CHARACTERISTICS OF BACTERIOCIN PRODUCING *LACTOCOCCUS* SPECIES ISOLATED FROM PROCESSED MEAT

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ABSTRACT

The bacteriocin producing *Lactococcus* species, isolated from processed meat (smoked beef) by serial dilution and poured plate inoculation were biochemically similar to *Latococcus lactis* subsp. *lactis* strain. The bacteriocin (s) produced by the three isolates have MW similar to nisin. They also have wider antibacterial range against Gram-positive pathogenic and spoilage bacteria similar to nisin. Plasmid profile showed UW1 and UW2 are similar but different from UW3 and all three isolates are different from the seven known nisin producing strain of *L. lactis* subsp*. lactis*. (DL, 150, 7690, Tis Sik, ATCC11454, 354/07, and 148). Furthermore, amino acid sequence analysis is needed to identify whether unknown bacteriocin is nisin or not.

Keywords: Bacteriocin, isolates

KARAKTERISTIK *Lactococcus* PENGHASIL BAKTERIOSIN YANG DIISOLASI DARI DAGING OLAHAN (Daging asap)

ABSTRAK

Bakteriosin yang dihasilkan oleh spesies *Lactococcus* hasil isolasi dari daging olahan (daging asap) dengan seri pengenceran dan penanaman pada cawan agar yang dilakukan secara duplo. Secara biokimia ketiga koloni isolate hampir sama dengan spesies *Lactococcus lactis* subsp. *lactis* (345/07 dan ATCC 11454) dan memproduksi bakteriosin yang menyerupai nisin. Bakteriosin ini juga mempunyai aktivitas antimikroba yang sama dengan nisin dengan kisaran yang luas melawan bakteri patogen dan bakteri perusak Gram-positif. Profil plasmid memperlihatkan bahwa *Lactococcus* hasil isolasi UW1 dan UW2 hampir sama tetapi berbeda dengan UW3 namun ketiganya berbeda dari ke 7 bakteri penghasil bakteriosin lainnya *L. lactis* subsp*. lactis*. (DL, 150, 7690, Tis Sik, ATCC11454, 354/07, dan 148). Analisis pemetaan asam amino perlu dilakukan untuk menentukan bakteriosin yang dihasilkan benar nisin atau bukan.

Kata kunci : Bakteriosin, isolat

INTRODUCTION

The lactic acid bacteria (LAB), used to produce many fermented foods, includes species and strain from several genera of Gram-positive bacteria such as N-group *Streptococcus* (currently designated as *Lactococcus*), *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Bifidobacterium*. During fermentation they metabolize food carbohydrates and produce high amounts of lactic acid. This lower the pH of the food environment and stabilizes the food against the bacteria associated with food spoilage and health hazard of food origin. These food grade starter culture bacteria have been used in food for many years and are considered safe. Beside lactic acid they also produce several other metabolites that are known to have an antimicrobial effect against bacteria associated with food spoilage and food borne illnesses. These antimicrobial metabolites include acetic acid, propionic acid, diacetyl, ethanol, and antimicrobial peptides or bacteriocin (Bhunia *et al*. 1988; Daeschel, 1989; Ray, 1994). Different subspecies of *Lactococcus lactis* produce bacteriocin such as diplococcin, lacticoccin A, B, and M, and nisin (Holo *et al*. 1991). Nisin from some strain of *Lactococcus lactis* subsp. *lactis* have been studied with respect to their spectrum of activity against Gram-positive bacteria, mode of bactericidal action and toxicity to animals as well as application in food (Hurst, 1983; Dodd *et al*. 1990). It is the only bacteriocin that has been permitted for use as a food preservative (Hurst, 1981).

They are three strains of *Lactococcus* spp. isolated from processed meat (smoked beef). Initial studies showed that the strains produced potent bacteriocin while growing slowly in refrigerated product and inhibited growth of undesirable psychotrophic spoilage and pathogenic bacteria. However, being homofermentative they did not produce gas and reduce the acceptance quality of food. It appears that these strains can be used as biological controls in refrigerated foods that are expected to have 4 to 8 weeks shelf–life. The objected of this study are to identify species/subspecies of the three isolates by comparing with the characteristics of nisin producing strains of *L. lactis* subsp ATCC 11454 and determine the bacteriocin producing ability and antibacterial spectrum of the bacteriocin they produce.

MATERIAL AND METHODS

Isolation of Bacterial Strains

10 g sample (smoked beef) were aseptically removed, placed into 90.0 mL of peptone water and homogenized for 60 second under aseptic condition in a stomacher (Lab Blender). Appropriate decimal dilution of homogenate were made in 0.1% peptone water for bacteria analysis. Bacteria were isolated by poured plate inoculation of 1,0 mL of diluted homogenates on TGE agar (Difco). Duplicate samples of each dilution were examined. Inoculated plates were incubated at 30^{o}C for 18-24h. From several colonies three of different colonies chosen as isolate

based on macroscopic, microscopic and morphological characteristic for further use, namely *Lactococcus* UW1, UW2 and UW3.

Nisin producing *Lactococcus lactis* subsp*. lactis* ATCC11454, and three isolates of *Lactococcus*, UW1, UW2, and UW3 were used in some studies, several other strains, known to produced nisin, were also used. These strains were grown in TGE (trypticase-glucose–yeast extract) broth at 30°C before being used in an experiment (Yang *et al.* 1992.).

Biochemical Characteristics of the Isolates

To determine biochemical characteristics of the isolates, the protocols describe by Schleifer *et al*. (1985) were used. These protocols provided the method of biochemical tests needed to identify lactic acid bacteria.

Carbohydrate fermentation patterns of the three isolates were compared against two known nisin producing strain of *Lactococcus lactis* subsp. *lactis* (ATCC 11454 and 345/07). A modified version of the method describe by Schleifer *et al.* (1985) was used. The basal medium was TGE buffer broth without glucose but with 0.004 bromocresol purple (pH 5.2 yellow; pH 6.8 red). After sterilizing the basal media and cooling to room temperature, filter sterilized carbohydrates were added to final concentration of 0.5% (w/v). Wells in sterile microtiter plates were filled in triplicate with 25 µL of broth containing each carbohydrate. The filling of microtiter plates and transfer of bacterial cell was done under hood.

To prepare cells of the three isolates and strains ATCC 11454 and 354/07 for inoculation, the strains were grown over-night in TGE broth 30°C. One mL of each culture was transferred to sterile centrifuge tubes (1.5 mL) and centrifuged for 3 to 5 minutes at 8000 rpm. The supernatant were discharded, the cells washed with 1mL sterile deionized water (dH_2O) and resuspend in 1 mL dH_2O. The micro titer plate wells containing the test carbohydrate were inoculated in triplicate with 10 µl of resuspended cells. The plates were incubated at 25 °C under unaerobic condition for 2 to 5 days, and checked daily. Positive results were indicated by turning of TGE buffer broth containing bromocresol purple to yellow in the micro titer wells.

Preparation of Purified Nisin and Unknown Bacteriocin(s)

The method developed by Yang *et al.* (1992) was used. *Lactococcus lactis* subsp.*lactis* ATCC 11454, and the three *Lactococcus* isolates were used in this study. The strains were initially grown over night at 30°C in TGE buffer broth which consisted of TGE broth plus 0.05% sodium citrate, 0.5% sodium acetate and 0.05% dipotassium phosphate (pH 6.5). For the production of nisin by ATCC 11454 and unknown bacteriocin by the isolates, buffer TGE broth (1L) was inoculated with the overnight cultures at 1% level and incubated at 30°C for 16 h. After incubation the culture broths were adjusted to pH 6,0 with 3N NaOH for adsorption of the bacteriocin on the surface of the producer cells, then heated in a water bath at 70°C for 15 minutes to kill the cells. The cell were collected by centrifugation and washed in 1/5 volume of sterile 5 M sodium phosphate (pH 6). The cells then

resuspended in 1/5 volume of sterile 0.1 M sodium chloride solution with pH adjusted to 1.5 by adding 5 % phosphoric acid. The cell suspension was stirred at 4^{o} C. over night for the release of bacteriocin from the cell surface into the saline solution. Acidified cell suspensions were centrifuge at 8000 x g for 15 minutes at 4^{o}C. Supernatant containing the bacteriocin were dialyzed in dH_2O with 2 to 3 changes (using dialysis membrane of molecule weight cut off 3,000 Da) for 24 h at 4^{o}C to remove NaCl and then freeze dried. The samples were kept in vial at -20^{o}C and used as purified preparation in different studies.

Antimicrobial Activity Assay of Nisin

The purified preparations were assayed for activity unit (AU)/mg dry material. 10 mg was dissolved in 1 mL dH_2O for a stock solution. Serial dilutions of the stock solution in sterile deionized water were prepared and 5 µL from each dilution was spotted in duplicates onto the surface of TGE agar plate containing a soft agar lawn of about 10^6 cell of *Lactobacillus plantarum* NCDO 955. The agar plates were then incubated at 30^{o}C for 24 h and examine for clear zone of inhibition around the spots where bacteriocins were applied. The highest dilution that gave a clear zone of at least 2 mm diameter was consider as the end point. To obtain the activity units (AU/mL) of stock solution (equivalent to 10 mg of dried preparations) the dilution factor of the end point dilution was multiplied by 200 (1000/5 µL) (Bhunia *et al*. 1988)

Identification of Bacteriocin Peptides by SDS-PAGE

Purified nisin and unknown bacteriocin preparation were dissolved in protein solubilizing solution (sample buffer) and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on (10–16.5%) discontinuous gradient gel designed to separate low molecular weight protein (De Wald *et al*. 1986; Yang *et al*. 1992). A 2 mg/mL of sample mixed with sample buffer (20% SDS, glycerol, 2 Marcaptoethanol, Briliant Blue G and M Tris. HCl, pH 6.8), heated at 65^{o}C for 1 minute and colled. 5 µL volumes were then transferred to the preformed gel. The samples were applied in duplicate. Molecular weight standard (MW, LKB producer AB, Bromma, Sweden) were applied in duplicate wells on the gel. After 3 h of electrophoresis at 60 mA, the gel was removed and was stained with coomassie blue for 2 h. The gel was distained and photographed. One half of the gel was washed in sterile dH_2O for 2 h to remove SDS, then placed on a prepoured agar-medium plate and overlaid with soft agar medium seeded with cells of an indicator bacteria. The plate was incubated at 30^{o}C for 24 h and examined for zone of growth inhibition and photographed.

Spectrum of Antimicrobial Activity

The disc assay method was use to determine the antimicrobial activity of purified and unknown bacteriocins against several food spoilage and food borne pathogenic bacteria as indicator strains (Bhunia *et al*., 1988). For lactic acid

bacteria TGE agar and for other bacteria TSY- agar (Tryptic Soy broth Difco+ 0.5% yeast extract and 1.5% agar) were used for prepoured plate. For soft agar overlay, same media with 0.8% agar were used. Incubation of the plates was carried out at the optimum temperature for each indicator species

RESULTS AND DISCUSSION

Biochemical characteristics of the isolates

Fermentation pattern of 11 carbohydrates and ability to hydrolyze arginin of the three isolates and 2 known (354/07 and 11454) *L. lactis* subsp. *lactis* strain were studied. Result presented in Table 1 indicated all three isolates have similar biochemical pattern as the two known strains.

Table 1. Characteristics most useful in differentiating *Lactococcus* spp.

Characteristics	*L. lactis* subsp. *lactis* (354/07)	*L. lactis* subsp. *lactis* (ATCC 11454)	*L. lactis* subsp. *lactis* (UW1)	*L. lactis* subsp. *lactis* (UW2)	*L. lactis* subsp. *lactis* (UW3)
Amygdalin	+	+	+	+	+
Galactose	+	+	+	+	+
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Melibiose	_	_	_	_	_
Raffinose	_	_	_	_	_
Ribose	+	+	+	+	+
Salicin	+	+	+	+	+
Sorbitol	_	_	_	_	_
Sucrose	+	+	+	+	+
Trehalose	+	+	+	+	+
Arginin dehydrolase	+	+	+	+	+

Refference from Scheifer *et al*.1985.
"+" indicates a positive reaction (yellow color); "_" indicates a negative reaction (purple or red color)

To determine the ability of the isolates to produce bacteriocins they were poured plated with TGE agar and incubated at 30^{o}C overnight to form colonies. The plates were overlaid each 5 mL melted soft agar seeded with *L. plantarum* NCDO 955, incubated at 30^{o}C overnight and examined for zone of growth inhibition around the colonies (Fig. 1)

All three isolates formed zones as the nisin producing strain ATCC 11454. This result indicated that all three strains are capable of producing bacteriocin

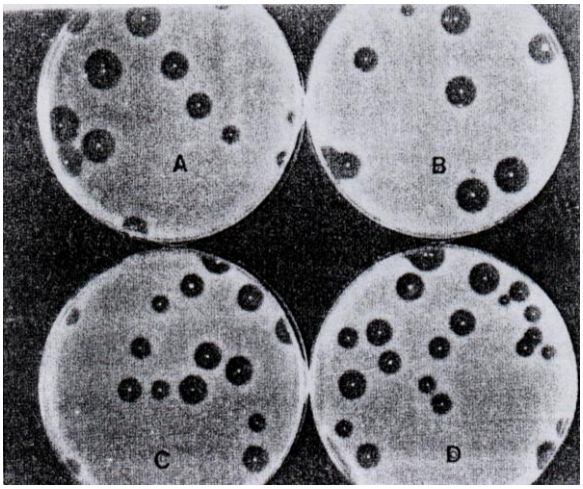

Figure 1. Bacteriocin production by *Lactococcus* isolates and *L. lactis* subsp. *lactis* ATCC11454 as indicated by zone of growth inhibitionof L. plantarum NCDO 955 around each colony (A). *L. lactis* subsp*. lactis* ATCC11454; (B) *L. lactis* subsp. *lactis* UW1; (C) *L. lactis* subsp*. lactis* UW2; and *L. lactis* subsp*. lactis* UW3.

Activity units purified bacteriocin preparation

Following freeze–drying the purified preparation were assayed for activity units against L. plantarum NCDO 955. The preparation contained AU/g of dried material: $50x10^6$ for UW1, $60x10^6$ for UW2, $60x10^6$ for UW3, and $60x10^6$ for ATCC 11454.

Identification of bacteriocin peptides by SDS-PAGE

Purified bacteriocin preparation from the three *Lactoccoccus* isolates and strain ATCC 11454 were analyzed for protein profiles by SDS-PAGE. Preparation From all four strains had several proteins between molecular weights about 2.5 to 14.4 kDa (Fig 2). The protein profiles of all four strains seemed to be very similar. Among these proteins, only the one with MW slight higher than 2.5 kDa is the protein with antibacterial activity as may be determined from Fig 3. Preparations from each strain gave zones of growth

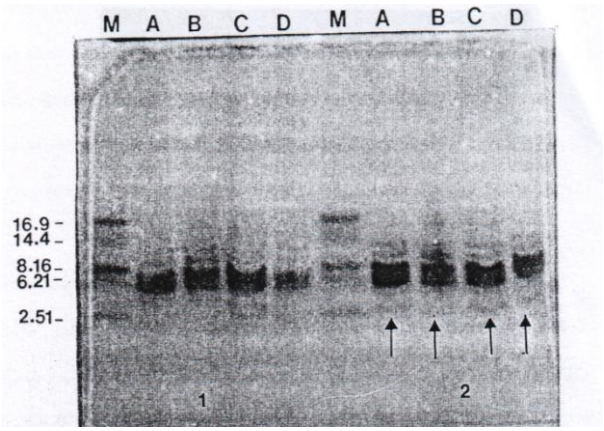

Figure 2. SDS-PAGE of nisin and three unknown bacteriocins preparations. Lane M, molecul standard (x 1000Da). Lane A,B,C, and D are *L. lactis* subsp*. lactis* ATCC11454, and UW1, UW2, and UW3, respectively. 1) 5μL; 2) 10μL sample loaded.

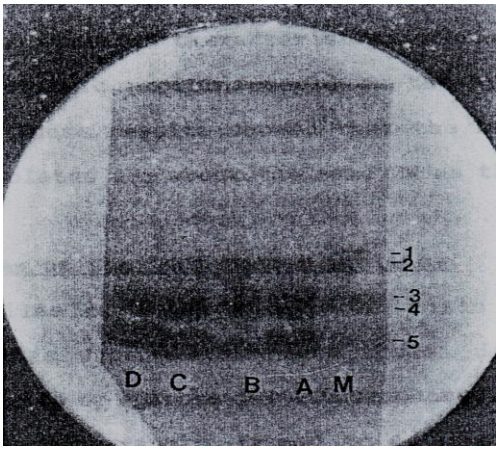

Figure 3. Direct detection of the band of nisin and unknown bacteriocin on SDS-PAGE by staining and by growth inhibition of *L. plantarum* NCDO 955. (A, B, C, and D) *L. lactis* subsp*. lactis* ATCC11454. and UW!, UW2, and UW3 with molecular weight standard (1) 16,950; (2) 14,400; (3) 8,160); (4) 6,210; and (5) 2,510 Da.

Inhibition only in the area if the lowest molecular weight protein. From the MW standards in the gel the MW of this protein is estimated to be about 3.0 kDa. Actual MW of nisin is 3.5 kDa (Buchman *et al.* 1988). Such a variation between

MW determined by SDS-PAGE and calculated MW from the amino acid composition has also been observed for several bacteriocins by Yang *et al*. (1992). This probably associated with the differences in the two methods used, namely actual amino acid sequences and SDS-PAGE. Also some specific characteristics of these small, hydrophobic and cation proteins could be the reasons for these differences. These results showed that the bacteriocins produced by the isolates are about the same MW as the nisin.

Plasmid Profile of the *Lactococcus* isolates

Plasmid profile of seven strains of nisin producing strains of *L. lactis* subsp. *lactis* and three *Lactococcus* isolates were analyzed by agarose gel electrophoresis (Fig. 4). All strains had plasmids ranging from 1 to 6. Strains DL 16, 7690, 150, and 148 seemed to have similar, each with about 6 plasmids. Strains Tik Sik 83 and 354/07 have similar profile each with only 1 large plasmid and strain ATCC 11454 has 3 plasmids. Isolate UW1 and UW2 have 5 plasmids and same plasmid profile while UW3 has only 2 plasmids. The seven nisin producing strains and the three isolates have several different plasmid profile. The three isolates harbored one or more plasmids that are present in the seven nisin producing strains. The nisin producing phenotype in *Lactococcus lactis* subsp. *lactis* is encoded in chromosomally linked transposons (Buchman *et al.* 1988).

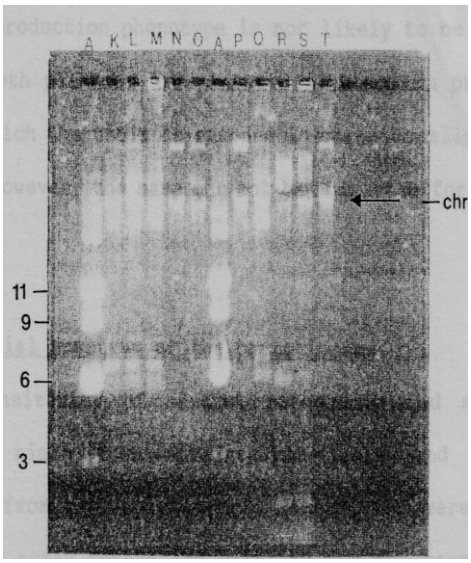

Figure 4. Plasmid profile of seven producing nisin and three unknown bacteriocin producing strains of *L. lactis* subsp*. lactis* strains and three isolates. Lane (K) DL 16; (L) 7690; (M) 150;(N) Tis Sik 83; (O) ATCC 11454; (P) 354/07; (Q) 148; (R) UW1; (S) UW2; (T) UW3

The 2 plasmids of the UW3 are quite different from those present in UW1, and UW2. Also among the five plasmids in UW1 and UW2, two are present in some nisin producing strains in which the trait is encoded in chromosomally linked transposon. However, the same can not be suggested for UW1 and UW2.

Antimicrobial Spectrum of the Bacteriocins.

The sensitivity of several pathogenic and spoilage bacteria to nisin from strain ATCC 11454 and unknown bacteriocins from the isolated UW1, UW2, and UW3 were determined by disc essay method and examined for the presence of zone of growth inhibition (Table 2). All the 12 test strains were sensitive to nisin as well the unknown bacteriocin(s) have a wide host range as strains from 7 Gram-positive genera were sensitive to them. It could be that the bacteriocin (s) produced by the three isolates are either nisin or very similar to nisin. It will be interesting to determine their amino acid sequence to identify wether they are nisin or not.

Table 2. Sensitivity of some pathogenic and spoilage bacteria to nisin and unknown bacteriocin produced by *Lactococcus lactis* subsp*. lactis*

Strains tested	11454	UW1	UW2	UW3
Staphylococcus aureus ATCC582	+	+	+	+
Staphylococcus epidermis 20[c]	+	+	+	+
Bacillus cereus SLR 779[b]	+	+	+	+
Bacillus subtilis 168	+	+	+	+
Lactobacillus M1	+	+	+	+
Leuconostoc 03	+	+	+	+
Propionicbacterium acne ATCC 6916[b]	+	+	+	+
Propionicbacterium[c]	+	+	+	+
Listeria momocytogenes Scott A[b]	+	+	+	+
Clostridium perfringens[b]	+	+	+	+
Clostridium sporogenes ATCC 7955[c]	+	+	+	+

[a] The bacterial strains were obtain from Food Microbiology Laboratory stock collection
[b] Pathogenic bacteria
[c] Spoilage bacteria, [d] +, sensitive as evidenced from zone of growth inhibition.

CONCLUSIONS

1. The three bacteriocin producing *Lactococcus* isolates were identified to be *L. lactis* subsp*. lactis* based on macroscopic, microscopic, morphological biochemical, peptide characteristic, plasmid profile and antimicrobial spectrum tested.
2. The three strains produce bacteriocins that are **similar to** nisin both in the MW and host range against Gram-positive spoilage and pathogenic bacteria.
3. The plasmid profiles of the three isolates are different from the seven nisin producing *Lactococcus* spp.

4. It is still needed to determine the amino acid sequence of unknown bacteriocin to identify whether they are nisin or not.

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