CHARACTERIZATION OF THE IMMUNITY FACTOR IN PRODUCER SELF PROTECTION AGAINST LEUCOCIN A

By

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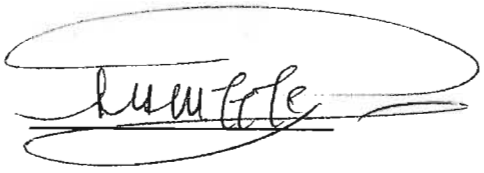
PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of Kwa-Zulu Natal, Pietermaritzburg, from January 2005 to November 2006, under the supervision of Dr Mervyn Beukes.

These studies represent the original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is dully acknowledged in the text.



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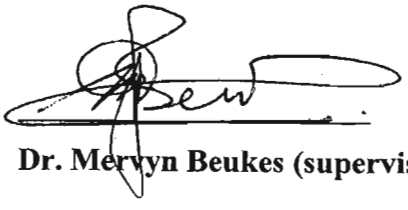

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ABSTRACT

Lactic acid bacteria produce pediocin-like bacteriocins designated as Class IIa. These antimicrobial peptides are antagonistic against *Listeria monocytogenes* and other closely related Gram-positive bacteria. Self-protection of the producer organism is attributed to the immunity proteins, encoded by genes that are co-transcribed with the structural gene that encode the bacteriocin. The lactic acid bacterium, *Leuconostoc gelidum* UAL 187-22 is immune to its own bacteriocin, leucocin A. This is accredited to its immunity protein and the possible absence of a receptor on its cytoplasmic membrane. Leucocin A was purified from the supernatant of *L. gelidum* to 90% purity by ion-exhange chromatography and C18 reverse phase High Pressure Liquid Chromatography (RP-HPLC) eluted with an acetonitrile, 0.1% Triflouroacetic acid (TFA) gradient. The immunity gene was isolated from the same producer using the polymerase chain reaction from the recombinant plasmid pJF 5.5 using primers EAL-2 and EAL-3. The amplicon was truncated into versions A and B by removing the C- and N-terminals, with *Hae*III and *Cla*I restriction enzymes, respectively. The amplicon and the truncated fragments A and B were cloned into pMAL-c2 to construct recombinant plasmids pKP1, pKP1A and pKP1B, correspondingly, which were transformed into *Escherichia coli (E. coli)* strain JM103. Clones were confirmed by colony PCR and Southern blot hybridization. The recombinant clones were subsequently expressed as MBP-IP, MBP-IPA and MBP-IPB fusion proteins that were verified by Western blot using the anti-MBP antibody. Factor Xa protease was used to cleave MBP from the proteins of interest. The resulting pure immunity protein versions had an approximate molecular weight of slightly more that 10 kDa. The binding interactions of the purified immunity protein constructs and leucocin A were compared on the Biacore 2000 instrument with surface plasmon resonance. None of the immunity constructs interacted with leucocin A, however, the N-terminal region of the immunity protein interacted with the cytoplasmic extract.

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LIST OF ABBREVIATIONS

APS	- Ammonium persulphate
ATP	- Adenosine 5'-triphosphate
ATZ	- Anilinothiazolinone
Bp	- Base pairs
BCA	- Bicinchoninic acid
BSA	- Bovine serum albumin
CAPS	- 3-[cyclohexylamino]-1-propansulfonic acid
CE	- Capillary electrophoresis
DNA	- Deoxyribonucleic acid
DTT	- Dithiothreitol
E. coli	- *Escherichia coli*
EDTA	- Ethylene diamine trifluro acetate
GC	- Gas chromatography
HBS	- 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM Na_2EDTA
HEPES	- 4-(2-hydioxyethyl)-1-peperazineethanesulfonic acid)
HK	- Histidine kinase
HPLC	- High pressure liquid chromatography
HRPO	- Horseradish peroxidase
IF	- Pre-induction peptide
IGVA	- Immunity gene version A
IGVB	- Immunity gene version B
IPTG	- Isopropyl-β-D-thiogalacto-pyranoside
kDa	- Kilo-Daltons
KOH	- Potassium hydroxide
LAB	- Lactic acid bacteria
Lan	- Lanthionine
LB medium	- Luria Bertani
L. gelidum	- *Leoconostoc gelidum*
L. monocytogenes	- *Listeria monocytogenes*
MA	- Micro BCA reagent A
MB	- Micro BCA reagent B

MBP	- Maltose binding protein
MBP-IP	- Maltose binding protein with immunity protein
MBP-IPA	- Maltose binding protein with version A immunity protein
MBP-IPB	- Maltose binding protein with version B immunity protein
MC	- Micro BCA reagent C
MS	- Mass spectrometry
MW	- Molecular weight
MeLan	- α-methyllanthionine
MRS	- De Man Rogosa Sharpe
NMR	- Nuclear magnetic resonance
ORF	- Open reading frame
PBS	- Phosphate buffered saline
Ppm	- Parts per million
PPPS	- Precision plus protein standards
PCR	- Polymerase chain reaction
PIPES	- 1,4-piperazinediethanesulfonic acid
PITC	- Phenylisothiocyanate
PG	- Phsphatidylglycerol
PMF	- Proton motive force
POD	- Peroxidase
PTC	- Phenylthiocarbamyl
PTH	- Phenylthiohydantoin
PTS	- Phosphotransferase system
PVDF	- Polyvinylidene difluoride
RP	- Reversed phase
RR	- Response regulator
RU	- Resonance units
SDS-PAGE	- Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	- Surface plasmon resonance
TAE	- Tris acetate EDTA
TE	- Tris EDTA
TFA	- Trifloroacetic acid
TOF	- Time-of-flight
TSA	- Tryptone soy agar

TSB	- Tryptone soy broth
X-gal	- 5 Bromo-4 chloro-3 indolyl-β-D-galactopyranoside

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CHAPTER ONE

LITERATURE REVIEW

1.1 BACTERIOCINS

1.1.1 Introduction to bacteriocins

Several pathogenic organisms give rise to outbreaks of food poisoning. Such organisms include salmonella, *E. coli* O157, Norwalk-like viruses and listeria. Consequently the biopreservative programmes are focussing on developing new approaches to inhibit bacterial pathogens associated with the outbreaks (Muriana and Klaenhammer, 1991; Leer *et al.*, 1995). Hence a growing interest is currently directed towards Gram-positive Lactic acid bacteria (LAB) represented by specific species of the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, *Bifidobacterium* and *Carnobacterium* that prevent spoilage by pathogenic microorganisms through acidification, competition for essential nutrients and the production of antimicrobial peptides, the so-called bacteriocins (McKay and Boldvin, 1990; Stiles, 1996; Eijsink *et al.*, 2002; Chen and Hoover, 2003).

Bacteriocins are produced by bacterial species and are defined as a heat stable heterogeneous group of extracellular bioactive peptides. These peptides are generally bacteriocidal against other bacterial species that are closely related to the producer strain, however, not the producer (Jacob *et al.*, 1953; Tagg *et al.*, 1976; Martinez-Cuesta *et al.*, 2000; Nes *et al.*, 2002). In a few cases, the bacteriocins have been observed to be active against more distant species (Patanker, 1985; Stevens *et al.*, 1991; Klaenhammer, 1993; Jack *et al.*, 1995; Cleverland *et al.*, 2001). These peptides usually target the envelope of bacterial cells by non-enzymatic mechanisms, in the process they disrupt the integrity of the cell membrane and/or inhibit cell wall synthesis (Twomey *et al.*, 2002). A variety of these bacteriocins have been identified, characterized and classified genetically and biochemically.

An insight has been gained in current biological studies of microbes, that is, utilizing bacteriocins to control detrimental microorganisms not only in the environment but also in food preservation for which antibiotics are prohibited. In order to fully utilize and trust bacteriocins, understanding the action mode of the peptides is essential, that is,

understanding the interaction mechanism between the bacteriocins and targeted cells and also the mechanism of self-protection from self-produced bacteriocins (Nissen-Meyer and Nes, 1997; Fimland, 2002b; Ennahar *et al.*, 2000b).

Gratia discovered the first bacteriocins in 1925 from *E. coli* a gram-negative bacteria strain "colicin V", which was active against another culture of *E.* coli (Waters and Crosa, 1991). Initially bacteriocins were termed bacteriocidal proteins due to their lethal biosynthesis, their narrow activity spectrum and their adsorption to specific cell envelope receptors (Jacob *et al.,* 1953; Chen and Hoover, 2003). The definition has since been modified to incorporate the properties of bacteriocins produced by gram-positive bacteria, which commonly do not possess a specific receptor for adsorption although exceptions exist (Tagg *et al.,* 1976; Gravesen *et al.,* 2002). To date bacteriocidal peptides produced by LAB are typically referred to as bacteriocins not antibiotics despite their bacteriocidal activity given the proteinaceous nature of newly characterized bacteriocins and their sensitivity to proteolytic enzymes (Axelsson and Holck, 1995; Twomey *et al.*, 2002; Johnsen *et al.*, 2004). These peptides are preferred to current therapeutic non-peptide antibiotics that can potentially illicit allergic reactions, for instance penicillin (Cleveland *et al.,* 2001).

The present study focuses on LAB bacteriocins most of which are cationic, hydrophobic or amphiphilic molecules composed of 20 to 60 amino acid molecules (Nilsen *et al.*, 1998; Chen and Hoover, 2003; Mathiesen *et al.*, 2005). The bacteriocins were classified into five classes by Klaenhammer (1993), the classification was based on their structural composition, mode of action, mechanism of export and inhibitory spectrum. However, classification may change as new bacteriocins are being discovered.

1.1.2 Classification of bacteriocins

The first class (Class I) consists of small bacteriocins (sizes approximately <5 kDa) termed lantibiotics (Chen and Hoover, 2003). Lantibiotics are distinguishable from other bacteriocins by their intra-molecular ring structure that is formed by the thioether amino acids lanthionine (Lan) or α-methyllanthionine (MeLan) (Sahl and Bierbaum, 1998; Brotz and Sahl, 2000; McAuliffe *et al.,* 2001).

Lantibiotics are not a homogenous group. They can be sub-grouped into Type A and B based on the chemical structure of their prepeptides and mature peptides, biosynthetic machinery and anti-microbial activity (Jung, 1991; Moll *et al.*, 1999; van Kraaij *et al.*, 1999; McAuliffe *et al.*, 2001). Type A lantibiotics which include nisin produced by *Lactococcus lactis*, subtilin produced by *Bacillus subtilin*, lacticin 481 produced by *Lacticin lactis* and epidermin and epilancin K7 produced by *Staphylococcus aureus,* are elongated, flexible and amphipathic/cationic peptides with a conserved arrangement of lanthionine-bridges with a net positive charge (Hurst 1981; Mortvedt *et al.*, 1991; Sahl, 1991; Piard *et al.*, 1992; Chen and Hoover, 2003) (Figure 1.1, Type A). Type B lantibiotics, which include mersacidin, duramycin, cinnamycin and actagardine, are smaller globular peptides and have a negative or no net charge (Sahl and Bierbaum 1998; Altena *et al.*, 2000; Chen and Hoover, 2003; Twomey *et al.*, 2002) (Figure 1.1, Type B).

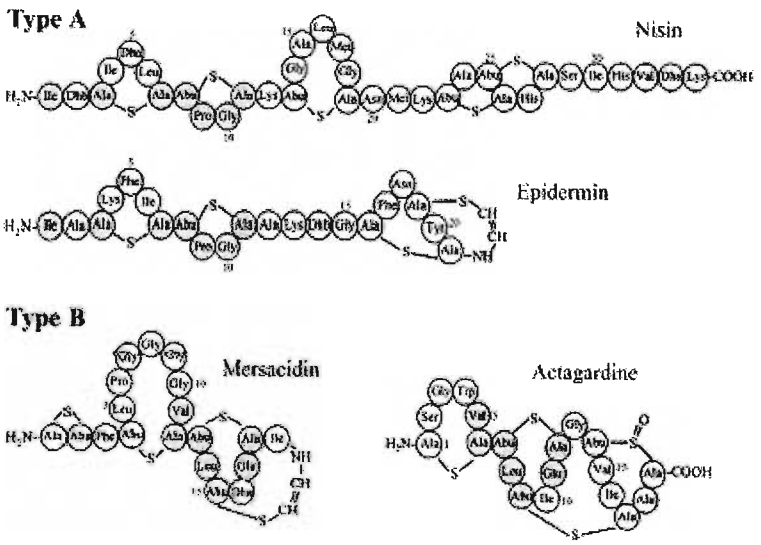


Figure 1.1. Structures of lantibiotics type A and type B.
Lantibiotics Type A are elongated with a corkscrew-like structure, e.g. nisin A and epidermin. Type B lantibiotics have globular cross-bridged C-terminus e.g. mersacidin and actagardine (Twomey *et al.*, 2002).

The second class (Class II) is the largest group with small sized (approximately <10kDa), heat-stable, non-lanthionine containing peptides that are anti-listerial (Biswas *et al.*, 1991; Henderson *et al.*, 1992; Ennahar *et al.*, 2000b; Morisset *et al.*, 2004). This class is sub-grouped into six smaller categories based on their different structural properties. The most characterized of these sub-groups are the first three Class IIa, Class IIb and Class IIc.

The first sub-group is Class IIa also known as pediocin-like bacteriocins. These have attracted more attention as they are single peptides with the strongest anti-listerial activity, the most well-known include pediocin PA-1/AcH produced by *Pediococcus acidilactici*, leucocin A UAL 187 produced by *Leuconostoc gelidum*, mesentericinY105 produced by *Leuconostoc mesenteroides,* sakacin P produced by *Lactobacillus sakei* strains Lb674 and LTH673 (Hastings *et al.*, 1991; Marugg *et al.*, 1992; Nieto *et al.*, 1992; Henderson *et al.*, 1992; Motlagh *et al.*, 1992; Ennahar *et al.*, 2000b; Mathiesen *et al.*, 2005).

The second sub-group is Class IIb bacteriocins that require two different peptides for full anti-listerial activity, for example lactococcin G produced by *Lacticin lactis* and plantaricin JK produced by *Lactobacillus plantarum* (Nissen-Meyer *et al.*, 1993; Anderssen *et al.*, 1998; Marco *et al.*, 2000). The third sub-group is Class IIc. These are thiol-activated peptides bacteriocins, which are produced by the cell's general secretory (*sec*) pathway (Klaenhammer, 1993). Class IIc include acidocin B produced by *Lactobacillus acidophilus*, enterocin B produced by *Enterococcus faecium* (Leer *et al.*, 1995; Marco *et al.*, 2000; Nes and Holo, 2000; Chen and Hoover, 2003).

The next three sub-classes of bacteriocins were recently characterized and their structural organization and production have not been studied thoroughly. Class IId are a small group of bacteriocins are synthesized without an N-terminal leader peptide or signal sequence and contain only one or no cysteine residues (Venema *et al.*, 1996). This class includes the two-component bacteriocin enterocin L50 and the single peptide bacteriocin enterocin Q, produced by *Enterococcus faecium* L50 as well as aurocin A70 produced by *Staphylococcus aureus* A70 (Cintas *et al.*, 1998; Netz *et al.*, 2001).

Class IIe do not contain cysteine residues and they have antibacterial spectra even more limited than the thiolbiotics, which contain a single cysteine residue (Jack *et al.*, 1995). These consist of two separate peptides that have a weak antibacterial activity when

functioning separately, nevertheless, when functioning together the activity is increased five-fold (van Belkum and Stiles, 2000). Class IIe bacteriocins include lactococcin M, plantaricins EF and JK, (McCormick *et al.*, 1998). The last sub-class of Class II bacteriocins is Class IIf, which are unmodified bacteriocins that do not contain the structural features or conserved motifs that are unique to any of the other subclasses (van Belkum and Stiles, 2000). These are cyclic peptides bacteriocins, cyclised by a head-to-tail peptide bond formation (Mendoza *et al.*, 1999; Kawai *et al.*, 1998). The bacteriocin AS-48 produced by *Enterococcus faecalis* is an example of the cyclic peptides (Samyn *et al.*, 1994).

The third class is Class III bacteriocins, these are non-lantibiotic, heat-labile peptides that are very large in size, >30kDa (Marco *et al.*, 2000). This class is not well characterized because they are not common among anti-listerial compounds of LAB and thus are of lesser interest to food scientists. Therefore only a few of these bacteriocins have been identified. These include helveticin J, helveticin V, acidophilucin A, lactacins A and B (Joerger and Klaenhammer, 1990; Toba *et al.,* 1991; Vaughan *et al.,* 1992).

The fourth class of bacteriocins, Class IV are composed of mixtures of proteins, lipids and carbohydrates moieties required for activity (Chen and Hoover, 2003). Klaenhammer, (1993) suggested that the complex bacteriocinogenic activities of Class IV bacteriocins maybe artefacts caused by the interaction between constituents from the cells or growth medium.

A new class of bacteriocins has been proposed, Class V. This class consist of ribosomally synthesized, non-modified, head-to-tail ligated, cyclic, antibacterial peptides such as circularin A (Klaenhammer, 1993; Fremaux *et al.*, 1995; Montville and Chen, 1998). Nonetheless, as with the fourth class these have not been thoroughly described and characterised.

1.1.3 Genetics and biosynthesis of LAB bacteriocins

The first two groups of bacteriocins in LAB have been mostly studied, well characterized and described due to the fact that they are of more interest for use in food preservation programmes than heat labile Class III bacteriocins and the less characterized Classes VI and V. These bacteriocins are encoded by genes that share many similarities, which have been shown to be located on chromosome fragments, plasmids or on transposons as operon-like structures (Rauch and de Vos, 1992; Axelsson and Holck, 1995; Altena, 2000). These operons are involved in the bacteriocin production and extracellular translocation, the immunity of the producers, and in several cases, the regulation of bacteriocin synthesis (Brurberg *et al.*, 1997; Quadri *et al.*, 1997a; Anderssen *et al.*, 1998).

1.1.3.1 Lantibiotics

1.1.3.1.1 Structural arrangement

To date about 30 lantibiotics have been discovered and described, but the most studied lantibiotic is nisin A that is produced by *L. lactis*. Nisin A was characterised in 1971 and has been accepted by the World Health Organisation as a preservative and used in the food industry (Hurst 1981; Delves-Broughton, 1990; Vandenbergh, 1993; Abee, 1995). Nisin A is an autoregulated bacteriocin with the genes that encode enzymes that are involved in post-translational modification.

Figure 1.2D show a typical genetic organisation of lantibiotics (Allgaier *et al.,* 1986; Piard *et al.,* 1992; Abee, 1995; Chen and Hoover, 2003). *NisA* encode the prebacteriocin of nisin A, prepronisin. The genes *nisB* and *nisC* encode proteins LanB and LanC, respectively play a role in pronisin modification (Augustin *et al.*, 1992). *NisT* encodes the ABC-transporter protein that is responsible for nisin externalization (Seizenn *et al.*, 1996) and *nisP* encodes the leader peptide protease that proteolytically removes the leader peptide (Havarstein *et al.*, 1995). *NisR* and *nisK* encode LanR and LanK, respectively that are involved in the nisin regulation mechanism (Klein *et al.*, 1993; Engelke *et al.*, 1994). Lastly the immunity genes are (i) *nisI,* which encodes a protein that is able to confer resistance to nisin and (ii) *nisF, nisE and nisG,* which encode individual domains of the

ABC-transporters that are involved in the externalization of the peptides (LanEFG) (Otto *et al.*, 1998).

1.1.3.1.2 Synthesis

Lantibiotics undergo extensive post-translational modification. They are produced as inactive prebacteriocin with an attached leader peptide that undergoes proteolytic cleavage resulting in the release of active mature peptides that are externalized to the outer cell by the ABC-transporter (McAuliffe *et al.*, 2001; Twomey *et al.*, 2002). The leader peptides of the two-lantibiotic groups differ. For type A the leader peptides are hydrophobic with charged residues and contain conserved FNLDV motifs between positions –20 and –15, which are suggested to play a role in modification and transport of the peptides (van der Meer *et al.*, 2001; Neis *et al.*, 1997). Type B leader peptides do not have FNLDV motifs, in contrast they contain the double-Gly (GA/GS/GG) motif which is responsible for their modification and transport (Fremaux *et al.*, 1993; Fath *et al.*, 1994; Havarstein, 1994; de Vos *et al.*, 1995). In addition, the leader peptides have been proposed to protect the producer strain by keeping the lantibiotic in an inactive state while it is inside the producer, and interact with the propeptide domain to ensure a suitable conformation essential for enzyme-substrate interaction (Sablon *et al.*, 2000; McAuliffe *et al.*, 2001).

1.1.3.1.3 Lantibiotics mode of action

Lantibiotics are generally active against Gram-positive bacteria, as they are too large to penetrate the outer cell lipids of Gram-negative organisms (Somma, *et al.*, 1977; Stevens *et al.*, 1991; Brotz *et al.*, 1995; Joosten *et al.*, 1996; Brotz and Sahl, 2000). The cytoplasmic membrane is the target of lantibiotics given that they function through the wedge-like mechanism that leads to formation of pores on the membrane (Abee, 1995; Moll *et al.*, 1999).

Type A lantibiotics and some of the Class II bacteriocins were observed to be membrane-active peptides, in that they exert their activity through the pore formation by depolarising the bacterial cytoplasmic membrane, which eventually leads to cell lysis (Benz *et al.*, 1991;

Sahl, 1991; Chen and Hoover, 2003). These peptides being positively charged (due to a lysine^{+} residue on the N-terminal) electrostatically associate with the negatively charged phospholipids and non-energized liposomes containing these lipids (Abee *et al.*, 1995).

Type B lantibiotics act by interfering with enzymatic reactions required for peptidoglycan biosynthesis of the targeted bacterial cells (Linnet and Strominger, 1973; Twomey *et al.*, 2002; Chen and Hoover, 2003). Brotz *et al.* (1998) reported that the N-terminal rings of Type B lantibiotics primarily bind to lipid II during the pore formation process. To complement the study by Brotz *et al.* (1998), Wiedemann (2001) concluded that while binding to the lipid II and forming pores Type B lantibiotics also independently inhibits cell wall synthesis.

1.1.3.2 Non-lantibiotics

These are the non-lanthionine containing peptides belonging to Class II, III, IV and V. As mentioned previously, Class II is the most studied class. Class II bacteriocins undergo minimal post-translational modification (Havarstein *et al.*, 1995; Nes *et al.*, 1996). These peptides are produced with a leader peptide that is proteolytically cleaved resulting a mature cationic, amphipathic and thermostable peptide (100°C-121°C heat-stable), which is translocated across the cytoplasmic membrane (Hancock, 1997; Sablon *et al.*, 2000). To date more than 50 Class II bacteriocins from LAB have been isolated and characterized.

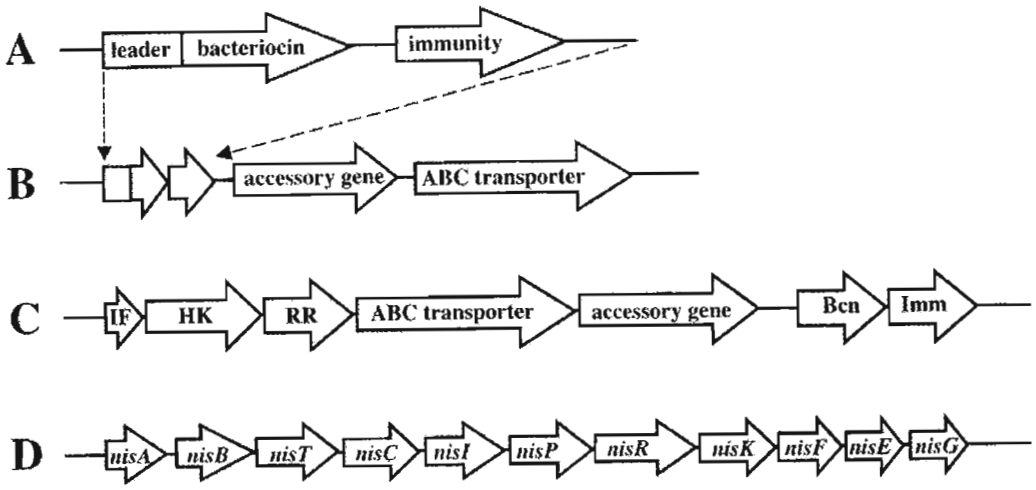


Figure 1.2. Operon structures required for the production of the bacteriocin classes. **A.** The Class IIc gene arrangement. **B.** Class IIb gene arrangement that consists of the ABC transporter and accessory genes in addition to the Class IIc arrangement. **C.** Class IIa have the Pre-induction peptide (IF), Histidine kinase (HK) and Response regulator (RR) in addition to genes found in the Class IIb array (Marugg *et al.*, 1992; Motlagh *et al.*, 1994). **D.** lantibiotics gene arrangement includes enzymes for post-translational modification (van Belkum and Stiles, 2000; Worobo *et al.*, 1995).

1.1.3.2.1 Structural arrangement

As mentioned previously genes that encode bacteriocins are arranged in operon-like structures, shown in Figure 1.2. Class IIa bacteriocins structural arrangement will be discussed in detail in Section 1.1.4.

Class IIb bacteriocins are also referred to as the two-peptide bacteriocins, as they require the combined action of two peptides for full antimicrobial activity (Allison *et al.*, 1994; Jimenez-Diaz *et al.*, 1995; Anderssen *et al.*, 1998). Moreover, Class IIb bacteriocins have double-glycine-type leader peptides that serve as recognition signals and hence they do not require the regulation system. Consequently in addition to Figure 1.2A gene array only the ABC-transporter and accessory genes are required for the bacteriocin production (Figure 1.2B) (Holo *et al.*, 1991; Peschel, 1993; Håvarstein *et al.*, 1994; Axelsson and Holck. 1995).

Class IIc bacteriocins have signal peptides, which allow them to be exported across the cytoplasmic membrane by the translocase general secretion {(*sec*)-dependent secretory} pathway (Leer *et al.,* 1995; Worobo *et al.,* 1995). Their gene clusters consist of the prebacteriocin and immunity gene (Figures 1.2A) (Ennahar *et al.*, 2000b). The bacteriocin precursor contains a canonical *sec* signal peptide consisting of a positively charged N-terminus, a hydrophobic core, and a defined cleavage site that is removed by a specific signal peptidase during translocation (von Heijne, 1983; Pugsley, 1993; Tomita *et al.,* 1996; Cintas *et al.,* 1997; Kalmokoff *et al.,* 2001).

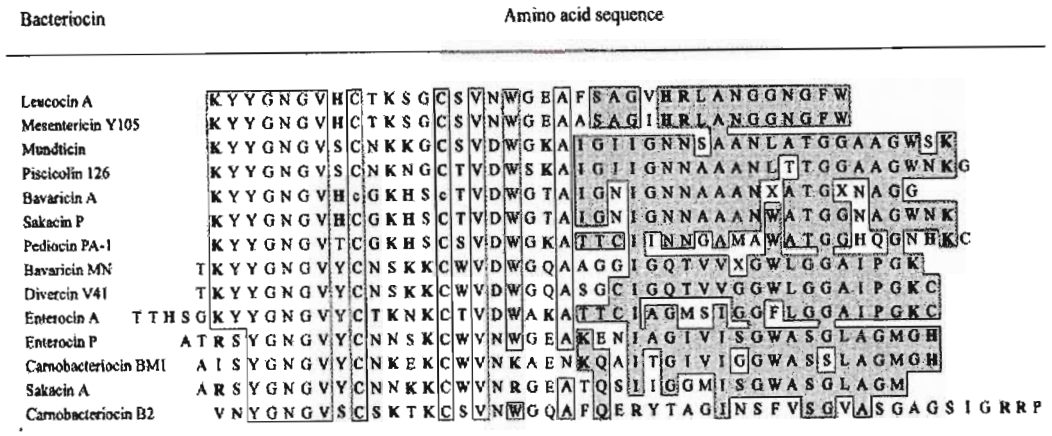
1.1.4 Pediocin-like bacteriocins

Due to the recurrence of serious listeriosis outbreaks caused by the food-borne pathogen *L. monocytogenes*, Class IIa bacteriocins have become a major focus in research for their novel naturally occurring biopreservatives "anti-listerial bacteriocins". *L. monocytogenes* is a Gram-positive bacterium, found in the division Firmicutes and named after Joseph Lister (Murray *et al.*, 1926). According to previous studies, at least 37 mammalian species both domesticated and feral, and at least 17 species of birds, fish and shellfish may carry *L. monocytogenes* (Roberts *et al.*, 2006). Furthermore about 1 to 10% of humans carry it in their intestines. "It is estimated that between 1,100 and 2,500 people in the United States develop listeriosis each year. This manifests itself as septicemia, meningitis, encephalitis, and intrauterine or cervical infections in pregnant women and that 20 to 25% of these *Listeria* infections are fatal" (Vadyavaloo *et al.*, 2002).

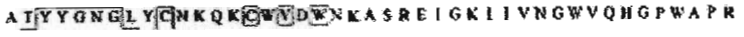
The classification of this class has been largely based on the presence of a conserved Tyr-Gly-Asn-Gly-Val-Xaa-Cys (KYYGNGV) amino acid motif near the N-terminal of the active peptide and the presence of cysteine residues that result in the formation of the disulfide bridge, which forms a six-membered ring over two cysteine residues (Figure 1.3A) (Nissen-Meyer and Nes, 1997). Consequently these bacteriocins have conserved disulfide bonds within the N-terminal regions (Ennhar *et al.*, 2000; Morisset *et al.*, 2004). The disulfide bond has been suggested to be very important for the activity and stability of the bacteriocins given that bacteriocins with two disulfide bridges that is formed by four

cysteine residues have a broader spectrum of antibacterial activity as compared to those with one disulfide bridge that is formed by two cysteine residues (Jack *et al.*, 1995; Montville and Chen, 1998; Eijsink *et al.*, 1998). A study by Miller *et al.* (1998a) reported that reducing the disulfide bond or cysteine residues mutation dramatically reduces bacteriocins activity.

Class IIa are single peptides referred to as "pediocin-like" or *"Listeria*-active" peptides. *"Listeria*-active" is misleading in that, bacteriocins that do not contain the KYYGNGV motif are still anti-listerial, and for example, bacteriocin 31 has an incomplete consensus motif and is active against *Listeria.* As a result researchers suggest that the consensus motif will evolve as the new bacteriocins are being discovered, in fact some authors consider the real motif to be YGNG since to date it is present in all anti-listerial bacteriocins (Figure 1.3A and B) (Eijsink *et al.*, 1998; McCormick *et al.*, 1998; Ennahar *et al.*, 2000b; van Belkum and Stiles, 2000). The function of the KYYGNGV sequence, if any, is not known, yet, any change in the motif drastically alters its bacteriocin activity (Tomita *et al.*, 1996; Papathanasopoulos *et al.*, 1997).



B. Pediocin-like bacteriocins



A. Bacteriocin 31

Figure 1.3. The protein sequences alignment of pediocin-like bacteriocins.
A. Illustration of the similarities of the consensus motifs on the N-terminal of the mature peptides (Sprules, *et al.*, 2004). **B.** Bacteriocin 31 mature peptide, which has an incomplete consensus motif (YYGNG) and is still anti-listerial (Ennahar *et al.*, 2000a).

1.1.4.1 Regulatory, immunity and transport modules

1.1.4.1.1 Regulation module

For transcriptionally regulated bacteriocins, additional open reading frames (ORFs) are present in the vicinity of the structural gene, always in the same order. These ORFs form a putative three-component signal-transduction autoregulatory cassette encoding membrane-bound signal producing proteins IF, HPK and RR that assist in the regulation mechanism (Figure 1.4) (Stock *et al.*, 1989; Parkinson, 1993; Nes *et al.*, 1996).

Little is known about this mechanism, however, authors have suggested that it is triggered by excess in IF concentration as a consequence of cell growth. The IFs are produced with the bacteriocins and are also, are small, heat-stable, cationic and hydrophobic peptides that are initially synthesized as prepeptides with leader sequences of the double-glycine type (Nes *et al.*, 1996; Nilsen *et al.*, 1998; Quadri *et al.*, 1997a; Eijsink *et al.*, 1998). The

prebacteriocin and pre-IF are processed and translocated to the outer cell by the ABC-transporter (refer to section 1.1.4.1.3), resulting in the release of mature bacteriocin and IF.

Researchers sustain the idea that LAB produce bacteriocins depending on environmental conditions such as the presence of DNA-damaging agents in the same media, for example, colicins production of *E. coli* is induced by mitomycin (Biswas *et al.*, 1991; de Vuyst *et al.*, 1996; Nilsen *et al.*, 1998). Other factors include the pH, temperature and the presence of other bacteria (Lazdunski *et al.*, 1988; Ahn and Stiles, 1990; Biswas *et al.*, 1991).

1.1.4.1.2 Immunity module

This module has been suggested to be responsible for the protection of the producers from self-produced bacteriocins. The entire mechanism is credited to immunity proteins (Axelsson and Holck, 1995; Dayem *et al.*, 1996; Johnsen *et al.*, 2004).

1.1.4.1.3 Transport module

This module is responsible for the modification and translocation of the bacteriocins, it consists of membrane bound ABC transport proteins and accessory proteins (Figure 1.4) (Franke *et al.*, 1996; Hühne *et al.*, 1996; Nes *et al.*, 1996; Sahl and Bierbaum, 1998). The accessory proteins are encoded by genes that are located adjacent to the ABC transporter gene. These genes generally consist of about 460 amino acids and are identical to HlyD (the accessory protein of the *E. coli* haemolysin A secretion apparatus) (Fath and Kotler, 1993; Marugg *et al.*, 1992; Axelsson and Holck, 1995; Fremaux *et al.*, 1995; Venema *et al.*, 1995; Diep *et al.*, 1996; O'Keeffe *et al.*, 1999; Varcamonti *et al.*, 2001). The exact function of accessory proteins in the translocation process is not yet fully understood. Nevertheless, they are required for the leader sequence processing and have been speculated to facilitate in the membrane translocation and they help in the processing of the N-terminal double glycine leader sequence (Venema *et al.*, 1995; Ennahar *et al.*, 2000b; van Belkum and Stiles, 2000).

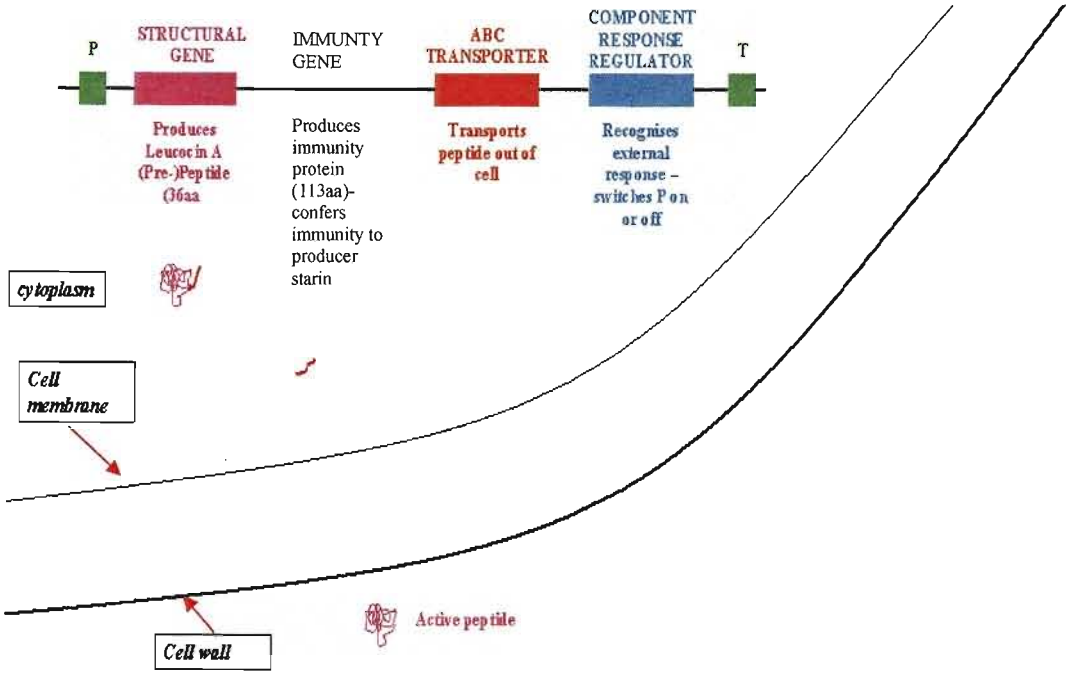


Figure 1.4. A typical schematic overview of a gene array for pediocin-like bacteriocin.
The gene array contains the genes that are important for the production and maturation of the bacteriocin. A promoter (P) that promotes the bacteriocin transcription, structural gene, which encodes the bacteriocin, the ABC transporter system, which is responsible for externalization of the peptide, a component response regulator, which is involved the bacteriocin production regulation and the terminator (T), which terminates the bacteriocin transcription.

The ABC transporter proteins have highly conserved ATP binding domains on their C-terminal regions, which are unique for every ABC transporter protein (Higgins, 1992; Fath and Kotler, 1993). Their N-terminal cysteine protease regions have dissimilar hydrophobic integral membrane domains of about 150 amino acids and are responsible for the cleavage of the leader sequence after the double-glycine motif (Havarstein *et al.*, 1995; Venema *et al.*, 1995). This cleavage occurs on the cytoplasmic side of the membrane during secretion of the bacteriocins (Franke, *et al.*, 1999). The ABC transporter binds to the proteolytic processing site (the C-terminal of the double glycine motif) of the leader sequence where ATP hydrolysis is triggered to provide energy for this translocation process. The ABC transporter then undergoes a conformational change and this result in the cleavage of the leader sequence and hence the release of a mature peptide to the outside of the cell (Nes *et al.*, 1996; Ennhar *et al.*, 2000; Aucher *et al.*, 2004). The leader sequence is thus considered a recognition signal for the translocation of the mature peptide (Havarstein *et al.*, 1995). Any mutation or deletion in the genes encoding any of the transport proteins would result in a complete loss of bacteriocin production (Diep *et al.*, 1994; Engelke *et al.*, 1994; Klein *et al.*, 1993; Fremaux *et al.*, 1995; Axelsson and Holck, 1995; Huhne, *et al.*, 1996).

1.1.4.2 Structural function

Figure 1.5 show a typical three-dimensional structure of Class IIa bacteriocins predicted by nuclear magnetic resonance (NMR) spectroscopy and mutagenesis in membrane mimicking environments (Gallagher *et al.*, 1997; Wang *et al.*, 1999; Ennahar *et al.*, 2000b; Fimland *et al.*, 2000; Uteng *et al.*, 2003; Morisset *et al.*, 2004). The mature bacteriocins are predicted to exist primarily in unstructured conformations, generally in random coils in aqueous solutions, whereas in non-aqueous solutions they adopt a partly helical (amphipathic) structure with varying amounts of hydrophobicity (Klaenhammer, 1993; Sailer *et al.*, 1993; Fremaux, *et al.*, 1995; Jack *et al.*, 1995; Fleury, 1996).

The N-termini of Class IIa bacteriocins are very similar and are believed to contain β-sheets maintained in a β-hairpin conformation that is stabilized by the disulfide bridge (Chen *et al.*, 1997a; Montville and Chen, 1998; Ennahar *et al.*, 2000b). These β-turn structured YGNGV motifs according to the current view is said to be involved in a

recognition step of the mechanism of action of Class IIa bacteriocins. The structures enable the bacteriocins to be easily exposed to and recognized by a putative membrane receptor on the targeted cell, which allows correct positioning of the bacteriocins on the membrane surface (Bhugaloo-Vial *et al.*, 1996; Gallagher *et al.*, 1997; Montville and Chen, 1998). Ennahar *et al.* (2000a) concluded that it is possible that modifications within the YGNGV perturb the β-turn structure and consequently the N-terminal β-sheet conformation, which ultimately affects activity not only against *Listeria* strains, but also against other target strains.

In contrast, the C-terminal regions of Class IIa bacteriocins show diversity and through construction of hybrid bacteriocins, Fimland *et al.* (2002a) suggested that these regions play a role in recognition. C-terminal regions are important determinants of their target cell specificities given that they are the regions that interact with the hydrophobic region of the membrane (Miller *et al.*, 1998b; Fimland, *et al.*, 2000; Uteng, *et al.*, 2003). These regions have been predicted to adopt an amphiphilic α-helix, spanning similar regions in different molecules and generally leave a non-helical portion of only one or two C-terminal residues (Fleury *et al.*, 1996; Gallagher *et al.*, 1997; Nissen-Meyer *et al.*, 1993). This helical portion is believed to be the transmembrane segment during pore formation in a sensitive cell membrane (Klaenhammer, 1993; Jack *et al.*, 1995; Bennik *et al.*, 1998; Abbe, 1995).

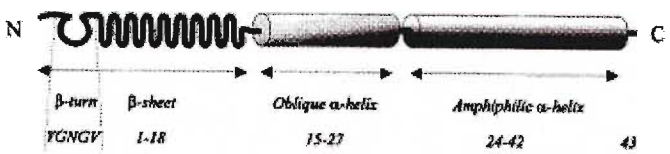


Figure 1.5. A typical Class IIa bacteriocin structure.
Class IIa bacteriocins have a conserved N-terminal the β-turn, β-sheet and the diverse C-terminal with the oblique α-helix and amphiphilic α-helix (Ennahar *et al.*, 2000b).

1.1.4.3 Mode of action

There is no concrete evidence with regards to the mode of action of Class IIa bacteriocins. However, researchers have made several conjectures (Bhunia *et al.,* 1992, Jack *et al.,* 1995; Chen *et al.,* 1997b). The generally narrow spectrum of antibacterial activity of Class IIa bacteriocins suggests the presence of a receptor molecule that is recognized by very specific bacteriocins (Chen and Hoover, 2003). In contrast, a study by Chen *et al.* (1997b) with lipid vesicles prepared from *L. monocytogenes* showed that pediocin PA-1 functions in the absence of a protein receptor. Chen *et al.* (1997b) later showed that the lipid composition of the target membrane is a determinant factor in modulating the pediocin PA-1 action, particularly the affinity of this bacteriocin for lipid vesicles increases with the increase in their anionic lipid content. Venema *et al.*, 1995 suggested that the receptors in the Class II bacteriocins targeted membranes act to determine specificity, where for lantibiotics, docking molecules (lipid II, the peptidoglycan precursor) heightens conductivity and stability of induced pores.

Most researchers believe in the "barrel-stave model", where the cationic Class IIa bacteriocins with polar residues bind to the cell membrane's anionic phospholipid head groups that serve as the primary receptors. This binding results in hydrophobic interactions between the hydrophobic/amphiphillic domain within the bacteriocin C-terminal and lipid fatty acid chains, resulting in insertion, followed by the electrostatic binding of the peptide to the membrane (Chikindas *et al.*, 1993; Abee 1995; Venema *et al.*, 1995; Montville and Chen, 1998; Chen *et al.*, 1997a; Moll *et al.*, 1999; Chen and Hoover, 2003). The bacteriocins then create the barrel-like pores whereby they orient parallel to the membrane surface and interfere with the membrane structure (Moll *et al.*, 1999). Van Belkum *et al.* (1991b) in their study on the action of purified Class IId bacteriocin, lactococcin A, concluded that the bacteriocin acts on intact cells and membrane vesicles but not on liposomes (lacking proteins and non-lipid constituents), suggesting that specific membrane receptors are involved in the recognition process between host and bacteriocin (Jack *et al.*, 1995).

Barrel-stave model for Class IIb bacteriocins with two peptides occurs as the lateral oligomerisation of bacteriocin monomers form two-component poration complexes. This occurs with the hydrophobic side of peptides facing the fatty acid chains of the membrane

lipids (Ennahar *et al.*, 2000b). The hydrophilic sides of α-helical peptides that consist of at least twenty amino acid residues form the inside wall of the water-filled pore, in order to be able to completely span the membrane, (Lear *et al.,* 1988; Ojcius and Young 1991).

These pores generally allow passage of hydrophilic solutes with molecular mass up to 0.5 kDa, which results in the leakage of inorganic phosphates, afflux of amino acids and dissipation of proton motive force (PMF) (van Belkum *et al.,* 1991b; Chikindas *et al.,* 1993; Abee, 1995; Venema *et al.,* 1995). The PMF is the electrochemical gradient over the cytoplasmic membrane composed of membrane potential (Ψ) and the pH gradient (ΔpH), which drives ATP synthesis and accumulation or extrusion of ions and other metabolites (Bhunia *et al.*, 1991; Kaiser and Montville, 1996; Bennik *et al.*, 1998) (Figure 1.6). Dissipation of PMF leads to the depletion of intracellular ATP since it is constantly consumed and the cells are unable to restore the ATP, which eventually leads to cell death through cessation of energy-requiring reactions (Venema *et al.,* 1994; Montvile and Chen, 1998; Vadyvaloo *et al.*, 2002).

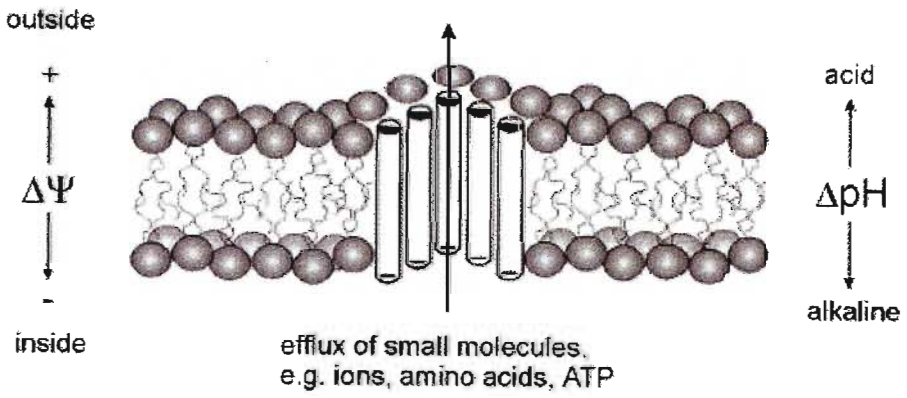


Figure 1.6. The Barrel-stave model.
An illustration of the PMF dissipation of a susceptible cell membrane caused by Class IIa bacteriocins. The pores formed allow the efflux of small molecules (< 0.5 kDa) (Klaenhammer, 1993).

1.1.4.4 Resistance to pediocin-like bacteriocins

The occurrence of bacteriocin tolerance and/or resistance among food spoilage and pathogenic bacterial species is a major concern with regards to the practical use of bacteriocins, since it may compromise the antibacterial efficiency of these compounds. The mechanism of resistance or tolerance has not been fully established. However, it has been suggested that in addition to the immunity that is genetically linked to bacteriocin production, bacteriocin resistance or tolerance can be due to a number of factors. Firstly, bacteriocin-resistant mutation, e.g. the appearance of spontaneous mutations following selection in the presence of bacteriocin, as found with therapeutic antibiotics in the environment. Secondly, some Class IIa bacteriocin producers possess one or more immunity genes for Class IIa bacteriocins that are not linked with bacteriocin production, the encoded immunity proteins may confer resistance to other bacteriocins in addition to their cognate bacteriocins (Aymerich *et al.*, 1996; Cintas, 2000). The latter factor builds a notion that connects bacteriocin resistance and immunity (Axelsson and Holo, 1995; Tomita *et al.*, 1996; Ramnath *et al.*, 2000).

Theoretically based, the mechanism of resistance is associated with the subunit IIAB of an enzyme EII_t^{Man} (permease) in a mannose-specific phosphotransferase system (PTS), which is encoded by the *mptACD* operon and is regulated by the δ^{54} transcription factor (Dalet *et al.*, 2001; Ramnath *et al.*, 2004; Vadyvaloo *et al.*, 2004). This was proved by Ramnath *et al.* (2004), they expressed the EII_t^{Man} in a resistant strain of *L. lactis* and upon the induction of the cloned operon, the strain became sensitive. The proposed mechanism behind this phenomenon is that upon the removal of the subunit or mutation of the site the bacteriocin cannot recognize the site and thus cannot bind, hence the strain becomes resistant (Ramnath *et al.*, 2000).

1.1.4.5 Bacteriocins produced by *Leuconostoc* species

Leuconostoc belongs to a Gram-positive, non-sporulating group of LAB called the 'sensu stricto' (Damelin *et al.*, 1995). These include *L. mesenteroides, L. gelidum, L. carnosum* and *L. lactis* and most of these bacteria grow optimally at temperature between 20°C and 30°C (Vadyvaloo *et al.*, 2002). Due to acid production of these bacterial species, during culture growth the pH drops from 6.5 to 4.4–5.0, a low pH that is ideal for the activity of the bacteriocins they produce (Stiles, 1994).

This study focuses on *L. gelidum* UAL 187 strain, which is used for food preservation due to its production of an antagonistic compound, a 24 amino acid leucocin A peptide. Leucocin A is a Class IIa bacteriocin that is active against some LAB and *L. monocytogenes* (Hastings *et al.*, 1991). The gene cluster of *L. gelidium* UAL 187 is of a typical Class IIa producer gene array (Figure 1.6)(Stiles, 1994). *Leuconostoc* species do not produce specific bacteriocins, i.e. peptides closely related to leucocin A may occur in several other *Leuconostoc* species (Garvie, 1986; Yang and Woese, 1989; Revol-Junelles *et al.*, 1996).

1.2 PEDIOCIN-LIKE BACTERIOCIN IMMUNITY

1.2.1 Bacteriocin immunity

As mentioned earlier the immunity proteins that protect the bacteriocin producers from their own bacteriocins are expressed concurrently with bacteriocins yet in contrast to bacteriocin biosynthesis they do not require the bacteriocin transport and processing system (Axelsson and Holck, 1995; Dayem *et al.*, 1996; Nes *et al.*, 1996; Johnsen *et al.*, 2004). However, the exact location of the genes that encode the immunity proteins differ, e.g. enterocin B immunity gene is located downstream of, but in the opposite orientation to the bacteriocin structural gene (Franz *et al.*, 1999), and carnobacteriocin A immunity gene is not orientated in close proximity to the bacteriocin structural gene (Franz *et al.*, 2000). The producer gene array of the two-component system bacteriocins (Class IIb), have only one immunity gene that is linked to both bacteriocin structural genes (van Belkum *et al.*, 1991a; Diep *et al.*, 1994, Quadri *et al.*, 1997b).

1.2.2 Immunity protein expression

To date the exact mode of action of immunity proteins remains unclear. Nonetheless, in order to uncover the action mode of immunity proteins, it is best to locate them within the cells. Studies by cell-fractionation concluded that the majority of immunity proteins are expressed in the cytoplasm compartment, with only a small proportion detected in the membrane (Chickindas *et al.*, 1993; Nissen-Meyer, 1993; Quadri *et al.*, 1995; Venema *et al.*, 1994; Abdel *et al.*, 1996). In addition, Venema *et al.* (1994) performed computational analysis of leucocin A, and reported that it contains a putative α-amphiphilic helix (residues 29 to 47), which could span the cytoplasmic membrane and that the C-terminal region of the protein is located outside of the cell (Eijsink *et al.*, 1998).

The immunity genes are expressed to produce cationic and principally hydrophilic immunity proteins that consist of 88 to 115 amino acid residues (Franz *et al.*, 2000; Johnsen *et al.*, 2004). Immunity proteins sizes vary, but generally those associated with one peptide bacteriocins consist of 51 to 113 amino acid residues that adopt an α-helical structure with one or two hydrophobic putative membrane spanning domains (van Belkum *et al.*, 1991a; Marugg *et al.*, 1992; Tichaczek *et al.*, 1993; Venema *et al.*, 1994; Quadri *et al.*, 1994; Eijsink *et al.*, 1998). Conversely, immunity proteins conferring immunity to two-peptide bacteriocins are normally larger, consisting of 110 to 154 amino acids as well as contain several putative transmembrane helices (van Belkum *et al.*, 1991b; Allison and Klaenhammer, 1996).

Based on literature the sequence identity observed between the immunity proteins ranges between 5 to 85% and does not correlate with similarities between the corresponding bacteriocins, i.e. the highest similarity between immunity proteins does not necessarily mean the highest similarity between the corresponding bacteriocins (Tichaczek *et al.*, 1993; Axelsson and Holck 1995; Aymerich *et al.*, 1996; Nes *et al.*, 1996; Eijsink *et al.*, 1998). However, as more bacteriocin loci are being characterized, it has become apparent that many of the identified immunity proteins do exhibit significant similarity to other immunity proteins, particularly in their C-terminal regions (Quadri *et al.*, 1995; Aymerich *et al.*, 1996; Cintas *et al.*, 1997; Franz *et al.*, 2000). Yet these proteins show a high degree of specificity with regards to the bacteriocin they recognize (Fimland *et al.*, 2002b).

1.2.3 Immunity protein mode of action

The mode of action of immunity proteins is still speculative, all that has been suggested is that interaction of the immunity protein with the membrane appears to protect the producer against its own bacteriocin. Previous studies show that the extent to which an immunity protein specifically recognizes, interacts with (directly or indirectly), and confers immunity to the cognate bacteriocin seems to depend on the C-terminals of both the immunity proteins and bacteriocins (Johnsen *et al.*, 2004). The C-terminal region of the pediocin-like bacteriocins is also the region that interacts with the hydrophobic region of the

membrane, which suggests that immunity proteins and bacteriocins directly or indirectly interact in this region of the membrane.

Immunity proteins act by either disturbing the process of bacteriocin aggregation and pore formation or disturbing the interaction between the bacteriocin and a putative membrane located bacteriocin receptor (Nissen-Meyer *et al.*, 1993; Venema, 1994). A Study by Fimland *et al.* (2000), showed that cytoplasm located immunity proteins might act by binding to the cytoplasmic side of this receptor, which somehow could block the receptor's ability to interact with the bacteriocin in a fashion that is necessary for bacteriocin action.

Studies by Quardri *et al.* (1995) and Dayem *et al.* (1996), on functional analysis of the immunity proteins conferring resistance to bacteriocins concluded that these proteins are localized intracellularly and are therefore not likely to act by directly binding to the extracellular bacteriocin. It was demonstrated that immunity proteins have poor affinity for the bacteriocins and that no direct interaction occurs in aqueous solution (in the cytoplasm) between the two proteins (Quardri *et al.*, 1995; Dayem *et al.*, 1996). In contrast, Johnsen *et al.*, 2004 observed that the immunity proteins associate themselves loosely with the cytoplasmic membrane as peripheral membrane proteins, which allows them to interact with their cognate bacteriocins. So there could be a direct interaction between the immunity proteins and cognate bacteriocins, however, the interaction only occurs on the membrane or membrane-mimicking environment.

In addition to providing total immunity against their cognate bacteriocin, immunity proteins may also appear to cross-immunize, i.e. provide total or partial protection against other Class IIa bacteriocins. Franz *et al.* (2000) showed that the immunity genes of carnobacteriocin A and enterocin B can be exchanged to confer cross protection against the corresponding bacteriocins.

1.2.4 Aims of this study

The known facts are that bacteriocin producers are immunized by immunity proteins that are encoded by genes that are co-transcribed in close vicinity to structural genes that encode bacteriocins. In a previous study of *Leuconostoc gelidum,* producer of class IIa leucocin A, it was reported that the leucocin A immunity protein interacts with an unknown ligand within the cytoplasm which influences the immunization mechanism (Kuvashie Pillay {not published}). The aim of this study was firstly to identify the unknown ligand that interacts with leucocin A immunity protein in the cytoplasm and secondly to uncover the region of leucocin A immunity protein involved in the interaction.

CHAPTER TWO

MAJOR TECHNIQUES OF THE STUDY

2.1 A RAPID TWO-STEP PROCEDURE FOR PURIFICATION OF BACTERIOCINS

There are various methods used in research for purification of bacteriocins, such as anion exchange chromatography, ammonium sulfate precipitation, and hydrophobic interaction chromatography (Mackay, *et al.*, 1997). For this study, leucocin A was purified from *L. gelidum* using a rapid two-step procedure. The procedure is simple and suitable for both small and large-scale purification of bacteriocins and cationic peptides. This method involves SP sepharose fast flow chromatography in conjunction with reverse phase High Pressure Liquid Chromatography (HPLC) (Uteng *et al.*, 2002).

2.1.1 Cation exchange chromatography SP sepharose fast flow

2.1.1.1 Principle

Ion-exchange chromatography is a technique that separates molecules on the basis of their charge, with the stationary phase being the actual ion-exchange resin, while the mobile phase is an aqueous solution that passes through the stationary phase. The technique is usually performed in columns, where charged molecules are retarded in their movement depending on the magnitude of their charge. Amersham Biosciences range of Bio-process Media SP Sepharose Fast Flow, a cation exchanger that binds cations (e.g. bacteriocins) due to its negatively charged ion exchanger sulphopropyl group, was used (Figure 2.1). On account of the wide pH range in which the ion exchanger is charged, elution of targeted peptides is not based on the pH but ionic strength of the buffers (Anderssen *et al.*, 1998).

SP sepharose resin contain highly cross-linked agarose base matrix that gives the media (in which the bacteriocins are grown) chemical and physical stability as well as rigidity (Amersham Biosciences, Uppsala, Sweden). This media property minimizes pH variations during during purification. This low pH provides the cation exchanger exceptional flow characteristics and low backpressures. A Linear flow rate of 400–700 cm/h through a bed

height of 15 cm at a pressure of 1 bar is the standard (Amersham Biosciences, Uppsala, Sweden).

Taking Le Chatelier's Principle into account a constant pH buffer with low ionic strength is used to equilibrate the column before a chromatographic run and wash off unattached peptides. The targeted peptide is then eluted with a higher ionic strength buffer in order to replace the ion of the targeted peptide that attached to the exchanger with the counter ion of the elution buffer since the bond is not a covalent linkage. The column regeneration is accomplished by eluting reversibly bound material either with a high ionic strength solution or by increasing the pH (Anderssen *et al.*, 1998). The eluted sample from the SP sepharose is an impure crude product, since it contains all cationic peptides.

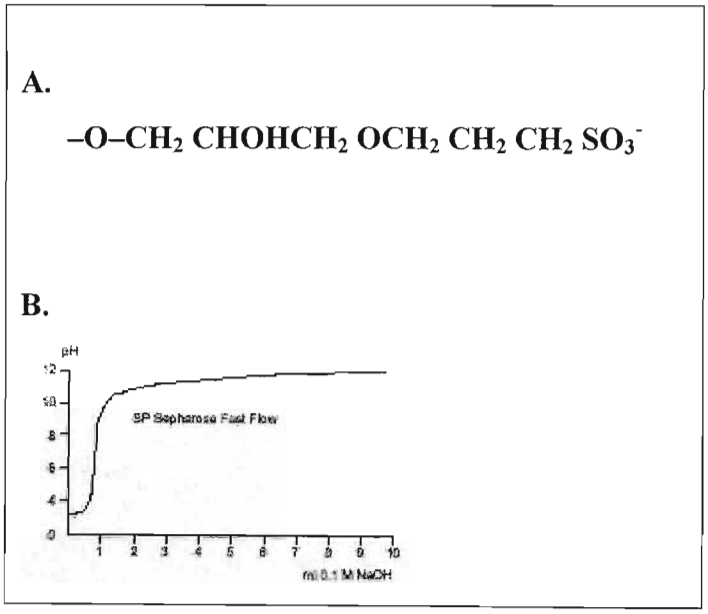


Figure 2.1. The SP sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden). **A.** An ion exchange group, sulphopropyl. **B.** An illustration of wide pH range in which the sulphopropyl group is charged.

2.1.2 Reverse Phase High Pressure Liquid Chromatography (RP-HPLC)

2.1.2.1 Introduction

Previously, a few reliable chromatographic methods were commercially available to laboratory scientists for separation of proteins. However, during 1970's a range of techniques were developed and used for chemical separations, these include open-column chromatography, paper chromatography, and thin-layer chromatography (Guo *et al.*, 1987; Chang *et al.*, 1994). Nevertheless, problems were encountered due to inadequacy of these chromatographic techniques with regards to quantification of compounds and resolution between similar compounds, consequently the HPLC was developed in the 1980's (Kunitani and Johnson, 1986). The instrument rose from theories that were originally developed for gas chromatography and were then applied to liquid chromatography. In addition, to purification of large biomolecules, the HPLC was used to separate polypeptides of closely identical sequence (that differ by one base pair), due to its high resolution. As a result the technique became the most commonly used chemical compounds separation method (Pearson *et al.*, 1982; Chang *et al.*, 1994). HPLC has improved since then with computers and automation added to the convenience of the technique (Kunitani and Johnson, 1986).

2.1.2.2 Principles

The RP-HPLC instrument functions according to the properties of the analytes (polypeptides) that are injected into the column. It retains the peptides until a certain concentration of the elution buffer is reached for the proteins to elute, i.e. gradient elution (McCroskey *et al.*, 1987).

RP-HPLC instruments (Figure 2.2A) consist of a reservoir of mobile phase - a solvent that is being continuously applied to the column, a stationary phase - a solid support contained within the column over which the mobile phase continuously flows, a pump that applies constant high pressure with no pulsating, an injector, a separation column, a detector and a recorder. The separation process starts by the injection of a sample solution through the

injector port, which is then carried by the mobile phase and adsorbed to the hydrophobic surface of the reversed-phase (RP) surface by the analyte's hydrophobic foot (which depends on the amino acid sequence) (Fahner *et al.*, 1999). During the interaction of the polypeptides and RP surface, it is the hydrophobic regions of the analytes that are involved in the interaction, where the rest of the peptide is in contact with the mobile phase (Hancock *et al.*, 1994).

Since in RP-HPLC the stationary phase (typically hydrocarbons, waxy liquids or bonded hydrocarbons such as silica that has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}) is nonpolar and the mobile phase (typically aqueous-organic mixtures such as methanol-water or acetonitrile-water) is polar with respect to the analyte, it is assured that the retention is the result of the interaction of the nonpolar components of the solutes and the nonpolar stationary phase (Pearson *et al.*, 1982). Polar molecules interact more strongly with the mobile phase than with the stationary phase and hence they elute from the column faster, and thus have shorter retention times. In contrast, the non-polar molecules, which have stronger interactions with the stationary phase than with the mobile phase that elute from the column less slowly, and thus have longer retention times. As a result different components, for example, polypeptides in a sample mixture pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase (Lu *et al.*, 2001).

The steady high pressures provided by the pumps drive the mobile phase through the column, and give the components less time to diffuse within the column, which leads to improved resolution in the resulting chromatogram (Lu *et al.*, 2001). Reversed phase columns use acids such as trifloroacetic acid at a very low percentage as high acid concentrations can corrode the metal parts of the HPLC instrument (Liu *et al.*, 2004). No aqueous bases are used as these will destroy the silica. Additionally, all solvents used on the instrument are degassed to eliminate formation of bubbles (McCroskey *et al.*, 1987; Fahner *et al.*, 1999).

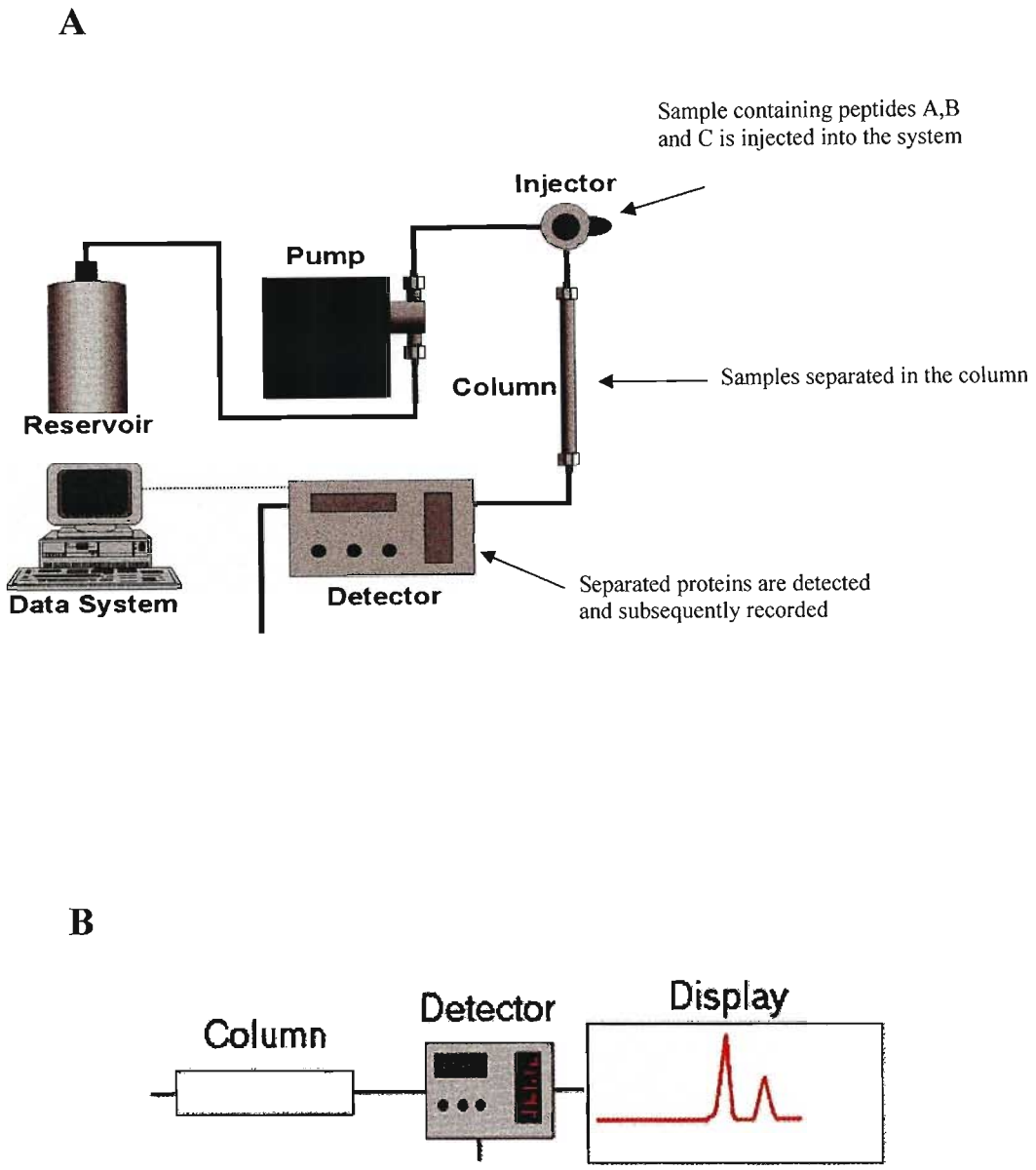


Figure 2.2. An illustration of the RP-HPLC instrument arrangement and formation of a chromatogram. (Modified from LC Resources, Walnut Creek, CA).
A. All the components of the RP-HPLC instrument are involved in the separation of peptides (LC Resources, Walnut Creek, CA). **B.** HPLC outputs the results in a form of a chromatogram, which is a plot of detector response as a function of time. As the column separates the polypeptides they are detected and displayed in the chromatogram.

As the proteins elute from the column they are detected by the detector. The detector emits a pulse in response to the eluting peptide, which registers as a peak on the chromatogram. The peak is recorded on the recorder (Figure 2.2B).

2.1.2.3 Applications

RP-HPLC inistrument is used in biotechnological, biomedical and biochemical research as well as for the pharmaceutical, food and environmental industries (Liu *et al.,* 2004). It will continue to be one of the most important laboratory separation techniques for analytical and preparative purposes such as analysis of protein therapeutic products, protein identity, detection of genetic changes and protein degradation and verification of intact protein conformational changes.

2.2 MASS SPECTROMETRY

2.2.1 Introduction

Mass spectrometry (MS) also known as Mass Spectroscopy, is an analytical spectroscopic instrument that is principally used for measuring the molecular weight (MW) of a sample by measuring the mass-to-charge ratio of ions within the sample. MS can be used in the analysis of many types of samples from elemental to large proteins and polymers to determine their composition by generating a mass spectrum representing the masses of sample components (Barber *et al.*, 1981). MS began in the early 20th century with J.J. Thomson, who used a vacuum tube to demonstrate the existence of electrons and positive rays. Thomson observed that the technique could also be used to analyse chemicals. For nearly thirty years this instrument was fundamentally used to discover a number of isotopes, to determine the relative abundance of isotopes, and to measure isotope masses (Dalton and Glish, 2003). However, these important fundamental measurements laid the foundation for later developments in diverse fields ranging from geochronology to biochemical research (Schramm, 2004).

The detector used in this study is the Time-of-flight (ToF) analyser, which uses an electric field to accelerate ions at the same potential and measure the time it takes for the ions to reach the detector.

2.2.2 Principle

Mass spectrometers can be divided into three fundamental parts, namely the ionisation source where sample under investigation has to be introduced, the analyser, which resolves the ions formed in the ionisation source according to mass, and lastly the detector, which produces a signal from the separated ions (Ashcroft, 2006). These mass spectrometer parts are maintained under high vacuum to give the ions a reasonable chance of travelling from one end of the instrument to the other without any hindrance from air molecules.

2.2.2.1 Ionisation source

There are two methods for sample introduction into the system (ionisation source). In the first method, the sample is inserted directly into the ionisation source. In the second method the sample is chromatographically separated, the *en route* to the ionisation source. The latter method usually entails the mass spectrometer being coupled directly to a high pressure liquid chromatography (HPLC), gas chromatography (GC) or capillary electrophoresis (CE) separation column, which separate the sample into a series of components which then enter the mass spectrometer sequentially for individual analysis (Glish *et al.*, 1980).

Inside the ionisation source the sample molecules are ionised, because ions are easier to manipulate than neutral molecules. MS produces charged particles (ions) from these samples by bombarding them with electrons, which result in the analysed chemical substances being positively charged since MS always work with positive ions, with the exception of electrospray MS, which uses negative ions (Dalton and Glish, 2003).

2.2.2.2 Analysis

Subsequent to the ionisation process, charged particles are accelerated to ensure that they all have the same kinetic energy and therefore will separate based on their masses. The ions are then subjected to a MS electric and/or magnetic fields, which deflects them according to their mass -to-charge ratios (m/z) ratios. The lighter they are, the more they are deflected and the faster they reach the detector as illustrated in Figure 2.3. The time the ions take to reach the detector is measured and can be used to determine the mass-to-charge ratio of the particle (Glish *et al.*, 1980).

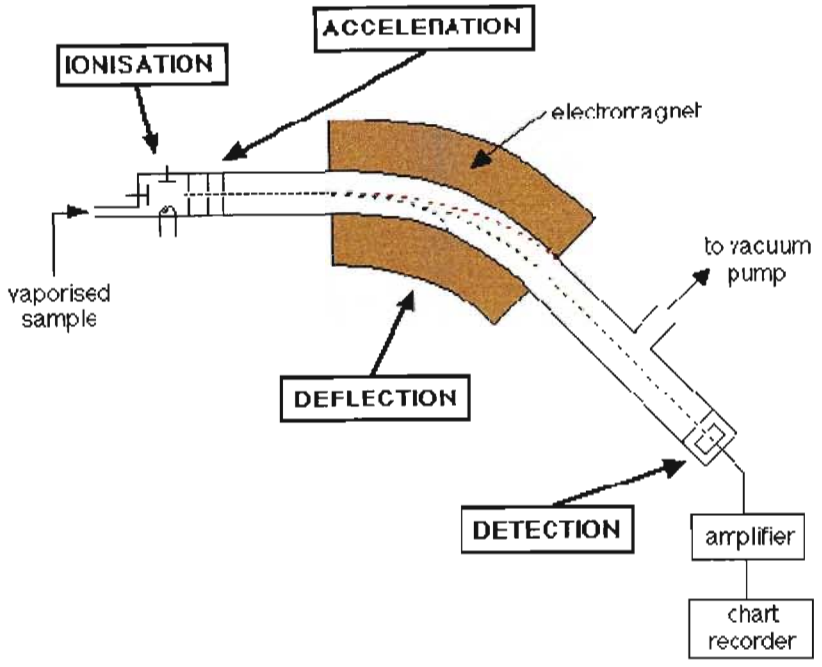


Figure 2.3. A Mass Spectrometer system.
(http://en.wikipedia.org/wiki/Mass_spectrometry).
The samples are introduced into the system and are ionised into positive charged particles and subsequently deflected by a magnetic field where they are analysed according to their masses that are interpreted to infer the amino acid sequence

2.2.2.3 Detection

The detector monitors the ion current and amplifies it. A signal is then transmitted to the data system where the m/z ratios are stored together with their relative abundance and are recorded in the form of mass spectra (m/z spectrum), which is interpreted with computer programs to determine the structure and elemental composition of the molecule (Glish *et al.*, 1980). The m/z values of the ions in the sample are plotted against their intensities on a stick-diagram. The above data is then processed by the instrument software to determine the molecular weight of each ion and is displayed on a stick-diagram (Glish and Vachet, 2003).

The accuracy of the techniques varies with the sizes of the molecules. For large molecules, the molecular weights can be measured to within 0.01% of the total molecular weight, which is sufficient to allow minor mass changes to be detected, e.g. the substitution of one

amino acid for another, or a post-translational modification. The molecular weights of smaller molecules can be measured to within an accuracy of 5 ppm, which is often sufficient to confirm the molecular formula of a compound.

2.2.3 Applications

Mass spectrometers are currently used in industry and academia scientific research for both routine and research purposes, as a sequencing tool. The technique generates structural information of peptides or oligonucleotides by fragmenting and analysing the molecule. It can be used for the detection of specific drug metabolites in biological matrices and in reaction monitoring, such as, enzyme reactions, chemical modification, protein digestion (Glish and Vachet, 2003). The technique is used in proteomics, mainly for protein identification, since the technique allows even tiny amounts of a protein to be identified both by its sequence and through database searches of protein fingerprints (Dalton and Glish, 2003; Glish *et al.*, 1980). Furthermore, the technique is used for all kinds of chemical analyses, ranging from environmental analysis to the analysis of petroleum products, trace metals and biological materials including the products of genetic engineering (Glish and Vachet, 2003).

2.3 SURFACE PLASMON RESONANCE

2.3.1 Introduction

Surface plasmon resonance (SPR) is an optical method for measuring biomolecular interactions. In this particular study, the technique was used to measure the kinetics of protein-ligand interaction of purified proteins. Figure 2.4 illustrates the most common geometrical set up of the instrument, which is the Kretschmann configuration (Biacore AB, Uppsala, Sweden). In the Kretschmann configuration of SPR for antibody interaction measurement, a very thin layer of the absorbate, for example an antibody is immobilised on a metal surface (sensor chip), which is placed onto a glass substrate. The appropriate analyte, for example, an antigen is injected with the flow cell, and applied across the metal surface. The absorbate - analyte interaction is measured directly and represented as a sensogram (van der Merwe and Barclay, 1994; Jonsson *et al.*, 1991).

Although there are several SPR-based systems, by far the most widely used one is the BIAcore, produced by BIAcore AB, which has developed into a range of instruments (Jonsson *et al.*, 1991; Biacore AB, Uppsala, Sweden). SPR is a powerful tool for the analysis of protein-protein interactions, particularly low affinity interactions, which are difficult to study using any other technique. Through binding analysis, the technique provides a quick way of evaluating macromolecules evaluation, for example, the structural integrity of recombinant molecules (MacKenzie *et al.*, 1997). SPR is also very useful for analysis of mutant proteins, as well as evaluation of the effects of the mutation on the binding properties, such as, affinity, enthalpy, stoichiometry kinetics, and thermodynamics of the immobilised protein (Jonsson *et al.*, 1991).

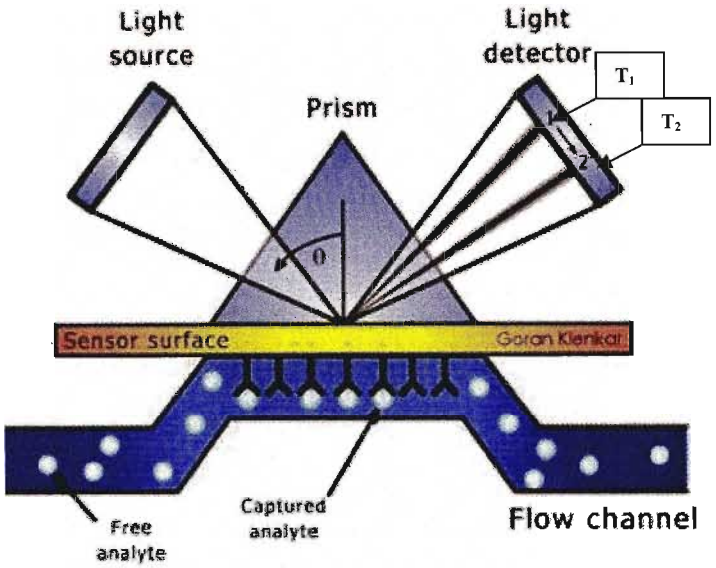


Figure 2.4. Depiction of surface plasmon resonance technology. (BIAcore Technical manual, Biacore AB, Uppsala, Sweden)
The sensor surface is a gold metal and is referred to as plasmon. The absorbates are attached to the plasmon. The analytes are injected with the flow cell. The two grey lines 1 and 2 in the reflected beam projected on to the detector symbolise the light intensity drop following the resonance phenomenon at time t1 and t2. The line projected at t1 corresponds to the situation before binding of analytes to the absorbates on the surface and t2 is the position of resonance after binding.

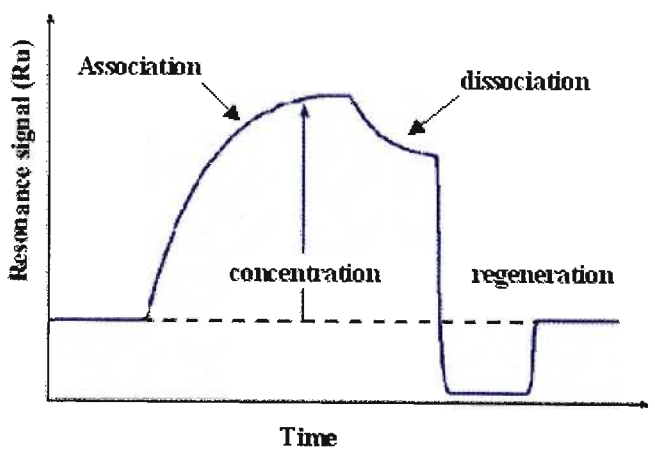


Figure 2.5. A SPR Sensorgram. (BIAcore Technical manual, BIAcore AB, Uppsala, Sweden).
The profile is a result of association and dissociation reactions that occur.

2.3.2 Principles

The incoming beam of light passes from material with a high refractive index (the sensor surface) into material with a low refractive index (flow cell) through which an aqueous solution (the running buffer) passes under continuous flow (O'Shannessy *et al.,* 1993). This movement of the light beam through different indexes causes some light to be reflected from the interface. When the angle at which the light strikes the interface (the angle of incidence or θ) is greater than the critical angle, the light is completely reflected (total internal reflection) and no light is refracted across the interface (Figure 2.4) (Biacore AB, Uppsala, Sweden).

As the incident light is totally reflected, the electromagnetic field component creates an exponentially detenuating evanescent wave by penetrating a short distance into a medium of a lower refractive index. If the surface of the glass is coated with a thin film of a noble metal, generally gold, this reflection is not total. The reflected light intensity is reduced at a specific incident angle producing a sharp shadow, called surface plasmon resonance due to the resonance energy transfer between evanescent wave and surface plasmons. This reduction is due to interaction of some of the light with the delocalised electrons in the metal film (Jonsson *et al.*, 1991). The angle greater than the critical angle at which this reduction of reflected light intensity is greatest and at which the intensity of reflected light reaches a minimum is referred to as a dip, and is called the surface plasmon resonance angle (spr) (Parsons and Stockley, 1997).

If complimentary analyte binds to the absorbate, the refractive index shifts and the SPR-dip moves to larger angles, as the accumulation of protein on the surface results in an increase in the refractive index (O'Shannessy *et al.,* 1993; MacKenzie *et al.*, 1997; van der Merwe *et al.*, 1997). The movement of the SPR-dip is the actual monitored signal, and the movement over time forms the sensorgram. This change in refractive index is measured in real time by detecting changes in the intensity of the reflected light, and the result is plotted as response or resonance units (RUs) versus time on a sensorgram (Figure 2.5). During injection of analyte, the changes in signal result from two processes, either the association to or dissociation from the surface. At the end of injection, running buffer continues to

flow over the chip. At this stage the change in signal results from dissociation only (Parsons and Stockley, 1997).

For the association and dissociation phases of the reaction the rates of change of the SPR signal can be analysed to yield apparent rate constants. The ratio of these values gives the apparent equilibrium constant (affinity). The size of the change in SPR signal is directly proportional to the mass being immobilised and can thus be interpreted crudely in terms of the stoichiometry of the interaction (MacKenzie *et al.*, 1997).

2.3.3 Advantages

A major advantage of SPR is that much smaller amounts of proteins can be used, since signals can be obtained from sub-microgram quantities of material. Given that the SPR signal depends only on binding to the immobilised template, it is also possible to study binding events from molecules in extracts and hence not necessary to have highly purified components (Biacore AB, Uppsala, Sweden). The technique also allows for the study of biomolecular interactions in real-time. There is no need for labelling for SPR, which helps save time and preserves biomolecular nativity of the compounds (O'Shannessy *et al.*, 1993).

2.3.4 Limitations

It is used less, due to its high cost (O'Shannessy *et al.*, 1993). SPR is not well suited to high-throughput assays or the analysis of small molecules (Mr < 1000). However, the pitfalls associated with SPR are easily avoided once they are understood (van der Merwe *et al.*, 1997; Assiongbon *et al.*, 2004).

2.4 N-TERMINAL SEQUENCING

2.4.1 Introduction

Perkin Elmer Applied Biosystems Model 494 Precise protein/peptide sequencer with an on-line Perkin Elmer Applied Biosystems Model 140C PTH Amino Acid Analyser was used for amino acid sequence determination. The instrument uses a chemical process that is derived from the degradation method developed by Pehr Edman (Edman, 1964). The Edman Sequencing service gives two services, namely, protein characterization and identification. The former was employed in this study for N-terminal sequence analysis using automated Edman degradation chemistry (Chen *et al.*, 2007).

2.4.2 Principles

Following in-gel or polyvinylidene difluoride (PVDF) bound protein digestions, the protein is introduced into the system. Inside the system, the protein is adsorbed onto a solid surface, generally a cationic polymer such as glass fiber coated with polybrene. The protein undergoes repetitive cycles that involve three steps. Firstly, the labeling step, where the Edman reagent phenylisothiocyanate (PITC) is coupled with the amine group of the N-terminal amino acid of the protein to form a phenylthiocarbamyl (PTC) group. Secondly is the cleavage of the terminal amino acid derivative by anhydrous acid, TFA (Sigma) to generate an anilinothiazolinone (ATZ) amino acid, which leaves the new amino terminus for the next degradation cycle. The last step is the conversion of the ATZ derivative to the more stable phenylthiohydantoin (PTH) derivative. The three stages are followed by PTH analysis using microbore HPLC. Each cycle takes 30-50 minutes depending on the instrument used and it produces a PTH chromatogram that identifies one amino acid. The chromatograms are collected using a computer data analysis system (Henzel *et al.*, 1999).

Regularly the first cycle of the sequencing run is a standard mixture of 19 PTH-amino acids, which is also introduced into the column for separation. This standard chromatogram provides standard retention times of the amino acids for comparison with each Edman degradation cycle chromatogram (Henzel *et al.*, 1999). This is done by overlaying one chromatogram of the unknown amino acids of the protein on top of the standard chromatogram, to see the corresponding amino acid (Figure 2.6). Only qualitative analysis is required, so the amino acid does not have to be eluted from the chromatography column.

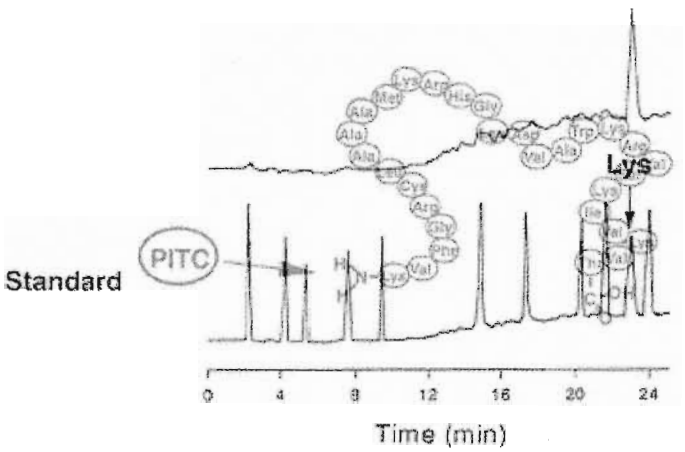


Figure 2.6. Typical chromatograms produced during N-terminal sequencing of peptides. (Procise LC Protein Sequencer, PE Applied Biosystems).
A chromatogram produced by an unknown amino acid is superimposed with a chromatogram produce by the standards in order to identify the amino acid. From the diagram it is clear that the amino acid is lysine. PITC reagent is coupled with the amine group of the N-terminal amino acid of the protein to form a PTC group, which occurs in the labeling step.

2.4.3 Limitations

Edman degradation proceeds from the N-terminal of the protein, therefore any modification of the N-terminal amino group will hinder the process (Perkin-Elmer Manual, 1995). Additionally, blockage of the N-terminal during sample preparation results in unobtainable data. Another limitation is that, the technique cannot determine the position of the disulfide bridges (Hirano *et al.*, 1992).

CHAPTER THREE

MATERIALS AND METHODS

3.1 STRAINS AND PLASMIDS

The strains and plasmids used in this study were obtained from the laboratory collection at the University of Kwa-Zulu Natal, Pietermaritzburg (Table 3.1A and 3.1B).

Table 3.1A. List of bacterial strains used in this study.

Strain	Attributes	Source or Reference
Leuconostoc gelidum		
UAL 187-22	LcnA$^+$ Imm$^+$ containing native plasmids pLG9.2 and pLG 7.6	Hastings and Stiles, 1991
UAL 187-13	LcnA$^-$ Imm$^-$ containing native plasmids pLG 9.2	Hastings and Stiles, 1991
Listeria monocytogenes		
	Leucocin A sensitive	Ramnath *et al.*, 2000
Ecsherichia coli		
JM103	*thr rpsL endA sbc-15 hsdR4* Δ (lac pro AB) F' *traD36 pro AB, lacZΔM15*	Sambrook *et al.*, 1989

Table 3.1B. List of plasmids used in this study.

Plasmids	Attributes	Source or Reference
pJF5.5	Amp^r, LcnA$^+$ Imm$^+$	Felix *et al.*, 1994
pMAL-c2	malE, lacZ α, Amp^r	New England BioLabs
pKP		
pKP 7.0	Amp^r , malE-Imm, pMALc-2 derivative	Pillay, 2004
pKP 7.0A	Amp^r, malE-Imm-versionA, pMAL-c2 derivative	This study
pKP 7.0B	Amp^r, malE-Imm-versionB, pMAL-c2 derivative	This study

3.2 BACTERIOCIN LEUCOCIN A

3.2.1 Production of leucocin A by *Leuconostoc gelidum*

The leucocin A producer *Leoconostoc gelidum* UAL 187-22 was cultured with De Man Rogosa Sharpe (MRS) broth and agar (Merck, South Africa, Gauteng). *L. gelidum* UAL 187-22 was grown in 1000 ml MRS broth at 25°C for a minimum of 2 days to an optical density λ_{600} of 0.5 and centrifuged at 10 000 x g for 15 minutes to obtain the supernatant containing crude bacteriocin. *L. gelidum* UAL 187-13, a leucocin negative strain (negative control), was grown under the same conditions (Hastings and Stiles, 1991).

3.2.2 Leucocin A characterization

3.2.2.1 Bacteriocin activity assay

The supernatant of both *L. gelidum* strains were tested for the presence of bacteriocin using the spot on-lawn assay as described by Hastings *et al.* (1991). A MRS agar plate was overlaid with TSA molten agar containing the indicator strain *Listeria monocytogenes,* a food isolate (Ramnath *et al.*, 2000). *L. monocytogenes* was grown in tryptone soy broth (TSB) (Merck, South Africa, Gauteng) at 30°C for 8 hours (Ramnath *et al.*, 2000). Aliquots of 10 µl of the supernatants were spotted onto the overlay and left to dry for 30 minutes, after which the plate was incubated overnight at 25°C. The colonies of both *L. gelidum* strains were assayed, by three-way streaking on a *Listeria* lawn and observing zones of growth inhibition after 8 hours to overnight incubation.

3.2.2.2 Proteinase K sensitivity assay

The supernatant containing the crude bacteriocin was exposed to proteinase K (Roche, Germany, Mannheim) by performing a half moon assay to confirm that the active

substance was indeed proteinaceous. The half moon assay was performed, where a sterile filter (Rotrand/Red; rim diameter, 0.2 mm) saturated with proteinase K (100 $mg.ml^{-1}$) was used. Proteinase K (10 µl) and filtered supernatant (10 µl) were spotted 0.5 cm apart on MRS agar plates seeded with an 8 hour *L. monocytogenes* culture. The plates were dried for 30 minutes and incubated overnight at 25°C. Protease sensitivity was observed as a half-moon-shaped zone of inhibition.

3.2.3 Purification of leucocin A

3.2.3.1 Cation exchange chromatography

Bacteriocin purification was achieved by following a two-step purification method described by Uteng *et al.* (2002). Initially the crude bacteriocin sample was passed through an ion exchange column, by loading the crude sample (1000 ml) into a 10 ml SP Sepharose Fast Flow column (Amersham Pharmacia Biotech) equilibrated with 20 mM sodium phosphate buffer pH, 5.8. Purification was performed at temperatures below 20°C. The column was subsequently washed with 15 column volumes of the phosphate buffer. The flow through and the washes were collected and assayed for activity. The bacteriocin was eluted with 20 mM sodium phosphate buffer, pH 5.8, containing 1 M NaCl. Elution was monitored at λ_{280}. The column was subsequently washed and regenerated with 20 mM sodium phosphate buffer, pH 5.8, containing 1.5 M NaCl and 20 mM sodium phosphate buffer, pH 5.8, respectively.

Eluted fractions were tested for bacteriocin presence by the spot on-lawn assay as described in 3.2.2.1. The fractions containing the leucocin A peptide were pooled, freeze dried and resuspended in 0.1% TFA. Freeze dried samples were stored at 4°C until further use.

3.2.3.2 Reverse Phase High Pressure Liquid Chromatography

Crude freeze-dried peptide samples in 0.1% TFA were further purified by reverse phase HPLC on a C-18 column (25 cm x 4.6 mm, 5 μm particle size, 125 Å; pore size, 12.5 nm) as described by Uteng *et al.* (2002). Aliquots (1 ml) of the crude samples were clarified by centrifugation at 4 000 x g for 10 minutes and injected and eluted at a flow rate of 1 ml per minute. The program used is outlined in Table 3.2. Leucocin A was eluted using a linear acetonitrile (BDH) gradient containing 0.1% TFA. The elution was monitored at λ_{220} and the fractions assayed for activity.

Table 3.2. Reverse Phase High Pressure Liquid Chromatography gradient used for leucocin A purification on a C-18 reverse phase column using an acetonitrile, 0.1% TFA.

Step#	Time (min)	Flow rate ($ml.min^{-1}$)	% Buffer A (Acetonitrile, 0.1%TFA)	Curvature
*Equil.	2.00	1.00	0.00	0.00
1	20.00	1.00	0.00	1.0.00
2	20.00	1.00	50.00	1.0.00
3	5.00	1.00	90.00	1.0.00
4	5.00	1.00	0.00	1.0.00

***Equil**: equilibration

3.2.4 Quantification of leucocin A

3.2.4.1 Double dilution assay

Leucocin A activity was tested at each of the following purification stages: *L. gelidum* supernatant, elute from the SP sepharose column, including the flow through and the wash, as well as HPLC purified bacteriocin. Aliquots (10 µl) of the fractions were diluted with 0.1% TFA in a dilution series to quantify leucocin A activity. Dilution series of all fractions were prepared by diluting the samples in a 2- fold series, i.e. the first dilution was 10 µl of sample + 10 µl of 0.1% TFA (dilution factor = ½), the second was 10 µl of the first dilution + 10 µl 0.1% TFA (dilution factor = ¼), repeated ten fold. All the dilutions were assayed by the spot on lawn assay and undiluted leucocin A extracts, were used as controls. The last dilution to produce an inhibition zone was used to calculate the activity, using formulae in Table 3.4.

3.2.4.2 Protein quantification

The concentrations of the purified peptides were determined using the Micro Bicinchoninic Acid (BCA) Assay Kit (Pierce).

BCA standards were prepared in test tubes by diluting a 200 $\mu g.ml^{-1}$ Bovine Serum Albumin (BSA) stock solution to different concentration dilutions listed in Table 3.3 (labeled A-H). In a different sterilin tube a working solution was freshly prepared, by mixing together Micro BCA reagent A (MA), Micro BCA reagent B (MB) and Micro BCA reagent C (MC) at a ratio 25:24:1, respectively. The working solution was dispensed in aliquots of 250 µl in different sterile eppendorfs into which 250 µl of BCA standards were added and the mixtures were incubated at 60°C for 60 minutes. Absorbance readings of the mixtures were taken at λ_{562} and used to construct a standard graph of absorbances at λ_{562} versus BCA standard concentrations (Figure 4.6). The peptides of unknown concentration were prepared under the same conditions as the BCA standards and their λ_{562} absorbance readings were used to extrapolate the concentration from the graph.

Table 3.3. Concentration of Bicinchoninic acid standards prepared by diluting the Bovine Serum Albumin stock solution with water.

Standard No.	H_2O *vol (ml)	BSA *vol (ml)	Final concentration (µg/ml)
A	8.00	0.20	40.00
B	4.00	4.00 (of A)	20.00
C	4.00	4.00 (of B)	10.00
D	4.00	4.00 (of C)	5.00
E	4.00	4.00 (of D)	2.50
F	4.80	4.00 (of E)	1.00
G	4.00	4.00 (of F)	0.50
H	8.00	0.00	0.00

*vol: volume

Table 3.4. Summary of the formulae used for quantification of leucocin A after purification.

Unknown	Formula
Activity ($Au.ml^{-1}$)	*Reciprocal of the dilution factor x (1000÷spot volume)
Total activity (Au)	Activity x (culture volume ÷ sample volume)
Protein concentration ($mg.ml^{-1}$)	*Protein concentration
Specific activity ($Au.mg^{-1}$)	Total activity ÷ protein concentration

***Reciprocal of the dilution factor**: e.g. last dilution to form a zone is 4^{th} dilution, which is 1/16, ∴the reciprocal is 16
***Protein concentration** : determined from BCA assay

3.2.5 Mass spectrometry

The purified leucocin A was confirmed by determining the size using Liquid Chromatography Mass Spectrometry (LCMS). The procedure was performed at Stellenbosch University using the Waters API Q-TOF Ultima instrument and the Phenomenex Prodigy ODS2, 1x100 mm, 5 µm column. Leucocin A (50 µl) was diluted in water to make a final concentration of 1 $\mu g.ml^{-1}$. The volume of sample injected was 10 µl, at a flow rate of 50 µl per minute. Table 3.5 show the outline of the method used for the analysis of the peptide.

Table 3.5. Mass spectrometry gradient for leucocin A analysis on a Phenomenex Prodigy ODS2 column using TFA and methanol solvents, at a constant flow rate of 50 µl per minute.

Step#	Time (min)	*% Buffer A	*% Buffer B	Curvature
1	0.00	95.00	5.00	1.00
2	11.00	0.00	100.00	1.00
3	12.01	0.00	100.00	1.00
4	12.02	95.00	5.00	1.00
5	15.00	95.00	5.00	1.00

***Buffer A**: TFA
***Buffer B**: Acetonitrile

3.3 LEUCOCIN A IMMUNITY PROTEIN

Leucocin A immunity gene was amplified using PCR from the recombinant plasmid pJF 5.5 (Felix *et al.*, 1994) haboured in *E. coli* JM103 (Sambrook *et al.*, 1989). The amplicon was cloned into pMAL-c2 vector as outlined in Figure 3.1 (New England BioLabs).

3.3.1 Plasmid DNA isolation

The plasmids pJF 5.5 and pMAL-c2 were isolated using a Nucleobond AX 100 Plasmid Purification Kit (Macherey-Nagel, Duren, Germany) for high-copy plasmids according to manufacture's instructions. A single bacterial colony of *E. coli* JM103 strain containing the appropriate plasmid was inoculated into 30 ml Luria Bertani (LB) broth containing ampicillin at a final concentration of 100 $\mu g.ml^{-1}$. The inoculated culture was grown at 37°C overnight in a vigorously shaking water bath. From the overnight culture, 700 µl was removed and added to 300 µl of 80% (v/v) glycerol for storage at –70°C in a 1.5 ml sterile microfuge tube. The remaining culture was harvested by centrifugation at 6 000 x g in a JA 20.1 rotor of a Beckman centrifuge (J2-21M) for 15 minutes at 4°C. The supernatant was discarded and the cells were resuspended in 4 ml of S1 buffer (50 mM Tris-HCl, 10 mM EDTA, 100 $mg.ml^{-1}$ RNase A, pH 8.0) and 4 ml of S2 buffer (200 mM NaOH, 1% SDS) was added. The suspension was mixed gently by inverting the tubes 6-8 times, followed by the incubation at room temperature for 5 minutes. Pre-cooled S3 buffer, 4 ml (2.8 M Potassium acetate, pH 5.1) was added and the suspension was incubated in ice for 5 minutes.

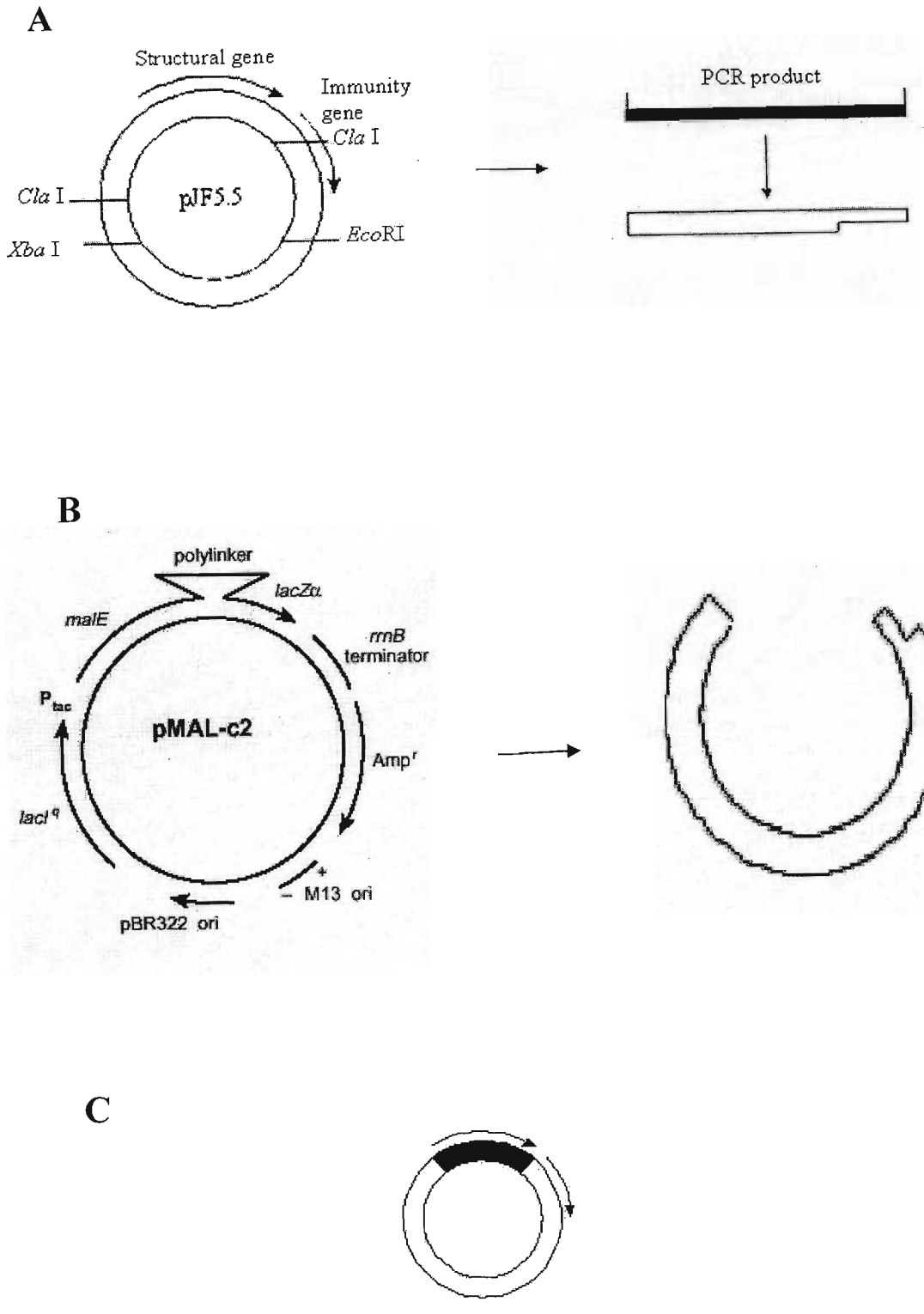


Figure 3.1. A flow diagram illustrating the amplification and cloning of the immunity gene into pMAL-c2 vector. **A.** Amplification and restriction digest of the immunity gene. The gene was amplified from the recombinant plasmid pJF 5.5, and cleaved with the enzyme (endonucleases) *Xba*I at the C-terminal, which resulted in the formation of a sticky-end. **B.** Restriction digest of the pMAL-c2 vector. The vector was cleaved at the polylinker site with restriction enzymes *Asp700* and *Xba*I to generate blunt and sticky ends respectively. **C.** The diagram of a recombinant pMAL-c2 vector with the immunity gene (black) inserted down-stream from the *mal*E gene. The arrows represent the transcription direction.

The lysed cells resulted in a formation of a white precipitate, which was filtered, using NucleoBond filters. The clear lysate was loaded onto a NucleoBond AX 100 column pre-equilibrated with 2.5 ml of N2 buffer (100 mM Tris, 15% ethanol, 900 mM KCl, 0.15% Triton X-100, pH 6.3). Following loading, the column was washed with 10 ml N3 buffer (100 mM Tris, 15% ethanol, 1.15 M KCl, pH 8.5). The plasmid DNA was eluted with 3.5 ml N5 buffer (100 mM Tris, 15% ethanol, 1 M KCl, pH 8.5) and the DNA was precipitated with 3.5 ml isopropanol. The DNA was pelleted by centrifuging the suspension at 15 000 x g for 30 minutes at 4°C. The supernatant was discarded and the pellet, which contained DNA, was washed twice in 2 ml of 70% ethanol and centrifuged at 15 000 x g for 10 minutes. The DNA was air-dried at room temperature for a maximum of 20 minutes, and resuspended in 50 µl of Tris-EDTA (TE) buffer (100 mM Tris-HCl, pH 7.5; 100 mM EDTA).

3.3.2 Analysis of DNA by agarose gel electrophoresis

The isolated plasmid DNA was analysed using agarose gel electrophoresis. An agarose gel of 0.8% (w/v) was prepared using electrophoresis grade agarose (Pionadisa), dissolved in Tris-acetate-EDTA (TAE) buffer (0.4 M Tris, 10 mM Na_2EDTA, 10 mM Na acetate, pH 8.5) by heating. After cooling to ≈55°C, ethidium bromide (0.5 $\mu g.ml^{-1}$ in distilled H_2O) was added and the gel was allowed to solidify. Purified plasmid DNA (5 µl) was mixed with 2 µl DNA loading buffer and 5 µl of sterile distilled H_2O and the mixture was loaded on the agarose gel. Gels are electrophoresed at 80 V for 90 minutes. DNA bands were visualized using a UV transilluminator of the Versadoc 2000 image analyser (BioRad).

3.3.3 Quantitation of purified DNA

Plasmid DNA was quantified through spectrophotometry. Purified DNA was diluted 1: 100 in TE buffer. The optical densities were determined at λ_{260} and λ_{280}, in order to determine the purity of the nucleic acids, which is obtained from the ratio of $\lambda_{260}/\ \lambda_{280}$

readings. A ratio of 1.8 denoted a pure DNA sample. The reading at λ_{260} was used to calculate the DNA concentration using the formula: λ_{260} x dilution factor x 50 = x $\mu g.ml^{-1}$.

3.3.4 Immunity gene isolation

3.3.4.1 Amplification of the immunity gene

The immunity gene was amplified from pJF 5.5 using primers EAL-2 and EAL-3 (Felix *et al.*, 1994) (Figure 3.1A) by the automated Perkin Elmer 9600 thermal cycler (Perkin-Elmer, Applied Biosystem). The PCR reaction mixture was composed of reagents set out in Table 3.6. The primer sequences are shown in Figure 3.2, the reverse primer has the *Xba*I site added on the 3' end.

Forward: EAL-2

5' **ATT CTA GAC AAA GTC ATT TAT CTT TCA AAG ATA** 3'

Reverse: EAL-3

5' **ATG AGA AAA AAT AAC ATT TAT TGG ACG** 3'

*Xba*I sit

Figure 3.2. The primer sequences for amplification of the immunity gene, *Xba*I site added on the 3' end of EAL-3.

The PCR amplification profile used was: (i) initial denaturation at 95°C (5 minutes), (ii) 30 cycles of denaturation at 94°C (30 seconds), annealing at 55°C (30 seconds), elongation at 72°C (30 seconds), (iii) final elongation at 72°C (7 minutes) and (iv) cooling to 4°C.

Table 3.6. Reaction components used for the immunity gene amplification from recombinant plasmid pJF 5.5.

Reagents	*Vol (µl)	*Quant	*TR	*Cont1	*Cont2
10x PCR Buffer with $MgCl_2$	10.00	1x	✓	✓	✓
10mM dNTPs mix	2.00	0.20 mM	✓	✓	✓
Primer EAL-2	1.00	300.00 nM	✓	×	✓
Primer EAL-3	1.00	300.00 nM	✓	×	✓
DNA template	2.00	0.12 $mg.ml^{-1}$	✓	✓	×
Taq (Roche)(1U μl^{-1})	2.50	1U	✓	✓	✓
Sterile dH_2O	81.50		81.50	83.50	83.50
Total	100.00		100.00	100.00	100.00

***Vol**: Volumes taken from the stock solutions
***Quant** : Quantity (final concentrations of the reagents)
***TR**: Test reaction
***Cont**: Control reaction

3.3.4.2 Purification of the amplicon

The amplicon was cleaned up with the NucleoSpin PCR Clean Up Kit (Macherey-Nagel Duren, Germany) according to the instruction manual. The supernatant contained the clean amplicon, which was quantified at λ_{260} and analysed on agarose gel electrophoresis. Molecular weight sizes were determined using the BioRad Quantity One software package with BioRad prestain MWM (Molecular Weight Marker) as internal standards. The rest of the amplicon was collected and stored at –20°C until further use.

3.3.5 Cloning of the immunity gene

3.3.5.1 Blunt ending of the Amplicon

The purified amplicon (138 $\mu g.ml^{-1}$) was blunt ended with the Klenow enzyme. The reaction mixture for blunt ending was prepared in a sterile microfuge tube and contained: (i) 16 µl of PCR product, (ii) 2 µl of reaction buffer H, (iii) 1 µl of dNTP (2 mM) and (iv) 1

µl Klenow enzyme. The reaction was incubated in a 30°C water bath for 15 minutes. The Reaction was stopped by heating for 10 minutes in a 75°C water bath and stored at -20°C, until further use.

The blunt-ended product was analysed on a 2% agarose gel and viewed with a UV trans-illuminator of the Versadoc 2000. Fastruler High Range DNA Ladder (Fermentas) was included on the gel to verify the approximate size of the amplicon.

3.3.5.2 Restriction digest of the amplicon

The blunt ended amplicon (Klenow reaction) was digested with *Xba*I to generate a 3' sticky end (Figure 3.1 A). The reaction mixture was made up in a sterile microfuge tube, which contained: (i) Klenow reaction (17 µl), (ii) 2 µl reaction buffer H (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM Dithioerythritol (2 µl) and (iii) restriction enzyme *Xba*I (1 µl). The reagents were mixed and incubated in a 37°C water bath for 2 hours. The reaction was stopped by heating in a 65°C water bath for 10 minutes.

3.3.5.3 Restriction digest of pMAL-c2 DNA

The pMAL-c2 vector was double digested with the restriction enzymes *Asp*700 and *Xba*I, which created "sticky" and "blunt" ends, respectively (Figure 3.1B). The reaction mixture (20 µl) in a sterile microfuge tube contained the enzymes, 1 µl each (10 U), 2 µl reaction buffer B (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 $mg.ml^{-1}$ BSA) and 16 µl pMAL-c2 DNA (3376 $\mu g.ml^{-1}$). The reaction was incubated in a 37°C water bath for 4 hours and subsequently stopped by heating in a 65°C water bath for 10 minutes. The restricted DNA samples were analysed by electrophoresis on a agarose gel (1% [w/v]) using the Molecular Weight Marker III (Roche) to estimate the approximate DNA fragment size obtained, the gel was viewed with a Versadoc UV transilluminator.

3.3.5.4 Ligation procedure

The immunity gene was cloned into the pMAL-c2 vector through a ligation procedure. The ligation reaction was prepared in a sterile microfuge tube containing the restricted DNA in a 1 : 5 (vector : insert) volume ratio. The reaction was prepared as follows: (i) 13.4 µl insert and (ii) 2.6 µl vector, were mixed and heated in a 65°C water bath for 5 minutes and subsequently cooled on ice, (iii) 2 µl 10x ligation buffer (660 mM Tris-HCl, 50 mM $MgCl_2$, 50 mM Dithiothreitol (DTT), 10 mM ATP, pH 7.5) and (iv) 2 µl T4 DNA ligase (U mg^{-1}) were added into the reaction. The reaction was incubated overnight (≈15 h) in a 16°C water bath, and stopped by heating at 65 °C for 5 minutes. Ligation reactions were stored at -20°C until further use.

3.3.5.5 Transformation procedure

3.3.5.5.1 Preparation of competent cells

A single colony of *E. coli* JM103 strain was inoculated into 10 ml LB broth and grown overnight in a 37°C water bath, with vigorous shaking. From the overnight culture, 500 µl was sub-cultured into 100 ml LB broth and grown in a 37°C water bath with vigorous shaking to λ_{600} of 0.3 - 0.4. The culture was dispensed into 2 x 50 ml pre-chilled sterile tubes and left to cool on ice for 10 minutes. The cells were pelleted by centrifugation at 5 000 x g at 4°C for 10 minutes, the supernatant was discarded and the pellets were dissolved in 10 ml cold buffer (10 ml, 60 mM $CaCl_2$, 10 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 7.0). Solutions containing the cells were combined and centrifuged at 4 500 x g at 4°C for 10 minutes, the supernatant was discarded and the pellet was resuspended in ice cold buffer (as described above). The competent cell were aliquoted in 200 µl volumes in sterile microfuge tubes and incubated on ice for 30 minutes before used, or stored in a -70°C freezer for later use.

3.3.5.5.2 Transforming into competent cells

Competent cells were thawed on ice and mixed by finger tapping. Transformation mixtures were prepared in sterile microfuge tubes. For each mixture, 100 µl of competent cells was mixed with 10 µl ligation mixture by finger tapping. The mixtures were incubated on ice for 30 minutes and subsequently heat shocked at 42°C for 90 seconds. Prewarmed LB broth, 900 µl aliquot was added into each mixture, and incubated at 37°C for 1 hour. Aliquots of 200 µl of the transformation mixtures were plated on LB agar plates containing 100 $\mu g.ml^{-1}$ ampicillin, 80 $\mu g.ml^{-1}$ 5 Bromo-4 chloro-3 indolyl-β-D-galactopyranoside (X-gal), dissolved in dimethyl formamide, and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) using the 'hockey-stick' streaking method. The plates were incubated at 37°C for 16 hours. An uncut pMAL-c2 vector was transformed into competent cells and this served as a transformation control. Competent cells were plated on ampicillin supplemented LB plates and on unsupplemented LB plates, which served as negative and positive controls for the competent cells aptitude. The plates were incubated at 37°C for 16 hours.

The overnight plates were observed for the presence of blue and white colonies (Sambrook *et al.*, 1989). The white colonies on the X-gal plates were speculated to be representatives of recombinant clones. Using sterile toothpicks, white colonies ($\approx < 10$) were picked from transformation plates and transferred into different fresh LB tubes containing 100 $\mu g.ml^{-1}$ ampicillin and grown overnight in a shaking 37°C water bath. Glycerol stocks (30%) of the successfully grown overnight cultures were prepared.

3.3.6 Truncation of the leucocin A immunity gene

The wild type immunity gene (blunt ended amplicon) was truncated into two versions by cleaving off the terminal regions that were cloned into pMAL-c2 vector and transformed into *E. coli* JM103 strain.

3.3.6.1 Cleavage of the C-terminal (Version A)

The *Hae*III enzyme (Roche) was used to cleave off the C-terminal region from the 342 bp immunity gene. A 20 µl reaction mixture was prepared, containing the following reagents: (i) 16 µl blunt ended amplicon, (ii) 2 µl (10x) restriction buffer M (100 mM Tris-HCl, 500 mM NaCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.5 at 37°C), (iii) 1 µl *Hae*III enzyme and (iv) 1 µl *Xba*I enzyme. The reagents were mixed by finger tapping and incubated at 37°C for two hours. The reaction was stopped by heating at 65°C for 15 minutes.

3.3.6.2 Cleavage of the N-terminal (Version B)

The *Cla*I enzyme was used to cleave off the N-terminal region from the immunity gene. The 20 µl reaction mixture constituted of: (i) 16 µl blunt ended amplicon, (ii) 2 µl (10x) restriction buffer and (iii) 2 µl *Cla*I enzyme. The reagents were mixed by finger tapping and incubated at 37°C for two hours. The reaction was stopped by heating at 65°C for 5 minutes.

3.3.6.3 Cloning of the immunity gene versions A and B

The pMAL-c2 vector was digested as in paragraph 3.3.5.3. The immunity versions were cloned into the vector (Figure 3.3) and transformed following the procedures described in paragraph 3.3.5.5.2.

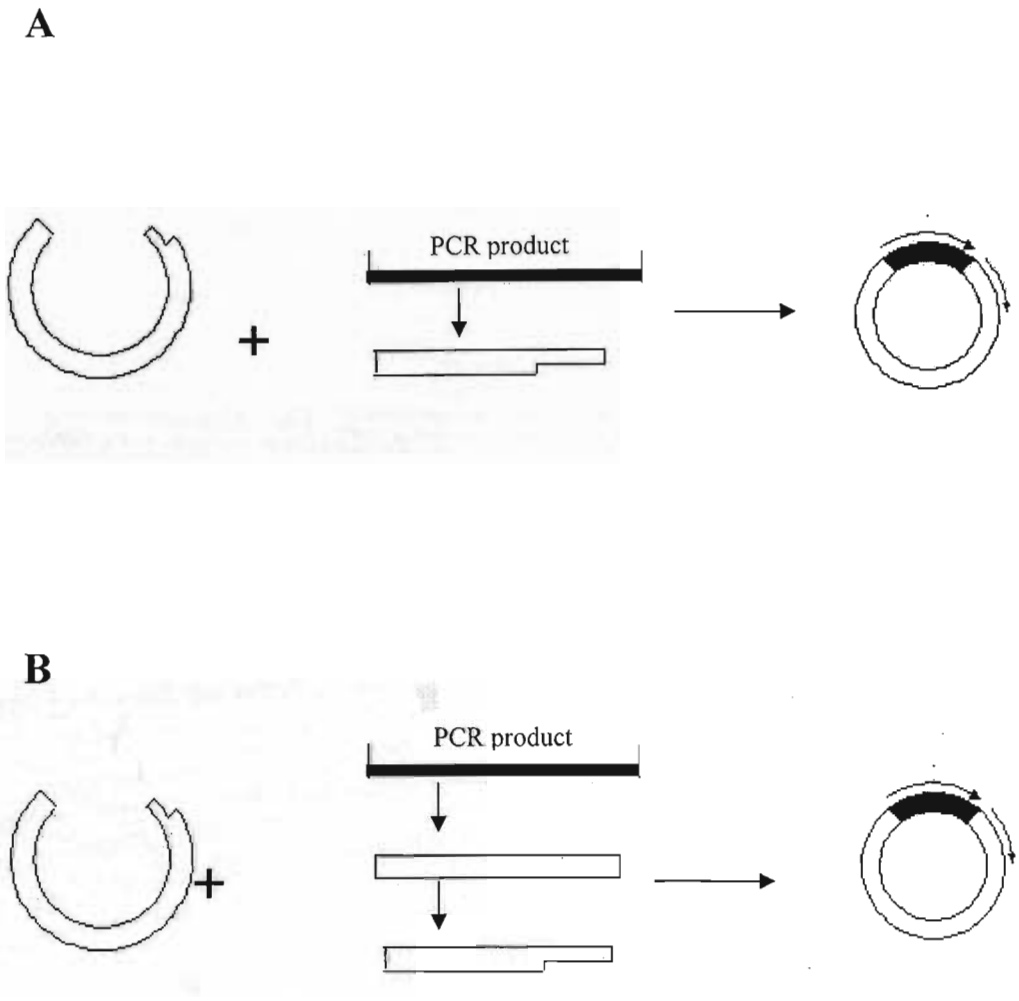


Figure 3.3. An illustration of the truncation and cloning of the immunity gene.

A. Cloning of version A into pMAL-c2 vector. The immunity gene was cleaved with *Hae*III to cleave off the C-terminal and *Xba*I to generate a sticky-end. B. Cloning of version B into pMAL-c2 vector. The immunity gene was digested with *Cla*I to cleave off the N-terminal and *Xba*I to generate a sticky-end.

3.3.7 Recombinant clones verification

3.3.7.1 Recombinant plasmid DNA isolations

Recombinant plasmid DNA isolations were performed using the alkaline lysis method (Sambrook *et al.*, 1989). A single bacterial colony or 100 µl from a glycerol stock of each of the recombinant clones were inoculated independently into 10 ml ampicillin (100 $\mu g.ml^{-1}$) supplemented LB broth and grown overnight at 37°C with vigorous shaking. The cells were centrifuged at 7 520 x g for 5 minutes in a JA 20.1 rotor at 4°C. The supernatant was discarded and the cells were resuspended in 200 µl GTE solution (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM ethylene diamine tetraacetic acid {EDTA}). The cell suspensions were transferred to microfuge tubes and left at room temperature for 5 minutes. RNase A (2 µl) from a 10 $mg.ml^{-1}$ stock and 400 µl freshly prepared 0.2 M NaOH, 0.1% SDS solution were added to the cell suspensions. The suspension was subsequently placed on ice for 5 minutes.

To each cold cell suspension 300 µl of potassium acetate solution (29.5 ml glacial acetic acid, KOH pellets to pH 4.8, H_2O to 100 ml) was added and mixed thoroughly. The suspensions were placed on ice for 5 minutes to precipitate the cell debris and chromosomal DNA. The mixtures were centrifuged in a microcentrifuge at 11 000 x g for 5 minutes at room temperature. The supernatant from each mixture (800 µl) were transferred to a fresh microfuge tube, into which 600 µl of isopropanol was added and the mixtures were left at -20°C for 30 minutes. The cells were microcentrifuged at 11 000 x g for 5 minutes to pellet the plasmid DNA. The supernatants were discarded and the pellets were washed in 0.5 ml ice-cold 70% ethanol and dried in a TurboVac (Virtis, USA) for a maximum of 15 minutes. Each dried pellet was resuspended in 50 µl TE buffer. From each DNA sample, 10 µl was quantified using a spectrophotometer at λ_{260} and λ_{280} and analysed by agarose gel electrophoresis, the remaining DNA was kept at -20°C until further required. The recombinant plasmids produced are listed in Table 3.1B.

3.3.7.2 Colony polymerase chain reaction

The procedure was performed according to Amberg, 2006. A small colony of a recombinant clone was picked with a toothpick and swirled in 5 µl of H_2O (or TE buffer) in a PCR tube. The suspension was microcentrifuge at 11 000 x g for 5 minutes. The supernatant (2 µl) was used as a template in a 25 µl PCR reaction. The reaction components were prepared as in Table 3.6, except the volume of water added to the reaction was 6.5 µl to make up the reaction volume to 25 µl. EAL2 and EAL3 primers that were used for amplification of the cloned immunity gene were used. The reaction controls were: 1. a reaction without the primers, 2. a reaction without the template.

3.3.7.3 Southern Hybridization

The VacuGene XL Protocol No. 1 (Pharmacia) was used to transfer DNA from a gel onto a nitrocellulose membrane. Initially plasmid DNA from putative clones isolated by the alkaline lysis method described in paragraph 3.3.7.1 and were analysed on agarose gel (1% [w/v]). The gel was subjected to subsequent hybridization steps: (i) depurination, 50 ml of solution I (0.2 N HCl) was poured onto the gel with a pipette and left for 20 minutes, (ii) denaturation, 50 ml of solution II (0.5 mM NaOH, 1.5 mM NaCl), was added and removed after 20 minutes, (iii) neutralization, 50 ml of solution III (0.5 mM Tris, pH 7.0, 3 M NaCl) was poured onto the gel and removed after 20 minutes.

Transfer of DNA: The nitrocellulose membrane was pre-treated prior to transfer by incubating it in a small container in sterile distilled water for 1 min and subsequently incubating it in Solution IV (20x SSC, pH 7.0 [3 M NaCl, 10 mM Na citrate, 0.1% SDS]). The nitrocellulose membrane was placed on a Vacu Gene XL apparatus, DNA from the gel was transferred onto the nitrocellulose membrane with 20 x SSC transfer solution for a period of 60min. Following transfer, the wells were marked. Agarose was removed from the membrane by washing with solution IV for 10 minutes. The membrane was washed twice with 2x SSC for 15 minutes and subsequently washed with 0.1x SSC containing 0.1% SDS. The membrane was air-dried in the oven for 30 minutes.

Hybridization: JHA 7 probe was used to detect the immunity genes as it has a short sequence that is complementary to a sequence near the 3' end of the immunity gene. The probe was labeled in microfuge tube using the Roche Tailing Kit. The 20 µl labeling reaction contained: (i) 4 µl $CoCl_2$ solution, (ii) 4 µl reaction buffer (1 M potassium cacodylate, 0.125 M Tris-HCl, 125 mg BSA, pH 6.6), (iii) 2 µl probe solution (0.3 µM), 1 µl DIG/dUTP solution, (iv) 1 µl terminal transferase (50 U) and (v) 8 µl sterile distilled H_2O. The reaction was mixed and incubated at 37°C for 15-30 minutes and cooled on ice. The blotted membrane was pre-hybridized overnight at 42°C in a Hybaid HB-OV-BL hybridization tube containing the pre-hybridisation solution (6x SSC, 0.5% SDS, 5x Denhardt's solution {2% Ficoll 400, 2% Polyvinylpyrrolidone, 2% BSA [fraction V]}). The pre-hybridization solution was replaced by the hybridization solution (10 ml pre-hybridizing solution containing 60 µl of the labeled JHA 7 probe (0.3µM)), and the membrane was left overnight at 42°C (Sambrook *et al.*, 1989). To remove the hybridization solution the membrane was washed twice with 2 x SSC, 0.1% SDS for 15 minutes and subsequently washed twice with 0.1 x SSC, 0.1% SDS and air-dried.

Detection: The membrane was washed with 50 ml of buffer 1 (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl) for 1 minute. Buffer 1 was removed and replaced by 100 ml buffer 2 (1% skim milk powder in 100 ml buffer 1) followed by incubation at room temperature for 30 minutes with constant shaking. Buffer 2 was washed off briefly with 100 ml buffer 1. The membrane was incubated in 20 ml diluted antibody conjugate solution (4 µl anti-digoxigenin-AP conjugate with 20 ml buffer 2) for 30 minutes on a shaker. Unbound antibodies were removed by washing with 50 ml buffer 1 for 15 minutes. The membrane was equilibrated with 20 ml buffer 3 (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 2 minutes, and subsequently incubated in freshly prepared colour substrate solution (200 µl NBT/BCIP in 10 ml buffer 3) in a dark container for 16 hours. The reaction was terminated by washing the membrane in 50 ml TE buffer, pH 8.0, for 5 minutes. The results were documented by photocopying the wet membrane.

3.3.8 Expression of the fusion proteins

The immunity gene versions were cloned into the pMAL-c2 vector, where the cloned genes were fused to the *malE* gene that encodes the maltose binding protein (MBP). Consequently these genes are expressed as MBP-immunity fusion proteins.

3.3.8.1 Expression

All three putative clones were expressed. For the expression of each recombinant protein, 10 ml LB broth containing ampicillin (100 $\mu g.ml^{-1}$) was inoculated with a single *E. coli.* JM 103 colony transformed with the plasmid harboring the recombinant constructs. The above mentioned was grown overnight at 37°C. From each overnight culture, 2 ml was sub-cultured into 200 ml LB broth containing glucose and ampicillin (100 $\mu g.ml^{-1}$) and grown at 37°C with agitation to λ_{600} of ~0.5. An aliquot of 500 µl was removed from each culture (non-induced cells) and placed in a microfuge tube. To the remaining cells, IPTG was added to the final concentration of 0.3 mM was added and the cultures were further incubated for 2 hours in a 30°C water bath with agitation. An aliquot of 500 µl was removed from each culture (induced cells) and placed in a microfuge tube. The 500 µl aliquots were centrifuged at 13 000 x g for 2 minutes. The supernatants were discarded and the cells were resuspended in 50 µl of 2x SDS-PAGE treatment buffer (0.125 M Tris-HCl, 4% SDS, 20% v/v Glycerol, 0.2 M DTT, 0.02% Bromophenol blue, pH 6.8.

The rest of the cultures containing induced cells were harvested by centrifuging at 4 000 x g for 20 minutes at 4°C. The supernatant was discarded and each pellet containing cells was resuspended in 5 ml column buffer, pH 7.2 (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA). The cells were stored overnight at -20°C, as a pre-treatment for sonication. The cells were thawed in cold water and sonicated using VirSonic 60 (Polychem Supplies CC, Laboratory Consumables) in short pulses of 30 seconds to release the proteins. Protein release was monitored by means of a Bradford assay as described by Bradford (1976), where 10 µl of sonicated cells was diluted with 1.5 ml Bradford reagent (10% Serva blue G; 85% (m/v) phosphoric acid; 4.8% ethanol) and the absorbance reading of the mixture taken at λ_{595}. Following sonication, the samples were centrifuged at 9 000 x g for 30

minutes at 4°C, the supernatant (crude extract) containing the fusion proteins was diluted with column buffer (1:5) and the pellets were discarded. A fraction of 50 µl was removed from each diluted crude extract and mixed with 50 µl 2x SDS-PAGE treatment buffer in a microfuge tube.

3.3.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gel (10% [w/v]) was prepared in a vertical slab gel unit in a dual casting stand (Hoefer, SE 600), using reagents listed in Table 3.7. The separating gel was made and pipetted between the two glass plates to a level about 4 cm from the top and allowed to polymerize. The stacking gel of 4% was freshly made and layered on top of the separating gel. A comb was inserted into the viscous stacking gel to form wells, and the gel was allowed to polymerize for 60 minutes. The comb was slowly removed. The wells and the lower chamber were filled with 1000 ml of tank buffer (0.025 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.3). The crude extracts in 2x SDS-PAGE treatment buffer (paragraph 3.3.8.1) were boiled for 5 minutes, cooled on ice and 20 µl from each sample was loaded into the gel. Electrophoresis was performed at 18 mA per gel and at maximum voltage, until the bromophenol blue tracker dye reached the bottom of the gel. The gels were visualized by means of Coomassie blue staining.

Table 3.7. Reagents used to prepare SDS-PAGE gel for protein samples analysis.

Solutions	Running Gel (10%)	Stacking Gel (4%)
*Acrylogel	3.15 ml	650.00 µl
Distilled H_20	5.40 ml	3.00 ml
0.5 M Tris, pH 6.8	0.00	1.25 ml
1.5 M Tris, pH 8.8	1.25 ml	0.00
Glycerol 50% (v/v)	200.00 µl	0.00
SDS 20% (w/v)	50.00 µl	25.00 µl
*TEMED	15.00 µl	5.00 µl
*10% (w/v) APS	36.00 µl	25.00 µl

***Acrylogel**: Merck product
***TEMED**: Promega product
***APS**: Ammonium persulphate

3.3.8.3 Coomassie blue staining

Following the SDS-PAGE, the gels were immediately placed in a container with the staining solution (0.025% Coomassie brilliant blue R250, 40% methanol, 7% acetic acid) for 4 hours or overnight. The stain was replaced with destaining solution I (40% methanol, 7% acetic acid, in distilled H_2O) and gently shaken at room temperature. A sponge was placed on the gel to absorb the stain. After 30 minutes, destaining solution I was replaced by destaining solution II (7% acetic acid, 5% methanol, in distilled H_2O) until the background was entirely clear (Laemmli, 1970).

3.3.9 Purification of the fusion proteins

3.3.9.1 Amylose resin

The large-scale purification of the expressed MBP-fusion proteins was performed at room temperature using the pMAL-c2 Protein Fusion and Purification System using amylose resin (Figure 3.4). Amylose resin was placed in a 2.5 cm^2 x 10 cm column, a 10 ml bed volume and was equilibrated with column buffer. Column buffers with different pH values were used to purify different fusion proteins (Table 3.8). The diluted crude extract was loaded onto the column, where 1 ml of the flow through was collected. The column was subsequently washed with 5 column volumes of the column buffer and 1 ml from the wash was collected. The fusion proteins were eluted using column buffer containing 10 mM maltose. Fractions of 2 ml were collected sequentially and monitored by Bradford assay.

All eluted fractions containing the proteins were pooled, freeze-dried and quantified by BCA assay. The 1 ml samples collected during the purification stages as well as the eluted proteins were analysed on a Tris tricine gel (10% [w/v]). Molecular weight sizes were determined by comparing with the Precision Plus Protein Standards (PPPS) (Biorad).

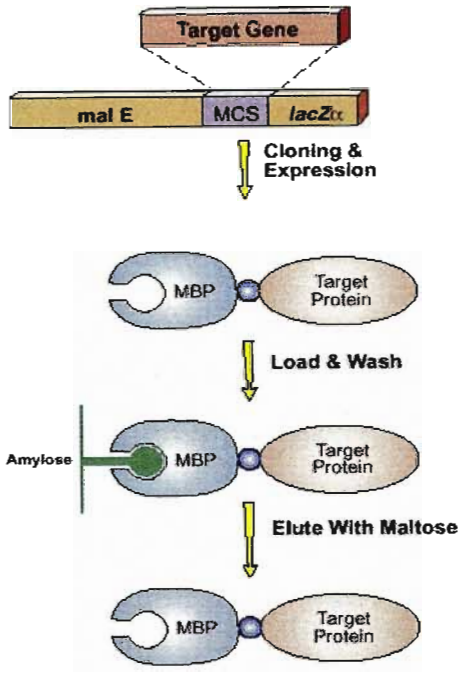


Figure 3.4. The expression and purification procedure of the MBP-fusion proteins. (New England BioLabs). MBP-fusion proteins were purified from bacterial lysate by affinity chromatography, using amylose resin and were eluted with maltose.

Table 3.8 Suitable pH values for purification of fusion proteins.

Fusion protein	Description	*Apt pH
MBP-IP	MBP fused to complete IP*	7.2
MBP-IPA	MBP fused to IP lacking the C-terminal	6.8
MBP-IPB	MBP fused to IP lacking the N-terminal	6.8

***Apt pH**: optimal pH for the purification
***IP**: Immunity protein

3.3.9.2 Tris tricine gel

Tris tricine gel was prepared for analysis of the purified fusion proteins, and for the size estimation. The gel was prepared in a vertical slab gel as for SDS-PAGE except different buffers were used for the preparation of the separating and stacking gels. The gel components are listed in Table 3.9.

The wells were filled with cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and the lower chamber was filled with 1x anode buffer (0.2 M Tris, pH 8.9). Similar to SDS-PAGE, protein samples (10 µl) were mixed with equal volumes of 2x Tris sample buffer (1 M Tris-HCl, pH 6.8, 8% SDS, 2.4% glycerol, 0.2% Coomassie blue G-250, 3.1% DTT) and boiled for 5 minutes before loading on the gel. Electrophoresis was performed at a constant voltage of 90 V and maximum current for 45 minutes or until the dye front reached the bottom of the gel. To view the band profiles the gel was stained overnight at room temperature with gently shaking in Coomassie blue staining solution for a period of 2-12 hours. The stain was removed from gel and replaced by destain I solution until the background was entirely clear.

Table 3.9. Composition of Tris tricine gel for separation of protein samples.

Stock Solutions	Separating Gel (10%)	Stacking Gel (4%)
Solution A	5.00 ml	1.50 ml
Solution B, pH 8.45	3.00 ml	500.00 µl
Distilled water	7.00ml	4.00 ml
10% (w/v) APS	50.00 µl	30.00 µl
TEMED	75.00 µl	15.00 µl

***Solution A**: Acrylogel
***Solution B**: 3 M Tris-HCl, 0.3% SDS

3.3.10 Western blotting analysis

The procedure was performed to confirm the presence of MBP on the purified fusion proteins.

3.3.10.1 Protein Transfer

Purified proteins were run on an SDS-PAGE gel. Two gels were prepared, one was used as a reference gel, which was stained and destained, and the other was used to transfer the proteins onto a nitrocellulose membrane. A sandwich consisting of two sponges, two blotting papers, the membrane and the SDS-PAGE gel (Figure 3.5) were soaked in ice-cold Towbin buffer (20 mM Tris, 192 mM Glycine, 20% (v/v) Methanol, pH 8.3) for 15 minutes to an hour in a glass container (Towbin *et al.*, 1979).

The sandwich was transferred to a Mini-Trans Cell (Biorad) filled with ice-cold TowBin buffer. Transfer was executed at a constant voltage of 90 V and maximum current for ± 3 hours. The membrane was removed from the sandwich and stained with Ponceau S (0.1% (w/v) Ponceau S, (1% (v/v) acetic acid). The membrane was destained with sterile distilled H_2O containing a few drops of 1 M NaOH. The membrane was air-dried by placing within two Whatmann filter papers for 1.5 hours.

3.3.10.2 Detection of MBP

The membrane was blocked by submerging it in 1x Phosphate Buffered Saline (PBS) (10 mM Phosphate, 0.15 M NaCl, pH 7.2) containing 5% (w/v) non-fat milk powder for 1 hour. The membrane was washed twice in PBS containing 0.1% (v/v) Tween 20. For detection of MBP, the membrane was incubated in 10 ml of PBS containing 0.5% (w/v) BSA and 10 µl of the anti-MBP monoclonal antibody (150 mM NaCl, 10 mM 4-(2-hydioxyethyl)-1-peperazineethanesulfonic acid (HEPES), pH 7.5, 50% glycerol) with HRPO conjugate attached, for 1 hour at room temperature with slight agitation. The membrane was washed with PBS containing Tween 20, and was placed in a dark container with 5 ml of a precipitating chromogenic substrate, BM blue peroxidase (POD) (Roche, country, city) until the bands were visible. The POD was removed by washing the

membrane with distilled H_2O and the membrane was dried between two Whatmann filter papers.

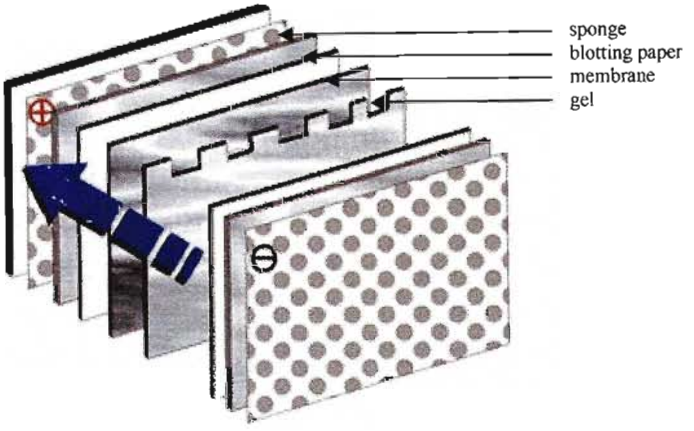


Figure 3.5. The Western blot layout.
(http://matcmadison.edu/biotech/resources/proteins/labManual/chapter_5/procedure5).
Western blot sandwich showing the route of protein transfer, from the SDS-PAGE gel to the nitrocellulose membrane.

3.3.11 The Factor Xa reaction

The purified fusion protein samples were exposed to Factor Xa (Biolabs) in order to cleave off the MBP from the immunity protein versions. The reactions were prepared in sterile microfuge tubes, where 6 µl of Factor Xa (20 mM HEPES, 500 mM NaCl, 2 mM $CaCl_2$, 50% glycerol, pH 8.0) was added to 60 µl of each amylose purified protein sample. The reactions were left for 24 hours at 23°C and analysed on Tris tricine gel (10% [w/v]).

3.3.12 Protein detection

The proteins were sequenced using N-terminal sequencing technique to confirm the sequences of the immunity protein versions. The proteins were sequenced as dried samples, where they were electroblotted on PVDF membrane and subjected to the sequencer.

The Protein blotting process is similar to the Western blot procedure, except the Polyvinylidene difluoride (PVDF) membrane was used and there is no immuno-detection step. The two SDS-PAGE gels that were prepared run the immunity protein versions were allowed to polymerise overnight. A concentration of proteins loaded on the gels ranged between 0.8 – 1.0 μg/lane. Subsequent to electrophoresis the reference gel was stained and destained, and the other was used to transfer the proteins onto the PVDF membrane. A sandwich consisting of two sponges, two blotting papers, the membrane and the SDS-PAGE gel were soaked in ice-cold 3-[cyclohexylamino]-1-propansulfonic acid (CAPS) buffer (10 mM CAPS, pH 11.0, 10% methanol) for 15 minutes to an hour in a glass container. The sandwich was transferred to a Mini-Trans Cell filled with ice-cold CAPS buffer. Transfer was executed at a constant voltage of 90 V and maximum current for 3 hours. The PVDF membrane was removed and stained in coomassie R-250 (0.025% Coomassie Brilliant Blue R-250 dissolved in a 40% methanol) with gentle agitation. The membrane was repeatedly destained in PVDF destain (50% methanol) until protein bands were visible and the PVDF membrane was air-dried by placing within two Whatmann filter papers for 1.5 hours.

N-terminal protein sequencing was performed on a 476A sequencer equipped with 120A HPLC system (Procise LC Protein Sequencer, PE Applied Biosystems) Edman degradation chemistry. The protein bands on the PVDF membrane corresponding to the three immunity protein versions were excised from the PVDF membrane, reconstituted in 0.1%TFA/20% acetonotrile and loaded directly on the sequencer. The program was modified to sequence 13 amino acid residues from the N-terminal regions of the proteins.

3.4 INTERACTION STUDIES

3.4.1 Biomolecular Interaction Assay

Interactions between the peptide, leucocin A, with the immunity protein versions and the cytoplasmic fractions of *L. gelidum* UAL 187-22 were investigated with a Biacore Model 2000 system, from Biacore (Biacore, Uppsala, Sweden). The degassed running buffer used was HBS (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM Na_2EDTA), supplemented with 0.05% (v/v) surfactant P20 (Biacore, Uppsala, Sweden).

For the first experiment, leucocin A was immobilised on a sensory chip CM5 that has a carboxymethyldextran surface (Amano *et al.*, 1999). The three immunity protein versions were injected with the flow channel that passed across the sensory surface. For the second experiment, the immunity protein versions were immobolised and the cytoplasmic extract was injected in the flow channel. The HBS Buffer served as a negative control.

Unreacted moieties were blocked on the surface using ethanolamine. The CM5 sensor chip was washed several times with 10 mM glycine, pH 2.2 to remove any unbound proteins. All the measurements were carried out in HBS at 25°C. Peptides were dissolved in HBS to approximately 6 $mg.\mu l^{-1}$. The surface of the sensor chip was regenerated upon injection of a 50 mM HCl solution after each run.

CHAPTER FOUR

RESULTS AND DISCUSSION

The self-protection of *L. gelidum* to self-produced leucocin A was speculated to be attributed to its leucocin A immunity protein. Additionally, the immunity protein reacts with a certain unidentified ligand in the cytoplasm, which might be the trigger for the immunity mechanism (Pillay, 2004). The objectives of this particular study were to identify the unknown ligand in the cytoplasm, to investigate the existence of a direct interaction between the immunity protein and leucocin A, and if there is, to ascertain the terminal region of the immunity protein involved.

4.1 BACTERIOCIN LEUCOCIN A

4.1.1 Bacteriocin production

The strain used in this study was *L. gelidum* UAL 187-22, which produces a leucocin A bacteriocin, confirmed by Hastings *et al.* (1991). The presence of the leucocin A in the *L. gelidum* supernatant was confirmed by performing the spot on lawn assay on a *Listeria* lawn, where it produced inhibitory zones (Figure 4.1A). Inhibiting the growth of *L. monocytogenes* verified that bacteriocin is either a Class IIa or Class IIb bacteriocin (Ennahar *et al.*, 2000a). *L. gelidum* UAL 187-13 served as the negative control and exhibited no inhibition zones. A successful colony assay is shown in Figure 4.1B, where *L. gelidum* UAL 187-22 colonies produced zones of inhibition on *Listeria* lawn, which proves that the strain is the actual host of leucocin A bacteriocin.

In the half moon assay, a half-moon-shaped zone of inhibition was observed, which pointed out that the bacteriocin was proteinase K sensitive and of a proteinaceous nature (Figure 4.1C).

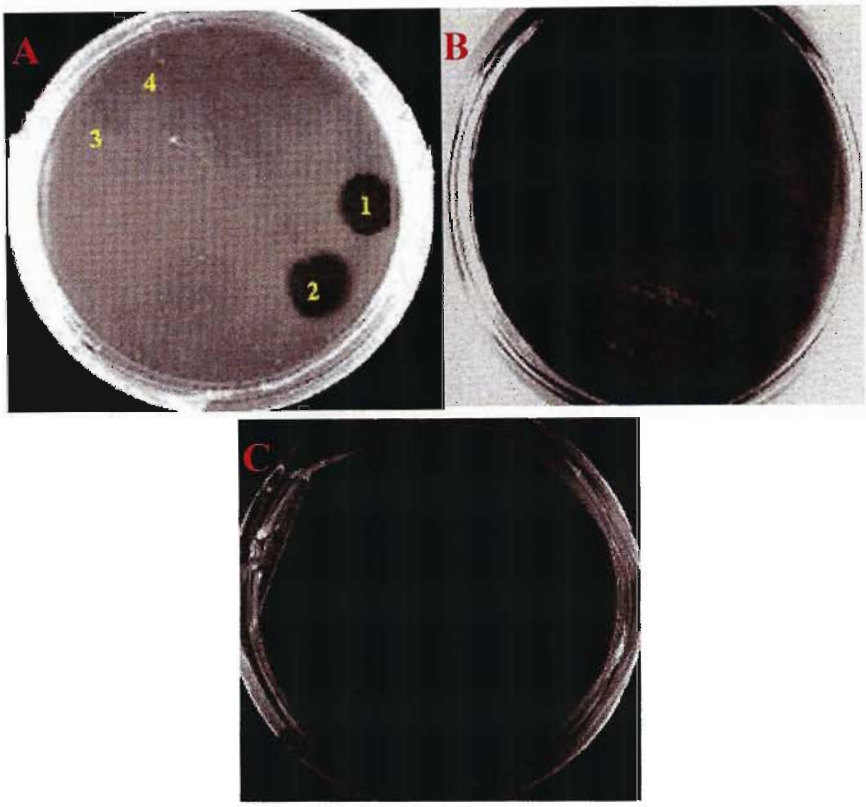


Figure 4.1. Illustration of leucocin A activity.
Activity of leucocin A is depicted by inhibition zones on a TSA plate overlaid with *L. monocytogenes.* **A.** Inhibition zones produced by the *L. gelidum* UAL 187-22 supernatant (spots 1 and 2). *L. gelidum* UAL 187-13 supernatant, a negative control produced no zones on the lawn (spot 3 and 4). **B.** The *L. gelidum* UAL 187-22 colony activity that confirmed the strain is a host of the active bacteriocin (plate). **C.** The half moon zones produced by proteinase K indicating the proteinaceous nature of the peptide.

4.1.2 Leucocin A purification

4.1.2.1 Cation exchange chromatography

In view of the fact that leucocin A was present in the *L. gelidum* UAL 187-22 supernatant the next step was to purify the peptide. A rapid two-step procedure was performed for a large-scale purification of leucocin A. The peptide is a cation with an isoelectric point of 6.0499 (http://www.embl-heidelberg.gde/cgi/pi-wrapper.pl). When the *L. gelidum* supernatant is passed through the SP sepharose resin, the peptide, in conjunction with other cations attaches to the anion group of the resin (Figure 2.1A). Bound peptides were eluted from the resin with phosphate buffer, pH 5.8 containing 1.0 M NaCl, which has a higher ionic strength that is able to replace the positive ions on the ion exchanger with Na^+ ion of the NaCl. Whilst the eluted crude sample was active, which was depicted by the inhibition zone, further purification of the sample was required as portrayed by the haziness of the inhibitory zones (Figure 4.2A).

4.1.2.2 Reverse Phase High Pressure Liquid Chromatography

Auxiliary purification of leucocin A was performed using HPLC in order to separate the peptide from other cations in the crude sample eluted from the SP sepharose chromatography resin. The crude sample (1.0 ml) was centrifuged before injecting into the reverse phase column, to clear solid contaminants in order to prevent column pollution.

Figure 4.3 and 4.4 show the HPLC chromatograms produced by the initial and the last runs. In both figures the presence of a number of peaks was due to differing retention times exhibited by the other peptides within the samples (as the samples are not completely pure after the Cation exchange chromatography). All peptides that were detected were collected and assayed for leucocin A activity. The peak at 47.997 minutes represented leucocin A, shown in Figure 4.3 and 4.4 (indicated by the arrow). It was eluted from the column in 80% acetonitrile, 0.1% TFA, and it was distinguished by its production of the inhibition zone on the *Listeria* lawn. The inhibition zone appeared to be very clear, which denoted the activity of the peptide (Figure 4.2B, zones 1 and 2). The similarity of the profiles illustrates the accuracy and consistency of the technique.

Shown in Figure 4.2B are the negative controls, phosphate buffer that was used to elute the peptide from the cation exchange column (zone 3) and acetonitrile containing 0.1% TFA that was used to resuspend the peptide after HPLC purification (zone 4) did not have the anti-microbial properties. This confirmed that the inhibition zones on *Listeria* lawn at zones 1 and 2 were as a result of the bacteriocin activity of leucocin A.

The purified peptides that eluted from the HPLC were pooled, freeze-dried, resuspended in 0.1% TFA and quantified by means of BCA assay. Leucocin A concentration was determined to be 5.7 $mg.ml^{-1}$. To maintain the structure and activity of leucocin A, the bacteriocin was kept at $-20^{o}C$ in an acidic buffer (TFA).

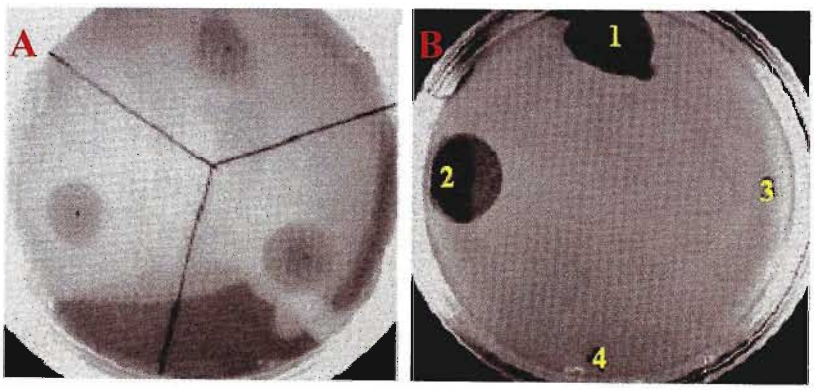


Figure 4.2. Activity assay of SP sepharose and HPLC purified leucocin A.
A. Assay of the partially purified leucocin A eluted from SP sepharose resin. The inhibition zones on the *Listeria* lawn are indistinct due to the impurity of the peptide. **B.** Assay of the purified leucocin A, and the buffers used in the process. Zones 1 and 2 the clear zones produced by the completely pure bacteriocin HPLC elutes. Zones 3 and 4 are the controls, phosphate buffer and acetonitrile containing 0.1% TFA, respectively.

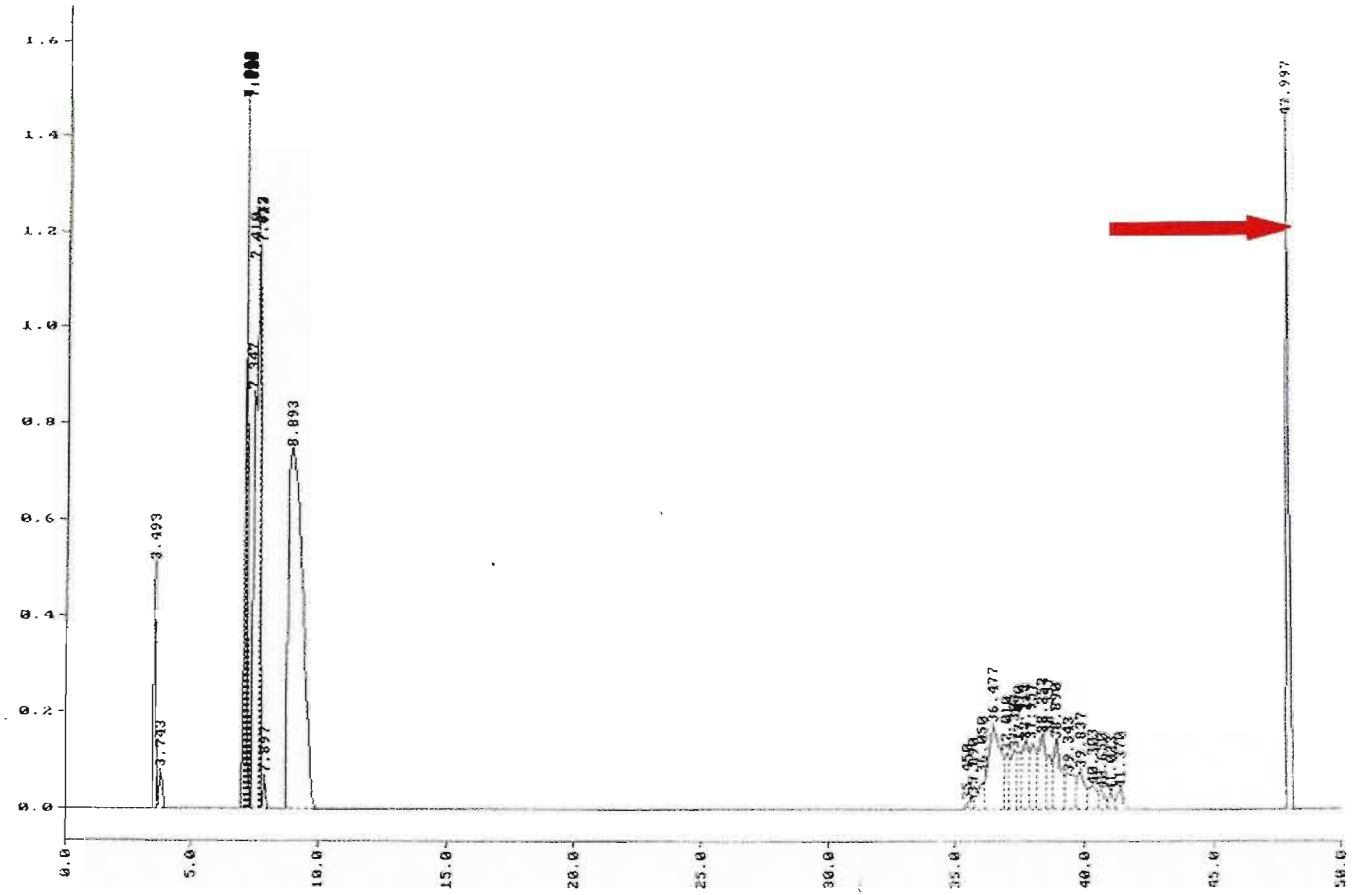


Figure 4.3. An initial HPLC Chromatogram from leucocin A purification.

The chromatogram was recorded through the first 50-minute HPLC chromatographic run of 1.0 ml leucocin A crude sample (3.2 $mg.ml^{-1}$). The bacteriocin eluted at 47.997 minutes in 80% acetonitrile, 0.1% TFA (indicated by the arrow). Aliquots of 1 ml were collected. The other peaks present in the chromatogram represent all the proteins detected in the sample.

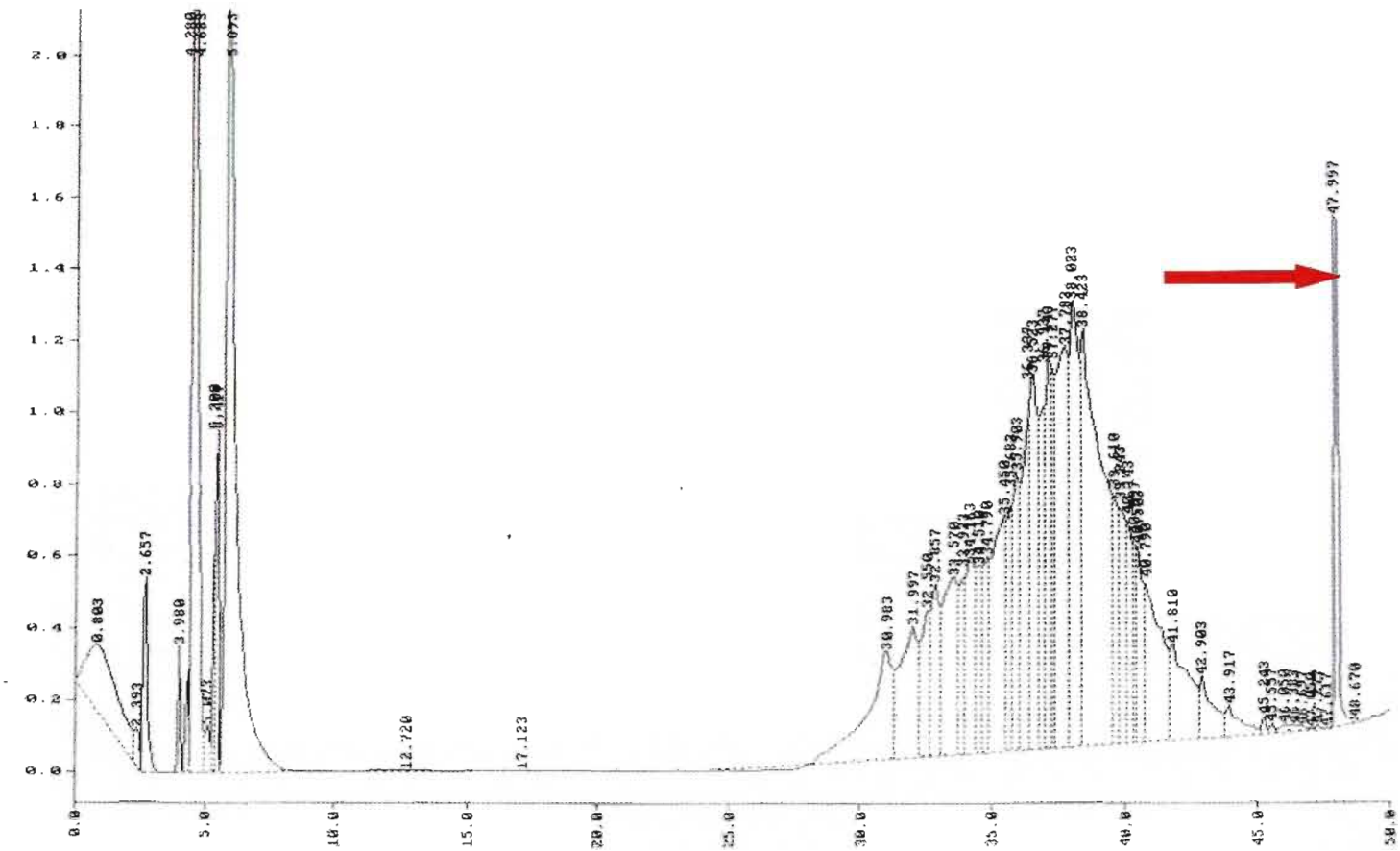


Figure 4.4. A final HPLC Chromatogram from leucocin A purification.
The last HPLC chromatographic run produced during 1.0 ml crude sample leucocin A purification.

4.1.3 Quantification of leucocin A

All elutes from different purification stages were quantified by a BCA assay to determine the peptide concentration in each elute by extrapolation from the BCA standard curve (Figure 4.6). The peptide concentrations are listed in Table 4.1.

A double dilution assay was performed on samples eluted from all purification stages with the aim of establishing the peptide activity as well as the amount of leucocinA activity lost during purification. A typical example of a double dilution result is shown in Figure 4.5 with the SP sepharose crude sample. The last inhibitory zone was formed by a 1/1000 dilution, which gave a specific activity of 1.5×10^5 Au.mg^{-1}. The HPLC purified peptide displayed a high specific activity (Table 4.1), which implies there was minimum bacteriocin loss during purification. The percentage of leucocin A recovered was 75.9%, which exemplified the competency of the technique.

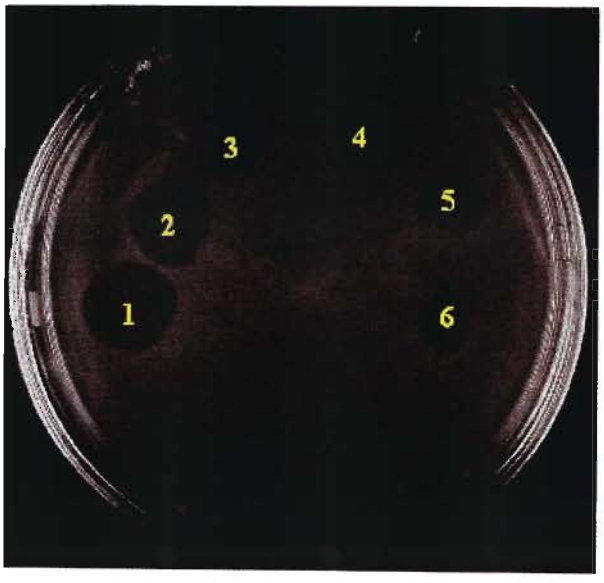


Figure 4.5. The Double dilution assay.
The double dilution assay for the quantification of the activity of the SP sepharose eluted crude product (3.2 mg.ml^{-1}) was doubly diluted six fold (represented 1-6 above). The last inhibition zone formed by the SP sepharose flow through was at a 10^{-3} dilution.

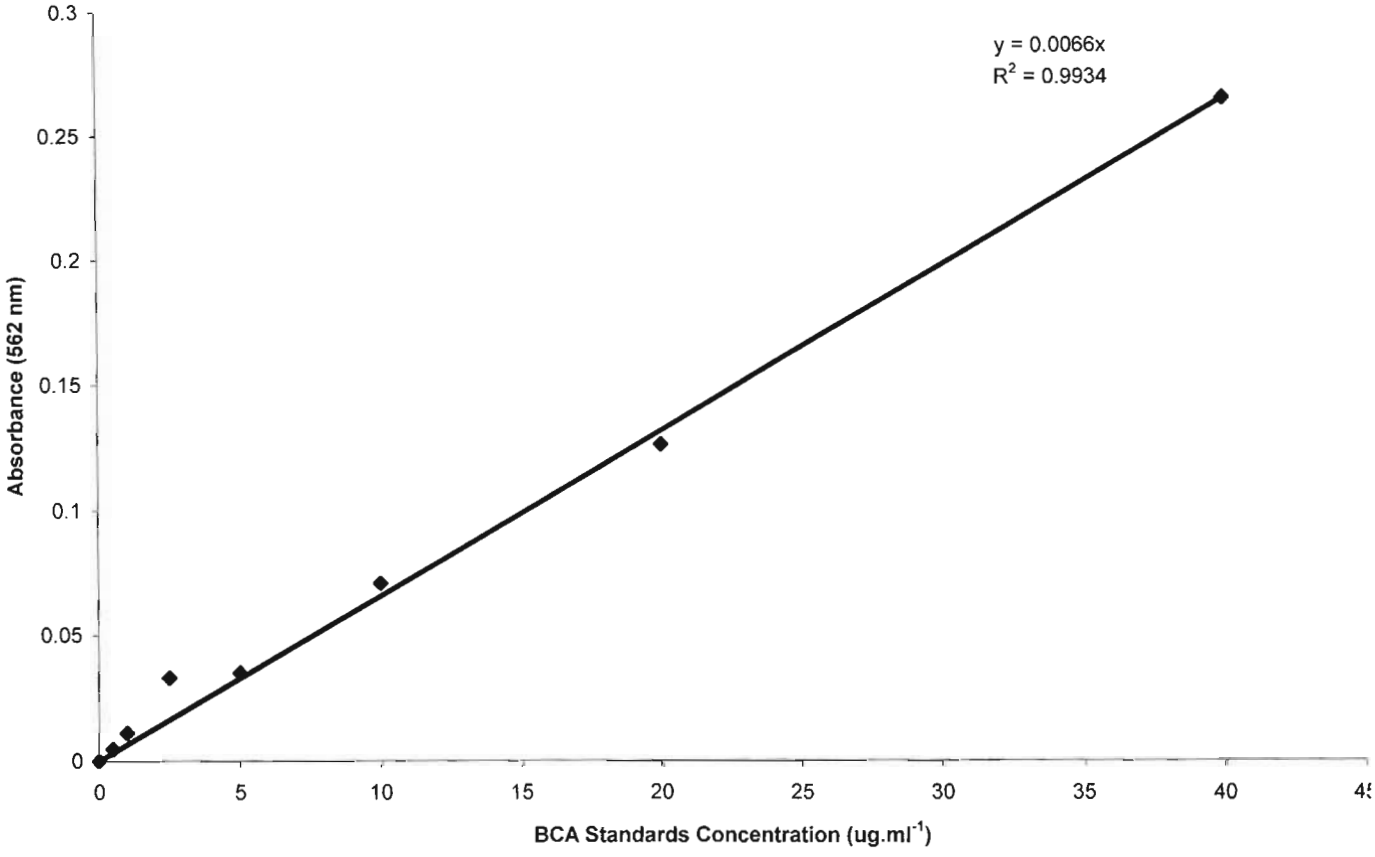


Figure 4.6. A standard curve produced by BCA standards at λ_{562}.

The curve was used to determine the unknown peptide or protein concentrations.

Table 4.1. Activity of the bacteriocin, leucocin A at different purification stages of its purification.

Purification Step	Vol (ml)	*Activicty ($Au.ml^{-1}$)	*Tot actv (Au)	*Protein conc ($mg.ml^{-1}$)	*Sp actv ($Au.mg^{-1}$)
***Supernatant**	250	25600	6.4×10^6	11.9	5.4×10^5
***S. Flow thro'**	250	1600	4.0×10^5	2.6	1.5×10^5
***S. Wash**	75	800	6.0×10^4	1.0	5.8×10^4
***S. Elute**	75	6400	4.8×10^5	3.2	1.5×10^5
***HPLC**	45	51200	2.3×10^6	5.7	4.1×10^5

***Au**: Activity units
***Au.mg^{-1}** : Activity units per mg
***Actv**: Activity
***Conc**: Concentration
***Vol**: Volume
***Tot**: Total
Sp: Specific

***Au.ml^{-1}** : Activity units per ml
*** HPLC**: HPLC purified bacteriocin
*** Super**: Supernatant
***S. flow through**: SP sepharose flow through
***S. wash**: Wash from Sepharose
***S. elute**: Elute from SP sepharose

4.1.4 Mass spectroscopy

Leucocin A consists of 37 amino acids and is 3.9303 kDa (http://ma.lindberg.gu.se/cutter2/). The size of the peptide was confirmed by Liquid Chromatography Mass Spectrometry, whereby the freeze-dried HPLC purified leucocin A was introduced into the mass spectrometer system and analysed.

The results were recorded on chart recorder, which is simplified into a chromatogram that is referred to as a "stick-diagram", which measures a signal intensity or relative intensity of a total number of ions in the sample against their mass-to-charge ratio (m/z). Figure 4.7A illustrates a stick-diagram obtained from mass spectroscopy analysis of leucocin A. The lines represent the detected ions, which are isotopes present in leucocin A sample. The most common ion was the leucocin A with a mass/charge ratio of 655.4515. The leucocin A ion was represented by a single line on a stick-diagram, entailing a charge of 1+. Thus the mass/charge ratio is the same as the mass of the ion, which is normally the case for ions passing through the mass spectrometer. However, in cases where the charge was 2+, the lines in the stick-diagram have another line half its intensity, for example, at m/z 983.4731, the second line has lower intensity than the 1+ ion line because the chances of forming 2+ ions are much less than forming 1+ ions (Glish and Vachet, 2003).

The relative atomic masses of the ions were calculated by the software of the spectrometer and are measured on the ^{12}C scale (Figure 4.7B). The figure illustrates the results obtained after the data was processed for mass determination. The most intense peptide had a mass of 3.92793 kDa, which was leucocin A, and it differed from the theoretical mass of 3.9303 kDa by 0.0025%.

The LCMS chromatogram in Figure 4.8 illustrates the peak that represents leucocin A, which was detected at 7.47 minutes. The peak appears to be very small due to the very low peptide concentration of 1 $\mu l.ml^{-1}$ as the sample was diluted prior to injection into the system.

LCMS proved to be very explicit and proficient, as it accurately verified the mass of the leucocin A peptide. In addition, the technique displaced impressive sensitivity since it was able to detect a number of proteins in the sample subsequent to HPLC purification.

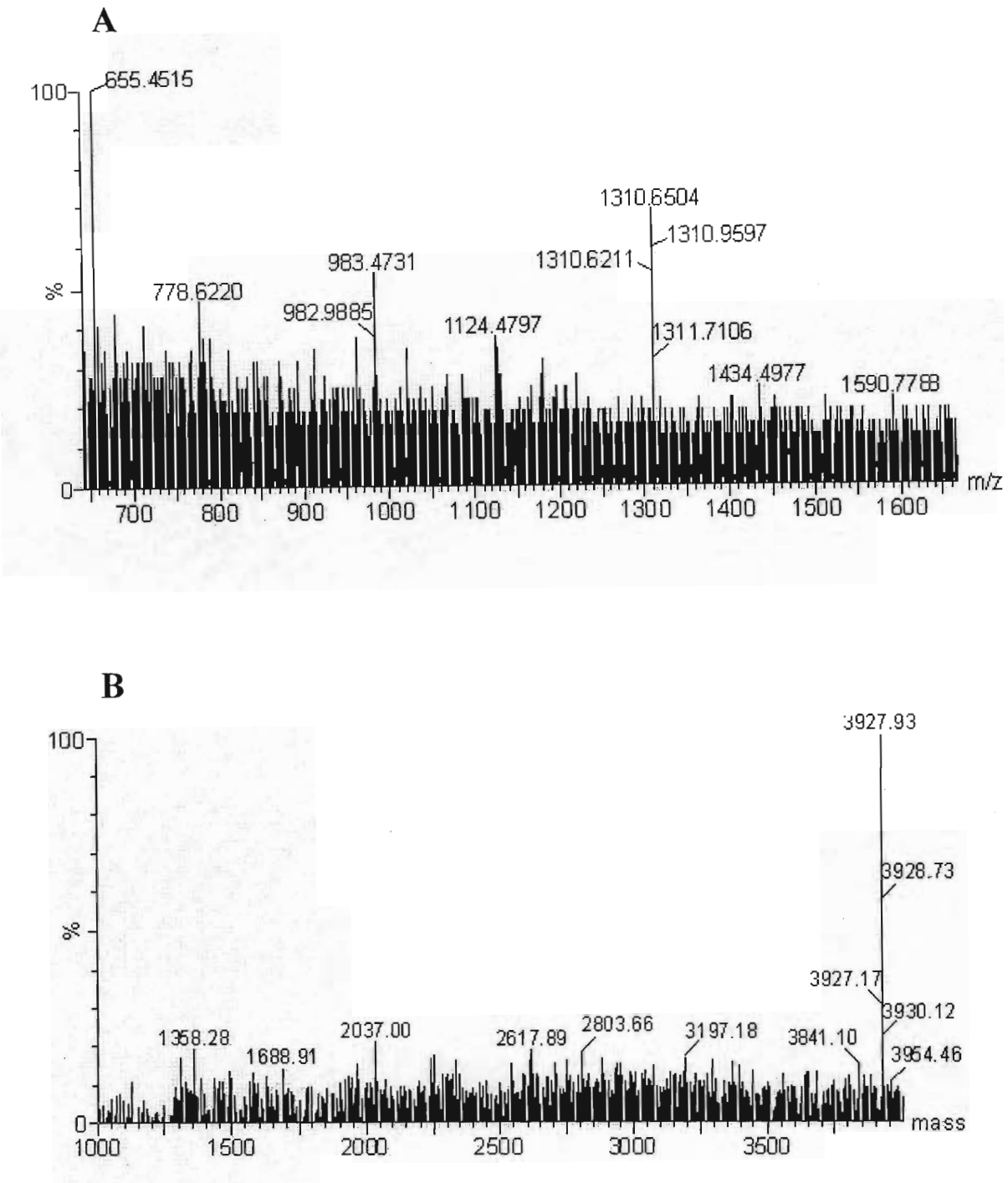


Figure 4.7. Data produced via the analysis of leucocin A using mass spectrometry to confirm the size of the peptide.

A. A stick-diagram from leucocin A analysis, relative insensities of ions of varying mass/charge ratio (m/z). **B.** A stick-diagram of ions masses present in the leucocin A sample.

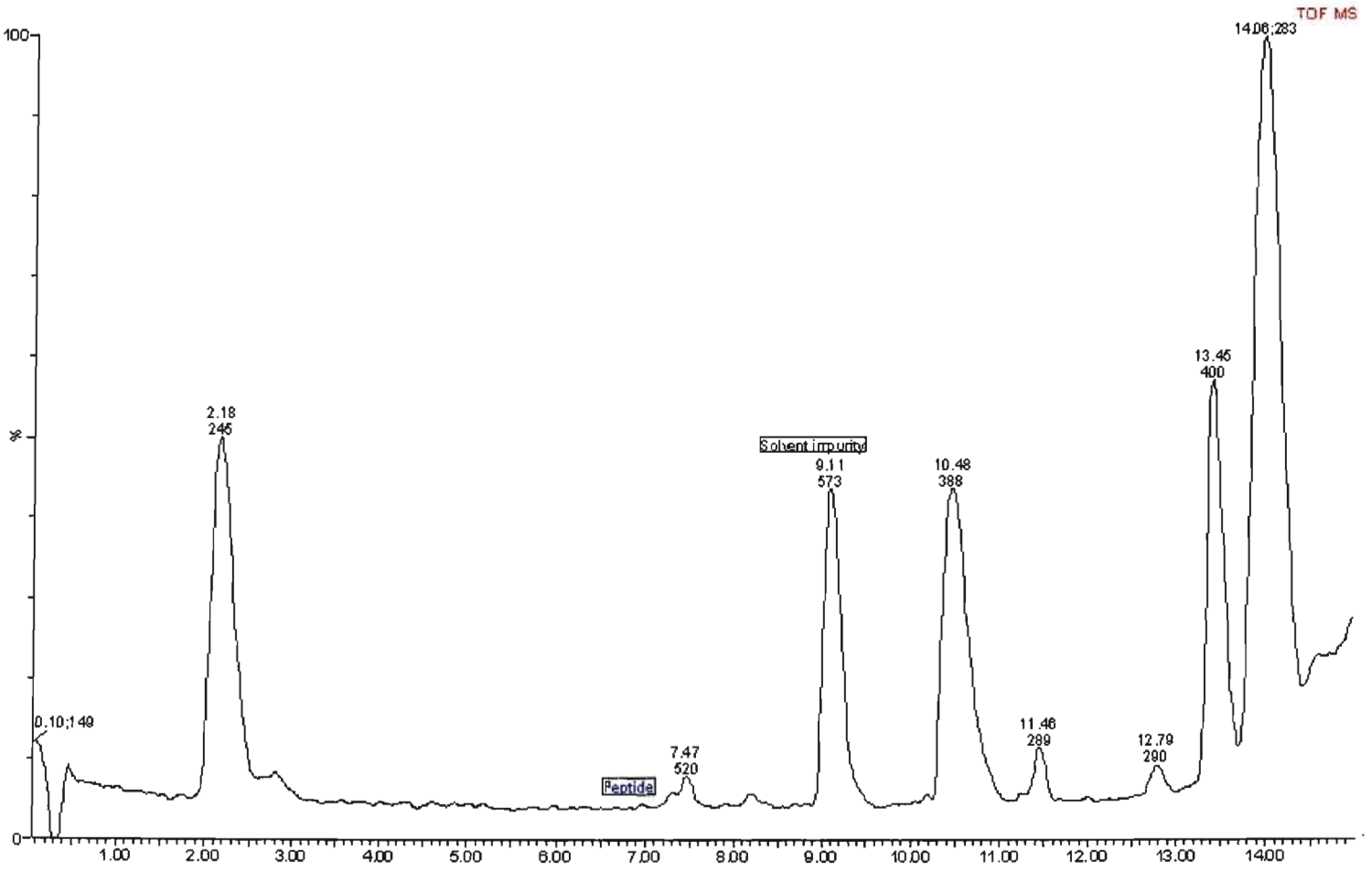


Figure 4.8. Liquid Chromatography Mass Spectrometry chromatogram produced during the analysis of leucocin A.
The HPLC purified leucocin A (10 µl of 1 mg.ml^{-1}) was analysed in a Phenomenex Prodigy ODS2 column. The small peak at 7.47 minutes represents the peptide, which was at a very low concentration.

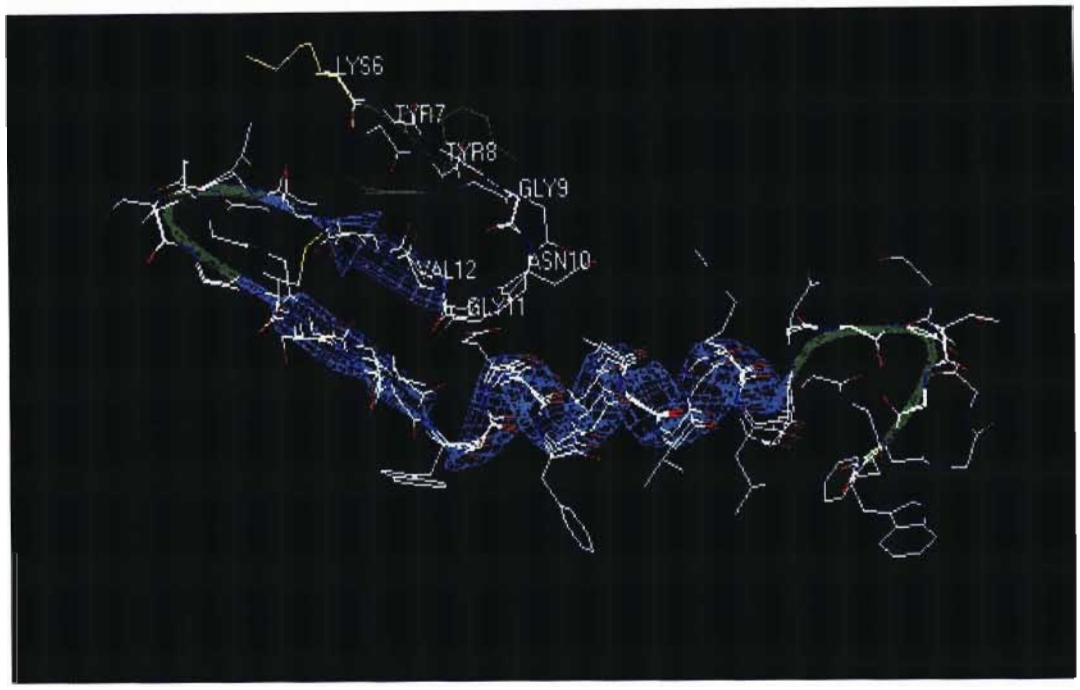


Figure 4.9. The Swiss-Pdbviewer constructed leucocin A structure.
The leucocin A structure constructed using Swiss-PdbViewer 3.7 program illustrates the conserved N-terminal amino acid motif (KYYGNGV), β-sheet region and the α-helix region.

The exact structure of leucocin A peptide has not been published as yet. The leucocin A sequence was downloaded from the NCBI data base (M64371, AAA68003.1) and used to predict the structure of the peptide using Swiss-PdbViewer 3.7 program (Figure 4.9).

A secondary predicted leucocin A structure showed a well-defined conformation that is similar to the leucocin A structure obtained from the leucocin A analysis in membrane mimicking environments using NMR spectroscopy (Chen *et al.*, 1997a; Morisset *et al.*, 2004). Leucocin A has an antiparallel β-sheet on the N-terminal region from residues 7-29, which has been suggested to govern the initial binding of the peptide to the targeted cells (Gallagher *et al.*, 1997; Uteng *et al.*, 2003). An amphipathic α-helix was observed from residues 17-31 followed by a C-terminal tail. It was suggested that the amphipathic α-helix is the domain that interacts with the phospholipid layer of susceptible cells (Miller *et al.*, 1998a).

4.2 LEUCOCIN A IMMUNITY PROTEIN

4.2.1 DNA isolation

Plasmid pJF 5.5 DNA was isolated from a transformed *E. coli* JM103 clone using the Nucleobond AX 100 Kit. The plasmid DNA hand a concentration of 122.5 $\mu g.ml^{-1}$ and a purity ratio of 1.9 after quantification at λ_{260} and λ_{280}. The highly pure pJF 5.5 DNA was used as a template for the amplification of the inserted immunity gene. The amplified immunity gene was cloned into a cloning, expression and purification ds-DNA pMAL-c2 vector. The pMAL-c2 DNA was isolated from *E. coli* using the same kit as for pJF 5.5 DNA isolation, which yielded a concentration of 117.6 $\mu g.ml^{-1}$ and a purity ratio of 2.2.

Figure 4.10A and B illustrate agarose gel analysis of the pure pJF 5.5 and pMAL-c2 DNA, respectively. Both plasmid DNA samples displayed three bands on the gels that represented different coiling states of plasmid DNA. Additionally, no RNA contamination was detected.

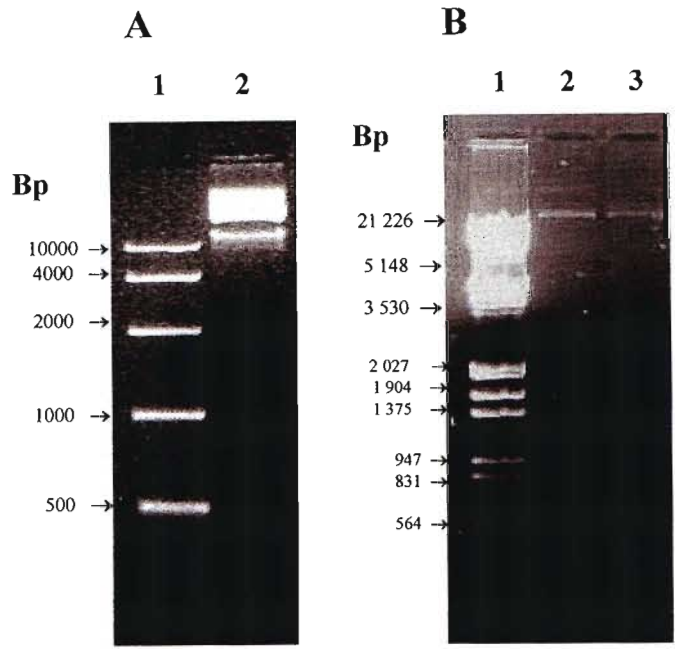


Figure 4.10. The plasmid DNA of pJF 5.5 and pMAL-c2 isolated with the Nucleobond AX 100 Kit.
A. Agarose gels (1% [w/v]), lanes, 1. Fastruler High Range DNA Ladder; 2. pJF 5.5 plasmid DNA (122.5 $\mu g.ml^{-1}$). **B.** Agarose gel (0.8% [w/v]), lanes, 1. Molecular Weight Marker (MWM III) (Roche), 2. Restricted pMAL-c2 vector (117.6 $\mu g.ml^{-1}$).

4.2.2 Immunity gene amplification

The immunity gene was amplified from the plasmid pJF 5.5, using EAL-2 and EAL-3 primers. The two primers were designed from the sequence of a previously cloned leucocin A immunity factor (Hastings *et al.*, 1991), and were confirmed to be effective by Pillay (2004). The *XbaI* site was added onto the 3' end of EAL-3 to ensure that the amplified immunity gene contained the *Xba*I recognition site, since the same site is found on the polylinker of the vector. A thermophilic DNA polymerase, called Taq polymerase, was used for amplification. Due its capability to work at 94°C, the temperature at which the DNA template was denatured (Roche). The resulting amplicon was cleaned with the NucleoSpin PCR Clean Up Kit, to ensure excess salts and other interfering reaction components were eliminated.

The pure amplicon (138.0 $\mu g.ml^{-1}$) was analysed on agarose gel (1% [w/v]) to determine the size by the BioRad Quantity One software. The size obtained was approximately 340 bp, which compared favourably with the theoretical immunity gene size of 342 bp (Figure 4.11A) (NCBI database).

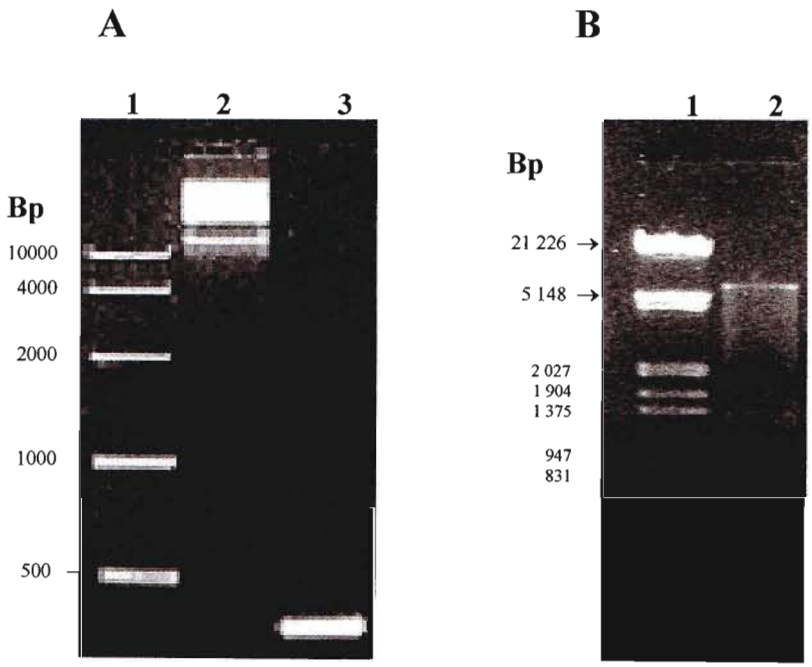


Figure 4.11. The Nucleobond AX 100 Kit isolated plasmid DNA and the restricted fragments of pJF 5.5 and pMAL-c2 and the PCR product.
A. Agarose gels (1% [w/v]), lanes, 1. Fastruler High Range DNA Ladder; 2. pJF 5.5 plasmid DNA; 3. A purified amplicon. **B.** Lanes, 1. MWM III; 2. Restricted pMAL-c2 vector.

4.2.3 Cloning of the immunity protein versions

The vector and the inserts were restricted in a manner that ensured complementary ends of either strand, to facilitate uni-directional insertion of the immunity gene into the pMAL-c2 vector. The immunity gene was blunt ended by Klenow enzyme, and subsequently restricted with *Xba*I that generated a sticky end on the C-terminal. The vector was double digested at the polylinker with *Asp700*, an isoschizomer for *Xmn*I that cleaves at position 2759 generating a blunt end and *Xba*I that cleaves at position 2782 generating a sticky end. Figure 4.11B illustrates the 6646 bp digested pMAL-c2 vector (Felix *et al.*, 1994).

The blunt-ended immunity gene was truncated by cleaving off the terminal regions. *Hae*III and *Cla*I endonucleases were used to cleave off the C-terminal and N-terminal regions producing immunity gene version A (IGVA) and immunity gene version B (IGVB), respectively (Figure 4.12). *Hae*III is a blunt end-producing endonuclease that cuts the gene at the 5' end, position 284 (GG↓CC) resulting in a blunt-ended 284 bp fragment, which was subsequently restricted with *Xba*I to generate a C-terminal sticky end. *Cla*I is a sticky end-producing endonuclease that cuts at the 3' end, position 68 (AT↓CGAT), resulting in a 274 bp fragment.

Cleavage of the terminal regions was a success as illustrated in Figure 4.13. The truncated fragments were compared to the wild type immunity gene, and the difference in the band sizes indicated the reduction of the fragment length. In Figure 4.13A, the 342 bp wild type immunity gene in lane 2 and the 284 bp IGVA is in lanes 3 and 4. In Figure 4.13B, lane 2 is the wild type immunity gene and lane 3 is the 274 bp IGVB.

The immunity gene versions were independently cloned into the pMAL-c2 vector by means of a ligation procedure. These genes were inserted at the polylinker site of the vector between the *mal*E and the lacZ α genes. Hence, they were fused to the *mal*E gene that encodes the MBP. The different constructs were transformed into *E. coli* JM103 strain.

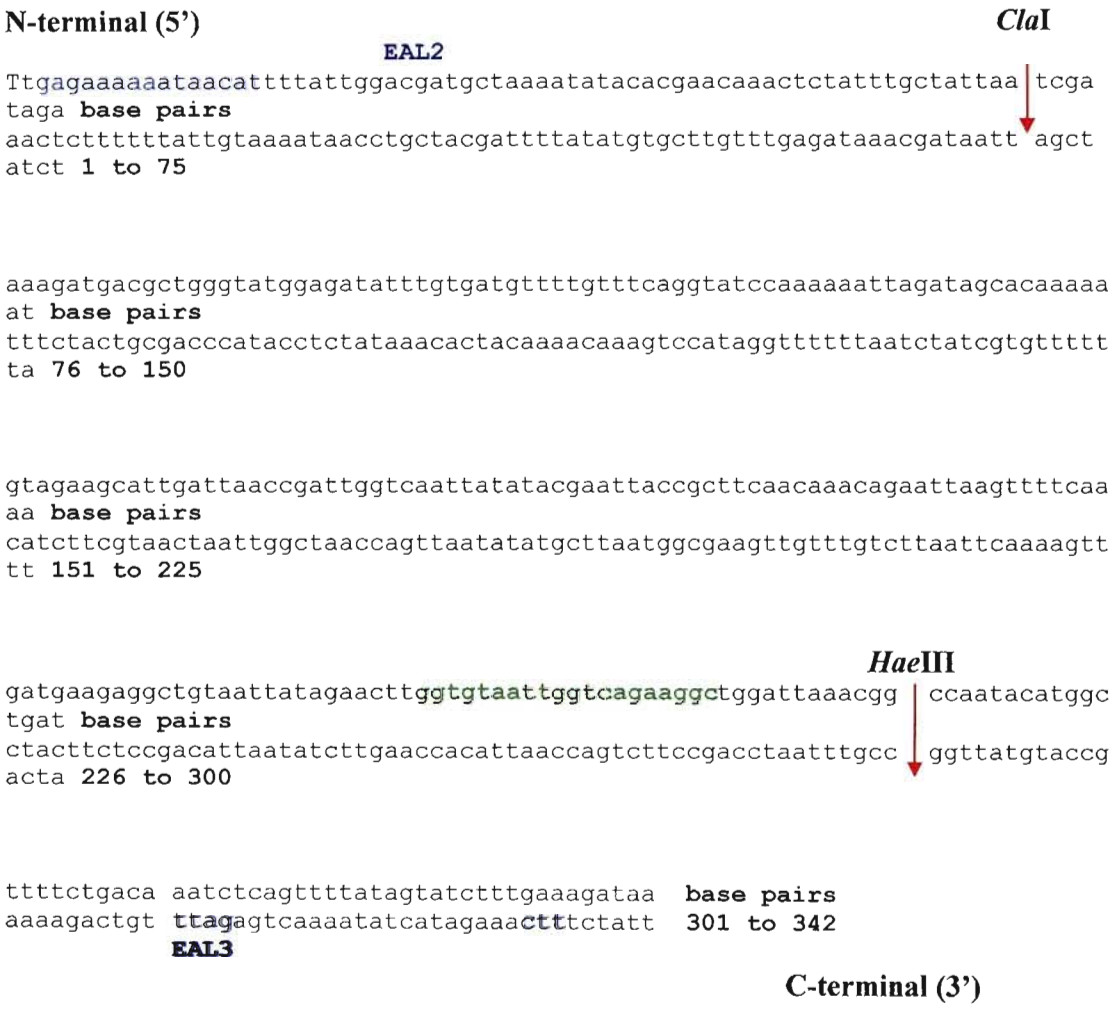


Figure 4.12. The 342 bp immunity gene sequence obtained from NCBI database (M64371, AAA68004.1).
The primer binding sites are shown on the sequence, EAL2 and EAL3 (in blue). The red arrows show the restriction sites for the *Hae*III enzyme and *Cla*I enzyme, which cleave off the C and N terminals, respectively. The binding site for the JHA 7 probe that was used for Southern blotting is highlighted in green.

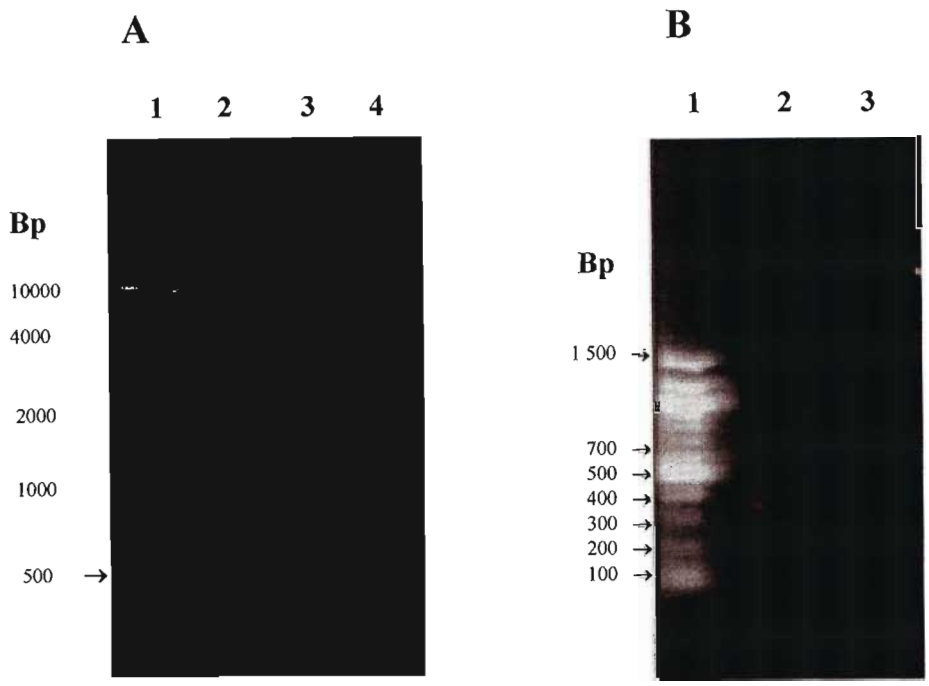


Figure 4.13. Digested immunity gene fragments in agarose gel (1% [w/v]).
A. Lanes, 1. Fastruler High Range DNA Ladder; 2. Wild type immunity gene; 3 & 4 *Hae*III digested immunity gene (284 bp). **B.** Lanes: 1. Fastruler O'Range DNA Ladder (Fermentas); 2. Wild type immunity gene; 3. *Cla*I digested immunity gene (274 bp).

Insertion of the gene onto the pMAL-c2 vector interrupts the *mal*E-*lac*Zα fusion activity, which allows a blue-to-white screen for inserts on X-gal supplemented LB agar plates. Blue colonies were indicative of β-galactosidase positive phenotypes, as the β-galactosidase breaks down lactose into glucose and galactose (Figure 4.14B). White colonies were indicative of β-galactosidase negative phenotypes (recombinant clones) due to deactivation of β-galactosidase (Figure 4.14A). The new recombinant plasmids were pKP1 (contains the wild-type immunity gene), pKP1A (contains IGVA) and pKP1B (contains IGVB).

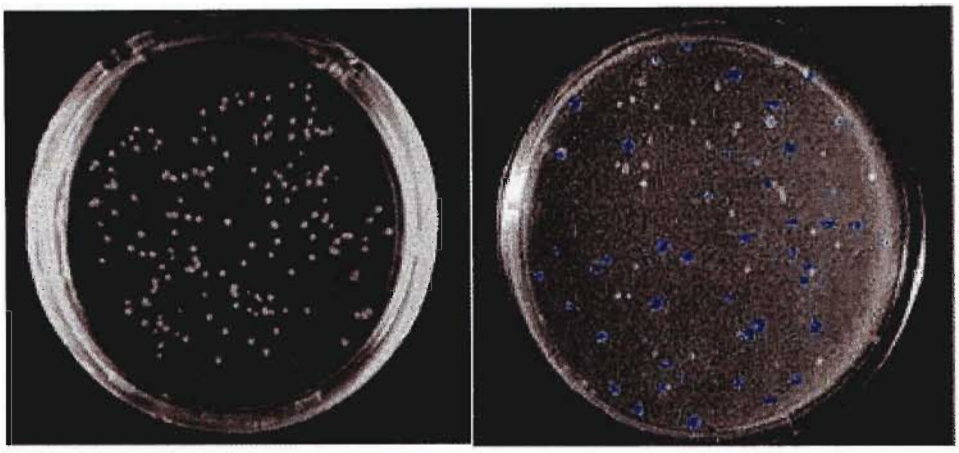


Figure 4.14. *E.coli* JM103 colonies that have been transformed with recombinant vectors and grown at 37°C on LB agar containing ampicillin, IPTG and X-gal.
A. White colonies that represent bacterial cells carrying only recombinant plasmid. B. Blue and white colonies that represent parental and recombinant clones.

4.2.4 Confirmation of recombinant clones

4.2.4.1 Recombinant vector DNA isolation

DNA from putative clones of pKP1, pKP1A and pKP1B that contain plasmids pKP1, pKP1A and pKP1B, respectively, were isolated using the alkaline lysis method as per Section 3.3.7.1. The spectrophotometric analysis of DNA indicated concentrations of 8.20 $\mu g.ml^{-1}$, 0.16 $\mu g.ml^{-1}$ and 0.13 $\mu g.ml^{-1}$, respectively. The DNA was analysed by agarose gel electrophoresis to verify the presence of plasmid DNA and to ensure that there was no RNA contamination (Figure 4.15).

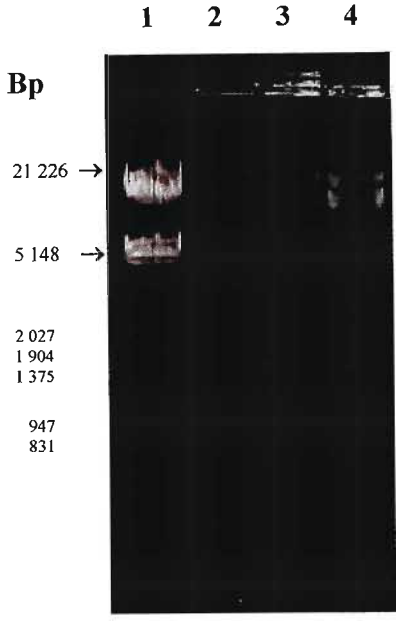


Figure 4.15. Analysis of plasmid DNA isolated from recombinant clones using the alkaline lysis method in agarose gel (1% [w/v]).
Lanes, 1. MWM III; 2. Clone pKP1A DNA; 3. Clone pKP1B DNA; 4. Clone pKP1 DNA.

4.2.4.2 Colony PCR

The procedure was performed in order to confirm presence of the inserted immunity gene fragment in the pKP1 plasmid. Primers EAL2 and EAL3 were used for amplification of the immunity gene. It was not feasible to do colony PCR on pKP1A and pKP1B, as the fragments no longer contained binding sites for the primers. Consequently, confirmation of the clones was performed by Southern hybridisation process.

The results of the colony PCR experiment are illustrated in Figure 4.16. Lanes 4 and 5, clearly show the PCR products (342 bp fragment). Lane 2, Control 1, a reaction without primers, had no bands. Lane 3, Control 2, a reaction without a template, produced a primer dimer.

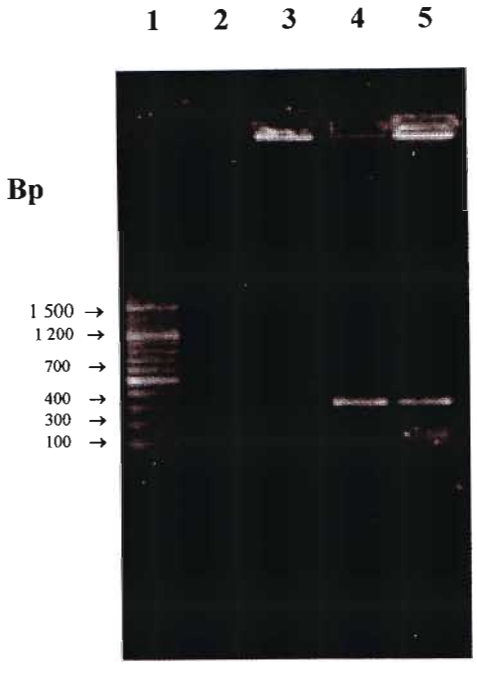


Figure 4.16. Agarose gel (1% [w/v]) showing a colony PCR products produced by the amplification of the immunity gene usinf clone pKP1 as a template.
Lanes, 1. Fastruler O'range DNA Ladder; 2. Control 1 (no primer); 3. Control 2 (no template); 4 & 5 amplified immunity gene inserts.

4.2.4.3 Southern blotting

Southern blotting procedure was performed to confirm the presence of the truncated immunity gene versions located within the recombinant DNA samples. The DIG-labelled oligonucleotide, JHA 7, was used as a probe, because it has a sequence complementary to the immunity gene (Figure 4.12). The JHA 7 probe is 20 bp in size, and hence tends to be very specific, as observed from the results depicted Figure 4.17.

The results obtained from the Southern blot experiment revealed that all three clones were true positive clones as they were detected by the JHA 7 probe (Figure 4.7A, lanes 3-5 and Figure 4.7B lanes 2-4). Plasmid pJF 5.5 in panel A, lane 2 and panel B, lane 1, served as a positive control as it has the immunity gene in it, and it was detected by the probe. The detection control was the DIG labelled control DNA in panel A, lane 7 and panel B, lane 6, which was successfully detected. The pMAL-c2 vector served as a negative control in

panel A, lane 6 and panel B, lane 5, which was not detected on the nitrocellulose membrane.

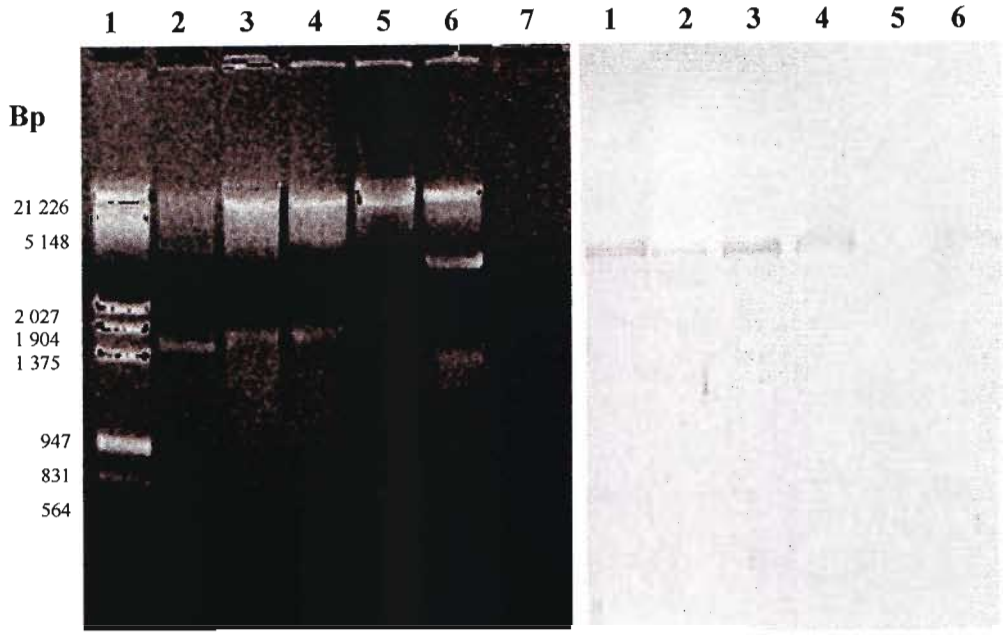


Figure 4.17. Recombinant clones on agarose gel (1.2% [w/v]) (A) and the equivalent Southern blot hybridization image (B).
A. Lanes, 1. MWM III (Roche); 2. pJF 5.5 DNA (positive control); 3. Clone pKP1A; 4. Clone pKP1B; 5. Clone pKP1; 6. pMal-c2 vector (negative control). 7. DIG labeled control DNA (Roche). **B.** Lanes, 1. pJF 5.5; 2. Clone pKP1A; 3. Clone pKP1B ; 4. Clone pKP1; 5.pMal-c2 vector; 6. DIG labeled control DNA. The JHA 7 probe was used at a concentration of 0.3 μM, which binds near the 3' end of the immunity gene.

4.2.5 Cytoplasmic expression of the fusion proteins

The cloned genes were fused to the *malE* gene and were thus expressed as the MBP-immunity fusion proteins (Kapust and Waugh, 2000). Due to the deletion of the *mal*E signal sequence on the pMAL-c2 series, the proteins are not expressed in the periplasm. Instead large amounts are expressed in the *E. coli* cytoplasm by the strong tac promoter with the translation initiation signals of MBP (Kellerman and Ferenci, 1982; Bessette, *et al.*, 1999; Kapust and Waugh, 1999). Translation is initiated efficiently due to the concurrence of the ribosome-binding site with the N-terminal MBP domain of the fusion protein.

In view of the fact that the pMAL–c2 vector carries a gene that encodes the *lac* repressor that maintains the expression of the P_{tac} at low levels, the induction of the fusion proteins was achieved by the addition of *lac*-inducer IPTG to the log phase culture. The addition of IPTG induces the activity of β-galactosidase, by binding and inhibiting the lac repressor and also increases the solubility of the proteins, provided the temperature is reduced (Fox and Waugh, 2003).

The fusion proteins expressed from putative clones were, MBP-IP (encodes the wild-type immunity gene), MBP-IPA (encodes the gene lacking the C-terminal), and MBP-IPB (encodes the gene lacking the N-terminal) were analysed on a SDS-PAGE gel. In Figure 4.18 are results obtained from the expression of clone pKP1, lanes 2 and 3 show the induced and non-induced samples of the expressed clone pKP1. A dark band that occurs in the induced sample and is absent in the non-induced sample usually indicates the occurrence of expression. The expressed fusion protein MBP-IP has a molecular weight of 58.55 kDa (indicated by the arrow), and is not intense in the non-induced sample. To confirm that the expressed protein fell within the expected size range, it was compared to MBP which has a theoretical mass of ± 43 kDa (pMAL Protein Fusion Purification System). Figure 4.18B indicates that the induced MBP-IP in lane 1 was slightly larger than the MBP in lane 3, as it contains the 15.55 kDa immunity protein (indicated by arrows).

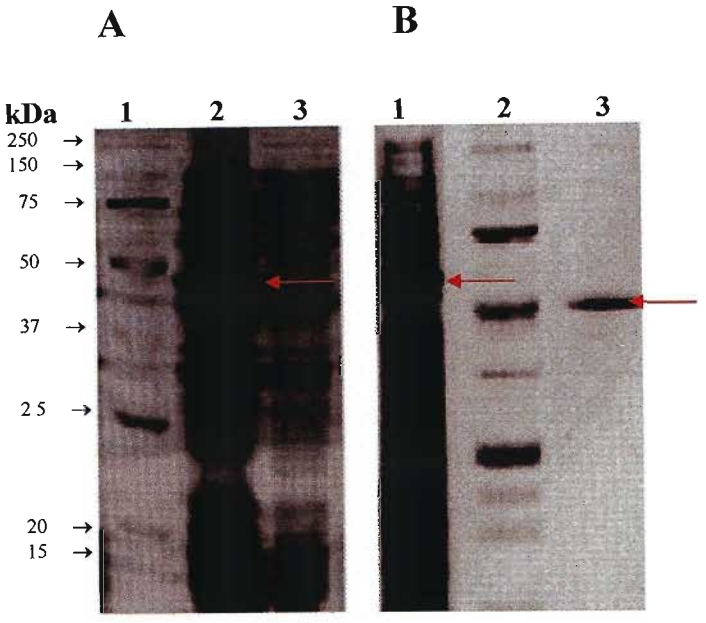


Figure 4.18. Coomassie-stained SDS-PAGE gel (10% [w/v]), showing the expressed fusion construct containing the entire immunity protein.
A. Lanes, 1. Precision Plus Protein Standards (PPPS) (Biorad); 2. Induced clone pKP1 (58.55 kDa); 3. Non-induced clone pKP1. **B.** Lanes, 1. Induced clone pKP1; 2. PPS (Biorad); 3. Purified MBP (43 kDa) (size control).

Figure 4.19 illustrates the induction of pKP1A and pKP1B for the expression of MBP-IPA and MBP-IPB, respectively. Shown in Figure 4.19A, lane 3 is the induced sample that has a thick 55.81 kDa MBP-IPA band, and lane 4 is the non-induced sample. Similarly Figure 4.19B, lane 1shows the induced 54.91 kDa MBP-IPB protein and the non-induced sample is in lane 2.

Following the induction of the putative clones, proteins were released from the cells by sonication. The Bradford assay was used to monitor protein release at λ_{595}, as it contains a red cationic CBBG dye that binds to the released protein at arginine, tryptophan, tyrosine, histidine and phenylalanine residues forming anionic blue CBBG complexes at this wavelength. The increase in the intensity of the blue colour indicates an increase in the CBBG complex concentration, which is depicted by the increase in the absorbance reading at λ_{595}. Consequently, the stability of the absorbance reading imply that maximum proteins have been released by the cell.

Lane 4 in Figure 4.19A, is a typical sonicated induced sample, in this case clone pKP1B was sonicated. The 54.91 kDa band of the sonicated sample is thicker than that of the unsonicated induced sample, which signifies more proteins are released by sonication.

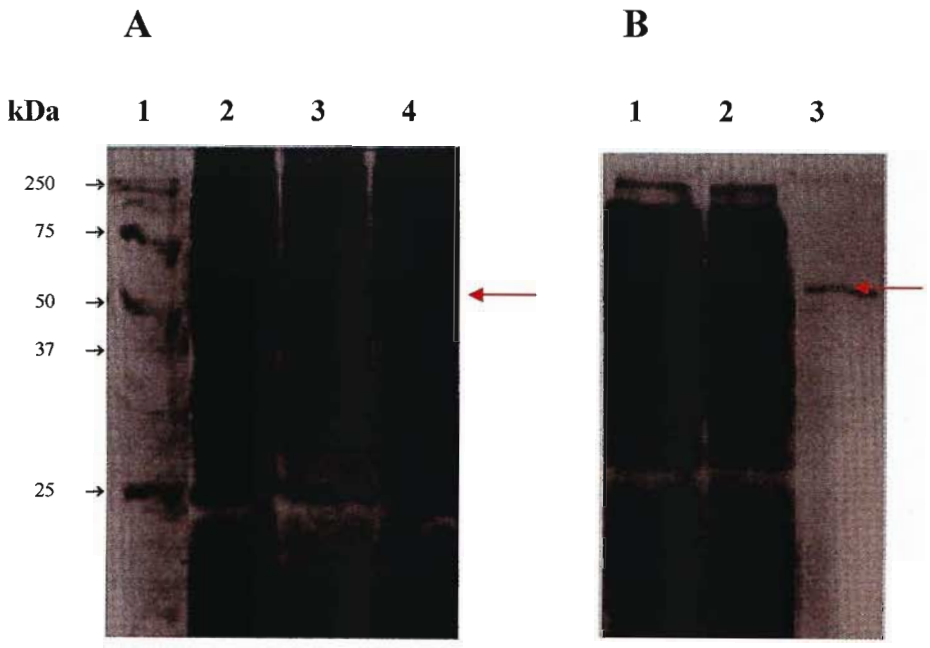


Figure 4.19. Coomassie-stained SDS-PAGE gel (10% [w/v]), showing the expression of the different fusion protein versions induced using 0.3 mM IPTG.
A. Lanes, 1. PPPS; 2. Induced clone pKP1A; 3. Non-induced clone pKP1A; 4. Sonicated clone pKP1A. **B.** Lanes, 1. Induced clone pKP1B; 2. Non-induced clone pKP1B; 3. MBP.

4.2.6 Purification of the immunity protein versions

The expressed fusion proteins were purified from the bacterial lysate in a one-step affinity chromatography on an amylose matrix. MBP has a strong affinity for amylose resin, and as it is connected to the N-terminal of the desired protein, this enables the fusion protein to bind to the amylose resin. The proteins were eluted from amylose resin by competitive displacement with maltose, as the MBP has an even higher affinity for maltose (Kapust and Waugh, 2000). Additionally, the MBP being an effective solubility enhancer improved the purification process (Routzahn and Waugh, 2002).

Theoretically, the column buffer with pH of 7.2 is used for the purification, which was true for the MBP-IP purification. Conversely, the MBP-IPA and MBP-IPB did not bind efficiently at pH of 7.2. The low affinity of MBP-IPA and MBP-IPB to amylose resin was attributed to their low solubility and aggregated state resulting from the fact that they did not fold properly, owing to the truncation of the proteins.

Nevertheless, after optimisation using different column buffer pH values, 6.8 was found to be optimal for purification of MBP-IPA and MBP-IPB (Table 3.8). Acidic conditions resulted in a lower affinity of the proteins for the amylose resin. Conversely, basic conditions resulted in a stronger affinity, which led to a high background on the SDS-PAGE gel. In Figure 4.20, lanes 2-4 illustrate the high background caused by the attachment of nonspecific proteins to the resin. Lanes 6 and 7 demonstrate the protein loss caused by the acidic buffer that decreased the affinity of the proteins to the amylose resin.

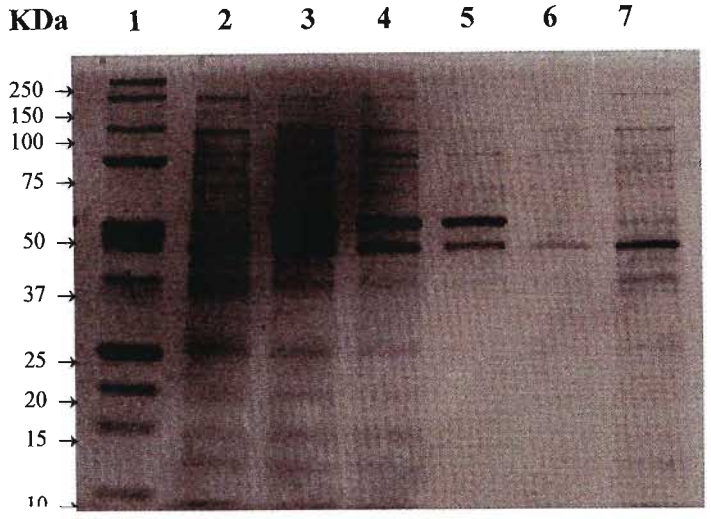


Figure 4.20. Coomassie-stained Tris tricine gel (10% [w/v]) illustrating the effect of column buffer pH values on the purification of the immunity protein versions.
Lanes, 1. PPPS; 2. MBP-IPA, pH 7.6; 3. MBP-IPA, pH 7.2; 4. MBP-IPA, pH 7.0; 5. MBP-IPA, pH 6.8; 6. MBP-IPA, pH 2.8; 7. MBP-IPB, pH 2.8.

Protein loss during the purification process was monitored by performing the BCA assay on the aliquots eluted from the amylose resin and reading their absorbances at λ_{562} Figure 4.21 indicates that only minimal amount of protein was lost with the flow through, whereas, no protein was lost during the washing stage.

The purified proteins were analysed on a Tris tricin gel to determine the sizes of the purified proteins. Tris tricine gels are generally used for analysis of smaller proteins (<15kDa) due to their higher resolution. Figure 4.22 indicates that the purified MBP-IPA, MBP-IPB and MBP-IP produce single bands on the gel in lanes 5, 6 and 7, respectively. The purification kit displayed effective purification aptitude, as denoted by the solubility and purity of the proteins.

Conversely, the yield was very low, which was portrayed by the faintness of the bands in Figure 4.22 and the decrease in the amount of proteins present in the elute as compared to that of the initial supernatant fraction in Figure 4.21. Figure 4.22, lanes 4 and 8 are the flow through from the purification of MBP-IPA and MBP-IP, respectively. It is evident

that a small amount of the desired proteins was lost during this stage as there are faint bands of the same size as the fusion proteins observed in the lanes 5-7. The concentrations of fusion proteins recovered were 15.64 $mg.ml^{-1}$, 17.78 $mg.ml^{-1}$ and 14.42 $mg.ml^{-1}$ for MBP-IP, MBP-IPA and MBP-IPB, respectively. The proteins were very stable, which can be accredited to the MBP as it has been observed to protect its passenger proteins from proteolytic degradation *in vivo* (Fox and Waugh, 2003; Fox *et al.*, 2003; Smith, *et al.*, 2003)

Figure 4.22, lane 2 is the MBP (43 kDa), which was loaded to compare its size to the sizes of the fusion proteins. The sizes of the fusion proteins ranged between 55 and 58 kDa. The presence of multiple bands in lane 2 may be due to protein dimerization caused by reaction-resistant complex formation, producing two bands one at 115 kDa and the second at 38 kDa.

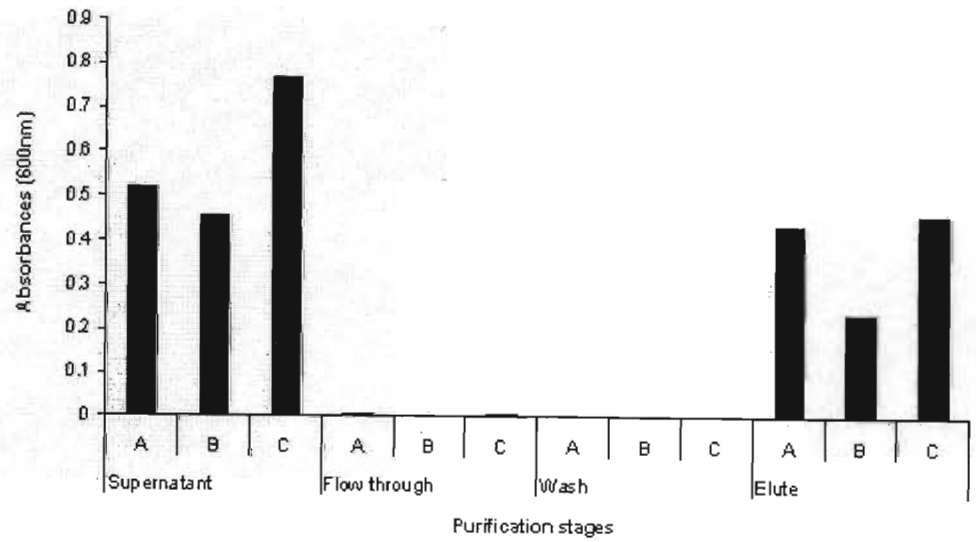


Figure 4.21. Graph used to demonstrate protein loss during the amylose purification stages.
On the graph A, B and C represent proteins MBP-IPA, MBP-IPB and MBP-IP, respectively. The proteins were purified at their optimum pH.

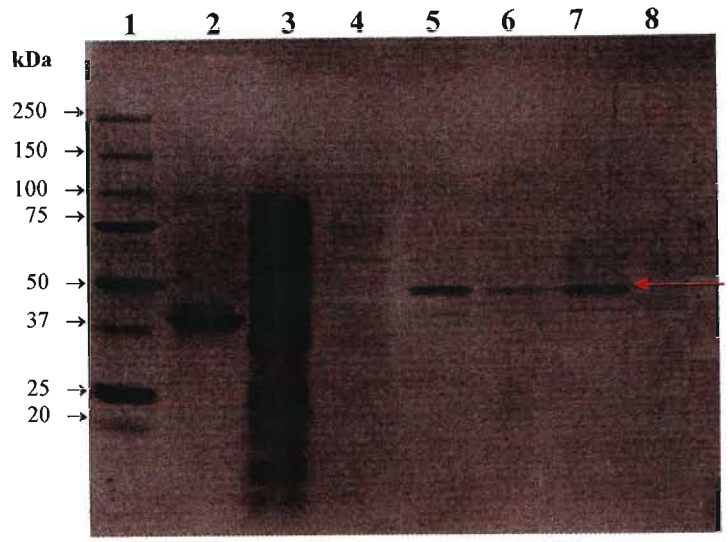


Figure 4.22. Coomassie-stained Tris tricine gel (10% [w/v]), for the analysis of purified fusion proteins.
Lanes, 1. PPPS; 2. MBP; 3. Clone pKP1B lysate flow through; 4. MBP-IPA flow through; 5. MBP-IPA elute, pH 6.8; 6. MBP-IPB elute, pH 6.8; 7. MBP-IP elute, pH 7.2; 8. Clone pKP1 lysate flow through.
NB: not all elutes from the purification process are shown

4.2.7 Detection of the protein of interest

Following the purification step, the presence of the proteins was verified by means of a Western blot analysis. Anti-MBP antibody was used to detect the immunity proteins, given that the proteins were fused to the MBP. The primary antibody was conjugated to HRPO, which facilitated the detection step and reduced the time required to perform the experiment since the addition of the secondary antibody conjugated to HRPO was eliminated from the procedure.

The protein transfer was confirmed by temporary staining of the nitrocellulose membrane with Ponceau S stain. The bands that symbolize the antibody-protein complexes were subsequently exposed by the addition of the POD. Figure 4.23 demonstrates the results of the Western blot, where lanes 2 to 5 of the SDS-PAGE gel (Figure 4.23A) correspond to lanes 1 to 4 on the Western blot image (Figure 4.23B). The anti-MBP antibody detected

the MBP as the dark bands appeared on the blot (Figure 4.23, panel B lane 4). It is clear that the anti-MBP antibody was highly sensitive since it was able to detect the previously described reaction-resistant complexes (Section 4.2.6) of the fusion proteins, which were not visible on the SDS-PAGE gel. The untagged protein standards served as a negative control (Figure 4.23, first lanes of panel A) and the pure MBP served as a positive control (Figure 4.23, lane 2 of panel A).

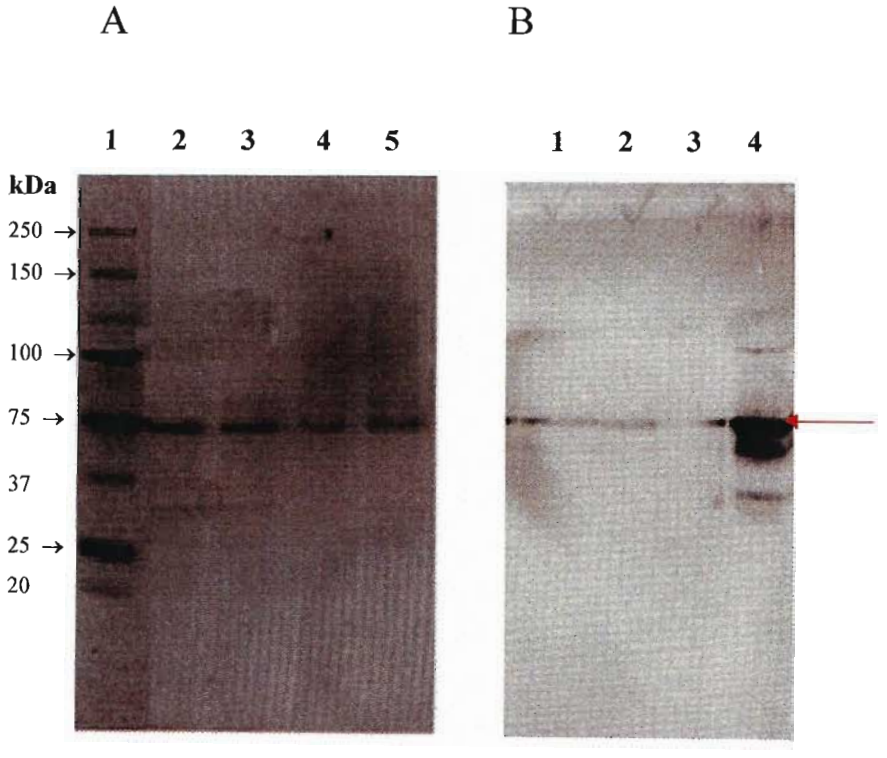


Figure 4.23. Coomassie-stained reference SDS-PGE (10% [w/v]) (A) and the corresponding Western Blot image (B) produced during the transfer of purified fusion proteins.
A. Lanes, 1. PPPS; 2. MBP; 3. MBP-IP; 4. MBP-IPA; 5. MBP-IPB. **B.** Lanes, 1. MBP-IPB; 2. MBP-IPA; 3. MBP-IP; 4. MBP (PPPS was not detected on the Western blot).

4.2.8 Cleavage of the MBP from the fusion protein

The fusion proteins were subjected to Factor Xa protease activity in order to cleave off the MBP. Figure 4.24 shows the Factor Xa cleavage site, Ile- (Glu or Asp)-Gly-Arg, encoded by the pMAL-c2 vector polylinker sequence as indicated by the arrow (New England Biolabs).

Figure 4.25 is a 20% Tris tricine gel showing the results obtained following the Factor Xa cleavage. Multiple bands were observed in the profiles of MBP-IPA, MBP-IPB and MBP-IP as shown in lanes 2, 3 and 4, respectively. The the immunity protein versions sizes could not be accurately estimated since the bands were very faint on the gel, however, their sizes ranged between 10 and 15 kDa which correlated to the theoretical sizes of 15.55 kDa, 12.807 kDa and 11.91 kDa for the wild type immunity protein, version A and version B, respectively. The MBP was observed in lane 2, 3 and 4 with an approximate size of 45 kDa (indicated by the green arrows), demonstrated that the MBP had been separated form the immunity protein versions. The multiple bands that appeared on the gel were speculated to be partially cleaved fusion proteins (indicated by the yellow arrows).

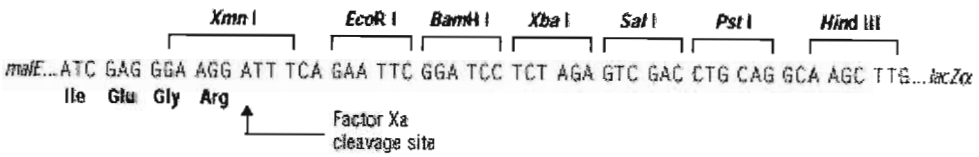


Figure 4.24. The polylinker of the pMAL-c2 vector, showing the accessible recognition sites including the Factor Xa protease cleavage site that facilitates the separation of the MBP and its partner protein (New England BioLabs, USA, Beverly).

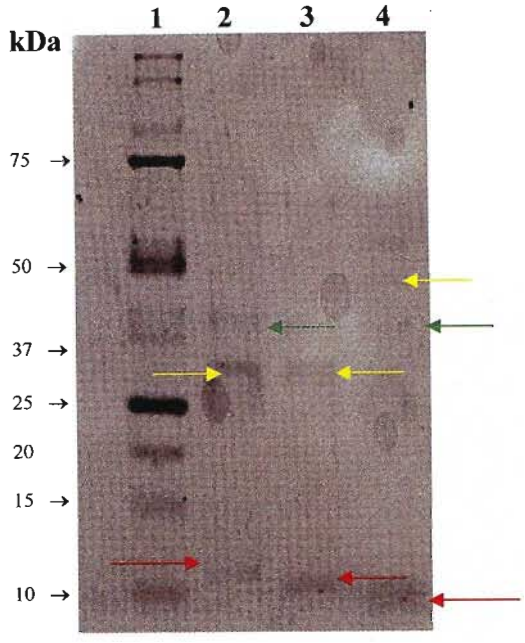


Figure 4.25. Coomassie-stained Tris tricine gel (20% [w/v]), illustrating the band profiles after the cleavage of the MBP from the immunity protein versions using Factor Xa enzyme.
Lanes, 1. PPPS; 2. MBP-IP; 3. MBP-IPA; 4. MBP-IPB.

4.2.9 Protein sequencing

Following Factor Xa digests, the proteins were subjected to N-terminal sequencing to confirm the orientation of the immunity protein versions on the pMAL-c2 vector. The proteins were initially separated on a SDS-PAGE gel and subsequently transferred to a PVDF membrane, which has a with a high protein binding capacity (0.1-0.22 μm pore size) to ensure that most proteins were retained.

Figures 4.26 shows the Factor Xa digested fragments on a SDS-PAGE gel (A) and the corresponding PVDF blot (B). All the proteins were successfully transferred. The MBP bands are indicated by the green arrows, the partially cleaveD fusion proteins are indicated by the yellow arrows and the immunity protein versions are indicated by the red arrows. The bands running on the dye front in lanes 2-4 were hypothesized to be the immunity protein versions and were thus sequenced. The wild type immunity protein and IPVA were successfully sequenced (Table 4.2). The obtained sequences were evident that the

proteins are the expected immunity proteins. However, no consistent sequences were obtained for IPVB, which can be attributed to the absence of the N-terminal as the Edman degradation process is hindered by any alteration in the N-terminal of the protein since the initial step in protein sequencing is the coupling of the free N-terminus of the protein with PITC (Perkin Elmer, 1995).

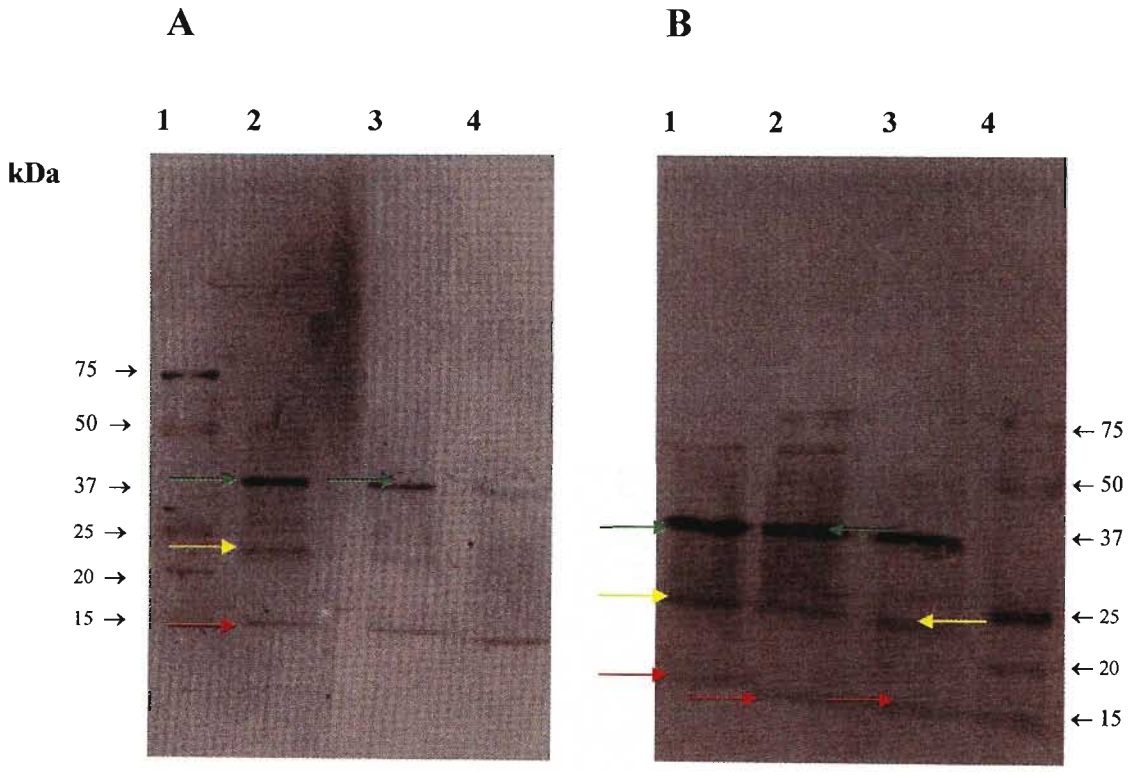


Figure 4.26. Coomassie-stained SDS-PAGE gel (10% [w/v]) and a corresponding PVDF membrane image of the band profiles of the fusion proteins following Factor Xa digestion.
A. Lanes, 1. PPPS; 2. Immunity protein; 3. Immunity version A; 4. Immunity version B.
B. Lanes, 1. Immunity version B; 2. Immunity version A; 3. Immunity protein; 4. PPPS.

Table 4.2. Protein sequences of the immunity protein versions obtained from the N-terminal sequencing.

Protein name	Generated sequence
Immunity protein	MRKNNILLDDAKI
IPVA	MRKNNILLDDAKI
IPVB	N/A

4.2.10 Interaction studies

The results obtained from SPR interaction studies showed that there were no direct interaction between the leucocin A peptide and the immunity protein versions. However, interaction was observed between the wild type immunity protein and the cytoplasmic fraction of *L. gelidum* (Table 4.3). By exposing the truncated immunity protein versions to the cytoplasmic fraction, only the IPVA, C-terminally truncated version showed interaction. It can therefore be concluded that the N-terminal region of the immunity protein is involved in the hypothesized immunity mechanism. Time limited the testing of the MBP interaction against the cytoplasmic fraction and should be performed in the future.

Table 4.3. Surface plasmon resonance studies confirming interaction between ligand and orphan binding partners.

Ligand constructs	Leucocin A	Cytoplasmic fraction
Full Immunity protein	*-ve	*++
C-terminally truncated (IPVA)	*-ve	*++
N-terminally truncated (IPVB)	*-ve	*-ve

*++: Interaction occurred
*-ve: No interaction occurred

CHAPTER FIVE

CONCLUSION AND FUTURE PROSPECTS

Research continues for more effective bacteriocins. Development is ongoing for optimization of existing bacteriocins to address both biological and economical concerns. Bacteriocins are able to serve as bacterial inhibitors similar to the low molecular weight antibiotics, toxins, lytic agents, and bacteriophages. Bacteriocins are generally regarded as safe for they have been in our food systems for centuries. Due to their protein nature, they are more acceptable than their synthetic chemical counterparts. Bacteriocins are produced by bacteria, which imply their genes could possibly be genetically engineered to encode novel ideal peptides. This gives researchers an opportunity to circumvent the problems associated with the resistance of strains, thus, enabling control of the growth of problematic food and clinical pathogens as well as increasing the inhibitory spectrum of the bacteriocins. Understanding the immunity mechanism will allow researchers to control the fate of sensitive as well as resistant strains when exposed to the bacteriocidal peptides.

This study formed the basis of a broader project to understand the mechanisms of immunity in the producer organism. The first objective was to clone and express the immunity protein, both in its full compliment as well as in truncated versions of the protein. This would lay the foundation for further investigations into the direct role of this immunity protein. We were able to satisfy the initial objective, to generate the immunity protein versions and material to be used in the rest of the project. Initial surface plasmon resonance (SPR) experiments indicate the absence of any direct interaction between the immunity protein and leucocin A. The possibility of a binding partner in the cytoplasm of the producer organism is being investigated. This was after initial studies showed a possible interaction with an orphan protein.

To date, not much research has focused on the role the immunity protein plays in producer protection against its peptide. Although it has already been shown that the high levels of resistance to Class IIa bacteriocins in *L. monocytogenes* are due to shut down of mannose phosphotranferase system, the scientific evidence seems to indicate that immunity proteins may also be involved. Future experiments aim to unravel some of these answers and we intend substantiating some of our speculations.

CHAPTER SIX

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