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Biochemical Identification of Bacteriocins from
Enterococcus faecalis 710C

by

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DEDICATION

To my parents,

Xinming Liu and Li Luo

ABSTRACT

Enterococcus faecalis 710C is a lactic acid bacterium that produces two bacterocins, ent7A and ent7B. Both ent7A and ent7B have strong activity against gram-positive food pathogens including *Listeria* spp., *Clostridium* spp., vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). Mass spectrometry analyses revealed that both ent7A (5201 Da) and ent7B (5207 Da) have formylated N-terminal methionine. The amino acid sequences, structural gene sequences of ent7 from nucleotide position 1-275 and immunity gene were determined. Circular dichroism data suggest that in aqueous solution ent7A and ent7B have 20 to 25% alpha-helical region. Addition of membrane-mimicking reagent (trifluoroethanol) did not significantly enhance the alpha-helical content in ent7A and ent7B. Chiral analysis by gas chromatography-mass spectrometry showed that the amino acid residues elucidated in ent7A and ent7B were all in L-configuration.

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LIST OF ABBREVIATIONS

AAFC Lacombe - Agriculture and Agri-Food Canada, Lacombe Research Station

ABC - ATP-binding Cassette

APT - All-Purpose Tween

ATCC - American Type Culture Collection

AU - Active unit

bp - Base pair

CD - Circular dichroism spectrometry

θ - Molar ellipticity

Dha - α, β-dehydroalanine

Dhb - α, β-dehydrobutyrine

DHB - 2, 5-dihydroxybenzoic acid

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

ESI - Electrospray ionization

FDA - Food and Drug Administration

FPLC - Fast protein liquid chromatography

FT-ICR - Fourier Transform Ion Cyclotron Resonance

GRAS - Generally recognized as safe

h - Hour

HCCA - α-cyano-4-hydroxy cinnamic acid

HK - Histidine kinase

IF - Induction factor

kb - Kilobase

kDa - Kilodalton

KE - Kinetic energy

LAB - Lactic acid bacteria

MALDI-TOF - Matrix-assisted laser desorption/ionization – time-of-flight

mCCDA - Modified-charcoal cefoperazone deoxycholate agar

MeLan - β-methyl-lanthionine

mg - Milligram

min - Minute

mL - Millilitre

MRSA - Methicillin-resistant *Staphylococcus aureus*

MS - Mass spectrometry

MS/MS - Tandem mass spectrometry

m/z - Mass-to-charge

nt - nucleotide (position)

PCR - Polymerase chain reaction

PMF - Proton motive force

Prov Lab - Provincial Laboratory for Public Health, Edmonton, AB

Q - Quadrupole

QS - Quorum-sensing

RCM - Reinforced Clostridial Medium

rDNA - Ribosomal DNA gene

RP – HPLC - Reverse-phase high performance liquid chromatography

s - Second

SA - Sinapinic acid

TBE - Tris-borate-EDTA buffer

TFA - Trifluoroacetic acid

TFE - Trifluoroethanol

TSB - Tryptic soy broth

UAFM - University of Alberta, Food Microbiology Laboratory Culture Collection

UAL - University of Alberta Lactic Acid Bacteria Collection

VRE - Vancomycin-resistant *Enterococcus faecium*

1. INTRODUCTION

Bacterial foodborne illness has been an escalating concern worldwide. In Canada, it is estimated that between 11 and 13 million Canadians suffer every year from illnesses caused by foodborne bacteria (Health Canada and the Public Health Agency of Canada).

To prevent bacterial foodborne illness, various food preservation methods have been used. These methods include canning, pasteurizing and addition of chemical additives. Due to concerns from consumers, there has been a rapidly emerging area of research and development of naturally occurring organisms that produce antimicrobial substances to control the growth of foodborne pathogens (Cleveland et al., 2001; Gálvez et al., 2007; Gálvez et al., 2008). Of special interest are lactic acid bacteria (LAB) that grow naturally on food. LAB are known for their probiotic use and the antimicrobial compounds they produce that inhibit the growth of many foodborne pathogens. One type of these antimicrobial compounds is bacteriocins (Franz et al., 2007; McMullen and Stiles, 1996; Stiles and Hastings, 1991; Stiles, 1996). Bacteriocins are proteinaceous compounds secreted by bacteria and they have antimicrobial activity against closely related species. Bacteriocins are proteinaceous compounds that are frequently are many orders of magnitude more potent than conventional antibiotics on specific bacterial strains (Cotter et al., 2005).

Enterococcus is a genus of LAB that has a large range of environmental niches. Some enterococci are bacteriocin-producers. They exhibit strong antimicrobial activity against a broad spectrum of foodborne pathogens, including *Listeria* spp., *Clostridium* spp. and *Staphylococcus aureus* (Franz et al., 2007). The chemical properties and modes of action of these bacteriocins have been of an increasing interest (Abee et al., 1995).

The strain of interest in the current research, *Enterococcus faecalis* 710C was isolated from a ground beef sample that had been plated onto KF Streptococcus agar and the strain was obtained from the University of Alberta Food Microbiology Culture Collection. Previous studies show that this organism has a broad spectrum of antimicrobial activity (this study), including common gram-positive foodborne pathogens such as *Clostridium* spp., *Listeria monocytogenes*, and methicillin-resistance *Staphylococcus aureus* (MRSA).

In previous work, the 16S rDNA of *E. faecalis* 710C was confirmed and PCR amplification of virulence-factor-associated genes had been conducted (Franz, C. M. A. P., personal communication). Presence of virulence factor genes or positive results on plate assays were obtained for ace (collagen adhesion), aggregation substance (*asa*1), enterolysin A, gelatinase, EfaAfc (endocarditis antigen from *E. faecalis*) and β-haemolysin. Negative results were obtained for vanB (vancomycin resistance) or EfaAfm (endocarditis antigen from *E. faecium*). Subsequent investigations showed that this organism produces bacteriocins.

In the present study, bacteriocins from *E. faecalis* 710C were isolated and purified. The chemical properties of these bacteriocins were characterized, including their amino acid sequences, structural gene sequence, secondary structure, configuration of amino acid residues and antimicrobial activity.

2. LITERATURE REVIEW

2.1 Lactic acid bacteria and food safety

2.1.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are gram-positive, non-sporulating bacteria that produce lactic acid as the major end product of carbohydrate fermentation. LAB include a diversity of bacteria from families *Lactobacillaceae, Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Leuconostocaceae* and *Streptococcaceae* (Garrity et al., 2005).

LAB are traditionally classified based on their morphology, growth temperatures, and metabolism patterns (Salminen et al., 2004). They can also be classified based on their 16S ribosomal RNA genes (16S rDNA), which contain regions that have variable sequences amongst different bacterial species.

LAB can be found in a wide range of ecological niches: soil, plants, animals and surface water, and many food products. Most LAB associated with food are non-pathogenic to humans. They are part of the healthy microflora of human and animal intestinal tract. Some LAB provide health benefits to humans and are known as probiotics. Only a few LAB strains carry virulence factors that are associated with human diseases.

2.1.2 Roles of lactic acid bacteria in food

Depending on the species, some LAB are food-spoilage microorganisms while others are essential to food manufacturing and preservation. LAB have been used for fermented foods for over 6000 years (Holzapfel et al., 1995). They participate in fermentation during the manufacture of food products such as yoghurt, sausages, sauerkraut and cheese. The major end product of fermentation, lactic acid, can assist the coagulation of milk by lowering the pH during cheese ripening. Lactic acid can also improve food flavour, help digestion and facilitate nutrient uptake in the human gut. It is known that *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* aid digestion and reduce the risk of intestinal infections by competing with pathogenic bacteria in the human digestive tract. Other metabolites of LAB, such as acetic acid and carbon dioxide can provide taste and prevent food from spoilage (Holzapfel et al., 1995). Certain LAB species produce antimicrobial compounds that suppress the growth of food spoilage organisms and extend the storage life of food.

2.1.3 Lactic acid bacteria and food preservation

Food preservation techniques involve both physical and chemical methods of preservation. Physical methods include canning, pasteurizing and non-thermal techniques such as high pressure or pulsed electric fields. Chemical methods

include addition of salt, sugar or chemical preservatives to the food to inhibit microbial growth. Due to the concern from consumers regarding the amount of daily salt and sugar intake, as well as the potential toxicity of chemical preservatives to the human body, there has been an increasing interest in cultivation of foods' natural microflora and/or their antibacterial substances (biopreservation). LAB have demonstrated their potential as biopreservatives because a few LAB strains are part of natural-occurring microflora on meat and many are generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA). Many of these LAB produce antimicrobial compounds including organic acids, carbon dioxide, non-proteinaceous antimicrobials and bacteriocins.

Bacteriocins have attracted great research interest because of their potency (effective concentration at nM range to achieve antimicrobial activity), especially against *Listeria monocytogenes*, which causes listeriosis.

2.2 Bacteriocins

Bacteriocins are peptides or proteins synthesized by ribosomes in bacteria. They are generally secreted out of the cell into the extracellular medium. Bacteriocins are often synthesized in the inactive form (pre-bacteriocin) and then processed into their mature (active) form during transport. Active bacteriocins exert antibacterial effects, either bactericidal or bacteriostatic, against closely or

non-closely related bacteria (Jack et al., 1995).

Bacteriocins were first identified from *Escherichia coli* V in 1925. This strain secretes a toxic substance (later named as colicin V) that inhibited *E. coli* S (Gratia, 1925). Since then, more and more antimicrobial substances like colicin V have been discovered. These substances have characteristics in common. They are all proteinaceous and secreted by bacteria for growth competition with other bacteria in the same ecological niche.

Bacteriocins have been discovered not only in gram-negative bacteria, but also in gram-positive bacteria such as LAB. Bacteriocins from gram-negative bacteria differ from those secreted by gram-negative bacteria in that they possess self-regulated biosynthesis and a dedicated transport system for excretion (Riley and Wertz, 2002).

2.2.1 Classifications of LAB bacteriocins

Classification of bacteriocins from LAB is complicated mainly due to different views on whether bacteriocins should be classified according to their chemical structures, methods of excretion, or the modes of action. A widely accepted classification was suggested by Klaenhammer in 1988 and reviewed by Nes et al. in 2007:

Class I bacteriocins: lantibiotics

Class II bacteriocins:

Class IIa: (pediocin-like bacteriocins);

Class IIb: bacteriocins, whose activity depends on the complementary activity of two peptides (two-peptide bacteriocins);

Class IIc: *sec*-dependent secreted bacteriocins;

Class IId: the remaining Class II bacteriocins;

Class III bacteriocins;

Class IV bacteriocins.

The Class I bacteriocins includes lantibiotics, which are further divided into Type A (linear) and Type B (globular) lantibiotics. Lantibiotics produced by LAB mostly belong to Type A. They comprise a group of small (19 to 37 amino acid residue) polycyclic bacteriocins containing uncommon post-translationally modified amino acids, including: α, β-dehydroalanine (Dha), α, β-dehydrobutyrine (Dhb), *meso*-lanthionine (Lan) and β-methyl-lanthionine (MeLan) (Sahl and Bierbaum, 1998). Examples include nisin A from *Lactococcus lactis* (de Vuyst and Vandamme, 1994b) and lactocin S from *Lactobacillus sakei* L45 (Mørtvedt et al., 1991).

Class II bacteriocins include small (< 10 kDa), heat-stable, cationic non-lantibiotics. They are further categorized into Class IIa bacteriocins, which have strong antilisterial activity and have a conserved N-terminal amino acid sequence (YGNGVXaaC). Class IIa bacteriocins, include leucocin A from *Leuconostoc gelidum* UAL187 (Hastings et al., 1991), pediocin PA-1/AcH produced by *Pediococcus acidilactici* PAC-1.0 (Henderson et al., 1992) and *Pediococcus*

acidilactici H (Bhunia et al., 1988); Carnobacteriocin BM1 from *Carnobacterium maltaromaticum* LB17B (Quadri et al., 1994), and enterocin A from *Enterococcus faecium* CTC492 (Aymerich et al., 1996). Class IIb bacteriocins include brochocin-C produced by *Brochothrix campestris* ATCC 43754 (Siragusa and Cutter, 1993) and plantaricin S from *Lactobacillus plantarum* C11 (Diep et al., 1996). Class IIc bacteriocins include enterocin P from *Enterococcus faecium* P13 (Cintas et al., 1997) and Class IId bacteriocins include lactococcins A and B from *Lactococcus cremoris* 9B4 (Holo et al., 1991) and *Lactococcus lactis* WM4 (Stoddard et al., 1992) and enterocins L50A, L50B and enterocin Q from *Enterococcus faecium* L50 (Cintas et al., 1998; Cintas et al., 2000). Some bacteriocins that do not have an N-terminal leader peptide are known as "leaderless" peptides. They are grouped into Class IId bacteriocins.

Class III bacteriocins are large (>30 kDa), heat-labile proteins (in some literature they are referred to as bacteriolysins). They include enterolysin A from *Enterococcus faecalis* LMG 2333 (Nilsen et al., 2003) and helveticin J from *Lactobacillus helveticus* 481 (Joerger and Klaenhammer, 1986).

Class IV bacteriocins are cyclic peptides including carnocyclin A from *C. maltaromaticum* UAL307 (Martin-Visscher et al., 2008), enterocin AS-48 produced by *Streptococcus* (*Enterococcus*) *faecalis* subsp. *liquefaciens* S-48. (Gálvez et al., 1986; Gálvez et al., 1989) and gassericin A from *Lactobacillus gasseri* LA39 (Kawai et al., 1998).

2.2.2 Bacteriocin biosynthesis, processing and secretion

Most bacteriocins are synthesized as a preprobacteriocin that is biologically inactive. A preprobacteriocin contains a C-terminal probacteriocin domain and an N-terminal extension (Nes et al., 1996). The N-terminal extension contains a specific amino acid sequence that is required for the recognition of preprobacteriocin by a dedicated transport system. The C-terminal probacteriocin then undergoes post-translational modifications, yielding mature bacteriocin (Hastings et al., 1991). However, in some bacteriocins, the N-terminal extension is absent, resulting in the characterization of “leaderless” bacteriocins. Examples include enterocin L50A and L50B from *Enterococcus faecium* L50 (Cintas et al., 1998).

Class I bacteriocins (lantibiotics) undergo extensive post-translational modifications including incorporation of the uncommon amino acid residues. Class II bacteriocins undergo minimal post-translational modifications such as disulfide bridge formation and methionine residue oxidation (Cintas et al., 1998).

In most cases, mature bacteriocins are secreted by the ATP-binding Cassette (ABC) transporters or by a bacterial preprotein translocase (*sec*). Bacteriocins secreted by ABC transporters contain double-glycine residues at the -2 and -1 positions, which are recognized by ABC transporter (Håvarstein et al., 1995). Bacteriocins secreted via the *sec* pathway have a signal peptide on their N-terminal extension that contains an amino acid sequence that is recognized by the

sec-translocase (den Blaauwen and Driessen, 1996). The secretion mechanism for leaderless peptides is so far unknown.

2.2.3 Mode of action of bacteriocins

Bacteriocins exert bacteriostatic or bactericidal effects on target cells. Bacteriostatic effect refers to the inhibition of growth or reproduction of target cells without causing cell death. Bactericidal effect include killing of target bacteria through primary and secondary modes of action: during primary modes of action, bacteriocin molecules insert themselves into the membrane and aggregate to form pores in the membrane. During secondary modes of action, bacteriocin molecules bind to target cell wall made of teichoic and lipoteichoic acids, where autolysins are normally immobilized. Since bacteriocins are strong cationic compounds, the binding of bacteriocins to cell wall competes the binding of autolysins to teichoic and lipoteichoic acids through cation-exchange process. Therefore, autolysins are released and the target cell is lysed.

2.2.4 Regulation of bacteriocin production

It has been suggested that bacteriocin production occurs throughout the growth phase of the producer strain (Parente et al., 1997). However, bacteriocin

activity is usually not detected while the producer strain is still at its lag- or early-log phase due to their low concentration. Bacteriocin concentration significantly increases when the producer strain reaches late-log or early-stationary phase (Fontaine et al., 2007).

The production of most class IIa bacteriocins is influenced by the density of producer strain in the growth medium. The model that explains the cell density-bacteriocin production relationship is called quorum-sensing (QS) (Kleerebezem and Quadri, 2001). This model describes a three-component regulatory system which includes induction factor (IF), histidine kinase (HK), and response regulator. The IF can be a peptide different from the bacteriocin, or can be the bacteriocin itself (Saucier et al., 1995). The IF is secreted outside the cell during bacterial growth. It is sensed by the sensor domain of the HK located on the exterior face of the cell membrane. The catalytic domain of HK, which is located on the interior face of the cell membrane, becomes phosphorylated and activated. Activated HK dephosphorylates cytosolic response regulator, which promotes the transcription of the bacteriocin gene.

It has been found that adequate amount of IF in the medium is needed for bacteriocin production, i.e., a minimal inoculum size of the bacteriocin producer strain with its culture supernatant that contains IF, is required (Saucier et al., 2008). Otherwise no bacteriocin production can be detected. For example, when the inoculum size of *C. maltaromaticum* LV17 is below 4 log CFU/mL, *C. maltaromaticum* LV17 does not produce bacteriocin (Saucier et al., 1995).

However, in this case, bacteriocin production can be restored by addition of producer-strain culture (Saucier et al., 1995) or the purified IF.

In addition to cell density, there are other factors influencing bacteriocin production. For example, the composition of nutrients in the bacterial growth media can influence pediocin PA-1 production in *P. acidilactici* NRRL B-5627 (Guerra et al., 2001). In some cases, bacteriocin production is temperature-sensitive. For instance, the production of sakacin A from *Lactobacillus sakei* 706 reaches maximum at 20ºC and ceases when temperature rises above 35ºC (Diep et al., 2000). Another example where temperature controls bacteriocin production is with *C. maltaromaticum* UAL26 where bacteriocins are not produced if the culture is grown at temperatures above 15°C (Gursky et al., 2006).

2.3 Chemical characterization of LAB bacteriocins

2.3.1 Purification of bacteriocins

Bacteriocins are secreted into culture medium during bacterial growth; therefore, purification of bacteriocins begins from extracting bacteriocins from culture supernatant. LAB bacteriocins are cationic and hydrophobic; therefore, most purification methods are based on these properties. There are non-chromatographic based and chromatographic-based methods to purify

bacteriocins. Non-chromatographic methods are generally used for partial purification of bacteriocins or “clean-up” of bacteriocins before they are further purified by chromatographic-based methods. Common non-chromatographic methods for purification of bacteriocins include organic solvent extraction, ammonium sulphate precipitation (Holo et al., 1991) and cell adsorption-desorption (Yang et al., 1992). Organic solvents, such as butanol, can extract bacteriocins from aqueous cell supernatant since bacteriocins are hydrophobic (Piva and Headon, 1994). Ammonium sulphate precipitation is based on the fact that proteins are soluble in low-salt solutions (salting-in) and that they precipitate in high-salt solutions (salting-out). Different proteins salt out at different concentrations of salt. In ammonium sulphate precipitation, bacteriocins with other protein impurities are dissolved in solution with a low concentration of ammonium sulphate. When the ammonium sulphate concentration in the solution increases, bacteriocins and protein impurities salt out at different concentrations of ammonium sulphate and are separated. Ammonium sulphate precipitation is a convenient method to concentrate and enrich bacteriocins from crude cell culture supernatant. Cell adsorption-desorption method is based on the interaction of the cationic bacteriocins and the anionic bacterial cell surface made of teichoic and lipoteichoic acids. In this method, the bacterial surface is washed with a high-pH solution so that cationic bacteriocins secreted into growth medium will bind to the cell surface. The cells are then harvested, and the bacteriocins are desorbed from the cell surface by washing cells with a low- pH solution (Yang et al., 1992).

Organic solvent extraction, ammonium sulphate precipitation and cell adsorption desorption can separate bacteriocins from most impurities in cell supernatant. Further purification of bacteriocins usually requires liquid chromatography. Various types of chromatography have been used, including cation-exchange, hydrophobic-interaction, solid-phase extraction, size-exclusion, and reverse-phase (RP) high performance liquid chromatography (HPLC). Cation-exchange chromatography was efficient for the purification of many LAB bacteriocins including pediocin PA-1 from *Pediococcus acidilactici* LMG 2351 (Uteng et al., 2002), plantaricin ST31 from *Lactobacillus plantarum* ST31 (Todorov et al., 2004), and bacteriocin ST15 from *Enterococcus mundtii* ST15 (Granger et al., 2005). Hydrophobic-interaction column and solid-phase extraction column have also been applied to purify durancin L28-1A from *Enterococcus durans* L28-1, which was partially purified using a Phenyl-TOYOPEARL hydrophobic column and solid-phase Sep-Pak C18 cartridges (Yanagida et al., 2005). Solid-phase extraction is usually a pretreatment step prior to the HPLC purification. Its main purpose is to desalt bacteriocins and remove impurities.

To obtain a high degree of purity of a bacteriocin, RP-HPLC or immunoaffinity chromatography is needed. RP-HPLC has been used to purify bacteriocins such as enterocin L50A, L50B (Cintas et al., 1998), and carnocyclin A (Martin-Visscher et al., 2008). Immunoaffinity chromatography has been developed for rapid purification of bacteriocins from bacterial culture supernatant.

Using the anti-pediocin PA-1 polyclonal antibody for pediocin PA-1 purification achieved 53.3% recovery (Naghmouchi et al., 2008), compared to 38% recovery using SP-sepharose strong cation-exchange column (Gaussier et al., 2002). However, so far many bacteriocins do not have commercially available antibodies and making antibodies using animals is time consuming and costly. For this reason application of immunoaffinity chromatography for bacteriocin purification is not as widely used as HPLC.

The choice of columns for HPLC separation depends on the bacteriocin and it is difficult to generalize which column will be more efficient. Trials of different columns are needed for efficient bacteriocin purification. Factors that need to be considered include stationary phase of the column, particle size, mobile phase composition, pH and temperature, sample preparation and programming (solvent flow rate and gradient).

2.3.2 MS analyses of bacteriocins

Mass spectrometry (MS) analyses involve ionization of analytes and measurement of these ions according to their mass-to-charge ratio. MS techniques used in the study of bacteriocins include matrix-assisted laser desorption/ionization (MALDI) MS and electrospray ionization (ESI) tandem MS (MS/MS) analyses. MALDI- MS has been utilized to detect the presence bacteriocins in a sample matrix and to determine the molecular weights of

bacteriocins. ESI-MS/MS analysis has been used for sequencing bacteriocins. This technique has also been used in studying chemical modifications (e.g., post-translational modification), as well as in the quantification of bacteriocins.

2.3.2.1 MALDI- MS

MALDI- MS has been widely used in protein analysis since its first introduction in 1988 (Karas and Hillenkamp, 1988). It is a convenient, sensitive and accurate method to study proteins (Rose et al., 1999). A major advantage of MALDI- MS is that it is a soft ionization method, since the analyte is co-crystallized with a matrix, which is usually a UV-absorbing weak organic acid to avoid undesired fragmentation of bacteriocins during the ionizing process. The UV-laser irradiates the crystal, resulting in an efficient transfer of laser energy transfer from the matrix to the analyte. The matrix can protect the analyte from fragmentation and can promote the ionization of the analyte. The analyte ions will be accelerated by an electric field and separated according to their mass-to-charge (m/z) ratio.

Common matrices employed for bacteriocin analyses include α-cyano-4-hydroxy cinnamic acid (HCCA), sinapinic acid (SA) or 2, 5-dihydroxybenzoic acid (DHB). Generally, HCCA is suitable for peptides under 5 kDa, SA for large peptides/protein above 5 kDa and DHB for protein digests (ProteoChem).

There are different methods to deposit the analyte and the matrix onto the

sample plate for analysis: 1) the matrix is spotted onto the plate and air-dried, a second layer (matrix: analyte = 1: 1) is applied on top of the first layer, air-dried and rinsed with water drop to remove salts in the matrix that can interfere the analysis (two-layer method); 2) similar to 1) except that the second layer only contains analyte (thin-layer method); and 3) matrix and analyte are mixed at 1: 1 ratio, spotted onto the plate and air dried (no rinsing). This method is also referred to as dried-droplet method (Kussmann et al., 1997). Method 1 is commonly used but Method 2 provides a greater tolerance to sample impurity such as salts and detergents. Method 3 is simplest but analyte detection may be easily interfered with by impurities in the sample.

MALDI- MS is usually done in conjunction with a time-of-flight (TOF) analyser. In MALDI-TOF MS, ions generated are accelerated by an electric field of known strength (Stephens, 1946). Thus, all ions with the same charge have the same kinetic energy (KE). According to the equation $KE = ½ mv^2$, ions of smaller masses have higher velocity. The time that takes an ion to reach the detector (fixed distance) is therefore dependent on the mass-to-charge (m/z) ratio. TOF analysers include linear and reflectron types (RETOF). In linear TOF, ions travel in straight line to the detector. In RETOF, ions are deflected by an energy-focusing mirror and then reach the detector. Generally, RETOF provide better resolution of ions than linear TOF.

2.3.2.2 ESI- MS/MS

ESI is a different ionization method than MALDI. In ESI, a solution that contains the analyte passes through a high-voltage capillary and forms charged droplets. These charged droplets are evaporated by inert gas (e.g, nitrogen) and become smaller and smaller. When the droplet size reaches the point that the droplet surface tension can no longer withstand the coulomb repulsion between the analyte ions within the droplet (the Rayleigh limit), the droplet breaks into even smaller droplets. This process repeats. Finally, the solvent-free ions are released.

With an ESI-MS/MS detector, usually a triple quadrupole (Q) analyzer is used. This analyzer consists of Q1, Q2 and Q3. Q1 is used to select the analyte ion (parental ion), Q2 is a collision cell where parental ions are fragmented into daughter ions by colliding with e.g., argon gas inside the collision cell, and Q3 filters and scans the daughter ions produced in Q2 for their m/z. The m/z of ion fragments can be used for peptide sequencing.

The parental ion can be identified based on the ion fragmentation pattern (the masses of the fragments), since ions from different analytes have unique ion fragmentation patterns. In most cases of low-energy collisions, only the amide bonds of a peptide break. The peptide breaks into two parts, one part contains the N-terminus and the other contains the C-terminus. The part of peptide with the N-

terminus is referred to as b-ion and that with the C-terminus is referred to as y-ion (Roepstorff and Fohlman, 1984). The mass information of all the b- and y- ions of a peptide can be compared to theoretical fragmentation pattern of peptides with known sequences from database to determine the amino acid sequences of a peptide. The difference between theoretical mass and experimentally measured mass may also indicate a specific type of chemical modification of the peptide (Delta Mass, the Association of Biomolecular Resource Facilities).

The limit of MS/MS method in peptide sequencing, however, includes that Leu and Ile cannot be differentiated, since leucine (Leu) and isoleucine (Ile) have the same mass. In this case, other techniques need to be employed to determine whether this residue is Leu or Ile, e.g., from the gene that codes the peptide.

ESI-MS/MS has been widely employed for proteins or peptides analyses because of its high sensitivity and accuracy. For example, the accurate mass spectra of protein digests at a picomole level sensitivity can be acquired by nano-ESI Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS (Kosaka et al., 2000). The Bruker 9.4T Apex-Qe FTICR used in this study has mass accuracy of ± 2 ppm over m/z range from 100 to 10,000 and can achieve accuracy of < 1 ppm upon internal calibration.

2.3.3 Circular Dichroism

Circular dichroism spectrometry (CD) is a fast way to obtain structural information of proteins or peptides. Proteins and peptides are made of chiral (L- or D-) amino acid residues (except glycine). Thus they are optically active and have CD absorption. CD records the differential absorption of the left- (L-) and the right- (R-) handed circularly polarized light from an optically active (chiral) molecule. The CD absorption of proteins and peptides is largely influenced by the spatial arrangement of their amino acid residues. Therefore, a protein or peptide adopting different configurations (secondary structures) or conformations (tertiary structures) will generate different CD signals. CD spectra are usually represented as the molar ellipticity as a function of wavelength. The CD signals in the near-UV range (250-350 nm) are influenced by protein tertiary structure, while the signals in the far-UV range (190-250 nm) are influenced by the secondary structures (α-helix, β-sheet or random coil).

The percentage of α-helix, β-sheet or random coil in a protein or peptide is usually calculated using computer software such as CDPro. The percentage of the α-helical content can also be calculated from formula: percentage of α-helical region = $(-[\theta]_{222nm} + 3000) / 39000$, in which θ is the molar ellipticity (Juban et al., 1997). Since bacteriocins normally contain only α-helical (structured) and random coil (unstructured) regions, the percentage of α-helix in a bacteriocin is

estimated using the formula above.

CD is a useful tool to monitor the conformational change of a bacteriocin after it transfers from an aqueous environment to a hydrophobic environment. In other research, CD technique can also be used in the study the thermal stability of bacteriocin, as bacteriocin will change its secondary structure (α-helical region will become random coil during heating) and result in different CD signals (Montalbán-López et al., 2008).

However, CD experiments have limitations: 1) no information on which region in the bacteriocin is either coiled or is an α-helix; 2) some common organic solvents are not compatible with CD analysis, e.g., acetonitrile, chloroform and dichloromethane.

2.3.4 Chiral analysis of bacteriocins

Chiral chromatography is a type of chromatography in which special stationary phases are used to separate chiral compounds. Chiral chromatography includes LC- and GC- based methods, both of which have been widely applied to screen and separate enantiomers.

Chiral GC analysis is a conventional method for L-/D- screening of peptides because of its robustness and short analysis time. Once a method is validated, it can be used to screen any hydrolyzed peptides consisting the common 20 amino

acids. Data processing is greatly simplified, as MS spectra of compounds that elute from the column are recorded in real-time. The MS spectrum of each chromatographic peak can be linked to a library database, which allows identification of compounds from electron ionization MS spectra.

In chiral GC analysis, a peptide sample is hydrolyzed into free amino acids and derivatized. Derivatization is used to increase sample volatility through alteration of the compound functional groups. The derivatized amino acids pass through a GC column. As L- and D- amino acid derivatives interact differently with the stationary phase, they elute at different times from the column, thus separation is achieved.

The GC columns used for L-/D- screening may have a chiral or non-chiral stationary phase. Common stationary phases include polymeric matrices of dimethylsiloxane or (2-carboxypropyl)-methylsiloxane (Frank et al., 1978). This type of stationary phase is thermal stable and can be used at a temperature up to 230 ˚C. The column allows for the separation of all the racemic protein amino acids in one chromatogram in about 30 min.

Depending on the choice of column, various methods have been developed to derivatize amino acids. For example, amino acids are derivatized to *N*-(O, S)-pentafluoro-propanoyl-isopropylesters for separation on a Chirasil-Val column (Abdalla et al., 1987). The Chirasil-Val column has a stationary phase made from a copolymer of dimethyl-siloxane and (2-carboxypropyl) methylsiloxane. It is

coated with chiral selector L-valine-*t*-butylamide, which separates amino acid enatiomers through hydrogen bonding (Abe et al., 1995). Amino acids are derivatized to *N*-(*O, S*)-ethoxycarbonyl 2-chloropropylesters for separation on on a CP-Sil 19 CB column (Bertrand et al., 2007).

Effective separation of L- and D- amino acid derivatives also depends on other factors such as temperature program and flow rate of carrier gas. These conditions need to be optimized to achieve the separation of amino acid enantiomers.

2.4 The enterococci

2.4.1 Overview

Enterococci belong to family *Enterococcaceae* of LAB under phylum Firmicutes. They are gram-positive, facultative anaerobic diplococci of intestinal origin (Thiercelin and Jouhaud, 1903). By 1997, 19 species of enterococci had been identified (Stiles and Holzapfel, 1997), including *E. faecalis*, *E. faecium*, *E. durans* and *E. mundtii*.

Enterococci can be found in a large range of environmental niches, including food (Eaton and Gasson, 2001), birds (Martín-Platero et al., 2006) and plants

(Mundt, 1963). Enterococci have also been isolated from human intestinal tract (Noble, 1978).

2.4.2 Bacteriocins from enterococci

Many enterococci are bacteriocin-producers. These bacteriocins are usually referred to as enterocins. Most enterocins belong to Class II bacteriocins, for example, enterocin L50A and L50B from *E. faecium* L50 (Cintas et al., 1998) and enterocin AS-48 from *E. faecalis* S-48 (Martínez-Bueno et al., 1994). There are some enterocins that do not belong to Class II bacteriocins. For example, cytolysin from clinical strains of *E. faecalis* (Huycke et al., 1991; Ike et al., 1987) is a lantibiotic and belongs to the Class I bacteriocins. Enterolysin A from *E. faecalis* LMG 2333 (Nilsen et al., 1993) belongs to the Class III bacteriocins. Some enterocins exhibit strong antimicrobial activity against a broad spectrum of foodborne pathogens, including *Listeria* spp., *Clostridium* spp. and *Staphylococcus aureus* (Franz et al., 2007).

Generally enterococci were regarded as food contaminants and can be associated with virulence factors for human diseases such as endocarditis and septicemia. However, certain strains of *Enterococcus* spp. are beneficial and are used in food fermentations. For example, enterococci are predominant strains in

the starter cultures in the manufacturing of southern European cheeses like Greek kefalotyri, Spanish cebreiro and Portuguese picante da beira baixa cheese (Tsakalidou et al., 1993). Other strains of *Enterococcus* spp. have been suggested to have probiotic effects. These effects include restoration of intestinal flora, reduction of serum cholesterols, reduction of lactose intolerance and stimulation of immune systems (Havenaar et al., 1992). One example, *E. faecium* SF68, has been found to shorten the period of diarrhea in pediatric patients and accelerate the normalization of the intestinal flora (Bellomo et al., 1980). In addition, bacteriocin-producing enterococci have a broad spectrum of antimicrobial activity against food spoilage bacteria. Whether these strains of *Enterococcus* spp. can be directly added to food as preservative or not needs cautious consideration. However, the use of purified bacteriocins from enterococci may provide a very effective hurdle to control the growth of pathogens in ready-to-eat foods.

2.5 Research Objectives

The objective of this study was to purify and identify enterocins from *E. faecalis* 710C. The bacteriocins from this strain have a broad spectrum antimicrobial activity against common food pathogens and some spoilage organisms. To achieve the objective, the study was designed:

1. To isolate enterocins (ent7A and ent7B) from *E. faecalis* 710C and purify them to homogeneity.
2. To determine the amino acid sequences, chemical modifications, secondary structures and configurations of amino acid residues of the isolated enterocins.
3. To determine the sequences for the structural and immunity genes of th enterocins.
4. To test the antimicrobial activity of purified enterocins.

3. MATERIALS AND METHODS[1]

3.1 Bacterial strains and culture.

Bacterial strains used in this study are listed in Table 3.1. *E. coli* AW1.7 was isolated from a commercial beef slaughter plant where cattle are processed at a rate of 250 heads/h (Aslam *et al.*, 2004). *E. coli* GGG10 (isolated from a commercial slaughter plant 20 years ago, prior to the commercial use of decontamination interventions). Stock cultures of bacteria were maintained at -74ºC in the appropriate broth with 40% v/v glycerol. *E. faecalis* 710C was obtained from the University of Alberta collection of lactic acid bacteria.

E. faecalis 710C was grown at 37ºC in All-Purpose Tween (APT) broth [Difco, Becton Dickinson (BD) Microbiology Systems, Sparks, MD]. *Lactobacillus* spp. were grown at 25ºC on APT agar or in APT broth. Viable cells of *Clostridium* spp. were grown anaerobically at 39ºC in Reinforced Clostridial Medium (RCM; Oxoid, England). *Campylobacter jejuni* ATCC 700819 and *Brevundimonas diminuta* were grown on modified charcoal cefoperazone deoxycholate Agar (mCCDA) agar plates (Oxoid). For use in antimicrobial activity assays, *Campylobacter* spp. and *B. diminuta* were grown on Tryptic soy

[1] *A version of this chapter has been submitted for publication in the Journal of Agricultural and Food Chemistry.*

agar (Difco) supplemented with 0.5% yeast extract (Difco) incubated at 39ºC in an anaerobic jar flushed with a mixture of 4.98% CO_2 with a balance of nitrogen to create microaerophilic conditions. All other indicator strains (Table 3.1) were grown in TSB (with 0.5% yeast extract) or on TSB agar plates.

The cultivation of *C. botulinum* spores and viable cells was performed by Melissa Haveroen as follows: Proteolytic strains of *C. botulinum* used in this study were *C. botulinum* 368B, IIB ATCC 13983, A6, and A62, and non-proteolytic strains were DB2, 2B, and 17B (University of Alberta Food Microbiology lab collection). Medium for spore production for strain DB2 was Sporulation Medium (SM; Health Protection Branch) containing 50 g $liter^{-1}$ tryptone (Difco) and 10 g $liter^{-1}$ peptone (Difco), while Reinforced Clostridial Medium (RCM; Difco, Becton Dickinson, Sparks, MD) was used for strain A6. Spores of all other strains were produced in Trypticase-Peptone-Glucose-Yeast Extract Broth (TPGY; USDA) containing, per liter: 50 g tryptone, 5 g peptone, 20 g yeast extract (Difco), 4 g glucose (Fisher Scientific Canada, Ottawa, ON), and 1 g sodium thioglycollate (Sigma-Aldrich Canada Ltd., Oakville, ON). Before use, all media was incubated in an anaerobic chamber for at least 24 h (Coy Laboratory Products Inc., Grass Lake, MI) under an atmosphere of 5% CO_2, 10% H_2, and balance N_2 (Praxair Canada, Edmonton, AB) to ensure anaerobic conditions.

For spore production, 100 µl of spore suspensions were inoculated into 5 ml of appropriate medium, heat-shocked in a water bath for 15 min at either 55°C (non-proteolytic) or 75°C (proteolytic), and incubated at 37°C under anaerobic conditions for 48 h. The 5-ml culture was then used to inoculate a 250-ml volume of the appropriate medium, and the culture was incubated at 37°C under anaerobic conditions for 14 days with periodic monitoring by phase contrast microscopy. Spores were harvested by centrifuging at 16270 x *g* for 20 min, followed by 5 washes (100 ml) and a final resuspension (10 ml) in sterile 0.9% NaCl, and a 1-h heat treatment at 65°C to destroy residual toxin and vegetative cells. *C. botulinum* strains were stored as spore stocks in 0.9 % (wt/vol) NaCl at 4°C, and were enumerated using RCM (Difco) containing 2.5 % (wt/vol) agar.

Table 3.1: Bacterial strains used in this study.

Strain	Source
Brochothrix campestris ATCC 43754	ATCC[1]
Brevundimonas diminuta UFM1	UAFM[2]
Campylobacter jejuni ATCC 700819	ATCC
Carnobacterium divergens UAL9	UAFM
Carnobacterium maltaromaticum UAL8A, UAL8B, UAL8C2, UAL26, JG126	UAFM
Clostridium botulinum spores and viable cells[3]	UAFM
Clostridium butyricum ATCC 8260, viable cells	ATCC
Clostridium difficile 3195, 76; viable cells	UAFM
Clostridium perfringens CL5626, R783; viable cells	UAFM
Clostridium sporogenes 25779, 7955; viable cells	ATCC
Enterococcus faecalis 710C	UAFM
Escherichia coli AW 1.7	Aslam et al. (2004)
Escherichia coli GGG10	AAFC Lacombe[4]
Enterococcus faecium BFE900 and VRE strains	UAFM
Vancomycin resistant *Enterococcus faecium* CL3745, E2155, E2217, E2352, M1008, S769, R493, R704, R846	Prov Lab[5]
Lactobacillus sakei DSM20017, 706	UAFM
Leuconostoc gelidum UAL 187	UAFM
Listeria innocua ATCC 33090	ATCC
Listeria monocytogenes ATCC 15313, CDC7762, FS-15	ATCC and UAFM
Pediococcus acidilactici PAC 1.0	UAFM
Salmonella enterica serovar Typhimurium 18	UAFM
Staphylococcus aureus ATCC 23235	ATCC
Methicillin resistant *Staphylococcus aureus* R468, R507, R667, R719, R776, R870, R948, R1230, R1262, R1578	Prov Lab

[1]American Type Culture Collection

[2] University of Alberta, Food Microbiology Laboratory Culture Collection

[3] Data provided by Melissa Haveroen. Proteolytic strains of *C. botulinum* used in this study were *C. botulinum* 368B, 2B, A6, and A62, and non-proteolytic strains were DB2, IIB ATCC 13983, and 17B

[4] Agriculture and Agri-Food Canada, Lacombe Research Station. Culture obtained from Dr. G. Gordon Greer.

[5] Provincial Laboratory for Public Health, Edmonton, AB. Data provided by Denise Carlson (CanBiocin Inc., AB)

3.2 Activity assays.

Bacteriocin activity was monitored by spot-on-lawn assays (Franz et al., 2000). Supernatant of a 24 h culture of *E. faecalis* 710C was filtered through 0.22 µm 25 mm mixed cellulose ester syringe filter (Fisher Scientific, Edmonton, AB) and spotted onto solid (1%) agar and allowed to air dry. Soft (0.5%) agar was inoculated with the indicator organism (1.2%) and poured onto the solid agar. Plates were incubated at appropriate temperatures depending on the indicator strain. After 24 to 48 h of incubation, plates were examined for zones of clearing.

3.3 Isolation and purification of bacteriocins.

E. faecalis 710C was grown in 1 L of APT broth (Difco; 5% inoculum) at 37ºC for 22 h. The culture was centrifuged at 10,000 x *g* in for 20 min at 4ºC. The supernatant was filtered through Millipore Express PLUS polyethersulfone membrane (Millipore Corporation, Billerica, CA). Cation-exchange purification of ent7A and ent7B were carried out on an ÄKTA™explorer Fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Uppsala, Sweden). The cell supernatant was loaded onto a cation-exchange column (HiPrep™ 16/10 SP FF, GE Healthcare Life Sciences, Uppsala, Sweden; pre-conditioned with 50 mM sodium acetate buffer, pH 4.6) at 8 mL/min. Detection

of peptides was monitored by a UV-detector at 280 nm. The column was washed with 5-column-volumes of sodium acetate buffer after supernatant was loaded onto the column. Bacteriocins were eluted in a gradient of 2 M NaCl in the same buffer. Active fractions were desalted using C18 cartridges [Sep-Pak® C18, Waters Corporation, Milford, MA; pre-washed with 2-propanol containing 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, Oakville, ON), conditioned with 0.1% TFA] and eluted in 40% to 70% 2-propanol with 0.1% TFA. The 2-propanol was removed by RotaVAP (Büchi Corp., New Castle, DE) at 35ºC. Active peptides were separated by reverse-phase HPLC (model 1200, Agilent Technologies Inc., Palo Alto, CA). The column used was an Eclipse XDB-C18 (Agilent) with a particle size of 5 µm, length 150 mm and inner diameter 4.6 mm. The column was pre-conditioned with 40% acetonitrile (Fisher Scientific, Fair Lawn, NJ) with 0.1% TFA. The peptides were separated in a linear gradient of 40% to 70% acetonitrile containing 0.1% TFA, at a flow rate of 1 mL/min. Detection of peptides was monitored by a UV-detector at 280 nm. Antimicrobial activity in flow-through and fractions from each purification step was tested using spot-on-lawn assays. Protein concentration measured at 280 nm with a NanoDrop spectrophotometer (NanoDrop® ND-1000, Thermo scientific).

3.4 Mass spectrometry analyses.

Active peptides were analyzed by MALDI-TOF MS for molecular weight detection. α-cyano-4-hydroxycinnamic acid (HCCA, Sigma Chemical, St. Louis, MO) or 2, 5-dihydroxybenzoic acid (DHB, Sigma Chemical) were used as matrices. Spectra were obtained on a Voyager Elite MALDI-TOF MS system (Applied Biosystems, Foster City, CA) operating in negative ion mode.

Peptide sequence information was obtained on a Q-TOF Premier nano-infusion ESI system (Waters). Active fractions from RP-HPLC were further purified by C4 ZipTip (MilliPore) to remove TFA. Eluents from C4 ZipTips were infused to the Q-TOF Premier mass spectrometer by nano-infusion ESI to produce MS/MS spectra. Data were processed using software MassLynx 4.1. The MS/MS spectra of ent7A and ent7B were compared with the theoretical fragment ions of enterocin MR10A and MR10B.

The exact mass of the intact peptide was measured by infusion on a Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS, model Bruker 9.4T Apex-Qe, Billerica, MA) by co-infusing with bovine insulin (Sigma-Aldrich) to provide an internal calibration. The peptides were dissolved at a concentration of approximately 0.5 µM in 1:1 acetonitrile/water with 0.2% formic acid for analysis.

3.5 Gene amplification and sequencing.

Total DNA of *E. faecalis* 710C was isolated using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). To amplify nucleotide (nt) position 1-275 of MR10A-MR10B gene (*mr10*) (GenBank: DQ366596.1), *ent* 7F forward primer: 5'-ATGGGAGCAATCGCAAAATTAG-3' and *ent* L50B reverse primer: 5'-TAGCCATTTTTCAATTTGATC-3' were used. The gene was amplified with 30 cycles of 94ºC for 30 s, annealing at 44ºC for 30 s, and elongation at 72ºC for 30 s. PCR product was purified by using a QIAquick PCR purification kit (Qiagen) and subjected to 2% agarose gel electrophoresis in TBE buffer. The PCR product was ligated into pGEM®-T vectors (Promega, San Luis Obispo, CA) and cloned into *E. coli* DH5α cells by electroporation (0.2-mm cuvettes, field strength 1.8 kV, 200 Ω, capacitance of 25 μF). *E. coli* cells were screened for the insert using X-Gal/IPTG (Isopropyl β-D-1-thiogalactopyranoside) method (Sambrook and Russell, 2001). To amplify pGEM®-T from T7 to SP6 transcription initiation site including the insert, T7 promoter primer: 5'-TAATACGACTCACTATAGGG-3' and SP6 promoter primer: 5'-TATTTAGGTGACACTATAG-3' were used. Amplification was performed by colony PCR with 30 cycles of 94ºC for 45 s, annealing at 45ºC for 30 s, and elongation at 72ºC for 30 s. The PCR product was detected and purified as above

and sequenced using an ABI Big Dye version 3.1 Terminator sequencing kit (Molecular Biology Service Unit, University of Alberta). Nucleotide sequence of ent7 was compared to that of MR10 using BLASTN program [National Center for Biotechnology Information (NCBI)].

The immunity gene of ent7A and 7B (*ent7Imm*) was amplified using total DNA of *E. faecalis* 710C as well. The forward primer 710CImF: 5'-GCAGAATTAGCAGGAGCGATAACAGCAT-3' and the reverse primer 710CIMR: 5'- CGTAGTCAGGAAGTGATTGTTTG-3' were designed from the upstream and downstream of enterocin immunity gene BacI (NCBI accession AB292312, unpublished data) with the aid of PCR primer design software (Primer3). The PCR was programmed with 35 cycles of 94ºC for 1 s, annealing at 53ºC for 2 s, and elongation at 72ºC for 1 min and 30 s. The PCR product was sequenced as above using 710CImF as the sequencing primer.

3.6 Activity assays of purified ent7A and ent7B.

Purified ent7A and ent7B (dissolved in water) were tested against foodborne pathogens and spoilage organisms: *C. jejuni* ATCC 700819 and *B. diminuta* UFM1 (isolated from a pig processing plant), viable cells of *C. sporogenes* ATCC 25779, viable cells and spores of *C. botulinum* (purified ent7A tested only) *E. coli* AW1.7, *E. coli* GGG10, *Listeria monocytogenes* FS-15 and *Staphylococcus*

aureus ATCC 23235 using spot-on-lawn assays. Activity of ent7A and ent7B were evaluated by measuring the diameter of growth inhibition zones using spot-on-lawn method.

The antimicrobial activity of purified ent7A was tested by Melissa Haveroen against both spores and viable cells of *C. botulinum*. Briefly, 5 µL of purified enterocin 7A (1.8 mg mL^{-1}) was spotted on APT agar (1.5% w/vol), dried for 10 min, and overlaid with 5 mL semi-solid (0.75% w/vol) RCM (Difco) seeded with either 3 x 10^3 CFU mL^{-1} heat-shocked clostridial spores or 50 µL of an overnight culture of vegetative cells. For the spore inhibition test, proteolytic and non-proteolytic strains were heat-shocked for 15 min at 75°C and 55°C, respectively. Plates were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products Inc.) under an atmosphere of 5% CO_2, 10% H_2, and balance N_2 (Praxair Canada) for 24 h. Plates were then examined for clear zones of inhibition.

3.7 Circular dichroism analysis.

Circular dichroism (CD) spectra of ent7A and ent7B were obtained on a spectrophotometer [model DSM17, On-Line Instrument Systems (Olis), Bogart, GA] in a thermally controlled quartz cell with a 0.02-cm path length. The instrument was checked against a 1 mg/ml solution of D-10-camphorsulfonic acid.

The peptide concentration for analysis was 0.5 mg/ml. Peptides were prepared in 100% 20 mM sodium phosphate buffer (~ pH 6) or in 50% trifluoroethanol (TFE) and 50% of the same buffer. The CD spectra were recorded at 20°C. Data were collected at every 1 nm. The bandwidth was set at 2.0 nm. For both sets of experiments, baseline spectra of the appropriate solvent system were subtracted from the sample spectra prior to calculating molar ellipticities. Point-by-point integration was performed as a function of the high voltage readings on the photomultiplier detectors. The results were expressed in units of molar ellipticity (θ; degrees cm^2 $dmol^{-1}$) and plotted against the wavelength. The α-helical content of the peptide was calculated according to the molar ellipticity at 222 nm (θ_{222nm}) by using the following equation (Morrow et al., 2000): percentage α-helix = $(-[\theta_{222nm}] + 3000) / 39000 \times 100\%$.

3.8 Chiral GC-MS analysis

3.8.1 Preparation of L- and D- amino acid standards

Amino acid residues in ent7A and ent7B include Ala, Gly, Asn, Asp, Glu, Gln, Ile, Leu, Lys, Thr, Tyr, Val, Met, Trp, Arg, Pro, Phe and His. Gly does not have enatiomers so it was included in amino acid standards. Two standards, one

containing the above amino acids in L- form, and the other standard, contained the amino acids in D- form, were prepared by mixing ca. 0.02 mmol of each amino acid. The L-amino acid standard and D-amino acid standard were dissolved separately in 3 mL of 0.2 N HCl and heated for 5 min at 100°C. The standards were dried under argon gas. Acetyl chloride (1.5 mL) was slowly added to 5 mL of 2-propanol, and the solution was added to each standard. The resulting mixture was heated in a pressure vessel for 45 min at 100°C. After cooling on ice, the mixture was dried as above. Three mL of dichloromethane and 1 mL of pentafluoropropyl anhydride (derivatizing reagent; Sigma Chemical) were added and the mixture was heated in pressure vessel for 15 min at 110°C followed by cooling and argon gas drying.

3.8.2 Preparation of ent7A and ent7B

The peptides were prepared in the same fashion as above except that instead of heating for 5 min at 100°C in 3 mL of 0.2 N HCl, 2 to 3 mg of ent7A and ent7B were separately dissolved in 5 mL 6 N HCl and heated overnight in pressure vessels at 110°C.

3.8.3 Instrumentation.

The analysis was performed on a 5890 series II gas chromatograph (Agilent) instrument coupled with 7070EVG analytical organic mass spectrometry (Waters). The MS instrument has: 1) m/z range up to 1000; 2) mass accuracy of 0.2 u; 3) resolving power of ~1,000 (10% valley) ~2,000 fwhm. A Chirasil-Val 50 m x 0.25 mm x 0.16 µm capillary GC column (Grace, Deerfield, IL) was used in this analysis. The sample was injected through split/splitless mode with helium as the carrier gas with head pressure of 17 psi. Two temperature programs were used: 1) the oven temperature was initially set at 30°C and ramped to 90°C at 15°C/min followed by increasing to 180°C at 4°C/min; 2) oven temperature increased from 80°C to 190°C, 3°C/min. The analytes were ionized by electron impact and detected by the mass spectrometer operating in low resolution mode.

3.8.4 GC-MS data processing

MS spectra of derivatized amino acids that were eluted from the column were recorded in real-time. MS spectrum of each peak was linked to National Institute of Standards and Technology (NIST) Mass Spectral Library, which allowed for interpretation of the peaks from the chromatogram.

4. RESULTS[2]

4.1 Bacteriocin activity and purification

4.1.1 Antimicrobial activity of *E. faecalis* 710C cell-free supernatant

Spot-on-lawn assays were used to monitor antimicrobial activity of the cell-free supernatant of an overnight culture of *E. faecalis* 710C and the purified ent7A and ent7B. *E. faecalis* 710C supernatant had strong antimicrobial activity against gram-positive foodborne pathogens such as *Listeria*, *Clostridium* spp., MRSA, and gram-negative *B. diminuta* (Table 4.1). The cell-free supernatant of *E. faecalis* 710C had only very weak activity against *C. maltaromaticum* UAL 307 and *L. mesenteroides* Y105. *E. faecalis* 710C supernatant had no activity against *C. jejuni*, *E. coli* or *Salmonella* spp.

[2] *A version of this chapter has been submitted for publication in the Journal of Agricultural and Food Chemistry.*

Table 4.1: Antimicrobial spectrum of *E. faecalis* 710C against a wide spectrum of indicator organisms.

Indicator strain	Result
Brochothrix campestris ATCC[1] 43754	+
Brevundimonas diminuta UFM[2]1	+
Campylobacter jejuni ATCC 700819	-
Carnobacterium divergens UAL9	+
Carnobacterium maltaromaticum UAL8A, UAL8B, UAL8C2, UAL26, UAL307, JG126	+
Clostridium botulinum spores and viable cells[3]	+
Clostridium butyricum ATCC 8260, viable cells	+
Clostridium difficile 3195, 76; viable cells	+
Clostridium perfringens CL5626, R783; viable cells	+
Clostridium sporogenes 25779, 7955; viable cells	+
Escherichia coli AW 1.7	-
Escherichia coli GGG[4]10	-
Enterococcus faecium BFE900 and VRE strains	+
Vancomycin resistant *Enterococcus faecium*[5] CL3745, E2155, E2217, E2352, M1008, S769, R493, R704, R846	+
Lactobacillus sakei DSM20017, 706	+
Leuconostoc gelidum UAL 187	+
Listeria innocua ATCC 33090	+
Listeria monocytogenes ATCC 15313, CDC7762, FS-15	+
Pediococcus acidilactici PAC 1.0	+
Salmonella enterica serovar Typhimurium 18	-
Staphylococcus aureus ATCC 23235	+
Methicillin resistant *Staphylococcus aureus* R468, R507, R667, R719, R776, R870, R948, R1230, R1262, R1578[5]	+

+: inhibition; - : no inhibition.

[1]American Type Culture Collection

[2] University of Alberta, Food Microbiology Laboratory Culture Collection

[3] Data provided by Melissa Haveroen. Proteolytic strains of *C. botulinum* used in this study were *C. botulinum* 368B, 2B, A6, and A62, and non-proteolytic strains were DB2, IIB ATCC 13983, and 17B

[4] Agriculture and Agri-Food Canada, Lacombe Research Station. Culture obtained from Dr. G. Gordon Greer.

[5] Provincial Laboratory for Public Health, Edmonton, AB. Data provided by Denise Carlson (CanBiocin Inc., AB)

4.1.2 Partial purification of bacteriocins by cation-exchange column and solid-phase extraction

Ent7A and ent7B were partially purified using cation-exchange chromatography from the supernatant of a culture of *E. faecalis* 710C grown in APT medium (Figure 4.1).

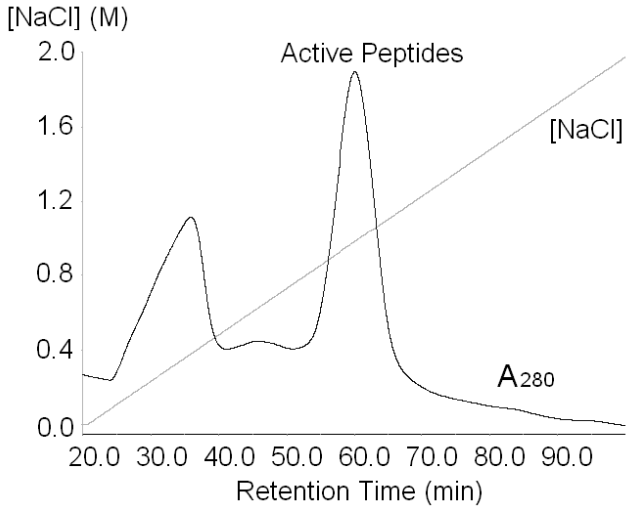


Figure 4.1: Cation-exchange chromatography for preliminary purification of ent7A and ent7B from the culture supernatant of *E. faecalis* 710C. Elution of peptides used a linear gradient up to 2 M NaCl and was monitored by a UV-detector at 280 nm. Activity of each fraction was tested against *L. sakei* DSM20017. All active peptides were eluted from 50.0-70.0 min.

The active peptide fraction was desalted on a C18 reverse-phase cartridge and subject to RP-HPLC purification.

4.1.3 Purification of bacteriocins by RP-HPLC

Ent7A and ent7B were further purified by RP-HPLC (Figure 4.2) using a linear gradient of 40% to 70% acetonitrile containing 0.1% TFA.

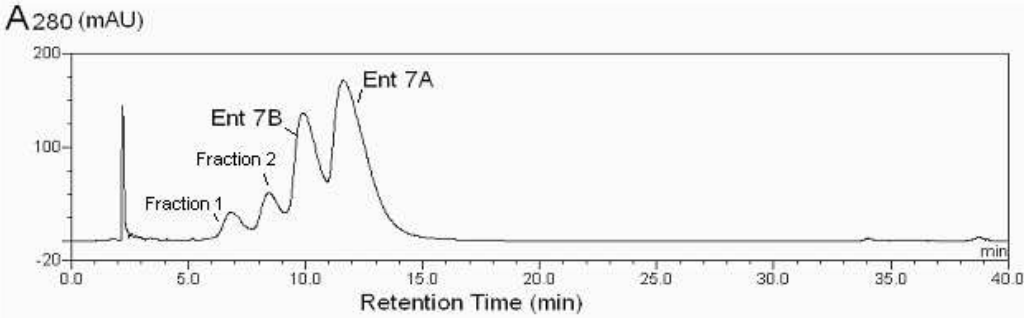


Figure 4.2: Separation of ent7A and ent7B by RP-HPLC. Elution of peptides was monitored by a UV-detector at 280 nm.

Ent7B and ent7A eluted with retention times of 9.9 and 11.6 min, respectively. Fraction 1 and fraction 2 indicated in Figure 4.2 are the oxidized forms of ent7B and ent7A, respectively (see 4.2.1).

4.1.4 Production and activity of purified ent7A and ent7B

Typically, one liter of culture yielded about 10 mg of ent7A and 8 mg of ent7B. The concentration of ent7A and ent7B that were needed to inhibit the indicator strain *L. sakei* DSM20017 on a spot-on-lawn assay were 4 µg/mL and 6 µg/mL, respectively. Ent7A and ent7B were not only active against gram-positive bacteria (*C. sporogenes* 25779, *L. monocytogenes* FS-15 and *S. aureus* 23235), but also against the gram-negative bacterium, *Brevundimonas diminuta* UFM1 (Figure 4.3). Ent7A was tested against all the spores and viable cells of *C. botulinum* in this study. Ent7A was active against the spores but not viable cells (tested by Melissa Haveroen). No synergistic effect between ent7A and ent7B was observed when *L. sakei* DSM20017 was used as the indicator organism.

In this study, both HPLC Fraction 1 (oxidized ent7B) and Fraction 2 (oxidized ent7A) were active against *L. sakei* DSM20017. However, the activity of oxidized ent7A/7B was only 25% of that observed for the non-oxidized ent7A/7B.

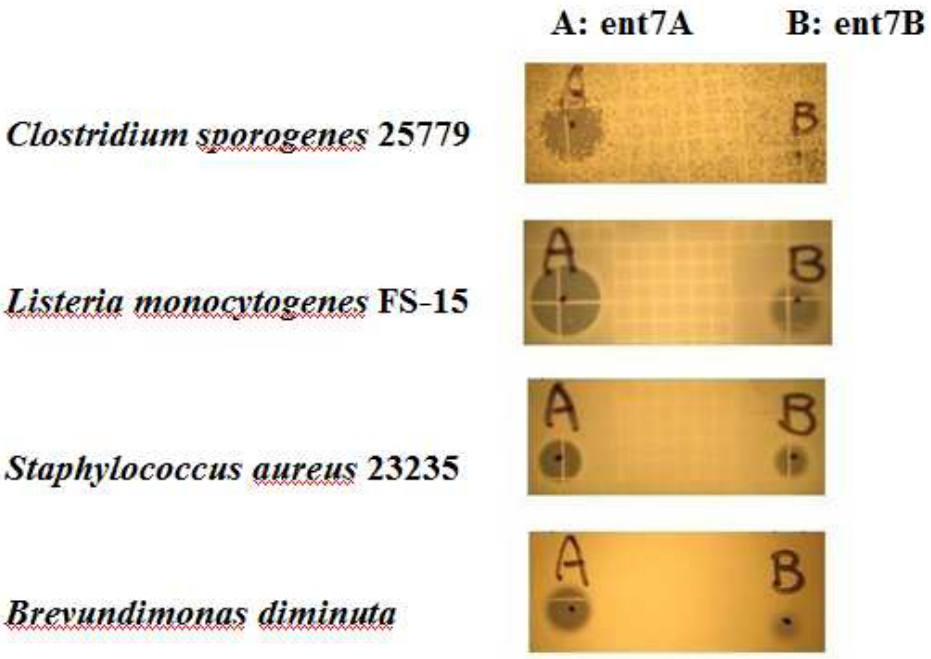


Figure 4.3: Antimicrobial effect of purified ent7A and ent7B on different indicator strains.

4.2 MS based analyses of bacteriocins

4.2.1 MALDI-TOF MS

MALDI-TOF spectra of ent7A/ ent7B showed single peaks at 5199.5 Da and 5205.3 Da, respectively (Figure 4.4a and b). The MALDI-TOF MS spectra were acquired in negative mode, hence the molecular weights are for the deprotonated forms ($[M - H]^-$) of ent7A and ent7B.

More accurate molecular weight determinations of ent7A and ent7B, as well as HPLC Fractions 1 and 2 (Figure 4.2) were done on a Q-TOF Premier Nano-ESI mass spectrometer. The molecular weight for the peaks observed were 5200.85 Da (ent7A, monoisotopic molecular ion), 5206.85 Da (ent7B, monoisotopic molecular ion), 5222.60 Da (Fraction 1, monoisotopic molecular ion), and 5216.60 Da (Fraction 2, monoisotopic molecular ion).

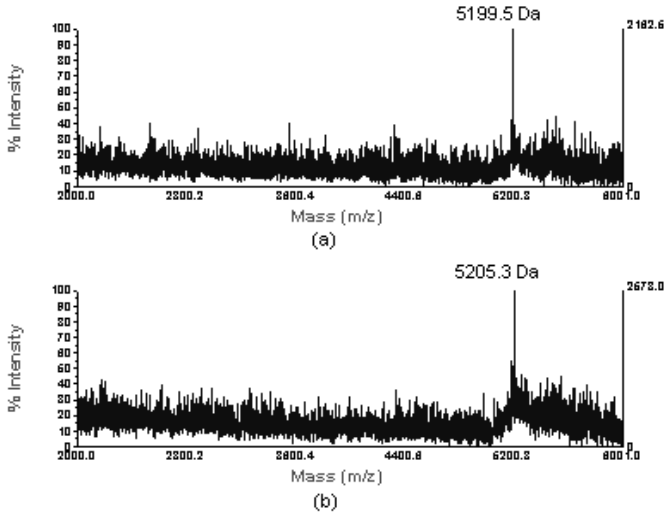


Figure 4.4: Negative-ion-mode MALDI-TOF MS spectrum of HPLC-purified [M – H]$^-$ ent7A. (a) [M – H]$^-$ ent7B (b).

4.2.2 ESI-MS/MS

4.2.2.1 Amino acid sequences of ent7A and ent7B

The molecular weights of ent7A and ent7B are close to the reported molecular weights of bacteriocin MR10A and MR10B (Martín-Platero et al., 2006). It was suspected that ent7A and ent7B might have similar amino acid sequences as bacteriocin MR10A and MR10B. To confirm this hypothesis, the MS/MS ion patterns of ent7A and ent7B were compared to the theoretical MS/MS ion patterns

of bacteriocin MR10A and MR10B. Comparison of MS/MS fragment peaks of ent7A with theoretical fragments from MR10A (Martín-Platero, 2006) showed that the observed y-ion series matched the theoretically predicted MR10A y-ion series from the y_2 to y_{43} ions (miss y_{42}). Similarly, MS/MS fragment peaks of ent7B with theoretical fragments from MR10B (Martín-Platero, 2006) showed that the observed y-ion series matched the theoretically predicted MR10B y-ion series from the y_2 to y_{43} ions (miss y_{29}, y_{40}- y_{43}). This suggested that ent7A and MR10A, ent7B and MR10B, may have identical amino acid sequences.

4.2.2.2 N-terminal formylation of ent7A and ent7B

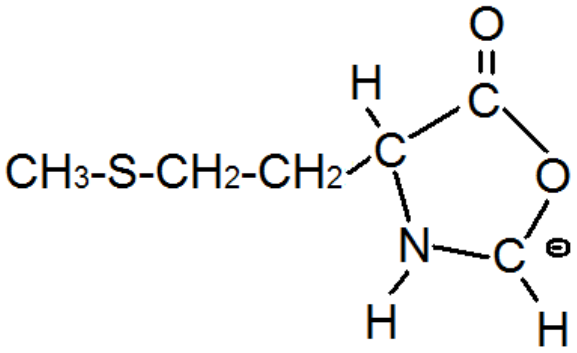
The b-ion series of ent7A was compared to those of MR10A. Every ent7A b-ion observed (from b_1 to b_{43}, b_{14} was missing) was 28 Da higher than the corresponding theoretical MR10A b-ions. This suggests a chemical modification on the N-terminal amino acid residue of ent7A, resulting an increase of 28 Da on b_1 ion [Figure 4.5 (a)]. Chemical modification with a functional group of 28 Da in mass can be either dimethylation (addition of 2 x CH_2) or formylation [Figure 4.5 (b), DeltaMass, http://www.abrf.org/index.cfm/dm.home]. Dimethylation and formylation can be differentiated, since the mass of a dimethyl group is 0.036 Da higher than that of a formyl group.

The masses of b_1-b_4 ions of ent7A were compared to theoretical masses of b_1-b_4 ions of MR10A (Table 4.2). The b_1 ion of ent7A is 160.043 ± 0.003 Da,

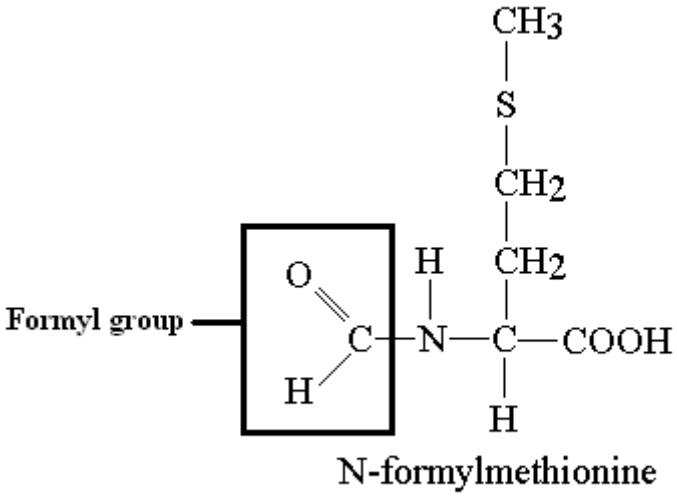
compare to 160.043 Da (formylated) and 160.079 Da (dimethylated). The Q-TOF instrument was pre-calibrated for an accuracy of 10 to 20 ppm for masses of 100-500 Da. It was clear that the chemical modification on ent7A b_1 ion (N-terminal amino acid residue) is formylation. The same analyses were performed on ent7B. Results revealed that ent7B has the same sequence as MR10B with a corresponding formylated Met on the N-terminus.

Table 4.2: Experimental molecular weights of b1 to b4 ion series of ent7A determined by nano-ESI -MS/MS and theoretical molecular weights of the formylated or dimethylated MR10A.

Sample		Ion Series (Da)			
		b1	b2	b3	b4
Experimental	ent7A	160.043 ± 0.003	217.062 ± 0.004	288.098 ± 0.006	401.178 ± 0.008
Theoretical	Met1 formylated MR10A	160.043	217.065	288.102	401.186
Theoretical	Met1 dimethylated MR10A	160.079	217.101	288.138	401.222



(a)



(b)

Figure 4.5: (a) The theoretical methionine b_1 ion structure. (b) Formyl-methionine structure.

N-terminal formylation of ent7A and ent7B was further confirmed by accurate mass measurements of the entire peptides. The instrument, Bruker 9.4T Apex-Qe FTICR was internally calibrated with bovine insulin to an accuracy of 1 ppm. The theoretical m/z value of N-terminal formylated ent7A ($[M+6H]^{6+}$) is 867.82405 Da and analysis revealed a mass of 867.823 Da, a difference of 0.88 ppm. Similarly, the theoretical m/z value of N-terminal formylated ent7B ($[M+7H]^{7+}$) is 744.83993 Da and analysis revealed a mass of 774.839 Da, a difference of 0.78 ppm. The resulting empirical formula calculated for ent7A ($C_{252}H_{390}N_{60}O_{55}S_2$) and ent7B ($C_{251}H_{380}N_{62}O_{55}S_2$) match with those of formylated MR10A and MR10B, respectively.

4.2.2.3 Oxidation of ent7A and ent7B

The molecular weights of 5222.60 Da (HPLC Fraction 1, monoisotopic molecular ion) and 5216.60 Da (Fraction 2, monoisotopic molecular ion) differ from those of 5206.85 Da (ent7B, monoisotopic molecular ion) and 5200.85 Da (ent7A, monoisotopic molecular ion) by 16 Da, respectively. MS/MS analysis was performed on the sample peaks of 5222.60 Da and 5216.60 Da to determine whether they were chemically modified ent7A and ent7B. The y-ion series of the 5222.60 Da peak matched with those of ent7B from y_1-y_{39} (y_{40}-y_{43} not observed), indicating that: 1) The compound with a mass of 5222.60 Da has the same amino

acid sequence as ent7B; and 2) chemical modification did not occur on the C-terminus of ent7B. Each of the b_1-b_{38} ions of the 5222.60 Da peak was 15.99 Da (atomic weight of oxygen) more than the corresponding b-ion series of ent7B (b_{39}-b_{43} not observed), indicating that Fraction 1 contained the mono-oxidized form of ent7B, and that the site of oxidation was the N-terminal methionine (which was also formylated). The same analysis was performed on the 5216.60 Da sample, indicating that Fraction 2 contained ent7A with an oxidized N-terminal formylated Met.

4.3 Sequencing the bacteriocin structural and immunity genes

The structural gene (*ent7*) for ent7A and ent7B [nucleotide (nt) 1-275, coding for the total 44 amino acid residues in ent7A and the first 40 amino acid residues out of 43 in ent7B] was amplified by *ent* 7F forward primer and *ent* L50B reverse primer (Figure 4.6). The PCR product was purified and ligated into pGEM®-T vectors, which were cloned into *E. coli* DH5α cells by electroporation. Positive clones were screened and the SP6 to T7 region of pGEM®-T with insert was amplified yielding a PCR product with size of approximately 400 bps (Figure 4.7). The PCR product was sequenced. The *ent7* nt 1-275 matched 100% with *mr10* gene (287 nts) from position 1-275 (Figure 4.8).

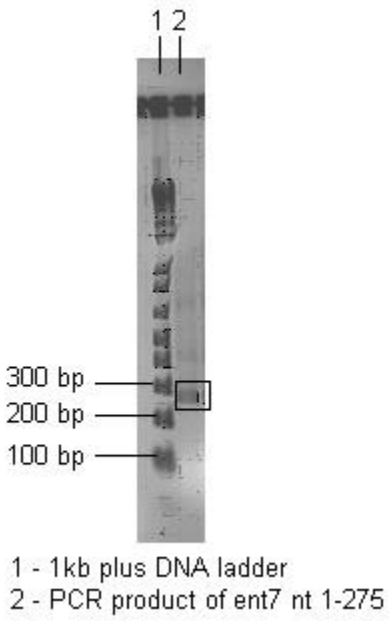


Figure 4.6: Amplification of *ent7* nt 1-275. The ent 7F forward primer and ent L50B reverse primer were used for PCR. The PCR product was subjected to electrophoresis using 2% agarose gel in TBE buffer. Box in lane 2 indicates the PCR product.

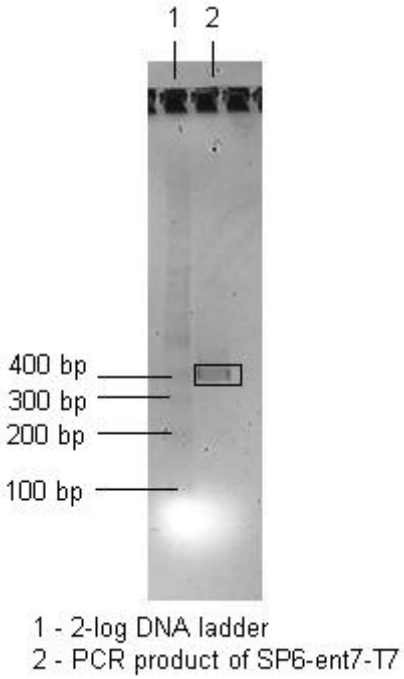


Figure 4.7: Amplification of SP6 and T7 region of pGEM®-T with insert ent7 nt 1-275. The SP6 and T7 primers were used for PCR. The PCR product was subjected to electrophoresis using a 2% agarose gel in TBE buffer. Box in lane 2 indicates the PCR product.

```
ent7  ATGGGAGCAATCGCAAAATTAGTAGCAAAGTTTGGATGGCCAATTGTTAAAAAGTATTAC  60
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mrl0  ATGGGAGCAATCGCAAAATTAGTAGCAAAGTTTGGATGGCCAATTGTTAAAAAGTATTAC  60

ent7  AAACAAATTATGCAATTTATTGGAGAAGGATGGGCAATTAACAAAATTATTGATTGGATC  120
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mrl0  AAACAAATTATGCAATTTATTGGAGAAGGATGGGCAATTAACAAAATTATTGATTGGATC  120

ent7  AAAAAACATATTTAAAAATAAGGATGTGTTAATGTATGGGAGCAATCGCAAAATTAGTAG  180
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mrl0  AAAAAACATATTTAAAAATAAGGATGTGTTAATGTATGGGAGCAATCGCAAAATTAGTAG  180

ent7  CAAAGTTTGGATGGCCATTTATTAAAAAATTCTACAAACAAATTATGCAGTTTATCGGAC  240
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
mrl0  CAAAGTTTGGATGGCCATTTATTAAAAAATTCTACAAACAAATTATGCAGTTTATCGGAC  240

ent7  AAGGATGGACAATAGATCAAATTGAAAAATGGCTA  275
      |||||||||||||||||||||||||||||||||||
mrl0  AAGGATGGACAATAGATCAAATTGAAAAATGGCTA  275
```

Figure 4.8: Nucleotide sequence alignment of *ent7* and *mr10* from nt 1-275.

The immunity gene of ent7A and 7B (*ent7Imm*) was amplified using primers from up- and downstream of the immunity gene. The PCR product (961 bps, Figure 4.9) was observed on a 2% agarose gel following electrophoresis. The sequence of the immunity gene (543 bps; data not shown) of ent7A and 7B matched 100% with that of ent NA and NB (GenBank: AB292312.2).

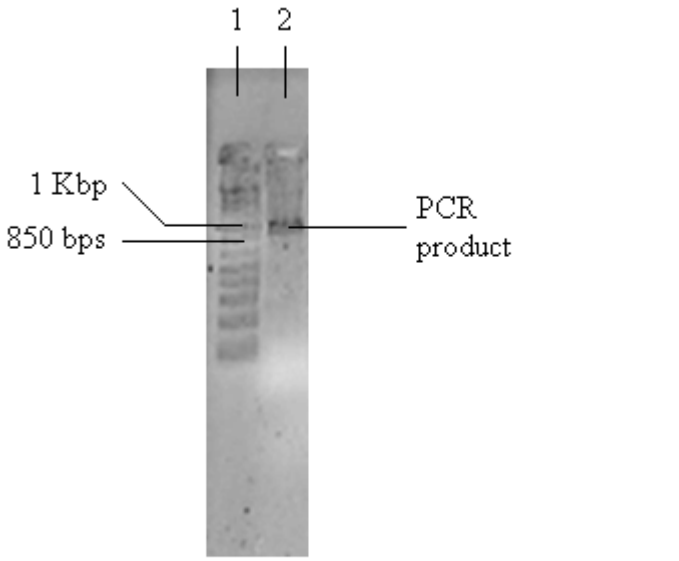


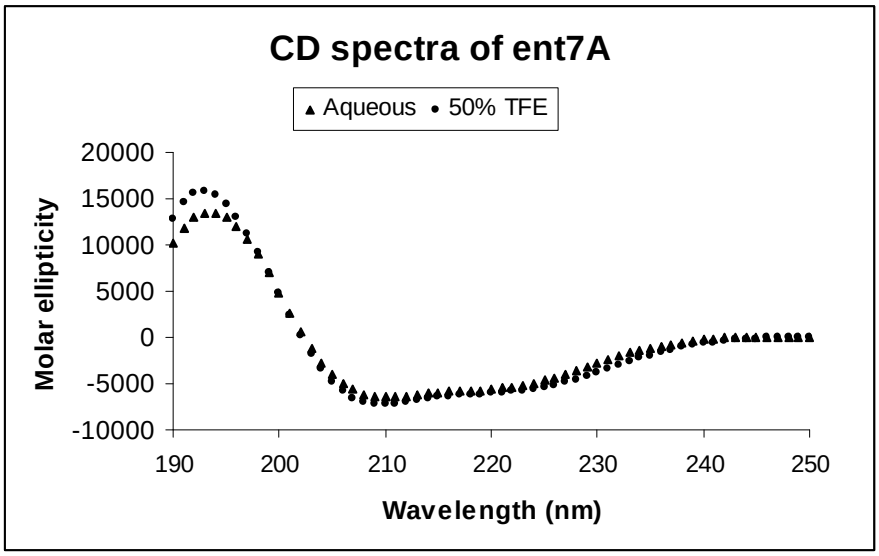
Figure 4.9: Amplification of *ent7Imm*. The 710CImF and 710CIMR primers were used for PCR. The PCR product was subjected to electrophoresis using 2% agarose gel in TBE buffer.

4.4 Circular dichroism

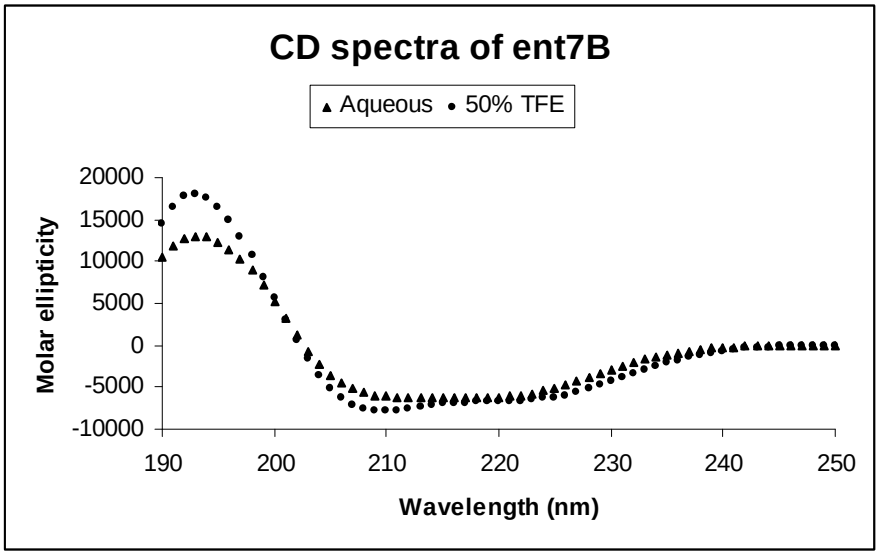
CD spectroscopy was performed to explore the structural characteristics of ent7A and ent7B. Peptides were prepared in both aqueous and more hydrophobic (upon addition of TFE) solutions. CD spectra of ent7A [Figure 4.10 (a)] and ent7B [Figure 4.10 (b)] were obtained.

Under aqueous conditions, CD results indicated that both ent7A and ent7B have defined structures and are largely α-helical (approximately 21% and 23%,

respectively). Upon the addition of TFE, a membrane-mimicking solvent (Kaur et al., 2004), the α-helical content of ent7A and ent7B was slightly enhanced (up to 23% and 25%, respectively).



(a)



(b)

Figure 4.10: CD spectra of ent7A (a) and ent7B (b). Ent7A and ent7B were dissolved in 20 mM sodium phosphate buffer and 50% trifluoroethanol (TFE) and 50% of the same buffer at 20°C.

4.5 Chiral GC-MS

Initial assessment was performed to ensure that L- amino acid derivatives could be separated from their corresponding D- enantiomers by the chiral column. The L- and D- amino acids were mixed and co-injected onto the instrument. The D- amino acid derivatives were eluted before their corresponding L- amino acid derivatives. All L- and D- amino acid derivatives were separated except for Pro. His, Arg and Trp were not observed on the chromatogram, indicating that they could not be eluted from the column.

The hydrolyzed and derivatized ent7A or ent7B was co-injected onto the GC-MS instrument with L- amino acid standards. There was no broadening or splitting of peaks from the L- amino acid standard, suggesting that the amino acid residues in ent7A and ent7B were in L- configuration except for Pro, His, Arg and Trp, which were not resolved. This was confirmed by co-injecting ent7A or ent7B with D- amino acid standard. The peaks from ent7A or ent7B amino acid residues separated from those of the standard.

The Asn/ Asp (derivatized into the same compound) could not be fully resolved. However, peak broadening was observed when L- and D- Asn/Asp were mixed, whereas this was not seen in analyte mixture comprised of L- amino acid standard and ent7A/7B. This suggests that the Asn/Asp in both ent7A and ent7B are all L- form.

In this analysis, 13 out of 18 amino acids (Ala, Glu, Gln, Ile, Leu, Lys, Thr, Tyr, Val, Met, Asn, Asp and Phe) were identified to be L- amino acids (Gly does not have enantiomer). The configurations of Pro, Arg, His and Trp residues could not be resolved. Both L- and D- Pro were coeluted, thus no conclusions regarding the form of Pro in the peptides can be made. Peaks from Arg, His and Trp were not observed on chromatogram. In literature on chiral analysis of peptides using Chirasil-Val column (20 m), these amino acid can elute from the column in a temperature program from 80°C to 190°C, 3°C /min using Hydrogen gas (Allenmark and Schurig, 1997). To assess whether this change in temperature program aided the elution of Arg, His and Trp, L- amino acids were analyzed using this temperature program but no elution of these amino acids was observed.

5. DISCUSSION

Lactic acid bacteria have played an important role in food industry for many years. They not only participate in food fermentation, but also protect food from spoilage. One of the protective effects comes from the bacteriocins that LAB produce and their activity against foodborne pathogens and spoilage organisms.

Enterococci are a group of LAB, many of which produce bacteriocins (enterocins) that have a broad antimicrobial spectrum against foodborne pathogens such as *Listeria*, *Clostridium* spp. and *Staphylococcus aureus* (Franz et al., 2007). Enterococci have been participated in the food industry for fermentation; however, there are concerns regarding the use of these organisms in ready-to-eat food, as many of the *Enterococcus* spp. are associated with virulence factors for human diseases including gelatinase, adhesion to collagen, aggregation substance and endocarditis antigen (Franz, C. M. A. P., personal communication). In light of this, it may be more promising to apply the purified enterocins to foods rather than using a live culture of *Enterococcus* spp. With this in mind, purification of enterocins becomes important.

This study proposed a method for purifying enterocins using chromatographic columns (cation-exchange and RP-HPLC). The enterocin-producing strain used was *E. faecalis* 710C, which secretes ent7A and ent7B. The purity of ent7A and 7B was examined through MALDI-TOF MS. The MS spectra of Ent7A and

Ent7B showed a single peak, at 5199.5 Da and 5205.3 Da, respectively, indicating that the enterocins had been purified to homogeneity.

For further study of ent7A and ent7B, chemical and molecular biology techniques were employed. The amino acid sequences of ent7A and 7B were determined via ESI-MS/MS and genetic sequencing. The Leu and Ile residues of ent7A and ent7B were confirmed by sequencing the ent7 gene from nucleotide position 1-275, which codes for ent7A and by sequencing part of the ent7B gene that has Leu and Ile residues.

During the ESI-MS/MS study, it was observed that the mass of b_1 ions (i.e. for the N-terminal residue) from both ent7A and 7B was 28 Da higher than that of the theoretical values. High-resolution MS data confirmed that this 28 Da increase in b_1 ion mass in both ent7A and ent7B were a result of formylation. A b_1 ion is not usually observed for most peptides because its formation would require the presence of a carbonyl group from the non-existent N-1 amino acid (Schlosser and Lehmann, 2000). However, if an acetyl or formyl group is present on the amino group at the N-terminus, a b_1 ion can be observed, as the carbonyl is available to form the cyclic oxazolone structure of a b-ion. In contrast, dimethylation on Met could not lead to formation of this type of stable structure. Although the formylated b_1 ion peak of ent7A/B is weak, its presence can be confirmed and distinguished from an N,N-dimethyl modification by the differences in exact mass and isotope patterns for two CH_2 groups vs. a CO group.

In most eukaryotes and prokaryotes, N-formylmethionine (fMet) is the first amino acid in a nascent peptide chain, because fMet is coded by the start codon (AUG). The fMet in eukaryotes is usually cleaved. The formyl group of fMet is removed or fMet is cleaved as a whole. In prokaryotes, fMet can be either cleaved or retained in the peptide.

As reviewed by Giglione et al. in 2004, the fMet in bacteria is generally processed co-translationally through N-terminal Met Excision (NME), during which the formyl group of fMet is removed by peptide deformylase (PDF), followed by the cleavage of Met by methionine aminopeptidase (MAP). NME requires the N-terminus of peptide to be exposed to PDF and MAP. When the N-terminus is buried inside of the peptide, or it is inserted into the membrane, or is sterically hindered, PDF/MAP cannot excise fMet (Dong et al., 1996).

Other N-formylated bacteriocins have been discovered, for example, enterocin L50A/B from *Enterococcus faecium* L50 (Cintas et al., 1998), enterocin A5-11A/B from *Enterococcus durans* (Batdorj et al., 2006), enterocin F-58A/B from *Enterococcus faecium* F58 (Achemchem et al., 2005), lacticin Q from *Lactococcus lactis* QU 5 (Fujita et al., 2007) and possibly enterocin Q from *Enterococcus faecium* L50 (Cintas et al., 1998). Very recently, enterocins L50A and L50B from *Enterococcus faecium* IT62, which have high sequence similarity (ca. 90%) with ent7A/MR10A and ent7B/MR10B, were shown to be formylated on the N-terminal Met (Izquierdo et al., 2008). Both MR10A and MR10B from *E.*

faecalis MRR10-3 are in the SwissProt Public protein database (UniProtKB/TrEMBL entry Q1A2D3 for MR10A and Q1A2D2 for MR10B). Their amino acid sequences were deduced from the structural genes that encode MR10A and MR10B (Martín-Platero et al., 2006). The authors observed a 28 Da difference between the masses based on genetic sequence and those seen by mass spectrometry, but these were "ascribed to oxidation in the methionine residues or other alterations in the molecules produced during the purification process." However, oxidation of methionine would give only a 16 Da increase in mass. In our study, we observed oxidized ent7A and ent7B, but they eluted in different HPLC fractions from ent7A/ent7B and their N-terminal Met is both oxidized and formylated. Oxidation and formylation of Met were result from distinct mechanisms. Oxidation of Met is mainly due to oxidative stress of the peptide, whereas formylation can a result from failure to deformylate the peptide. The first 10 residues of MR10A and MR10B were successfully sequenced by Edman degradation, which implied that the N-terminal amino group was not modified. However, it should be noted that N-formyl groups are easily removed by mild aqueous acid treatment in contrast to most other N-acyl groups (Dong et al., 1996; Sheehan et al., 1958). Because such conditions are frequently used during HPLC purification of peptides (e.g. TFA/water/CH_3CN), partial deformylation to liberate the N-terminus as a free amino group can occur after collection of peptide-containing fractions if they are permitted to stand in the solvent even for modest periods of time. When ent7A and ent7B were exposed to 3 N HCl at room

temperature for 5 days, a 28 Da decrease in the mass of ent7A and ent7B was observed in the MALDI-TOF MS spectra. Hence, we propose that MR10A and MR10B from *E. faecalis* MRR 10-3 are also formylated at the N-terminal amino group of methionine.

A similar situation exists with the closely homologous enterocins L50A and L50B, which were recently found to be N-formylated and in part oxidized at methionine sulfur when isolated from *E. faecium* IT62 (Izquierdo et al., 2008). When these bacteriocins were initially isolated from another strain, *E. faecium* L50, only a single bacteriocin ("pediocin L50") with its N-terminus blocked for Edman sequencing and having a mass of 5250 Da was reported (Cintas et al., 1995). Subsequent revision by the authors reported the currently accepted sequences of enterocins L50A and L50B, but without mass spectral data and with the statement that the N-termini were blocked, possibly by an N-formyl group on the methionine (Cintas et al., 1998). Interestingly, the addition of two oxygen atoms (one on each of methionine sulfur) as well as an N-formyl group to the sequence of L50A would give the mass of 5250 that was initially reported, and in accordance with the modifications recently seen in a different strain by Izquierdo et al. in 2008. Enterocins 62-6A and 62-6B from *E. faecium* 62-6 have been reported to be N-formylated at their terminal methionine and to have the identical amino acid sequence as L50A and L50B (the region of their plasmid-encoded operon containing the structural genes has three silent nucleotide mutations)

(DeZwaan et al., 2007). Oxidation of methionine to a sulfoxide was also observed. In this regard, enterocin Q, a 34 amino acid bacteriocin with an unrelated sequence isolated from the same strain as L50A and L50B, was reported to have a cysteine disulfide as well as two oxidized methionines (Cintas et al., 2000). However, the predicted mass difference for such modification would be 30 Da, whereas the observed difference was 28 Da (equivalent to an N-formyl group). Enterocin Q was amenable to Edman sequencing, initially indicating no block at the N-terminus, but its prior purification was done under acidic aqueous conditions. As indicated above, this can lead to loss of the N-formyl group from a portion of the peptide to give a free amino terminus. Because Edman sequencing is very sensitive, and a small fraction of deformylated species in mostly N-formylated peptide, would not be seen by mass spectrometry, we propose that enterocin Q might be N-formylated.

An fMet appeared to be present in ent7A, ent7B, enterocin L50A/B, enterocin F-58A/B, enterocin Q and lacticin Q. It has been suggested that bacteriocins that lack a leader peptide are expected to have fMet at their N-terminus (Fujita et al., 2007). The presence of formyl group on the N-terminal methionine of ent7A and 7B indicated that the secretion of ent7A and ent7B could be through a mechanism that does not require the recognition of the leader peptide.

During the aerobic growth of *E. faecalis* 710C, Met was prone to oxidation by active oxygen species (AOS) into methionine sulfoxide (Grimaund et al., 2001).

This oxidation process was reversible because methionine sulfoxide reductase (Msr), an enzyme present in all living organisms (Brot et al., 1981; Brot et al., 2000), can reduce methionine sulfoxide to methionine (Grimaund et al., 2001). Oxidation of Met can also occur following exposure of ent7A and ent7B to environmental oxidizing agents (Stadtman, 1992), such as ozone (Berlett et al., 1991).

Addition of oxygen to ent7A/ent7B decreases their hydrophobicity. Therefore, oxidized ent7A/ent7B eluted with a lower concentration of acetonitrile than ent7A/ent7B during the RP-HPLC purification procedure.

In the current study, the secondary structures of ent7A and 7B were examined. CD data revealed that both ent7A and ent7B have over 20% of structured (alpha-helical) region in aqueous solution, whereas most Class II bacteriocins have random coils in aqueous solution except for bacteriocins like carnocyclin A from *Carnobacterium maltaromaticum* UAL307 (Martin-Visscher et al., 2008). The alpha-helical content of both ent7A and 7B did not increase significantly upon addition of TFE. Since the mechanism by which TFE can induce an alpha-helical structure in a protein/peptide remains an unknown, the reason why the alpha-helical content of ent7A and 7B did not increase significantly upon addition of TFE is yet unknown.

The configuration of amino acid residues of ent7A and ent7B was screened using chiral GC-MS. The configuration of most amino acid residues were

resolved except for Pro, His, Arg and Trp. This could be due to the upper temperature limit of the column. These amino acids do not readily elute at low temperatures. While the temperature limit of the column is reported to be 230ºC, column bleed is excessive above 180ºC such that compound detection is very difficult as they must be present above the level of background coming from the column. As a result, detection of Pro, His, Arg and Trp, which elute above 180ºC, is difficult.

Although chiral GC analysis is efficient for L-/D- screening of amino acids, in this study, it was found that chiral GC analysis has a number of limitations: 1) it is not efficient for screening Arg, His and Trp, as these amino acids have difficulty passing through the column; 2) a peak corresponding to a D- amino acid does not give information on which amino acid is D- form. For example, if a peptide has 4 alanines and only one is in the D-form, and a D-Ala peak is observed, it is impossible to determine which Ala is in D- form. In this situation, additional experiments must be performed; if the amino acid sequence of the peptide is known, specific peptidyl enzymes such as trypsin can be selected to digest the peptide, so that the four alanines can be separated onto separate fragments. These fragments can be screened separately using chiral GC to find out which fragment has a D-Ala. Alternatively, enzymes that specifically recognize D-Ala and its adjacent amino acid residue can be employed to locate D-ala in this peptide, 3) chiral GC columns are generally more susceptible to column bleed than other

types of GC columns due to the manufacturing process. When the oven temperature rises above 160°C, column bleed becomes obvious and a rise in the baseline of the chromatogram baseline and peaks from the stationary phase material, can suppress signals from analytes and causes difficulty in interpretation of the chromatogram, 4) chiral GC columns are very sensitive to air leaks, which generally prevents the use of a guard column. Even subtle installation imperfections in the junction between a guard column and a chiral GC column can cause complete dysfunction of the chiral column. If compounds that cannot be eluted enter into the column, the only way to clean the column is to trim off the part of column where these compounds are trapped and 5) Columns are costly and when columnsare taken out of the GC instrument for storage, the open end must be sealed to prevent impurities from entering.

Purified ent7A and ent7B were active against common gram-positive food pathogens. Ent7A was tested against spores and viable cells of *C. botulinum*, and was active against both spores and viable cells. Ent7A and 7B were against gram-negative *B. diminuta*, which has been suggested to cause infections in immune-suppressed population, such as cancer patients (Han and Andrade, 2005). Neither ent7A nor ent7B was not active against *S.* Typhimurium or *E. coli*. Most bacteriocins from gram-positive bacteria are effective against gram-positive bacteria and only a few have been shown to be effective against gram-negative organisms (Jack et al., 1995). One explanation for this observation is that gram-

negative bacteria have an outer membrane that prevents the entry of most bacteriocins into the cell. In addition, *S.* Typhimurium 18 has a deformylase, which removes the formyl group on the N-terminal Met of ent7A and ent7B, so that peptidase M, which can target peptides beginning with Met followed by Gly (Miller et al., 1987), can degrade both enterocins.

This study has confirmed the amino acid sequence of ent7A and 7B from *E. faecalis* 710C. The configuration of the majority of amino acid residues from these two bacteriocins has been determined. Both ent7A and 7B have 20-25% of defined structures (alpha-helix) in aqueous and 50% TFE solution. The above information leads to further study on ent7A and 7B include: 1) complete sequence of *ent7* structural gene; 2) secretion mechanism of ent7A and ent7B; 3) the mechanism of ent7 activity and how it is related to the partial or overall structure of ent7A and ent7B molecules.

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