria in addition to a fungus were used as indicator organisms in this study. Proteus vulgaris 1753, Escherichia coli 1357, Enterobacter cloacae, Serratia marcescens 921/79, Kibblesa pneumonia, Bacillus cereus, Bacillus subtilis, Citrobacter freundii and Staphylococcus aureus, in addition to Candida albicans were used. These microorganisms were kindly provided by Bacteriology Unit, Botany Department, Faculty of Science, Tanta University. The indicator organisms were grown on nutrient agar plates at 37°C for 24 h [12].

Assay for antibacterial activity

Antimicrobial activity was confirmed by using the agar-spot test method, Overnight cultures of the test organisms were prepared in MRS broth at 30°C ± 2°C and then centrifugated at 7000 rpm for 30 min at 4°C and the cell-free supernatants were filter sterilized (Millipore/0.22 µm). Overnight culture of the indicator organisms were prepared and 5×10^5 cfu ml^{-1} in 100 µl Potassium-Sodium-Phosphate buffer, (pH 7) were spread onto nutrient agar plates. 100 µl of sterilized cell free supernatant of the test organisms (adjusted to 6.0 with sterile 1 N NaOH) was loaded into well made in inoculated plates. The plates were incubated at suitably temperature (37°C) overnight. The antimicrobial activity was showing a clear zone of growth inhibition [13]. The largest inhibition zone of the indicator bacterium indicates the most antimicrobial-producing test organisms.

Strain identification

Strain was largest inhibition zone against indicator organisms was selected for further studies. Strain was identified according to the physiological and biochemical characteristics described by [14]. Pigment formation was tested on Tryptic Soy Broth (Merck) as described by [15]. Test for motility was done according to [16]. Catalase test was performed as recommended by [17]. Mannitol salt agar (MSA) [18], hemolytic activity [19]. Sugar fermentation reactions were recorded by using the API 20 Strep and compared with reactions listed for enterococci [20]. Further identification was by DNA banding pattern generated with primers specific for Enterococcus according to the methods described by [21].

Determination the molecular weight mass by (SDS-PAGE)

Enterococcus sanguinicola was grown in MRS broth for 20 h at 30°C. The cells were harvested by centrifugation (7000rpm, 30 min) and the bacteriocin precipitated from the cell-free supernatant with 70% saturated ammonium sulphate [22]. The precipitate was re suspended in 25 mM ammonium acetate (pH 6.5), desalted by using a 1000 Da cut-off dialysis membrane and separated by SDS–PAGE, as described by [23]. A low molecular weight marker with sizes ranging from 2.5 to 45 kDa was used. The gels were fixed and stained, the position of band was determined.

Chemical structure of the antimicrobial materials the infra red spectra (IR)

The infra red spectra of the antimicrobial material were carried out using infra red spectrophotometer Perkin-Elmer1430. Small discs were made from the mixture of about 1 mg of the tested material and 300 mg of pure K Br, followed by pressing into a disc and used for determination of the infra red spectra. The measurements were carried out at infra red spectra between 400 and 4000 nm [24].

MODE OF ACTION OF ANTIMICROBIAL MATERIAL

Adsorption studies

Adsorption of the bacteriocins by producer strain was studied by using the method of [25]. After 20h of growth in MRS broth at 30°C, 300 ml of the culture was adjusted to pH 6.0, centrifuged (7000 rpm, 4°C 30 min), washed in sodium phosphate buffer (pH 6.5), and resuspended in 10 ml of 100 mM NaCl (pH 2.0, 4°C) by slowly stirring. After 1 h the cell suspension was centrifuged, the pH of supernatant adjusted to 7.0 and then tested for antibacterial activity as described by [26].

Leakage of potassium ions of indicator organisms

Cells of indicator organisms of each culture were harvested, washed twice with phosphate buffer (pH 7) and resuspended in sterile deionized water. 0.5 ml of the suspension (5×10^5 CFU/ml) were mixed to 4.5 ml buffer containing 0.04, 0.1 and 5 mg/ml of the antimicrobial materials and incubated at room temperature in rotary shaker at 150 rpm for 30 min. The incubated samples were analyzed for potassium ion using flame photometer (Clinical flame photometer 410C) [27].

Respiration of indicator bacteria

The indicator bacteria were grown in nutrient broth containing different concentrations of the tested materials (bacteriocin) (0.125, 0.250 mg/ml) in rotary shaker (150 rpm), then bacterial pellet were collected by centrifugation (7000 rpm for 30 min at 4°C), and washed twice in phosphate buffer. 0.5
The isolated bacteria were screened for their antimicrobial activities against different microbial indicators Gram-negative bacteria (Proteus vulgaris 1753, Escherichia coli 1357, Enterobacter cloacae, Serratia marcescens 921/79 and Klebsiella pneumonia) and Gram-positive bacteria (Citrobacter freundii, Bacillus cereus, Bacillus subtilis and Staphylococcus aureus), in addition to Candida albicans as a fungus. Antimicrobial activities expressed in inhibition zones against all of the tested indicator organisms. The antimicrobial-producing isolate was identified by biochemical test by using API 20 and DNA analysis shown as Gram-positive cocci, absence of catalase, hemolytic and mannitol salt agar was showed as negative results. The sequencing of PCR products (using species-specific primers) and the Blast program used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny showed 94% identity to Enterococcus sanguinicola (accession no.Gu460396) from Sires. Further identification to species level, E. sanguinicola was based on pigment formation on Tryptic Soy Agar, and carbohydrate fermentation reactions recorded with and API 20 Strep (not shown). The cells were non-motile, which distinguished the strain from Enterococcus strains.

The SDS-PAGE analysis of the purified antimicrobial material was performed (Figure 1). Separation by SDS–PAGE indicated that bacteriocin of E. sanguinicola is a peptide of approximately 42 KDa compared to marker proteins. The maximum activity has been recorded at the beginning of stationary growth have performed on cultures of 24-h-old. The recovery of bacteriocin after ammonium sulfate precipitation was approximately 80%. Active fractions collected from the equilibrated column of Sephadex G-150. The separation of these fractions yielded an active peak which eluted at 40–45 min (Figure 2). No antimicrobial activity was recorded after treatment of the cells with NaCl at pH 2.0 (data not shown), suggests that antimicrobial material (bacteriocin) does not adhere to the cell surface. The structure of antimicrobial materials compound under investigation, it is
necessary to have an assignment for the IR-absorption bands corresponding to the active groups in the compound. IR spectra of the two antimicrobial materials showed the same functional groups (Figure 3). The spectrum was subdivided into different regions: 3000-3500 cm⁻¹ was hydroxyl groups (OH), 2926 cm⁻¹ was aliphatic group (C-H), 2082 cm⁻¹ was (C=O), 1647 cm⁻¹ was (C=N), 1548 cm⁻¹ was (C=C), 1346-1201 cm⁻¹ was aromatic system and region 1120-618 cm⁻¹ was aliphatic group (C-H).

Different concentration of the bacteriocins produced by *E. sanguinicola* affected on flow of K⁺ outside the cells of some indicator organisms. (Figure 4) showed that the leakage of K⁺ ion increased as the concentration of bacteriocin increased. Effect of bacteriocins produced by *E. sanguinicola* on respiration of some indicator organisms with different crude bacteriocins concentration (0.0, 0.125 and 0.250 mg/ml). The data presented in (Figure 5) indicated the bacteriocins had a negative effect on the respiration of the tested indicators, where the amount of consumed oxygen decreased by increasing the bacteriocins. The amount of oxygen was determined as dissolved oxygen expressed in mill mole.

No toxic effect of the tested bacteriocins (10 and 100 mg/ml) against *Artemia salina* larva was recorded under the experimental conditions. The effect of bacteriocins of *E. sanguinicola* on the growth of indicator organisms was investigated using 3-h-old cultures of the indicators, resulted in growth inhibition for two hours followed by a slow recovery of growth (Figure 6).

Complete inactivation of antimicrobial activity was observed after treatment of the cell-free supernatant of strain *E. sanguinicola* with Proteinase K and trypsin, respectively, after incubation for 2 h at pH settings between 2.0 and 12.0, this indicates that the antimicrobial materials produced by is protein in nature (Bacteriocin). The antimicrobial activities at the different pH were determined after cell free extracts of *E. sanguinicola* was adjusted to pH 3, 4, 5, 6, 7, 8, 9 and 10 incubated at 37°C for 20 min. The data presented in (Figure 7) showed that no antimicrobial activity was recorded at pH values from 5 to 10. On the other hand, high antimicrobial activity was obtained at pH 3.

Heat treatment on the activity of the bacteriocins produced by *E. sanguinicola* showed that, non significant decrease in the antimicrobial activity was reported at 70°C or 100°C after incubation for 15 min. compared to control (at room temperature) while complete loss of the antimicrobial activity after incubation at 70°C, 100°C for 30 min and by autodaving (Figure 8). Bacteriocin *E. sanguinicola* remained stable when treated with 1% (v/v, final concentration) Tween 20 and urea (1%, w/v) where, the activity reduced to zero after treatment for 5 h at 37°C. On the other hand, SDS increase the bacteriocin activity compared to control (Figure 9).

DISCUSSION

Bacteriocins are natural antimicrobial agent produced by food fermentative bacteria [31] Bacteriocins and the organisms that produce them have potential in food and feed industry as natural preservatives [5,8,32]. In this study, the antimicrobials active isolates were isolated from traditional Egyptian food from Sires. The bacteriocin isolated from Sires was identified as *Enterococcus sanguinicola*. The *Enterococcus* genus, a member of the lactic acid bacteria (LAB) is found in various environments and are also found in vegetables, plant materials and fermented foods products [32,33]. MRS, pH 6.5, temperature 30ºC and incubation period of 20 hours were the optimum conduction for the production bacteriocin indicated that this medium was the most convenient and contains specific nutrients that are required for the production of desired material [34]. Bacteriocin of *E. sanguinicola* inhibited the growth of indicator bacteria and conforms to the description of a bacteriocin as defined by [35]. However, the activity recorded against Gram-negative bacteria is unusual, and has thus far only been reported for a few bacteriocins of lactic acid bacteria [36]. As far as we could determine, bacteriordan produced by *E. mundtii* with activity...
against Gram-positive and Gram-negative bacteria suggested that specific nutrients are required for antimicrobial material production [34]. The antimicrobial material obtained by the isolated bacterial species *E. sanguinicola* was separated by precipitation the cell free supernatant which contains the antimicrobial material by 80% saturation ammonium sulphate. The precipitates were collected, drained and then dissolved in small volume of isopropanol in 25 mm ammonium acetate pH 6.5. The dissolved crude antimicrobial material was purified by passing into a column of Sephadex G-150. The SDS-PAGE analysis of our purified bacteriocins showed the molecular weight of bacteriocin isolated from *E. sanguinicola* was 42 KDa (De Kwaadsteniet et al., 2005). This may indicate that the increase in the total biological activity might result from multimolecular dissociation for *E. sanguinicola* bacteriocins [3]. The active fractions which contain the active peptide were tested against indicator bacteria and give inhibition zone its diameter between (8-17 mm) for *E. sanguinicola*, these results clearly
Figure 6 Effect of bacteriocins produced by *E. sanguinicola* on growth of some indicators.

Figure 7 Effect of pH on the antimicrobial activity of bacteriocins produced by *E. sanguinicola*. (Control = pH: 7).

Figure 8 Effect of heat on the antimicrobial activity of bacteriocin produced by *E. sanguinicola*. 
demonstrated that the antimicrobial materials was produced by traditional Egyptian fermented food bacteria [37]. Infrared (IR) spectroscopy of the antimicrobial materials indicated the presence of many functional groups in the two antimicrobial materials as follow OH, C=O, C-H, C=N, C=C and aromatic group.

The mode of action of the produced antimicrobial materials not detected after treating with NaCl at pH 2.0, suggesting that the antimicrobial materials did not adhere to the surface of the producer cells. Similar results have been reported for plantaricin ST31 [25], bozacin B14 [13] and pediocin ST18 [25]. The bacteriocins were destroyed by proteolytic enzymes produced by the indicators [25]. In the cell lysis showed the growth of indicator bacteria was inhibited for 2 h, followed by a slow recovery of growth. Similarity to the bacteriocin produced by Lactobacillus plantarum which isolated from molasses. Absence of cell lysis suggests that the mode of action of two antimicrobial materials is not impairment of cell wall biosynthesis. This is in agreement with [38]. The common mechanism of action which has been determined for other bacteriocins of lactic acid bacteria (LAB) is disruption of the electrochemical gradient across the cytoplasmic membrane by pore formation [39].

The bacteriocin of E. sanguinicola had no toxic effect on Artemia salina. So, bacteriocin may be safe to use in food industry [40]. The mode of action also showed that the different concentration of antimicrobial materials increased the flow of potassium ion from the susceptible cells by made increase in membrane permeability which allow the passive efflux of ions (K⁺) [41]. The results agreed with those obtained by [42] found that lactacin 3147 interact with cytoplasmic membrane, leading to formation of pores. These pores were shown to be selective for K⁺ ions. Also, it found that lactocinc (G) formed small pores, which allowed potassium efflux, resulting in ATP hydrolysis and dissipation of membrane potential. The effect of antimicrobial materials on the indicator bacteria respiration suggested that the respiration of some indicator bacteria decreased in the presence of the different concentration of the produced antimicrobial materials from E. sanguinicola.

Treatment of antimicrobial materials with proteolytic enzymes proteinase K and Trypsin resulted in complete inactivation (digestion) of antimicrobial materials activity, confirming their proteinaceous nature and also lends support their characterization as bacteriocins [43]. Similar characterization has been reported for the bacteriocins from Enterococcus sp. [43,44]. Antimicrobial materials production was strongly dependent on pH and temperature as claimed by [36]. Activity of antimicrobial materials was observed in our study at acidic and neutral pH levels (5.5:7.5), but maximum antimicrobial materials activity was noticed at pH 6.0 and 6.5. Similar results were observed by [45]. The bacteriocins produced by our test bacteria exhibited antimicrobial activity against the indicator organisms at pH 3-4 and no activities were recorded at pH values below 3 and above 4. The finding that the antimicrobial material is pH unstable was perhaps due to more rapidly degraded by subtle changes in pH and is common among investigators.

The activity of antimicrobial materials observed at different growth temperatures, suggested that the temperature play an important role in antimicrobial materials production, similarity plantaricin [46]. The inhibitory activity was seen only in narrow pH range (3:4) and above that loss its activity, this result assertion that the antimicrobial materials were a protein in nature and as such were a bacteriocin. When the antimicrobial materials of E. sanguinicola was submitted to heat treatment, most of the activity was maintained at temperatures 70 °C and 100°C for 15 min. and one third of the activity was lost after 30 min, so our bacteriocins considered to be heat stable [47]. Heat stability could be considered a very useful characteristic as many of these bacteriocins may find a potential use in food preservation. Similar types of results have been reported for other bacteriocins [44,48], but the antimicrobial materials activity were inactivated after treatment at 121°C for 20 min. Also bacteriocin ST15, produced by Enterococcus mundtii ST15, bacteriocin N15 produced by Enterococcus faecium N15 [49]. Production of antimicrobial materials at acidic and neutral pH levels, over a wide temperature range (30°C:45°C), and with inhibition of food-borne pathogens including E. coli, B. cereus and Staphylococcus aureus will be quite promising for development of
a wider applications of these antimicrobial materials in various foods with control of these pathogens.

Bacteriocin produced by *E. sanguinicola*, sodium dodecyl sulfate (SDS) enhance its antimicrobial activity. Suggesting that the detergents may dissociate the bacteriocin, thereby releasing more active subunits. Similar observations were recorded for enterocin EJ97 produced by *E. faecalis* EJ97. Antimicrobial material which produced by *E. sanguinicola*, Sodium dodecyl sulfate (SDS) had no effect on antimicrobial material activity which produced, suggesting that the detergents may dissociate the bacteriocin, thereby releasing more active subunits.

**CONCLUSION**

The bacteriocins from food grade appear to qualify as ideal food biopreservative and it used at low concentration, active under refrigerated storage, not alter the nutritional properties and non-toxic to humans.

**REFERENCES**


