AU J.T. 16(2): 74-80 (Oct. 2012)

Property and Antibacterial Spectrum of Partially Purified Enterocin Produced by Entrocinogenic *Enterococcus faecalis* Isolated from the Gut of Cockroach

Oluwole Moses David, Anthonia Olufunke Oluduro* and Oladiran Famurewa

Department of Microbiology, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria
E-mail: <davidoluwole5@gmail.com; ofamurewa@yahoo.com>

Abstract

Enterocins are small, heat stable and antimicrobial agents are directed against a broader spectrum of bacteria. Isolation, partial characterization and antibacterial activity of partially purified enterocin from Enterococcus faecalis *form the aim of this study. The protein content of crude enterocin of the enterocin from highest producing strain was 16.6 mg/ml and its activity was 170 AU/ml. The antibacterial activity of crude enterocin produced by* Enterococcus faecalis *was tested on both Gram-positive bacteria which include* Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus *and* Bacillus cereus, *while Gram-negative bacteria were* Klebsiella pneumoniae, Serratia marcescens, Pseudomonas aeruginosa, Proteus mirabilis, Listeria monocytogenes *and* Salmonella *Typhi. The effect was pronounced on Gram-negative than Gram-positive pathogens. The enterocin was stable at 40, 50, 60 and 70^{o}C and over a pH range between 4.0 and 8.5. It has bactericidal effect and cause rapid inhibition of the growth of the sensitive strains. No change was observed in the enterocin bioactivity after treatment with sodium chloride, magnesium chloride, sodium dodecyl chloride, ethylene diamine tetra-acetic acid and urea. In conclusion, since it can withstand a broad range of temperature and pH, it can be used as biocontrol of both pathogenic and spoilage organisms.*

Keywords*: Cockroach, bacteriocin, characterization, Gram-positive bacteria, Gram-negative bacteria, biocontrol, spoilage organisms.*

Introduction

Bacteriocins are a diverse group of gene encoded antibacterial agents proteinaceous in nature (Batdorj *et al.* 2006). They are produced by bacteria to inhibit the growth of similar or closely related bacterial strain(s) (Rasool *et al.* 1996; Franz *et al.* 1997; Heng *et al.* 2006) They are typically considered to be narrow spectrum antibiotics (Herranz and Driessen 2005) and are structurally, functionally and ecologically diverse. Bacteriocins are categorized in several ways and into different classes based on the producers (Nazef *et al.* 2008).

Enterocin is a class of bacteriocin produced by *Enterococcus* species. Enterocins are small and heat stable which production is an important characteristic of most *Entero-coccus* spp. Enterocinogenic strains have been isolated from different sources like sous-vide cooked fish fillets (Ben Embarek *et al.* 1994), Italian ryegrass (Izquierdo *et al.* 2008), intestinal track of ostrich (Jennes *et al.* 2000) and chicken ceca (Line *et al.* 2008). Enterocins are grouped in the current bacteriocin classification systems (Balla *et al.* 2000; Franz *et al.* 2007). Antibacterial potencies of the enterocins have been tested on both Gram-positive and Gram-negative bacteria of medical significance (Line *et al.* 2008).

The antimicrobial activities of enterocin are directed against a broader spectrum of bacteria than is seen for other bacteriocins produced by Gram-negative bacteria (Drider *et*

* Department of Microbiology, Obafemi Awolowo University, Osun State, Nigeria.
E-mail: <oluduro2003@yahoo.co.uk>.

al. 2006). Due to activity of enterocins against food-borne pathogens, they have been reported to be good candidates for bio-preservatives in food. Their use in food or in model food studies has received much attention in recent years (Herranz *et al*. 2001).

There paucity information on the property of the enterocin produced by *E. faecalis* isolated from arthropods and their antibacterial spectrum. This work therefore investigates the properties of enterocin produced from cockroach.

Materials and Methods

Isolation and Identification of *E. faecalis*

Cockroaches were collected from the Government Hostel of the Ekiti State University, Ado-Ekiti, Nigeria. They were surface-sterilized in 1.0% sodium hypo-chloride for one minute and rinsed in three changes of sterile distilled water according to Amund *et al.* (1986). The surface sterilized samples were aseptically dissected to remove the gut, which was macerated and serially diluted. The inoculum was plated on Bile aesculin agar (Oxoid) and incubated at 37^{o}C for 24hrs. Representative colonies were picked and sub-cultured to get pure culture. Colonial, morphological and biochemical characteristics as of the isolates were done as described by Holt *et al.* (1994).

Source of Test Bacteria

Bacterial isolates, which include *Salmonella* Typhi, *Klebsiella pneumoniae, Bacillus cereus, Serratia marcescens, Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus faecalis, Enterococcus feacium, Staphylococcus aureus* and *Listeria monocytogenes*, were collected from the Department of Microbiology, Ekiti State University, Ado-Ekiti, Ekiti State, Nigeria. The culture of the test pathogens were standardized using the method of Bauer *et al.* (1966).

Screening for Enterocin Production

Deferred antagonism method was used for the preliminary testing of isolated *E. faecalis* for the production of enterocin. *Enterococcus faecalis* strains were evaluated for anti-microbial activity against indicator organisms by the agar block method (Stern *et al.* 2006). Approximately 10^7 CFU/ml of each isolate was plated on the surface of MRS agar, and incubated for 24hr at 37°C. Agar blocks (diameter, 5mm) containing growth were aseptically excised from the MRS agar and placed upside down on the surfaces of agar plates seeded with 10^7 cells of test organisms. Plates were incubated for 48h at 37°C. Enterocin production was quantified by measuring the diameters of the resulting inhibition zones for the growth of the test organisms.

Preparation of Cell Free Supernatant (CFS)

The strain of the selected *E. faecalis* isolates which showed the highest inhibition zone for at least three indicator organisms during the primary screening were used for further studies. CFS from the strains was obtained and its inhibitory activity against the indicator organisms was assayed by the agar well-diffusion test. CFS was obtained from MRS broth cultures after 18 hr incubation at 32ºC by centrifugation at 10,000 *g* for 10 min at 4ºC. To rule out inhibition due to pH reduction caused by organic acids, the pH of the supernatants was adjusted to 6.2 using 1 N NaOH. Inhibitory activity from hydrogen peroxide was also eliminated by addition of catalase (Sigma Chemical Co., St. Louis, MO, USA) at 130 U/mL. To ensure sterility, the supernatant was filter through 0.22-µm pore-size filters (Carl Schleicher & Schüll).

Determination of Protein Content of the Enterocin Produced

The protein content of the crude extract was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The enterocin was harvested from culture of early stationary phase of *E. faecalis*. The cells were removed by centrifugation at 5,000 × *g* for 15 min at 48^{o}C. Ammonium sulfate was added to the supernatant at the rate of 4 g/10 ml of culture supernatant. The protein precipitate was pelleted by centrifugation at 10,000 × *g* for 20 min and dissolved in 500 ml of 20 mM sodium phosphate buffer (pH 5.0).

The supernatant was transferred to a clean sterile container.

Evaluation of *In vitro* Activity of Partially Purified Enterocin

For detection of enterocin activity, an agar-well diffusion assay was used according to the method of Muriana and Luchansky (1993). Soft MRS agar (0.7%, w/v), containing indicator organisms, was overlaid onto MRS plates. Wells were made in the lawn of hardened soft agars. Aliquots (50 µl) of supernatant of overnight cultures (16 h) were poured in the wells. The plates were incubated overnight at 37 °C. A clear zone of inhibition around the well was taken for enterocin production.

Determination of Stability of Enterocin

The sensitivity of the of partially purified enterocin from *E. faecalis* to different pH was estimated by adjusting the pH of the cell free supernatant to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 with 1M NaOH or 1M HCl and testing against the indicator strains after 30 minutes and 2 hours of incubation. The sensitivity to heat was also tested by heating cell free supernatant samples to 30, 40, 50, 60, 70, 80, 90, 100 and 121^{o}C and assay the residual activity after 5, 10, 15, 20, 30 and 40 min of incubation.

The stability of the enterocin to some chemicals was tested on cell-free supernatant treated for 2 hours with 0.1 mg/ml and 1ml final concentration of the following chemical substance sodium chloride, magnesium chloride, sodium dodecyl, sulphate (SDS), ethylene diamine tetra acetic acid (EDTA) and urea, while the active supernatant was used as control.

Results

The antimicrobial producing *E. faecalis* strains were isolated from the gut of cockroaches. They were non-motile, Gram-positive, spherical bacterium, facultative anaerobe with a fermentative metabolism.

Out of the seven strains of *E. faecalis* screened for the production of enterocin, only one was not enterocigenic. *E. faecalis* E18 had the highest zone of inhibition on the test organisms (Table 1). This suggested that it was able to produce the highest amount of enterocin. Next to it was *E. faecalis* E8. The enterocin produced has a highest effect on *S. aureus.* The least effect was demonstrated on *B. cereus.*

The protein content of crude enterocin E18 sample was 16.6 mg/ml and the crude enterocin activity was 170 AU/ml. Ammonium sulfate precipitation concentrations was 233 AU/ml. Table 2 shows that after purification of crude enterocin on ammonium sulphate precipitation and ion exchange the resultant volume was 5 ml and 3 ml, respectively. After purification, the protein concentration was 166.6 mg/ml.

Table 1. Preliminary antibacterial screening of enterocin produced by *E. faecalis* (zone of inhibition in mm).

Isolate	*S. Typhi*	*K. pneumoniae*	*B. cereus*	*S. marcescens*	*E. coli*
E. faecalis E45	26	0	0	10	0
E. faecalis E22	22	10	0	0	0
E. faecalis E8	15	20	8	10	10
E. faecalis E70	0	0	0	0	0
E. faecalis E91	0	10	0	0	5
E. faecalis E7	10	0	0	0	0
E. faecalis E18	21	6	9	16	12

Table 2. Purification of crude enterocin from *E. faecalis* E 18.

Step	Crude	$(NH_4)_2SO_4$ Precipitation	Ion Exchange
Volume (ml)	10	5	3
Activity (AU/ml)	170	233	144
Protein cone (mg/ml)	16.6	8.4	2.6
Total Activity (AU)	1700	1165	342
Total Protein (mg)	166	42	7.6
Specific Activity (AU/mg)	10.2	27.7	45
Yield	100	68.5	20.1
Fold purification	1	2.71	4.39

The inhibitory activity of crude enterocin produced by *E. faecalis* E18 was tested on both Gram-positive (*E. faecalis, E. faecium, S. aureus* and *B. cereus*) and Gram-negative (*K. pneumoniae, S. marcescens, P. aeruginosa, P. mirabilis, L. monocytogenes* and *S.* Typhi) pathogens.

Table 3 shows the crude enterocin zone of inhibition on test organisms. The effect was pronounced on Gram-negative more than Gram-positive pathogens. The highest zone of inhibition was found in *S. marcescens* (24mm), followed by *S. aureus* (24 mm). There was no inhibitory effect on *B. cereus*.

Table 3. Antibacterial activity of enterocin E18 against selected pathogens.

Gram stain reaction	Test Organism	Zone of inhibition (mm)
Positive	*E. faecalis*	6
	E. faecium	10
	S. aureus	24
	B. cereus	0
Negative	*K. pneumoniae*	20
	S. marcescens	30
	P. aeruginosa	8
	P. mirabilis	9
	L. monocytogenes	12
	S. Typhi	22

Table 4. Effect of chemical substance on the inhibitory activity of enterocin.

Salt	Concentration	*Enterocin activity*
Sodium chloride	0.1	+
	0.5	+
	1.0	+
Magnesium chloride	0.1	+
	0.5	+
	1.0	+
Sodium dodecyl chloride	0.1	+
	0.5	+
	1.0	+
EDTA	0.1	+
	0.5	+
	1.0	+
UREA	0.1	+
	0.5	+
	1.0	+

Figure 1 presents the effect of pH on the activity of enterocin from *E. faecalis*, the activity of enterocin increase from pH 3.0 and reached the peak value at pH 5.0, thereafter there was a decline in the activity of the enterocin. Effects of temperature on the activity of partial purify enterocin was shown in Fig. 2. The activity increases with increase in temperature till 60^{o}C, after which the enterocin activity reduced gradually. The effect of different chemical substances such as sodium chloride, magnesium chloride, sodium dodecyl chloride, EDTA and urea on enterocin is shown in Table 4. The tested chemicals compounds had no distinct effect on the activity of enterocin.

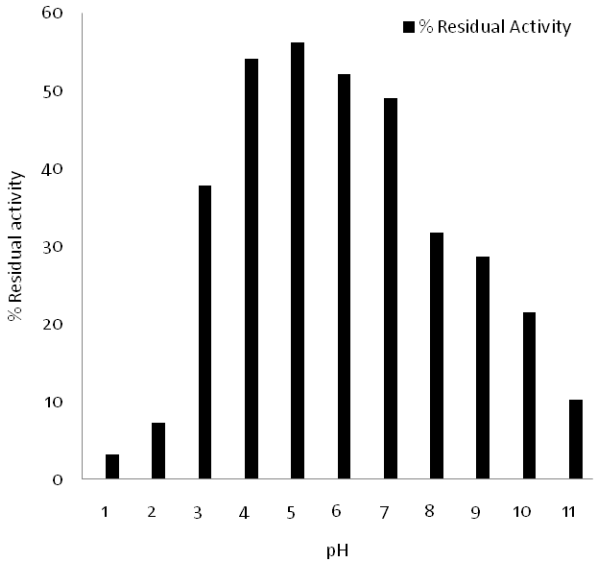

Fig. 1. Effect of pH on the activity of partial purified enterocin E18.

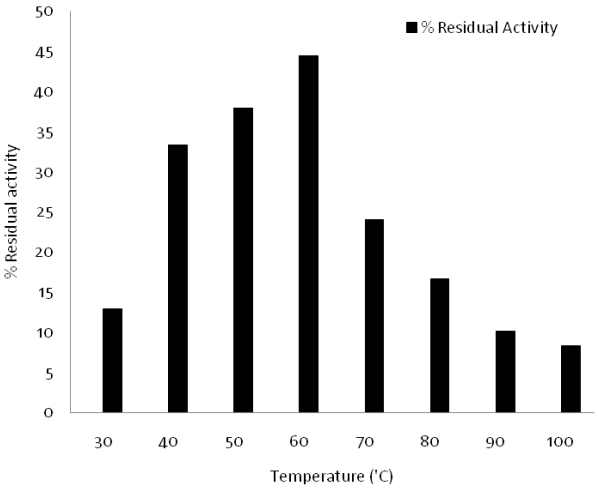

Fig. 2. Effect of temperature on the activity of partial purified enterocin E18.

Discussion

Enterococcus spp are able to grow in 0.9 M NaCl, hydrolyze aesculin in the presence of 40% bile salts as earlier reported by Gilmore *et al*. (2002). The result of this work showed that 14 (29.17%) of 48 strains of isolates of *E. faecalis* from gut of cockroach produced enterocin. The enterocin assessed in this study was tested against human pathogen of primary importance which has been implicated in different human and animal infections. The pathogens were susceptible at varying degree to the enterocin-like others earlier identified (Ben Embarek *et al.* 1994).

Enterocin had a broad-spectrum inhibitory activity similar to the one reported by Jennes *et al.* (2000). As against the findings of Line *et al.* (2008) that reported a pronounced activity of enterocin against Gram-negative bacteria, the enterocin studied in this study was more effective against Gram-positive bacteria. The activity of the enterocin has broad-spectrum against both Gram-positive and Gram-negative bacteria except for *B. cereus.* The activity observed against Gram-negative organisms is particularly unusual and has thus far been reported for only a few bacteriocins (Drider *et al.* 2006). The enterocins are usually active against other enterococci, LAB, *L. monocytogenes, S. aureus*, and *Clostridium* spp., including *Cl. botulinum, Cl. perfringens* and *Cl. tyrobutyricum* (Franz *et al.* 1999). In this study, the enterocin inhibited the growth of *Enterococcus* spp., *L. monocytogenes*, *K. pneumoniae* and *S. marcescens*. However, it was not effective against *B. cereus*. Enterocins are small and heat stable peptides that are ribosomally synthesized and insensitive to catalase. The inhibitory activity of enterocin was not inactivated by different salts at different concentrations. The inhibitory activity was stable at 100^{o}C for 30 min, and had a pH range from 2 to 10.

The result obtained from purification of crude enterocin produced by *E. faecalis* E 18 showed the protein concentration of crude enterocin to be 16.6 and after addition of ammonium sulphate precipitation the protein content increased to 42. The activities reduce from 170 AU/ml to 114 AU/ml during the purification process (Table 2), in parallel with an increase of specific activity, which reaches 45 AU/mg. This phenomenon is largely described by the spectrum of partially purified enterocin with stronger activities as noted by Balla *et al.* (2000).

Conclusion

Enterococcus faecalis can produce enterocins, which are various, having great diversity in their structure, and are active against numerous microorganisms, especially food borne pathogens and against microorganisms of environmental and medical interests. These studies pointed out the distribution and importance of *E. faecalis* as sources of enterocin.

The enterocin so far studied exhibited activity towards Gram-positive and Gram-negative bacteria, the data gathered from this study let us think of the fact that the enterocin produced was stable at different pH, high temperature and no distinct effect of chemical substance on inhibitory activity of enterocin. It can be used as useful chemotherapeutic agent. This study has revealed that the enterocin produced by the *E. faecalis* from the gut cockroach has a wide antibacterial spectrum and is stable over varying conditions, hence making it a good candidate for medical and industrial applications. Further investigations of this claim are hereby recommended.

References

Amund, O.O.; Yakubu, O.S.; and Malaka, S. L.O. 1986. A study of bacteria from the digestive system of two advanced termites (*Isoptera*, *Termitidae*) in Nigeria. Nigerian Journal of Biological Sciences 1(1): 19-24.

Balla, E.; Dicks, L.M.T.; Du Toit, M.; van der Merwe, M.J.; and Holzapfel, W.H. 2000. Characterization and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. Applied and Environmental Microbiology 66(4): 1298-304.

Batdorj, B.; Dalgalarrondo, M.; Choiset, Y.; Pedroche, J.; Métro, F.; Prévost, H.; Chobert, J.-M.; and Haertlé, T. 2006. Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. Journal of Applied Microbiology 101(4): 837-48.

Bauer, A.W.; Kirby, W.M.M.; Sherris, J.C.; and Turck, M. 1966 Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology 45(4): 493-6.

Ben Embarek, P.K.; From Jeppesen, V.; and Huss. H.H. 1994. Antibacterial potential of *Enterococcus faecium* strains isolated from sous-vide cooked fish fillets. Food Microbiology 11(6): 525-36.

Drider, D.; Fimland, G.; Héchard, Y.; McMullen, L.M.; and Prévost, H. 2006. The continuing story of class IIa bacteriocins. Microbiology and Molecular Biology Reviews 70(2): 564-82.

Franz, C.M.A.P.; Du Toit, M.; von Holy, A.; Schillinger, U.; and Holzapfel, W.H. 1997. Production of nisin-like bacteriocins by *Lactococcus lactis* strains isolated from vegetables. Journal of Basic Microbiology 37(3): 187-96.

Franz, C.M.A.P.; Holzapfel, W.H.; and Stiles, M.E. 1999. Enterococci at the crossroads of food safety? International Journal of Food Microbiology 47(1-2): 1-24.

Franz, C.M.A.P.; van Belkum, M.J.; Holzapfel, W.H.; Abriouel, H.; and Gálvez, A. 2007. Diversity of enterococcal bacteriocins and their grouping in a new classification scheme. Federation of European Micro-biological Societies (FEMS) Microbiology Reviews 31(3): 293-310.

Gilmore, M.S.; Clewell, D.B.; Courvalin, P.M.; Dunny, G.M.; Murray, B.E.; and Rice, L.B. 2002. The Enterococci: Pathogenesis, Molecular Biology, Antibiotic Resistance, and Infection Control. American Society for Microbiology (ASM) Press, Washington, DC, USA.

Heng, N.C.K.; Swe, P.M.; Ting, Y.-T.; Dufour, M.; Baird, H.J.; Ragland, N.L.; Burtenshaw, G.A.; Jack, R.W.; and Tagg, J.R. 2006. The large antimicrobial proteins (bacteriocins) of streptococci. International Congress Series 1,289: 351-4.

Herranz, C.; Chen, Y.; Chung, H.-J.; Cintas, L.M.; Hernández, P.E.; Montville, T.J.; and Chikindas, M.L. 2001. Enterocin P selectively dissipates the membrane potential of *Enterococcus faecium* T136. Applied and Environmental Microbiology 67(4): 1,689-92.

Herranz, C.; and Driessen, A.J.M. 2005. Sec-mediated secretion of bacteriocin enterocin P by *Lactococcus lactis*. Applied and Environmental Microbiology 71(4): 1,959-63.

Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T.; and Williams, S.T. (eds.). 1994. Bergey's Manual of Determinative Bacteriology. 9th ed. Williams & Wilkins, Baltimore, MD, USA.

Izquierdo, E.; Bednarczyk, A.; Schaeffer, C.; Cai, Y.; Marchioni, E.; Van Dorsselaer, A.; and Ennahar, S. 2008. Production of enterocins L50A, L50B, and IT, a new enterocin, by *Enterococcus faecium* IT62, a strain isolated from Italian ryegrass in Japan. Antimicrobial Agents and Chemotherapy 52(6): 1,917-23.

Jennes, W.; Dicks, L.M.; and Verwoerd, D.J. 2000. Enterocin 012, a bacteriocin produced by *Enterococcus gallinarum* isolated from the intestinal tract of ostrich. Journal of Applied Microbiology 88(2): 349-57.

Line, J.E. Svetoch, E.A.; Eruslanov, B.V.; Perelygin, V.V.; Mitsevich, E.V.; Mitsevich, I.P.; Levchuk, V.P.; Svetoch, O.E.; Seal, B.S.; Siragusa, G.R.; and Stern, N.J. 2008. Isolation and purification of enterocin E-760 with broad antimicrobial activity against Gram-positive and Gram-negative bacteria. Antimicrobial Agents and Chemotherapy 52(3): 1,094-100.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193(1): 265-75.

Muriana, P.M.; and Luchansky, J.B. 1993. Biochemical methods for purification of bacteriocins. *In*: Hoover, D.G.; and Steenson, L.R. (eds.). Bacteriocins of Lactic Acid Bacteria. Academic Press, New York, NY, USA, pp. 41-61.

Nazef, L.; Belguesmia, Y.; Tani, A.; Prévost, H.; and Drider, D. 2008. Identification of lactic acid bacteria from poultry feces: evidence on anti-*Campylobacter* and anti-*Listeria* activities. Poultry Science 87(2): 329-34.

Rasool, S.A.; Ahmed, S.; and Iqbal, A. 1996. Streptococcins of indigenous hemolytic streptococci. Natural Product Letters 8(1): 67-74.

Stern, N.J.; Svetoch, E.A.; Eruslanov, B.V.; Perelygin, V.V.; Mitsevich, E.V.; Mitsevich, I.P.; Pokhilenko, V.D.; Levchuk, V.P.; Svetoch, O.E.; and Seal, B.S. 2006. Isolation of a *Lactobacillus salivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacter jejuni* in the chicken gastrointestinal system. Antimicrobial Agents and Chemotherapy 50(9): 3,111-6.