Cloning, expression, and characterization of lactic acid bacteria recombinant prolidases

A Thesis Submitted

to the College of Graduate Studies and Research

in Partial Fulfilment of the Requirements

for the Degree of Master of Science

in the Department of Applied Microbiology and Food Science

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

By

Soo In Yang

PERMISSION TO USE

In presenting this thesis in partial fulfilment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purpose may be granted by the professor who supervised my thesis work or, in their absence, by the head of the department or the dean of the college in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Applied Microbiology and Food Science
University of Saskatchewan,
51 Campus Drive,
Saskatoon, SK, Canada, S7N 5A8

ABSTRACT

Lactobacillus plantarum (*Lb. plantarum*) NRRL B4496 and *Lactococcus lactis* (*Lc. lactis*) NRRL B1821 prolidase genes were isolated, cloned, and sequenced. The sequence-confirmed genes were subcloned into the expression systems. The recombinant prolidases from the pKK223-3 systems were purified through ammonium sulphate precipitation and anion-exchange column chromatography. Recombinant *Lb. plantarum* prolidase, however, demonstrated a loss of activity during the purification. The following characterization work was performed on purified recombinant *Lc. lactis* prolidase.

The mass spectroscopic result and the molecular modelling suggested a 80 kDa homodimer with two metal cations at the catalytic centre of the prolidase. The optimum temperature was 50 ºC and showed more than 50% activities between 40 and 55 ºC. The enzyme was most stable at 30 ºC and withstood 20 min of heat-treatment up to 60 ºC, however, lost activity over 70 ºC. Circular dichroism indicated a denaturation temperature of 67 ºC. The optimum pH was 6.5 for hydrolyzing Leu-Pro and the enzyme did not display any activity below pH 5.5 nor above pH 7 with this peptide. However, Phe-Pro was hydrolyzed the fastest at pH 7 and Arg-Pro had a maximum rate at pH 9. This metallopeptidase exhibited a broad range of metal cation preference, hydrolyzing Leu-Pro with Mn^{++}, Co^{++}, Zn^{++}, Ca^{++}, and Mg^{++}. Further kinetic analysis showed

unusual allostery of the enzyme (Hill coefficient: 1.3). The unique substrate intakes on Glu-Pro and tripeptides were observed while Val-Pro was not hydrolyzed. The molecular modelling of this prolidase suggested a difference in the substrate specificity resulting from a loop structure, L33 to R40, near the substrate binding site.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. Takuji Tanaka for his ceaseless endeavour on the research, supervision, and friendship. I deeply appreciate guidance and expertise from the advisory committee members, Dr. Phyllis Shand and Dr. Darren Korber. Dr. George Khachatourians, Dr. Mike Ingledew, and Dr. Nicholas Low are acknowledged for their enthusiastic and generous advice.

I thank my colleagues, Anil Illathu, Anula Perera, Bill Reid, Bei Zhang, Delia Mavaragani, Dinka Besic, Edwin Pensaert, Ioannis Mavragani, Kelly Aasen, Rick Green, Samira Dadgar, Sinisa Vidovic, Sohail Qazi, Tara Rema, and Xuesong Li for their kindness and sincere friendship. I am also grateful to previous summer students, Takayuki Yoshimura, Shuanghui Liu, and Lili Liu, for their assistance and companionship.

I thank Dr. Michael Nickerson and Gerald Henriksen for their friendly attitudes every time. Other thanks go to Patty Posnikoff, Sharon Greenough, and Bobbie Gormley for their help on personal and administrative works.

I am also indebted to my brother and sister for their invaluable encouragement. I finally would like to express my acknowledgement to my parents for their lofty love and motivation throughout the master program.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES

LIST OF ABBREVIATIONS

Å	Angstrom
ABC	ATP-binding cassette
APpro	proline aminopeptidase
ATP	adenosine triphosphate
CD	circular dichroism
CspA	cold shock protein A
DTT	dithiothreitol
DEAE	diethyl-aminoethyl
Δ	deletion
dNTP	deoxynucleotide triphosphate
EDTA	ethylene diamine tetra acetic acid
ESI-MS	electrospray ionization mass spectrometry
FPLC^{TM}	Fast Protein Liquid Chromatography
fs	femtosecond
g	gravitational force
G^+	Gram positive
h	Hill coefficient
Hsp	heat shock protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LAB	lactic acid bacteria
LB	lysogeny broth
Lb.	Lactobacillus
Lc.	Lactococcus
MCS	multiple cloning site
MetAP	methionine aminopeptidase
M_r	relative molecular mass
MS	mass spectrometry
M_w	molecular weight
MWCO	molecular weight cut-off
NFS	non-fat-solid
m/z	mass-to-charge ratio
NRC-PBI	National Research Council-Plant Biotechnology Institute
ORF	open reading frame
ps	picosecond
PCR	polymerase chain reaction
PDB	Protein Data Bank
PEG	polyethylene glycol

*pep*Q	prolidase coding gene
PepQ	prolidase
Pfpol	Pyrococcus furiosus prolidase
pm	picometre
Pmf	proton motive force
PNK	polynucleotide kinase
PRT	proton motive force-driven peptide transport family
PrtP	proteinase
rpm	revolutions per minute
SAP	shrimp alkaline phosphatase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethylenediamine
UV	ultraviolet
V_{max}	maximum velocity
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
Xaa	amino acid
Yaa	amino acid

1. INTRODUCTION

Nutritionally fastidious lactic acid bacteria (LAB) require a complex and rich medium (Axelsson, 1998). Milk might be a good choice for their habitat with respect to their evolutionary development, consisting of 80% of water and 20% fat and non-fat-solid (NFS). The proteinous substances, mainly proline-rich casein in the NFS are essential amino acids and nitrogen sources for growth (www.foodsci.uoguelph.ca, 2006).

The enzymatic degradation of proteinous materials in dairy products generally results in the formation of small peptides. These diverse and randomly digested products are further hydrolyzed by peptidases, generating free amino acids to meet the nutritional demands of starter microorganisms in dairy processing (e.g. LAB). However, the structural uniqueness of proline-containing regions of proteins diminishes the susceptibility towards proteinases due to the conformational restriction caused by the imine rings of prolines. Therefore, it causes difficulties in the hydrolysis processes of the polypeptides (Morel *et al*., 1999a,b).

Many proteinases and peptidases do not affect the hydrolysis of proline-containing proteins; on the contrary, some peptidases are specifically responsible for the hydrolysis of proline-containing peptides. LAB rely mainly on certain types of peptidases for this. Peptidases reported to have specific hydrolysis of peptidases of proline-containing peptides are proline iminopeptidase, prolinase, X-prolyl dipeptidyl

aminopeptidase (PepX; XPDAP), and prolidase (PepQ) (Christensen *et al.*, 1999). Prolinase and prolineiminopeptidase share similar substrates, Pro-Xaa or Pro-Xaa-Xaa, respectively (i.e. proline at the N-terminus of the peptide). X-prolyl dipeptidyl aminopeptidase generates Xaa-Pro dipeptides from the N-terminus of polypeptides. Prolidase (EC 3.4.13.9) possesses specificity on Xaa-Pro dipeptides, specifying its unique catalytic role (Kunji *et al*., 1996).

In fermented food products, such as cheese, proteolytically released amino acids and peptides are key to flavour formation. Among the generated peptides, hydrophobic fragments are known to be the main bitter-taste factor (Lindsay, 2000). Proline-containing peptides are hydrophobic and exhibit a bitter taste in cheese (Singh *et al*., 2003). The function of prolidase is important in cheese-making since it is the enzyme that hydrolyses these hydrophobic proline dipeptides. Prolidase also possesses another important characteristic in human-related physiological matters, iminopeptiduria; a rare, but lethal disease induced by prolidase deficiency (Shrinath *et al*., 1997). The disease is characterized by various clinical symptoms, such as chronic skin ulcers, recurring infections, mental retardation, and splenomegaly (Kokturk *et al*., 2002).

This research aimed at cloning the prolidase gene, *pep*Q, from genomes of LAB, in order to characterize the recombinant enzymes. This research is expected to provide a better understanding on the physiological characteristics of prolidases and their practical applications in food industry with respect to removal of cheese bitterness.

Genomic DNA from *Lactobacillus plantarum* NRRL B4496 and *Lactococcus lactis* NRRL B1821 were isolated, and the *pep*Q genes amplified and cloned into cloning vectors. Recombinant strains carrying the *pep*Q genes were determined by restriction enzymes and confirmed by DNA sequencing. Cloned genes were subcloned

into expression vectors and the optimum conditions of soluble recombinant prolidase expression were examined in *E. coli* systems. Purification of the expressed prolidases was done using with ammonium sulphate precipitation and anion exchange column chromatography. The characterization studies were conducted on recombinant *Lc. lactis* prolidase since the recombinant *Lb. plantarum* prolidase lost its activity during the purification process. Kinetic and structural characteristics of recombinant *Lc. lactis* prolidase were examined and demonstrated considerably unique features (e.g. novel allostery and notably different substrate specificities).

2. LITERATURE REVIEW

2.1 Lactic acid bacteria and protein utilization systems

LAB produce lactic acid as a major end product of their carbohydrate metabolism, preventing the growth of other microbes by lowering the pH in the external environment. There are several genera of LAB, classified by their morphological and physiological characteristics: *Enterococcus, Lactococcus, Streptococcus, Vagococcus, Pediococcus, Leuconostoc, Lactobacillus*, etc. Since the main growth medum, milk, rarely contains free essential amino acids, LAB have developed efficiently-organized proteolytic systems (i.e. proteinases, transporters, and peptidases) in order to meet their auxotrophic demands. Proteolytically generated oligopeptides are transported into the cell and undergo further hydrolysis to provide the building blocks and nitrogen (Axelsson, 1998; Lehninger, 1984).

The proteolytic system of LAB is comprised of three distinctive parts: extracellular serine-proteinases, transport systems, and intracellular peptidases. They are systematically and connectively organized to perform protein utilization through peptide formation by proteinases, delivery by the transport systems, and hydrolysis by peptidases (Figure 2.1). According to genetic mutation studies, the system has proteinases with broad substrate specificities and oligopeptide transport is the main route of entry for nitrogen followed by systematic hydrolysis by peptidases in the cell (Kunji *et al*., 1996).

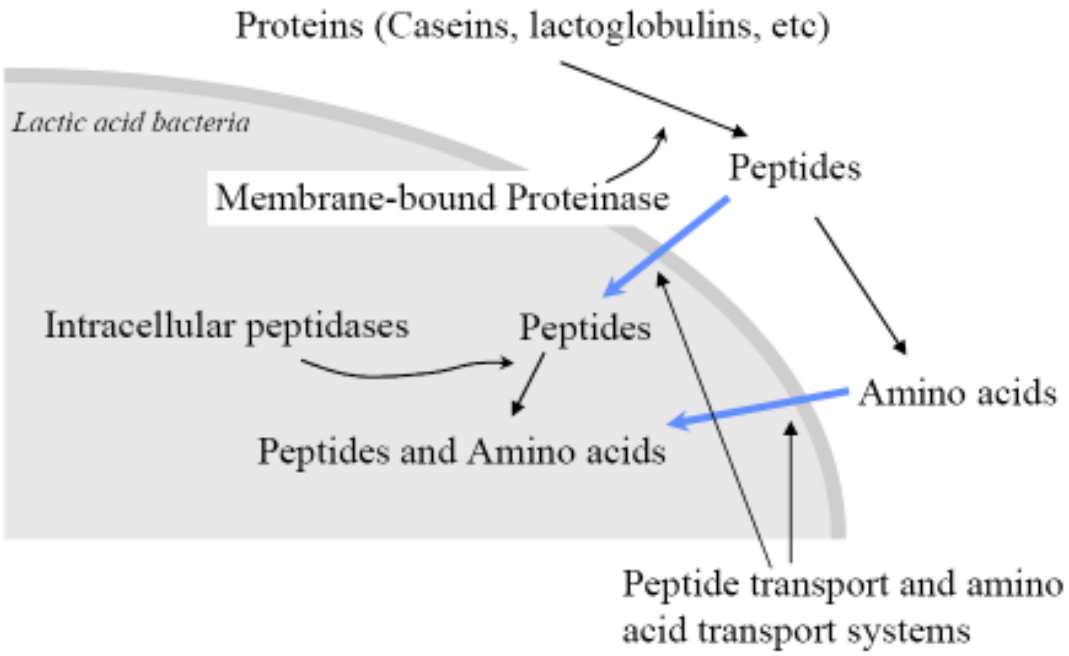


Figure 2.1 Protein utilization system of lactic acid bacteria.

2.1.1 Proteinase

For auxotrophic growth, LAB require 4 to 14 amino acids depending on species (Juillard *et al*., 1995; Mills and Thomas, 1981). Casein, a main protein in milk, is hydrolyzed into simpler forms, including di-, tri-, and oligopeptides (Schmidt, 1982). Cell wall-associated LAB proteinases (PrtP) are responsible for this primary proteolytic activity and function cooperatively with transport systems (Doeven *et al*., 2005).

Casein contains all of the amino acids required for cell growth and certain required portions of casein proteins are hydrolyzed by PrtP into oligopeptides to meet the nutritional requirements of LAB (Kunji *et al*., 1996). As a consequence, numerous studies have examined the preferential sites of proteinase attack on caseins (α-, β-, and κ-casein: De Vos and Siezen, 1994; Kok and De vos, 1994; Pritchard and Coolbear, 1993; Smid *et al*., 1991; Tan *et al*., 1993). Proteinases from diverse species of LAB have

demonstrated molecular masses ranging from 180 to 190 kDa (Laan and Konings, 1989) with high homologies observed in the conserved sequences (> 95%) when compared to the reference *Lb. casei* proteinase (Kunji *et al*., 1996). When the structures of the LAB proteinases were compared to a well-known serine proteinase, subtilisin, all exhibited similar catalytic N-terminal domains, consisting of amino acid residues to accommodate catalytic and substrate positioning functions. Ser433, His94, Asp30, and Asn196 in *Lc. lactis* SK11 proteinase are examples of such residues (De vos and Siezen, 1994).

The differences of primary sequences between the expected and mature proteinases from LAB revealed the presence of translocalization-signal sequences at the N-terminal sites and post-translational processing (Gilbert *et al*., 1996). In other words, the signal sequence transports the immature protein to the outside of the cell and is removed before performing full catalytic functions (Kok and De vos, 1994).

2.1.2 Peptide transport systems

Biochemical analyses have confirmed the presence of three different functional peptide transport systems in *Lc. lactis* MG1363 (Charbonnel *et al*., 2003; Hagting *et al*., 1994; Lamarque *et al*., 2004; Tynkkynen *et al*., 1993). These three systems are classified as hydrophilic di- and tripeptide (DtpT; Hagting *et al*., 1994), oligopeptide transport systems (Opp; Kunji *et al*., 1993), and hydrophobic di- and tri-peptide transport systems (Dpp; Sanz *et al*., 2001).

The Opp system is a member of the ATP Binding Cassette (ABC) superfamily that requires ATP to transport oligopeptides (Higgins, 1992). Kunji *et al.* (1993) showed *Lc. lactis* possesses an Opp system using mutant analyses. Tynkkynen *et al.* (1993) also evidenced the presence of the systematic transporters in a chemically defined system.

The sole functionality of Opp system was proven by an *opp* knockout mutant (Δ*opp*) study, demonstrating complete failure of the oligopeptide uptake (Tynkkynen *et al*., 1993). Kunji *et al*. (1993) and Tynkkynen *et al*. (1993) elucidated that the Opp system can transport peptides up to 35 residues-long.

According to Smid *et al.* (1989), DtpT is a membrane-bound transporter and is responsible for the transport of hydrophilic di- and tripeptides. This system is distinguished from the ABC transport system by virtue of a proton motive force-driven peptide transport family (PRT-family) which was previously considered to only function in eukaryotic transport systems (Steiner *et al*., 1995). The DtpT is driven by the pH gradient-force across the cytoplasmic membrane. Additionally, a knockout (Δ*dtpT*) mutant study proved its exclusive role in the transport of hydrophilic di- and tripeptides (Foucaud *et al*., 1995).

Analyses of single and double mutants (Δ*opp*; Δ*dtpT*; Δ*opp* and Δ*dtpT*) showed a third peptide transport system, Dpp (Foucaud *et al*., 1995; Sanz *et al*., 2001). The Dpp system preferentially transports hydrophobic di- or tripeptides (Foucaud *et al*., 1995) and is energized by ATP or energy-rich phosphorylated intermediates (Doeven *et al*., 2005). This ABC system shares a similar structure with the Opp system, including peptide-binding proteins, transmembrane proteins, and ATP binding proteins (Sanz *et al*., 2001).

LAB proteolytic systems are regulated through the inhibition of transcription in the transport system operons. The transport systems are meticulously controlled by a molecule called Cod Y, a pleiotropic transcriptional repressor that senses the branched chain amino acid pool in the cell (Guédon *et al*., 2001). That is, a high level of branched chain amino acids activates the transcriptional repression by Cod Y on the Opp operon.

2.1.3 Amino acid transport systems

Amino acid transport systems are more complex and diverse than the peptide transport systems. *Lactococcus* spp. possesses at least ten different amino acid transporters and these are highly specific for structurally similar amino acids (e.g. Lys, Arg, or Orn). Among the known transporters, some transporters (Glu and Gln) are dependent on ATP and some rely on proton-motive-force (pmf)-dependent transporters (Ala, Gly and Met) (Konings *et al.*, 1989; Kunji *et al*., 1996).

2.1.4 Localization of peptidases in lactic acid bacteria

The pathways of utilization of casein-derived peptides are intracellular events and are carried out by peptidases in the cytoplasm (Table 2.1). It was believed that peptidases were secreted into the growth media; however, this assertion has been nullified by a series of experiments where the intracellular existence of these enzymes was confirmed (Christensen *et al*., 1999; Detmers *et al*., 1998; Kunji *et al*., 1998).

2.1.5 Peptidases in lactic acid bacteria

According to the information elucidated from *Lactobacillus* and *Lactococcus* spp., almost all known LAB peptidases exist in the cytoplasm and possess unique specificities on different lengths of peptides. The oligopeptides introduced through the cell membrane are hydrolyzed into amino acid units by intracellular peptidases. The resultant amino acids are destined for building blocks of protein synthesis, metabolic energy generation, and recycling of cofactors (Christensen *et al*., 1999).

Several of the known peptidases in LAB are categorized in Table 2.2 according to their functional characteristics. Among the peptidases listed, X-prolyl dipeptidyl

aminopeptidase, proline iminopeptidase (PepI), prolinase (PepR), and prolidase are the enzymes which specifically hydrolyze proline-containing substrates.

Table 2.1 Localization of peptidases in lactic acid bacteria (data modified from Kunji *et al*., 1996).

	Localization	Sources
Aminopeptidase N	intracellular	*Lc. lactis* sub. *cremoris* Wg2
	intracellular	*Lc. lactis* sub. *cremoris* MG1363
	intracellular	*Lc. lactis* sub. *cremoris* HP
	intracellular	*Lb. casei* sub. *casei* LGG
	cell-wall	*Lb. helveticus* LHE511
	intracellular	*Lb. helveticus* CNRZ32
	intracellular	*Lb. helveticus* SBT2171
Aminopepdiase C	intracellular	*Lc. lactis* sub. *cremoris* AM2
	intracellular	*Lc. lactis* sub. *cremoris* WG2
	intracellular	*Lb. delbrueckii* sub. *lactis* DSM7290
	intracellular	*Lb. delbrueckii* sub.*bulgaricus* B14
Tripeptidase	intracellular	*Lc. lactis* sub. *cremoris* Wg2
	intracellular	*Lc. lactis* sub. *cremoris* AM2
	intracellular	*Lc. lactis* sub. *cremoris* MG1363
	cell-wall	*Lc. lactis* sub. *cremoris* IMN-C12
Dipeptidase	intracellular	*Lc. lactis* sub. *cremoris* H61
	intracellular	*Lc. lactis* sub. *cremoris* Wg2
	intracellular	*Lc. lactis* sub. *cremoris* MG1363
	intracellular	*Lb. delbrueckii* sub. *lactis* DSM7290
	intracellular	*Lb. delbrueckii* sub.*bulgaricus* B14
	intracellular	*Lb. helveticus* SBT2171
	intracellular	*Lb. helveticus* CNRZ32
	intracellular	*Lb. helveticus* 53/7

Table 2.2 Peptidases from lactic acid bacteria (data modified from Christensen *et al*., 1999; Clein *et al*., 1995).

Peptidase	Name
Aminopeptidase	PepA, PepC, PepN, PepP
Leucyl aminopeptidase	PepL
Dipeptidase	PepD, PepV
Proline iminopeptidase	PepI
Prolidase	PepQ
Prolinase	PepR
Tripeptidase	PepT
Endopeptidase	PepE, PepF1, PepF2, PepF3, PepG, PepO
X-prolyl dipeptidyl aminopeptidase (XPDAP)	PepX
Unclassified peptidases	

2.1.5.1 Aminopeptidases

Aminopeptidases are characterized by their broad specificities and categorized into four different classes: PepA, PepC, PepN, and PepP. All peptidases release amino acids from the N-terminal ends of oligopeptides. PepA shows notable catalytic activity on acidic amino acids located at the N-terminus of peptide chains (Rul *et al*., 1995). PepC demonstrated significant hydrolytic activities on basic, acidic, and hydrophobic amino acids at Xaa located at the N-termini of the peptide chain; however, no observable catalytic activity has been recorded for the proline residues and Xaa-Pro-(Yaa)n (Chapot-Chartier *et al*., 1994; de Palencia *et al*., 2000; Mistou *et al*., 1994; Neviani *et al*., 1989; Wohlrab and Bockelmann, 1993). PepN and PepP share a common catalytic property, actively cleaving Arg and Lys at the N-termini. However, PepN prefers Leu

and Ala at the second position from the N-terminus and PepP favours specifically Arg or Lys that is directly followed by the two amino acids, Pro-Pro.

2.1.5.2 Dipeptidases

The two known dipeptidases in LAB, PepD and PepV, are dipeptide-specific and characterized by their broad substrate specificities. The substrate dipeptides are basic and hydrophobic residues at the N-termini. No significant activities on Pro-containing dipeptides have been reported for either PepV or PepD. The difference of PepV and PepD is notable in the hydrolysis pattern (i.e. Val-Xaa and Ile-Xaa are hydrolyzed only by PepV, and not PepD) (Christensen *et al*., 1999).

2.1.5.3 Tripeptidases

Bosman *et al.* (1990) and Bacon *et al.* (1993) reported the presence of tripeptide-specific peptidases in *Lc. lactis* subsp. *cremoris* Wg2 and *Lc. lactis* subsp. *cremoris* AM2, respectively. The specificities to tripeptides were very high and PepT could not hydrolyze any other peptides. The presence of PepT activities in *Lactobacillus sake* (Sanz *et al*., 1998) and *Pediococccus pentosaceus* K 9.2 (Simitsopoulou *et al*., 1999) have also been reported.

Leucyl aminopeptidase (PepL) is Leu-specific (Leu-Xaa or Leu-Xaa-Yaa) at the N-termini, liberating Leucine residues. The activities on Ala-Xaa and Ala-Xaa-Xaa have also been reported (Klein *et al*., 1995).

2.1.5.4 Endopeptidases

Endopeptidases are the largest group of LAB peptidases and occupy diverse functionality in the generation of oligopeptides. There are several endopeptidases: PepE, PepO, PepG, and PepF. PepE (Fenster *et al*., 1997) demonstrates endopeptidyl hydrolysis on Enkephalin (Tyr-Gly-Gly-Phe-Met) at the Gly_3-Phe_4 bond and Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) at the Gly_4-Phe_5 bond. PepO (Lian *et al*., 1996; Pritchard *et al*., 1994) has specificity on oligopeptides ranging from 5- to 13- amino acids. PepG (Klein *et al*., 1997) possesses catalytic activity to cleave N-terminal dipepeptides from oligopeptides with the exception of oligopeptides, containing proline at the N-terminal site. PepG also showed PepC activity on Ala-$(Xaa)_n$, however, it never shares other characteristics with PepC (Klein *et al*., 1997). The endopeptidase PepF is known to be responsible for the release of tri- to heptapeptides from heptapeptides to heptadecapeptides substrate (Monnet *et al*., 1994).

2.1.5.5 Proline-specific peptidases

Among the peptidases shown in Table 2.2, PepI, PepR, XPDAP, and PepQ recognize proline-containing peptide substrates. XPDAP (PepX) participates in a unique reaction among LAB peptidases. XPDAP releases N-terminal Xaa-Pro dipeptides from 3 to 7 residue-length peptides (Lloyd and Pritchard, 1991; Tsakalidou *et al*., 1998), where Xaa can be Arg, Ala, Ile, Val, and Gly (Tsakalidou *et al*., 1998). The generated dipeptides are subsequently digested by PepQ.

PepI is reported to liberate prolines from Pro-Xaa dipeptides or Pro-Xaa-Xaa tripeptides, where Xaa are hydrophobic, acidic, or aromatic amino acids (Baankreis and Exerkate, 1991). PepR is specific to Pro-Xaa dipeptides. In this case, Xaa is relatively

similar to the substrates of PepI (Shao *et al*., 1997). Conversely, Xaa-Pro dipeptides are only cleaved by PepQ characterizing prolidase as a unique and important enzyme. Furthermore, prolidase can release proline at the C-terminus while PepR and PepI cleave proline at the N-terminus. The detailed information on prolidases is provided in section 2.2.

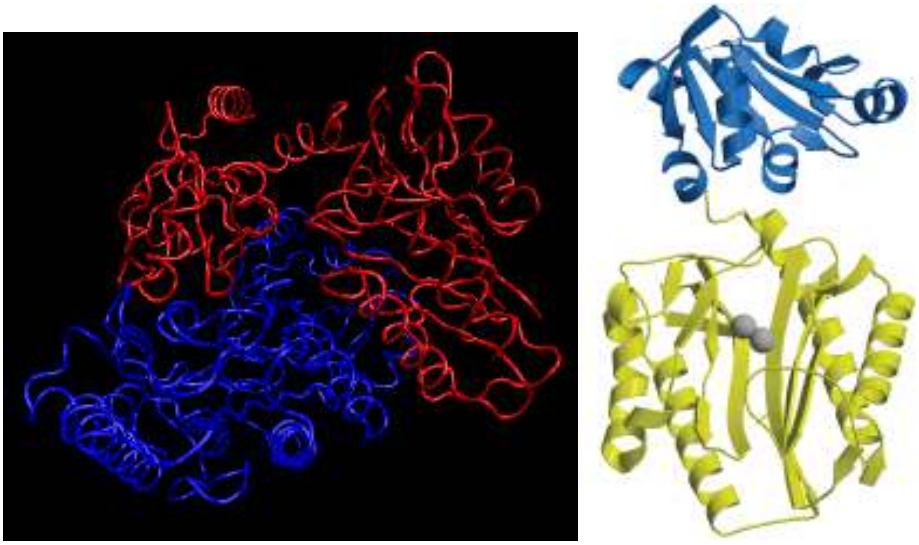
2.2. Prolidase

2.2.1 General characteristics of prolidases

Prolidase is a physiologically important enzyme and commonly present in organisms ranging from bacteria to human and take part in the final steps of the degradation of proteins (Morel *et al*., 1999a,b). Purified and characterized prolidases (*Streptococcus cremoris*, Kaminogawa *et al*., 1984; *Lb. casei*, Fernández-Esplá *et al*., 1997; *Lb. helveticus,* Shao *et al.,* 1997) have shown their strict dipeptidase characteristics. Prolidases have demonstrated amino-terminal activities on the Xaa-Pro dipeptide substrates where Xaa are hydrophobic (Ala, Ile, Leu, Val), basic (His), aromatic (Phe, Tyr), and sulphur-containing amino acids (Met) (Hart *et al*., 1998). Prolidase, with a 1.1kb open reading frame (ORF), is monocistronic and shows a high transcription level between the exponential and stationary phases in *Lb. delbrueckii* (Christensen *et al*., 1999; Rantanen and Palva, 1997). A few prolidases have shown activity on Pro-Pro, but none on Gly-Pro (Christensen *et al*., 1999). It has also been indicated that LAB rely on PepQ for the hydrolysis of Met-Pro, Leu-Pro and Phe-Pro (Christensen *et al*., 1999). The importance of prolidase in LAB was also verified by a deletion mutant (Δ*pep*Q), corresponding to 13% longer generation time (Christensen and Steele, 1999), suggesting its functionally critical role in LAB.

2.2.2 Structural overview of prolidases

In Protein Data Bank (PDB, http://www.rcsb.org), four molecular structures of prolidases are available (PDB accession code: 1PV9, 1WY2, 1WN1, and 2IW2). Two are from *Pyrococcus. Horikoshii* (*P. horikoshii*) Ot3 (Mitutani *et al*., 2005; Jeyakanthan *et al*., 2005) and the others are from *P. furiosus* (Maher *et al*., 2004) and *Homo sapiens* (Mueller *et al*., 2006). According to Maher *et al.* (2004), recombinant prolidase (*Pf*prol) of the hyperthermophilic archaeon *P. furiosus* showed the following characteristics. The prolidase was a homodimer and the molecular mass of each subunit was 39.4 kDa. The enzyme was a metalloprotein with a dinuclear metal cluster at its active sites, where two Co^{++} were located. The cobalt cations can be replaced with Mn^{++}, but not with other divalent cations. In most cases, both Co^{++} are required for full catalytic function of the prolidase (Maher *et al*., 2004). Each subunit consists of an N-terminal domain (residues 1-112), an α-helical linker (113-123), and a C-terminal domain (124-348), where the active site is located. The prolidase shares metallopeptidase motifs with *Escherichia coli* (*E. coli*) methionine aminopeptidase (MetAP; Roderick and Matthews, 1993) and *E. coli* proline aminopeptidase (APPro; Wilce *et al*., 1998) (i.e. MetAP and APPro have Co^{++} and Mn^{++} in their active sites, respectively). Residue 291-300 in the C-terminal domain of *Pf*prol form a bent loop and show close bending to the active site, providing a smaller space for substrate binding. This is the reason that the prolidase substrates are dipeptides rather than oligopeptides or larger substrates (Maher *et al*., 2004) (Figure 2.2).



A **B**

Figure 2.2 Three-dimensional structure of *Pyrococcus furiosus* prolidase (A) and the ribbon model of a subunit (B) (Maher *et al*., 2004). **A**: Blue and red chains represent two identical subunits. **B**: N-terminal domain (blue), C-terminal domain (yellow), and cobalt ions (gray) are displayed.

2.2.3 Prolidase deficiency in humans

Iminopeptiduria, caused by prolidase deficiency, is an autosomal recessive disorder and is characterized by highly variable symptoms including rashes, dysmorphic features, anaemia, splenomegaly, and skin ulceration. This biochemical abnormality is a common symptom caused by a deficiency of prolidase in the body (Phang *et al*., 1995). Additionally, immunological disorders (i.e. an excessive level of immunoglobulins in the serum and recurrent infections) are known symptoms in patients suffering from prolidase deficiency (Cleary *et al*., 1994).

2.2.4 Prolidases in the food industry

In the food industry, proline-containing peptides in cheese (Agboola *et al*., 2004) or fermented fish sauce (Park *et al*., 2002) are known to be main sources of bitter taste, according to sensory evaluation. As a consequence, prolidase is postulated to be a bitter-taste remover in this respect.

2.2.4.1 Prolidases in the cheese making industry

Cheese making starts from the clotting process of milk. Coagulant enzymes change the colloidal structure of proteins, forcing gelation. The resultant curd is collected, pressed, moulded into different shapes, and stored for ripening (Wolfgang, 2004). During the cheese ripening period, a high level of proteolysis occurs via enzymes originated from LAB, developing flavour from their hydrolysates (i.e. aromatic amino acids and peptides) (Singh *et al*., 2003).

In order to curtail the time-consuming process of ripening in cheese making, starter culture methods have been employed (Peterson *et al*., 1975). However, the starter culture often results in over-ripened cheese, thus attenuation methods are used to prevent unwanted acidification by starter LAB during cheese making (Wolfgang, 2004). The attenuation methods include hot, and cold temperature shock, or exposure of the microbes to microwaves in order to decrease their viability (Wolfgang, 2004). The typical attenuation method is performed by the sub-lethal heat-treatment of the bacterial cell suspension. A crucial point for this step is to maintain viability of the potential ripening enzymes (Klein *et al*., 1999), which include peptidases and proteinases. This method is a better choice for cheese flavour than utilizing active LAB, which can

produce excessive acidity; however, the attenuation still causes the bitterness problem derived from undigested peptides.

Bitterness, a common problem in cheese, is generated as a by-product of the hydrolysis process (Fernandez-Garcia *et al*., 1988; Vafopoulou *et al*., 1989). Sullivan and Jago (1972) reported that bitterness is common in Cheddar cheese when the 2-23 residue-long hydrophobic peptides are formed. Chymosin is believed to be implicated in the formation of the hydrophobic peptides (Visser *et al*., 1977a, b; Singh *et al*., 2003). According to McGugan *et al.* (1979), McSweeney *et al.* (1994), and Cliff *et al.* (1993), the water soluble fraction (hydrophilic portion) of cheese favourably affected the development of cheese flavour, whereas the hydrophobic water insoluble fraction did not act as a flavour enhancer and even formed a bitter taste. The degree of bitterness has been evaluated by examination of the hydrophobic and hydrophilic peptide fractions of Cheddar cheese (Cliffe *et al*., 1993) and proline-containing synthetic peptides (Ishibashi *et al*., 1988). It has been found that the degree of bitter taste was much higher in hydrophobic peptides, especially proline-containing peptides. The relationship between peptide size and degree of hydrophobicity has not been clearly revealed (Agboola *et al*., 2004).

According to Ishibashi *et al.* (1988), the structural changes of peptides highly affect taste. In their experiment, pure amino acid L-proline was sweet. However, proline-containing peptides were all bitter. In the taste comparison, Pro-Pro and Pro-Pro-Pro showed 2.75- and 6.25-fold bitterness of free proline. Moreover, casein derivatives BPI-a (Arg-Gly-Pro-Pro-Phe-Ile-Val) demonstrated forty-fold bitterer taste than a shorter pentapeptide (Arg-Gly-Pro-Pro-Phe). It has been postulated that structural differences, caused by hydrophobic amino acids and the folding of peptides with imine

ring structures, increases bitterness. For instance, Phe-Pro showed one of the highest values in bitterness and was 25-fold more bitter than Pro-Phe (Ishibashi *et al*., 1988). It is apparent that the imine ring of proline residue at the C-terminus is actively involved with hydrophobic amino acid at the N-terminus in the formation of bitter taste (Ishibashi *et al*., 1988). Even though the recognition mechanism of bitterness is a hypothesis, a distance (4.1 Å) of each taste recognition unit on the bitter taste receptor is believed to be related to the intensity of bitterness. That is, the interactions of binding, stimulating units, peptides, and the distance between the units are relatively involved (Ishibashi *et al*., 1988).

Mistry *et al*. (1999) reported the favourable influence of salt on the reduction of cheese bitterness. According to Singh *et al*. (2003), high concentration of salt inhibited the activity of chymosin in Cheddar cheese. The role of chymosin in the bitterness of cheese is mentioned in this section. Excessive salt, however, can cause nutritional unbalance in consumers since an excessive intake of salt triggers geriatric diseases, such as hypertension and cardiovascular disease. Prolidase offers the potential to resolve the high salt problem in cheese through hydrolysis of bitter peptides.

2.2.4.2 Prolidases in the fishery industry

Out of several fermented proteinous seafood, fish sauce has been renowned for its high level of oligopeptides formed as a consequence of beneficial microbial fermentation. Park *et al.* (2002) reported that twelve peptides that were isolated using HPLC had proline residues at their C-terminus. The peptides were mainly di- and tripeptides and showed bitter and sour taste in sensory evaluation. It is believed that the

peptide conformation caused by proline significantly participates in bitterness as shown in the proline-containing hydrophobic peptides (Ishibashi *et al*., 1988).

2.2.5 Activity tests for prolidases

The activity of prolidase can be determined through two photometric methods.

The enzymatic method, employing the L-amino acid oxidase and peroxidase reactions, is convenient to use (Lewis *et al*., 1967). In detail, L-amino acids and prolines generated from prolidase catalytic reactions are deaminated by L-amino acid oxidase, forming hydrogen peroxides. These hydrogen peroxides are then converted into water by peroxidases using *o*-dianisidine as the oxygen receptor. The resultant oxidized *o*-dianisidine yields a dark brown colour and is measured at 436 nm spectrophotometrically (Figure 2.3, A).

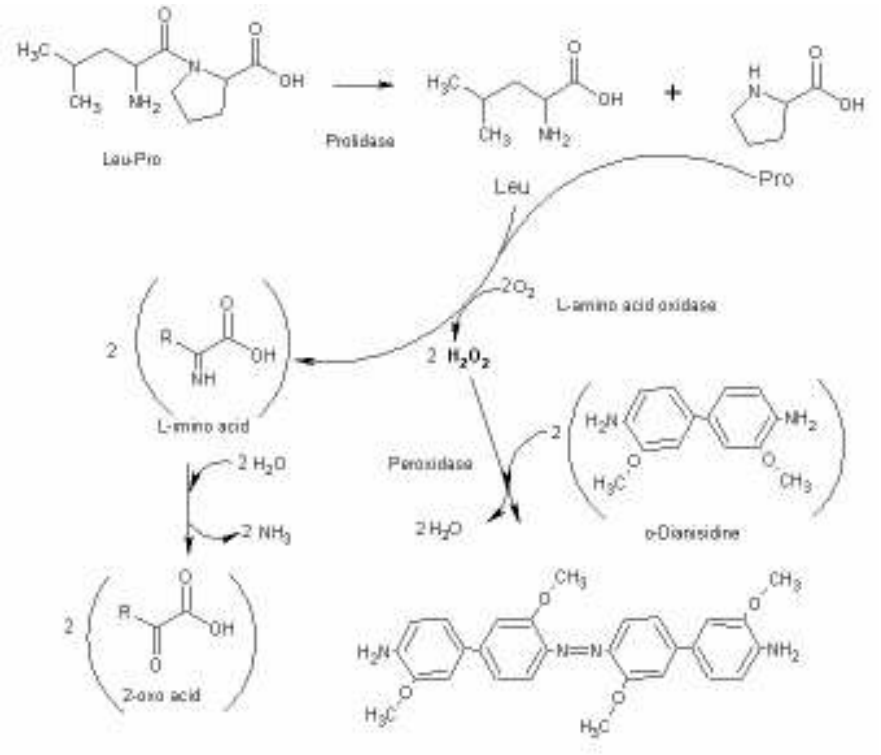
A photometric ninhydrin method (Figure 2.3, B) has also been widely used for amino acid and peptide analyses. This is based on the chromogenic formation of a purple product from the colorless ninhydrin via the released amine groups from amino acids. The resultant purple compound is easily detected by spectrophotometry (Creighton, 1993b).

2.3. Methods for analyzing protein structure

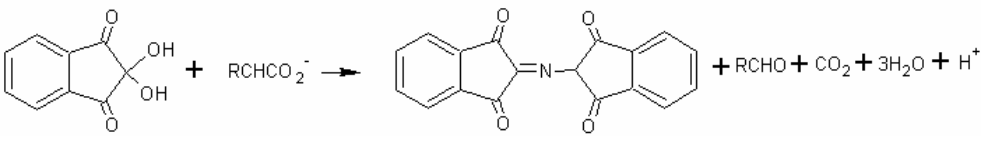
2.3.1. Far-UV circular dichroism (CD) spectroscopy

The amino acid side chains are considerably free from the polypeptide backbone because their conformational positioning is less strict than the backbone. The protein backbone is capable of absorbing UV light and the diverse groups of the protein contribute to vibrational spectra. In protein structure, L-amino acids of polypeptide

chains absorb left- and right-polarized light differently. Circular dichroism (CD) is based on the different absorption of a polarized beam of light by chiral molecules. As a result, CD spectra reflect the conformation of the polypeptide backbone regardless of effects from side chains (Creighton, 1993a).



A



B

Figure 2.3 Schematic illustrations of enzymatic (A) and ninhydrin (B) activity assays. Leu-Pro is shown as an example in panel A. The figures were generated with ACD/Chemsketch version 10.0.

2.3.2 Mass spectrometry

Mass spectrometry (MS) is highly useful in protein structural determination. MS is done by evaporation of a protein sample, which is the fragmented non-specifically by an electron beam to generate assorted protein fragments in the spectra. Only positively-charged fragments can be detected and the separation is based on the mass-to-charge ratio (Creighton, 1993b).

MS for protein analysis has the considerable advantages of accuracy and sensitivity. Only picomoles of samples are required for detection and up to 100 kDa can be detected. However, the low volatility of proteins and peptides limits an accurate detection. As a consequence, electrospray ionization, characterized by protein suspension in volatile solvent, has been employed. Proteins protonated by pH change have a net positive charge and are differentiated by various intact protein molecules, which possess varying numbers of charges, by detector; therefore, the mass-to-charge ratio furnishes spectral variations. Multimeric proteins and cofactors are easily dissociated so it is believed that protein unfolding occurs during the measurement and the measured values are accurate enough to estimate the relative molecular mass (M_r) of monomeric polypeptides (Creighton, 1993b).

3. HYPOTHESIS AND OBJECTIVES

As Sullivan and Jago (1972) reported, bitterness is a normal drawback of cheese products and affects the choices of the customers. This bitter taste is strongly increased by hydrophobic amino acid-proline dipeptides (Ishibashi *et al*., 1988). Since prolidase is responsible for the hydrolysis of those bitter taste-causing dipeptides, the elucidation on its characteristics is believed to be informative for the practical applications in cheese industry.

The research is aimed to clone, purify, and characterize prolidases from *Lb. plantarum* and *Lc. lactis* to provide a concrete foundation of characteristic information. The research will be conducted with following processes:

1. Isolations of prolidase genes.
2. Constructions and optimizations of prolidase expression systems.
3. Characterizations of prolidases and their interpretations.

The resultant information is believed to be useful for future site-directed mutation on the structural and functional residues as well as the determination of precise structure of the prolidase utilized by X-ray crystallography. Main objectives of this research include cloning, expression, purification, and characterization of *Lb. plantarum* and *Lc. lactis* prolidases.

4. MATERIALS AND METHODS

4.1 Sequences of putative prolidase genes

The presence of putative prolidase genes of *Lb. plantarum* and *Lc. lactis* were based on the reference strains of *Lb. delbrueckii* (Rantanen *et al*., 1997; Morel *et al*., 1999a,b) and *Lb. helveticus* (Rantanen *et al*., 1997). The genetic information on prolidases were from *Lb. plantarum* WCFS1 (GenBank accession code: NC004567) and *Lc. lactis* Il1403 (GenBank accession code: NC002662).

4.2 Preparation of microorganisms and isolation of genomic DNA from lactic acid bacteria

4.2.1 Cultivation of lactic acid bacteria and host microorganisms.

Lactic acid bacteria (*Lb. plantarum* NRRL B4496, *Lc. lactis* NRRL B1821; ARS Culture Collection, Peoria, IL, USA) were cultivated in 100 mL of MRS medium (BD, Franklin Lakes, NJ, USA) in a 200 mL flask for 24 hours at 37 ºC without shaking. Host *E. coli* GI698, GI724 (Invitrogen, Burlington, ON, Canada; genotype: F^-, λ^-, *lac*I^q, *lac*PL8, *amp*C::P_{trp} *c*I), and Top10F' (laboratory stock; genotype: F′{lacIq Tn10 (TetR)} mcrA Δ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG) were incubated from 15 to 37 ºC. Unless otherwise stated, all the chemicals were ACS-grade and purchased from VWR international (Mississauga, ON, Canada). The restriction enzymes and modifying enzymes were

obtained from Fermentas (Burlington, ON, Canada) and Invitrogen (Burlington, ON, Canada).

4.2.2 Genomic DNA isolation from lactic acid bacteria

The cultivated culture were harvested by centrifugation (Sorvall SS-34 rotor, model RC5C Plus, Ashville, USA), 4,000 g for 5 min and genomic DNA isolated by a modified genomic DNA isolation protocol (Sambrook and Russell, 2001). The cell pellet was resuspended in 3 mL of buffer solution (pH 8.0, 50 mM Tris-HCl, 50 mM EDTA) and frozen at -20 °C for 1 hour. To the frozen cells, 0.4 mL of lysozyme solution (10 mg/mL) was added and the cells were thawed on ice for 1 hour. When the suspension was thawed, proteinase K solution (1 mg/mL, pH 8.0, 50 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, 0.5% SDS) was added. The cell suspension was incubated at 50 °C for 1 hour with gentle shaking. The solution was emulsified gently with 0.5 mL of TE buffer saturated-phenol. After centrifugation (Hettich Zentrifugen, GmbH, Tuttlingen, Germany) at 16,200 g for 5 min, the aqueous phase was transferred into a new tube. Nucleic acids were precipitated by adding 10% volume of 3 M sodium acetate solution (pH 5.2) and 250% volume of absolute ethanol. The ethanol precipitated sample was stored at -20 °C for 1 hour. The cold specimen was then centrifuged for 10 min and the recovered nucleic acids were dissolved in 200 µL of TE buffer solution (pH 7.5, 50 mM Tris-HCl, 1 mM EDTA, 200 µg/mL RNase). After incubation for 2 hours at 37 °C, the purified genomic DNA was stored at -20 °C.

4.3 Preparation of insert DNA

4.3.1 Polymerase chain reaction (PCR) amplification of prolidase genes

Two pairs of primers for PCR were designed, based on the genomic DNA sequences of prolidase in GenBank and then custom synthesized (Integrated DNA Technologies Inc, Coravile, IL, USA). The primers for *Lb. plantarum* possess an *Eco*RI restriction enzyme site in the forward primer and a *Hind*III restriction enzyme site in the reverse primer (Table 4.1). The primers for *Lc. lactis* have *Eco*RI and *Pst*I restriction enzyme sites on the flanking ends of the ORF. The PCR reaction mixtures (total volume of 100 μL) contained 20 pmol of each primer pair, 20 μg of the genomic DNA, 0.04 mM of each 2'-deoxynucleotide 5'-triphosphate (dNTP), and 0.5 U of *Pfu* polymerase (*P. furious* DNA polymerase).

Table 4.1 Forward and reverse PCR primers used for prolidase-coding gene amplification.[+]

Organism	Name	Primer
Lb. plantarum	Forward primer	GGAGAATTCGAA**ATG**ACAGATTATACGAAAC *
	Reverse primer	CTTTGAAGCTTTTAA**TTA**AAGATCTAATAC **
Lc. lactis	Forward primer	GGAGAATTC**ATG**AGCAAAATTGAACGTATT *
	Reverse primer	ATTCTGCAG**TTA**GAAAATTAATAAGTCATG ++

[+] Restriction enzyme sites are underlined. Initiation and termination codons are indicated in bold.
* *Eco*RI restriction enzyme site
** *Hind*III restriction enzyme site
++ *Pst*I restriction enzyme site

The PCR program (thermal cycler model FTgene-5D, Techne, Burlington, NJ, USA) included 1 min at 95 °C for denaturation, 1 min at 45 °C for annealing, and 3 min (added 5 sec for each cycle) at 72 °C for extension step for 50 cycles (*Lb. plantarum*) and 30 cycles (*Lc. lactis*). When the amplification process was finished, the reaction mixtures were held at 4 °C.

4.3.2 Recovery of amplified prolidase-coding genes

The amplified genes of *Lb. plantarum* and *Lc. lactis* were separately prepared. Gene fragments of *Lb. plantarum* prolidase were digested with *Eco*RI *and Hind*III for adhesive end ligation. The amplified ORF fragments of *Lc. lactis* genome were prepared with *Eco*RI and *Pst*I restriction enzyme digestions. Digested sticky-end ORF fragments were applied for ligation procedure described in section 4.3.4. The digested PCR products were run on 1% agarose gels (unless otherwise stated) for 100 min under an electrical field of 40 mA. Among the separated bands on the agarose gel, 1.1 kbp bands of prolidase coding genes were excised using a sharp knife. The DNA fragments were recovered from the gel slices using QIAEX II gel extraction kit (QIAGEN, Mississauga, ON, Canada).

4.3.3 Preparations of cloning vectors

pUC18 cloning vectors (Fermentas, Burlington, ON, Canada) were digested with *Eco*RI-*Hind*III for *Lb. plantarum* and *Eco*RI-*Pst*I for *Lc. lactis* prolidase genes. The enzyme digestions were performed according to protocols from the manufacturers. The digested DNA was dephosphorylated with shrimp alkaline phosphatase (SAP) to prevent self-ligation (Ausubel *et al*., 1990). The thirty minutes of SAP treatment at 37 °C was

followed by inactivation of the enzyme at 70 °C for 5 min. The purification of vectors were then prepared with the same method mentioned 4.3.2.

4.3.4 Ligation of purified DNA

All purified DNA underwent ligation for 12 hours at 14 °C for adhesive end ligation and 16 hours at 11 °C for blunt-end ligation with T4 DNA ligase as recommended by the manufacturer. The constructed recombinant genes were stored at -20 °C until required.

4.4 Transformation and plasmid mini preparation

The host *E. coli* were transformed as follows: The ligated recombinant gene (10 μL) and *E. coli* Top10F' competent cell (100 μL) mixture were placed for 30 min on ice followed by 1 min at 42 °C and placing on ice for 1 min (Hanahan, 1983; Sambrook and Russell, 2001). The transformants were plated onto ampicillin LB (Lysogeny-Broth, also called Luria-Bertani) plates (1% peptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar, 150 μg/mL of ampicillin). The plates were incubated at 37 °C overnight. Propagated colonies were picked with autoclaved tooth picks and cultivated in 2 mL LB broth (composition is the same as LB plate without agar). Recombinant plasmid DNA was isolated by a modified alkali-lysis method (plasmid mini-preparation; Sambrook and Russell, 2001). Overnight cultures of the recombinants were harvested by microcentrifugation (1mL in a 1.7 mL micro-tube) at 16,200 g for 30 sec, and the recovered pellet was resuspended in 100 μL of deionized water. After adding 300 μL of the alkali SDS solution (1% SDS, 0.1 N NaOH), the sample was mixed gently. The mixture was held for 2-5 min at room temperature. Sodium acetate (5 M, pH 4.8, 225

μL) was added into the solution followed by a brief mixing. The cellular debris was removed by centrifugation at 16,200 g for 5 min. The supernatant was liquid-liquid extracted (100 μL; phenol: chloroform = 50 μL:50 μL) and centrifuged at 16,200 g for 5 min. The separated upper solution was then mixed with 450 μL of isopropyl alcohol (99%) and centrifuged at 16,200 g for 10 min. After removing the supernatant, RNase A (DNase free, 50 ng/μL final concentration) was added and the sample incubated at 37 °C for 1 hour. After addition of 30 μL of polyethylene glycol-NaCl (20% PEG-8000, 2.5 M NaCl) to the solution, the mixture was kept at -20 °C for 20 min and centrifuged at 16,200 g for 10 min. The supernatant was removed and the precipitated DNA was dissolved in 50 μL of TE buffer (1 mM EDTA; pH 7.6, 10 mM Tris-HCl). The orientation and sequence of recombinant DNA were confirmed with DNA sequencing at the National Research Council Canada-Plant Biotechnology Institute (NRC-PBI, Saskatoon, SK, Canada; AB3730x capillary electrophoresis DNA analyzer). The confirmed DNA sequences are provided in appendix A.

4.5 Cloning of prolidase genes into protein expression systems

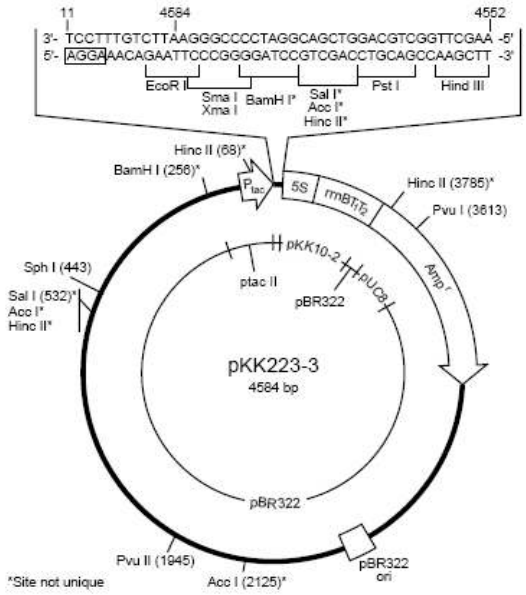
The sequenced recombinant prolidase genes were isolated and purified by the modified plasmid mini preparation method mentioned above; afterward, the prolidase genes were excised using appropriate restriction enzymes and purified on 1% agarose gel. The genes were then subcloned into protein expression systems to produce recombinant prolidases. To clone into the recombinant DNA expression systems, pHIL-S1, and pPIC-9 expression vectors (Invitrogen, Burlington, ON, Canada) in *Pichia pastoris* were considered as well as pKK223-3 and pTrxFus (Invitrogen, Burlington, ON, Canada) expression vectors. The directions of the genes were confirmed with several

combinations of restriction enzyme analyses since the genes must be inserted after a promoter in the proper orientation. The multiple cloning sites (MCS) are provided (Figure 4.1)

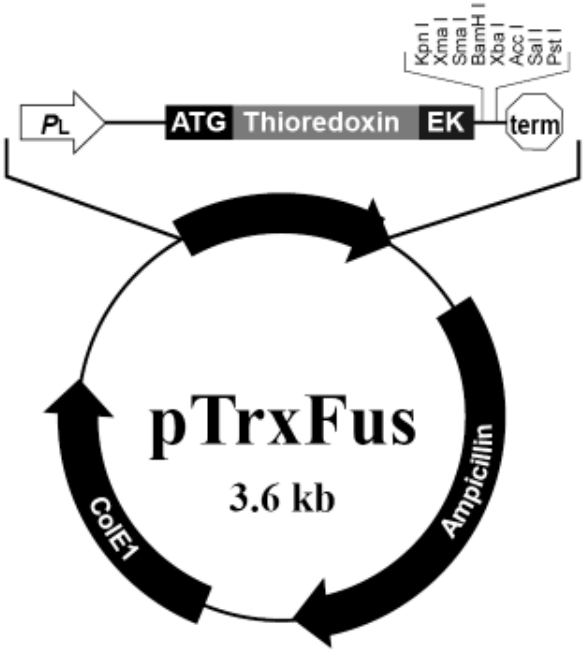
4.5.1 Cloning of recombinant *Lactobacillus plantarum* prolidase into protein expression systems

During the cloning of the pKK223-3-*pep*Q recombinant system, the prolidase-gene was cleaved out with *Eco*RI and *Hind*III. This DNA was blunt-ended with Klenow Fragment at 37 °C for 30 min followed by its inactivation at 70 °C for 10 min. The insert DNA was isolated from 1% agarose gel and purified using the QIAGEN gel extraction kit. *Sma*I-digested pKK223-3 vector was dephosphorylated by SAP and purified from the agarose gel. The digested insert DNA and the vector fragments were ligated.

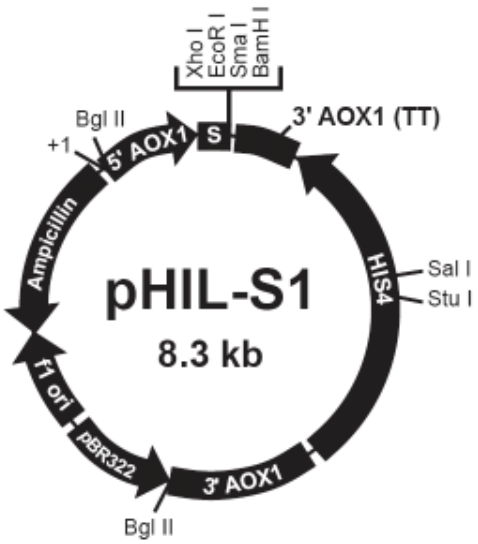
The insert DNA for pTrxFus was isolated from the pUC18-*pep*Q clone with *Eco*RI-*Bam*HI and blunt-ended with Klenow Fragment. This fragment was introduced into the Klenow Fragment-SAP-digested *Bam*HI site of pTrxFus. For pHIL-S1, the *Eco*RI-*Bam*HI-digested fragment was ligated into *Eco*RI-*Bam*HI site. *Hind*III-*Eco*RI-Klenow Fragment-digested insert DNA gene from the pUC18 clone was inserted into the pPIC-9 system (prepared with *Eco*RI–Klenow Fragment-SAP). All ligations were carried out with T4 DNA ligase at 14 °C for sticky-end and at 11 °C for blunt-end.


11
4584
4552
3'- TCCTTTGTCTTAAGGGCCCCTAGGCAGCTGGACGTCGGTTCGAA -5'
5'- AGGAAACAGAATTCCCGGGGATCCGTCGACCTGCAGCCAAGCTT -3'
EcoR I
Sma I
Xma I
BamH I*
Sal I*
Acc I*
Hinc II*
Pst I
Hind III
Hinc II (68)*
BamH I (256)*
P lac
5S
rrnBT1t2
Hinc II (3785)*
Pvu I (3613)
pKK10-2
pUC ori
Amp r
ptac II
pBR322
Sph I (443)
Sal I (532)*
Acc I*
Hinc II*
pKK223-3
4584 bp
pBR322
Pvu II (1945)
Acc I (2125)*
pBR322
ori
*Site not unique

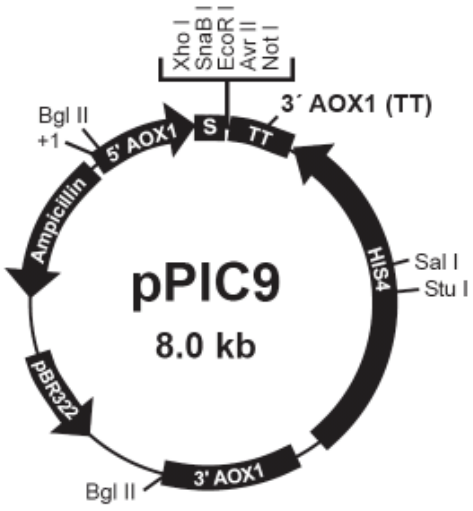
A


Kpn I
Xma I
Sma I
BamH I
Xba I
Acc I
Sal I
Pst I
PL
ATG
Thioredoxin
EK
term
pTrxFus
3.6 kb
ColE1
Ampicillin

B



C



D

Figure 4.1 Expression vectors with multiple cloning sites (A: pKK223-3, adapted from European Molecular Biology Lab, 2006; B: pTrxFus; C: pHIL-S1; D: pPIC-9, Invitrogen, 2006). The multiple cloning sites are indicated on the circular maps.

4.5.2 Cloning of recombinant *Lactococcus lactis* prolidase into protein expression systems

For recombinant *Lc. lactis* prolidase, only the pKK223-3 and pTrxFus protein expression systems were used. The insert DNA for the expression vectors were prepared with *Eco*RI-*Pst*I restriction enzymes, blunt-ended, and introduced into the *Sma*I site of the pKK223-3 vector. The pTrxFus vector was digested with *Cfr*9I and blunt-ended. Blunt-ended *Eco*RI-*Bam*HI insert DNA was ligated with the prepared vector. The recombinant DNA was examined using restriction enzymes and the positive clones were preserved at -20 °C for testing protein expression.

4.5.3 Transformation of *E. coli* with recombinant DNA

pKK223-3 and pHIL-S1 protein expression vectors possessing prolidase genes were transformed into *E. coli* Top10F'. *E. coli* GI698 and GI724 host cells were prepared for the pTrxFus system. After transformation, Top10F' host cells bearing pKK223-3 and pHIL-S1-prolidase recombinant systems were incubated on ampicillin-LB plates at 37 °C. RMG plates (modified from Invitrogen manual; laboratory protocol; 2% casamino acids, 1×M9 salts, 1.3% glycerol, 1 mM $MgCl_2$, 1.5% agar, and 150 μg/mL ampicillin) were also used for the pTrxFus recombinants. Individual plates were incubated at 37 °C (Top10F' and GI724) and 30 °C (GI698). Colonies that formed after overnight incubation were randomly selected and incubated in 2 mL cultures (LB: Top10F'; RMG media: GI724 and GI698). Cells were harvested approximately at stationary phase and the plasmid DNA was recovered for further DNA physical mapping. After screening by restriction enzymes and small scale (2 mL) expression tests, positive clones were preserved in 50% glycerol (v/v) at -70 °C.

4.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of prolidase

Protein expression and purification process were examined with SDS-PAGE. Throughout all experiments, 10% SDS-PAGE gels were utilized to examine the size of prolidases. Stock solutions used for SDS-PAGE were prepared as follows: Acrylamide (30%, w/v)/bis-acrylamide (0.8%, w/v) solution was prepared with deionized water. Gel buffers were separately prepared for resolution gels (1.5 M Tris-HCl, pH 8.8) and loading gels (0.5 M Tris-HCl, pH 6.8). A 10% SDS stock solution was also prepared.

For 2 sets of gels, 30% acrylamide/bis-acrylamide solution (3.33 mL for a resolution gel; 0.65 mL for a stacking gel), the buffers (2.5 mL for a resolution gel; 1.25 mL for a stacking gel), 10% SDS solution (100 μL for a resolution gel; 50 μL for a stacking gel), deionized water (4 mL for a resolution gel; 3 mL for a stacking gel), ammonium persulphate (4-5 mg to each solution), and N, N, N', N'-tetramethylethylenediamine (TEMED: 10 μL for resolution gel, 5 μL for stacking gel) were mixed gently (modified from Laemmli, 1970). The stacking gel was cast on the top of the resolution gel. The constructed gel was run under 18 -50 mA in the running buffer (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS). Protein samples were mixed with an equal volume of 2x protein loading buffer (20 mM Tris-HCl, pH 7.6; 2% SDS; 0.01% bromophenol blue; 50% glycerol) and denatured at 95 °C for 10 min. Ten to twenty five microliter was loaded for each sample. When the dye in the sample reached the bottom of the separation gel, the gel was removed from the plates and stained with 110 mL of staining solution (0.25% of Coomassie Brilliant Blue G-250 or R-250; glacial acetic acid: methanol: deionized water = 1: 5: 5). Slow agitation was provided for

homogeneous staining. Destaining of the gel was done in 7% acetic acid and 10% methanol solution (modified from Sambrook and Russell, 2001).

4.7 Expression of prolidases

The expression of recombinant prolidases from different host *E. coli* was examined under diverse growth conditions. Temperature, antibiotics, aeration, induction time, incubation time, pH, and salt concentration were considered. The conditions of the consideration include as follows: (1) media selection (LB and 2YT media), (2) 0.1 to 1 mM of the inducer, IPTG, (3) 0.1 to 1 μg/mL chloramphenicol, (4) 0.085 and 0.35 M NaCl, (5) induction at Abs $_{600}$ 0.4, 0.5, 0.8, and 1.2, (6) culture size (100 mL to 10 L culture), (7) incubation temperature (16, 18, 22, 29, 30, 32, and 37 °C), (8) culture pH (5.5 and 7.5), (9) aeration and shaking. These conditions were examined individually and in combinations. Detailed conditions are given in the results and discussion section.

4.8 Purification of prolidases

Since recombinant proteins failed to bind to the anion exchange column with the conditions employed for other prolidases, the following conditions were examined: (1) purification without metallic ions to prevent the oxidization of zinc at higher pH, (2) adjustment of buffer pHs to enhance binding capacity to the column, (3) weak or strong anion or cation exchange resins (4) prevention of tailing in the chromatography process using additives (e.g. 1.5 M urea, low-amount (1 mM) of DTT, or high concentration of sugar), (5) examination on different metallic ions, and (6) removal of nucleic acids, which decrease resolution of column chromatography, by protamine sulphate, RNase, and DNase.

The harvested cells possessing recombinant prolidase-protein expression vectors were resuspended in lysis buffer (20 mM citrate buffer; pH 6.0 for *Lc. lactis*; pH 6.5 for *Lb. plantarum* containing 1mM $ZnSO_4$, 100 mM NaCl, 8 μg/mL of RNase, 0.2 mg/mL lysozyme; Fernández-Esplá and Martin-Hernandez, 1997). The amount of buffer was adjusted to a 10-fold volume of the wet cell weight (e.g. 100 ml for 10 g of recombinant cells), and the cells were disrupted using a sonicator (model 450, Sonifier, Branson Ultrasonics Co., CT, USA; 30 sec burst and 30 sec pause operations over 10 min) or a French Press (SLM Instruments, IL, USA; 20,000 psi, 3 passes). The cell lysate was centrifuged at 17,200 g, 4 °C for 20 min using a Sorval SS-34 rotor and the supernatant collected. The protein concentration of this crude extract was determined using the BioRad Protein Assay kit (Mississauga, ON, Canada), and the concentration was adjusted to 2 mg/mL (or 2.65 mg/mL) using the same buffer. The crude extract was placed at 4 °C overnight after the addition of ammonium sulphate (10% to 80% saturation) and then centrifuged at 17,200 g, 4 °C for 20 min with a Sorvall SS-34 rotor. The supernatant was stored separately and the pellet dissolved in the same buffer and dialyzed against 4L (2 × 2 L) of the same buffer at 4 °C for more than 12 hours (Spectra/Por, MWCO 3,500, 45 mm width). Protamine sulphate (10% of the total protein amount) was added and the sample centrifuged at 17,200g, 4 °C (Sorvall SS-34 rotor). The supernatant was then filtered through 0.2 μm nitrocellulose syringe filter (VWR international) and applied to a DEAE-Sephacel anion exchange column (3 cm diameter × 15 cm lengths; General Electrics Healthcare Bio-sciences Co., Mississauga, ON, Canada). The column was washed with 20 mM citrate buffer (pH 6.0 for *Lc. lactis*; pH 6.5 for *Lb. plantarum*) containing 1 mM $ZnSO_4$ and eluted using an increasing NaCl

gradient from 0 M to 0.5 M, operating under a 2 mL/min flow rate with 1000 mL gradient elution at 4 °C using an FPLC^{TM} system (Pharmacia, Uppsala, Sweden). The eluent was fractionated and each fraction was examined on a 10% SDS-PAGE gel to determine the fraction containing prolidase. Fractions were pooled and concentrated (also filtered) using the Amicon ultra membrane filtration system (YM-30, MWCO 30 kDa, 45 mm width, Millipore, MA, USA).

4.9 Characterization of purified recombinant *Lactococcus lactis* prolidase

The pH dependence, temperature dependence, substrate specificity, denaturation temperature, metallic ion dependence, secondary structure measurement by Far-UV spectrum, molecular size, and kinetic values were determined for the purified recombinant prolidases.

4.9.1 Examination of prolidase activity using the coupled enzyme method

The colorimetric coupled-enzyme methods (Lewis *et al*., 1967; Wohlrab and Bockelmann, 1992) were employed to measure the prolidase activity (excluding kinetics constant analyses) for all recombinant prolidase activity determinations (Figure 2.3; page 20). A total of 600 µL reaction mixture (the 550 µL enzyme cocktail, 50 µL substrate) was used with each solution component prepared separately. The enzyme cocktail mixture included 10 µL of L-amino acid oxidase (2 mg/mL), 10 µL of peroxidase (5 mg/mL), 25 µL of *o*-dianisidine (11.5 mM), 10 µL of prolidase (15 ng), and 495 µL of 20 mM citrate buffer (pH 6.5) with 1 mM $ZnSO_4$. Five minute pre-incubations at 50 °C for the substrate and the enzyme cocktail in separate tubes were made before the initiation of the reaction. Fifty microliter of preheated 20 mM

dipeptides (dissolved in pH 6.5, 20 mM citrate buffer) was mixed with 550 μL of the preheated enzyme cocktail and incubated at 50 °C. This optimum temperature was determined from preliminary tests. Measurements were performed continuously for 5 min at Abs 436nm. Depending on the characterization study, the details of the methodology was modified.

4.9.2 Substrate specificity of recombinant prolidase

A variety of substrates were examined, included Arg-Pro, Val-Pro, His-Pro, Phe-Pro, Lys-Pro, Leu-Pro, Asp-Pro, Glu-Pro, Gly-Pro, Leu-Val-Pro, and Leu-Leu-Pro. The peptides were hydrolyzed at 50 °C, pH 6.5 for 5 min. The concentration of peptides was adjusted to 1.67 mM and expressed as relative activities to the most preferred substrate (Leu-Pro).

4.9.3 Effect of temperature on enzymatic stability and activity

To examine heat stability, recombinant prolidase was preheated at the designated temperature for 20 min in a water bath. The measurement of the residual activity was carried out for 5 min at 50 °C and absorption was continuously measured (every 15 sec) at Abs 436 nm.

To test the effect of temperature on prolidase activity, the reaction was measured for 15 min at the target temperature after 5 min of pre-incubation at the same temperature. In the pre-incubation, prolidase and substrates were held in separate tubes. Every 5 min, a 30 μL aliquot of prolidase reaction mixture was heat inactivated at 90 °C for 5 min, cooled on ice for 5 min, and 270 μL of the enzyme reaction mixture (L-amino acid oxidase and peroxidase cocktail) was added. The enzyme mixture was incubated at

37 °C for 20 min and the absorbance was read at 436 nm. The activity was determined from the linear regression of the plot of readings.

4.9.4 Effect of pH on enzymatic activity

For the examination of pH dependence, the test pH was prepared with 20 mM citrate buffer (pH 4, 5, and 6.5), 20 mM HEPES buffer (pH 7.0), 20 mM Tris-HCl buffer (pH 7.5, 8.0, and 8.5) and 20 mM glycine-NaOH buffer (pH 9.0, 10, and 10.5). The temperature was set to 50 °C and all reactions were measured for 5 min at 15 sec intervals. For these trials, activity was analyzed using Leu-Pro as the substrate.

4.9.5 Effect of metallic ion on enzymatic activity

For dependence on metallic ions (Mn^{++}, Co^{++}, Ca^{++}, Mg^{++}, and Zn^{++}) were examined as the same method stated in 4.9.1 by substituting metals.

4.9.6 Effect of substrate concentration on reaction rates

In these experiments, the reaction rates of recombinant *Lc. lactis* prolidase were measured by the ninhydrin method (Yaron and Mlynar, 1968). The substrate (Leu-Pro) was prepared from 0.1 mM to 8 mM in the 20 mM citrate buffer (pH 6.5). The reaction was carried out at 50 °C for 15 min. Every 5 min, 50 µL of the mixture was transferred into 125 µL of ninhydrin reagent (0.6 g of ninhydrin in 12 ml of glacial acetic acid and 8 ml of 6 M phosphoric acid) and 125 µL of glacial acetic acid. The mixture was heated at 100 °C for 30 min and cooled on ice. Spectrophotometry measurements were taken at 480 nm. All the experiments were performed in triplicate.

4.9.7 Secondary structure and thermal denaturation temperature measurement of recombinant prolidase

For secondary structure and thermal denaturation temperature analysis, Far UV-circular dichroism (CD) spectroscopy (PiStar-180, Applied Phytophysics Ltd, Leatherhead, Surrey, UK) was employed (Creighton, 1993a). In order to desalt the purified recombinant prolidase solution, 1 mL (1.5 mg/mL) of recombinant *Lc. lactis* prolidase sample was filtered (0.2 μm syringe filter) and loaded to a Sephadex G-50 gel filtration column (1 cm × 30 cm; Biorad) equilibrated with 20 mM of sodium phosphate buffer (pH 6.0) as a mobile phase. The elution was at a 0.1 mL/min flow rate. Desalted samples were pooled, concentrated to 1.12 mg/mL with MicroSep column (MWCO 10 kDa; Pall Life Science, East Hills, NY, USA) at 17,200 g for 30 min using a Sorval SS-34 rotor and divided into two groups for the secondary structure analysis (300 μL) and the thermal denaturation examination (700 μL). The sample was analyzed with the CD at the SSSC. The CD spectrum was measured between 180 nm and 250 nm. The resultant spectrum was analyzed using CDNN software (Bohm *et al*., 1992) to calculate the composition of the secondary structure. The thermal denaturation temperature was determined from the change of CD spectrum over the temperature change from 25 °C to 90 °C. The change of the spectrum was plotted and the rate of the change calculated for determining the temperature denaturation measurement, which was defined as the temperature where the rate of change reached its maximum.

4.9.8 Mass spectrometry (MS) measurement of recombinant prolidase

A Sephadex G-50 size exclusion column (1 cm × 30 cm) was equilibrated with deionized water and 100 μL (1.5 mg/mL) of 0.2 μm filtered recombinant *Lc. lactis* prolidase was desalted at a flow rate of 0.3 mL/min. Fractions were examined on 10% SDS-PAGE (20 mA, 90 min), and peak fractions pooled and mixed with the same volume of acetonitrile and acetic acid (99:1, v/v). The final concentration of enzyme was 0.5 mg/mL. The prepared sample was measured by the mass spectrometer (API Q-star XL Hybrid MS System) at the SSSC.

4.9.9 Computational molecular modelling of prolidase

The deduced protein sequence (appendix B) from *Lc. lactis* prolidase-coding gene was submitted to the JicSaw server (Bate *et al*., 2001) and the generated protein coordinate file was used for energy minimization which is the process of searching local minimum energy landscape of a protein molecule (Phillips *et al*., 2005). The energy minimized computational model of *Lc. lactis* prolidase was then calculated using the NAMD molecular modelling program (Phillips *et al*., 2005). The experiment was employed according to a manual from the provider. The initial model was energy-minimized using topology data provided with the program in a computationally generated water-filled box. The energy force-field parameters were adjusted to a 15 Å cut-off distance and 2 fs per step. The calculation was performed through 5,000 runs for 10 ps. Topological identity with *Pyrococcus* spp. prolidase was computationally calculated using a visual molecular graphics viewer VMD (Humphrey *et al*., 1996).

Protein sequence alignments of prolidases were visually created with CLC Free Workbench 3.2.1 (CLC bio, Aarhus, Denmark, 2007).

5. RESULTS AND DISCUSSION

5.1 PCR Amplification and gene cloning

5.1.1 Cloning of prolidase genes

5.1.1.1 Cloning of *Lactococcus lactis* prolidase gene

After the empirical searching for annealing temperature (45-55 °C), the best result was shown at 45 °C and this was used for PCR amplification. PCR using the forward and reverse primers successfully amplified the predicted 1,089 bp gene fragment under the conditions described in the method section. The cloned gene was confirmed using restriction enzyme analyses and further verified with DNA sequencing (confirmed sequence posted in the appendix A).

5.1.1.2 Cloning of *Lactobacillus plantarum* prolidase gene

As similar to *Lc. lactis* gene application, several annealing temperature were examined and PCR primers possessing *Eco*RI and *Hind*III restriction sites were successful in amplifying the *Lb. plantarum* prolidase gene using annealing at 45 °C. The length of the ORF was confirmed as 1110 bp using DNA sequencing and is provided in appendix A.

5.1.1.3 Homologies of prolidase genes

The sequence-verified *Lc. lactis* NRRL B1821 prolidase gene showed 100%

homology to *Lc. lactis* spp. Il1403 (GenBank accession code: NC002662) and showed 89% homology with *Lc. lactis* spp. cremoris SK11 prolidase (GenBank accession code: cp000425). With the exception of these two prolidase genes, all the knownprolidase DNA sequences showed less than 20% homology to the sequenced *Lc. lactis* prolidase gene. The sequence of *Lb. plantarum* NRRL B4496 prolidase gene shared 99%homology to the *Lb. plantarum* WCFS1 prolidase (GenBank accessioncode: NC004567) gene, 86% with *Lb. pentosus* (GenBank accession code: AF176799) and less than 17% with the others.

The deduced amino acid sequences (appendix B) from the gene sequences were used to examine the structural relationships of prolidases. The sequences of *Lb. plantarum* NRRL B4496 and *Lc. lactis* NRRL B1821 demonstrated 54% of homology with each other (Figure 5.1). The known prolidase sequences and human prolidase were also analyzed (Figure 5.2). The protein sequences from *Lb. pentosus* (50%), *Lb. helveticus* CNRZ32 (GenBank accession code: AAC24966; 52%), *Lb. acidophilus* NCFM (GenBank accession code: AAV42321; 51%), *P. furiosus* (GenBank accession code: NP 5709072; 36%) and *Homo sapiens* (*H. sapiens*, PDB accession code: 2IW2; 32%) showed high homologies to *Lc. lactis* prolidase.

5.1.2 Cloning of recombinant prolidase genes into protein expression systems

Four different protein expression systems (pTrxFus, pHIL-S1, pPIC-9, and pKK223-3) were utilized in parallel to examine the efficiency and degrees of expression for *Lb. plantarum* prolidase. As mentioned in section 4.5.2, the recombinant *Lc. lactis* prolidase was examined only with the pKK223-3 and pTrxFus systems. The prolidase gene was engineered with different restriction enzymes to meet the requirements for

each vector system (i.e. ease of sub-cloning, gene orientation, and matching reading frame).

5.1.2.1 Cloning of recombinant prolidase gene into the pTrxFus expression system

In order to provide higher solubility using a helper protein, thioredoxin, a fusion protein expression system, pTrxFus, was considered. The *Lb. plantarum* prolidase gene was excised using *Eco*RI-*Hind*III from the pUC18 vector and then blunt-ended. Since the pTrxFus system employs a protein fusion expression, the inserted gene should be in frame with the thioredoxin gene to prevent frame shift errors (Invitrogen, 2006). The blunt-ended gene possessed two extra nucleotides upstream of the ORF and the blunt-ended *Bam*HI site of pTrxFus had an extra base on the downstream of the thioredoxin gene. These extra bases created an extra codon between two genes and resulted in a fused gene of thioredoxin and *pep*Q. The resultant recombinant DNA was examined with the restriction enzymes *Nhe*I-*Pst*I in order to confirm the orientation of the inserted DNA (Figure 5.3). The correct direction should result in 3.6 kbps and 1.0 kbps (4.6 kbps and 170 bps expected in the reverse orientation).

The *Lc. lactis* prolidase gene was introduced into the blunt-ended *Cfr*9I site of the pTrxFus in order to create an in-frame connection of the two genes. *pep*Q-pTrxfus digested with *Hinc*II digestion proved its right orientation, showing 405, 1656, and 2642 bp bands.

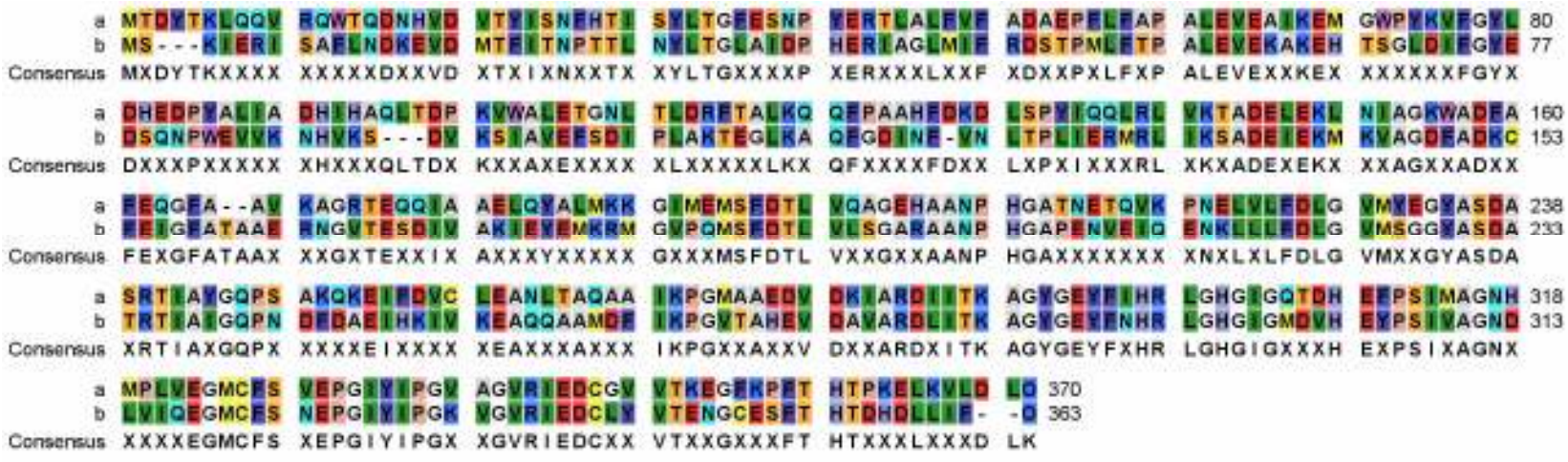


Figure 5.1 The Sequence alignment of *Lactococcus lactis* NRRL B1821 and *Lactobacillus plantarum* NRRL B4496 putative prolidase amino acid sequences. *Lb. plantarum* (a) *and Lc. lactis* (b) prolidase genes are shown. The consensus is based on the 100% matching sequences. X stands for non-matching amino acid. The Figure was generated using the CLC Free Workbench^{TM}. Each colour represents a group of amino acids that show similar characteristics.

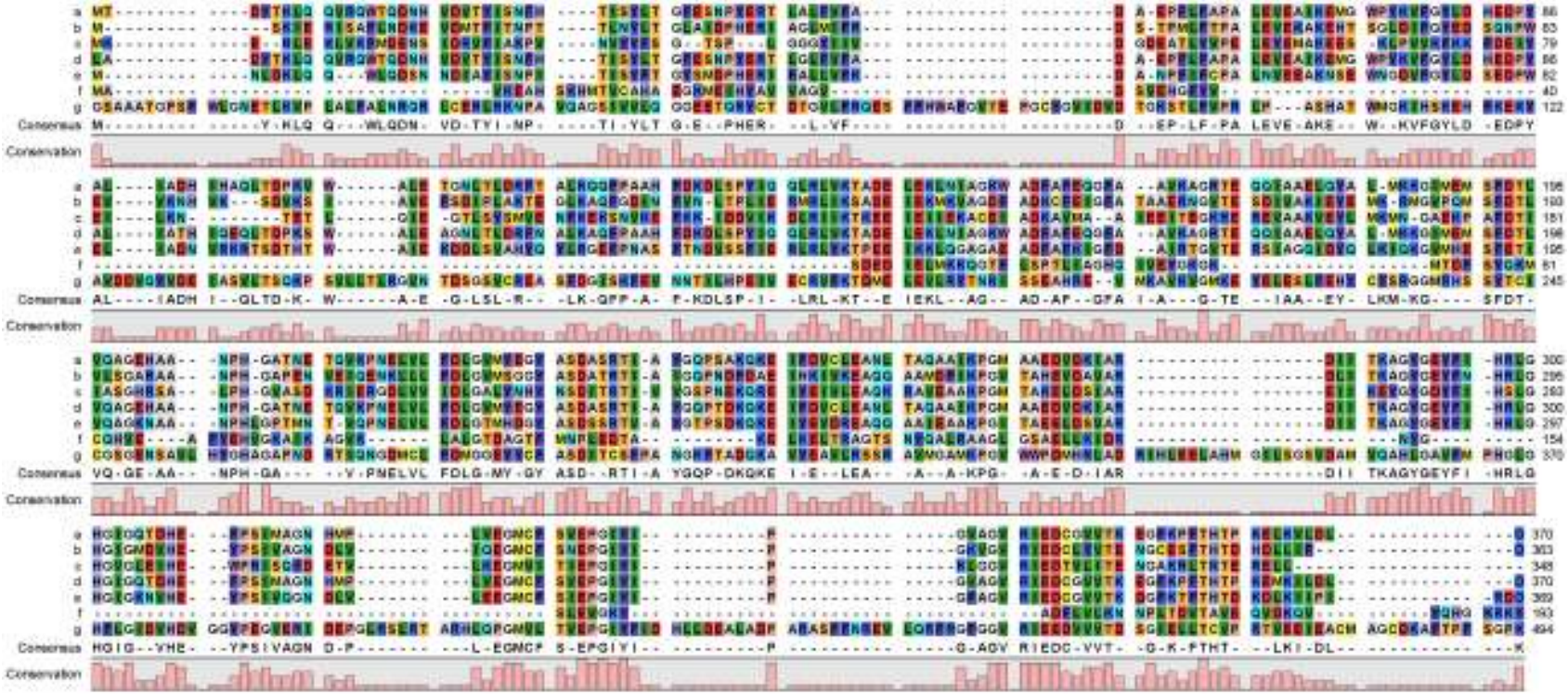


Figure 5.2 Amino acid sequence alignment results of prolidases (a: *Lb. plantarum* NRRL B4496; b: *Lc. lactis* NRRL B1821; c: *P. furiosus*; d: *Lb. pentosus*; e: *Lb. helveticus*; f: *Lb. acidophilus*; g: *H. sapiens*). Amino acids that are conserved in majority of sequences ($\geq 50\%$) were considered to be consensus. The conservation colour represents the degree of the conserved residues among compared sequences. The figure was generated using the CLC Free Workbench™.

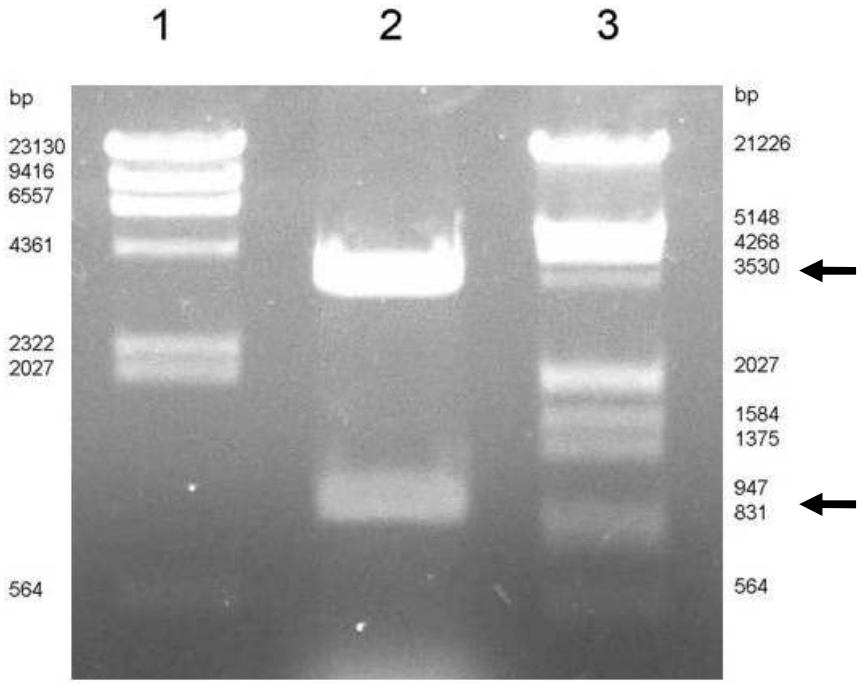


Figure 5.3 The ethidium bromide-stained agarose gel of recombinant *Lactobacillus plantarum* prolidase gene-pTrxFus digestion using restriction enzyme physical mapping. The pTrxFus recombinant DNA digested with *Nhe*I-*Pst*I is shown. The *Nhe*I site is on the prolidase ORF and the *Pst*I site is on the pTrxFus system. Two bands (3.6 kbps and 1.0 kbps; a positive orientation) are shown with DNA molecular size markers (lane *1* and *3*). The restriction enzyme physical mapping of pTrxFus clones of *Lb. plantarum*.

5.1.2.2 Cloning of recombinant *Lb. plantarum* prolidase gene into the pHIL-S1 expression system

The positive clones of pHIL-S1-*Lb. plantarum* prolidase recombinant were examined by *Eco*RV (772, 3828, and 4798 bps), *Hinc*II (1041, 1677, 1954, and 4726 bps), and *Eco*RI-*Bam*HI (1148 and 8250 bps) (Figure 5.4). Each assay showed positive results of cloning into the pHILS-1 system.

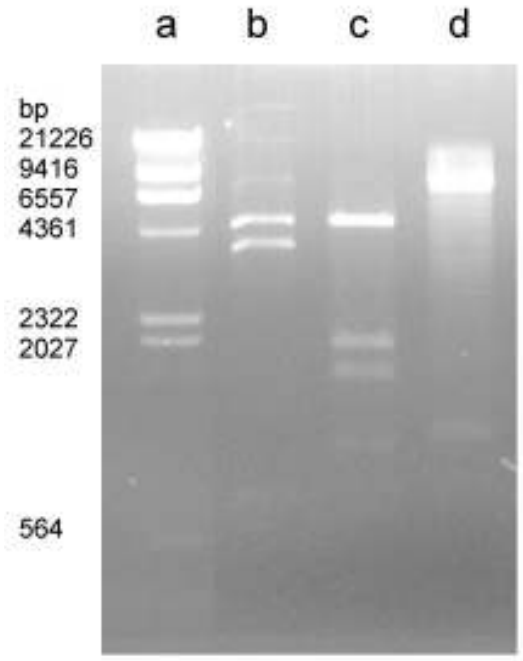


Figure 5.4 Ethidium bromide-stained agarose gel showing restriction enzyme digestion of recombinant *Lactobacillus plantarum* prolidase gene-pHIL-S1. Fragments of restriction enzyme digestions were examined on the agarose gel (*a*: DNA molecular size marker; *b*: *Eco*RV; *c*: *Hinc*II; *d*: *Eco*RI-*Bam*HI digestion). The positive fragments by *Eco*RV (4798, 3828, and 772), *Hinc*II (1041, 1677, 1954, and 4726), and *Eco*RI- *Bam*HI (1148 and 8250) digestions are presented.

5.1.2.3 Cloning of recombinant *Lactobacillus plantarum* prolidase genes into the pPIC-9 expression system

The pUC18 fragment (5'-*Hind*III-3'-*Eco*RI) of *Lb. plantarum* was blunt-ended, then introduced into filled *Eco*RI site of the pPIC-9 vector and examined using restriction enzyme analysis. The cloning of pPIC-9 was not successful and did not result in any positive clones.

5.1.2.4 Cloning of recombinant prolidase genes into the pKK223-3 expression system

The cloned pKK223-3 systems possessing prolidase genes were examined with *Hinc*II (*Lb. plantarum* recombinant; Figure 5.5) and *Pst*I-*Hind*III and *Nco*I-*Hind*III restriction enzyme analysis (*Lc. lactis* recombinant; Figure 5.6). The *Hinc*II-digested recombinant DNA yielded four diagnostic bands (3253, 1079, 781, and 464 bps), indicating positive clones. *Nco*I-*Hind*III and *Hind*III-*Pst*I restriction enzyme digestions also demonstrated expected fragments sizes of 5,340 bps with 237 bps and 5,605 bps with 28 bps, respectively (Figure 5.6).

5.2 Optimization of recombinant prolidase expression

5.2.1 Expression of recombinant prolidase under standard conditions

The expression of foreign genes in prokaryotic systems can be difficult and the solubilization of recombinant protein usually requires a lot of time and efforts. As Singh *et al.* (2005) noted, the inclusion bodies produced in *E. coli* are densely packed, causing denaturation of the expressed proteins in the cell. These inclusion bodies do not allow the renaturation of the molecules under physiological conditions. Once they are packed, the loss of secondary structures occurs and the reduction of recovery becomes usually less than 15-25% (Datar *et al.*, 1993). Three types of *E. coli* expression systems, pKK223-3/Top10F', pTrxFus/GI724, and pTrxFus/GI698 were examined under the conditions as recommended by their suppliers. Top10F' harbouring the pKK223-3 recombinant system was incubated in LB broth at 37 °C. Host cells, GI724 and GI698, harbouring the pTrxFus recombinants were incubated in induction media at 37 °C and 30 °C, respectively. The expressions in pTrxFus and pKK223-3 systems under these conditions failed to express the recombinant prolidase (Figure 5.7, 5.8, and 5.9).

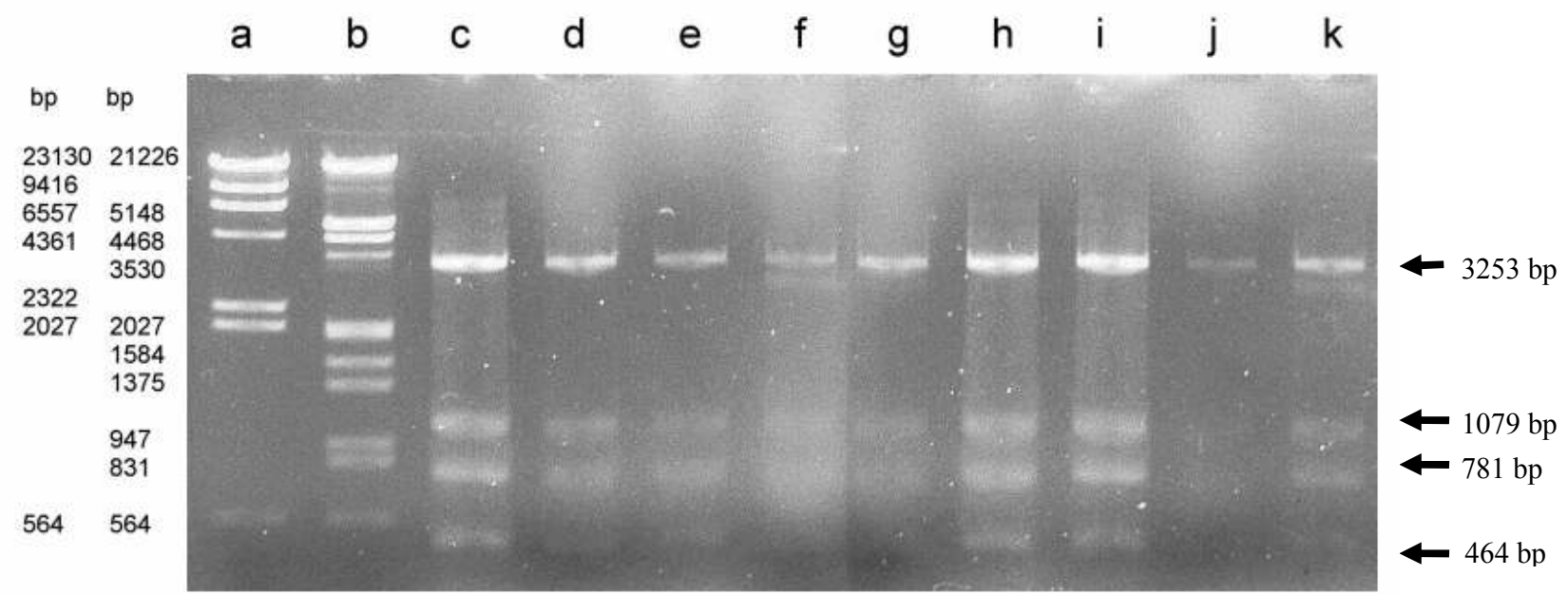


Figure 5.5 Ethidium bromide-stained agarose gel showing *Hinc*II digestions of recombinant *Lactobacillus plantarum* DNA. Distinctive four fragments (464, 781, 1079, and 3253 bps) are indicative with arrows. Molecular size makers (lane *a* and *b*) are shown with digested recombinant DNA (lanes *c* to *k*).

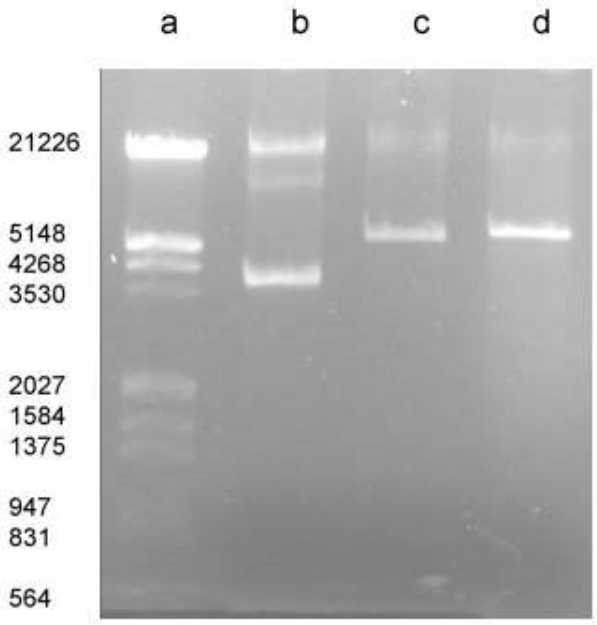


Figure 5.6 Ethidium bromide-stained agarose gel showing *Hind*III-*Nco*I and *Pst*I-*Hind*III digestions of *Lactococcus lactis* recombinants. (*a*) DNA molecular size marker, (*b*) undigested recombinant DNA, (*c*) *Nco*I-*Hind*III digestion (5,340 bps), and (*d*) *Pst*I-*Hind*III digestion (5,605 bps) are shown.

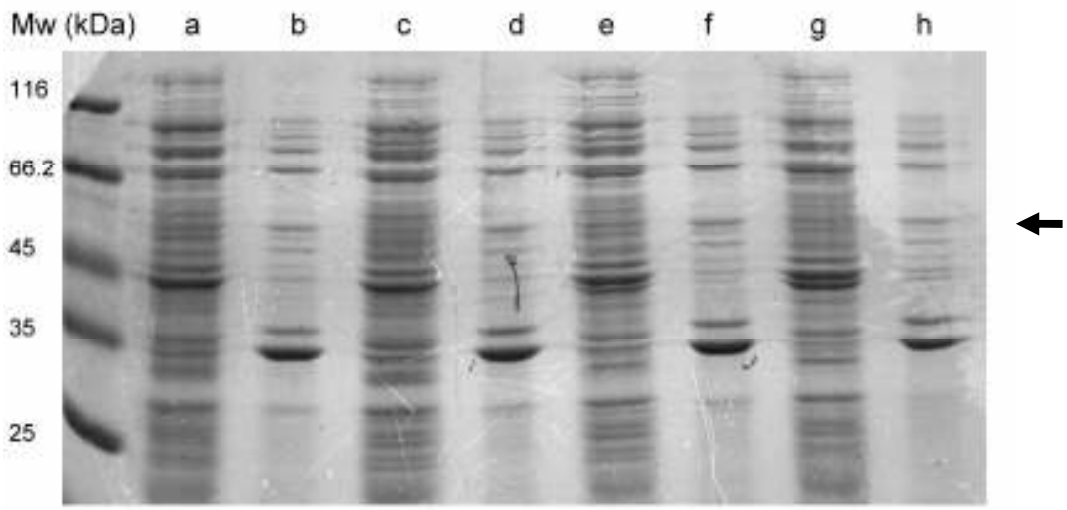


Figure 5.7 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the expression of recombinant *Lactococcus lactis* prolidase in the GI698 system harbouring pTrxFus recombinant *Lactococcus lactis* prolidase constructs. Host cells possessing *Lc. lactis* recombinants were cultured in 10 mL of induction media at 30 °C with 100 rpm shaking. Four cultures were harvested at 17,200 g for 20 min. After disruption with sonication, soluble fractions and precipitate fractions were recovered. Lanes *a*, *c*, *e*, and g represent soluble fractions from an individual culture. Lane *b*, *d*, *f*, and h are insoluble fractions. The induction of expression was at Abs $_{600}$ 0.52 (*a*: GI698; *b*: non-induced standard; *c*, *d*, *e*, and f: induced samples). The arrow indicates the size of the predicted fusion proteins (51 kDa). Five microgram of protein was loaded on the SDS-PAGE and stained with Coomassie Brilliant Blue G-250. The recombinant systems did not express the expected recombinant prolidase.

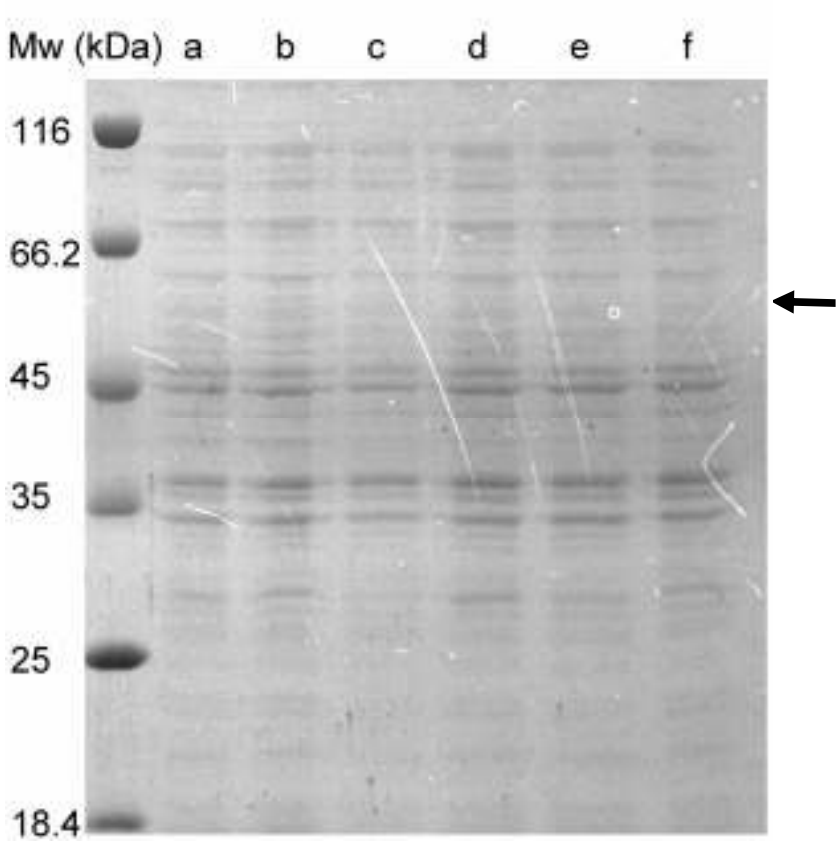
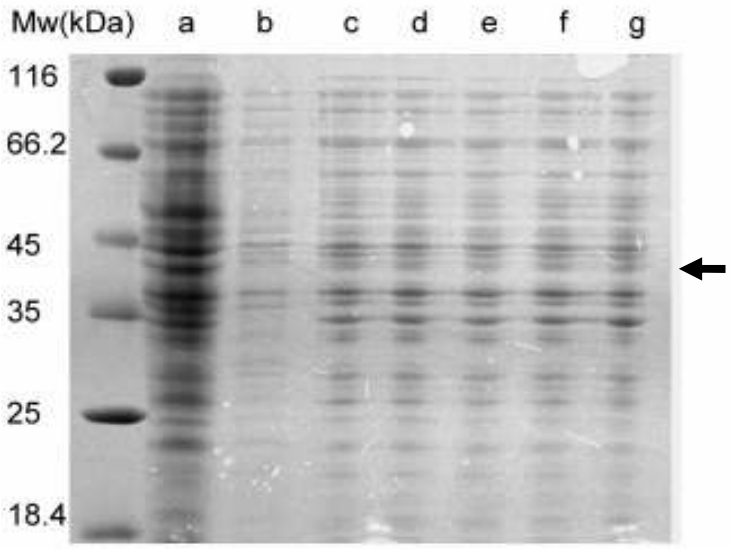
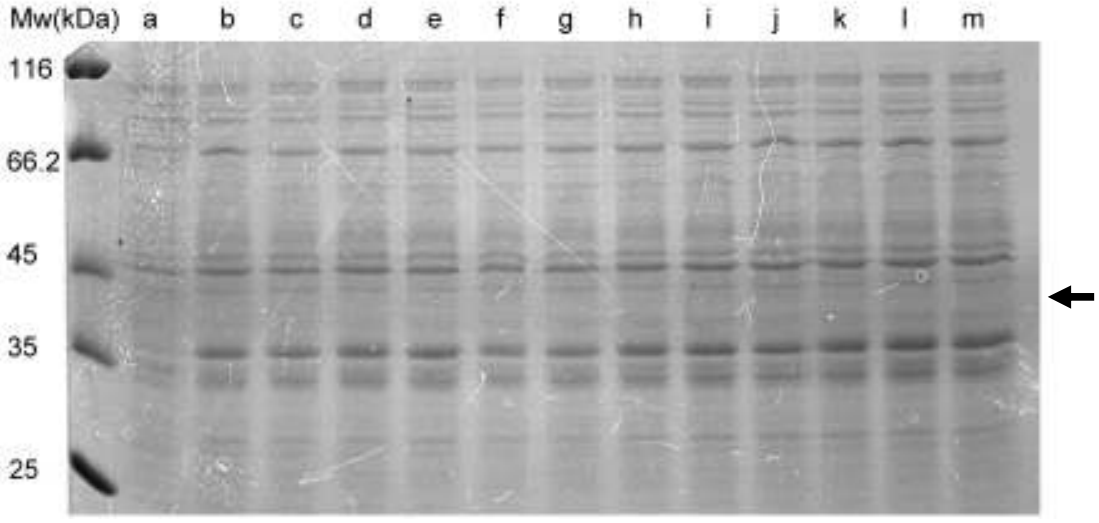


Figure 5.8 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing expression in GI698 system harbouring pTrxFus recombinant *Lactobacillus plantarum* prolidase constructs. Host cells possessing *Lb. plantarum* were cultured in 10 mL of induction media at 30 °C with 100 rpm shaking. Four cultures were harvested at 17,200 g for 20 min. After disruption with sonication, soluble fractions and precipitate fractions were recovered. Lane *a*, *c*, and *e* represent soluble fractions from an individual culture. Lane *b*, *d*, and *f* represent insoluble fractions. The induction of expression was at Abs $_{600}$ 0.52 (*a*: GI698; b: non-induced standard; *c*, *d*, and *e*: induced samples). The arrow indicates the size of the predicted fusion proteins (52 kDa). Five microgram of protein was loaded on the SDS-PAGE and stained with Coomassie Brilliant Blue G-250. The recombinant systems did not express the expected recombinant prolidase.



A



B

Figure 5.9 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the expression of Top10F' recombinant systems harbouring pKK223-3. Five micrograms of *Lc. lactis* (A) and *Lb. plantarum* (B) whole cell extracts were loaded. Five and twelve individual clones of recombinant *Lc. lactis* and *Lb. plantarum* prolidases, respectively, were compared for their expression. **A**: Each lane has Top10F' (*a*), no induction (*b*), and whole cell extracts (*c*, *d*, *e*, *f*, and *g*). **B**: Each lane has non-induced standard (*a*) and whole cell extracts (*b*, *c*, *d*, *e*, *f*, *g*, *h*, *i*, *j*, *k*, *l*, and *m*). The predicted sizes (40 kDa of recombinant *Lc. lactis* prolidase and 41 kDa of recombinant *Lb. plantarum* prolidase) are noted with the arrows. Two millilitre cultures were carried out at 37 °C and aerated by 100 rpm shaking.

5.2.2 Achievement of an optimized recombinant prolidase expression level

Expression of recombinant prolidase in the pKK223-3 system of *Lb. plantarum* is shown in Figure 5.10. After 17 hours of incubation at 37 ºC, approximately 50% of the total protein expressed from *E. coli* Top10F' was recombinant *Lb. plantarum* prolidase according to a densitometry method using the Image J program, a Java-based graphical calculation program (Abramoff *et al*., 2004). However, under that condition, the recombinant proteins formed an insoluble pellet after centrifugation. This misfolded protein aggregate could be derived from the failure of the repair control system in the cell and known to be an indicator of a cellular stress (Gottesman *et al*., 1997).

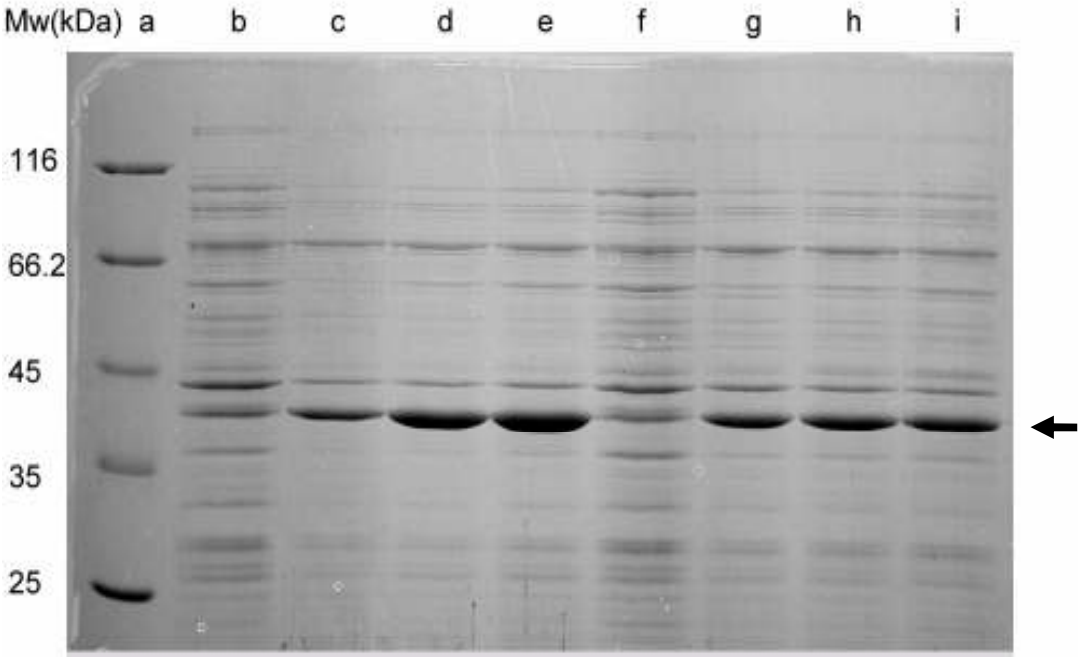


Figure 5.10 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the expressed recombinant prolidase. Two pKK223-3 recombinant *Lb. plantarum* prolidase are shown. # 1 culture (*a*: molecular marker, *b*: non-induced culture, *c*: 1.5 hours, *d*: 3 hours, *e*: 17 hours) and # 2 culture (*f*: non-induced culture, *g*: 3.5 hours, *h*: 6.5 hours, *i*: 19 hours) were incubated in 10 mL at 37 °C, 100 rpm, and 1 mL aliquots were collected each time.

5.2.3 Effect of culture conditions on enzyme solubility

To achieve soluble expression in the *E. coli* system harbouring pKK223-3 recombinants, diverse conditions were examined. Several modifications to the culture medium and physical incubation conditions were considered together (i.e. pH, temperature, induction point, incubation time, antibiotics, aeration, IPTG concentration, and NaCl concentration). The alteration was aimed to alleviate stress from the forced protein overexpression by providing favourable growth conditions or reducing the growth rate.

Modifications were first approached using 2YT media (Sambrook and Russell, 2001) in 10 mL. This enriched medium (60% and 100% more tryptone and yeast extract respectively than LB medium) was not effective and the host cell generated a higher level of insoluble protein than grown in LB medium. Another approach to increase expression by using a larger amount of IPTG, an inducer, was devised. One or ten millimolar IPTG was applied into 10 mL cultures at 37 °C. Incubation and induction was made at Abs_{600} 0.25, 0.3, and 0.4. Solubility was visually examined on SDS-PAGE every hour for 4 hours. The amount of expressed soluble recombinant prolidase exhibited a slight increase with 10 mM IPTG in all of the induction batches stated above; however, the degree was not noteworthy. As Jespers *et al*. (1991) reported, a higher NaCl concentration can reduce the growth rate and release overexpression stress. From preliminary tests, 0.34 M of NaCl (normally 0.085 M in LB medium) showed a subtle increase of soluble recombinant protein (data not shown).

To achieve a higher recovery of soluble enzyme, the effect of pH (Koretzki *et al*., 1989) and antibiotics (Lee and Beckwoth, 1986) were also inspected. Temperature

was also reduced to 33 °C from 37 °C since enhanced solubility was observed from precursory tests (data not shown). The experimental scheme is represented in Table 5.1.

Table 5.1 Modified expression conditions for *E. coli* hosts harbouring the pKK223-3 recombinant prolidase construct.

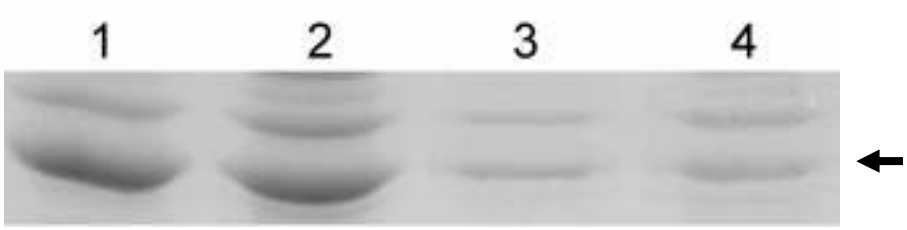
	1	2	3	4	5
Temperature (°C)	33	33	33	33	33
pH	5.5	5.5	5.5	5.5	5.5
IPTG (mM)	1	1	0.1	0.1	0
Chloramphenicol (μg/mL)	1	0.1	1	0.1	0
Ampicillin (μg/mL)	150	150	150	150	150

Ten millilitre cultures with each condition (1 to 5 in Table 5.1) were prepared in 125 mL flasks and incubated at 33 ºC with 100 rpm shaking. As shown in Figure 5.11, condition #1 at log phase, Abs $_{600}$ 0.4 and 0.8, expressed soluble recombinant protein. However, soluble recombinant protein decreased when incubation was extended overnight. In an attempt to recover more protein with reduced culturing time, culture sizes were increased to 3 L and 10 L using condition #1. However, these larger cultures failed to yield soluble recombinant proteins under condition #1.

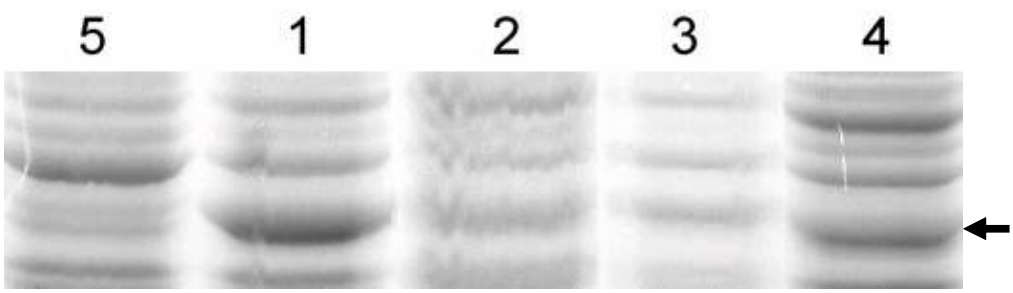
5.2.3.1 Effect of cold shock on enzyme solubility

To increase solubility and reduce expression stress of the host cell, lower temperature was considered. Various temperatures were tested (37, 32, 30, 29, 22, 18, and 16 °C) overnight in 1 L culture and inductions were made at Abs $_{600}$ 0.5. The best

expression was shown under the conditions: pH 5.5, 1 mM IPTG, 1 μg/mL chloramphenicol, forced aeration (or 150 mL culture in a 500 mL flask at 250 rpm shaking), and 150 μg/mL ampicillin at 16 °C for 25 to 40 hours of incubation. Under these conditions, recombinant prolidase accounted for approximately 40% of the total soluble protein (Figure 5.12). Solubility of the recombinant protein increased to a great extent when the culture was incubated at 16 ºC than at a higher temperature (over 16 ºC).



A



B

Figure 5.11 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the expression of recombinant *Lactobacillus plantarum* prolidase by the pKK223-3 recombinant system in the modified conditions. Ten millilitre modified cultures were induced at Abs $_{600}$ 0.4 (A) and Abs $_{600}$ 0.8 (B). The numbers on the figures indicate the conditions shown in Table 5.1. All of the 100 mL cultures were incubated for 5 hours, crude extracts (soluble fractions) after cell destruction (refer to section 3.8) were examined. Five microgram of protein was applied. The expressed recombinant proteins (41 kDa) are indicated by arrows.

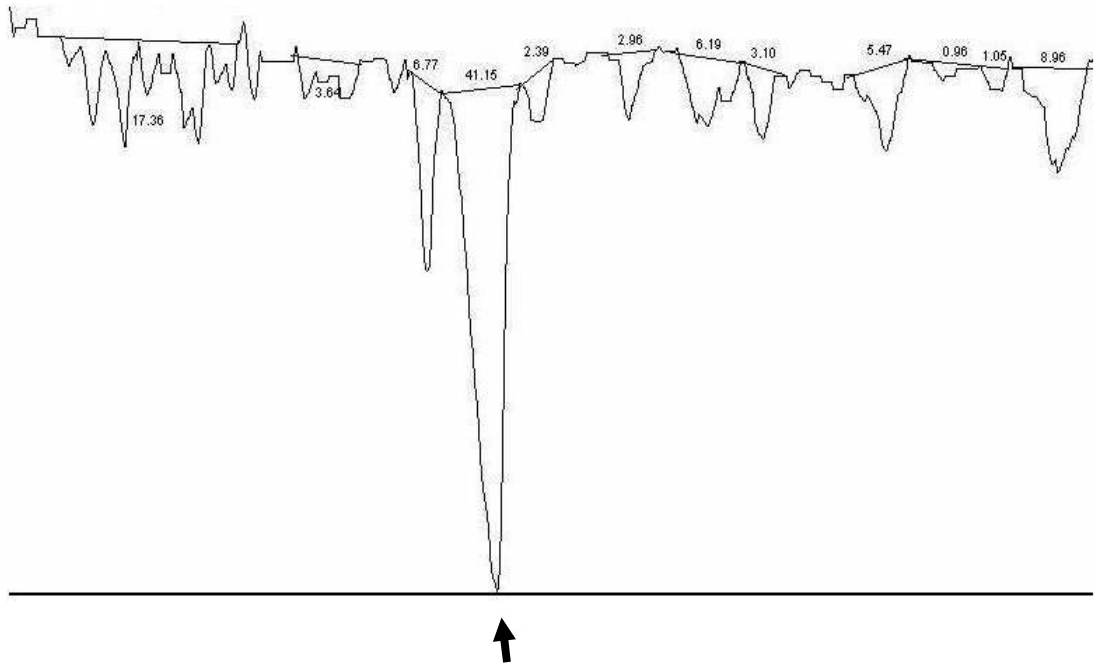
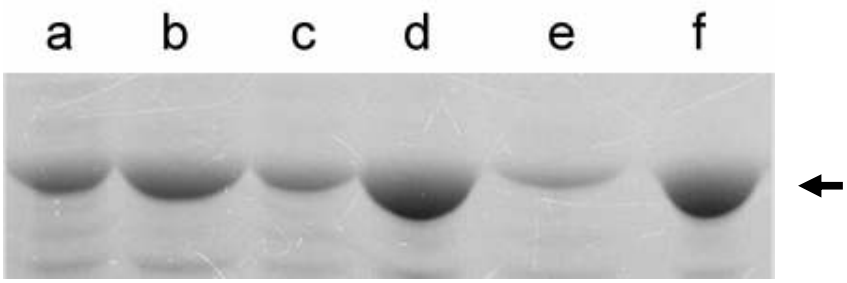


Figure 5.12 Densitometric measurements of expressed *Lactobacillus plantarum* soluble protein. One hundred millilitres of modified LB medium culture (pH 5.5, 1 mM IPTG, 1 µg/mL chloramphenicol, and 150 µg/mL ampicillin) in a 500 mL flask was incubated at 16 °C, 250 rpm shaking. Induction was made at Abs $_{600}$ 0.5. After 36 hour incubation, cells were harvested and disrupted. The harvested supernatant after centrifuging at 17,200 g was loaded on the SDS-PAGE and stained with Coomassie Brilliant blue G-250. The destained gel was used for densitometric measurement by Image J. The recombinant *Lb. plantarum* prolidase is displayed and indicated by the arrow. The peak area represents the percent ratio of the total soluble proteins.

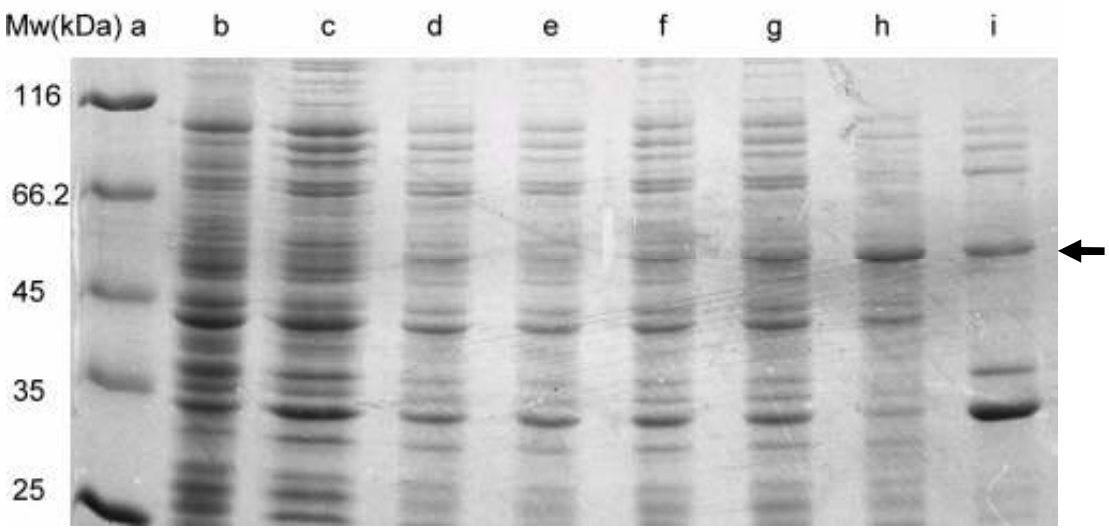
5.3 Expression of recombinant prolidase in the pTrxFus system

The recombinant prolidase expression in pTrxFus/*E. coli* GI724 and GI698 hosts system was examined with the methods used for the pKK223-3 system. Under normal growth temperatures for GI724 (37 ºC) and GI698 (30 ºC), they did not express soluble protein as shown in the pKK223-3 system. According to the supplier, *E. coli* GI698 is adequate for growing at temperature lower than GI724, thus *E. coli* GI698 was used for the modified cultures. The modified conditions (Table 5.1) with the exception

of temperature, which was lowered to 16 ºC, allowed the strong expression in both recombinant *Lb. plantarum* and *Lc. lactis* prolidases (Figure 5.13, A and B).



A



B

Figure 5.13 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the expression of recombinant *Lactobacillus plantarum* (A) and *Lactococcus lactis* (B) prolidases harbouring the pTrxFus system. **A**: soluble (*a*, *c*, *e*) and insoluble proteins (*b*, *d*, *f*) after incubation at 16 °C are shown. Incubation for 24 hours (*a*, *b*), heat shock of 24 hour-specimen at 80 °C for 1 min (*c*, *d*), and 48 hour incubation (*e*, *f*) are shown. **B**: Soluble proteins recovered under various conditions are shown (a: *E. coli* GI698 without vector; *b*: non-induced; *c*: 2 hour incubation; *d*: 4 hour incubation; *e*: 6 hour incubation; *f*: 8 hour incubation; *g*: soluble protein after 24 hour incubation; *h*: insoluble protein after 24 hour incubation). Both A and B culture sizes are 1.5 L of induction medium with 5 µg of proteins loaded. The arrows indicate 52 kDa (A) and 51 kDa (B) of thioredoxin-recombinant fusion proteins.

The degree of expression in both recombinant prolidases was high, approximately 30% of total soluble protein as estimated by the Image J program. Under these modified conditions, less than 40 hours of incubation at 16 ºC was essential to maintain a higher level of solubility (Figure 5.13, A). When the culture was incubated for 40 hours, it generated a much higher level of insoluble recombinant prolidases than at the 24 hour incubation.

5.4 Purification of recombinant prolidase

5.4.1 Ammonium sulphate precipitation of recombinant prolidase

Prolidase from *Lb. plantarum* showed precipitation in the range of 10-40% ammonium sulphate (Figure 5.14). In the process of resolublizing the salting-out fraction, however, the recombinant prolidase demonstrated permanent denaturation. A variety of pH (pH 6, 6.5, 7, 7.5) and metallic ions (Co^{++}, Mg^{++}, and Zn^{++}) were considered together; however, this variety did not provide a successful result. In all conditions examined, *Lb. plantarum* prolidase formed a denatured aggregate rather than the crystal-like form.

Conversely, ammonium sulphate precipitation purified the recombinant *Lb. lactis* prolidase. Recombinant *Lc. lactis* prolidase demonstrated high compatibility with ammonium sulphate precipitation and the proteins were resolubilized after dialysis. Most of the recombinant protein precipitated at 60% ammonium sulphate precipitation in 1 mM ZnSO4, 20 mM citrate buffer, pH 6.0 (Figure 5.15). Forty one milligrams of soluble *Lc. lactis* prolidase was recovered from 272 mg of protein in the crude extract.

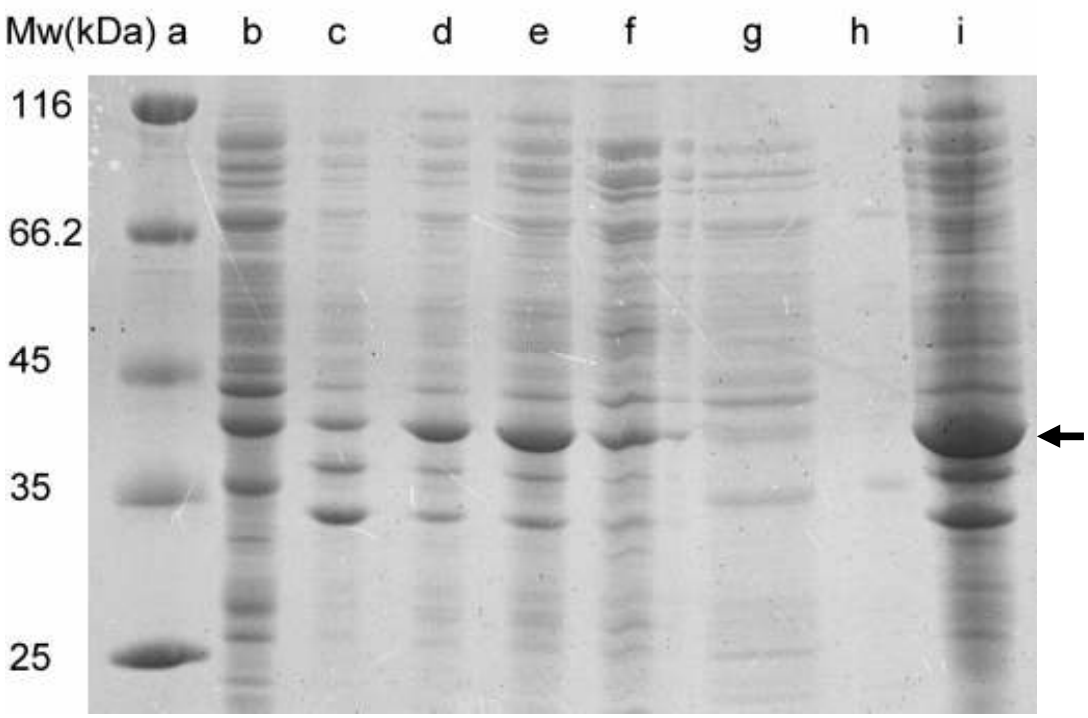


Figure 5.14 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the ammonium sulphate precipitations of recombinant *Lactobacillus plantarum* prolidase produced by the pKK223-3 system. Ammonium sulphate precipitated fractions are shown. Each lane shows crude extract (*a*) from 1.5 L LB modified medium, 10% (*b*), 20% (*c*), 30% (*d*), 40% (*e*), and 60% (*f*) of ammonium sulphate precipitates. The precipitates were recovered by centrifugation (17,200 g for 20 min) and dissolved into 5 mL of 20 mM Tris-HCl buffers (pH 8.0). The combined 20% and 30% saturated solutions were dialyzed against 4 L (2 × 2 L) of the same Tris-HCl buffer overnight. The recovered supernatant (*g*) and precipitates (*h*) after centrifugation (17,200 g for 20 min) are shown. Five micrograms of protein was loaded. The arrow indicates 41 kDa (the recombinant prolidase).

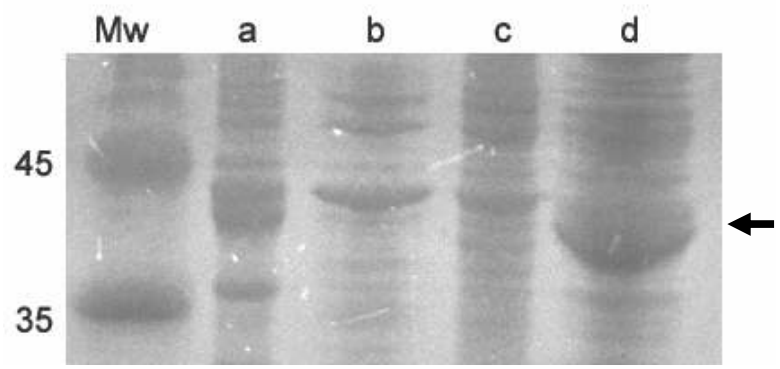


Figure 5.15 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the ammonium sulphate precipitations of recombinant *Lactococcus lactis* prolidase produced by pKK223-3 system. The crude extract (*a*) from 900 mL LB modified medium, 20% (*b*), 40% (*c*), and 60% ammonium sulphate saturations (*d*) are shown. Ammonium sulphate saturation was processed overnight, centrifuged (SS-34 rotor, 17,200 g), and the precipitates were dissolved in 5 mL of 20 mM citrate buffer (pH 6.0) containing 1 mM $ZnSO_4$. Dialysis against 4 L (2 × 2L) of the same citrate buffer overnight was done. Five micrograms of protein was loaded. The recombinant protein is indicated with an arrow at 40 kDa.

5.4.2 Column chromatography of recombinant prolidase

5.4.2.1 Effect of metallic ions on enzyme stability in the purification process

Prolidases from *P. furiosus* (Co^{++} or Mn^{++}; Ghosh *et al*., 1998), *Lb. casei* (Mn^{++}; Fernández-Esplá, 1997), and *Lb. delbrueckii* (Zn^{++}; Morel *et al*., 1999a, b) demonstrated different metal requirements for their catalytic activities. Since metallic ions were related to catalytic activities of these metalloenzymes, they were necessary during the purification. A loss of activity was observed without metallic ions in recombinant *Lc. lactis* prolidase purification. The precipitation of metallic ions and recombinant proteins, however, was found. Therefore, activities of the prepared crude extract of the recombinant prolidases were preliminarily examined with Zn^{++}, Mg^{++}, Mn^{++}, Ca^{++}, and Co^{++}. From this preliminary result, Zn^{++} was used throughout the purification.

5.4.2.2 Column chromatography of recombinant *Lactobacillus plantarum* prolidase

Since low recovery of recombinant *Lb. plantarum* prolidase was observed in the ammonium sulphate precipitation, the crude extract was directly examined with diverse columns under different conditions (i.e. pH and metallic ions). The recombinant prolidase did not bind to all resins in 20 mM of pH 6.0 (citrate), 6.5 (citrate), 7.5 (MES or Tris-HCl), and 8.0 (MES or Tris-HCl) buffers in both the presence and absence of metallic compounds (1 mM of $ZnSO_4$, $ZnCl_2$, $Co(NO_3)_2$, $CaCl_2$ $MgCl_2$, or $MnCl_2$). Zinc yielded a milky coloured precipitate above neutral pH 7.0 and manganese and magnesium could not stabilize the recombinant prolidase.

The final optimal buffer was pH 6.5, 20 mM citrate buffer containing 1 mM $ZnSO_4$ with 1 mM DTT. The crude extract (40 mL, 3.4 mg/mL) was applied to the DEAE-Sephacel column and 39 mg of protein was recovered. This sample was immediately loaded on the Phenyl Sepharose column and 6.3 mg of protein was eluted. Over the purification process, tailing on the chromatogram and impurity on the SDS-PAGE were observed.

The final sample was examined using diverse substrates (i.e. Leu-Pro, Gly-Pro, Phe-Pro, Arg-Pro, Val-Pro, and His-Pro); however, the sample could not hydrolyze these dipeptides.

5.4.2.3 Column chromatography of recombinant *Lactococcus lactis* prolidase

The purification process described in the methods yielded 18 mg of protein through the two step purification (Table 5.2, Figure 5.16, and Figure 5.17). From the crude extract, 79% of activity was recovered and the purified sample showed 461 U/mg of specific activity.

Table 5.2 Purification of recombinant *Lactococcus lactis* prolidase

Purification process	Total protein (mg)	Total activity (unit [+])	Specific activity (unit/mg)	Yield (% activity)	Purification fold
cell extract	272	10642	39	100	1
60% $(NH_4)_2SO_4$	41	8530	209	80	5.3
DEAE-Sephacel [++]	18	8381	461	79	11.8

[+] One unit is defined as μmole of substrate consumed by one μg of enzyme per minute
[++]DEAE-Sephacel result is after concentration through an ultra membrane filtration (MWCO 30 kDa)

5.4.3 Purification of pTrxFus fusion recombinant prolidase

The thioredoxin fusion protein of prolidase from *Lb. plantarum* demonstrated similar results to the pKK223-3 system. The expressed protein did not provide an ionic differentiation from other proteins in all ion exchange resins examined, generating a long tailing over the chromatogram. The author suspects that the partially denatured conformational structure of recombinant prolidase inhibited consistent distributions of charged molecules on the surface of the protein, and thus binding to the resins.

The fusion protein of *Lc. lactis* prolidase was eluted at the early stage of the NaCl gradient (0 M to 0.1 M) in the anion exchange resins tested from pH 6.0 to pH 8.5 with 1 mM of metallic ions. Enterokinase, a fusion site cleaving enzyme, has not successfully released the recombinant prolidase.

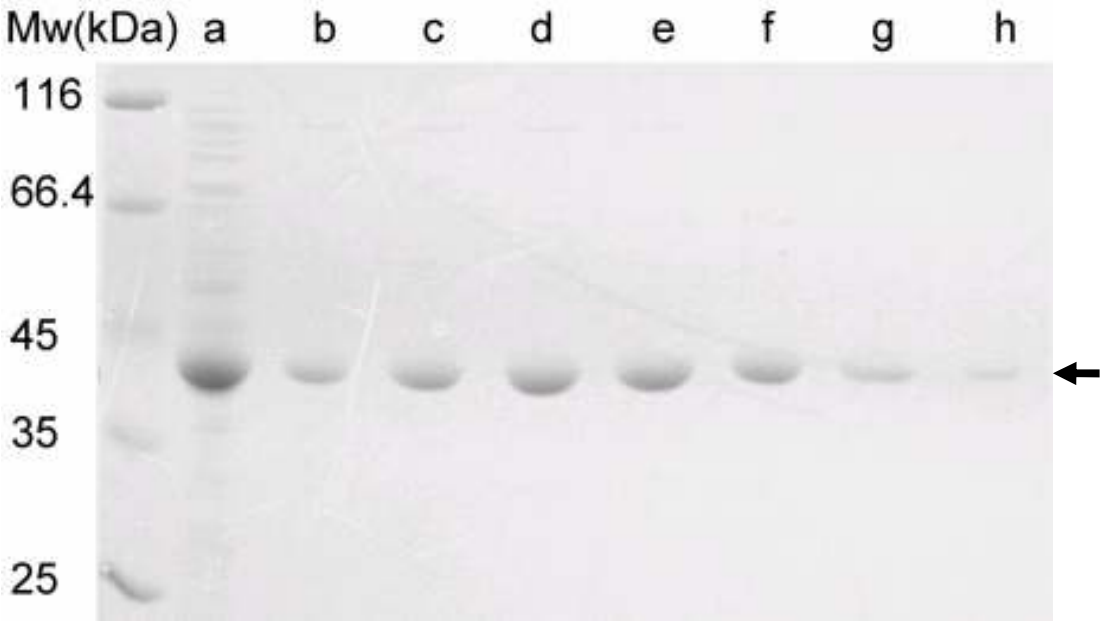


Figure 5.16 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the DEAE-Sephacel 0.3 M NaCl gradient elution of recombinant *Lactococcus lactis* prolidase. The column (3 × 15 cm) was equilibrated with 3 bed volumes of running buffer (pH 6.0, 20 mM citrate buffer with 1mM $ZnSO_4$). A flow rate of 2 mL/min was maintained and a linear gradient elution was made from 0 to 0.5 M NaCl (total 1L). Recombinant *Lc. lactis* prolidase was eluted at 0.3 M NaCl. The sample after ammonium sulphate purification (*a*) was used as a positive standard. The fractions from # 27 to 33 are shown. The 40 kDa recombinant proteins (*b*-*h*) are indicated by the arrow. All operations were made at 4 °C. The linear gradient elution and flow rate were controlled by the FPLC™. Five micrograms of protein was loaded per lane.

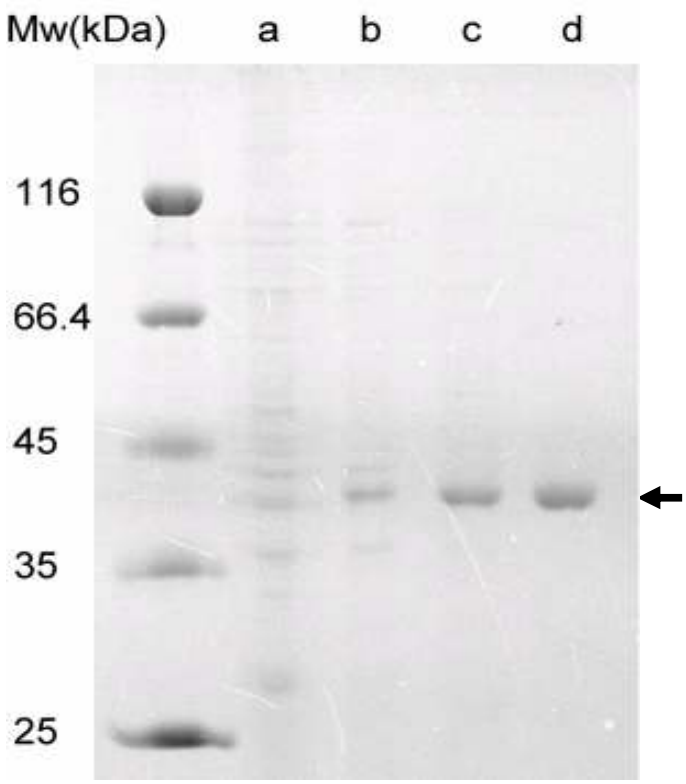


Figure 5.17 Coomassie Brilliant Blue G250-stained 10% SDS-PAGE gel showing the final purification data of recombinant *Lactococcus lactis* prolidase. The negative standard (*a*; no induction), crude extract (*b*), 60% of ammonium sulphate precipitation (*c*), and ultra-membrane (30 kDa MWCO) filtered DEAE-Sephacel fraction concentrate (*d*) are displayed. Three micrograms of protein was loaded and the 40 kDa recombinant *Lc. lactis* prolidase is indicated by the arrow.

5.5 Characterization of recombinant *Lactococcus lactis* prolidase

Characteristics of *Lc. lactis* prolidase were examined for the following parameters: pH dependence, temperature dependence, substrate specificity, thermal stability, metallic ion dependence, secondary structure, thermal denaturation temperature, molecular modelling, molecular mass determination, and enzyme kinetics. According to the reports from *Lb. delbrueckii* (Morel *et al*., 1999a,b) and *Lb. casei* (Fernández-Esplá *et al*., 1997) prolidases, 40 °C to 55 °C were considered as the optimal temperature. A

preliminary substrate determination of the recombinant prolidase showed that Leu-Pro exhibited the highest activity (Table 5.3). In the following characterization, Leu-Pro was used as the standard substrate.

Table 5.3 Preliminary substrate specificity determination [a]

Substrate	Abs change at 436 nm in 10 min
Gly-Pro	0.02 ± 0.007
Phe-Pro	0.02 ± 0.005
Val-Pro	0.02 ± 0.005
Lys-Pro	0.18 ± 0.012
Leu-Pro	0.71 ± 0.027
Arg-Pro	0.00 ± 0.067

[a] Total reaction mixture was 300 μL including 10 μL of L-amino acid oxidase (2 mg/mL), 10 μL of peroxidase (5 mg/mL), 50 μL of *o*-dianisidine (11.5 mM), 1 μl of $ZnSO_4$ (300 mM), and substrate (volume was adjusted to 15 μL). Afterward, the mixture was adjusted to 297 μL with pH 6.0, 20 mM Tris-HCl. To the final cocktail solution, 3 μL of the crude extract (2.65 mg/mL) was added and incubated at 40 °C for 10 min. The reaction mixture was inactivated at 80 °C for 10 min, cooled on ice for 5 min, and absorbance was read at 436 nm.

5.5.1 Effect of temperature on recombinant *Lactococcus lactis* prolidase activity

Thermal dependence of recombinant *Lc. lactis* prolidase activity was examined from 20 to 70 °C with 5 or 10 °C intervals. Temperature dependence profile showed the highest activity at 50 °C (Figure 5.18). The optimum temperature was similar to that of *Lb. delbrueckii* prolidase (Morel *et al*., 1999a,b) and 5 °C lower than *Lb. casei* prolidase (Fernández-Esplá *et al*., 1997). In the temperature ranges, lower than 40 °C and higher than 55 °C, activities dropped drastically to a negligible level. Temperatures between 40 and 55 °C showed more than half of the activity at 50 °C. This range of the working

temperature was wider than the counterpart of *Lb. delbrueckii* prolidase (40-50 °C, Morel *et al*., 1999a,b). In the following characterization, this optimum temperature (50 °C) was used unless otherwise stated.

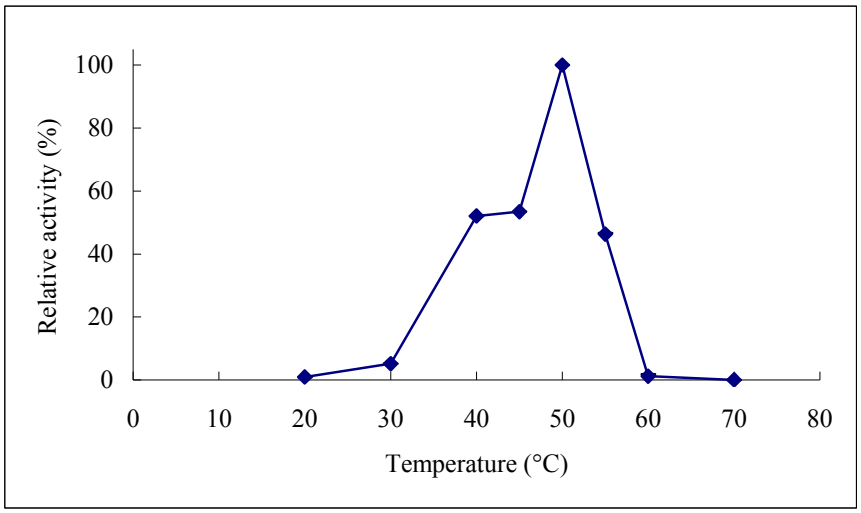


Figure 5.18 Effect of temperature on recombinant *Lactococcus lactis* prolidase activity. Relative activities were determined by specific activities on 1.67 mM Leu-Pro for 15 min after pre-incubation in pH 6.0, 20 mM citrate buffer at the designated temperature for 5 min. Every 5 min an aliquot of the reaction was heated at 90 °C, cooled, and mixed with the reaction enzyme cocktail. Further reaction by the enzyme cocktail was processed at 37 °C for 20 min and measurements were read at Abs 436 nm. The results were expressed as relative percent to the maximum activity at 50 °C. Standard errors for each temperature were less than 1%, thus the errors were not shown in the plot.

5.5.2 Effect of temperature on recombinant *Lactococcus lactis* prolidase stability

The temperature stability of recombinant *Lc. lactis* prolidase was measured between 20 to 100 °C. The highest stability was shown at 30 °C and the activity decreased below and above 30 °C (Figure 5.19). At temperatures higher than 60 °C, residual activity dropped sharply and was negligible above 70 °C. The highest temperature that recombinant prolidase withstood was approximately 10 °C lower than

Lb. casei prolidase (Fernández-Esplá *et al*., 1997), which showed a sharp drop of activity above 70 °C and demonstrated a decrease of activity at temperature lower than 20 °C as similarly shown in this experiment (below 30 °C).

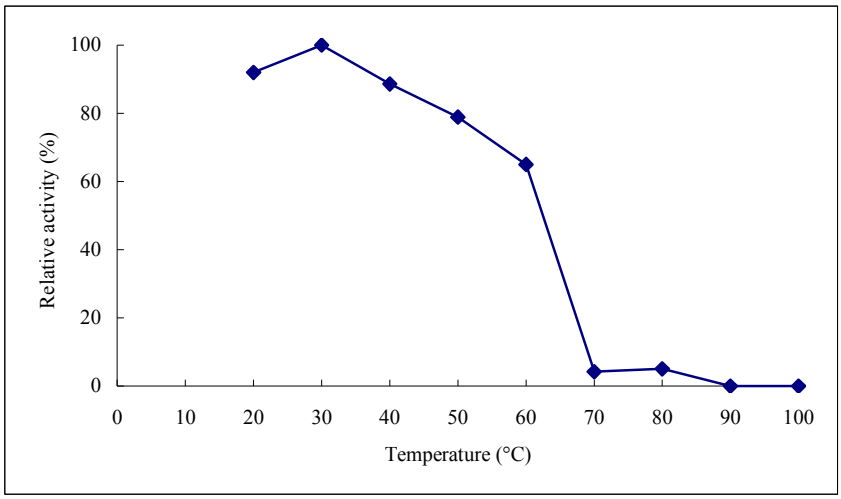


Figure 5.19 Effect of temperature on recombinant *Lactococcus lactis* prolidase stability. Twenty minute pre-heated recombinant prolidase at designated temperatures were preheated (50 °C) for 5 min and mixed with the 5 min pre-heated (50 °C) Leu-Pro (1.67 mM) containing enzyme cocktail. Measurements were performed at 50 °C continuously for 5 min. The calculated specific activities were converted into relative percent activities to the highest activity. Standard errors for each temperature were less than 1%, thus the errors were not shown in the plot.

In these thermal characteristic analyses, the author found that the stability differed by the presence of the substrates. In the regular activity analyses, prolidase was pre-incubated at 50 °C for 5 min without any substrate. In the thermal stability analyses, prolidase was pre-incubated for 20 min in the absence of the substrates, and then 5 min pre-incubated to determine activity at 50 °C. The observed activity of the latter sample (incubation for 20 min) was lower by almost 50%. After the substrate was added, the 5 min incubated sample showed a linear increase of liberated amino acids for 5 min at

50 °C indicating that the activity did not decrease during incubation with the substrate (data not shown). Considering these observations, the author believes that the recombinant *Lc. lactis* prolidase was stabilized in the presence of the substrates.

5.5.3 Effect of pH on recombinant *Lactococcus lactis* prolidase

The pH range between 4 and 9 was employed for the pH dependence examination (Figure 5.20). The optimum pH was 6.5 with activity decreasing below pH 6.0 and above pH 6.5. The results indicated that recombinant *Lc. lactis* prolidase was a weak acidic peptidase. The optimum pH of the recombinant prolidase is slightly different from *Lb. delbrueckii* prolidase (pH 6.0, Morel *et al*., 1999a,b) and the *Lb. casei* prolidase (pH 7.0, Fernández-Esplá *et al*., 1997).

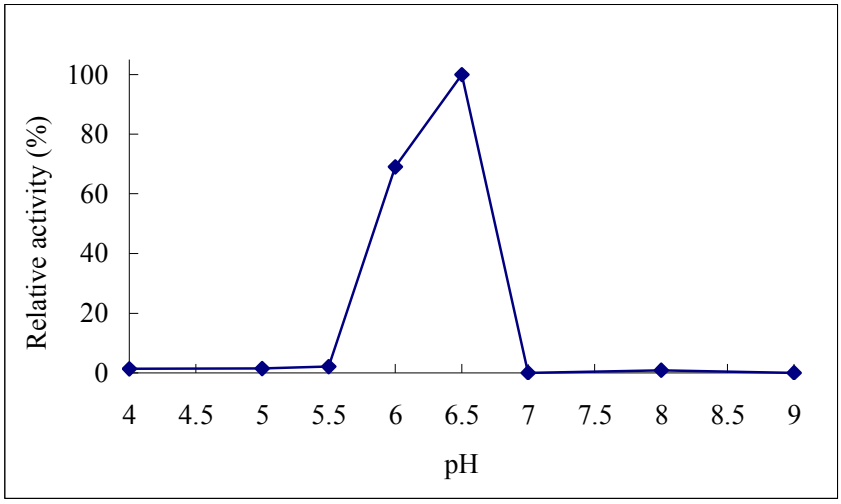


Figure 5.20 Effect of pH on recombinant *Lactococcus lactis* prolidase activity. Different pH buffers (20 mM) were utilized as follows: sodium citrate buffer (pH 4-6.5), MES buffer (pH 7), Tris-HCl buffer (pH 7.5, 8.0), and glycine-NaOH buffer (pH 9.0). Leu-Pro (1.67 mM) was hydrolyzed at 50 °C for 5 min and measurements were read at Abs 436 nm. Calculated specific activities were expressed as percent activities relative to the activity at pH 6.5. Standard errors for each pH were less than 1%, thus the errors were not shown in the plot.

5.5.4 Effect of divalent cations on recombinant *Lactococcus lactis* prolidase activity

Since prolidase is a metallopeptidase, the effects of metallic ions were examined with divalent cations (i.e. Ca^{++}, Co^{++}, Zn^{++}, Mn^{++}, and Mg^{++}). Prolidases have demonstrated different preferences on metals; for instance, the highest preferences of *P. furiosus* are for Co^{++} or Mn^{++} (Ghosh *et al*., 1998), *H. sapiens* for Mn^{++} (Myara *et al*., 1984), *Lb. delbrueckii* for Zn^{++} (Morel *et al*., 1999a,b), and *Lb. casei* for Mn^{++} (Fernández-Esplá *et al*., 1997).

Among examined metallic ions, Mn^{++} exhibited the best catalytic activities of recombinant *Lc. lactis* prolidase. Zn^{++} recorded 93% with respect to relative activity to Mn^{++}. Co^{++} and Ca^{++} were also considerable (i.e. 86% and 61%, respectively) (Table 5.4). Recombinant *Lc. lactis* prolidase demonstrated a wide range of metallic ion compatibility compared to other known prolidases. For instance, prolidase from *Lb. casei* (Fernández-Esplá *et al*, 1997) demonstrated 49% activity with Co^{++} and 24% with Mg^{++} to full catalysis with Mn^{++}.

Table 5.4 Effect of divalent cations on recombinant *Lactococcus lactis* enzyme activity.[a]

Metals	Relative Activity (%)
Ca^{++}	61 ± 0.001
Co^{++}	86 ± 0.001
Mg^{++}	45 ± 0.001
Mn^{++}	100 ± 0.001
Zn^{++}	93 ± 0.001

[a]: One millimole of metallic ions were provided as salt forms ($CaCl_2$, $Co(NO_3)_2$, $MgCl_2$, $MnCl_2$, and $ZnSO_4$) and measured activities were converted into relative activities.

According to the protein sequence alignment among several prolidases (detailed data are provided in section 5.5.10), potential chelating residues (D221, D232, H296, E325, and E339 in *Lc. lactis* NRRL B1821 prolidase) were well conserved. However, preferences for metallic ions and consequent catalytic efficiency showed differences among prolidases. It is not clear what determined the metal dependence. However, some interpretations are possible: All preferred metallic ions in prolidases were transition metals (Mn, Co, and Zn) rather than alkali earth metals (Mg and Ca). The ionic radii of Mn^{++}, Co^{++}, Zn^{++}, Mg^{++}, and Ca^{++} are 0.80, 0.72, 0.71, 0.65, and 0.99 pm, respectively. The electronegativities for each metal are 1.55, 1.88, 1.65, 1.31, and 1.00, respectively. The preferential metals are relatively similar in size and electronegativity (Table 5.5). In addition, the author suggests that preferential selections on metallic ions among compatible metallic ions in prolidases are probably matters related to substrates they utilize and the residues around the metallic ions.

Table 5.5 Summary of densities, atomic radii, and electronegativity of metals used in this experiment.

	Ionic form	Ionic radius (picometre)	Electronegativity (Pauling scale)
Transition metals	Mn^{++}	0.8	1.55
	Zn^{++}	0.71	1.65
	Co^{++}	0.72	1.88
Alkali earth metals	Mg^{++}	0.65	1.31
	Ca^{++}	0.99	1

5.5.5 Substrate specificity of recombinant *Lactococcus lactis* prolidase

Recombinant *Lc. lactis* prolidase demonstrated the highest activity with Leu-Pro, which was almost 6 times higher than the second highest substrates, Arg-Pro and Phe-Pro at the optimal pH 6.5 (Figure 5.21). Phe-Pro does not have a charged side chain while the protonation of Arg (p*K*a 12.5) in Arg-Pro decreases with pH increase, yielding varying different characteristics of Arg-Pro. This decrease of protonation can lead to different behaviour toward pH changes and responses to residues around it. To investigate the possible pH effects on both substrate and enzyme, Phe-Pro and Arg-Pro were examined under diverse pH conditions (Figure 5.21). Interestingly, activity of recombinant prolidase increased for Phe-Pro at pH 7 by 3.5 times and for Arg-Pro at pH 9 by 5.3 times relative to their activities at pH 6.5. At pH 9.0 recombinant prolidase hydrolyzed Arg-Pro at 37% of full activity recorded at optimum pH 6.5 on Leu-Pro. However, no activity was observed on Leu-Pro at pH 9.0 (Figure 5.21 and Table 5.6).

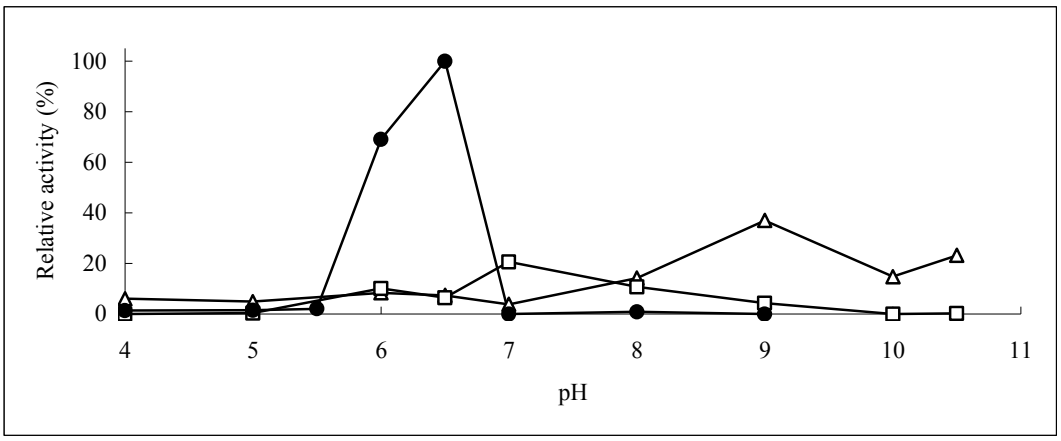


Figure 5.21 Relative activities of recombinant *Lactococcus lactis* prolidase on Leu-Pro, Phe-Pro, and Arg-Pro along with pH changes. The activities are expressed relatively to the highest activity on Leu-Pro at the optimum pH 6.5. Leu-Pro (●), Phe-Pro (□), and Arg-Pro (Δ) are presented. Standard errors for each pH were less than 1%, thus the errors were not shown in the plot.

Table 5.6 Relative activities of recombinant *Lactococcus lactis* prolidase on various substrates [a]

Substrate	Relative activity (%)		
	pH 6.5	pH 7 [b]	pH 9 [c]
Gly-Pro	0		
Val-Pro	0		
Lys-Pro	0		
Arg-Pro	7 ± 0.001	4 ± 0.001	37 ± 0.001
Phe-Pro	6 ± 0.001	21 ± 0.001	4 ± 0.001
Leu-Pro	100 ± 0.001	0	0
Asp-Pro	0		
Glu-Pro	7 ± 0.001		
Leu-Val-Pro	4 ± 0.001		
Leu-Leu-Pro	8 ± 0.001		

[a] Activities were measured with 20 mM citrate buffer (pH 6.5, 7, 9.0). All contained 1 mM of $ZnSO_4$ and incubated at 50 °C for 5 min. The calculated activities were converted relatively to activity on Leu-Pro at pH 6.5.
[b] 20 mM MES buffer
[c] 20 mM Tris-HCl buffer

Even though the measured activity was lower than the maximum activity shown, this holds some meaningful facts (i.e. conformational changes in the active sites and the putative existence of pH sensitive residues).

Lc. lactis prolidase activity on Val-Pro was not observable unlike other LAB prolidases (Christensen *et al*., 1999; Ghosh *et al*., 1998). To date, prolidase has been called a strict dipeptidase. However, considerable activities were shown on tripeptides, Leu-Leu-Pro and Leu-Val-Pro. Even though hydrolytic activity on Val-Pro-Leu was shown from *Lb. delbrueckii* prolidase (Rantanen *et al*., 1997), the position of proline was in the middle and the degree of activity was not reported. An additional important characteristic was the fact that recombinant *Lc. lactis* prolidase also hydrolyzed Glu-Pro

at a noticeable rate (7%). Glutamic acid is categorized as a hydrophilic and negatively charged amino acid and has not been considered as a substrate for prolidase (Table 5.6).

5.5.6 Kinetic analysis of recombinant *Lactococcus lactis* prolidase

The revealed kinetic behaviour of recombinant *Lc. lactis* prolidase indicated allostery with a typical sigmoidal curve in velocity against substrate concentration plot (Figure 5.22).

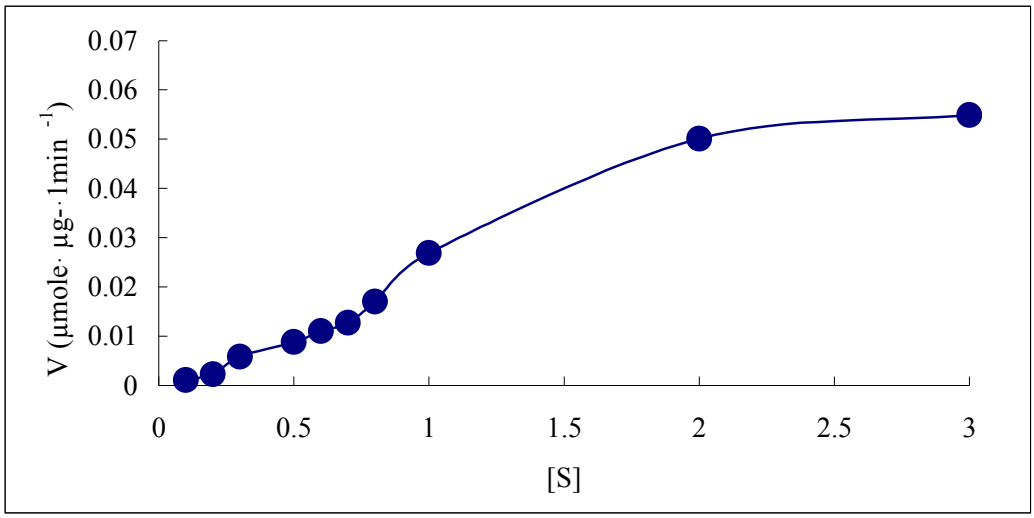


Figure 5.22 Effect of substrate concentration on the velocity of recombinant *Lactococcus lactis* prolidase. Substrate concentration ([S]) and enzyme activity (V) are expressed in millimolar concentration and micromoles of Leu-Pro per microgram of recombinant prolidase per minute, respectively. Standard errors at each [S] were less than 0.0001, thus the errors were not shown in the plot.

Positive cooperativity has not been observed from other prolidases (Morel *et al*., 1999a,b; Ghosh *et al*., 1998; Hu *et al*., 2003), which all follow Michaelis-Menten kinetics. Substrate inhibition, reported in *Lb. casei* (Fernández-Esplá *et al*., 1997) and partially purified *Lc. lactis* prolidase (Booth *et al*., 1990a and b) was not observable. We

hereby report the first positive allosteric prolidase. The Hill coefficient of this novel allosteric enzyme was cooperative at 1.3 (Figure 5.23). The allosteric effectors were presumably substrates since the enzymatic activities were measured in a chemically defined system. The effect of cooperativity degree by each amino acid or substrate and the location of the allosteric site are not yet clear.

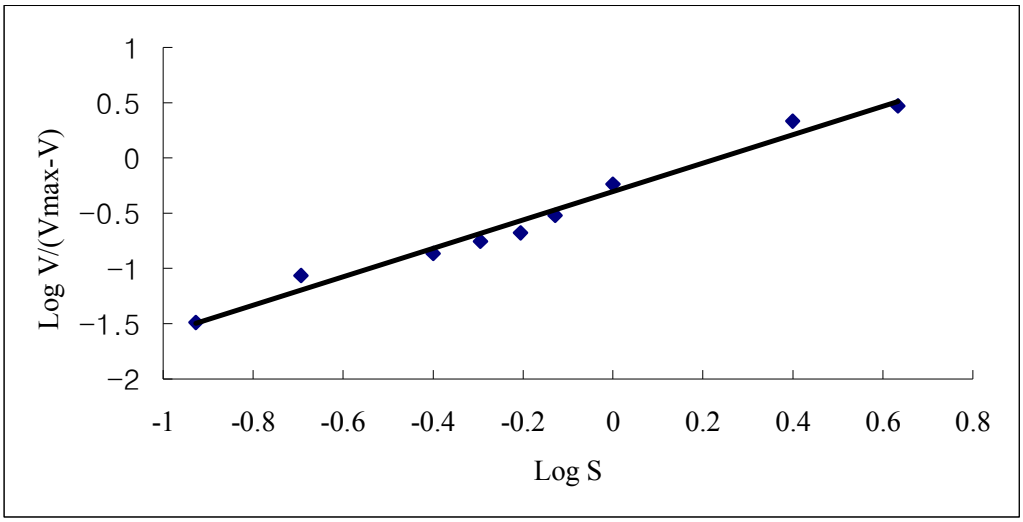


Figure 5.23 Hill plot of recombinant *Lactococcus lactis* prolidase activity on Leu-Pro. The observed velocities were used to calculate logarithmic values. Hill coefficient (*h*) was 1.3. Data were analyzed using Proc Corr command in SAS. The r^2 value was 0.99064 and the p-value was 0.001.

5.5.7 Molecular mass determination of recombinant *Lactococcus lactis* prolidase

Molecular mass of a recombinant *Lc. lactis* prolidase subunit was confirmed by electrospray ionization mass spectrometry. The spectrum showed two deconvoluted major peaks with 130 Da difference. The determined molecular mass was 40,164 and 40,294 Da (appendix C). This difference is considered as a Zn^{++} cluster (2 zinc ions: 130

Da) bound at the active site as similarly shown by ESI-MS (electrospray ionization mass spectrometry) analysis of a bovine zinc-bound protein (Fundel *et al*., 1996).

5.5.8 Secondary structure analysis and denaturation temperature determination of recombinant *Lactococcus lactis* prolidase

The CD spectrum of recombinant *Lc. lactis* prolidase was measured in the Far-UV range, 180 to 250 nm (Figure 5.24). The spectrum showed the ratio among secondary structures was α-helices (16%), β-sheets (32%), β-turns (17%), and random coils (35%). Prolidase from the hyperthermophilic archaeon *P. horikoshii* OT3 (PDB accession code: 1WY2) demonstrated 45% α-helices, 24% of β-sheets, and 31% of β-turns and random coils (calculations were based on the PDB structural data file; Mizutani *et al.*, 2005).

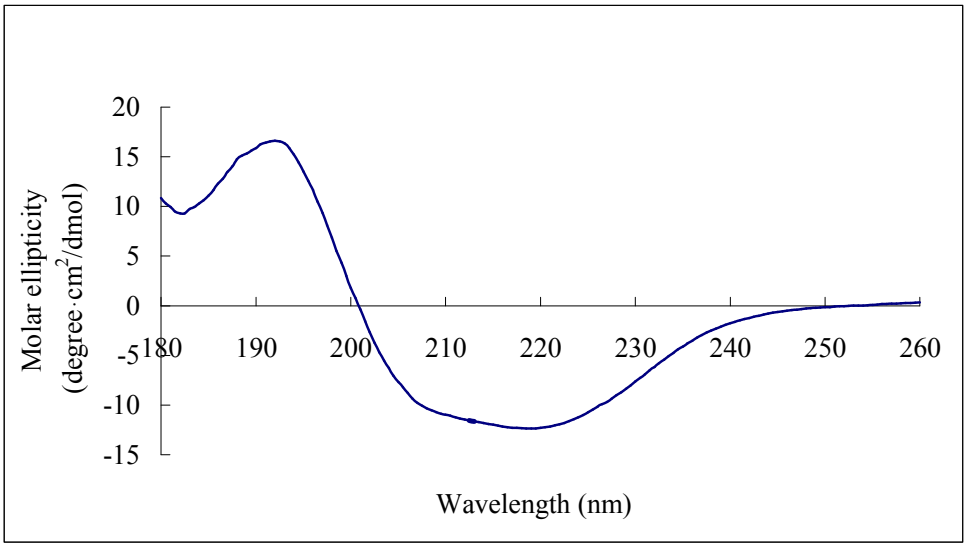


Figure 5.24 Far-UV CD spectrum of recombinant *Lactococcus lactis* prolidase. The experiment was performed at pH 6.0, 20 mM sodium phosphate buffer using Sephadex G-50 gel filtration column (1 × 30 cm). One millilitre of desalted sample was concentrated to 1.12 mg/mL using MicroSep™ (MWCO 10 kDa) and filtered with a 0.2 µm nitrocellulose syringe filter. Five hundred microliters was measured in a 0.1 cm path length cuvette. The sample was scanned 5 times from 180 nm to 260 nm.

Another prolidase from *P. horikoshii* OT3 (Jeyakanthan *et al*., 2005; PDB accession code: 1WN1) also showed almost the same composition, α-helices (42%), β-sheets (23%) and other structures (35%). The shown differences between prolidases from *P. horikoshii* OT3 and *Lc. lactis* were approximately 29% in α-helices and 8% in β-sheets, however, this data is a reference until accurate crystallographic structure of recombinant *Lc. lactis* prolidase can be elucidated.

The denaturation temperature was determined through the CD spectrum change by *in situ* increasing temperature. The calculation of data indicated that the denaturation temperature was 67 °C (Figure.5.25). This result was parallel to the thermal dependence of the activity (Figure 5.18). The recombinant prolidase lost its activity between 60 and 70 °C and its denaturation temperature was 67 °C.

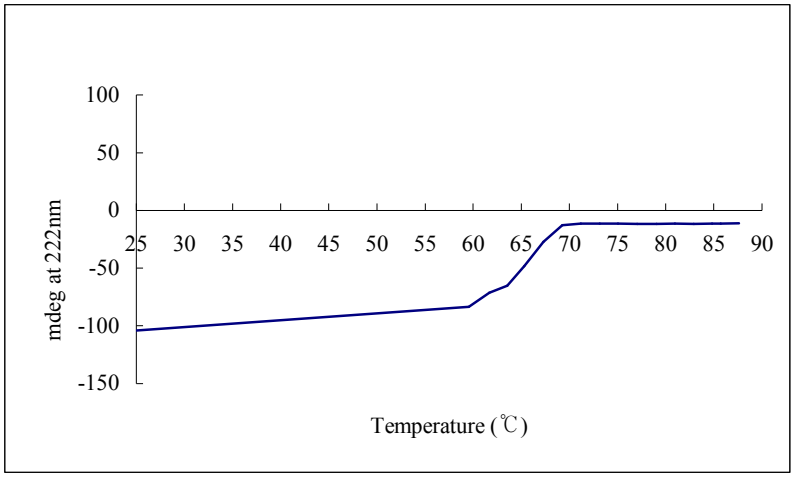


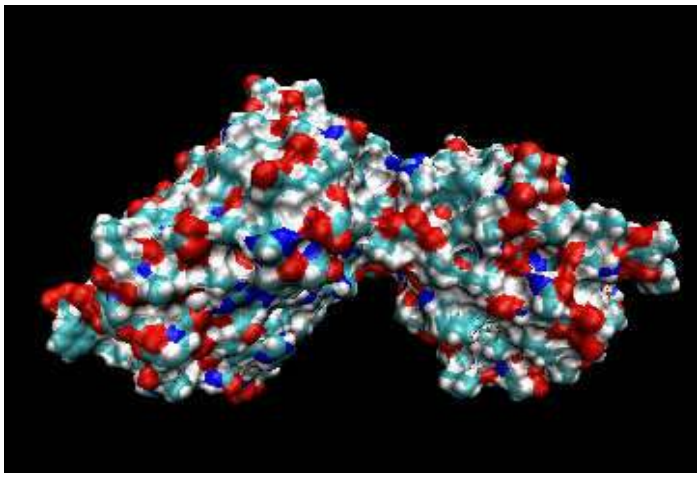
Figure 5.25 Thermal denaturation profile of recombinant *Lactococcus lactis* prolidase. The experiment was carried out at pH 6.0, 20 mM sodium phosphate buffer using a Sephadex G-50 gel filtration column (1 × 30 cm). One millilitre of de-salted sample was concentrated to 1.12 mg/mL using MicroSep^{TM}, MWCO 10 kDa and filtered with a 0.2 µm syringe filter. Seven hundred microliter was measured in a 0.01 cm path length cuvette. Fifty microliter of sample was scanned 3 times between 25 °C to 90 °C. Far-UV CD spectrum of recombinant *Lc. lactis* prolidase was measured at 222 nm.

Therefore, the decrease of activity was the result of the enzyme denaturation. This result also implied a relationship between thermal stabilities and the structural composition (i.e. especially α-helical structure) of enzymes between *Lc. lactis* prolidase (67 °C) and *Pyrococcus* spp (over 100 °C; Ghosh *et al*., 1998).

5.5.9 Structural analysis on the molecular model of recombinant *Lactococcus lactis* prolidase

Energy minimization of the recombinant prolidase was computationally processed to predict its natural topological form. The minimized energy landscape computationally generated an entire molecule in water (Figure 5.26, a). This model demonstrated a similar topological superposition to the *Pyrococcus* spp. prolidase crystallographic structures (Maher *et al*., 2004, Jeyakanthan *et al*., 2005; Mitutani and Kunishima, 2005; Figure 5.26, b). Despite the secondary structural differences in CD analyses, conformational structures of the compared prolidases were almost identical. Even though some monomeric prolidases have been reported (*Lb. casei* prolidase: Fernández-Esplá *et al*., 1997; *Streptococcus cremoris* prolidase: Kaminogawa *et al*., 1984), the structural similarities with known conformations were confirmatively indicative of the homo-dimeric structure of *Lc. lactis* prolidase. These similar prolidases (i.e. *P. furiosus*: Ghosh *et al*., 1998; *P. horikoshii* OT3: Jeyakanthan *et al*., 2005, Mitutani and Kunishima, 2005; *H. sapiens*: Mueller *et al*., 2006) have homo-dimeric structures. Unless otherwise stated, subunit A is one of the identical subunits and the other subunit will be stated as subunit B and noted with B at the end of residue name in the rest of this thesis.

A



B

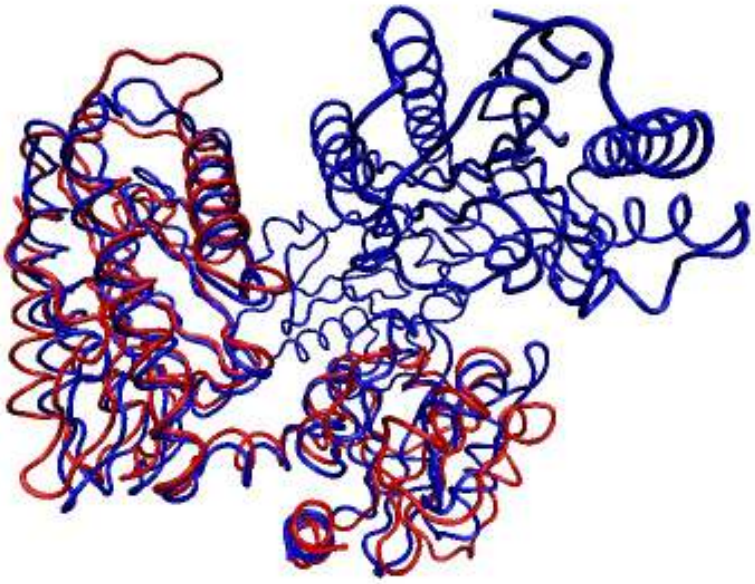


Figure 5.26 Molecular models of recombinant *Lactococcus lactis* prolidase (A) and superposition with *Pyrococcus horikoshii* OT3 prolidase (B). The presented molecular model based on the deduced amino acid sequence of recombinant *Lc. lactis* prolidase was generated by JicSaw server and energy-minimized using the NAMD molecular program. Molecular superposition of recombinant *Lc. lactis* and *P. horikoshii* OT3 prolidases (Jeyakanthan *et al*., 2005) is shown. The blue coloured homo-dimeric ribbon structure represents *P. horikoshii* OT3 prolidase. Monomeric *Lc. lactis* prolidase is shown in red (It is considered dimeric as well).

5.5.10 Conserved metal binding residues of recombinant *Lactococcus lactis* prolidase

X-ray crystallographic work by Maher *et al.* (2004) indicated that *P. furiosus* prolidase possesses five charged residues at the active site (i.e. D209, D220, H284, E313, and E327) as metal ion chelating sites for the catalytic Co^{++} atoms. All five residues were conserved among the examined prolidase protein sequences. In *Lc. lactis* prolidase, the corresponding chelating residues were D221, D232, H296, E325, and E339 and identical to *P. furiosus* prolidase (Table 5.7). This clearly indicates an important architecture for prolidases to maintain functionality regardless of their phylogenetic distances by evolution.

Table 5.7 Five highly-conserved amino acid residues at the active sites of prolidases

Origins of prolidases	Chelating residues					References
Lc. lactis NRRL B1821	D221	D232	H296	E325	E339	this research
Lb. plantarum NRRL B4496	D226	D237	H301	E330	E344	this research
*P. furiosus**	D209	D220	H284	E313	E327	1PV9[b]
P. horikoshii OT 3	D212	D223	H283	E316	E330	1WY2[b]
P. horikoshii OT 3	D215	D226	H286	E319	E333	1WN1[b]
Lb. casei ATCC 334	D223	D334	H298	E327	E342	ABJ69644[a]
Lb. pentosus	D226	D237	H301	E330	E344	AF176799[a]
Lb. helveticus CNRZ32	D223	D234	H298	E327	E341	AF012084[a]
Lb. delbrueckii sub. bulgaricus	D223	D234	H294	E327	E341	CAB07978[a]
S. pyogenes MGAS 10750	D218	D229	H293	E322	E336	ABF37386[a]
S. pneumoniae D 39	D218	D229	H293	E322	E336	ABJ54419[a]
H. sapiens	D277	D288	H372	E413	E453	2IW2[b]

* *P. furiosus* metal binding residue information based on Maher *et al*. (2004).
[a] GenBank accession code
[b] PDB accession code

5.5.11 Substrate binding site of recombinant *Lactococcus lactis* prolidase

All known prolidases, including *Lc. lactis* prolidase, mainly hydrolyze hydrophobic amino acids at the P_1 (N-terminus) and proline at the P_1' (C-terminus) positions (nomenclature of positions is based on Lowth and Mattews, 2002). Maher *et al*. (2004) indicated that *P. furiosus* prolidase possesses 7 substrate binding residues, L37B, F178, I181, I290, H280, Y317, and R325, in the active site. The first 4 residues are responsible for binding P_1 amino acid and the latter 3 residues are for the P_1' amino acid (Table 5.8). Residues binding the P_1' residues of the dipeptides were 100% conserved among all examined prolidases. The residues in the S_1, P_1 amino acid binding site, were mainly hydrophobic amino acids. This hydrophobicity is probably the decisive factor to limit substrate preferences of prolidases to hydrophobic dipeptides.

The superposition of recombinant *Lc. lactis* prolidase models to *P. furiosus* prolidase crystallographic structure (Maher *et al*., 2004) showed high homology of the active sites between two prolidases (Figure 5.27, A). Seven residues in the S_1 and S_1', P_1' amino acid binding residues, sites of *Lc. lactis* prolidase were well superposed with the exception of I41B and L193 (Figure 5.27, 5.28). I41B of *Lc. lactis* prolidase did not show close positioning to L37B of *P. furiosus* prolidase, rather A34B showed closer positioning to L37B (Figure 5.28). The limiting factors for substrate size were defined by the conserved residues 36-39 of *P. furiosus* position (Figure 5.28). In the sequence alignment, the sequence 36-39 of *P. furiosus* demonstrated high homology to *Lc. lactis* residues 37-40. However, the superposition of the models indicated residues 33-36 of *Lc. lactis* were more appropriately positioned to limit the size of substrates. The exact positions of the responsible residues should be further examined in the 33B-40B region. Additional substrate size-limiting residues (P306, S307, and I308) of *Lc. lactis* prolidase

were homologous to the size-limiting factors (P294, R295, and I296) reported in *P. furiosus* prolidase (Maher *et al*., 2004) showing tight positioning toward the active site. The side chain of S307 of *Lc. lactis* prolidase was shorter than R295 of *P. furiosus* as shown in Figure 5.28. The shorter side chain of S307 explains why *Lc. lactis* prolidase hydrolyzes tripeptides. These observations should be investigated through site-directed mutagenesis in order to reveal the functionality of the substrate binding site.

Table 5.8 Conserved residues in prolidase active sites*

Origins of prolidases	S_1 residues				Size limiting residues			S_1' residues			References
Lc. lactis NRRL B1821	$I41^+$	F190	L193	V302	P306	S307	I308	H292	Y329	R337	this research
Lb. plantarum NRRL B4496	$T44^+$	F195	L198	D307	P311	S312	I313	H297	Y334	R342	this research
P. furiosus	$L37^+$	F178	I181	I290	P294	R295	I296	H280	Y317	R325	1PV9[b]
P. horikoshii OT 3	$L41^+$	F184	I187	I296	P297	R298	V299	H286	Y323	R331	1WY2[b]
P. horikoshii OT 3	$L40^+$	F181	I184	V293	P300	Y301	I302	H283	Y320	R328	1WN1[b]
Lb. casei ATCC 334	$I42^+$	F193	I196	T305	P309	S310	I311	H295	Y332	R340	ABJ69644[a]
Lb. pentosus	$T44^+$	F195	L198	D307	P311	S312	I313	H297	Y334	R342	AF176799[a]
Lb. helveticus CNRZ32	$F40^+$	F192	I195	V304	P308	S309	I310	H294	Y331	R339	AF012084[a]
Lb. delbrueckii sub. bulgaricus	$F40^+$	F192	I195	V304	P308	S309	I310	H294	Y331	R339	CAB07978[a]
S. pyogenes MGAS 10750	$Q41^+$	F187	M190	V299	P303	S304	I305	H289	Y326	R334	ABF37386[a]
S. pneumoniae D 39	$Q41^+$	F187	M190	V299	P303	S304	I305	H289	Y326	R334	ABJ54419[a]
H. sapiens	$F71^+$	Y242	I245	V377	G381	G382	Y383	H367	Y417	R451	2IW2[b]

a: Accession code of GenBank
b: Accession code of PDB
*: Sequence alignment is based on *P. furiosus* active site residues (Maher *et al*., 2004).
+: Residues from the other subunit.

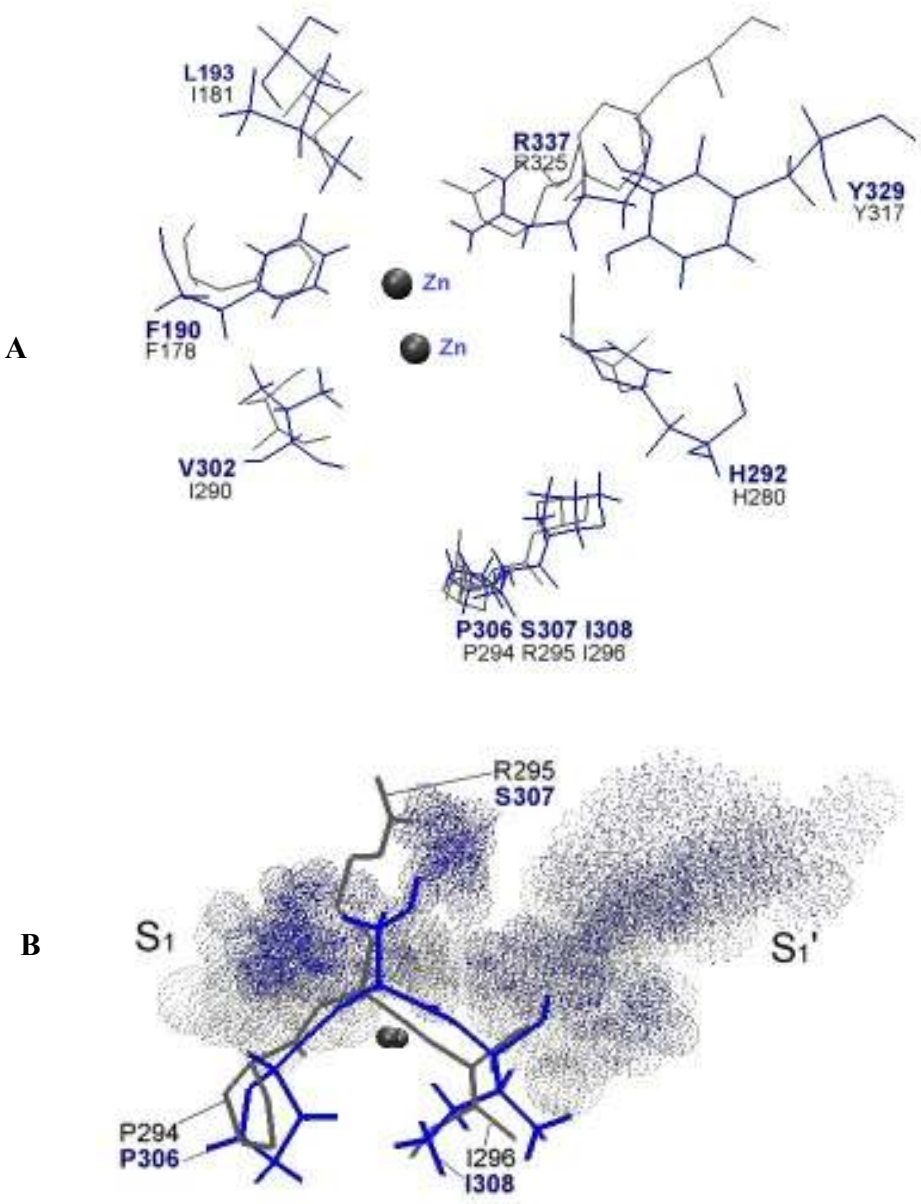


Figure 5.27 Active site superposition of the *Lactococcus lactis* prolidase molecular model and *Pyrococcus furiosus* prolidases. A: The S_1, S_1', and substrate size-limiting residues in the active sites of *P. furiosus* (gray) and *Lc. lactis* (blue) prolidases are presented. *P. furiosus*: S_1 site (F178, I181, and I290; L37B is not shown) S_1' site (H280, Y317, and R325), Zn metal ions (gray balls), and substrate size-limiting residues (P294, I296, and R295) are noted. *Lc. lactis*: S_1 site (F190, L193, and V302; A34B is not shown), S_1' site (H292, Y329, and R337), and substrate size-limiting residues (P306, S307, and I308) are noted. **B**: The superposed substrate size-limiting residues of *P. furiosus* (gray) and *Lc. lactis* (blue) are shown. The corresponding residues are indicated. The illustration was generated with VMD molecular modelling program.

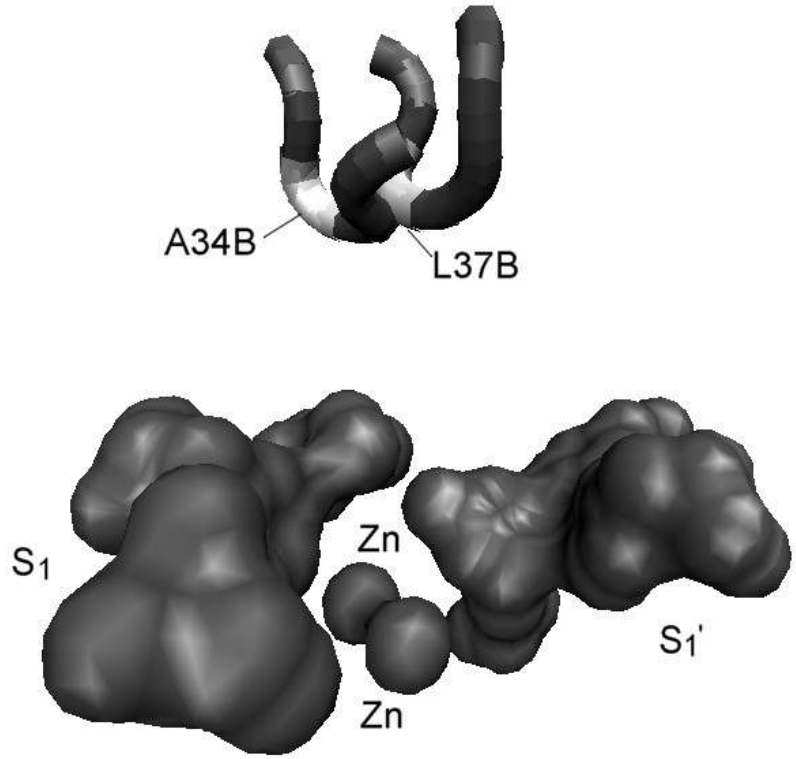


Figure 5.28 Substrate size-limiting residues from subunit B of *Lactococcus lactis* prolidase. The residues (P36B, L37B, G38B, and G39B) of *P. furiosus* prolidase are depicted and L37B is noted in white. The corresponding residues (L33B, A34B, I35B, and D36B) of recombinant *Lc. lactis* prolidase subunit B are illustrated and A34B is noted in white. The area below the loop is an active site of subunit A in the C-terminal domain. Only the *P. furiosus* active site is shown. S_1, S_1' sites and 2 Zn metal ions are illustrated. This figure was generated by the VMD molecular modelling program. The sequence alignment is shown in Figure 5.2.

6. FINAL DISCUSSIONS AND CONCLUSIONS

This research was conducted to provide fundamental information for food applications of prolidases in order to reduce bitterness caused by proline-containing peptides. The research successfully cloned and expressed two prolidase genes from LAB. However, difficulties were found in the solubility of both recombinant prolidases. Under favourable conditions for growth of the hosts, these prolidases formed inclusion bodies that were insoluble and inactive. Unfortunately there were too many problems to purify the prolidases from these inclusion bodies (e.g. uncertainty of refolding). After a long attempt, prolidases were successfully expressed as soluble proteins. The optimized temperature was 16 °C where the recombinant grows very slowly. It is proposed this phenomenon could be explained by either: 1) less stress because of the low expression rate of recombinant prolidase at low temperatures and/or 2) the contribution of cold and heat shock proteins as chaperones (Jiang *et al*., 1997; Neidhardt and; Newkirk *et al*., 1994; VanBogelen, 1987). Further investigation of these two hypotheses was not studied since it was not the focus of the present study. Though the reduced activity at this low temperature would have helped to lower the interference on the host cell activity, we believe temperature was not the sole factor to induce the soluble protein. It is likely that both factors synergistically assisted the soluble expression of *Lc. lactis* prolidase achieved in this research. The author believes the process and the conditions employed in this optimization of soluble expression can be applied for many recombinant enzymes

in cases when similar difficulties are encountered. The success of soluble expression seemed to promise progress in the characterization of prolidases. Nevertheless, the purification met with further difficulties, especially in *Lb. plantarum* prolidase, demonstrating the formation of insoluble clusters. It was considered that this prolidase possessed ill-defined structures and acted as mixed proteins. As a result, recombinant *Lb. plantarum* prolidase could not be purified into a pure state. It is not clear why this prolidase had these ill-defined structures. The reason was probably derived from unfavourable expression conditions, which did not allow for full-folding of this prolidase. In contrast, recombinant *Lc. lactis* prolidase was successfully purified into pure preparation. There was less difficulty in the purification process.

The purified prolidase was further characterized which revealed some interesting results. Metallic preference indicated unique characteristics from other prolidases. Ca^{++}, Co^{++}, Mg^{++}, and Zn^{++} showed at least over 45% activities relatively to Mn^{++} with the recombinant prolidase. This was considered to be a broad range of specificities, compared to other known prolidases, which were compatible with only one or two metallic ions (e.g. *P. furiosus* prolidase on Co^{++} or Zn^{++} (Ghosh *et al*., 1998), *H. sapiens* on Mn^{++} (Myara *et al*., 1984), *Lb. delbrueckii* on Zn^{++} (Morel *et al*., 1999b) and *Lb. casei* on Mn^{++} (Fernández-Esplá *et al*., 1997). The residues responsible for binding metallic ions among prolidases are provided in Table 5.7. Protein sequence alignments among several prolidases showed that potential chelating residues were well conserved among them. It was not clear what determined the metal preference. However, transition metals (Mn^{++}, Co^{++}, and Zn^{++}) showed higher activities than alkali earth metals (Mg^{++} and Ca^{++}) among examined metals. In addition, preferential selections on metallic ions among preferred ions in prolidases are probably related to peptide-substrates and

residues around the metallic ions in the catalytic centre. For the decisive conclusion, the chelating residues must be analyzed as a part of structure-function relationship study of this prolidase.

Another interesting feature was shown in its substrate specificity (Table 5.6). Substrate specificities for prolidases have been limited to mainly their dipeptidase characteristics (Fernández-Esplá *et al*., 1997; Ghosh *et al*., 1998; Morel *et al*., 1999b). However, considerable activities were shown on *Lc. lactis* prolidase for tripeptides, Leu-Leu-Pro and Leu-Val-Pro. The only example, to our knowledge, was *Lb. delbrueckii* prolidase, which reportedly took Val-Pro-Leu as the substrate, though the degree of the activity was not mentioned (Rantanen *et al*., 1997). Another unique characteristic was the fact that recombinant *Lc. lactis* prolidase hydrolyzed Glu-Pro at a noticeable rate (7%). Glutamic acid is categorized as a hydrophilic and negatively charged amino acid and has not been considered as a substrate for prolidase.

All known prolidases, including recombinant *Lc. lactis* prolidase, mainly prefer hydrophobic amino acid in P_1, and proline in P_1' positions. According to Maher *et al*. (2004), *P. furiosus* prolidase possessed ten important substrate binding residues, and four of them were responsible for binding P_1 amino acid, three residues limit the size of substrate and the other three residues were P_1' amino acid (Table 5.8). Three residues in the S_1' site (H292, Y329 and R337 of *Lc lactis* prolidase) were 100% conserved among examined prolidases. They obviously contribute the strict recognition of the P_1' residue, Pro. All four residues in the S_1 site of prolidases conserve their hydrophobicity among prolidases. This hydrophobicity was believed to be the decisive factor to limit substrate preference of prolidases to hydrophobic dipeptides. Considering the absolute conservation in S_1' site, the residues in S_1 site should be responsible for the differences

of substrate specificities among prolidases. These four residues were suggested to contribute to the crucial determination process of their substrates.

The superposition of recombinant prolidase structure to *P. furiosus* prolidase (Maher *et al*., 2004) showed that active site of the recombinant prolidase possessed very well conserved structure (Figure 5.27 and 5.28). The above-mentioned seven residues in the S_1 and S_1' sites were well superposed with the exception of I41B and V302 of *Lc. lactis* prolidase. I41B of *Lc. lactis* prolidase did not show close positioning relative to L37B of *P. furiosus* prolidase, rather D36B was positioned closer to L37B. Maher *et al*. (2004) suggested that a loop structure (36B-39B), which includes L37B, limited the size of substrates of *P. furiosus* prolidase. In the sequence alignment, this region demonstrated homology to 37B-40B of *Lc. lactis* prolidase. However, this region had an insertion in the *Lc. lactis* sequence and was modeled as a large loop. Thus it is expected that the contribution of this *Lc. lactis* loop would be different from the smaller *P. furiosus* 36B-39B loop. Moreover, this region has sequence varieties among LAB prolidases. It suggested that the region would affect the differences of the substrate specificities among LAB prolidases.

Three residues of *Lc. lactis* prolidase, P306, S307, and I308, were indicated as additional substrate size-limiting factors showing a tight positioning toward the active site, similar to the positioning of P294, R295 and I296 found in *P. furiosus* prolidase (Maher *et al*., 2004), as shown in Figure 5.27. The side chain of S307 was shorter than corresponding R295 of *P. furiosus* and it can explain the reason why recombinant *Lc. lactis* prolidase also takes tripeptide recognizably. These observation are based on the theoretical model, thus it must be proved with the site-directed mutagenesis in the future.

pH-dependent substrate specificity was another distinctive characteristic of the recombinant prolidase. The degrees of activities on Phe-Pro and Arg-Pro were considerably changed by pH modifications (Figure 5.21). Their activity shifts were presumably affected by a protonation degree in the charges at the active site and a peptide residue, arginine. The molecular model showed some potential residues that can be protonated/deprotonated by pH changes, e.g., D36, H38, R293, around the active site. It would be possible that these residues change the P_1 residues preference in both hydrophobicity and charge interactions. These shifts will be useful for the selective removal of the bitterness-causing dipeptides in proteinous foods by controlling pH.

One of the most prominent features of this prolidase was an allosteric characteristic. Other known prolidases (Morel *et al*, 1999a,b; Ghosh *et al*., 1998; Hu *et al*., 2003) follow Michaelis-Menten kinetics. However, the unusual allostery of this prolidase can be a hurdle for the reduction of the bitter peptides since the allostery does not allow scavenging the peptides in a low concentration. Substrate inhibition, reported in *Lb. casei* prolidase (Fernández-Esplá *et al*., 1997) and partially purified *Lc. lactis* prolidase (Booth *et al*., 1990a,b), was not observable.

The denatured temperature was determined as 67 °C from circular dichroism observation. This temperature agreed with the inactivation temperature (above 70 °C, Figure 5.25) determined through the heat stability analysis. The loss of activities at a high temperature was, thus, likely caused by the denaturation of the active structure. This denaturation temperature was comparable to other LAB prolidases, but much lower than thermostable *P. furiosus* prolidase which was stable above 100 °C (Ghosh *et al*., 1998). From structural analysis by circular dichroism, recombinant *Lc. lactis* prolidase demonstrated approximately 30% lower α-helical structure and much higher level of

random structures than *Pyrococcus* spp. prolidases (PDB accession code: 1WN1, 1WY2). These low α-helical contents could be the reason of the lower stability of *Lc. lactis* prolidase.

This research has shown several interesting characteristics of *Lc. lactis* prolidase for the applications of bitterness removal in foods. For example, targeting of specific peptides is possible by controlling pH. In addition, the allosteric characteristic can be used to limit bitter peptide concentration at a certain level.

7. FUTURE RESEARCH

For further research, site-directed mutations should be considered in the active site in order to examine the conclusions indicated in this study. It may potentially broaden substrate specificity toward bitterness reduction in proteinous foods, such as cheese, protein dietary supplements, fish sauce, and soybean products. In addition, the allostery can be a difficulty to overcome to reduce the amount of proline-containing peptides, therefore, site-directed mutagenesis based on molecular modelling should be considered on the putative allosteric sites. For broad industrial applications, heat stability is a crucial matter. Residues responsible for structural stability of prolidase should be scrutinized and modified to increase heat stability. Finally, pharmaceutically applicable prolidase should be considered: microencapsulated prolidase for prolidase-deficient patients is possible. Using viral vectors, such as retroviruses or adenoviruses, gene therapy can be also considered (Pfeifer and Verma, 2001). The human prolidase-coding gene is located in chromosome number 19, locus q12-q13.2 (GenBank accession code: NC000019). This non-functional gene of a prolidase-deficient patient can be possibly restored by insertion or replacement of a genetically engineered prolidase gene using viral vectors.

8. BIBLIOGRAPHY

Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image Processing with Image J. Biophotonics International. **11:**36-42.

Agboola, S., S. Chen, and J. Zhao. 2004. Formation of bitter peptides during ripening of ovine milk cheese made with different coagulants. Rait. **84:**567-578.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. A. Seidman, J. A. Smith, and K. Struhl. 1990. Current Protocols in Molecular Biology. Green Publishing Associates and Wiley-Interscience. New York, USA.

Axelsson, L. 1998. Lactic acid bacteria: classification and physiology, p. 1-72. *In* S. Slminen and A. von Wright (ed.), Lactic acid bacteria. Macrcel Dekker, Inc. New York, USA.

Baankreis, R. and F.A. Exterkate. 1991. Characterization of a peptidase from *Lactococcus lactis* ssp. *cremoris* HP that hydrolyses di-and tripeptides containing proline or hydrophobic residues as the amino-terminal amino acid. Syst. Appl. Microbiol. **14:**317-323.

Bacon, C. L., M. Wilkinson, P. V. Jennings, I. N. Fhaolain, and G. O'Cuinn. 1993. Purification and characterization of an aminotripeptidase from cytoplasm of *Lactococcus lactis* subsp. *cremoris* AM2. Int. Dairy J. **3:**163-177.

Bartels, H. J., M. E. Johnson, and N. F. Olson. 1987. Accelerated ripening of Gouda cheese. Effect of heat-shocked thermophilic *Lactobacillus* and *Streptococcus* on proteolysis and flavour development. Milchwissenschaft. **42:**83-88.

Bates, P. A., L. A. Kelley, R. M. MacCallum, and M. J. E. Sternberg. 2001. Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. Proteins. **5:**39-46.

Bertani, G. 1951. Studies on lysogenesis: The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62:**293-300

Bohm, G., R. Muhr, and R. Jaenicke. 1992. Quantitative analysis of protein far UV circular dichroism spectra by neural networks. Protein Eng. **5:**191-195.

Booth, M., W. J. Donnelly, I. N. Fhaolain, P. V. Jennings, and G. O'Cuinn. 1990a. Proline –specific peptidases of *streptococcus cremoris* AM2. J. Dairy Res. **57:**79-88.

Booth, M., V. Jennings, N. Fhaolain, and G. O'Cluinn. 1990b. Prolidase activity of *Lactococcus lactis* ssp. *cremoris* AM 2: Partial purification and characterization. J. Dairy Res. **57:**245-254.

Bosman, B. W., P. S. T. Tan, and W. N. Konings. 1990. Purification and characterization of a tripeptidase from *Lactococcus lactis* ssp. *cremoris* Wg2. Appl. Environ. Microbiol. **56:**1839-1843.

Chapot-Chartier, M. P., F. Rul, M. Nardi, and J. Gripon. 1994. Gene cloning and characterization of PepC, a cysteine aminopeptidase from *Streptococcus thermophilus*, with sequence similarity to the eukaryotic bleomycin hydrolase. Eur. J. Biochem. **224:**497-506.

Charbonnel, P., M. Lamarque, J. C. Piard, C. Gilbert, V. Juillard, and D. Atlan. 2003. Diversity of oligopeptide transport specificity in *Lactococcus lactis* species: a tool to unravel the role of OppA in uptake specificity. J. Biol. Chem. **278:**14832-14840.

Christensen, J. E., E. G. Dudley, J. A. Pederson, and J. L. Steele. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie van Leeuwenhoek. **76:**217-246.

Christensen, J. E., and J. L. Steele. 2003. Impaired growth rates in milk of *Lactobacillus helveticus* peptidase mutants can be overcome by use of amino acid supplements. J. Bacteriol. **185:**3297-3306.

Cleary, M. A., M. Haeney, J. M. Couriel, and J. H. Walter. 1994. Immune function in prolidase deficiency. J. Inherit. Metab. Dis. **17:**345-348.

Cliffe, A. J., J. D. Marks, and F. Mullholl. 1993. Isolation and characterization of non-volatile flavours from cheese: peptide profile of flavour fractions from Cheddar cheese, determined by reverse-phase high performance liquid chromatography, Int. Dairy J. **3:**379-387.

Creighton, T. E. 1993a. Conformational properties of polypeptide chains. p. 171-198 *In* Proteins: Structures and molecular properties, 2nd ed. W. H. Freeman and company. New York, USA.

Creighton, T. E. 1993b. Chemical properties of polypeptides. p. 1-48 *In* Proteins: Structures and molecular properties, 2nd ed. W. H. Freeman and company. New York, USA.

Daniel, R. M., M. Dines, and H. H. Petach. 1996. The denaturation and degradation of stable enzymes at high temperature. Biochem. J. **317:**1-11.

Datar, R. V, T. Cartwright, and C. G. Rosen. 1993. Process economics of animal cell and bacterial fermentation: a case study analysis of tissue plasminogen activator. Bio/Technology. **11:**349-357.

de Palencia, P. F., F. L. de Felipe, T. Requena, and C. Peláez. 2000. The aminopeptidase C (PepC) from *Lactobacillus helveticus* CNRZ32. A comparative study of PepC from dairy lactic acid bacteria. Eur. Food Res. Technol. **212:**89-94.

Detmers, F. J. M., E. R. S. Kunji, F. C. Lanfermeijer, B. Poolman, and W. N. Konings. 1998. Kinetics and specificity of peptide uptake by the oligopeptide transport system of *Lactococcus lactis*. Biochem. **37:**16671-16679.

De vos, W. M. and R. J. Siezen. 1994. Engineering pivotal proteins for *Lactococcal* proteolysis. p. 56-71. *In* Biochemistry of milk products. Royal Society of Chemistry. Cambridge. Cambridge. England.

Doeven, M. K., J. Kok, and B. Poolman. 2005. Specificity and selectivity determinants of peptide transport in *Lactococcus lactis* and other microorganisms. Mol. Microbiol. **57:**640-649.

Doi, E., D. Shibata and T. Matoba. 1981. Modified colorimetric ninhydrin methods for peptidase assay. Anal. Biochem. **118:**173-184.

European Molecular Biology Lab. http://www.embl-hamburg.de-on Web, Nov, 2006

Fenster, K. M., K. L. Parkin, and J. L. Steele. 1997. Characterization of a thiol-dependent endopeptidase from *Lactobacillus helveticus* CNRZ32. A comparative study of PepC from dairy lactic acid bacteria. J. Bacteriol. **179:**2529–2533.

Fernández-Esplá, M. D., M. C. Martín-Hernández, and P. F. Fox. 1997. Purification and characterization of a prolidase from *Lactobacillus casei* subsp. *casei* IFPL 731. Appl. Environ. Microbiol. **63:**314-316.

Fernandez-García, E., M. Ramos, C. Polo, M. Juarez, and A. Olano. 1998. Enzyme accelerated ripening of Spanish hard cheese. J. Food Chem. **28:**63-80.

Foucaud, C., E. R. Kunji, A. Hagting, J. Richard, W. N. Konings, M. Desmazeaud, and B. Poolman. 1995. Specificity of peptide transport systems in *Lactococcus lactis*: Evidence for a third system which transports hydrophobic di- and tripeptides. J. Bacteriol. **177:**4652-4657.

Fundel, S. M., D. L. Pountney, R. Bogumil, P. M. Gehrig, D. W. Hasler, P. Faller, and M. Vašák. 1996. Isolation and characterization of a novel monomeric zinc- and heme-containing protein from bovine brain. FEBS Lett. **395:**33-38.

Gerhart, J. C. and A. B. Pardee. 1962. The Enzymology of control by feedback inhibition. J. Biol. Chem. **237:**891-896.

Ghosh, M., A. M. Grunden, D. M. Dunn, R. Weiss, and M. W. W. Adams. 1998. Characterization of native and recombinant forms of an unusual cobalt-dependent proline dipeptidase (prolidase) from the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **180:**4781-4789.

Gilbert, C., D. Atlan, B. Blanc, R. Portailer, J. E. Germond, L. Lapierre, and B. Mollet. 1996. A new cell surface proteinase: sequencing and analysis of the *prt*B gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*. J. Bacteriol. **178:**3059-3065.

Gottesman, S., S. Wickner, and M. R. Maurizi. 1997. Protein quality control: triage by chaperones and proteases. Genes Dev. **11:**815-823.

Guédon, E., P. Renault, S. D. Ehrlich, and C. Delorme. 2001. Transcriptional pattern of genes coding for the proteolytic system of *Lactococcus lactis* and evidence for coordinated regulation of key enzymes by peptides supply. J. Bacteriol. **183:** 3614-3622.

Hagting, A., E., R. S. Kunji, K. J. Leenhouts, B. Poolman, and W. N. Konings. 1994. The di- and tripeptide transport protein of *Lactococcus lactis*. J. Biol. Chem. **269:**11391-11399.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**577-580.

Hart, H., L. E. Craine, and D. J. Hart. 1998. Amino acids, peptides, and proteins. p. 483-518. *In* Organic chemistry. 11th ed. Houghton Milfflin company. NewYork. USA.

Hockney, R. C. 1994. Recent developments in heterologous protein production in *Escherichia coli*. Trends Biotechnol. **12:**456-463.

Holmgren, A. 1985. Thioredoxin. Ann. Rev. Biochem. **54:**237-271.

Hu, M., Z. Cheng, and L. Zheng. 2003. Functional and molecular characterization of rat intestinal prolidase. Pediatr. Res. **53:**905-914.

Humphrey, W., A. Dalke, and K. Schulten. 1996. VMD - Visual Molecular Dynamics. J. Mol. Graphics. **14:**33-38.

Invitrogen. 2006. http://www.invitrogen.com-on Web, Nov, 2006

Ishibashi, N., T. Kubo, M. Chino, H. Fukui, I. Shinoda, E. Kikuchi, H. Okai, and S. Fukui. 1988. Taste of proline-containing peptides. Agric. Biol. Chem. **52:**95-98.

Jespers, L., E. Sonveaux, J. Fastrez, A. Phanapoulos, and J. Davidson. 1991. Overexpression of the phage lambda lysozyme cloned in *Escherichia coli*: Use of a degenerative mixture of synthetic ribosome binding sites and increase of protein stability *in vivo*. Protein Eng. **4:**485-492.

Jeyakanthan, J., J. Taka, Y. Kitaguchi, Y. Shiro, S. Yokoyama, and K. Yutani. X-ray crystallographic structure of prolidase from *P. furiousus* OT3. PDB accession code (1WN1).

Jiang, W., Y. Hou, and M. Inouye. 1997. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA Chaperone. J. Biol. Chem. **272:**196-202.

Juillard, V., D. Le Bars, E. R. Kunji, W. N. Konings, J. C. Gripon, and J. Richard. 1995. Oligopeptides are the main source of nitrogen for *Lactococcus lactis* during growth in milk. Appl. Environ. Microbiol. **61:**3024-3030.

Junker, J. P., K. Hell, M. Schlierf, W. Neupert, and M. Rief. 2005. Influence of substrate binding on the mechanical stability of mouse dihydrofolate reductase. Biophys. J. **89:**L46-L48.

Kaminogawa, S., N. Azuma, I. K. Hwang, Y. Suzuki, and K. Yamauchi. 1984. Isolation and characterization of a prolidase from *Streptococcus cremoris* H 61. Agric. Biol. Chem. **48:**3035-3040.

Klein, J. R., A. Dick, J. Schick, H. T. Matern, B. Henrich, and R. Plapp. 1995. Molecular cloning and DNA sequence analysis of pepL, a leucyl aminopeptidase gene from *Lactobacillus delbrueckii* subsp. *lactis* DSM7290. Eur. J. Biochem. **228:**570-578.

Klein, J. R., J. Schick, B. Henrich, and R. Plapp. 1997. *Lactobacillus delbrueckii* subsp. *lactis* DSM7290 *pepG* gene encodes a novel cysteine aminopeptidase. Microbiol. **143:**527-537.

Klein, N. and S. Lortal. 1999. Attenuated starters: an efficient means to influence cheese ripening- a review. Int. Dairy J. **9:**751-762.

Kok, J. and W. M. De Vos. 1994. The proteolytic system of lactic acid bacteria. p. 169-210 *In* Genetics and biotechnology of lactic acid bacteria. Blackie and Professional. London. England.

Kokturk, A., T. I. Kaya, G. Ikizoglu, and A. Koca. 2002. Prolidase deficiency. Int. J. Dermato. **41:**46-48.

Konings, W. N., B. Poolman, and A. J. M. Driessen. 1989. Bioenergetics and solute transport in *Lactococci*. CRC Crit. Rev. Microbiol. **16:**419-476.

Kopetzki, E., G. Schumacher, and P. Buckel. 1989. Control of formation of active soluble or inactive insoluble baker's yeast α-glucosidase PI in *Escherichia coli* by induction and growth conditions. Mol. Genet. Genomics. **216:**149-155.

Kunji, E. R., E. J. Smid, R. Plapp, B. Poolman, and W. N. Konings. 1993. Di-tripeptides and oligopeptides are taken up via distinct transport mechanisms in *Lactococcus lactis*. J. Bacteriol. **175:**2052-2059.

Kunji, E. R. S., G. Fang, C. M. Jeronimus-Stratingh, A. P. Bruins, B. Poolman, and W. N. Konings. 1998. Reconstruction of the proteolytic pathway for use of beta-casein by *Lactococcus lactis*. Mol. Microbiol. **27:**1107-1118.

Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings. 1996. The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek. **70:**187-221.

Laan, H. and W. N. Konings. 1989. Mechanism of proteinase release from *Lactococcus lactis* subspecies *cremoris* Wg2. Appl. Environ. Microbiol. **55:**3101-3106.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227:**680-685.

Lamarque, M., P. Charbonnel, D. Aubel, J. Piard, D. Atlan, and V. Juillard. 2004. A multifunction ABC transporter (Opt) contributes to diversity of peptide uptake specificity within the genus *Lactococcus*. J. Bacteriol. **186:**6492-6500.

Lian, W., D. Wu, W. N. Konings, I. Mierau, and L. B. Hersh. 1996. Heterologous expression and characterization of recombinant *Lactococcus lactis* neutral endopeptidase (Neprilysin). Arch. Biochem. Biophys. **333:**121-126.

Lindsay, R. C. 2000. Controlling cheese flavour during accelerated ripening. Dairy Pipeline. **12:**2-5.

Lee, C. A. and J. Beckwith. 1986. Suppression of growth and protein secretion defects in *Escherichia coli sec*A mutants by decreasing protein synthesis. J. Bacteriol. **166:**878-883.

Lehninger, A. L. 1982. Oxidative degradation of amino acids: the urea cycle. p. 531-560. *In* Principles of biochemistry. Worth Publishers, Inc. New York. USA.

Lewis, W. H. P. and H. Harris. 1967. Human red cell peptidases. Nature. **215:**351-355.

Lloyd, R. J. and G. G. Pritchard. 1991. Characterization of X-prolyldipeptidylaminopeptidase from *Lactococcus lactis* ssp. *lactis*. J. Gen. Microbiol. **137:**49-56.

Lowther, W. T. and B. W. Matthews. 2000. Structure and function of the methionine aminopeptidases. Biochem. Biophys. Acta. **1477:**157-167.

Lunn, C. A. and V. P. Pigiet. 1982. Localization of thioredoxin from *E. coli* in an osmatically sensitive compartment. J. Biol. Chem. **257:**11424-11430.

Maher, M. J., M. Ghosh, A. M. Grunden, A. L. Menon, M. W. W. Adams, H. C. Freeman, and J. M. Guss. 2004. Structure of the prolidase from *Pyrococcus furiosus*. Biochemistry. **43:**2771-2783.

McGugan, W. A., D. B. Emmons, and E. Larmond. 1979. Influence of volatile and non-volatile fractions on intensity of Cheddar cheese flavour. J. Dairy Sci. **62:**398-403.

McSweeney, P. L. H., S. Pochet, P. F. Fox, and A. Healy. 1994. Partial identification of peptides from the water-insoluble fraction of Cheddar cheese, J. Dairy Res. **61:**587-590.

Mills, O. E. and T. D. Thomas. 1981. Nitrogen sources for growth of lactic acid *Streptococci* in milk. New Zeal. J. Dairy Sci. **16:**43-55.

Mistou, M. Y., P. Rigolet, M. P. Charpot-Chartier, M. Nardi, J. C. Gripon, and S. Brunie. 1994. Crystallization and preliminary X-ray analysis of PepC, a thiol aminopeptidase from *Lactococcus lactis* homologous to bleomycin hydrolase. J. Mol. Biol. **237:**160-162.

Mistry, V. V. and K. M. Kasperson. 1998. Influence of salt on the quality of reduced fat Cheddar cheese. J. Dairy Sci. **81**:1214-1221.

Mizutani, H and N. Kunishima. 2005. Crystal structure of the prolidase from *Pyrococcus horikoshii* Ot3. PDB accession code: 1WY2.

Monnet, V., M. Nardi, A. Chopin, M. C. Chopin, and J. C. Gripon. 1994. Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. J. Biol. Chem. **269:**32070-32076.

Moore, S. and W. H. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. **211:**907-913.

Morel, F., C. Gilbert, C. Geourjon, J. Frot-Coutaz, R. Portalier, and D. Atlan. 1999a. The prolyl aminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* belongs to the alpha/beta hydrolase fold family. Biochem. Biophys. Acta-Protein Struct. Molec. Enzym. **1429:**501-505.

Morel, F., J. Frot-Coutaz, D. Aubel, R. Portalier, and D. Atlan. 1999b. Characterization of a prolidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with an unusual regulation of biosynthesis. Microbiol. **145:**437-446.

Mueller, U., F. H. Niesen, Y. Roske, F. Goetz, J. Behlke, K. Buessow, and U. Heinemann. 2006. Crystal structure of human prolidase. PDB accession code 2IW2.

Myara, I., A. Myara, M. Mangeot, M. Fabre, C. Charpentier, and A. Lemoonnier. 1984. Plasma prolidase activity: A possible index of collagen metabolism in chronic liver disease. Clin. Chem. **30:**211-215.

Neidhardt, F. C. and R. A. VanBogelen. 1987. *Escherichia coli* and *Salmonella typhimurium*. p. 1334-1335. *In* Cellular and Molecular Biology. American Society for Microbiology Press. Washington, DC. USA.

Neviani, E., C. Y. Boquien, V. Monnet, L. P. Thanh, and J. C. Gripon. 1989. Purification and characterization of an aminopeptidase from *Lactococcus lactis* ssp. *cremoris* AM2. Appl. Envrion. Microbiol. **55:**2308-2314.

Newkirk, K., W. Feng, W. Jiang, R. Tejero, S. D. Emerson, M. Inouye, and G. T. Montelione. 1994. Solution NMR structure of the major cold shock protein (CspA) from *Escherichia coli*: Identification of a binding epitope for DNA. Proc .Nat. Acad. Sci. USA. **91:**5114-5118.

Park, J. N., K. Ishida, T. Watanabe, K. I. Endoh, K. Watanabe, M. Murakami, and H. Abe. 2002. Taste effects of oligopeptides in a Vietnamese fish sauce. Fisheries Sci. **68:**921-928.

Petterson, H. E. and G. Sjostrom. 1975. Accelerated cheese ripening: a method for increasing the number of lactic starter bacteria in cheese without detrimental effect to the cheese-making process and its effect on the cheese ripening. J. Dairy Res. **42:**313-326.

Pfeifer, A. and I. M. Verma. 2001. Gene therapy: promises and problems. Annu. Rev. Genom. Hum. G. **2:**177-211.

Phang, J. M., G. C. Yeh, and C. R. Scriver. 1995. Disorders of proline and hydroxyproline metabolism. p. 1125-1146. *In* The metabolic and molecular basis of inherited disease. 7th ed. McGraw-Hill. New York. USA.

Phillips, J. C., R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L. Kale, and K. Schulten. 2005. Scalable molecular dynamics with NAMD. J. Comput. Chem. **26:**1781-1802.

Pritchard, G. G., A. D. Freebairn, and T. Coolbear. 1994. Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* SK11. Microbiol. **140:**923-930.

Pritchard, G. G. and T. Coolbear. 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. **12:**179-206.

Rantanen T. and A. Palva. 1997. *Lactobacilli* carry cryptic genes encoding peptidase-related proteins: characterization of a prolidase gene (*pepQ*) and a related cryptic gene (*orfZ*) from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Microbiol. **143:**3899-3905.

Roderick, S. L. and B. W. Matthews. 2002. Structure of the cobalt-dependent methionine aminopeptidase from *Escherichia coli*: a new type of proteolytic enzyme. Biochemistry. **32:**3907-3912.

Rul, F., J. C. Gripon, and V. Monnet. 1995. St-PepA, a *Streptococcus thermophilus* aminopeptidase with high specificity for acidic residues. Microbiol. **141:**2281-2287.

Sambrook, J. and D. W. Russell. 2001. Molecular cloning: A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press. New York, U.S.A.

Sanz, Y., F. C. Lanfermeijer, P. Renault, A. Bolotin, W. N. Konings, and B. Poolman. 2001. Genetic and functional characterization of *dpp* genes encoding a dipeptide transport system in *Lactococcus lactis.* Arch. Microbiol. **175:**334-343.

Sanz, Y., F. Mulholland, and F. Toldrá. 1998. Purification and characterization of a tripeptidase from *Lactobacillus sake*. 1998. J. Agric. Food. Chem. **46:**349-353.

Schmidt, D. G. 1982. Association of caseins and casein micelle structure. p. 61-68. *In* Developments in dairy chemistry. Elsevier, London. U.K.

Shao W., G. U. Yuksel, E. G. Dudley, K. L. Parkin, and J. L. Steele. 1997. Biochemical and molecular characterization of PepR, a dipeptidase, from *Lactobacillus helveticus* CNRZ32. Appl. Environ. Microbiol. **63:**3438-3443.

Shrinath, M., J. H. Walter, M. Haeney, J. M. Couriel, M. A. Lewis, and A. L. Herrick. 1997. Prolidase deficiency and systemic lupus erythematosus. Arch. Dis. Child. **76:**441-444.

Simitsopoulou, M., A. Vafopoulou, T. Choli-Papadopoulou, and E. Alichanidis. 1997. Purification and partial characterization of a tripeptidase from *Pediococcus pentosaceus* K9.2. Appl. Environ. Microbiol. **63:**4872-4876.

Singh, S. M. and A. K. Panda, 2005. Solubilization and refolding of bacterial inclusion body proteins. J. Biosci. Bioeng. **99:**303-310.

Singh, T. K., M. A. Drake, and K. R. Cadwallader. 2003. Flavor of Cheddar cheese: a chemical and sensory perspective. Compr. Rev. Food Sci. F. **2(4):**166-189.

Smid, E. J., A. J. Driessen, and W. N. Konings. 1989. Mechanism and energetics of dipeptide transport in membrane vesicles of *Lactococcus lactis.* J. Bacteriol. **171:**292-298.

Smid, E. J., B. Poolman, and W. N. Konings. 1991. Casein utilization by *Lactococci*. Appl. Environ. Microbiol. **57:**2447-2452.

Stucky, K., J. R. Klein, A. Schueller, H. Materm, B. Henrich, and R. Plapp. 1995. Cloning and DNA sequence analysis of *pepQ*, a prolidase gene from *Lactobacillus delbrueckii* subsp. *lactis DSM7290* and partial characterization of its product. Mol. Genet. Genomics. **247:**494-500.

Sullivan, J. J. and G. R. Jago. 1972. The structure of bitter peptides and their formation from caseins. Aust. J. Dairy Tech. **27:**98-104.

Tan, P. S. T., B. Poolman, and W. N. Konings. 1993. Proteolytic enzymes of *Lactococcus lactis.* J. Dairy Res. **60:**269-286.

Tsakalidou, E., R. Anastasiou, K. Papadimitriou, E. Manolopoulou, and G. Kalantzopoulos. 1998. Purification and characterization of an intracellular X-prolyl-dipeptidyl aminopeptidase from *Streptococcus thermophilus* ACA-DC 4. J. Biotech. **59:**203-211.

Tynkkynen, S., G. Buist, E. Kunji, J. Kok, B. Poolman, G. Venema, and A. Haandrikman. 1993. Genetic and biochemical characterization of the oligopeptide transport system of *Lactococcus lactis*. J. Bacteriol. **175:**7523-7532.

University of Guelph. www.foodsci.uoguelph.ca-on Web, Nov, 2006.

Vafopoulou A., E. Alichanidis, and G. Zerfirids. 1989. Accelerated ripening of Feta cheese, with heat-shocked cultures or microbial proteinases. J. Dairy Res. **56:**285-296.

Visser, F. M. W. 1977. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. Neth. Milk Dairy J. **31:**188-239.

Wilce, M. C. J., C. S. Bond, N. E. Dixon, H. C. Freeman, J. M. Guss, P. E. Lilley, and J. A. Wilce. 1998. Structure and mechanism of a proline-specific aminopeptidase from *Escherichia coli*. P. Natl. Acad. Sci. USA. **95:**3472-3477.

Wohlrab, Y and W. Bockelmann. 1992. Purification and characterization of dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. **2:**345-361.

Wohlrab, Y and W. Bockelmann. 1993. Purification and characterization of a second aminopeptidase (PepC-like) from *Lactobacillus delbrueckii* subsp. *bulgaricus* B14. Int. Dairy J. **3:**685-701.

Wolfgang A. 2004. Industrial enzymes. p. 137-141. *In* Enzymes in industry: production and applications. 2nd ed. Wiley-VCH. Weinheim. Germany.

Yaron, A. and D. Mlynar. 1968. Aminopeptidase P. Biochem. Biophys. Res. Commun. 32**:**658-663.

9. APPENDICES

Appendix A: DNA sequencing results

Lc. lactis NRRL B1821 prolidase coding gene sequence from DNA sequencing.

```
   1 ATGAGCAAAA TTGAACGTAT TTCAGCTTTT CTGAATGACA AGGAAGTCGA TATGACTTTC
  61 ATCACTAATC CGACAACTCT AAATTATCTA ACAGGGCTAG CAATTGATCC CCATGAACGA
 121 ATTGCTGGGC TGATGATTTT TAGAGATTCA ACTCCGATGT TATTTACTCC TGCTTTGGAA
 181 GTGGAAAAAG CTAAAGAACA CACAAGTGGT CTGGATATTT TCGGATATGA AGATTCTCAA
 241 AACCCTTGGG AAGTTGTTAA AAATCATGTA AAATCAGATG TTAAATCAAT TGCTGTTGAA
 301 TTTTCAGATA TTCCTTTAGC TAAAACTGAA GGGTTGAAAG CACAATTTGG TGATATTAAT
 361 TTTGTAAATT TGACTCCTTT GATTGAACGG ATGCGTTTGA TTAAGTCAGC AGATGAAATT
 421 GAAAAAATGA AAGTTGCTGG TGATTTTGCT GACAAGTGTT TTGAAATTGG TTTTGCCACT
 481 GCTGCTGAAC GTAATGGTGT GACTGAATCT GATATCGTTG CAAAAATTGA ATACGAAATG
 541 AAACGTATGG GTGTTCCACA GATGTCATTT GACACGCTTG TTTTATCAGG AGCTCGCGCT
 601 GCTAATCCAC ATGGTGCACC TGAAAATGTT GAAATTCAAG AAAATAAACT TTTGCTCTTT
 661 GACTTGGGTG TGATGAGTGG CGGATATGCT TCTGATGCGA CACGTACAAT CGCTATTGGT
 721 CAACCTAATG ACTTTGATGC TGAGATCCAT AAAATCGTTA AGGAAGCTCA ACAAGCAGCC
 781 ATGGACTTCA TTAAACCTGG TGTAACTGCA CATGAAGTTG ATGCTGTTGC TCGTGATTTA
 841 ATTACTAAAG CAGGATATGG TGAATATTTC AATCACCGCC TTGGACATGG TATCGGAATG
 901 GACGTTCACG AATATCCATC TATTGTTGCC GGAAATGACC TCGTAATTCA AGAAGGAATG
 961 TGCTTCTCTA ACGAACCTGG AATTTATATT CCTGGTAAAG TAGGTGTGCG TATTGAAGAC
1021 TGTCTTTACG TAACAGAAAA TGGTTGTGAA AGCTTCACTC ATACTGACCA TGACTTATTA
1081 ATTTTCTAA
```

Lb. plantarum NRRL B4496 prolidase coding gene sequence from DNA sequencing.

```
   1 ATGACAGATT ATACGAAACT GCAACAAGTC CGTCAATGGA CCCAAGACAA TCACGTTGAC
  61 GTGACCTACA TCAGTAACTT TCACACAATT TCATATTTGA CTGGTTTTGA GAGCAACCCC
 121 TACGAACGGA CGCTAGCACT GTTTGTTTTC GCGGATGCGG AACCATTTCT CTTTGCACCC
 181 GCTCTCGAAG TTGAAGCAAT CAAGGAGATG GGCTGGCCTT ACAAAGTTTT CGGCTACCTC
 241 GACCATGAAG ATCCATACGC GCTAATTGCT GATCACATTC ACGCACAATT GACTGATCCT
 301 AAAGTTTGGG CCCTTGAAAC TGGGAACTTG ACGTTAGACC GCTTTACCGC GCTCAAACAG
 361 CAATTTCCAG CTGCCCACTT CGATAAGGAT CTATCACCTT ATATTCAGCA ATTACGGCTG
 421 GTCAAAACAG CTGATGAACT TGAAAAACTA AACATTGCCG GTAAATGGGC CGACTTTGCT
 481 TTTGAACAAG GATTTGCTGC TGTCAAGGCG GGTCGCACCG AACAACAGAT TGCTGCCGAA
 541 TTACAGTATG CCTTAATGAA AAAGGGAATT ATGGAAATGA GTTTCGACAC TTTAGTCCAA
 601 GCTGGTGAAC ATGCCGCCAA CCCCCATGGT GCAACTAACG AAACCCAAGT CAAGCCAAAT
 661 GAATTAGTGC TATTCGACCT CGGCGTCATG TATGAAGGCT ATGCTTCTGA TGCTTCCCGG
 721 ACAATTGCTT ATGGCCAACC GAGTGCCAAG CAAAAGGAAA TCTTTGACGT CTGTCTGGAA
 781 GCCAACTTGA CTGCTCAGGC AGCCATTAAA CCAGGCATGG CAGCCGAAGA TGTCGATAAA
 841 ATCGCACGCG ATATCATCAC CAAAGCGGGC TATGGCGAAT ACTTCATTCA CCGGTTAGGT
 901 CACGGAATCG GCCAAACTGA CCACGAATTT CCTTCGATTA TGGCTGGTAA CCACATGCCA
 961 CTCGTTGAAG GCATGTGCTT CTCAGTAGAA CCTGGGATCT ACATTCCCGG CGTAGCTGGT
1021 GTTCGAATCG AAGACTGTGG GGTCGTTACT AAGGAAGGCT TTAAGCCATT TACCCATACG
1081 CCAAAAGAAT TAAAAGTATT AGATCTTTAA
```

Appendix B: Deduced amino acid sequences

Deduced amino acid sequence of *Lc. lactis* NRRL B1821 prolidase coding gene.

```
  1 MSKIERISAF LNDKEVDMTF ITNPTTLNYL TGLAIDPHER IAGLMIFRDS TPMLFTPALE
 61 VEKAKEHTSG LDIFGYEDSQ NPWEVVKNHV KSDVKSIAVE FSDIPLAKTE GLKAQFGDIN
121 FVNLTPLIER MRLIKSADEI EKMKVAGDFA DKCFEIGFAT AAERNGVTES DIVAKIEYEM
181 KRMGVPQMSF DTLVLSGARA ANPHGAPENV EIQENKLLLF DLGVMSGGYA SDATRTIAIG
241 QPNDFDAEIH KIVKEAQQAA MDFIKPGVTA HEVDAVARDL ITKAGYGEYF NHRLGHGIGM
301 DVHEYPSIVA GNDLVIQEGM CFSNEPGIYI PGKVGVRIED CLYVTENGCE SFTHTDHDLL
361 IF
```

Deduced amino acid sequence of *Lb. plantarum* NRRL B4496 prolidase coding gene.

```
  1 MTDYTKLQQV RQWTQDNHVD VTYISNFHTI SYLTGFESNP YERTLALFVF ADAEPFLFAP
 61 ALEVEAIKEM GWPYKVFGYL DHEDPYALIA DHIHAQLTDP KVWALETGNL TLDRFTALKQ
121 QFPAAHFDKD LSPYIQQLRL VKTADELEKL NIAGKWADFA FEQGFAAVKA GRTEQQIAAE
181 LQYALMKKGI MEMSFDTLVQ AGEHAANPHG ATNETQVKPN ELVLFDLGVM YEGYASDASR
241 TIAYGQPSAK QKEIFDVCLE ANLTAQAAIK PGMAAEDVDK IARDIITKAG YGEYFIHRLG
301 HGIGQTDHEF PSIMAGNHMP LVEGMCFSVE PGIYIPGVAG VRIEDCGVVT KEGFKPFTHT
361 PKELKVLDL
```

Appendix C. Mass spectrometry result of recombinant *Lc. lactis* prolidase.

The electron spray ionization method was used for molecular mass measurement using API Q-star XL Hybrid MS System and mass–to-charge ratios were deconvoluted into relative molecular weights.