Acta Veterinaria (Beograd), Vol. 60, No 1, 59-66, 2010.

DOI: 10.2298/AVB1001059V UDK 619:637.5.04/.07

ANTIMICROBIAL PROPERTIES OF INDIGENOUS *LACTOBACILLUS SAKEI* STRAIN

VESKOVIĆ-MORAČANIN SLAVICA*, OBRADOVIĆ D**, VELEBIT B*, BOROVIĆ BRANKA*, ŠKRINJAR MARIJA*** and TURUBATOVIĆ L*

**Institute of Meat Hygiene and Technology, Belgrade, Serbia*
***Agricultural Faculty, University of Belgrade, Serbia*
****Faculty of Technology, University of Novi Sad, Serbia*

(Received 7th August 2009)

The strain I 154 of Lactobacillus sakei has been isolated from traditionally fermented sausages in the course of the realization of the international project (INCO PROJECT Nº ICA4-CT-2002-10037). This strain exhibited the ability for bacteriocin production. Antimicrobial properties of the isolated bacteriocin (sakacine), its sensibility towards proteolytic enzymes, as well as the effect of increased to high temperatures on its stability have been examined in this work. Semi purified bacteriocin (sakacine) has been isolated from bacteriocin - producing strain Lactobacillus sakei I 154 by the method of saturated precipitation with up to 70% ammonium mono-sulphate solution. The activity of isolated sakacine was examined towards Listeria monocytogenes, Staphylococcus aureus and Escherichia coli 0157:H7. Pepsine, Papaine and Proteinase K were used as proteolytic enzymes. The influence of increased and high temperatures on the bacteriocin activity was examined at different temperatures and exposition periods including autoclaving effects.

Key words: Lactobacillus sakei, bacteriocin, antilisterial efects

INTRODUCTION

Growing needs for naturally safe and healthy food have caused the increased interest for the use of bacteriocin-producing LAB, which are nowadays used as protective cultures for the production of fermented products in the meat industry (Schillinger and Lücke, 1989; Mc Mullen and Stiles, 1996; Hugas, 1997). The principle of biological protection is based on the decrease of the health consumer risk by acting primarily towards undesirable spoilage or food poisoning bacteria, without changing the quality of the final product.

Bacteriocines of LAB represent the natural antimicrobial peptides or proteins that have a very interesting potential for the application in the food processing industry. Hurst (1981) has named the bacteriocines as "biological food preservatives" and this term shortly become widely used. The possibility of their application as bio-protectors (Cleveland *et al*., 2001), in the service of health

protection (Turcotte *et al*., 2004), has been established along with the simultaneous increasing of food shelf life (De Vuyst and Vandamme, 1994). These are ribosomal proteins that have bactericidal effects and are rapidly digested by the proteinases of the human digestive tract (Joerger *et al*., 2000). In the literature they are often, considering their antibacterial properties, compared to antibiotics (Hansen, 1993; Hurst, 1981). However, unlike therapeutic antibiotics, their application, by the rule, avoids the possibility of occurrence of unwanted allergic reactions in humans (Cleveland *et al*., 2001). What was for the human race, directly, the discovery of penicillin by Alexander Fleming in 1929, that are, indirectly, bacteriocines in the terms of natural protection and food safety.

During the realization of the international project "Safety of traditional fermented sausages: Research on protective cultures and bacteriocines" (INCO PROJECT Nº ICA4-CT-2002-10037), the strain *Lactobacillus sakei* I154 was isolated from traditionally fermented sausages. For this strain the ability of bacteriocin production has been previously detected.

The aim of this research was to examine the properties of the bacteriocin isolated from *Lb. sakei* I154 in terms of its strength and antimicrobial spectrum, its sensitivity to proteolytic enzymes, as well as its activity at increased to high temperatures.

MATERIAL AND METHODS

Cultures

The range of the antibacterial action of the protective *Lb. sakei I 154* culture was determined by the Agar Well Diffusion Assay (AWDA) method against the chosen test microorganisms (*L. monocytogenes* NCTC 10527, *Staphylococcus aureus* NCBF 1499 and *Escherichia coli* 0157:H7 NCTC 12079).

Determination of the ability of bacteriocin production by Lb. sakei I 154

Test microorganisms were added to BHI agar (with 0.5% agar) in the amount that ensured the concentration of 10^7–10^8 cfu/mL in the medium. The incubated activity of created H_2O_2 was eliminated by the addition of catalase (5 mg/mL), and experimental confirmation was done by the proteinase test (50 μL of proteinase K with an activity of 10-25 mg/mL, was added to 50 μL of the examined, neutralized broth culture). After one hour incubation at 37°C the antimicrobial activity was determined.

The existence of the test microorganism growth inhibition zone was considered as a positive result.

Isolation of the semi purified bacteriocin from Lb.sakei I 154 and determination of its activity

Isolation of semi purified bacteriocin from *Lb.sakei* I 154 was done by the method of saturated precipitation with ammonium-sulphate (Schillinger and Lücke, 1989) was adjusted to individual laboratory conditions (Veskovic-Moracanin, 2005; 2007). Several days plating of the broth culture, with the aim to achieve a *Lb. sakei* concentration of 10^{10}-10^{11} cfug^{-1}, was performed by

centrifuging at 10000 rpm for 30 minutes at 4°C (MSE, "High Speed 18", England). After separation and neutralization up to pH 6,5 – 7,0 of the supernatant with 10 N NaOH, the precipitation of bacteriocin with ammonium-sulphate (472.2 g/L) was done with the aim of obtaining a 70% saturated solution. Separated bacteriocin in the shape of whitish pellets was suspended in 0.05 M of sodium-phosphate buffer – pH 7.

The activity of the isolated, semi purified bacteriocin of *Lb. sakei* was determined by Agar Well Diffusion Assay (AWDA) method against the selected test microorganism – *L. monocytogenes* NCTC 10527.

The isolated bacteriocin, as well as its series of suitable dilutions in the amount of 50 μL, were sterilized by filtering through 0.22 μm microbiological filter (Acrodisc, Germany) and subsequently were spotted into the pre-made and marked basins in agar. Dilutions were made with sterile deionized water. After one hour incubation at 4°C, the plates were incubated at 30°C for 24h in order to stimulate diffusion of the examined bacteriocin.

The activity of the isolated bacteriocin was expressed as absolute value, marked as arbitrary units (AU/mL). Arbitrary values were determined by the following formula: $AU/mL = 2^n \times (1000\ \mu L/50\ \mu L)$, where n represents the maximum dilution of the bacteriocin – bacteriocin portion of 50 μL which gives the growth inhibition zone for *L. monocytogenes* (test microorganism) wider than 2 mm.

The influence of increased temperatures and proteolytic enzymes on the activity of the isolated bacteriocin

The examination of the influence of increased and high temperatures to the activity of bacteriocin was done with the aim of establishing the functional properties of the isolated bacteriocin, as well as comprehension of the ability of its application during the production of certain meat products that require these temperature rates in their technology. The isolated semi purified bacteriocin (1 mL) was exposed to the following temperatures: 65°C, 80°C, 90°C and 100°C, as well as to the effect of high temperatures (121°C) and high pressure (1.2 Ba) as during sterilization. Cuvettes containing 1 mL of bacteriocin were dipped into the water bath heated at 65°C, 80°C and 90°C during 10 and 30 minutes. The influence of 100°C was measured after 10, 30 and 60 minutes. High temperature (121°C) and increased pressure (1.2 Ba) effects during sterilization, were determined after 15 minutes. After the predicted time of exposition, the activity of the isolated bacteriocin was determined by the agar diffusion method (AWDA) with the selected test microorganism (*L. monocytogenes* NCTC 10527). Results were read after 24h incubation at 30°C.

Determination of the protein nature of the isolated bacteriocin was done in the reaction with proteolytic enzymes. In this research Pepsin – TS (with activity of 1:10000) NF "Galenika", Papaine (≥30000 USP-U/mg) "Merck" and Proteinase K (600 m Ansou U/mL) "Applichem" were used. Enzyme preparations were added separately in 1 mL of the examined bacteriocin in a concentration of 1 mg/mL and incubated for one hour at 37°C. After that, the suitable amount (50 μL) of the sample (bacteriocin + enzyme) was tested by the agar-diffusion method (AWDA)

in order to determine its antilisterial activity (same as in the previous experiment). Results were taken after 24h of incubation at 30°C.

RESULTS AND DISCUSSION

Compared to the confirmative tests of bacteriocin activity, the examined *Lb. sakei* I154 strain, in a medium with *L. monocytogenes* NCTC 10527, has had the typical profile of bacteriocin producing strain (Table1).

Table 1. Typical profile of bacteriocin-producing *Lb. sakei I 154*

Broth culture	Neutralized broth	Catalase test	Proteinase test
+++	+++	+++	– – –

The determined ability for the bacteriocin production, in examined *Lb. sakei*, is in accordance with the literature data proving that different strains of *Lb. sakei,* isolated from meat or meat products (fermented sausages), have the ability to produce secondary metabolites – bacteriocines (Rodrigues *et al.*, 1995 Aymerich *et al*., 2000; Schillinger and Lücke; 1989; Hugas *et al*., 1995). However, genetic and biochemical characterization has showed that there are only three bacteriocines synthesized by *Lb. sakei*, and these are: sakacine A, sakacine P and lactocine S (Nettles and Barefoot, 1993). Sakacine A and sakacine P are the ribosomal proteins having characteristic amino sequences at the N-terminal end of the peptide (Tyr-Gly-Asn-Gly-Val-Cys), which corresponds to the IIa class of LAB bacteriocines (Ennahar *et al*., 1999). This group of bacteriocines possesses the emphasized antilisterial effect and their application in the food industry is very important (Drider *et al*., 2006). More detailed analyses (Cocolin *et al*., 2005) have established that isolated bacteriocin corresponds, according to its properties, to sakacine P.

Results of the examination of antibacterial activity of bacteriocin producing *Lb. sakei* I 154 strain have showed the intensive inhibitory activity towards *L. monocytogenes* NCTC 10527, while the inhibitory effect towards *S. aureus* NCBF 1499 and *E. coli* 0157:H7 NCTC 12079 was missing.

Established bacteriocin activity of *Lb. sakei* I 154 towards *L. monocytogenes* is in accordance to other author's findings that implies the fact that inhibitory activity of the LAB bacteriocines is dominant mostly towards Gram positive bacteria, primarily towards bacteria belonging to genus *Listeria* (Schillinger*,* 1990; Abee*,* 1995). Actually, a high taxonomic relationship of the genuses *Listeria* and *Lactobacillus*, determines the high sensitivity of *Listeria* species towards bacteriocines produced by *Lactobacillus spp.* (Ludwig *et al*., 1984; Wilkinson and Jones, 1997). This is the reason for having the research mostly aimed at the examination of LAB bacteriocin action towards *L. monocytogenes* as a test microorganism (Deaschel, 1989; Schillinger and Lücke, 1989).

Maximum dilution of bacteriocin isolated from *Lb. sakei* I 154, which gave the antilisterial effect, was 1:32 (2^5). By the above stated formula the calculated

activity of this bacteriocin was cca 640 AU/mL. The mechanism for determination of bacteriocin activity by the critical dilution test is shown in Figure 1.

Figure 1. Mechanism for determination of activity of bacteriocin isolated form *Lb. sakei* by the critical dilution principle

Results of the examination of high temperatures on the activity of bacteriocin isolated from *Lb. sakei* I 154 are implying its emphasized thermo resistance (Table 2). Generally, as the temperature increases, at 100°C, the decrease of its activity occurs. Antilisterial activity of bacteriocin, slightly diminished, is established even after sterilization (121°C, 1.2 Ba; 15 minutes of exposition). The established thermal resistance indirectly discovers the nature of isolated bacteriocin. Actually, literature data evidencing that bacteriocines isolated from *Lb. sakei* mostly belong to the I and IIa classes (Holck *et al*., 1992; Cintas *et al*., 1998; Guyonnet *et al*., 2000), which are classes of very small globular proteins featured with high thermostability.

Table 2. Activity of bacteriocin isolated from *Lb. sakei* I 154 after exposure to increased temperatures

bacteriocin *Lb.sakei* I 154	65°C		80°C		90°C		100°C			121°C 1.2 Ba
	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min	60 min	15 min
	+++	+++	+++	+++	+++	+++	++	++	++	++

Results of the examination of the influence of proteolytic enzymes on the activity of isolated bacteriocin have shown the absence of the antilisterial effect. In this way its protein nature was confirmed, which is basically the property of bacteriocines produced by LAB (Klaenhammer, 1993; Nes and Holo, 2000).

The protein nature of bacteriocin determines their destiny in the human digestive tract. Humans are the final consumers of the products with added bacteriocines or bacteriocines produced by LAB. Experts from this area of food safety (Sanders, 1993; Joerger *et al*., 2000) while defining the bacteriocines of LAB, emphasize their protein nature, showing the fact that these can be degraded by the human digestive proteinases.

CONCLUSION

The established functional properties of the bacteriocin isolated from *Lb. sakei* I154, as well as the proved inhibitory effect against the *Listeria monocytogenes,* represents the interesting potential that should be the basis for further investigations in the aim of establishing the possibility of its application as bioprotector in the food industry, primarily during the production of fermented meat products – Sremska sausage (Veskovic-Moracanin, 2005; 2007).

On the other hand, significant persistence of the consumers, in terms of a negative attitude when the utilization of chemical substances – additives in food production is concerned, opens the possibility of introduction of bacteriocin as an addition to the "barrier" approach of food protection. Determined thermostability of isolated bacteriocin enables its application even in those products that are produced at increased or high temperatures. At the same time, protein nature of the bacteriocin and its sensitivity to the action of proteolytic enzymes fulfills with optimism when thinking of its possible application as a food bioprotector. Any other destiny in the human organism (accumulation, partial degradation, resorption, etc.) wouldn't be desirable. On the other hand, suppression of pathogenic bacteria in the human digestive tract caused by the activity of directly added bacteriocines as additives should be suspicious due to inactivation with proteolytic enzymes.

Due to all of these facts and the obtained data the recommendation for potential application of the bacteriocin isolated from *Lb. sakei* I 154 in the meat industry, as an addition to the barrier approach of food protection in the aim of ensuring the food safety can be given.

ACKNOWLEDGMENT:
This research was funded by Serbian Ministry of Science through the project, № 20217: "Technological and protective characteristics of autochthonous strains of LAB isolated from traditionally fermented sausages and the possibility of their appliance in meat industry".

Address for correspondence:
Vesković-Moračanin Slavica, PhD
Institute of Meat Hygiene and Technology
Kaćanskog 13
11000 Belgrade, Serbia
E-mail: slavica@inmesbgd.com

REFERENCES

1. *Abbe T*, 1995, Pore-forming bacteriocines of gram-positive and self-protection mechanisms of producer organisms, *FEMS Microbiol Lett*, 129, 1-10.
2. *Aymerich MT, Garriga M, Monfort JM, Nes IF, Hugas M*, 2000, Bacteriocin-producing lactobacilli in Spanish-style fermented sausages: characterization of bacteriocines, *Food Microbiol*, 17, 33-45.
3. *Cintas LM, Casaus P, Havarstein LS, Hernandez PE, Nes IF*, 1998, Biochemical and genetic characterization of enterocin P, a novel sec dependent bacteriocin from *Enterococcus faecium* P 13 with a broad antimicrobial spectrum, *Appl Environ Microbiol*, 63, 4321-30.
4. *Cleveland J, Montville T, Nes F, Chikindas L*, 2001, bacteriocines: Safe, natural antimicrobials for food preservation, *Int J Food Microbiol*, 71, 1-20.
5. *Cocolin L, Urso R, Rantsios K, Comi G*, 2005, Identification, sequencing and characterization of lactic acid bacteria genes responsible for bacteriocin production. Research project "Safety of traditional fermented sausages: Research on protective cultures and bacteriocines". Workshop for Dissemination of the Project Results, Proceedings, 35-52.
6. *Daeschel MA*, 1989, Antimicrobial substances from lactic acid bacteria for use as food preservatives, *Food Technol*, 43, 1, 164-7.
7. *De Vuyst L, Vandamme E*, 1994, Nisin, an antibiotic by *Lactococcus lactis* subsp. *lactis*: properties, biosynthesis, fermentation and applications. In: DeVuyst L, Vandamme E (Eds), bacteriocines of lactic acid bacteria, Blackie, London, Glasgow, New York, Tokyo, Melbourne, Madras, 151-221.
8. *Drider D, Fimland G, Hechard Y, Mcmullen LM, Prevost H*, 2006, The continuing story of class IIa bacterrocins, *Microbiol Mol Biol Rev*, 70, 2, 564-82.
9. *Ennahar S, Sonomoto K, Ishizaki A*, 1999, Class IIa bacteriocines from lactic acid bacteria: antibacterial activity and food preservation, *J Biosci Bioeng*, 87, 6, 705-16.
10. *Guyonnet D, Fremaux C, Cenatiempo Y, Berjeaud JM*, 2000, Method for rapid purification of class IIa bacteriocines and comparison of their activities, *Appl Environ Microbiol*, 66, 1744-8.
11. *Hansen JN*, 1993, Antibiotics synthesized by post translational modification, *Annu Rev Microbiol*, 47, 535-64.
12. *Holck AL, Axelsson L, Birkeland S, Aukrust T, Blom H*, 1992, Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706, *J Gen Microbiol*, 138, 2715-20.
13. *Hugas M*, 1997, Biopreservation of meat and meat products, Actes du collague lactic, Caen, France, 213-27.
14. *Hugas M, Garriga M, Aymerich M, Montfort M*, 1995, Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lactobacillus sake* CTC494. *J Appl Bacteriol*, 79, 322-30.
15. *Hurst A*, 1981, Nisin, *Adv Appl Microbiol*, 27, 85-123.
16. *Joerger RD, Hoover DG, Barefoot SF, Harmon KM, Grinstead DA, Nettles-Cutter CG*, 2000, bacteriocines, In: Lederberg, editor. Encyclopedia of microbiology, 1, 2nd edition, San Diego: Academic Press, Inc, 383-97.
17. *Klaenhammer TR*, 1993, Genetics of bacteriocines produced by lactic acid bacteria, *FEMS Microbiol Rev*, 12, 39-85.
18. *Ludwig W, Schleifer KH, Stackebrandt E*, 1984, 16S rRNA analysis of *Listeria monocytogenes* and *Brochothrix thermosphacta*, *FEMS Microbiol Lett,* 25, 199-204.
19. *McMullen IM, Stiles E*, 1996, Potential use of bacteriocin – producing lactic acid bacteria in the preservation of meats, *J Food Protect,* (Suppl) 64-71.
20. *Nes IF, Holo H*, 2000, Class II antimicrobial peptides from lactic acid bacteria, *Biopolymers*, 55, 50-61.
21. *Nettles G, Barefoot F*, 1993, Biochemical and genetic characteristics of bacteriocines of food-associated lactic acid bacteria, *J Food Protection*, 56, 339-56.
22. *Rodriguez M, Cintas M, Casaus P, Horn N, Dodd M, Hernandez E, Gasson J*, 1995, Isolation of nisin-producing *Lactococcus lactis* strains from dry fermented sausages, *J Appl Bacteriol*, 78, 109-1152.

23. *Sanders M*, 1993, Effect of consumption of lactic acid cultures on human health, *Adv Food Nutr Res*, 37, 67-127.
24. *Schilinger U*, 1990, bacteriocines of lactic acid bacteria, *Biotechnol*, *Food Qual*, 55-74.
25. *Schillinger U, Lucke K*, 1989, Antibacterial activity of *Lactobacillus sakei* isolated from meat, *Appl Environ Microbiol,* 5, 1901-6.
26. *Turcotte C, Lacroix C, Kheadr E, Grignon L, Fliss I*, 2004, A rapid turbidometric microplate bioassay for accurate quantification of lactic acid bacteria bacteriocines, *Int J Food Microb*, 90, 283-93.
27. *Veskovic-Moracanin S*, 2005, The Influence of bacteriocines isolated from *Leuconostoc mesenteroides* E 131 and *Lactobacillus sakei* I 154 on *Listeria monocytogenes* during production of "Sremska" sausage, MSc Thesis, Agricultural Faculty, University of Belgrade, Serbia.
28. *Veskovic-Moracanin S*, 2007, The Influence of *Lactobacillus sakei* I 151, bacteriocin of Leuconostoc mesenteroides E 131 and MAP to the sustainability of "Sremska" sausage, PhD Thesis, 2007. Agricultural Faculty, University of Belgrade, Serbia.
29. *Wilkinson BJ, Jones D*, 1997, A numerical taxonomic survey of *Listeria* and related bacteria, *J Gen Microbiol*, 98, 399-421.

ANTIMIKROBNA SVOJSTVA AUTOHTONOG IZOLATA *LACTOBACILLUS SAKEI*

VESKOVIĆ-MORAČANIN SLAVICA, OBRADOVIĆ D, VELEBIT B, BOROVIĆ BRANKA, ŠKRINJAR MARIJA i TURUBATOVIĆ L

SADRŽAJ

Soj *Lactobacillus sakei* I 154 izolovan je iz tradicionalno fermentisane kobasice u sklopu međunarodnog projekta (INCO PROJECT № ICA4-CT-2002-10037). Soj je ispoljio sposobnost proizvodnje bakteriocina. U radu su ispitivana antimikrobna svojstva izolovanog bakteriocina (sakacin) kao i njegova osetljivost ka proteolitičkim enzimima i aktivnost i stabilnost pri visokim temperaturama. Sakacin je izolovan metodom precipitacije zasićenim 70% rastvorom amonijum sulfata. Ispitivan je efekat na rast *Listeria monocytogenes*, *Staphylococcus aureus* i *Escherichia coli* 0157:H7. Za ispitivanje proteolitičke stabilnosti korišćeni su enzimi pepsin, papain i proteinaza K. Ispitivana je i aktivnost bakteriocina pri različitim temperaturama i ekspoziciji uključujući i efekte autoklaviranja.