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Ph.D. Dissertation

Anti-microbial and anti-oxidant mechanism study of a novel peptide YD1 from *Bacillus amyloliquefaciens*

Graduate School of Chosun University

College of Pharmacy

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Bacillus amyloliquefaciens 에서 생산된 펩타이드 YD1 의 항균 및 항산화 작용기작연구

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Advisor: Prof. Jin Cheol Yoo

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Table of Contents

List of Tables

List of Figures

ABSTRACT

Anti-microbial and anti-oxidant mechanism study of a novel peptide YD1 from *Bacillus amyloliquefaciens*

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The current study reports that many fermented foods possess both nutritive and non-nutritive components, which have the modulating potential to particular molecular function relevant to well-being and health of the consumer. However, around 90% of naturally fermented foods and alcoholic beverage in different regions of the world are still relied on traditionally home made production. Foods and beverages are fermented naturally contain both functional and non-functional microorganism. During the fermentation functional microorganisms convert the chemical constitutions of materials of animal/plant sources; thereby enhancing the bio-availability of nutrients, sensory quality of the food, imparting bio-preservative effects and betterment of food safety, toxic components degradation, and anti-nutritive factors, producing antimicrobial and antioxidant compounds, stimulation the probiotic functions, and to increase the defenses of, with some bioactive health promoting compounds.

In this dissertation, we report the isolation, purification, and complete biochemical characterization of a novel peptide YD1 from the strain *Bacillus*

amyloliquefaciens CBSYD1 isolated from Korean traditional fermented food kimchi. In addition, we report that the probiotic characteristics of *Bacillus amyloliquefaciens CBSYD1*. Moreover, upon on confirmation of the novelty of peptide YD1, we studied the antimicrobial and antioxidant mechanism study of YD1.

Newly isolated the strain *Bacillus amyloliquefaciens CBSYD1* displayed high probiotic nature in *in-vitro* studies. The growth of the strain was adapted to the bile-salt condition, and a clear halos-zone was observed in a bile-salt plate assay and viable in different conditions of the digestive track.

Antimicrobial peptides (AMPs), low-molecular-weight peptides with broad-spectrum antimicrobial effects, are the most promising candidates for the novel antimicrobials. An influential cationic glycine-rich AMP YD1 (molecular-weight ~1.0 kDa) was purified from the strain *Bacillus amyloliquefaciens CBSYD1* isolated from conventional Korean fermented food kimchi. The YD1 is a potential candidate for the treatment of multidrug-resistant (MDR) bacteria infection. The 16S rRNA sequence analysis showed that CBSYD1 was identified as 99.79% similar to *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42. The amino acid sequence was determined by Edman degradation method, and residues of YD1 were found to be Ala-Pro-Lys-Gly-Val-Gln-Gly-Pro-Asn-Gly. After experiencing computational and sequence analysis using different biological servers suggested that YD1 possesses entirely novel amino acid sequence. YD1 exhibited antimicrobial effects against both Gram-positive and negative bacteria. The minimal inhibitory concentrations (MIC) of pure YD1 for *methicillin-resistant vancomycin-resistant enterococci (VRE), Staphylococcus aureus* B15 *(MRSA)*, and *Escherichia coli* KCTC1923 *(E. coli)*, concentration ranged from 8 to

64 μg/mL, representing higher potency over commercial reference antibiotics. The antimicrobial mechanism of action of YD1 was measured to involve cell-penetrating translocation inside the bacterial cell as well as interaction with the DNA leading ultimately to bacterial cell death. Analogously, Q-G-P-N-G is the likely predictable cell-penetrating motif for YD1.

The YD1 treatment on RAW 264.7 cells increased the translational and transcriptional activities of NF-E2-related factor-2 (Nrf-2) corresponding to the enhanced levels of heme oxygenase-1 (HO-1). Furthermore, the YD1-treated group showed higher levels of antioxidant enzymes compared to the oxidative stress group. YD1 demonstrated a high antioxidative activity by decreasing reactive oxygen species (ROS) and nitric oxide (NO) generation in RAW 264.7 cells along.

This study suggests that YD1 and the strain could be a natural antioxidant, antimicrobial agent and a probiotic candidate respectively. YD1 could be a promising antimicrobial candidate for the therapeutic application. Also, suggest that peptide YD1 from probiotics like *Bacillus amyloliquefaciens CBSYD1* could be used as natural foods and in preventing the oxidation reaction in food processing.

국문 초록

Bacillus amyloliquefaciens 에서 생산된 펩타이드 YD1 의 항균 및 항산화 작용기작연구

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최근 연구 보고들에 따르면 많은 발효식품들이 영양 성분들과 비영양 성분들을 두루 함유하고 있으며 이 성분들은 소비자의 웰빙과 건강에 관련된 특정 분자들의 기능을 조절할 수 있는 능력이 있다. 한편 세계 각지의 자연 발효식품들 및 술들의 90%는 전통적인 방식으로 가정에서 생산되고 있다. 자연적으로 발효된 식품과 술은 기능성 및 비기능성 미생물들을 함유하고 있다. 발효 과정 중에 미생물들은 동식물에서 유래된 물질들의 화학적 조성을 바꿈으로서 영양분들의 생이용성 및 식품의 관능적 품질 향상, 생물학적 보존 효과 발생, 식품 안전도 향상, 독성분 분해, 항영양요인 제거, 항균 및 항산화 화합물 생성, 프로바이오틱 기능 촉진 및 일부 생활성(bio-active) 건강증진 화합물들의 방어능력 향상에 기여한다.

본 논문은 전통적인 한국발효식품인 김치로부터 분리된 *Bacillus amyloliquefaciens CBSYD1* 균주에서 유래된 새로운 펩타이드 YD1 의 추출, 분리정제 및 전반적인 생화학적 특성들과 함께 *Bacillus amyloliquefaciens CBSYD1* 의 프로바이오틱 특성들을 보고하고 있다. 또한 새로운 펩타이드인 YD1 를 발견하고 YD1 의 항균 및 항산화 기전을 연구하였다.

새로 분리된 *Bacillus amyloliquefaciens CBSYD1* 균주는 *in-vitro* 연구에서 높은 프로바이오틱 특성을 보였다. 이 균주는 여러 담즙염 조건들 하에서 배양되었는데, 담즙염 플레이트 에세이에서 투명환(clear halos-zone)을 보여주었고 소화관의 다양한 조건들 하에서 생존하였다.

항세균 펩타이드는 광범위한 항균활성을 가진 저분자 단백질들로서 가장 유망한 새 항균제 개발 후보들이다. 다내제성 세균들을 처리하기 위해 양이온성이면서도 글라이신이 풍부한 강력한 항균제인 분자량 1kDa 이하의 펩타이드를 전통 한국발효식품인 김치로부터 분리된 *Bacillus amyloliquefaciens CBSYD1* 로부터 정제하였다. 16S rRNA 염기서열 분석 결과 *CBSYD1* 균주는 *Bacillus amyloliquefaciens* subsp. Plantarum FZB42(T)와 99.79%의 유사도를 보였다. 에드만 분해법을 사용하여 분석한 결과 YD1 의 아미노산 서열은 Ala-Pro-Lys-Gly-Val-Gln-Gly-Pro-Asn-Gly 이었다. 몇 개의 생물정보 서버들을 이용하여 검색한 결과 YD1 의 아미노산 서열이 독특하다고 판단되었다. YD1 은 그람 음성 및 그람 양성 세균들에 대하여 항균활성을

보였다. *Escherichia coli KCTC1923 (E. coli), methicillin-resistant Staphylococcus aureus B15 (MRSA)* 및 *vancomycin-resistant enterococci (VRE)* 에 대한 YD1 의 최소억제농도는 8 - 64 μg/mL 의 범위 내에 있었으며, 이는 시판 중인 기준 항생제들보다 더 강력한 항균활성을 갖고 있음을 보여준다. YD1 의 항균 기전은 YD1 이 세균 세포 내로 투과해 들어간 후 DNA 와의 상호작용을 통해 결국 세균 세포를 사멸시키는 것이라고 판단되었다. YD1 의 아미노산 서열 중 세포투과성 모티프로서 가장 가능성이 높은 부분은 Gly-Pro-Asn-Gly 이다.

RAW 264.7 를 YD1 으로 처리한 결과 Nrf-2 의 전사 및 번역 활성이 높아졌으며, 이에 상응하여 heme oxygenase-1 (HO-1)의 수준이 높아졌다. 또한 YD1 처리군은 산화 스트레스군에 비해 항산화 효소들의 수준이 높게 나타났다. YD1 은 RAW 264.7 세포 내에서 일산화탄소(NO) 와 활성산소종(ROS)의 생성을 감소시킴으로써 높은 항산화 활성을 보여주었다.

본 연구는 YD1 은 천연 항균/항산화제가, 해당 균주는 프로바이오틱 후보가 될 수 있음을 시사한다. YD1 은 임상 적용이 가능한 유망한 항균제가 될 수 있을 것이다. 또한 본 연구는 *Bacillus amyloliquefaciens CBSYD1* 와 같은 프로바이오틱(유익균)으로부터 유래된 펩타이드 YD1 이 천연 식품 제조 공정에서 산화반응을 억제하는 용도로 사용될 수 있음도 시사한다.

Abbreviations

ABTS : 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic

ACE : Activity of The Angiotensin-Converting Enzyme

AMP : Antimicrobial peptide

APD : Antimicrobial Peptide Database

APP : Avian Pancreatic Polypeptide

AU : Arbitrary Unit

AUC : Area Under the Curve

BLAST : Basic Local Alignment Search Tool

BSH : Bile Salt Hydrolase

CaM : Calmodulin

CAT : Catalase

CFU : Colony Forming Unit

CPP : Calcium Binding Phosphopeptides

CUPRAC : Cupric Reducing Antioxidant Capacity

DC : Dendritic Cells

DCFDA : Cellular Reactive Oxygen Species Detection Assay

DPPH : 2,2-Diphenyl-1-picrylhydrazyl

EDTA : Ethylenediaminetetraacetic acid

FICI : Fractional Inhibitory Concentration Index

FRAP : Ferric reducing antioxidant power

GPx : Glutathione Peroxidase

HDP : Host Defense Peptide

IEC : Intestinal Epithelial Cells

LAB : Lactic Acid Bacteria

MDR : Multi Drug Resistance

MH : Mueller-Hinton

MHA : Mueller Hinton Agar

MHB : Mueller Hinton Broth

MIC : Minimum Inhibitory Concentration

MPC : 2% Maltose, 1% peptone, and 0.01% $CaCl_2$

MRS : Media-De Man-Rogosa-Sharpe

MRSA : *Methicillin-resistant Staphylococcus aureus*

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMDAR : N-Methyl-d Aspartate Receptors

NPY : NeuroPeptide Y

NQO1 : NAD(P)H-Quinone Dehydrogenase 1

Nrf-2 : NF-E2-related factor-2

NRP : Nonribosomal Peptide

NUC : Nucleic Acid

OM : Outer Membrane

ORAC : Oxygen radical absorbance capacity

PDB : Protein Data Bank

POMC : Proopiomelanocortin

PPY : Pancreatic PolYpeptide

PYY : Peptide YY

RNS : Reactive Nitrogen Species

RONS : Reactive Oxygen and Nitrogen Species

ROS : Reactive Oxygen Species

SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SOD : Superoxide Dismutase

TDCA : Tauroglycocholic Acid

TEM : Transmission Electron Microscopy

VRE : *Vancomycin-Resistant Enterococci*

CHAPTER ONE

INTRODUCTION

1.1. Bioactive peptide

Peptidomics is the comprehensive qualitative and quantitative study of all peptides in a biological sample (Schulte *et al.*, 2005), is an emerging arena derived from proteomics and enabled by modern separation, analytical and computational technologies. Bioactive peptides are proteins synthesized in the form of prepropeptides in the cell; these prepropeptides are then cleaved and turned to give effective products. The bioactive peptides, as signaling molecules, play vital roles in pathogenesis and physiological function. The rapid development of bioinformatics, especially the project of human genome project in the 1980s and 1990s, have inspired to rapid accumulation of an enormous amount of experimental biological data well organized in many biological databases. In addition, large protein and gene sequence repositories such as GeneBank (Benson *et al.*, 2013), PIR (George *et al.*, 1997), Swiss-Port (Bairoch and Apweiler, 2000). Some other databases are also there with their specific focuses on protein or gene sequences such as nuclear protein databases (Dellaire *et al.*, 2003) and peptide database of immunology (Blythe *et al.*, 2002).

Bioactive peptides differ widely in their chemical structure, amino acid composition, and, therefore, in their biological function. These compounds commonly have cholesterol-lowering effects, in addition antiviral, antiprotozoal, antiinflammatory, antithrombotic, antioxidant, antihypertensive and antimicrobial activities (Bairoch and Apweiler, 2000, Benson *et al.*, 2013, Blythe *et al.*, 2002, Cé *et al.*, 2012), which make them striking for application to pharmaceuticals and foods.

Bioactive peptides may contain from 2 to 20 amino acid residues per molecule and may be encrypted within the sequence of protein. Though few have been reported to be 20 amino acid more residues. For example, Lunasin, a food derived peptide with anticancer activity, is composed 43 AA. When encrypted peptides released from the protein sequence become active (Möller *et al.*, 2008, Erdmann *et al.*, 2008) mostly by alkaline and acid chemical hydrolysis (Harnedy and FitzGerald, 2012), the proteolytic action of microorganisms or enzymatic hydrolysis of proteins.

Bioactive peptides act as metabolism modulators and regulatory compounds, hormone-like activities, once liberated as independent entities (Korhonen and Pihlanto, 2003b). Orally administrated bioactive peptides may affect the major body systems and a positive impact on body conditions or functions have been noticed and ultimately influence health (Kitts and Weiler, 2003). Due to distinct potential dietary peptide sequences to promote human health by minimizing the risk of chronic diseases or developing natural immune protection has stimulated a lot of scientific interest over the past few years. The therapeutic application of peptides especially in the field of the treatment of infections, cancer, immunological system disorders and cardiovascular disorders is the primary focus of many scientists although, apart from therapeutic applications these are also endorsed as functional components, i.e., food with designed properties. As a result of their physicochemical and physiological versatility, meat, milk, and egg-borne bioactive peptides are observed as highly prominent ingredients for health promoting pharmaceutical preparations or functional foods.The use of technologies for industrial scale production of such peptides have recently been

developed and already there are with supplementary peptides with specific bioactivities on international markets (Korhonen, 2009).

1.2. Bioactive peptides as pharmaceutical ingredients

By regulating, directing and/or coordinating Intra- and inter-cellular functions and communications, protein as well as peptides play vital roles in living body systems (Danquah and Agyei, 2012). Low molecular weight peptides are higher bioavailable than proteins or amino acids from nutritional point of view (Park, 2009), and it is also known to be less allergenic than their innate proteins which justify their use widely in the preparation of hypoallergenic infant food formulations (Danquah and Agyei, 2012, Høst and Halken, 2004). Moreover, the toolkit of nature, the diverse physiological roles of peptides make them suitable candidates for therapeutic agent developments (Agyei and Danquah, 2011, Lax, 2010). Consequently, in the light of the sizable side effects of synthetic drugs and with the delicate attention to fresher and 'greener' foods and nutraceuticals possessing heath-preventing or promoting properties, the bioactive peptides seems to be most appropriate candidates in the new era of pharmaceutical products (Danquah and Agyei, 2012).

1.3. Peptide and peptide classes

Peptides and protein are polymers which made up of amino acid units (also called residues) that are linked to each other through the formation of peptide bonds (amide bonds) after the amino group of one residue as well as the carboxylate of a second residue. Peptides are biologically happening short chains of amino acid monomers which linked by peptide (amide) bonds. The dipeptides are the shortest peptides (figure

1.1), containing two amino acids linked by a single peptide bond, then followed by tripeptides, tetrapeptides, etc. The polypeptide is a continuous, long, and unbranched peptide chain. Hereafter, peptides can categorize under the broad chemical classes of biological polymers and oligomers, together with nucleic oligosaccharides, acids, and polysaccharides, etc.

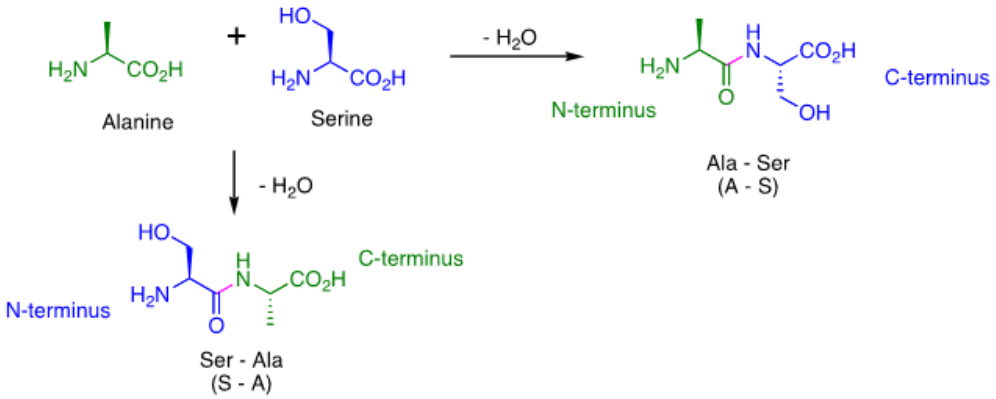

Figure 1.1: A dipeptide (example Ser-Ala or Ala-Ser) with green marked amino (Alanine) and blue marked carboxyl (Serine).

Peptides are notable from proteins on the basis of size, and as an arbitrary benchmark can be understood to contain 50 amino acid or fewer amino acids approximately (Korhonen and Pihlanto, 2006). Proteins are composed of one or more polypeptides arranged in a biologically functional way, often bound to ligands for instance coenzyme and cofactors, or to another protein or other macromolecule (DNA, RNA, etc.), or too complex macromolecular assemblies (Ardejani and Orner, 2013). Peptides are classified into several class, depending on how they are produced -

1.3.1. Milk peptides

Milk peptides, also called Lactotripeptides, are two naturally occurring peptides: Isoleucine-Proline-Proline (IPP) as well as Valine-Proline-Proline (VPP). These lactotripeptides are obtained from casein, a milk protein found in dairy products. Although dairy products mostly contained lactotripeptides, they are remained inactive in the original milk proteins.

Dairy peptides are reported to prevent the activity of the angiotensin-converting enzyme (ACE), belongs to the renin-angiotensin system, a natural mechanism that helps regulate blood pressure within the body. Under some conditions such as stress, an unhealthy diet, and/or unhealthy lifestyle ACE becomes overactive and converts angiotensin-II, cause blood vessels to constrict, and so increases blood pressure, from angiotensin-I (Boelsma and Kloek, 2008).

1.3.2. Ribosomal peptides

Translation of mRNA synthesizes these type peptides. Most commonly approximate 100 amino acids long peptide that is posttranslationally modified by numerous catalytic enzymes that catalyze the formation of a vast number of different chemical motifs.

Ribosomal peptides are often subjected to proteolysis to produce the mature form. These function, classically in a higher organism, as hormones and signaling molecules. Some organism produces peptides as antibiotics such as microcins (Duquesne *et al.*, 2007). As they are translated, the amino acid residues involved are restricted to those exploited by the ribosome.

However, posttranslational modifications of these peptides frequently happened such as hydroxylation, phosphorylation, sulfonation, glycosylation, palmitoylation, and disulfide formation. In overall, they are linear, although lariat structures have been observed (Pons *et al.*, 1991). Additional unusual manipulations do occur, such as racemization of L-amino acids to D-amino acids in platypus venom (Torres *et al.*, 2002).

1.3.3. Nonribosomal peptides

These peptides are a type of secondary peptide metabolites, usually produced by microorganisms like fungi and bacteria. NRPs are also found in higher organisms, such as nudibranchs, but it is thought to be made by bacteria inside these organisms (Li *et al.*, 2012). Glutathione is the most common NRP, which is a component of the antioxidant defenses of most aerobic organisms (Meister and Anderson, 1983). Other NRPs are commonly in unicellular organisms, plants, and fungi and are synthesized by modular enzyme complexes called nonribosomal peptide synthetases (Hahn and Stachelhaus, 2004). NRPs have possessed a diverse family of natural products with an enormous variety of pharmacological properties and biological activities. There are some examples of NRP shown in Table 1.1.

1.3.4. Peptones

Peptones are derived from meat and animal milk digested by proteolysis. In addition to comprising small peptides, the resulting material includes metals, fats, vitamins, salts,

and many other biological compounds. Peptones are used as nutrient media into bacteria and fungi media (Payne, 1976).

1.4. Production of peptides

A several numbers of methods are used to produce biologically active peptide from precursor proteins. The most common ones are –

a. enzymatic hydrolysis with digestive enzymes,
b. by means of the microbial activity of fermented food,
c. by the action of enzymes which derived from proteolytic microbes.

Upon the confirmation of structure of the bioactive peptide, it is possible to synthesize peptides. At present, three approaches are used commonly;

i. chemical synthesis
ii. recombinant DNA technology and
iii. Enzymatic synthesis

(Korhonen and Pihlanto, 2003b)

1.4.1. Enzymatic hydrolysis

This is the most common method to produce bioactive peptide by the enzymatic hydrolysis of whole protein molecules and using digestive enzymes numerous known bioactive peptides have been produced as well as enzyme combinations of different proteinases like pepsin, alcalase, trypsin, pancreatin, chymotrypsin, and thermolysis (figure 1.2).

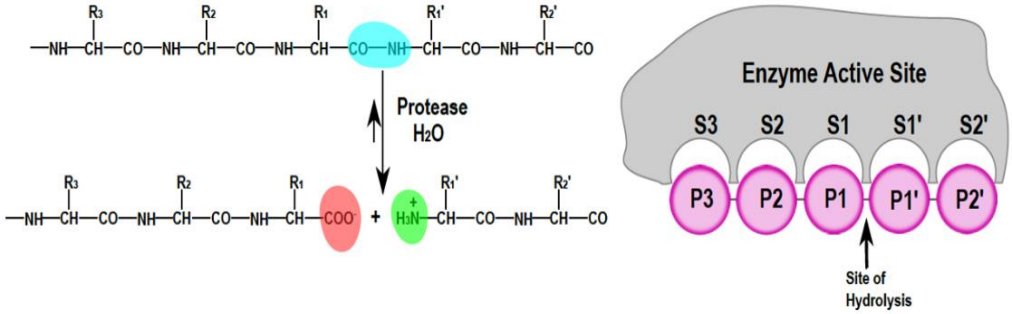

Figure 1.2: A protease regulated hydrolysis of a peptide bond. (A) A scheme of hydrolytic reaction. (B) The active of protease is composed of subsites (S). Every S has an affinity for respective residues (P). By the mean of a mechanism called "Lock and Key" directions protease specificity.

Studies have demonstrated that bioactive peptides can be produced by hydrolysis of proteins from milk using digestive enzymes (Korhonen, 2009, Korhonen and Pihlanto, 2006). Enzymes, pepsin, trypsin, and chymotrypsin, being the most prominent enzymes that has been displayed to release a number of calcium-binding phosphopeptides (CPPs), antihypertensive peptides, immunomodulatory, antibacterial, and opioid peptides both from diverse casein (α-, β- and j-casein) and whey proteins, e.g β-lactoglobulin (β-lg), α-lactalbumin (α-la), and glycomacropeptide (GMP) (Gobbetti *et al.*, 2007, FitzGerald *et al.*, 2004, Yamamoto *et al.*, 2003, Meisel and FitzGerald, 2003, Korhonen, 2009).

Table 1.1: Examples of nonribosomal peptides.

Antibiotics	Antibiotics precursors	Cytostatics	Immuno-suppressant	Siderophores	Pigments	Toxins	Nitrogen storage polymers	Phytotoxins
• Actinomycin • Bactracin • Calcium dependent antibiotic • Teixobactin •Zwittermicin A • Daptomycin • Vancomycin • Gramicidin • Tyrocidine	• ACV-Tripeptide	• Epothilone • Bleomycin	• Cyclosporine	• Pyoverdine • Enterobactin • Myxochelin A	• Indigoidine	• Microcystins, • Nodularins, • cyanotoxins from cyanobacteria	• Cyanophycin	• HC-toxin • AM-toxin • Victorin

1.4.2. Microbial fermentation

Since many dairy starter cultures possess highly proteolytic nature, production of bioactive peptides can be expected while the manufacture of fermented dairy products. Upon milk proteins proteolysis through microbial different bioactive peptides are released (Fitzgerald and Murray, 2006, Matar *et al.*, 2003, Gobbetti *et al.*, 2007, FitzGerald *et al.*, 2004). The strains, *Lactobacillus helveticus*, have been mainly reported for the production of antihypertensive peptides by many researchers, the inhibitory tripeptides Val-Pro-Pro (VPP), as well as Ile-Pro-Pro (IPP), are best known. Several animals and human studies have established the antihypertensive capacity of these peptides (Nakamura *et al.*, 1995, Hata *et al.*, 1996, Masuda *et al.*, 1996, Sipola *et al.*, 2002, Seppo *et al.*, 2003, Mizushima *et al.*, 2004, Aihara *et al.*, 2005, Jauhiainen *et al.*, 2005, Hirota *et al.*, 2007, Korhonen, 2009). Different bioactive peptides production in milk during fermentation by cheese starter bacteria, yogurt bacteria, and commercial probiotic have also been reported that the fermentation of milk with a commercial starter culture (a mixture of five *Lactobacillus* bacterial strains) followed by hydrolysis with a microbial protease enzyme to increase the ACE inhibitory activity of the hydrolysate.

Existing research data show that many fermented foods possess both nutritive and non-nutritive components, which have the modulating potential to particular molecular function relevant to well-being and health of the consumer. However, it is nearly 90% of naturally fermented foods, and alcoholic beverage in different regions of the world are still relied on traditionally home made production. Foods and beverages are fermented naturally contain both functional and non-functional microorganism (Tamang

et al., 2016). During the fermentation functional microorganisms convert the chemical constitutions of materials of animal/plant sources; thereby enhancing the bio-availability of nutrients, sensory quality of the food, imparting bio-preservative effects and betterment of food safety, toxic components degradation, and anti-nutritive factors, producing antimicrobial and antioxidant compounds, stimulation the probiotic functions, and to increase the defenses of, with some bioactive health promoting compounds (Thapa and Tamang, 2015, Parvez *et al.*, 2006, Tamang *et al.*, 2009). Among bacteria related with fermented foods and alcoholic beverages, lactic acid bacteria (LAB) frequently species of *Lactobacillus, Enterococcus, Pediococcus, Lactococcus, Leuconostoc, Weissella*, etc. are extensively present in many fermented foods and beverages (Holzapfel and Wood, 2014, Kubo *et al.*, 2011). Microorganisms from some genera and species are used as commercial starters in food fermentation (Table 1.2), and some of the products are commercialized globally as health foods, functional foods, nutraceuticals foods, and therapeutic foods (Bourdichon *et al.*, 2012, Thapa and Tamang, 2015, Bernardeau *et al.*, 2006).

1.5. Fermented food of Korea

Many fermented foods and beverages, which are the indispensable element of the Korean cuisine which are consumed by the Koreans and many people around the world throughout the year. Korean fermented food products have also become popular and are well appreciated by the people around the world. The primary fermented food items, except the alcoholic beverages that are consumed nowadays in Korea, are fundamentally categorized into three broad categories as bellow (Korean fermented foods, Kimchi and Doenjang, in Handbook of Fermented Functional Foods).

1.5.1. Category one: In this category, fermented food consists of the soy-based products, that includes doenjang (soybean paste), chongkukjang (quickly fermented soybean paste), gochujang (hot pepper-soybean paste), and ganjang (soy sauce) (figure 1.3) (Surh *et al.*, 2003). Conventionally, these types of fermented products are prepared once in a year; large clay pots are used to store and consumed throughout the year.

1.5.2. Category two: These type of fermented food is popular and prepared from fish and shellfish (figure 1.4. A). These products are consumed as such or are combined with kimchi (Surh *et al.*, 2003). Fish, shellfish, and its products provide essential vitamins, high-quality proteins, minerals, and polyunsaturated fatty acids source to consumer (Prester, 2011).

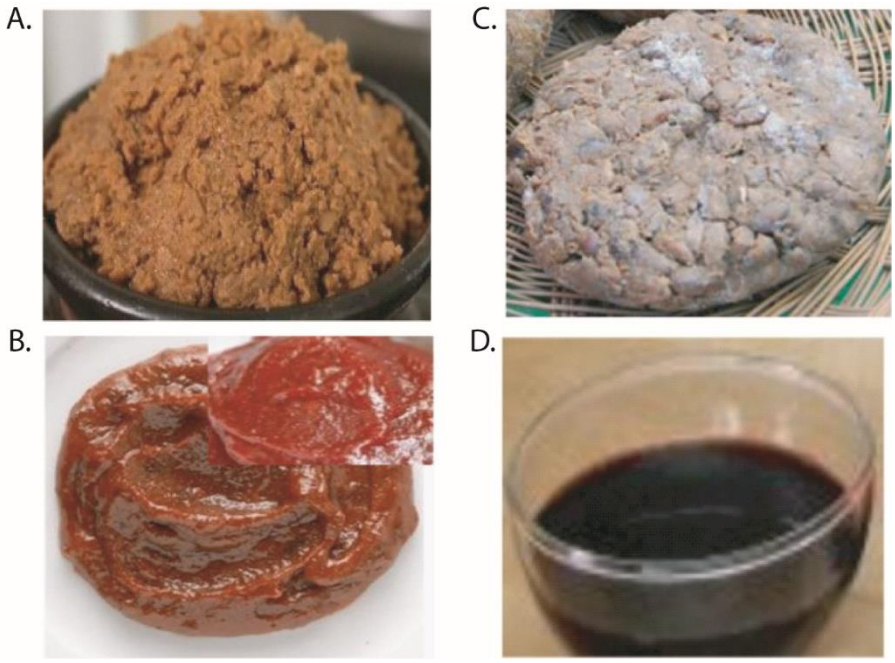

Figure 1.3: Different fermented food in Korea (Category one). In this category, fermented food consists of the soy-based products, which includes doenjang (soybean paste) (A), chongkukjang (quickly fermented soybean paste) (B), gochujang (hot pepper-soybean paste) (C), and ganjang (soy sauce) (D).

1.5.3. Category three: The kimchi is the third type, which is most extensively and popularly consumed not only in Korean but around the world (figure 1.4B). It is a primary Korean traditional fermented food. It is prepared from the Chinese cabbage (*Brassica rapa L*. spp. pekinensis [Lour.] Han) and/or radish as its main ingredient, sideways with different kind of vegetables (Surh *et al.*, 2003). Kimchi fermentation process takes a short period to complete.

Figure 1.4: Fermented food prepared from fish and shellfish (A). Korean traditional fermented kimchi (B).

1.6. Kimchi fermentation and its microorganisms

Commonly, fermentation of kimchi is divided into four stages based on acidity; early stage (acidity<0.2), immature stage (acidity 0.2–0.4), optimum-ripening stage (acidity 0.4–0.9), and finally, over-ripening or rancid stage (acidity>0.9) (Commission, 2001). The LAB profile during the process of kimchi fermentation varies with pH and acidity. *Leuconostoc mesenteroides* is observed during initial fermentation (pH 5.6–4.2 and acidity 0.48–0.89%), and later on, *Lactobacillus sakei* dominates in the fermentation (pH 4.15 and acidity 0.98%). A separate subset of LAB linked to kimchi fermentation is importantly influenced by temperature. *Lab. sakei* predominates in kimjang kimchi (as a long term stored kimchi for the winter season), and the strain appears to be suitable for low fermentation temperatures at 5–9°C and storage at −2°C temperatures (Lee *et al.*, 2008). The standard identification of bacterial isolates from kimchi showed that *Lactobacillus plantarum* and *Leu. mesenteroides* were the predominant species (Lim *et al.*, 1989). A recent report from Park and his colleagues present that microbial

communities of 13 kinds of Korean commercial kimchis at pH 4.2–4.4 using the method pyrosequencing, bacteria *Wei. koreensis* (27.2%) accounted for the uppermost proportion among total LAB in kimchis; followed by 14.7% of Lab. sakei, 8.7 of % *Weissella cibaria*, *Lab. graminis* (13.8%), *Lactobacillus gelidum* (6.3%), and other species followed (figure 1.5).

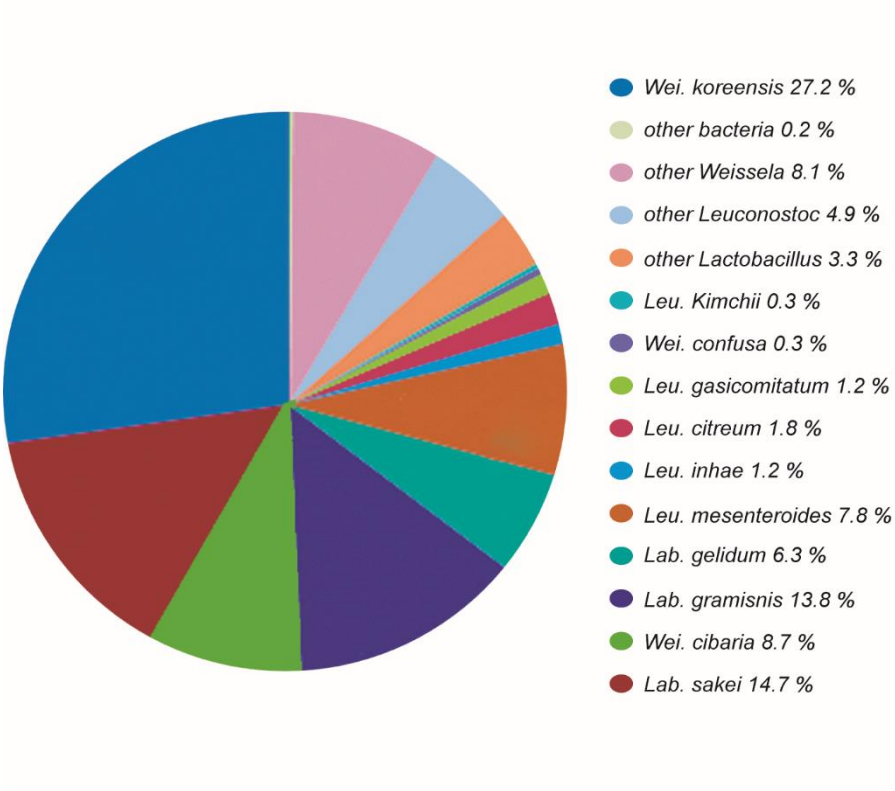

Figure 1.5: Microbial communities of 13 kinds of Korean commercial kimchi.

The yellow-green vegetables are used mainly for preparing fermented kimchi, which has been displayed to prevent cancer, increase immune function, antimicrobial activity, retard the aging process, and avert constipation (Park, 1995). Fermented kimchi enhanced its taste and functionality and became a good probiotic food.

Table 1.2: Microorganisms are used as commercial starters in food fermentation.

Group	Genera/species	Product/application(s)
Bacteria	*Acetobacter aceti subsp. aceti*	Vinegar
	A. pasteurianus subsp. pasteurianus	Vinegar, cocoa
	Bacilllus acidopullulyticus	Pullulanases (food additive)
	B. coagulans	Cocoa; glucose isomerase (food additive), fermented soybeans
	B. licheniformis	Protease (food additive)
	B. subtilis	Fermented soybeans, protease, glycolipids, riboflavin-B2 (food additive)
	Bifidobacterium animalis subsp. lactis, B. breve	Fermented milks with probiotic properties; common in European fermented milks
	Brachybacterium alimentarium	Gruyère and Beaufort cheese
	Brevibacterium flavum	Malic acid, glutamic acid, lysine, monosodium glutamate (food additives)
	Corynebacterium ammoniagenes	Cheese ripening
	Enterobacter aerogenes	Bread fermentation
	Enterococcus durans	Cheese and sourdough fermentation
	E. faecium	Soybean, dairy, meat, vegetables
	Klebsiella pneumoniae subsp. ozaenae	Tempe; production of vitamin B12
	Lactobacillus acetototolerans	Ricotta cheese, vegetables
	L. acidophilus	Fermented milks, probiotics, vegetables
	L. alimentarius	Fermented sausages; ricotta; meat, fish
	L. brevis	Bread fermentation; wine; dairy
	L. buchneri	Malolactic fermentation in wine; sourdough
	L. casei subsp. casei	Dairy starter; cheese ripening; green table olives
	L. delbruecki subsp. bulgaricus	Yogurt and other fermented milks, mozarella
	L. fermentum	Fermented milks, sourdough, urease (food additive)
	L. ghanensis	Cocoa
	L. helveticus	Starter for cheese; cheese ripening, vegetables
	L. hilgardii	Malolactic fermentation of wine
	L. kefiri	Fermented milk (kefir), decrease of bitter taste in juice (citrus)
	L. kimchii	Kimchi
	L. oeni	Wine

	L. paracasei subsp. paracasei	Cheese fermentation, probiotic cheese, probiotics, wine, meat
	L. pentosus	Meat fermentation and biopreservation of meat; green table olives; dairy, fruits, wine
	L. plantarum subsp. plantarum	Fermentation of vegetables, malolactic fermentation, green table olives; dairy, meat
	L. sakei subsp. sakei	Fermentation of cheese and meat products; beverages
	L. salivarious subsp. salivarius	Cheese fermentation
	L. sanfranciscensis	Sourdough
	L. versmoldensis	Dry sausages
	Lactococcus lactis subsp. lactis	Dairy starter, Nisin (protective culture)
	L. lactis, L. mesenteroides subsp. Cremoris, L. mesenteroides subsp. Dextranicum, L. mesenteroides subsp. mesenteroides	Dairy starter
	Oenococcus oeni	Malolactic fermentation of wine
	Pediococcus acidilactici	Meat fermentation, bio-preservation of cheese starter, meat
	P. pentosaceus	Meat fermentation and biopreservation of meat
	Propionibacterium acidipropionici	Meat fermentation and biopreservation of meat
	P. arabinosum	Cheese fermentation; probiotics
	P. freudenreichii subsp. freudenreichii	Cheese fermentation (Emmental cheese starter)
	Streptococcus natalensis	Natamycin (food additive)
	Weisella ghanensis	Cocoa
	Zymomonas mobilis subsp. mobilis	Beverages
Yeasts	*Candida famata*	Fermentation of blue vein cheese and biopreservation of citrus; meat
	C. guilliermondii	Citric acid (food additive)
	C. krusei	Kefir fermentation; sourdough fermentation
	Debaryomyces hansenii	Ripening of smear cheeses; meat
	Geotrichum candidum	Ripening of soft and semisoft fermented milks, cheeses, meat
	Kluyveromyces marxianus	Cheese ripening; lactase (food additive)
	S. bayanus	Kefir fermentation; juice and wine fermentation
	S. cerevisiae	Beer, bread, invertase (food additive)

	S. cerevisiae subsp. boulardii	Used as probiotic culture
	S. florentius	Kefir fermentation
	S. pastorianus	Beer
	S. sake	Sake fermentation
	S. unisporus	Kefir fermentation
	Schizosaccharomyces pombe	Wine
	Zygosaccharomyces rouxii	Soy sauce
Filamentous moulds	*Aspergillus flavus*	α-amylases (food additive)
	A. niger	Beverages; industrial production of citric acid; amyloglucosidases, pectinase, cellulase, glucose oxidase, protease (food additives)
	A. oryzae, A. sojae	Soy sauce, beverages; α-amylases, amyloglucosidase, lipase (food additives)
	Penicillium camemberti	White mold cheeses (camembert type)
	P. notatum	Glucose oxidases (food additive)
	P. roqueforti	Blue mold cheeses
	Rhizopus oligosporus	Tempe fermentation
	R. oryzae	Soy sauce, koji

1.7. Peptide families

In this section are mentioning ribosomal peptides, typically with hormonal activity. These type peptides are synthesized by cells as longer "proproteins" or "propeptides" and shortened before exiting the cell. Peptides are released into the bloodstream and perform their signaling functions. Some well-known peptides name is shown in Table 1.3.

1.7.1. Opioid peptides

In the brain, opioid peptides are bind to opioid receptors. Opiates and opioids mimic these peptides effect. Maybe the body itself produces these peptides, for instance, endorphins. The effects of opioid peptides may vary, but they all resemble those of opiates. In the brain, opioid peptide systems are known to play a vital role in emotion, attachment behavior, motivation, the response to pain, stress, and the control of the intake of food (figure 1.6). Some examples of opioid peptides are Proopiomelanocortin (POMC) peptides, Enkephalin pentapeptides, Prodynorphin peptides, etc.

1.7.2. Immunomodulating peptides

Immunomodulating peptides have been found in human and in cow milk proteins (Migliore-Samour and Jolles, 1988). Two peptides, α-lactalbumin f51-f53 and β-casein f54-f59, from human milk protein, improve the phagocytic activity of macrophages in both humans and mice and also develop resistance to certain bacteria in mice (PARKER *et al.*, 1984, Migliore-Samour *et al.*, 1989). The immunomodulating peptides isolated, the αs1-casein C-terminal hexapeptide (f194-f199), β-casein f191-f193 from bovine caseins stimulate macrophages. β-casein f63-f68 stimulate in vitro phagocytosis, but

this peptide and β-casein f191-f193 failed to exert protection of mice *in vivo* (Fiat *et al.*, 1989).

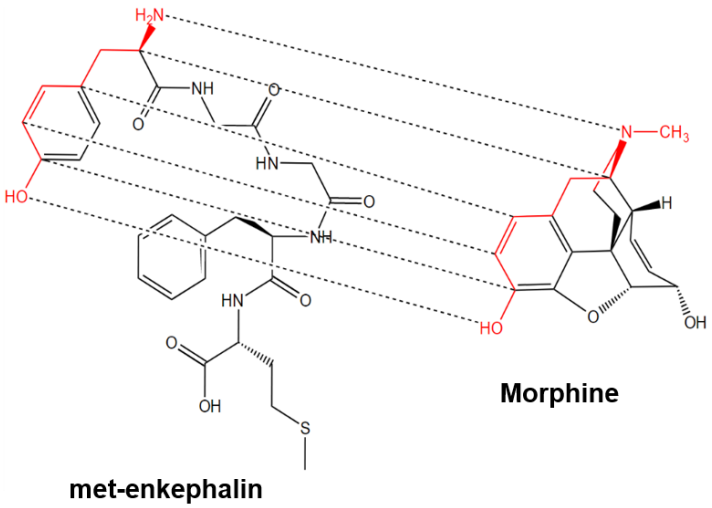

Figure 1.6: Structural correlation between met-enkephalin, an opioid peptide, (left) and morphine, an opiate drug, (right).

Table 1.3: Some well-known peptides

Peptides Types	Example
Antimicrobial	Magainin family Cecropin family Cathelicidin family Defensin family
Tachykinin	Neurokinin B Substance P Neurokinin A Kassinin Eledoisin
Vasoactive intestinal	Peptide PHI 27 (Peptide Histidine Isoleucine 27) VIP (Vasoactive Intestinal Peptide; PHM27) Secretin PACAP Pituitary Adenylate Cyclase Activating Peptide Glucagon GHRH 1-24 (Growth Hormone Releasing Hormone 1-24)
Pancreatic polypeptide-related	PPY Pancreatic PolYpeptide NPY (NeuroPeptide Y) APP (Avian Pancreatic Polypeptide) PYY (Peptide YY)
Opioid	Enkephalin pentapeptides Proopiomelanocortin (POMC) peptides Prodynorphin peptides
Calcitonin	Calcitonin Amylin AGG01
Other	Lactotripeptides - Lactotripeptides blood pressure (although the evidence is mixed), B-type Natriuretic Peptide (BNP) - produced in myocardium and useful for medical diagnosis

1.7.3. Mineral-binding peptides

Numerous phosphopeptides are comprising the cluster sequence -Ser-Ser-Ser-Glu-Glu- have been recognized from whole bovine casein. Peptides with these sequences provide the unique capacity to keep Ca, P and another mineral in a solution at intestinal pH. From enzymatic digest of milk proteins, several phosphopeptides have been identified, for example: f59-f79, αs1-casein f43-f58, αs2-casein f1-f24, f43-f79, and f46-f70 and β-casein f1-f28, f1-f25, f2-f28, f33-f48 (Tamang *et al.*, 2009, Thapa and Tamang, 2015).

1.7.4. Antithrombotic peptides

Milk and blood coagulation possess the functional similarities, as well as sequence homologies, exist between the fibrinogen g-chain and k-casein (Jollès and Caen, 1991). In 19896, Jolles *et al.* reported that bovine k-casein f106-f116 repressed platelet aggregation as well as mutual with the receptor binding site, therefore stopping fibrinogen binding with blood platelets. This inhibition mechanism was reliant on peptide amount (concentration). The two smaller tryptic peptides such as f113-f116 and k-casein f106-f112 exerted a minimal effect on platelet aggregation as well as did not prevent fibrinogen binding. These peptides are called casoplatelins. The actions of k-casein f106-f116 are likely that of the C-terminal peptide of the human fibrinogen g-chain (Fiat *et al.*, 1989).

1.7.5. Antimicrobial peptides

Antimicrobial peptides (AMPs), also referred as host defense peptides (HDPs) are the vital part of the innate immune response found amongst all classes of a living being (figure 1.7). Essential

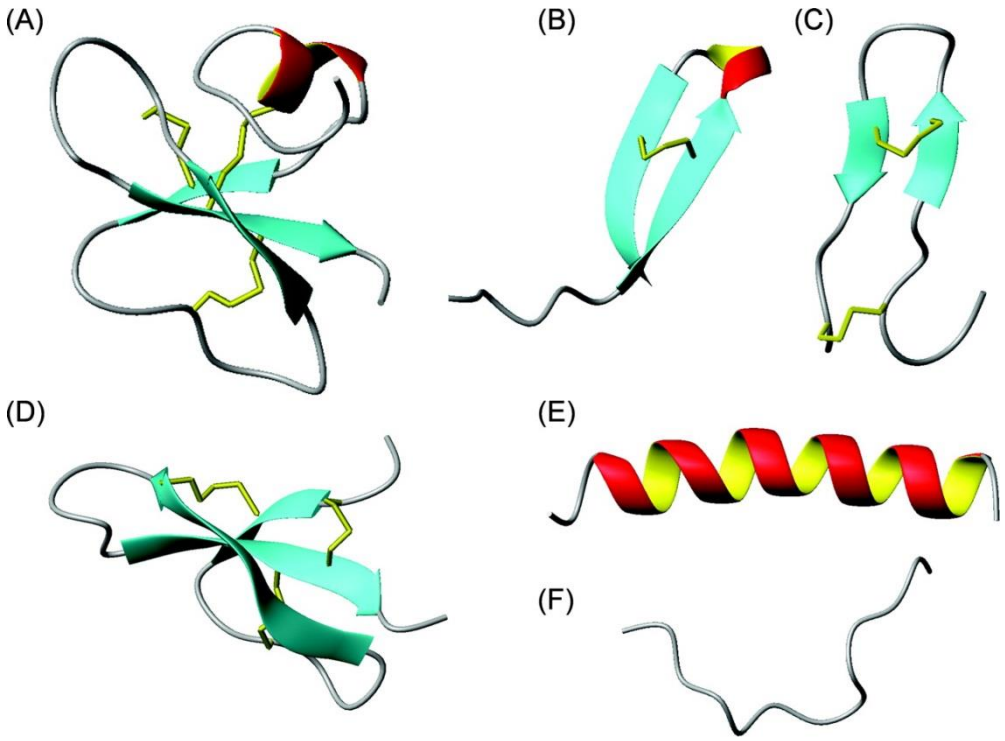

Figure 1.7: Antimicrobial peptides structural classes. Diverse structure of human β-defensin-2 (PDB: 1FQQ) (A); looped thanatin (PDB: 8TFV) (B); β-sheeted polyphemusin (PDB: 1RKK) (C); rabbit kidney defensin-1 (PDB: 1EWS) (D); α-helical magainin-2 (PDB: 2MAG) (E); extended indolicidin (PDB: 1G89) (F). Disulfide bonds are designated in yellow.

differences exist between eukaryotic and prokaryotic cells that may represent targets for antimicrobial peptides. These peptides are potent, broad spectrum antibiotics which display potential as novel therapeutic agents. AMPs have been demonstrated to kill both Gram negative and positive bacteria, enveloped fungi, viruses, and even transformed or cancerous cells (Reddy *et al.*, 2004). Unlike most conventional antibiotics, it may also

improve immunity by regulating as immunomodulators. Compounds from Marine fish sources have high levels antimicrobial properties with in vivo testing confirming the efficacy of fish peptides used in food/feed ingredients (Jia *et al.*, 2000).

1.7.6. Antioxidative peptides

It is widely recognized the importance of oxidation in our body as well as a food stuff. Metabolism of oxidative is an essential process for the survival of cells. Excessive of free radicals' production, they can overwhelm protective enzymes like catalase, superoxide dismutase (SOD), and peroxidase which cause damaging and lethal cellular effects (e.g. apoptosis) by cellular proteins, DNA, oxidizing membrane lipids, and enzymes thus shutting down the cell process. Studies have reported that antioxidative peptides from caseins in hydrolysis by digestive enzymes and in the fermentation of milk with proteolytic LAB strains (Korhonen and Pihlanto, 2003a). Most identified peptides are derived from αs-casein and have been shown free radical scavenging activities and to inhibit enzymatic and non-enzymatic lipid peroxidation, most likely by being a favored target over fatty acid-free radicals (Rival *et al.*, 2001). More and more additional exploration is required to elucidate the role of peptides in the in oxidative stress condition.

AIMS OF THE STUDY

More than 200 bacterial strains previously isolated from Korean fermented foods which were collected from different provinces of Korea in our laboratory. About 90% of naturally fermented foods and alcoholic beverage in different regions of the world are still relied on conventionally domestic manufacture. Foods are fermented naturally contain both functional and non-functional microorganism. Functional microorganisms convert the chemical constitutions of materials of animal/plant sources after being fermented; thus enhancing the sensory quality of the food, bio-availability of nutrients. Also produce antimicrobial and antioxidant compounds, stimulation the probiotic functions, and to increase the defenses of, with some bioactive health promoting compounds. Thus, goals of this study are,

- to screen microbial strain producing relatively higher bacteriocin of interest and to identify the strain,
- to purify the enzymes with maximum purity and yield by using least possible purification steps,
- to biochemically characterize the purified peptide
- upon confirmation of the novelty of purified YD1, to investigate the antimicrobial mechanism,
- to investigate the antioxidant mechanism,
- a literature review of future perspective.

CHAPTER TWO

THE STRAIN CBSYD1 AND PROBIOTIC

2. 1. INTRODUCTION

The history of fermented vegetable is old enough. Since about 2000 years ago in Korea salted and fermented vegetable has been consuming. It is sensible to undertake that the kimchi has been prepared and consumed from the 4th century A.D in Korea by considering the many ancient records of kimchi preparation. The taste of kimchi is reliant on upon the ingredients used, fermentation condition, and lactic acid bacteria (LAB) utilized in the fermentation as well.(Cheigh *et al.*, 1994, Kwon and Kim, 2007, Lee *et al.*, 1992). The standard ingredients of kimchi preparation are *Baechu* cabbage as well as radish; however, other vegetables such as green onion, leek, cucumber, and so on are also used for the making of different types of kimchi. High levels of minerals, vitamins, dietary fibers and other functional components are in kimchi. Many previous studies have reported that kimchi has anticancer, antioxidant, antiatherosclerotic, antidiabetic, antiobesity effects, and so on (Kim *et al.*, 2011, Kim *et al.*, 2007, Islam and Choi, 2009, Park, 1995). The most significant feature of kimchi for human health is that it comprises very high levels of bacteria produce lactic acid (10^8 to 10^9 cfu/g) (Chun and Woo, 1999) such as *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *Enterococcus faecalis*, *Lactobacillus plantarum*, *Streptococcus faecalis*, *Pediococcus cerevisiae*, *Pediococcus pentosaceous*, *Lactobacilli* app, and *Weissella koreanis* (Han *et al.*, 1990, Kim and Chun, 1966, Mheen and Kwon, 1984). Therefore, eating kimchi is a good way to include more vegetable and probiotics in the diet to improve health.

Bile salt hydrolase (BSH), an enzyme called Choloylglycine hydrolase (EC 3.5.1.24) that hydrolyzes bile salts to form taurine or glycine, as well as steroid core (Begley *et al.*, 2006, Liong and Shah, 2005). Hydrolyzed bile salts are less absorbed in the human

intestine compared to non-hydrolyzed ones, leaving more free bile acids to be excreted out of human bodies via feces (Begley *et al.*, 2006). Excessive excretions of bile salt decrease the total amount of bile salts availability in human bodies. The lost bile salts can be replenished via *de novo* synthesis from cholesterol, which could later reduce the level of serum cholesterol in the human body (Begley *et al.*, 2006). It is found that several bacterial genera like *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium*, *Bacteroides*, etc. showed BSH activity (Franz *et al.*, 2001, Stellwag and Hylemon, 1976, Kishinaka *et al.*, 1994, Noriega *et al.*, 2006, Lee *et al.*, 2011, Dashkevicz and Feighner, 1989, Liong and Shah, 2005). By considering their definition, a good number of microbial species that may exert probiotic properties is remarkable. To be a probiotic strain, it must able to exert beneficial effects and must have the capability to survive as well as metabolize in the gut. They must, therefore, be resistant to GI levels of acid.

Probiotic involves several mechanisms of action although but the exact manner in which they exercise their effects is still not fully known. These range from various bacteriocin and short chain fatty acid production, make the lowering of gut pH and promote nutrient competition to stimulation of mucosal barrier function as well as immunomodulation. Kimchi has received considerable attention internationally and in known as representative food of Korea for its various beneficial purposes. To control pathogenic bacteria including the production of antimicrobial peptides (AMP), “bacteriocins” are highly considered. AMPs are ubiquitously produced by a broad range of microorganism. *Bacillus* is an interesting genus to investigate since it provides a diverse array of antimicrobial peptides representing several different basic chemical structure (Bizani and Brandelli, 2002). A good number of the peptides with biological activities have been

reported increasingly from *Bacillus* and have become a significant attention for antimicrobial study (Dischinger *et al.*, 2009, Teixeira *et al.*, 2009, Wu *et al.*, 2005). Bacteriocin production or bacteriocin-like substances have also been described for some other important pathogens such as *Listeria monocytogenes* and *Streptococcus pyogenes* (Cherif *et al.*, 2001).

The action mechanism of a probiotic may include the modulation of microflora in the host, e.g., by enhancement of the microbial balance through the interaction of orally applied viable microorganisms with the microflora in the digestive tract lumen, host metabolic activities are being modulated, e.g., by immunomodulation, and stabilizing digestive enzyme pattern, by activating and regulating of systemic immune system responses, and mucosa-associated (figure 2.1). These mechanisms of action are also strain-dependent. Microflora provides in intestinal protection against a broad range of pathogens, including certain forms of *Escherichia Coli, Clostridia, Shigella, Salmonella, and Pseudomonas,* and yeasts as well such as *Candida albicans.*

There is no evidence has found *Bacillus amyloliquefaciens* subsp. plantarum as a probiotic bacterium. Few number of probiotic bacteria like *Bacillus subtills, Bacillus clausii, Bacillus cereus, Bacillus coagulans,* and *Bacillus licheniformis* are being used for human medicinal and food supplement (Cutting, 2011). In our study, we paid interest on Kimchi, a traditional Korean vegetable food that was fermented in different conditions by various microorganisms such as *Bacillus* and *Lactobacillus.* Thus, fermented food is a good source of microorganisms, and many scientists have tried to isolate different microbes from it capable of producing antimicrobial and other useful compounds. The

present study reports on the identification probiotic optimized the media for bacteriocin producing probiotic, *Bacillus CBSYD1*, isolated from fermented Kimchi and biochemical characterization of the crude bacteriocin designated as Cd.YD1.

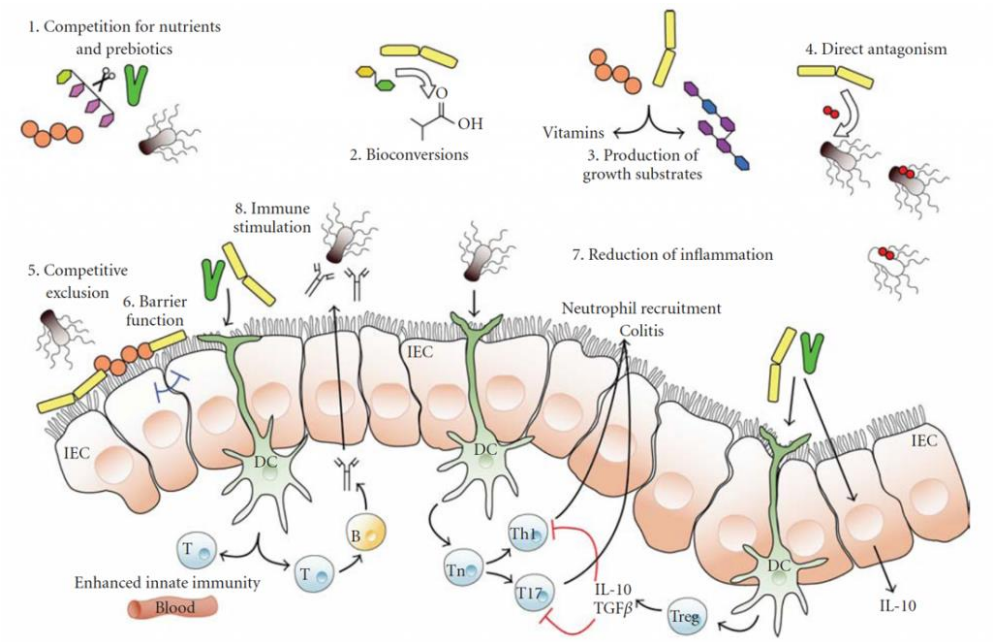

Figure 2.1: The schematic diagram demonstrating the potential or known mechanisms of action whereby probiotic bacteria on the microbiota. (1) as growth substrates competition for dietary ingredients, (2) bioconversion of, for instance, fermentation products with inhibitory properties from sugar, (3) Growth substrates production, for example, vitamins, for other microorganisms, (4) regulate antagonism by bacteriocins like compounds, (5) competitive prohibiting for binding sites, (6) enhanced the barrier function, (7) decrease of inflammation, thus changing properties of intestinal for persistence and colonization within, and (8) innate immune response stimulation (mechanisms unknown). DC: dendritic cells, IEC: epithelial cells, T: T-cells.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Bacterial media-de Man-Rogosa-Sharpe (MRS) and Mueller-Hinton (MH) were purchased from Becton Dickinson, Spark, USA. Agar was purchased from Daejun Chemicals and Metals Co, Gyeonggi-do, South Korea. All other reagents were of the extra pure grade. Strain *CBSYD1* was isolated from fermented kimchi.

2.2.2. Bacterial strain isolation and identification

Cabbages, from different provinces of Korea, were collected and processed for biochemical and molecular identification of microorganisms. The strain identification, based on morphological characteristics, was made according to Bergey's manual of systematic bacteriology (Lechevalier, 1989). Furthermore, the identification was confirmed by 16S rRNA sequence analysis (GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGG GAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAAC CTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGG TTGTCTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTAC AGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA CGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGT AAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACCT TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG

TAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCG
CAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG
TCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACG
TGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA
GGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGG
GGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAG
AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGG
CACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAG
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CAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGT
AATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCT
TTTAGGAGCCAGCCGCCGA) and phylogenetic tree. The nucleotide sequence of strain *CBSYD1* was submitted to the GenBank (ncbi.nlm.nih.gov/Genbank) under the accession no. KY062987.

2.2.3. Tolerance towards bile salt

To determine the tolerance towards bile salt, MRS broth (pH 6.5) with 0; and 0.3 % (w/v) of natrium salt of the tauroglycocholic acid (TDCA (Merck)) was used. These media were inoculated with fresh culture (1% v/v), and at every interval of 3, 6, 12, and 24 h, the absorbance at 560 nm was measured. The results were analyzed as perceptual growth compared to the control sample (0% of bile salt).

2.2.4. Bile Salt plate assay

We used agar plate assay to detect bile salt hydrolase activity in *Bacillus CBSYD1* as well as for screening probiotic. MRS-agar prepared bile salt plate (87 mm x 15 mm) with 0.5% (w/v) of the TDCA. Once the plate was prepared, we made three different spots using the replicator to put 30μL bacterial strains and plates were incubated under an anaerobic atmosphere at 37°C for 48 hours. After the incubation period, plates were kept with iodine-potassium iodide solution (1gm iodine, 5gm potassium iodine, and 330 mL deionized water) for 15 minutes to detect a clear halos zone. *E.coli* KCTC 1923 and L. acidophilus were considered as a negative and positive control bacteria respectively in the BSH activity assay.

2.2.5. Gastrointestinal tract condition tolerance of *Bacillus CBSYD1*

An overnight-cultured MRS broth (pH 5.6, at 37°C in the CO2 atmosphere (5% v/v)) were centrifuged (8690 g, 15 min, 5°C), and washed in buffer two times. After that, isolated cells were transferred into a solution simulating the stomach conditions (50 mL HCl, pH 2, 0.5% NaCl (w/v), 0.3% pepsin (w/v); Sigma-Aldrich, St. Louis, USA). The cells were incubated at 37°C in the CO2 atmosphere (5% v/v) with occasional stirring. In the intervals of 0, 1, 2, and 3 h, the counts were determined on MRS agar by the plate

method. After 3 h of cultivation, pH was adjusted to 7.2 ± 0.2 (by 10% w/v NaOH), and Ox bile (0.3% w/v; Merck) and pancreatin (0.1% w/v; Sigma-Aldrich) were added to simulate the conditions existing in the ileum. The incubation continued under the same conditions, the number of cells (CFU/mL) was determined in the intervals of hourly after the environmental change.

2.2.6. Bacteriocin activity assay

Bacteriocin activity was determined as described in our previous reports (Yoo *et al.*, 2007, Sohng *et al.*, 2008). A filter paper disc (8 mm, Toyo Roshi Kaisha, Japan) saturated with the anti-microbial sample (40 µl) was placed on the surface of Petri dish (87 mm x 15 mm) containing Muller Hinton Agar (MHA). The plate was incubated at 37°C, and a clear zone of inhibition surrounding the paper disc was measured in millimeter (mm).

An arbitrary unit per milliliter (AU/ mL) was defined as the reciprocal serial dilution after the last giving an inhibition zone. The titer of the antimicrobial substance solution, in AU/mL, was calculated as (1000/d) D, where D is the dilution factor, and d is the dose, the amount of antimicrobial substance solution was put on every paper disc, (Parente *et al.*, 1995). AU and zone of inhibition were observed against *Mycobacterium smegmatis* in every step of bacteriocin production.

2.2.7. Media optimization

The influence of various carbon sources on the antimicrobial compound production was determined with the media supplemented with 1% peptone as a nitrogen source and different carbon sources (1%) such as lactose, fructose, maltose, glucose, sucrose,

sorbitol, starch and mannitol. Fermentation was carried out in 50 mL media in 250 mL Erlenmeyer flasks with constant shaking at 160 rpm. Afterward, the influence of nitrogen source on the antimicrobial compound production was determined with the media supplemented with 1% maltose as a carbon source and several nitrogen sources (1%) for example oatmeal, soybean, yeast extract, beef extract, tryptone, peptone, and malt extract. Furthermore, the effect of metal ions on antimicrobial compound production was evaluated with media composed of 1% maltose, 1% peptone and various metal ions (0.01%) such as $MgCl_2$, $FeSO_4$, $CaCl_2$, Na_2HPO_4, NaH_2PO_4, $ZnSO_4$, KH_2PO_4, and NaCl.

Far along, the strain was cultured in the 1% peptone and 0.01 % $CaCl_2$ as nitrogen and metal ions source respectively, combined with the different percentage of 0.5 -2 of maltose. Similarly, 1% maltose and 0.01 % $CaCl_2$ as carbon and metal ions source respectively were connected with the different rate of 0.5 -2 of peptone. The culture was continued up to 72 h, and the sample was taken in every 12 h and cell-free supernatant was collected using centrifugation at 10000 rpm for 4 min. Zone of inhibition was observed against *Mycobacterium smegmatis* in every step of media optimization. Commercially available MRS and MH broth media were used, known as control media.

2.2.8. Bacteriocin production and protein precipitation

Bacillus CBSYD1 was cultured for 36 h in MPC medium (2% maltose, 1% peptone, and 0.01% $CaCl_2$). The culture supernatant (in 36 hours) was mixed with ammonium sulfate (at ca. 30–80% saturation) and was kept at 4 °C with overnight stirring. On the following day, the protein precipitated was recovered using centrifugation at 10000 rpm for 30 min

and was re-suspended in 10 mM Tris–HCl buffer (pH 7.0). Protein content was calculated by Bradford protein assay.

2.3. RESULTS AND DISCUSSION

2.3.1. Strain isolation and identification

Screening and characterization of these novel bacteriocin-like antimicrobial peptides producing probiotic have drawn the attention of many researchers due to their potential applications in therapeutic and food industry. Our current study describes the characterization of a bacteriocin produced by probiotic bacteria *Bacillus CBSYD1*, which resembles with *Bacillus amyloliquefaciens* sp. plantarum (99.79% similarity), newly isolated Korean traditional fermented food Kimchi (figure 2.2). The initial screening showed the BSH and antibacterial activity leading us to carry out the current and further study. The primary goal of our research group is to find out probiotic and its action mechanism. From the very recent probiotic review, it is reported that few number of *Bacillus* probiotic bacteria like *Bacillus subtills*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, and *Bacillus licheniformis* are being used for human medicinal and food supplement (Cutting, 2011). We found strain, *Bacillus CBSYD1* having 99.79% similarity with *Bacillus amyloliquefaciens* subsp.

2.3.2. Tolerance towards bile salt

It is found that all strains had tested the ability to adapt to the bile salt environment; the same growth pattern was observed after this period as in the control culture. In the figure 2.3a, *Bacillus CBSYD1* shows, at 6th-hour incubation it was nearly 70% growth to control and growth was adapted up to ~87% while incubated for 24 hours. Some scientific papers (Burns *et al.*, 2008, Mainville *et al.*, 2005) have reported the effects of different bile salts

concentrations on growth inhibition. It was confirmed previously that the adaptability of bifidobacteria to high levels of bile salts during an extended period cultivation.

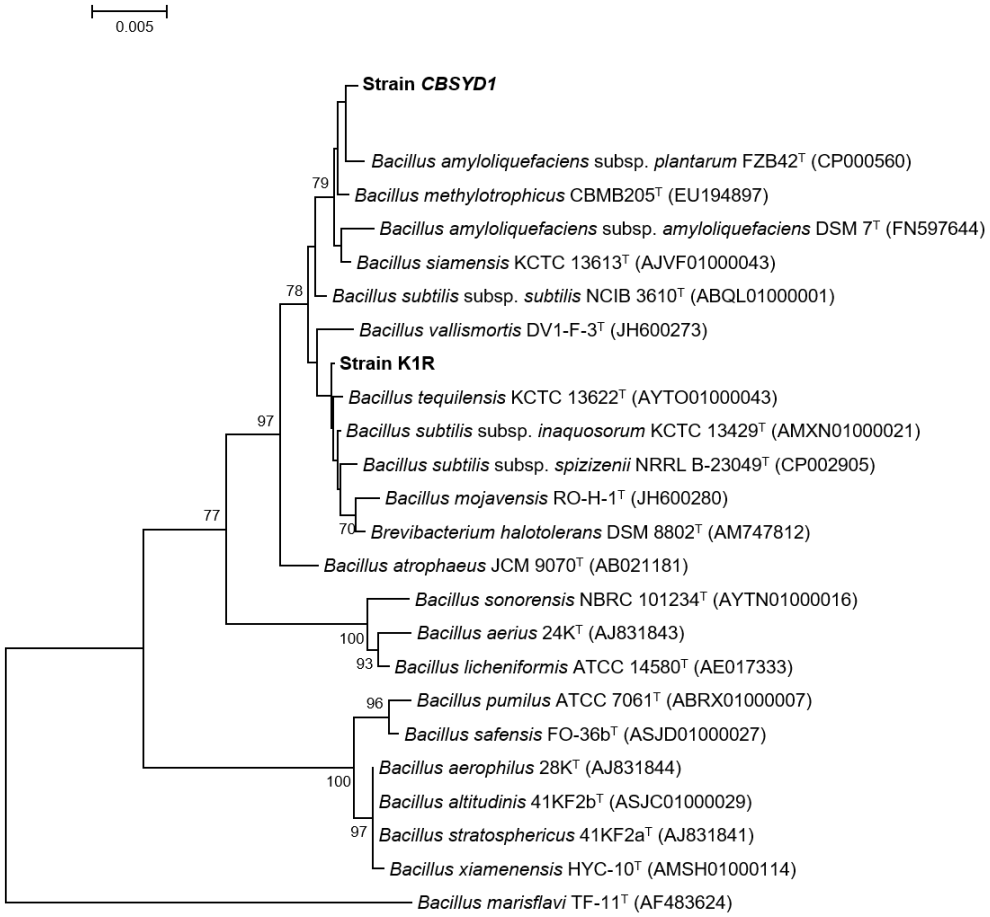

Figure 2.2: Phylogenetic tree based on closely complete 16S rRNA gene sequences showing relations between *CBSYD1* and some closely related taxa of the genus *Bacillus*.

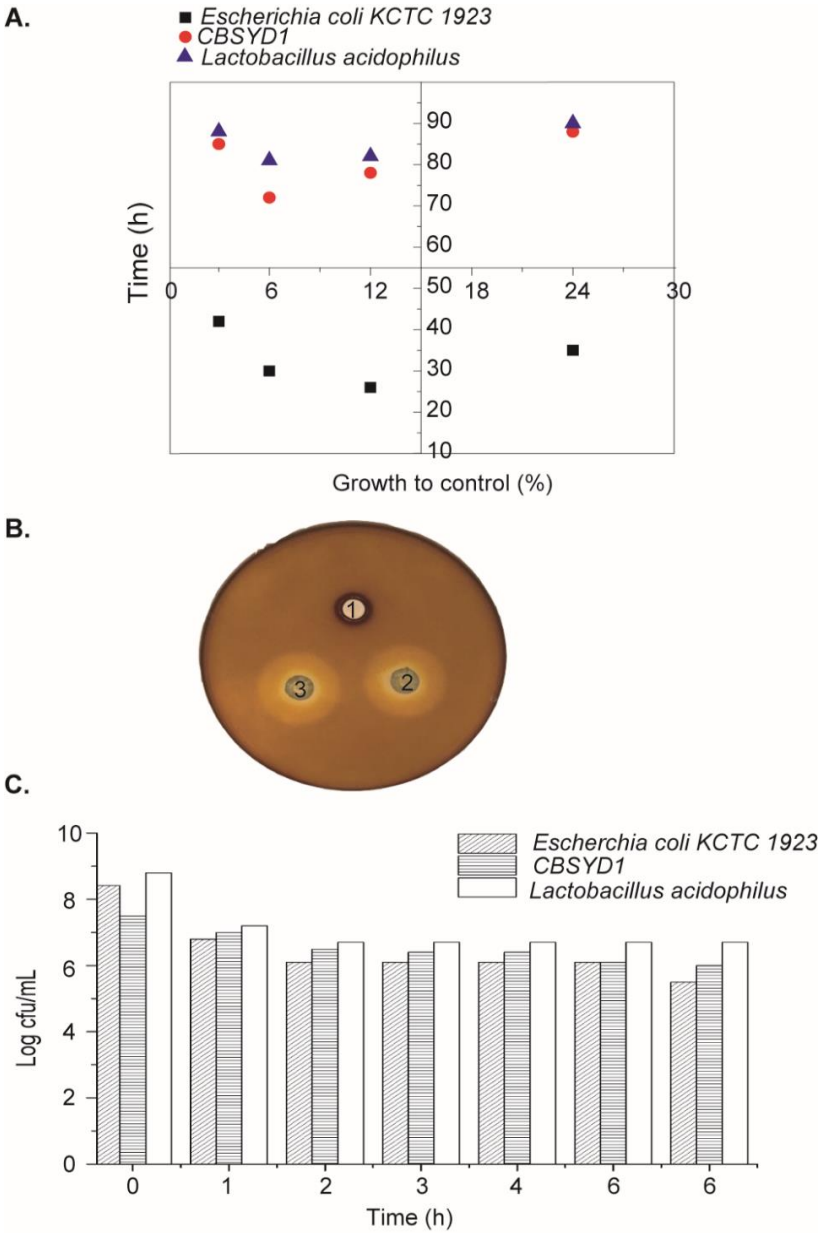

Figure 2.3: The culture media without bile salt as a control culture. Probiotic bacteria *Lactobacillus acidophilus* and non-probiotic *Escherichia coli* KCTC *1923* were considered as a positive and negative control bacterium. Growth of tested strains in MRA

broth with 0.3% (w/v) bile salt compared to control culture (A). Plate assay for the bile salt hydrolase (BSH) activity determination in soft MRS agar with 0.5% (w/v) of the taurodeoxycholic acid (B). Stability of strains cell in the conditions simulating gastrointestinal (stomach 0-3, pH 2; 0.3% w/v pepsin, small intestine 3-7, pH 6.8; 0.1% w/v pancreatin) (C).

2.3.3. Bile salt hydrolase

When strains were put on MRS plates containing 0.5% TDCA, to observe the taurin-conjugated bile acid was deconjugated, producing deoxycholic acid. The deconjugation activity of strains was manifested in figure 2.3b, a clear halos zone were shown for *Bacillus CBSYD1*, positive control *L. acidophilus*, and except negative control *E.coli* KCTC 1923.

2.3.4. Gastrointestinal tract condition tolerance of *Bacillus CBSYD1*

The survival of the digestive tract conditions is crucial for the following effects of probiotics in the gut. Different parts of the digestive track have different conditions. Probiotics are exposed to low pH salts and enzymes like lysozyme and pepsin when resting in the stomach. These enzymes play a significant role in affecting the survival of microorganism, including probiotic (Pinto *et al.*, 2006). In the small intestine probiotic exposed to different conditions such as slightly alkaline pH and the presence of bile salt. The tested strains showed a very different viability at low pH (figure 2.3c). Our proposed probiotic bacteria *Bacillus CBSYD1* sustained 97~89% viability in the condition stomach

pH 2; 0.3 % w/v pepsin for 1~3 hours in compared to *L. acidophilus* viability (100~93%) and good enough in compare to *E.coli, Bacillus CBSYD1* continued its viability till 83% in small intestine condition pH 6.8; 0.1% w/v pancreatin for next 4~6 hours. Whereas, *L.acidophilus* and *E.coli* continued 93 % and 76 % viability respectively. Botes et al. (Botes *et al.,* 2008) studied with 5 different lactobacilli, and the similar trend was observed.

2.3.5. Media optimization

Growth media temperature played a significant part and was frequently correlated with bacteriocin production (Todorov *et al.,* 2006). Bacteriocin activity was much higher at 37°C, which was a substantial agreement with outcomes from Lisboa reports' on bacteriocin produced by *B.amyloliquefaciens.* The maximal activity was evaluated at 37°C (Lisboa *et al.,* 2010).

The influence of various nutrition media on the production of the antimicrobial compounds in the crude sample has presented in figure 2.4. In carbon sources, maltose or lactose caused maximum production followed by mannitol or sorbitol (figure 2.4A) at 36 hours. Among nitrogen sources, peptone induced maximum production of antimicrobial compounds (figure 2.4B) at 36 hours. Magnesium Chloride or Sodium chloride (in 36 h) seemed to be the best among minerals (figure 2.4C). After finding the best C, N, and mineral source individually, different percentage of peptone and maltose were taken to observe the impacts of antimicrobial production. In figure 2.5A, 1% maltose and 0.01% $CaCl_2$ were combined with the different percentage of peptone (0.5~2%) where 1% of peptone showed maximum production at 36 hours. In the same manner, 1% of peptone

and 0.01% $CaCl_2$ were combined with the different percentage of maltose (0.5~2%), and 2% of maltose was having the highest production at 36 hours (figure 2.5B). Finally, the optimized media was considered as 1%peptone, 2% maltose, and 0.01% $CaCl_2$ for the maximum antimicrobial production at 37^{o}C after 36 hours over commercially available MRS and MH broth media.

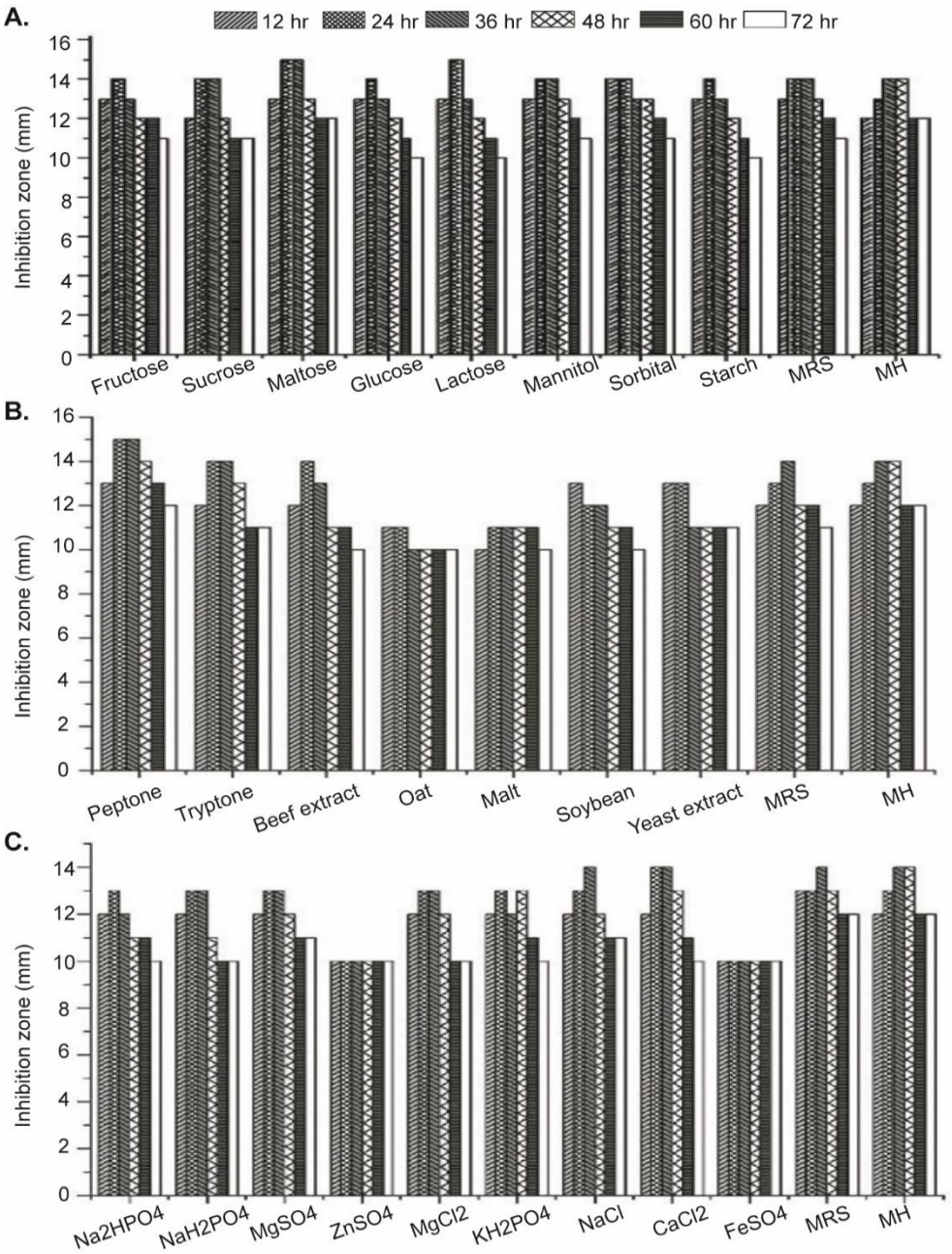

Figure 2.4: Effects of various nutrition sources in the production of antimicrobial compounds from *Bacillus CBSYD1*. (A) carbon sources (1%), (B) nitrogen sources (1%), (C) metal ion sources (0.01%). In all the cases, cultivation was performed in 250 mL flasks with 50 mL medium, at pH 7 and 37 °C, with shaking at 160 rpm.

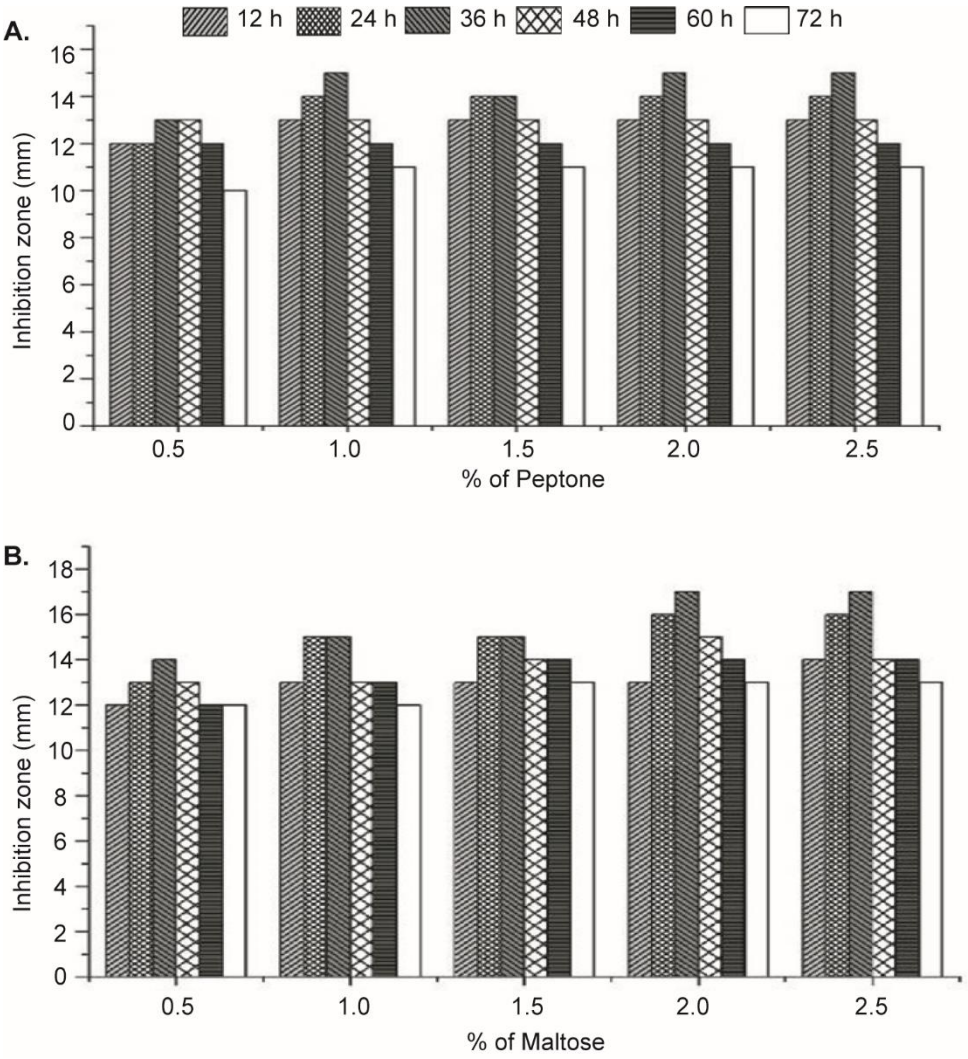

Figure 2.5: (A) 1% maltose and 0.01% $CaCl_2$ were combined with different percentage of peptone (0.5~2%) where 1% of peptone and 1% of peptone and 0.01% $CaCl_2$ were coupled with various percentage of maltose (0.5~2%) and (B) 2% of maltose. In all the cases, cultivation was performed in 250 mL flasks with 50 mL medium, at pH 7 and 37 °C, with shaking at 160 rpm.

2.3.6. Antibacterial activity of Cd.YD1

Ammonium sulfate (30–80% saturation) of bacteriocin was assayed against 33 different bacterial strains, and 40μL sample spotted on the disc containing 50μg protein. Table 2.1 shows as "+" for inhibition zone (mm) and "-" means not activity. Among 33 bacteria, Cd.YD1 showed an area of inhibition activity against 14 both Gram positive and negative bacterial strains. The highest activity showed against *Mycobacterium smegmatis* and followed by *Micrococcus luteus, Escherichia coli, Alcaligenes faecalis.* More importantly, it showed activity against resistant bacteria *Methicillin-resistant Staphylococcus aureus* S3 (*MRSA*) and *Vancomycin-resistant enterococci* 98 (*VRE*) and no activity against *Vancomycin-resistant Staphylococcus aureus* (*VRSA*).

Table 2.1: Anti-bacterial activity of bacteriocin Cd.YD1.

No.	Microorganism	Inhibition [a]	
1	*Staphylococcus aureus* KCTC 1928	+	Gram Positive
2	*Mycobacterium smegmatis* ATCC 9341	+++	
3	*Enterococcus faecalis* ATCC 29212	++	
4	*Micrococcus luteus* ATCC 9341	+++	
5	*Bacillus subtilis* ATCC6633	++	
6	*MRSA S3*	+	
7	*MRSA B15*	-	
8	*MRSA PB*	-	
9	*MRSA S1*	-	
10	*MRSA S3*	-	
11	*MRSA U4*	-	
12	*MRSA 4-5*	-	
13	*MRSA 5-3*	-	
14	*MRSA 693E*	-	
15	*VRSA*	-	
16	*VRE 2*	+	
17	*VRE 3*	-	
18	*VRE 4*	+	
19	*VRE 5*	+	
20	*VRE 6*	-	
21	*VRE 82*	-	
22	*VRE 89*	-	
23	*VRE 98*	+	
24	*IMP 100 (Pseudomonas aeruginosa)*	-	
25	*IMP 102 (Pseudomonas aeruginosa)*	-	
26	*IMP 120 (Pseudomonas hormaechei)*	+	
27	*IMP 123 (Pseudomonas baumanii)*	-	
28	*IMP 129 (Pseudomonas beteli)*	-	
29	*Candida albicans*	-	
30	*Escherichia coli* KCTC 1923	+++	Gram Negative
31	*Alcaligenes faecalis* ATCC 1004	+++	
32	*Pseudomonas aeruginosa* KCTC 1637	++	
33	*Salmonella Typhimurium* KCTC 1925	-	

[a] Expressed by the diameter of inhibition zones: -, no inhibition; +, <10 mm; ++,10-15 mm; +++, 16–18 mm;

2.4. CONCLUSION

In conclusion, it may be stated that strain *Bacillus amyloliquefaciens CBSYD1* was found as a potential probiotic candidate, which is mostly identical to *Bacillus amyloliquefaciens CBSYD1* might join at *Bacillus* probiotic short list. The *Bacillus amyloliquefaciens CBSYD1* could be designed as a probiotic candidate by producing bacteriocin. Further studies regarding purification, N-terminal sequence and hydrophilic nature of peptide are followed by to be sure of AMP. Relying on theses results, further investigations are being carried out.

CHAPTER THREE

ANTIMICROBIAL MECHANISM STUDY

3.1. INTRODUCTION

Antimicrobial peptides kill microorganisms in a relatively short time and disrupt cell integrity leaving the very little possibility for development of resistance in the target microbes (Brogden, 2005, Yeaman and Yount, 2003). On the other hand, in the case of conventional antibiotics, the mechanism of action is often more specific wherein they hinder the growth of bacteria, either by inhibiting protein synthesis, DNA synthesis, or bacterial cell wall synthesis or acting as metabolites or enzyme inhibitors (Brogden, 2005, Yeaman and Yount, 2003). Consequently, the chances of developing bacterial resistance are much greater using conventional antibiotics as compared to peptide antibiotics. Accumulating data suggest that antimicrobial peptides wield their cell lytic activity by several mechanisms, such as the carpet mechanism, barrel-Stave and through the toroidal mechanism (figure 3.1) (Brogden, 2005, Jenssen *et al.*, 2006, Yeaman and Yount, 2003). Other mechanisms have also been proposed such as the “self-promoted uptake of cationic peptides” or “aggregate model” (Jenssen *et al.*, 2006). Also, some of the antibacterial peptides inhibit bacterial cell growth similar to conventional antibiotics (figure 3.2) (Hale and Hancock, 2007, Nicolas, 2009).

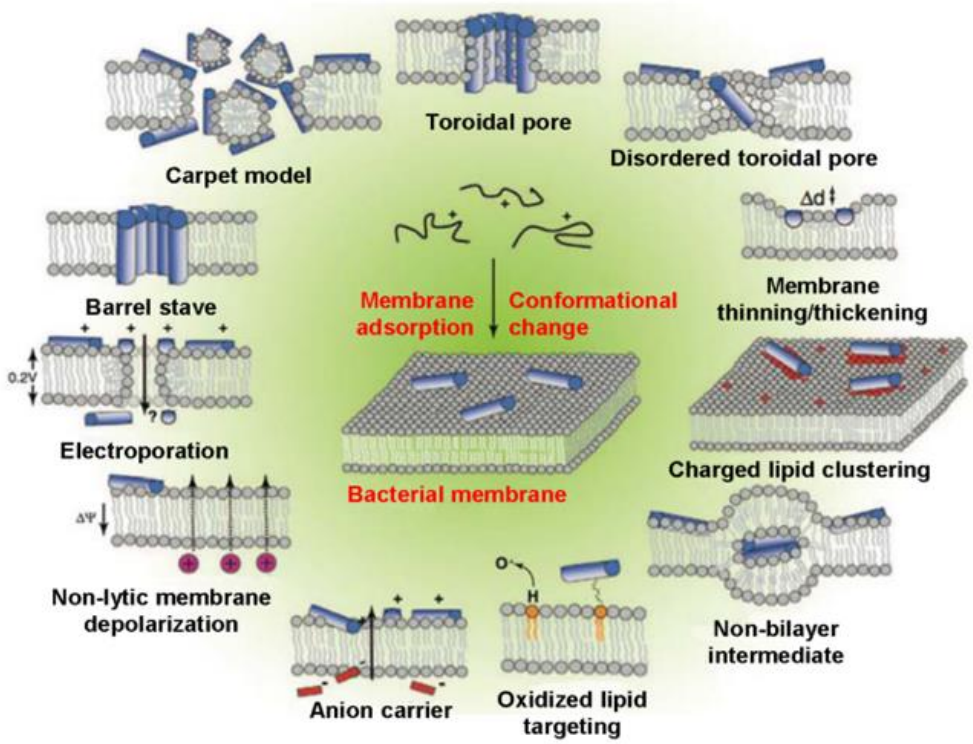

Figure 3.1: The carpet model, the barrel-stave model and the toroidal model for the antimicrobial peptide-induced killing of bacteria. Hydrophilic and hydrophobic regions of the peptide are shown in blue and red, respectively.

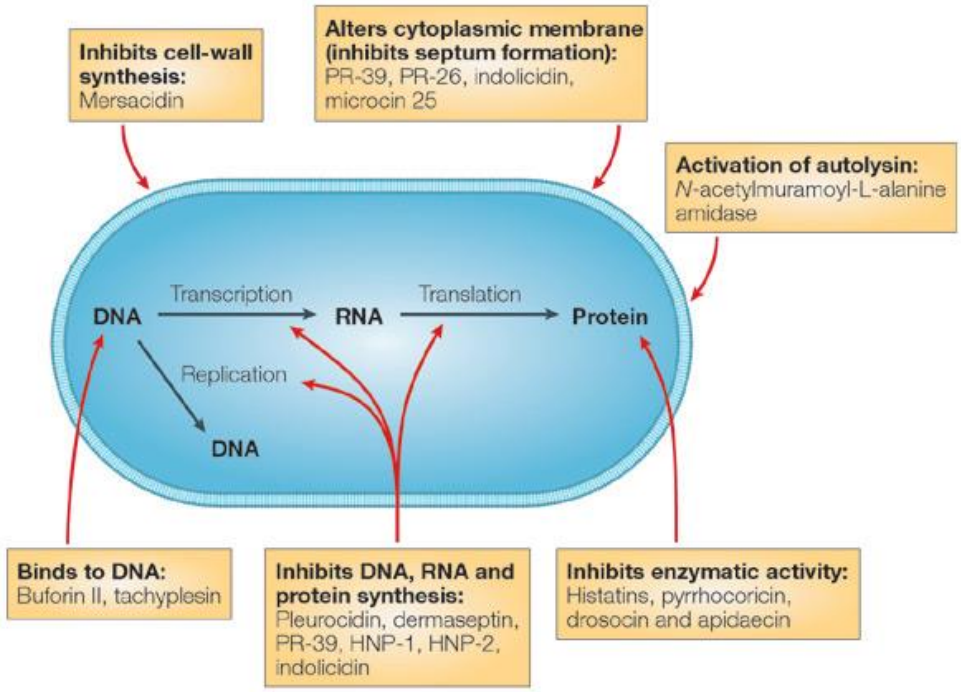

Figure 3.2: Alternative mechanisms of action of antimicrobial peptides.

Antimicrobial peptides cause bacterial cell lysis either by the formation of pores in the membrane or by destabilization of the entire membrane. Besides their direct antimicrobial function, antimicrobial peptides play a multifunctional role in animals including anti-inflammation, immunomodulation, wound healing, cytokine release, chemoattraction, angiogenesis and histamine release from mast cells (figure 3.3)

To understand the molecular basis of action and functioning of antimicrobial peptides, the determination of the structures and conformations of these peptides in solution, in lipid vesicles, or in the presence of membrane mimetic environment is essential.

Circular dichroism has been used to study their conformations or secondary structures. Information obtained from these studies depict that some antimicrobial peptides like BMAP-27, BMAP-28, cecropins, magainins, and others have unordered/random coil structures in an aqueous medium and adopt helical structures in a structure-promoting environment or the presence of lipid vesicles. Several other techniques like Fourier transformed infrared spectroscopy, X-Ray crystallography, Raman spectroscopy and oriented circular dichroism spectroscopy have also been used successfully to investigate the structure of several antimicrobial peptides. The structure obtained from these techniques in a particular environment is well correlated with their biological activities.

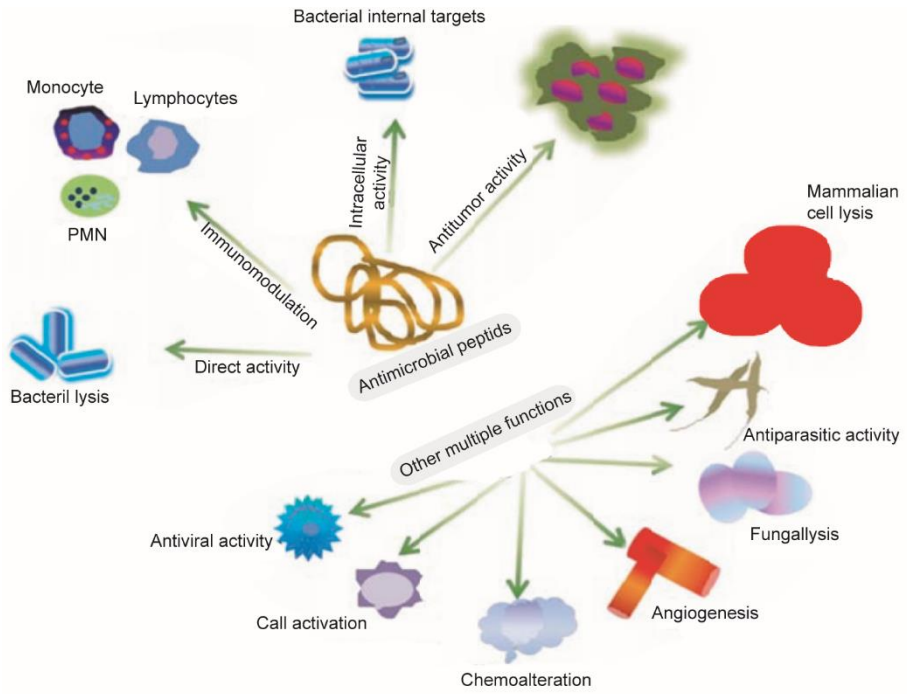

Figure 3.3: Demonstration of multifunctional activity of antimicrobial peptides.

Natural products are extensively used in our society. More than 60 % of approved drugs and drug candidates either are natural goods and/or derived from them. About 100,000 secondary metabolites (organic compounds, not directly involved in the growth, development, or reproduction of an organism) with molecular weight less than 2500 Da have been characterized; half from microbes and the other half from plants (Strobel and Daisy, 2003). AMPs and "bacteriocins" have increased attention for their possible application in controlling pathogenic microorganisms and infectious diseases. AMPs are ubiquitously produced by an extensive range of bacteria. *Bacillus*, a genus of significant attention to human health and therapeutic uses, provides a varied array of AMPs with different basic chemical structures. Numerous peptides with biological activities have been recognized from *Bacillus* and are under active study for their antimicrobial activity (Dischinger *et al.*, 2009, Teixeira *et al.*, 2009, Wu *et al.*, 2005). Bacteriocin or bacteriocin-like substances have also been described for some other important pathogens such as *Listeria monocytogenes* and *Streptococcus pyrogens* (Cherif *et al.*, 2001).

The history of use fermented vegetables as a fundamental source of beneficial bacteria is extensive. For over 20 decades, Koreans have consumed salted as well as fermented vegetables for example kimchi. The primary ingredients of kimchi are *Baechu* cabbage and radish; though, other vegetables like green onion, leek, and cucumber are also used for preparing various types of kimchi. Kimchi comprises high amounts of dietary fibers, vitamins, minerals, and other functional ingredients. Many previous studies regarding kimchi have reported that kimchi has exerted important effects like antioxidant,

antiatherosclerotic, anticancer, antimicrobial, antidiabetic, and antiobesity effects among others (Kim *et al.*, 2007, Islam and Choi, 2009, Park, 1995).

The rapid development and feast of bacterial resistance and the emergence of growing multi-drug resistant pathogens have created an essential need for the finding of novel classes of antimicrobial agents active against newly resistance microbial targets while evading current resistance action mechanisms. Elucidation of the mechanism(s) of action of antimicrobial drugs will help to characterize the interface of the pathogen with both the chemical as well as the host, design enhanced antimicrobials, allow to determine effective combinations of drugs, and also understand the development of microbial resistance. The examination of action mechanisms of developing compounds, not originated from target-based discovery, should be started early in their development stage to ease the alteration of the drug scaffold for betterment the selectivity of action and pharmaceutical profile. The production of AMPs from microbes isolated from fermented foods as well as their mechanisms of action have not been widely investigated.

In this part of work, we intended a two-step study. Initially, we isolated the strains from kimchi, screened, identified, characterized, and purified the potential AMP, and further characterized the purified AMP designated as YD1. Finally, upon the confirmation of the novelty of purified YD1, we investigated the antimicrobial mechanism of action.

3.2. MATERIALS AND METHODS

3.2.1. Culture media for YD1 production

The impact of various nutrient sources (carbon, nitrogen, and metal ion) on the antimicrobial compound production was determined, and media optimization was performed according to our previous report (Cho *et al.*, 2012). Fermentation was performed in 50 mL media in 250-mL Erlenmeyer flasks with continuous shaking at 160 rpm. Zone of inhibition was detected against *Mycobacterium smegmatis ATCC* 9341 at every step of purification and media optimization. Commercially available MRS and MH broth media were used as control media.

3.2.2. Antimicrobial activity

A filter paper disc (8 mm, Toyo Roshi Kaisha, Japan), is then placed on the surface of the petri dish (87 mm x 15 mm) containing Mueller Hinton Agar (MHA), saturated with the antimicrobial sample (40 µL). The plate was incubated at 37 ^{o}C, and a clear zone of inhibition adjacent the paper disc was measured in millimeter (mm).

An arbitrary unit per milliliter (AU/ mL) was defined as the reciprocal serial dilution after the last giving an inhibition zone. The titer of the antimicrobial substance solution, in AU/mL, was calculated following formula

$$AU/mL = (1000/d)\ D,$$

Where,

D = the dilution factor, and

d = the dose, the amount of antimicrobial substance solution added to each spot.

The minimal inhibitory concentration (MIC) was measured according to the method described by Weigand et al. (Wiegand *et al.*, 2008).

3.2.3. Purification of YD1

Sephadex G-25 and DEAE-Sepharose Fast Flow columns were obtained from Pharmacia (Uppsala, Sweden). CBSYD1 was cultured for 36 h in optimized media (1 % peptone, 2 % maltose, and 0.01 % $CaCl_2$). The culture supernatant was mixed with ammonium sulfate (30-80% saturation) and kept at 4 °C with overnight stirring. The precipitate was collected by centrifugation at 10,000 rpm for 30 min and re-dissolved in 10 mM Tris-HCl buffer (pH 7). The dialyzed sample was applied to a DEAE-Sepharose Fast Flow column (2.5 x 14 cm) pre-equilibrated with ten mM Tris-HCl buffer, pH 7. The column was washed with the same buffer and eluted with a linear gradient of KCl (0-1 M). Fractions of 3 mL were collected at a flow rate of 0.3 mL/min. Active fractions were collected, concentrated, and followed by for further purified with Sephadex G-25 column (1.5 x 28 cm) using the same buffer system.

3.2.4. Electrophoresis and In-situ analysis

The molecular weight of peptide was determined by Tricine SDS-PAGE (Schägger, 2006). The in-situ analysis was performed against indicator organism (~5 x 10^5 cfu/mL) by overlaying the processed gel from tricine SDS-PAGE [after washes with 50 mM Tris/HCl buffer (pH 7.5) containing 2.5 % Triton X-100 for several times] on 0.6 % agar on Muller-Hinton (DIFCO, USA) media and incubated at 37 °C.

3.2.5. Amino acid sequencing and computational analysis

Amino acid sequence of YD1 was determined by Edman degradation using a Procise Model 492 protein sequencer (Applied Biosystems, CA, USA). The amino acid sequence was analyzed using BLAST search against GenBank (http://www.ncbi.nlm.nih.gov/BLAST) and Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). The 3D structure projection was predicted by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) under the job ID S281576.

3.2.6. Stability of YD1

The thermal stability of YD1 samples was determined by exposure to 20, 40, 60, 80, and 100 ℃ for 30 min and 121 ℃/105 kPa for 15 min before analyzing the residual activity. Similarly, pH stability was determined over a range of pH 2–10 using 1 M NaOH or HCl. The effect of protease enzyme on YD1 stability was determined at two different enzyme concentrations (1 and 2 mg/mL).

3.2.7. Cytotoxicity

The cytotoxicity assay was performed according to our previous report by Choi et al. (Choi *et al.*, 2016). The murine macrophage cell lines Raw 264.7 were seeded in 96-well plates and 24 h later, treated with purified YD1, concentration ranging 8-120 μg/mL.

3.2.8. Synergism or antagonism of YD1 with antibiotics

YD1 was investigated for the interaction with antibiotics such as erythromycin (a protein synthesis translocation inhibitor), ceftriaxone sodium (a cell wall synthesis

inhibitor), and a quinolone (ciprofloxacin) that interferes with DNA gyrase supercoiling.

Log phase-grown *E. coli* and *MRSA* were cultured in MHB at 37 °C and diluted to a final inoculation density of 1 x 10^5 - 1 x 10^6 cfu/mL in a total of 200μL. The inhibition pattern indicates the interaction between the two compounds and the method enables the calculation of a fractional inhibitory concentration index (FICI), a numerical interpretation of the type of communication displayed.

For wells containing the lowest inhibitory combination of drugs, a fractional inhibitory concentration (FIC) is derived for each well from the following calculation:

$$= \frac{\text{MIC of compound A with B}}{\text{The MIC of compound A alone}} + \frac{\text{MIC of compound B with A}}{\text{The MIC of compound B alone}}$$

The FIC Index of ≤ 0.5 was considered to indicate synergism, a value ≥ 4 to indicate antagonism and all values > 0.05 to < 4.0 showed an indifferent interaction (Williams, 2001, Pasquale and Tan, 2005, Kadota, 1996).

3.2.9. Time-kill interaction between an antibiotic and YD1

Time-kill assays were conducted with concentrations corresponding to the MIC values of YD1 and erythromycin for reference strain, *E. coli*. Concentrations ranging from 8 to 256 μg/mL of YD1 or erythromycin were added to a bacterial suspension (1 x 10^5 - 1 x 10^6 cfu/mL) of the tested bacterial strain. Then, one mL of the tested suspension sample was collected every one hour for viable cell counting in MHA plate followed by incubation at 37 °C for 24 h.

3.2.10. Lysis of Gram-negative spheroplasts

Spheroplasts are Gram-negative bacteria in which nearly all of the outer membrane (OM) has been removed. Lysozyme destroys the peptide bonds in peptidoglycan and weakens the cell wall. *E. coli* was grown in 10 mL of MHB, incubated overnight at 37 °C on a shaker at 180 rpm. Each culture (100 µL) was used to inoculate 20 mL of fresh media and incubated at 37 °C for two h at 180 rpm. Spheroplasts were prepared as described by Kikuchi et al. (Kikuchi *et al.*, 2015)

Re-suspended spheroplasts and whole cells suspension were adjusted to OD 570 nm of 0.2, and 100 µL of each was added to a clear, flat-bottomed microtiter plate in duplicate wells. Ten microliters of YD1 in 10 mM Tris-HCl buffer (pH 7) was added to test wells for both preparations (spheroplasts and total cells) at the indicated different final concentrations. To the control, 10 µL of 10 mM Tris-HCl buffer (pH 7) was added instead of YD1. The percentage of intact spheroplasts or total cells was calculated as:

$$= \left(\frac{\text{Sample OD at time X}}{\text{Sample OD at time 0}}\right) \text{x } 100$$

The decrease in the OD of the suspension after addition of a membrane-active agent indicates lysis of spheroplasts.

3.2.11. DNA binding assay

The plasmid DNA (150 ng) of *E. coli* was incubated with increasing concentration of peptides in 20 µL of binding buffer [5 % glycerol, 10 mM Tris-HCl (pH 7), one mM EDTA, one mM DTT, 20 mM KCl, and 50 µg/mL BSA]. The reaction mixtures were kept at room temperature for 30 min, followed by addition of 4 µL of native loading buffer. An aliquot of 12 µL was applied to a 1 % agarose gel, and electrophoresis was performed in 0.5 X tris-borate-EDTA buffer.

3.2.12. Transmission electron microscopy (TEM)

TEM analysis was performed according to the method described by Lee et al. (2013) (Lee *et al.*, 2013). Ten milliliters of 10^6 cfu/mL *E. coli* suspension were exposed to 5x MIC (40 µg/mL) of YD1 to observe morphological changes and calculate the percentage killing of *E. coli* cells.

3.3. RESULTS AND DISCUSSION

3.3.1. Media of culture

The impact of various components of nutrition media on during the production of the antimicrobial compounds in the crude sample were highlighted. Among carbon sources, 2 % maltose endorsed the maximum production, and in the case of nitrogen sources, 1 % peptone promoted the maximum production. Sodium chloride or magnesium chloride (in 36 h) appeared to be the best among minerals. Lastly, media containing 1 % peptone, 2 % maltose, as well as 0.01 % $CaCl_2$ were determined to be optimum media for the maximum production of antimicrobial at 37 ˚C after 36 h over commercially available media, MRS and MH broth (figure 2.4 and 2.5). Characterization and screening as well as of novel AMPs are attractive owing to their possible applications for in the therapeutic use and food industry. Additionally, we conducted a preliminary investigation of the antimicrobial mechanism of action of purified AMP. Up to date, a few AMPs producing strains have been reported as isolated from kimchi (Teixeira *et al.*, 2009, Mah *et al.*, 2001, Wu *et al.*, 2005). In present experiment, we have reported the isolation of AMP producing bacterial strain, CBSYD1, from kimchi and studied the effect of several growth media and culture conditions for the production of maximum AMP, as well as determined the optimized media (2 % maltose, 1 % peptone, and 0.01 % $CaCl_2$) that promotes better production of YD1 over the commercially available media such as MRS and MH (figure 2.4 and 2.5). Growth media temperature and nutrients played a significant role in the production of bacteriocin (Todorov *et al.*, 2006). Antimicrobial activity was pointedly higher at 37 °C, consistent with the results from Lisboa *et al.* on bacteriocin, produced by *B. amyloliquefaciens*, (Lisboa *et al.*,

2010) as well as bacteriocin was very much stable at pH 4 to 9 and temperature up to 80°C, consistent with the outcomes by Todorov reports (Todorov *et al.*, 2006). The protease enzymes failed to change the antimicrobial activity of the pure YD1, which was not uncommon (Korenblum *et al.*, 2005).

3.3.2. Production, purification, and antimicrobial activity of YD1

The maximum production of YD1 was achieved in optimized media at 37 ˚C with shaking at 160 rpm for 36 h as shown in figure 3.4. The antibacterial activity (AU/mL) of YD1 lasted for 36 h (figure 3.4A), and up to 16 mm of a clear zone of inhibition was observed (figure 3.4B). Protein concentration increased considerably from 24 h (figure 3.4A) onwards. The antibacterial effects of YD1 against various Gram-positive and Gram-negative pathogenic bacteria were evaluated. YD1 was effective against *Staphylococcus aureus*, *MRSA*, and *VRE* with MIC values of 32 µg/mL, 16 µg/mL, and 32 µg/mL respectively; *E. coli* was found to be very sensitive (8 µg/mL) to YD1 whereas reference commercial antibiotics exhibited MIC values > 128 µg/mL (Table 3.1). Production of YD1 was carried out in optimized media. The purification of YD1 from the 36-h cultured supernatant (ammonium sulfate; 30-80 % saturation) of CBSYD1 is summarized in Table 3.2. The YD1 was purified to homogeneity by a two-step procedure (figure 3.5A-B), resulting in 40-fold purification and 12 % activity recovery. Tricine SDS-PAGE analysis showed a single band of YD1 corresponding to a molecular weight of ~1 kDa (figure 3.6). In the bioassay (in-situ) step, YD1 presented a zone of inhibition corresponding to the same protein band as that observed in Tricine SDS-PAGE. Stability studies revealed that YD1 remained completely stable at pH 4-9

and up to 80 °C, and its activity decreased sharply at or above 100 °C. The protease enzymes failed to alter the

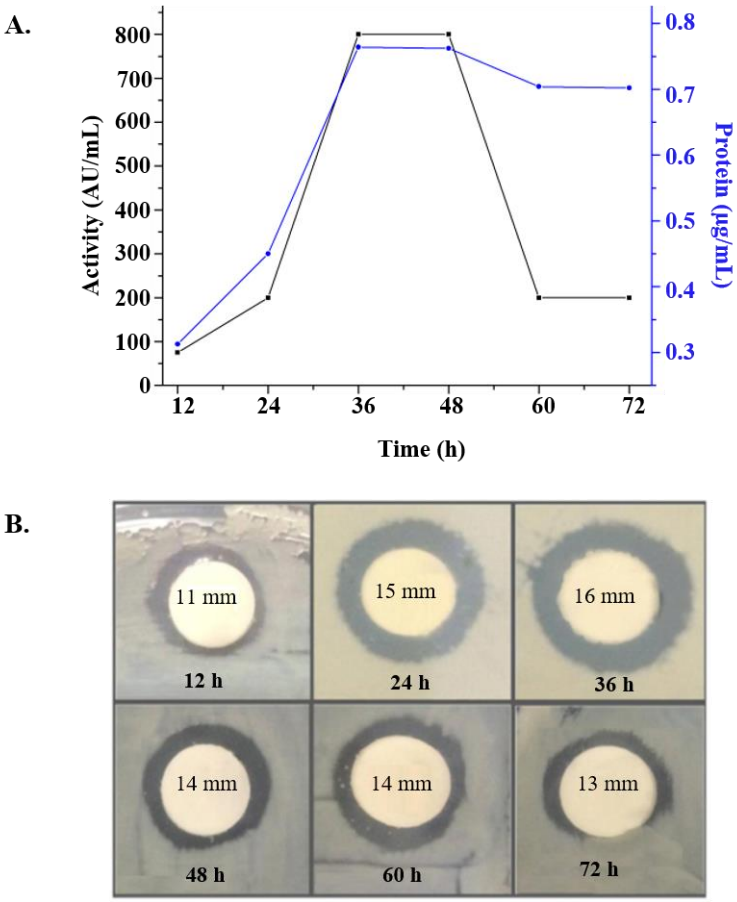

Figure 3.4: Production of bacteriocin and the inhibition activity against *Mycobacterium smegmatis ATCC* 9341. Total protein content (■) production increased hourly, and antibacterial activity AU/mL (▾) is maximum at 36 h

Table 3.1: Antimicrobial spectrum of YD1.

Microorganism	MIC (μg/mL)		
	YD1	Bacitracin	Vancomycin
Gram-negative bacteria			
Alcaligenes faecalis ATCC 1004	>128	>128	>128
Salmonella Typhimurium KCTC 1925	32	64	32
Escherichia coli KCTC 1923	8	>128	32
Pseudomonas aeruginosa KCTC 1637	16	>128	>128
Gram-positive bacteria			
Enterococcus faecalis ATCC 29212	64	2	0.5
Bacillus subtilis ATCC 6633	64	64	2
Staphylococcus aureus KCTC 1928	32	>128	>128
Micrococcus luteus ATCC 9341	64	64	1
Mycobacterium smegmatis ATCC 9341	8	>128	1
MRSA B15	32	64	>128
VRE 2	64	32	>128
VRE 5	64	64	>128
VRSA	>128	>128	>128

antimicrobial activity of the YD1. The homogeneity of YD1 was obtained by a two-step purification procedure, which resulted in 40-fold purification with 12 % activity recovery. The YD1 has lower molecular weight compared to a bacteriocin-like substance (BLS) as well as some other *Bacillus* AMPs (Teixeira *et al.*, 2009). YD1 was displayed to be effective against both Gram-positive and negative microbes as presented in Table 3.1. YD1 showed a better antagonistic effect over reference antibiotics (vancomycin and bacitracin) against MDR pathogens such as *VRE* and *MRSA*. Antimicrobial activity of YD1 was protuberant in comparison to reports of AMPs against MDR, and non-MDR pathogens of *Bacillus* have been published (Sandiford and Upton, 2012, Dischinger *et al.*, 2009, Zheng and Slavik, 1999).

Table 3.2: Purification steps of YD1.

Purification steps	Vol (mL)	Specific Activity (AU/mL)	Total activity (AU)	Fold	Recovery (%)
Cell free supernatant	1000	800	8×10^5	1	100
Ammonium sulphate	52	6400	3.328×10^5	8	41.6
DEAE-Sepharose	7	25600	1.792×10^5	32	22.4
Fast Flow Sephadex G-25	3	32000	0.96×10^5	40	12

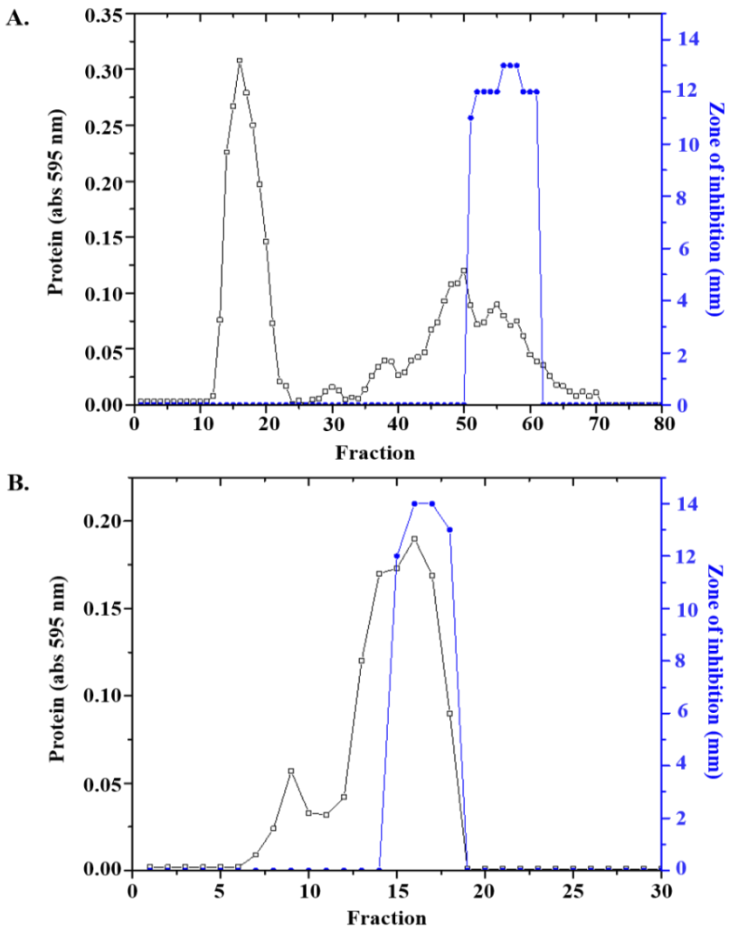

Figure 3.5: The elution profile of YD1 from a DEAE Sepharose Fast Flow ion exchange column (2.5 x 14cm) (A) and followed by the column Sephadex G-25 permeation column (1.5 x 28cm) (B).

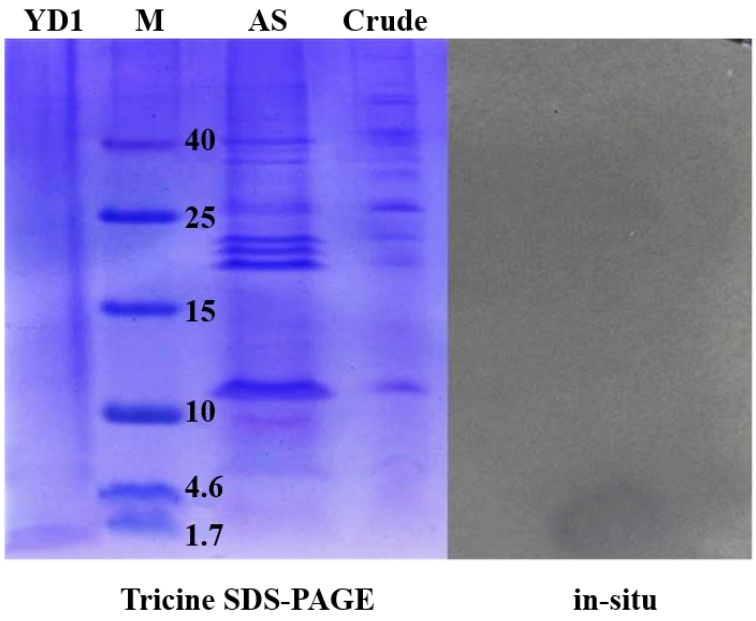

Figure 3.6: Tricine SDS-PAGE electrophoresis and in-situ analysis. Lane 1, Sephadex G-25 pooled pure YD1; lane 2, protein marker; lane 3, precipitation fraction of 30-80% ammonium sulfate (AS); and lane 4, broth sample. An inhibition zone appears in the in-situ assay is shown in the right panel.

3.3.3. Effects of YD1 in synergism and *E. coli* cell

Synergism activity was determined for YD1 and erythromycin with a mean FICI of 0.48, which suggested that YD1 may possess characteristics similar to erythromycin (Table 3.3 and figure 3.7). Afterward, TEM was employed to investigate the effects of peptide treatment on membrane integrity and intracellular changes in *E. coli*.

Table 3.3: Average FIC indices are resulting from chequerboard titrations between YD1 and selected antibiotics.

Antibiotic	**Ceftriaxone Na**		**Erythromycin**		**Ciprofloxacin HCl**	
Bacteria	Average FICI	Interpretation	Average FICI	Interpretation	Average FICI	Interpretation
***E. coli* KCTC 1923**	2.19	Indifferent	0.48	Synergism	0.92	Indifferent
***MRSA* B15**	1.15	Indifferent	0.51	Weak Synergism / Indifferent	0.99	Indifferent

FIC Index Interpretations: ≤0.5, synergism; >4, antagonism; >0.5 to <4.0 indifferent interaction.

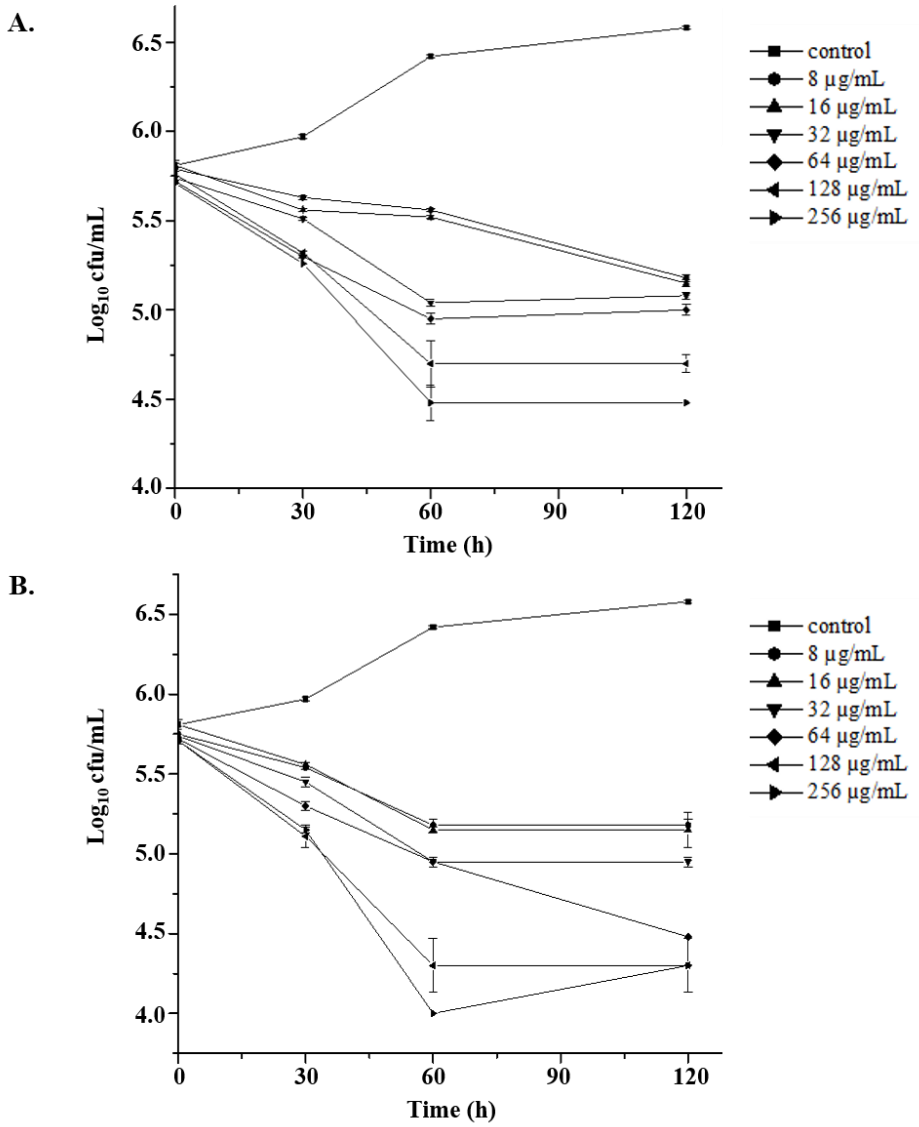

Figure 3.7: Effect of YD1 and erythromycin in combination on the rate of kill of *E. coli*. (A) Effect of YD1 (µg/mL) alone on *E. coli*.; (B) Effect of YD1 & erythromycin (µg/mL) in equal proportions on *E. coli*.

3.3.4. Amino acid sequencing, computational analysis, and antimicrobial study

The amino acid sequence residues of YD1 were determined as Ala-Pro-Lys-Gly-Val-Gln-Gly-Pro-Asn-Gly (Appendix-1). The amino acid sequence was determined by Edman degradation method and analyzed using BLAST search against GenBank (http://www.ncbi.nlm.nih.gov/BLAST) as well as Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). After experiencing computational and sequence analysis using different biological servers suggested that YD1 possesses entirely novel amino acid sequence with a coil-shaped secondary structure (figure 3.8) and has a net positive charge of +1 (Table 3.4). Comparison of YD1 sequence along with other closely related AMP sequences, presented in Table 3.4, showed differences. I-TASSER prediction analysis has suggested the presence of ligand binding sites of YD1 in a nucleic acid (NUC).

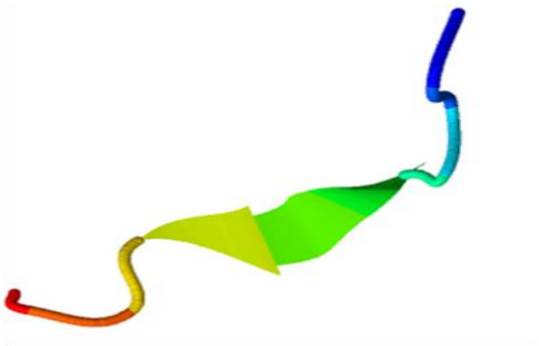

Figure 3.8: Three-dimensional secondary structure projections of YD1.

The sequence of YD1 was Ala-Pro-Lys-Gly-Val-Gln-Gly-Pro-Asn-Gly. APD search showed that the most neighboring similarity (40%) of YD1 was with Leucrocin I (AP02344), an AMP isolated from white blood cell extracts of crocodile, *Crocodylus*

siamensis (Pata *et al.*, 2011). Moreover, sequence alignment suggested other AMPs (Table 3.4) showed ≤ 40% similarity. Sequences comparison with other AMP sequences displayed that YD1 possesses unique and sequence and characteristics as well. It was reported that most of the AMPs are strongly cationic and minuscule (Yeung *et al.*, 2011). The features of the YD1 protein suggests that it is an AMP include: i) small size (0.924 kDa) with 10 amino acids; ii) cationic character (net charge

Table 3.4: Alignment between antimicrobial peptides and YD1.

Alignment	APD ID[a]	Similarity (%)	HR[b] (%)	Net charge	Gram activity	MW[c] (Da)
+ + N **G V Q** + **P** K Y A P K **G V Q** G **P** N G	AP02344	40	14	+1	+, -	798.89
A V **P** L I Y **K** R P **G** V Y **V** T K **P** K **G** K **A** + **P** + + + **K** + + **G** + + **V** Q G **P** N **G** +	AP02672	36.84	31	+5	+, -	2114.6
G I V D F **A** + **K G V** L **G** K I K **N** V L **G** I + + + + + **A** P **K G V** Q **G** + + P **N** + + **G** +	AP00320	35	52	+3	+, -	1941.3
F F C F **K G** + + T **P** C **G** + + A P **K G** V Q G **P** N **G**	AP02606	33.33	50	+1	+, -	1106.3
YD1 **-A-P-K-G-V-Q-G-P-N-G-**	Current study	100	20	+1	+, -	924.0

[a] Antimicrobial Peptide Database Identification
[b] Hydrophobic Residue
[c] Molecular weight (Dalton)

+1); and iii) theoretical pI value of 8.80 (calculated on based on amino acids sequences using ProtParam; server: http://web.expasy.org/protparam). Owing to its broad spectrum of antimicrobial effects and unique mechanism of action. Initial results from our study showed synergism between YD1 and known antibiotic erythromycin, a protein synthesis translocation inhibitor, indicating a strong relationship between their mechanisms of action (Odds, 2003) as well as the property of YD1 to interrelate with intracellular macromolecules rather than the cell wall machinery. Using an experimental microorganism *E. coli* (10^6 cfu/mL bacterial suspension) as a model organism, antimicrobial mechanism of action study was performed.

Typical cell membrane and intracellular contents were appeared in the YD1-untreatedbacterial cells (figure 3.9 i and ii). The *E. coli* cells exposed to 5x MIC (40 µg/mL) of YD1 exhibited several morphological changes after 4 h incubation. The treated cells showed a decrease in size and became irregular shape; seemed the loss of cytoplasm and light staining; likely plasmolysis; and appearance of bubbles in some (figure 3.9 iii). The microscopic images also have suggested that YD1 did not cause any disruption of the cytoplasmic membrane of *E. coli* cell. Further confirmation was established by observing the lack of lytic action of YD1 in whole cells of *E. coli* and > 88% intact surviving spheroplasts (figure 3.9 iv and figure 3.10A). We hypothesized that the mechanism activity of YD1 was associated with the inhibition/prevention of macromolecular synthesis or migration rather than with the damaging to the bacterial cell wall. To clarify the molecular mechanism of action, the plasmid DNA, from *E. coli*, the binding affinity of YD1 was determined by analyzing the electrophoretic movement of the DNA band. As shown in figure 3.10B, YD1 suppressed the migration of DNA in

a concentration-dependent manner, at 40µg/mL concentration, the DNA migration was suppressed 80% as well as completely suppressed the migration of the DNA at 10x MIC (>80 µg/mL). Cytotoxicity or cell viability investigation showed that YD1 was not toxic to RAW 264.7 macrophage cells. As shown in figure 3.11, YD1 did not display cytotoxicity at 120 µg/mL and found ~90% of the cells were viable.

This antibacterial activity might result from effects on cellular metabolism. Kang et al. (2015) and Li et al. (2014) also reported the similar observation of the morphological variation of *E. coli* cells when treated with berberine as well as α-terpineol respectively (Li *et al.*, 2014, Kang *et al.*, 2015). In figure 3.9, suggested that YD1 possesses cell-penetrating and translocation ability, which makes the YD1 responsible for its antimicrobial activity without damaging bacterial cell wall and causing without cytotoxicity in RAW 264.7 macrophage cells (figure 3.11). A recent review of Splith and Neundorf (2011) enlisted the commonly used cell-penetrating peptides (CPP) as well as AMPs (Splith and Neundorf, 2011). Furthermore, the report from Suet al and Boman et al. and their colleagues that positively charged residues in the α-helical sequence, and Arg-rich peptide, associate with the lipid phosphate groups to neutralize the Arg residue and thus allow the peptide translocation across the membrane (Su *et al.*, 2009, Boman, 1995, Åmand *et al.*, 2008). Recently, another report by Xie et al. (2011) suggested that the position of Pro residue in buforin II was more important than the overall α-helical content for the peptide translocation (Xie *et al.*, 2011).

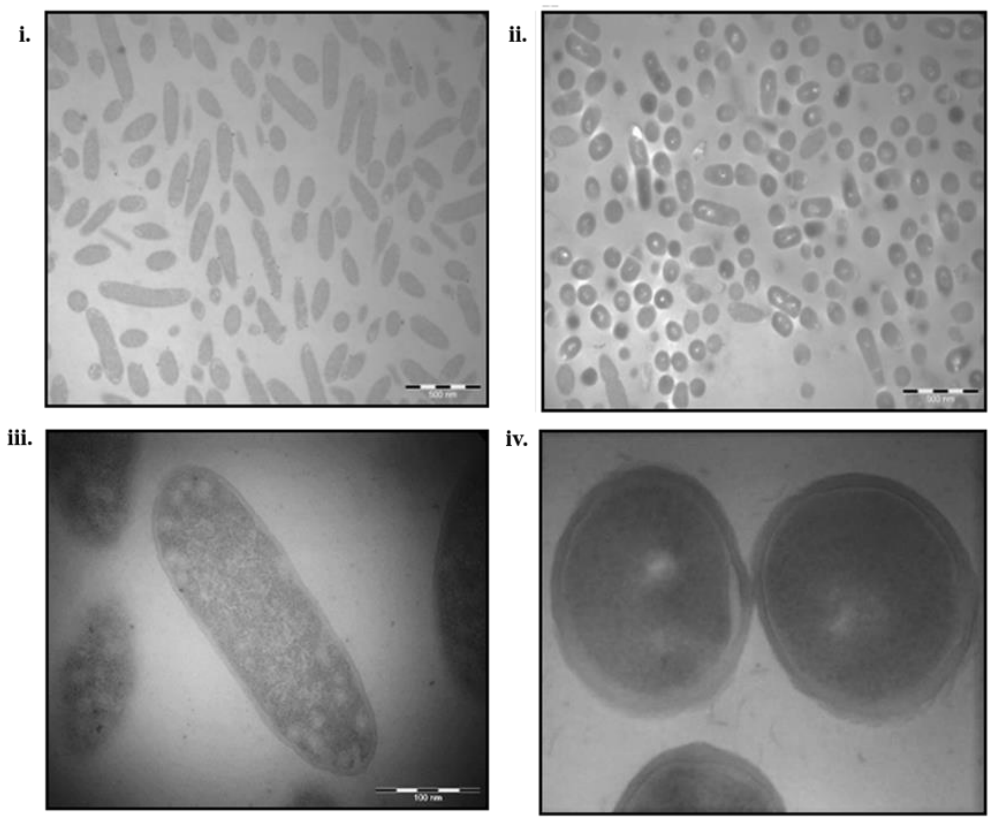

Figure 3.9: Transmission electron micrographs (TEM) of *E. coli* cells. i) and ii), untreated control of whole (x 40K zoom) and single cell (x 100K zoom) bacterial suspension, respectively, showing typical *E. coli* shapes; iii) and iv), *E. coli's* whole (x 40k zoom) and single cell (x 100K zoom) suspension, respectively, treated with 5x MIC (40 µg/mL) of YD1.

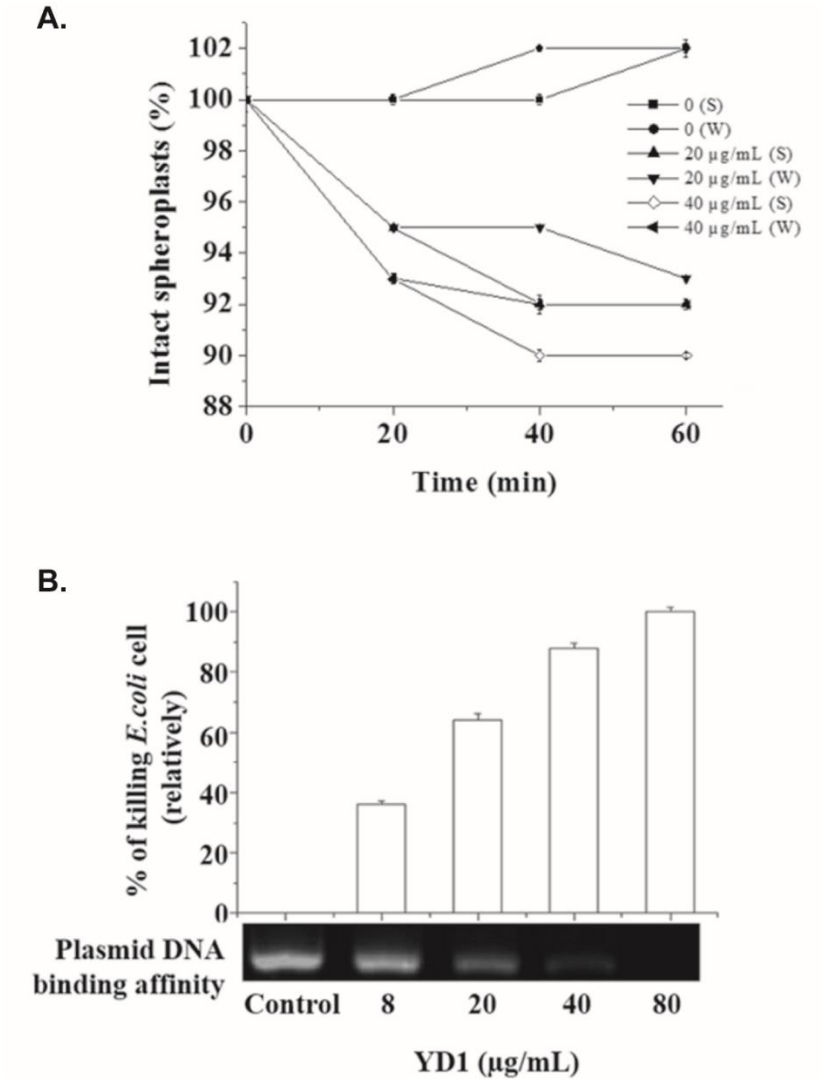

Figure 3.10: (A) Percentage (%) of *E. coli* spheroplasts and total cells remaining intact during exposure to YD1. Assays were performed in Tris buffer with 20 % w/v sucrose at OD 570 nm. (B) Plasmid DNA binding affinity measured for increasing amounts of YD1 (concentration ranging from 8-80 μg/mL).

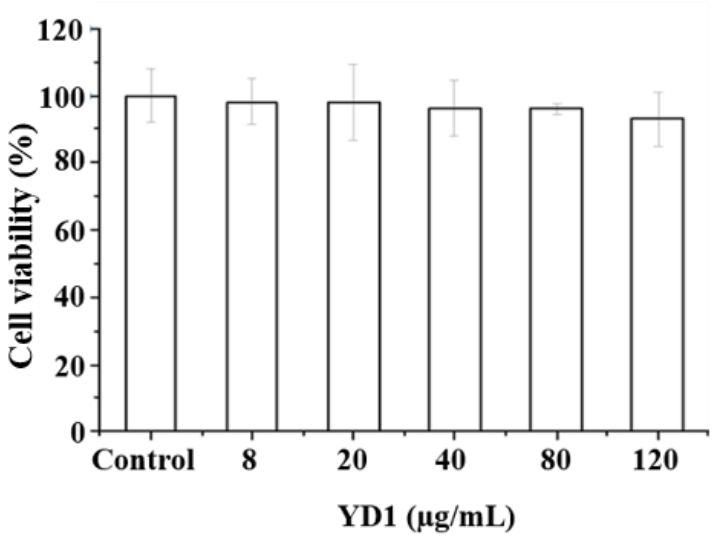

Figure 3.11: Effects of YD1 on cytotoxicity or cell viability. Cell viability was measured after 24 h incubation. Percentage of viable cells were tested with MTT assay in Raw 264.7 cells. Raw 264.7 cells were incubated in the presence or absence of 8-120 μg/mL YD1 for 24 h. Each bar shows the mean ± S.D of three independent experiments performed in triplicate.

The outcomes are consistent with these previous reports, YD1, a positively charged peptide, contains 3 glycines ([$G^{4,7,10}$]) as well as 2 proline ([$P^{2,8}$]) residues out of 10 amino acids. The antimicrobial strength of YD1 might not be determined solely by the coil-shaped secondary structure, but other factors, for instance, the type of positive charge and the location of Pro might also be necessary. In 2012, Jang and his co-workers (Jang *et al.*, 2012) reported two cell-penetrating motifs Q-F-P-V-G and Q-W-P-V-G for peptides Buforin IIb and Buforin IIIa, respectively. Analogously, Q-G-P-N-G is the likely predictable cell-penetrating motif for YD1. Additionally, I-TASSER

prediction analysis has shown the presence of ligand binding sites in YD1 sequence for a nucleic acid (NUC) (figure 3.12) which was a substantial agreement with the DNA binding affinity result shown in figure 3.10B.

In spite of the numerous proposed molecular processes regulated by AMPs, it is still uncertain which, if any, of the hypothesized mechanisms, is answerable for their biological activity (Pálffy *et al.*, 2009). Based on our results, the antimicrobial mechanism of YD1 may be

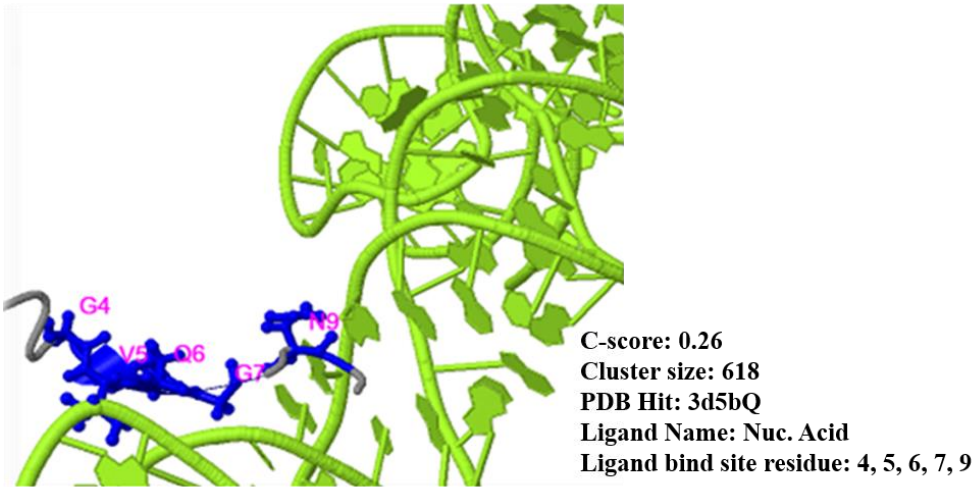

Figure 3.12: The biological annotations of the target proteins by COACH based on the I-TASSER structure prediction. Predicted ligand binding sites of YD1.

driven by cell-penetration as well as translocation inside the cell via a voltage-gated ion channel possibly or another mean (currently in process) and followed by interaction with bacterial DNA and ultimately leading to bacterial cell death (figure 3.13). After

elucidation of complete structural information of the YD1 will allow to study the in-depth antimicrobial mechanism of action as well as synthesize AMP analogs.

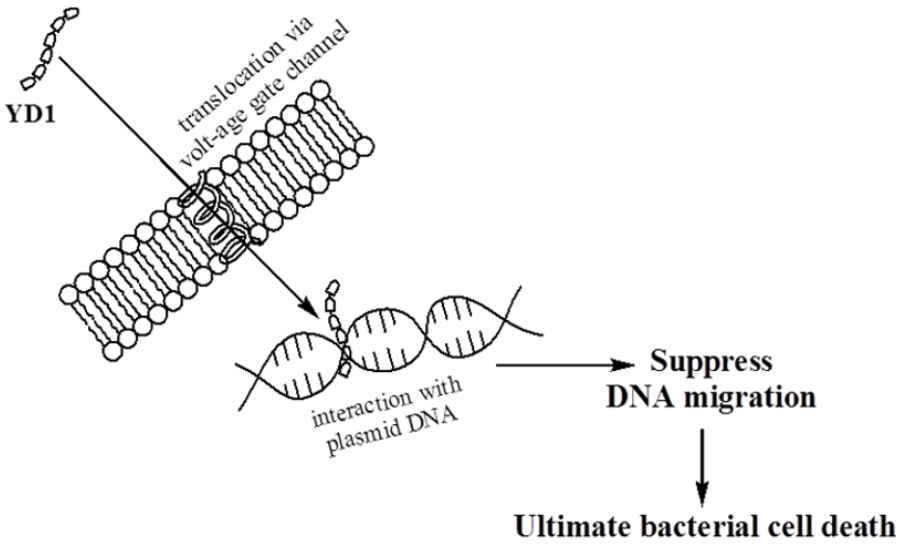

Figure 3.13: A diagram of the hypothetical antimicrobial mechanism of action of novel AMP YD1

3.4. CONCLUSION

In summary, the results presented in this study demonstrate the activity of the novel AMP YD1 purified from *Bacillus amyliquefaciens CBSYD1* isolated from Korean fermented kimchi. A broad-spectrum glycine-rich, low-molecular-weight AMP YD1 displays a unique antimicrobial mechanism of action characterized by its affinity for bacterial DNA, without causing any damage bacterial cell wall. Strong antimicrobial effects of YD1, against resistant pathogens, for example, *MRSA*, *VRE*, and *E. coli*, were observed.

Our results collectively propose that YD1 may serve as a promising agent for developing therapeutic candidate for the treatment of bacterial infections and endotoxin shock.

CHAPTER FOUR

ANTIOXIDANT MECHANISM STUDY

4.1. INTRODUCTION

The arena of free radical research is experiencing a tremendous progression in last few years. The formation of free radicals *in vivo* is primarily originated by the taking of molecular oxygen, which, because of its structure, itself a radical species since oxygen is the ultimate electron acceptor in the flow system of the electron that produces energy ATP. However, problems may take place when the electron flow is unpaired (free radical). The free radical theory was first coined by Denham Harma (Harman, 1955). A free radical is any species capable of existence, containing one or more unpaired electrons in an atom (Bhalodia *et al.*, 2011). Free radical species include reactive oxygen species (ROS) and reactive nitrogen species (RNS), which play a vital role in the process of aging (Harman, 1955). A natural by-product of oxygen (O_2) is constantly formed in aerobic organisms as metabolism, reactive oxygen species (ROS) are involved in many cellular complex processes, such as gene expression, signal transduction mitogenesis, and the regulation of cell proliferation. Various pathological conditions such as aging, cancer, inflammation, and hepatic diseases, are associated with high ROS, and therefore, interest has recently been growing in the use of antioxidants for the upkeep of human health as well as the prevention as well as treatment of some diseases (Martin *et al.*, 1996, Migliore and Coppedè, 2009, Reuter *et al.*, 2010).

In a normal condition of the healthy human body, the formation of pro-oxidants in the form of RNS and ROS is efficiently reserved in check by the numerous antioxidant defense levels. ROS and RNS include radicals such as superoxide ($O_2\cdot-$), hydroxyl (OH·), peroxyl ($RO_2\cdot$), hydroperoxyl ($HO_2\cdot$), alkoxyl (RO·), peroxyl (ROO·), nitric

oxide (NO·), nitrogen dioxide (NO_2·), and lipid peroxyl (LOO·) and non-radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($1O_2$), peroxynitrate ($ONOO^-$), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), and lipid peroxide (LOOH) (Pham-Huy *et al.*, 2008), radicals which are generated spontaneously in cells through metabolism (Sarma *et al.*, 2010).

4.1.1. Environmental stress and factors

Environmental stresses can cause metabolic changes in animals, plants, and humans that either increase the production of reactive oxygen and nitrogen species (RONS) or decrease the antioxidant production (Alessio and Hagerman, 2006). Occupational exposure to metals, benzene, cement dust, and multiple other agents is bounded with increased DNA oxidation, lipid peroxidation, and decreased levels of vitamin C and E (Yoshioka *et al.*, 2008, Taipau *et al.*, 2008, Rossner *et al.*, 2008, Aydin *et al.*, 2004, Kim *et al.*, 2004) (figure 4.1) that leads to diseases such as neurotoxicity, cancer, liver damage, cardiovascular diseases, inflammation, respiratory diseases and through the mutation in gene expression that promote apoptosis within cells, and inflammation systemically.

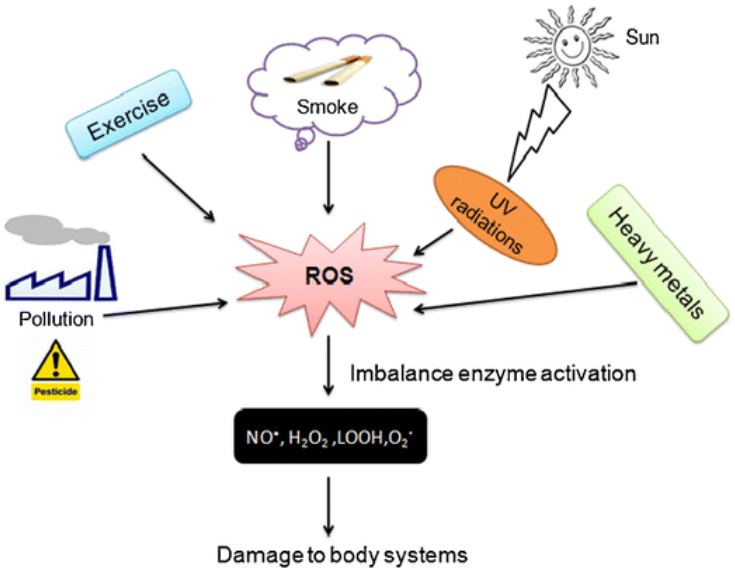

Figure 4.1: Overview of generation of ROS by various factors.

4.1.2. ROS and its complication in humans

Exposure to environmental or pathological agents such as atmospheric pollutants, cigarette smoking, ultraviolet rays, radiation, and toxic chemicals results in an imbalance between the pro-oxidants and antioxidants, which leads to oxidative stress (Videla, 2009). Pro-oxidant antioxidant balance in the cell is shifted towards the pro-oxidants if the formation of oxygen species is greatly increased or when the number of antioxidants is reduced. This state is called oxidative stress. Oxidative stress is basically caused by the following two main mechanisms: (1) reduction of antioxidant concentration due to mutated antioxidant enzymes, toxins, or the reduced intake of natural antioxidants; (2) the number of oxygen-, nitrogen-, or carbon-based reactive species derived from activated phagocytes is increased in the case of chronic inflammation (Somogyi *et al.*, 2007). The oxidative stress can damage important biomolecules such as lipids, nucleic acids, proteins, polyunsaturated fatty acids, and

carbohydrates and of DNA damage (Jeyadevi *et al.*, 2013, Yildirim *et al.*, 2001) which can lead to mutations. Cellular constituents do not successfully scavenge these ROS; they can stimulate free radical chain reactions, subsequently damaging the cellular biomolecules and finally leading to disease conditions (Halliwell and Gutteridge, 2015) such as Alzheimer's disease, chronic degenerative diseases, stroke, rheumatoid arthritis, diabetes, and cancer (Yildirim *et al.*, 2001, Sabir *et al.*, 2012).

Antioxidants have also been shown to provide cellular protection against oxidative stress, by direct or indirect pathways depending on their mechanism. In the case of direct pathways, antioxidants scavenge the reactive oxygen and nitrogen species by consumption or chemical modification. In contrast, upregulation of phase II detoxifying and antioxidant enzymes are involved in indirect pathways. To defend against oxidative stress, aerobic organisms have effective antioxidant networks that involve primary enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), as well as inducible phase II detoxifying enzymes (heme oxygenase-1 (HO-1) and NAD(P)H-quinone dehydrogenase 1 (NQO1) through the activation of NF-E2-related factor-2 (Nrf-2) (Kim and Jang, 2014). Furthermore, some antioxidants display their activity both directly and indirectly and are therefore called bifunctional antioxidants (Dinkova-Kostova *et al.*, 2007).

Synthetic antioxidants are widely used in the treatment of oxidative damage. However, their long-term use and high doses are associated with side effects including cancer (Kahl and Kappus, 1993). Hence, scientists have continued to show interests in screening novel biological compounds from various organic sources. Peptides have

different antioxidant activities based on the amino acid composition of their sequences, which varies depending on the proteinase used (Pena-Ramos and Xiong, 2001). Different amino acids, for instant tryptophan, histidine, leucine, and cysteine have been reported to display radical-scavenging activities (Park *et al.*, 2001, Chen *et al.*, 1996). Moreover, it has been indicated that the amino and carboxylic group in branches of acidic (aspartic acid and glutamic acid) and basic (arginine, lysine, and histidine) amino acids enhanced metal chelation through their charge properties (Saiga *et al.*, 2003, Liu *et al.*, 2010). The history of using fermented vegetables as a source of the beneficial bioactive compound is extensive.

4.1.3. Role of exogenous antioxidants in oxidative stress

Exogenous antioxidants are antioxidants that we get from our diet by eating antioxidant-rich foods. Exogenous antioxidant includes carotenoids, tocopherols, ascorbate, bioflavonoids, anthocyanidins, phenolic acids, etc. (Bouayed and Bohn, 2010, Urso and Clarkson, 2003). Phenolics and Flavonoids are the primary antioxidant compounds of plant origin. Phenolic compounds are supposed to defend the plants against tissue damages as they oxidize as well as combine with proteins. Similarly, flavonoids can act as antioxidant agents by acting as hydrogen donors or chelating metals. Considering all of these factors, there is no doubt that exogenous antioxidants are going to become a part of the food system. On the other hand, excess intake of polyphenols also leads to severe damage as follows. It was reported that long-term treatment of cells with polyphenols could increase endothelial nitric oxide synthase expression and calcium (Ca^{2+})/calmodulin (CaM) complex (Stoclet *et al.*, 2004) which also leads to cell damage. At the same time, many polyphenols like resveratrol and quercetin have been

reported to exhibit a protective effect against aging (Belinha *et al.*, 2007, Markus and Morris, 2008). An antioxidant response element mediates the activation and induction of c-fos and c-jun mRNAs by phenolic antioxidants in a specific and dose-dependent manner (Choi and Moore, 1993). Indeed, scientific studies report a major inverse association between polyphenol intake and aging. Antioxidants may prevent the production of oxidants/scavenge the radicals by chelation of transition metals, inhibit oxidants from attacking cellular targets, block the propagation of oxidative reactions, induce the expression of endogenous antioxidants, and modulate the pathways of signal transduction as well as gene expression via their reducing properties (figure 4.2). The free radical scavenging mechanism which exists already in the system is inefficient, and hence dietary intakes of antioxidant-rich compounds become important (Asseervatham *et al.*, 2012). Herbal products could be a better choice to meet the objective of the conclusion an appropriate treatment for reducing the free radicals generated from environmental and physiological factors.

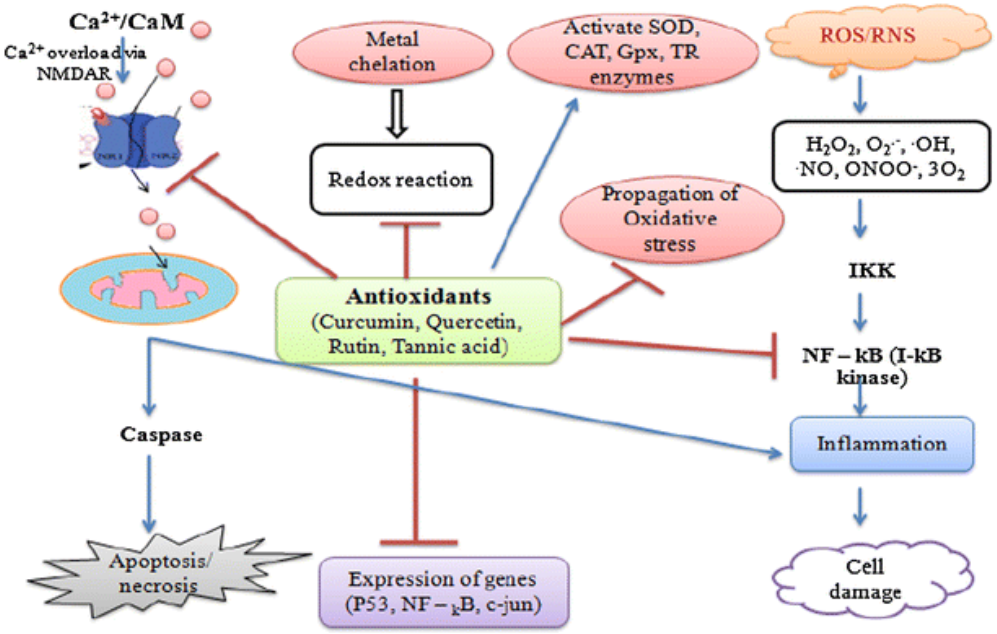

Figure 4.2: Mechanism of antioxidant defenses against free radical-induced damage in humans. Polyphenols or antioxidants affect glutamate-mediated Ca^{2+} influx through NMDAR, thereby reducing glutamate-induced Ca^{2+} influx into mitochondria; antioxidants interfere caspase pathway and inhibit cell damage; antioxidants inhibit the propagation of oxidative stress; it activates the expression of antioxidant enzymes CAT, SOD, GPx, TR; it interferes metal chelation and inhibit redox reaction; antioxidants inhibit the expression of redox genes. N-Methyl-D aspartate receptors (NMDAR), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), thioredoxin reductase (TR), calcium (Ca2+), calmodulin (CaM), IKK (I-kB kinase), NF-kB (nuclear factor), reactive oxygen species (ROS), reactive nitrogen species (RNS), hydroxyl radical (·HO), superoxide anion ($O_2 \cdot -$), hydrogen peroxide (H_2O_2).

4.1.4. Antioxidant peptides from fermented food kimchi

For over 2,000 years, Koreans have consumed salted and fermented vegetables such as kimchi, which contains high levels of vitamins, minerals, dietary fibers, and other functional ingredients. Studies have reported that kimchi has anticancer, antimicrobial, antioxidant, antiatherosclerotic, antidiabetic, and antiobesity effects amongst others. For a long time, microbial fermentation has been one of the most important sources of proteolytic enzymes, which can efficiently hydrolyze proteins to prepare bioactive peptides (He *et al.*, 2012). No evidence has found *Bacillus amyloliquefaciens* as a probiotic bacterium, and few probiotic bacteria like *Bacillus subtills*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, and *Bacillus licheniformis* are being used for human medicinal and food supplement.

In the current study, we identified the probiotic-like *Bacillus* strain from kimchi and purified an antioxidant peptide. After which, we characterized the antioxidant peptide designated as YD1 and studied the antioxidant mechanism of action after confirming the novelty of purified YD1.

4.2. MATERIALS AND METHODS

4.2.1. Radical-scavenging activity assays

4.2.1.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH radical scavenging assays were used for the evaluation of free radical scavenging activity of YD1 and were conducted following protocols described elsewhere with minor modifications (Tailor and Goyal, 2014). Various concentrations (1.5-18 µg/mL) of YD1 were studied. Ascorbic acid was tested as a standard antioxidant compound. The capacity of DPPH radical scavenger was determined using the following formula:

$$\text{DPPH radical-scavenging activity (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100$$

Where $\text{Abs}_{\text{control}}$ = absorbance of the control and $\text{Abs}_{\text{sample}}$ = absorbance of the sample. All samples were analyzed in triplicate.

4.2.1.2. 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical scavenging assay

A previously described method for the ABTS assay was adopted with slight modifications (Re *et al.*, 1999). The stock solution which could stand in the dark room for 16 h at standard room temperature and equal volumes of 7 mmol/L ABTS contained salt as well as 2.5 mmol/L potassium persulfate. Diluted the resultant $ABTS^{\bullet+}$ solution with methanol until an absorbance adjusted to 0.705 ± 0.001 at 735 nm was obtained. Variable concentrations of the sample could react with 198 µl of the $ABTS^{\bullet+}$ solution and the absorbance 734 nm was used to record the reading. Ascorbic acid was tested as

a standard antioxidant compound. The capacity to scavenge the $ABTS^{\bullet+}$ was estimated using the following formula:

$$\text{ABTS}^{\bullet+}\text{-scavenging activity (\%)} = \left[\frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}}\right] \times 100$$

Where $Abs_{control}$ = absorbance of the control and Abs_{sample} = absorbance of the sample. All samples were analyzed in triplicate.

4.2.1.3. Ferric reducing antioxidant power (FRAP) assay

For the measurement of reducing power, a FRAP assay was carried out as described previously (Benzie and Strain, 1996) with slight modifications. In short, the FRAP reagent contained acetate buffer (300 mM, pH 3.6), ferric solutions (20 mM $FeCl_3 \cdot 6H_2O$), and 2,4,6-Tris-(2-pyridyl)-s-triazine (TPTZ) solution of 10 mM mixed in 40 mM HCl at a proportion of 84:8:8. An aqueous sample of 2 µl at various concentrations and 198 µl of reagent FRAP were mixed, as well as the absorbance 595 nm was used to record the reading. Ascorbic acid was also tested as a standard antioxidant compound.

4.2.1.4. Cupric-reducing antioxidant capacity (CUPRAC) assay

The CUPRAC of YD1 was determined according to a previously described method (Apak *et al.*, 2005) with slight modifications. A solution of 7.5 mM neocuproine, 10 mM $CuCl_2$, and buffer (pH 7.2) 1 M ammonium acetate was mixed, and this final solution was added to the samples. Following one hour incubation of mixture at standard room temperature and 450 nm the absorbance was measured. Ascorbic acid was used as a standard compound.

4.2.1.5. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was carried out according to a previous report (Ou *et al.*, 2002). Trolox, an analog of vitamin E which is water-soluble, was used for positive control. The ORAC assay was conducted at 37 °C in pH 7.4 with a blank sample parallelly. The analyzer was automated to record the fluorescence of 200 mM fluorescein every minute after adding of 20 mM AAPH with an excitation (480 nm) as well as an emission wavelength (520 nm). The outcomes were calculated by using the variances in the areas under the curves of fluorescence decay among the blank and experimental sample as well as the area under the curve (Net AUC) values were expressed.

4.2.2. Cell viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Choi *et al.*, 2016). RAW 264.7 cells, were used to experiment cell viability, were seeded into a 96-well flat bottom microplate (density of 2×10^4 cells per well) and then incubated for one hour at 37 °C. The cells were treated later with several concentrations of YD1(1.5-72µg/mL). After 24 h of incubation, MTT (5 mg/ml in PBS) of 20 µl solution was added to every well, and afterward, the plate was incubated for next 2 h. The microplate reader (Victor3, PerkinElmer) was used to record absorbance at 450 nm.

4.2.3. Measurement of cellular NO generation

The concentration of nitric oxide (NO) in the medium was measured by an indicator, Griess reagent, of NO production. RAW 264.7 cells were seeded into a 96-well flat bottom microplate (at a density of 2×10^4 cells per well) and then incubated at 37°C for

one hour. The freshly seeded cells were then treated with lipopolysaccharide (LPS)of 1 μg/mL at concentrations (1.5-18μg/mL) of YD1. Afterward, the mixture incubates 24 h, NO concentration in the supernatants was estimated adding Griess reagent. The mixtures absorbance was determined using a microplate reader at a wavelength of 520 nm.

4.2.4. Measurement of intracellular ROS generation

Cellular oxidative stress is developed due to reactive oxygen species (ROS) generated by LPS was measured spectrofluorimetrically using the DCFDA method. Raw 264.7 cells were first cultured at a density of 2×10^4/mL in 96-well plates with DMEM for 24 h. Cells were pre-treated with various concentrations of YD1. After one hour, cells were stimulated with LPS and incubated for an additional 24 h. After washing two times with phosphate-buffered saline (PBS), cells were then treated with 25 μM DCFDA for 30 min at 37 ℃. The intensity of fluorescent was recorded at an excitation wavelength (485 nm) as well as an emission wavelength (528 nm) by using a fluorescence microplate reader (Victor3, PerkinElmer, Waltham, MA, USA).

4.2.5. Preparation of cell lysates and western blot

According to standard protocol, Raw 264.7 cells lysates were prepared and mixed in sample buffer (250 mM Tris-HCl, pH 7.2), 10% SDS, 0.5% bromophenol blue, 0.5 M DTT, 5% 2-mercaptoethanol, 50% glycerol), and denatured for 5 min at 100 ℃. For nuclear protein extraction, a nuclear/cytosolic fractionation kit (Cell Biolabs, San Diego, USA) was used. 10% SDS-PAGE was used to separate sample proteins (20 μg). Following placed to nitrocellulose membranes which were obtained from Whatman,

(Dassel, Germany), the membranes were then incubated for overnight with 5% skimmed milk and a range of antibodies. Anti-SOD1, anti-catalase (CAT), anti-HO-1, anti-glutathione peroxidase 1 (GPx-1), anti-Nrf-2, and β-actin antibodies (Santa Cruz Biotechnology, CA, USA) were used as primary antibodies. Anti-goat IgG-horse radish peroxidase (HRP) also from Santa Cruz, USA and as secondary antibodies, anti-rabbit IgG-HRP (Santa Cruz) were used. The antigen-antibody reaction was determined using a solution system, ECL, (Perkin Elmer).

4.2.6. Gene expression and reverse transcription PCR (RT-PCR) analysis

According to the manufacturer's (Life Technologies, Gaithersburg, MD, USA) protocol using TRIzol reagent, total RNA was isolated from RAW 264.7 cells. An RT-&GO Mastermix (MP Biomedicals, Seoul, Korea) was used for the RNA (1–10 μg) transcription into first-strand cDNA using, as well as PCR templates were the product transcription.

RT-PCR was carried out using a Takara PCR thermal cycler, in addition the following primer *Cat*, *Sod1*, *Gpx1*, *Nqo1*, and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) sequences were used (Table 4.1). Genes for *Cat*, *Sod1*, *Nqo1*, *Gpx1*, and *Gapdh* were amplified with a step denaturation at 94 °C for 30 secs, an annealing step at 58 °C for 30 secs, and an extension step at 72 °C for 30 secs for 30 cycles. The levels of mRNA were normalized to the gene, *Gapdh* (housekeeping gene).

4.2.7. Statistics

All experiments were performed three times (n=3), as well as the results, were expressed as mean±standard deviation (SD). To determine, the statistical significance

results Student's *t*-test was carried out using the program IBM SPSS statistics. The levels of statistically significant results were defined as $p^* < 0.05$, or $p^* < 0.01$, and $p^{**} < 0.001$.

Table 4.1: Oligonucleotides used in RT-PCR analysis.

Primers	Primer sequences	
	Forward	Reverse
CAT	5′-AGG CTC AGC TGA CAC AGT TC-3′	5′-GCC ATT CAT GTG CCG ATG TC-3′
GAPDH	5′-GCG AGA TCC CGC TAA CAT CA-3′	5′-AGT GAT GGC ATG GAC TGT GG-3′
GPx1	5′-GCT CAC CCG CTC TTT ACC TT-3′	5′-GAT GTC GAT GGT GCG AAA GC-3′
GST	5′- TGA GAG GAA CCA AGT GTT TGAG-3′	5′- CAG GGG GAC TTT AGC TTT AGAA-3′
HO-1	5′- TGA GAG GAA CCA AGT GTT TGAG-3′	5′- CAG GGG GAC TTT AGC TTT AGAA-3′
NQO1	5′-ATT GTA TTG GCC CAC GCA GA-3′	5′-GCA CTC TCT CAA ACC AGC CT-3′
Nrf-2	5′- CTT TAG TCA GCG ACA GAA GGAC-3′	5′- TCC AGA GAG CTA TTG AGG GACT-3′
SOD1	5′-AGG GCG TCA TTC ACT TCG AG-3′	5′-TCC TTT CCA GCA GCC ACA TT-3′

4.3. RESULTS AND DISCUSSION

4.3.1. Purified YD1 as an antioxidant

A Tricine SDS-PAGE analysis showed a single band of YD1 corresponding to the molecular weight of ~ 1 kDa (figure 3.5), and MALDI-TOF/MS analysis confirmed the presence of a single pure compound (Appendix-2). The amino acid residues of YD1 were Ala-Pro-Lys-Gly-Val-Gln-Gly-Pro-Asn-Gly. After sequence analysis using number of bioinformatics servers, YD1 showed an entirely new amino acid sequence, which was rich in glycine (Gly) and proline (Pro). Table 4.2 illustrates the comparison between the YD1 sequence and other closely related antimicrobial and antioxidant peptides. Hydrophobic amino acid residues such as alanine (Ala), valine (Val), leucine (Leu), or isoleucine (Ile) at the N-terminus have been known as a typical structural feature of antioxidant and other peptides (Sarmadi and Ismail, 2010, Guo *et al.*, 2009). The bioactivity of a peptide is majorly dependent on the position of amino acids in the sequence and the length of the peptide (Morgan *et al.*, 2012). Amino acids including Pro, Val, Ala, Leu, and sulfur-containing amino acids methionine (Met) and cysteine (Cys) have also reported contributing to the antioxidant properties of peptides (Rajapakse *et al.*, 2005, Li *et al.*, 2008). The pyrrolidine ring of proline can interact with the secondary structure of a peptide, thereby increasing the flexibility and is also capable of quenching singlet oxygen due to its low ionization potential (Chen *et al.*, 2012). Similarly, the single hydrogen atom of the Gly can provide the peptide backbone more flexibility as well as contribute to antioxidant properties. Moreover, glutamic acid (Glu) is an active cation chelator that forms a complex with calcium, iron, and zinc and may contribute to the antioxidant activity (Díaz *et al.*, 2003).

4.3.2. Antioxidant activity and mechanism study

Compounds become an antioxidant if they (i) have electron- or hydrogen-donating capacity, (ii) have the capacity to delocalize and stabilize the unpaired electron, and (iii) have metal-chelating potentiality. $ABTS^{+\bullet}$ and $DPPH^{\bullet}$ are well spectrophotometric methods for the measuring the antioxidant ability of test molecules. In a DPPH assay, the antioxidant provides an electron or hydrogen radical to the unstable free radical DPPH and reduces to become an even diamagnetic non-radical molecule.

Table 4.2: Comparison of YD1.

Name	Source	APD ID	[a]Rich AA residue	Length	Theoretical MW (Da)	Net charge	[a]Activity
YD1	*Bacillus amyloliquefaciens CBSYD1, fermented kimchi*	Current study	Gly	10	924	1	AGPN, AOx
Pleurain-A1	Skin secretions,*Rana pleuraden*,	AP00570	Ile, Leu	26	3053.6	3	AGPN, AF, AOx
SL21	*Homo sapiens*	AP00857	Ser, Arg, Gly	21	2326.7	4	AGPN, AF, AOx, CHE
Parkerin	Xizang plateau frog, *Nanorana parkeri*	AP01583	Ala	20	1945.2	2	AGPN, AF, AOx
Snakin-Z	fruits, *Zizyphus jujuba*	AP02258	Cys	31	3320.8	3	AGPN, AF, AOx, EI
Pleurain-A1-Thel	Skin secretions, tree frog, *Theloderma wangsiensis*	AP02260	Lys	26	3199.0	7	AGPN, AF, AOx
Brevifactin	Marine actinobacterium, *Brevibacterium aureum*	AP02261	Gly	4	~500	0	AGPN, AF, AOx
Pleurain-D1	Skin secretions, *Rana pleuraden*	AP02264	Leu	23	2478.1	3	AGPN, AF, AOx
Pleurain-E1	Skin secretions, *Rana pleuraden*	AP02265	Pro	26	2831.4	3	AGPN, AF, AOx
Pleurain-G1	Skin secretions, *Rana pleuraden*	AP02266	Lys	31	3360.9	2	AGPN, AF, AOx
Pleurain-J1	Skin secretions, *Rana pleuraden*	AP02267	Val	26	2779.4	3	AGPN, AF, AOx
Pleurain-M1	Skin secretions, *Rana pleuraden*	AP02268	Leu	31	3186.8	2	AGPN, AF, AOx
Pleurain-R1	Skin secretions, *Rana pleuraden*	AP02269	Thr, Ala	16	1775.0	1	AGPN, AF, AOx
Hispidalin	Seeds, winter melon, *Benincasa hispida*	AP02407	Asn	49	5700.2	1	AGPN, AF, AOx
Temporin-TP1	Skin secretions, *Hylarana taipehensis*	AP02458	Leu	16	1666.1	3	AGPN, AF, AOx
Brevinin-1TP1	Skin secretions, *Hylarana taipehensis*	AP02461	Lys	24	2461.1	4	AGPN, AF, AOx, MC
Brevinin-1TP2	Skin secretions, *Hylarana taipehensis*	AP02462	Lys	24	2495.1	4	AGPN, AF, AOx, MC

a: APD, Antimicrobial peptide database; AA, Amino Acid; AGPN, Anti-Gram positive and negative; AF, Anti-fungal; AOx, Anti-oxidant; CHE, Chemotatic; EI, Enzyme inhibitor; MC, Mammalian cells

DPPH-H molecule with consequent discoloration and decrease in absorbance. The degree of discoloration directly correlates with the magnitude of the scavenging potential of an antioxidant compound regarding its hydrogen donating ability. Moreover, in an $ABTS^{+\bullet}$ scavenging assay, a combined electron transfer as well as hydrogen atom transfer assay, the pre-formed $ABTS^{+\bullet}$ is formed when ABTS is oxidized with potassium persulfate and can be reduced while presenting of hydrogen-donating antioxidants. Surprisingly, YD1 significantly scavenged both the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical in a dose-dependent manner (figure 4.3 A-B). Afterward, we considered the FRAP and CUPRAC assays to assess whether YD1 has electron donating capacity. In this study, YD1 showed appreciably higher reduction capacity, suggesting that YD1 exhibited remarkable reduction capacity in a concentration-dependent manner (figure 4.4A-B). The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) (Ou *et al.*, 2002). Net AUC values of Trolox and YD1 were increased in a dose-dependent manner (figure 4.5).

4.3.2.1. Inhibitory effects of YD1 on cellular NO and ROS generation in RAW 264.7 cells

The consequences of YD1 on cell viability in RAW 264.7 cells were extensively investigated (figure 4.6A-B). Cell viability with various doses (1.5-72 μg/mL) of YD1 was measured with an MTT assay. The results showed no cytotoxic effects at doses up to 36 μg/mL. We then tried to evaluate the antioxidant activity of YD1 by examining NO generation and ROS production. LPS stimulation significantly increased the accumulation of NO to 100% in the medium compared with untreated control cells

while pre-treatment with YD1 notably decreased the NO release to 65% (**$p < 0.001$), 48% (**$p < 0.001$) and 24% (**$p < 0.001$) at doses of 1.5, 3, and 9 μg/mL, respectively (figure 4.7A). As YD1 has strong radical scavenging activity, the cellular ROS-scavenging activity of YD1 induced by LPS was tested where gallic acid as a positive control, a known antioxidant. YD1 treatment inhibited the generation of ROS in LPS-induced Raw264.7 cells in a dose-dependent manner (figure 4.7B).

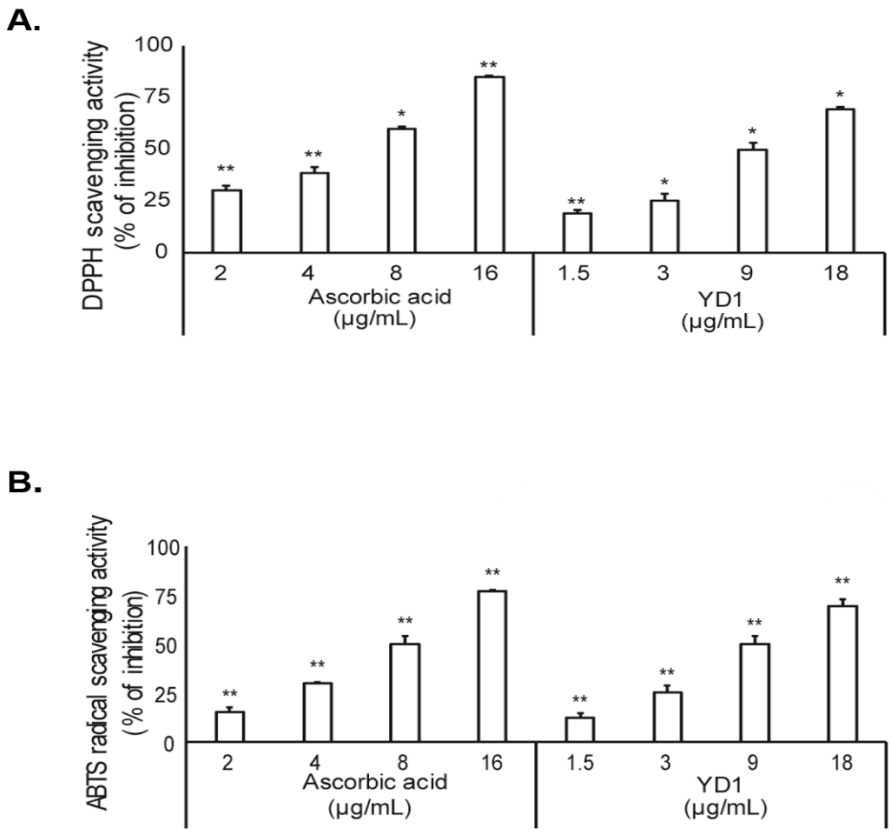

Figure 4.3: Radical scavenging effects of YD1 *in vitro*. The DPPH radical scavenging assay (A), ABTS $^{\bullet+}$ radical scavenging assay (B) were conducted with various

concentrations of YD1, and ascorbic acid was tested as a standard antioxidant compound.

4.3.2.2. Effects of YD1 on gene expression of antioxidants and Phase II antioxidant enzymes

Antioxidants and Phase II antioxidant enzymes play a pivotal role during cell proliferation, and ROS scavenging activity helps in maintaining cellular homeostasis. Enzymes such as GPx-1, SOD, and CAT are related to the elimination of presenting these free radical species within cells. Some degenerative diseases are results of damage to these enzymes. Cytosolic superoxide (O_2^-) is normally generated by the one-electron reduction of O_2, which happens mostly due to the slippage of electrons from electron carriers of the mitochondrial electron transport chain. It is well recognized that O_2^- is quickly converted into H_2O_2 by the presence of SOD in these circumstances. Additionally, H_2O_2 could be detoxified to H_2O by the scavenging enzymes such as CAT and GPx. These enzymes act together in the metabolic pathway of free radicals (Reczek and Chandel, 2015). In this study, YD1 treatment significantly increased both the mRNA and protein level of antioxidant enzymes such as SOD1, CAT and GPx-1 (figure 4.8A-B) in RAW264.7 cells, revealing that YD1 can maintain cellular homeostasis and protect the cell from oxidative stress.

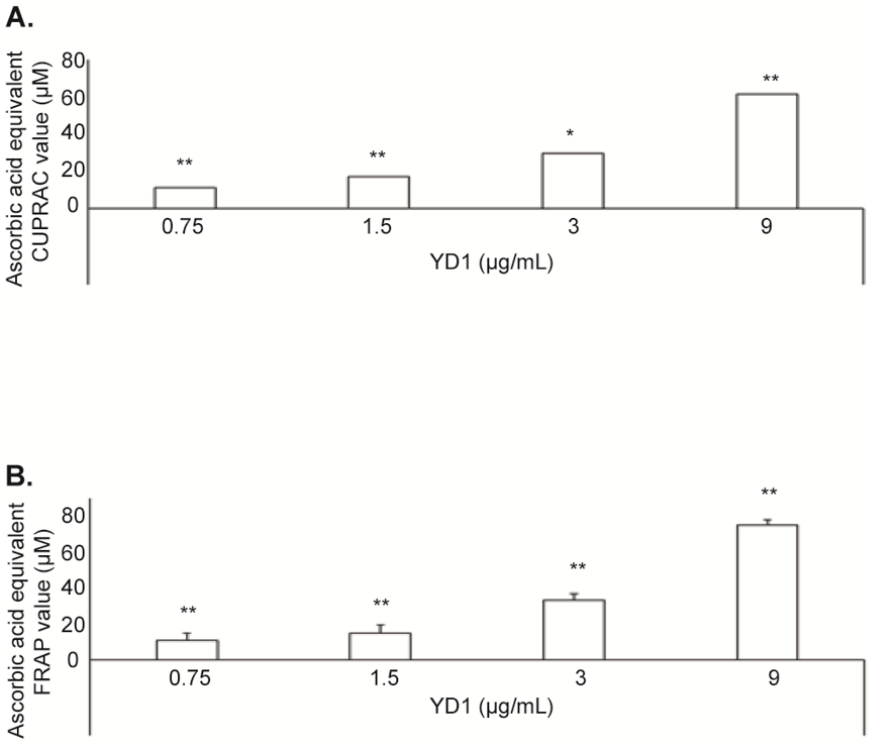

Figure 4.4: FRAP assay (B), and CUPRAC assay (C) were conducted with various concentrations of YD1, and ascorbic acid was tested as a standard antioxidant compound.

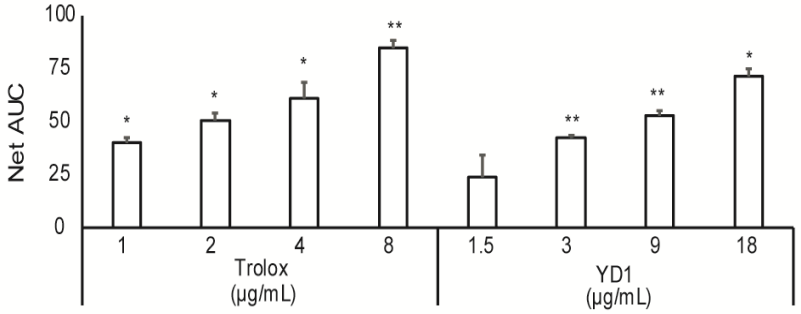

Figure 4.5: The ORAC activities of the samples were calculated by subtracting the area under the blank curve from the area under the sample curve, to obtain the net area under the curve (Net AUC)

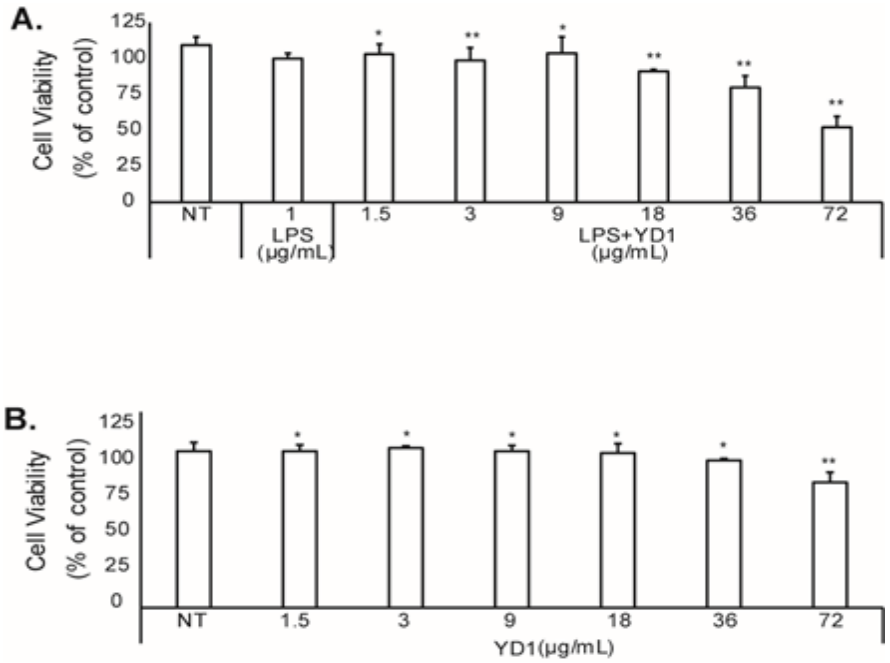

Figure 4.6: At a density of 2×10^4 cells per well (96-well plate), RAW 264.7 cells were seeded and Cell viability with LPS and YD1 treatment (A) and YD1 treatment alone (B). $^{**}p < 0.001$, significantly different from negative control, using a one-way ANOVA.

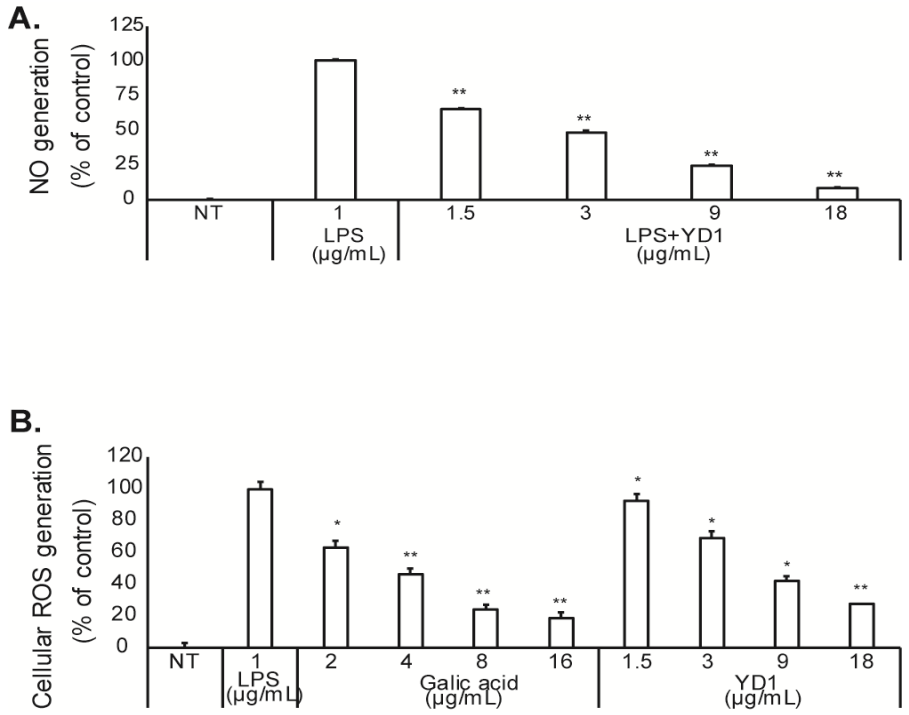

Figure 4.7: The inhibition of NO and ROS generation in RAW 264.7 cells (A and B). ***p* < 0.001, significantly different from negative control, using a one-way ANOVA

Under normal conditions, Nrf-2 is tethered in the cytoplasm by the Keap1 protein and plays a pivotal role in the activation of phase II enzymes when Nrf-2 is released and translocates to the nucleus by electrophiles and antioxidants (Zou *et al.*, 2014). We showed in figure 4.9 that YD1 treatment was able to increase the transcriptional and translational levels of HO-1 and promote the translocation of Nrf-2 into the nucleus in a concentration-dependent manner (figure 4.10A-B), in agreement with previous results (Chun, Alam, Son & Lee, 2016). In RAW264.7 cells, this indicates that the antioxidant activity of YD1 is in part attributable to the induction of HO-1 in a concentration-dependent manner (figure 4.11A-B), which is controlled by the activation of a Nrf-2

transcriptional factor in a concentration-dependent manner. HO-1 plays its antioxidant role by converting heme into the powerful pro-oxidant biliverdin and finally a strong antioxidant bilirubin (Zou et al., 2014). We postulate that the mechanism of interaction between YD1 and Nrf-2 may mimic the action of another Nrf-2 inducer such as sulforaphane and 5-O-caffeoylquinic acid, which modulates Nrf-2 nuclear translocation and ARE-dependent gene expression on targets such as HO-1, Nrf-2, NQO-1 in HT29 cells (Boettler *et al.*, 2012).

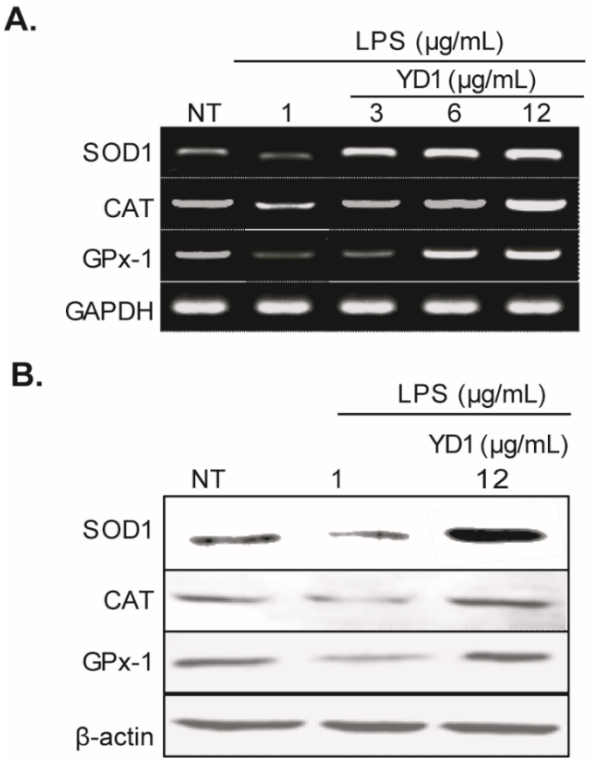

Figure 4.8: Analysis of primary and Phase II antioxidant and detoxifying enzyme mRNA levels in RAW 264.7 cells. Pre-treated the RAW 264.7 cells for 24 h with specified concentrations of YD1. RT-PCR measured the mRNA expression of the primary antioxidant enzyme (SOD, CAT, GPx-1) and phase II antioxidants, in a concentration dependent, (A) and the same concentrations used to perform western blot (B).

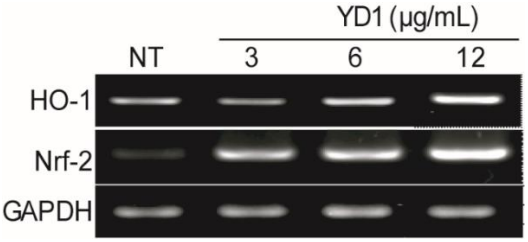

Figure 4.9: Effect of YD1 on Nrf-2 activity. RAW 264.7 cells were pretreated for 24 h with specified concentrations of YD1. The mRNA level of Nrf-2 and detoxifying enzyme (HO-1) were measured by RT-PCR, in concentration dependent manner.

Despite several proposed molecular processes regulated by antioxidant peptides, based on our findings, the antioxidant mechanism of YD1 may be driven by translocation inside the nucleus and ARE-dependent gene expression (figure 4.12). After elucidation of the complete structural information of the YD1 peptide and evaluating the following results such as phosphorylation of MAPKs and PI3K/Akt, and luciferase assays, we will study the antioxidant mechanism of action by different synthesized antioxidant peptide analogs. To the best of our knowledge, a peptide, which shows both antioxidant and antimicrobial activity, characterization and purification from a *Bacillus* strain isolated from fermented kimchi has never been reported.

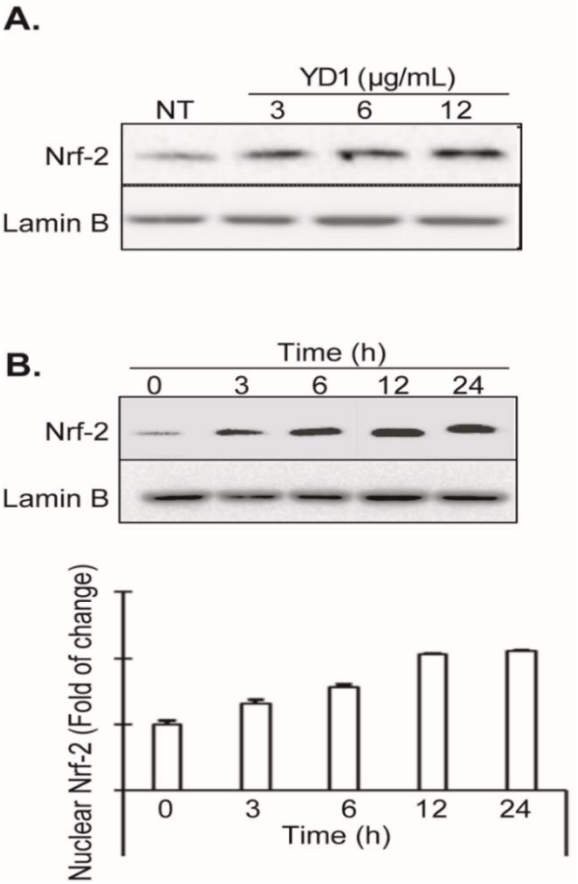

Figure 4.10: The nuclear translocation of Nrf-2 at concentration dependent (A), and time dependent (B) were measured by western blot analysis.

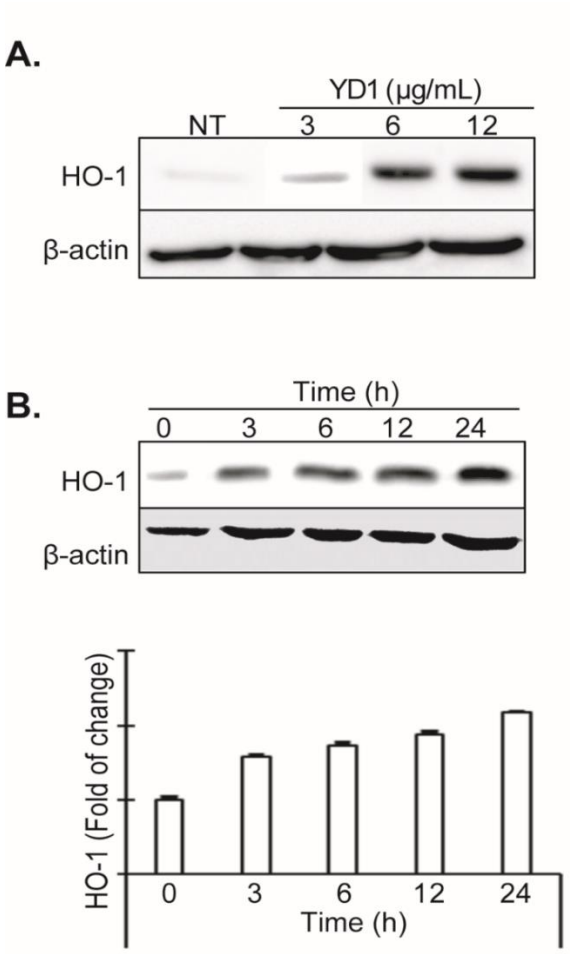

Figure 4.11: The expression of the detoxifying enzyme (HO-1) at concentration dependent (A), and time dependent (B) were measured by western blot.

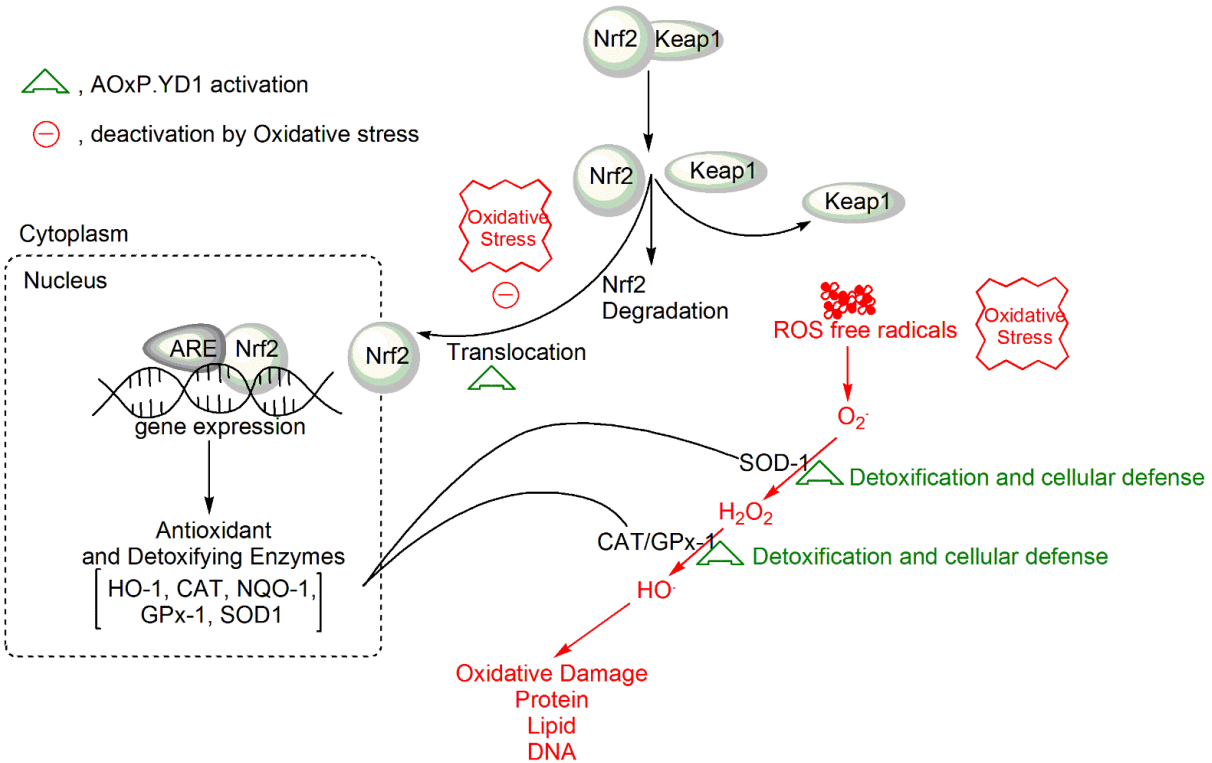

Figure 4.12: A diagram of a hypothetical antioxidant mechanism for novel antioxidant YD1

4.4. CONCLUSION

The present study demonstrates that YD1 has a high antioxidant activity, hydrogen atom transferability, and the potential candidate to prevent H_2O_2-induced oxidative stress and damage caused in RAW 264.7 cells via the increase of cellular thiol content, GST activity, and the antioxidant enzymes expression as well as the redox-sensitive transcription factor Nrf-2. Moreover, YD1 also has the potential to protect AAPH-induced oxidative stress damage as confirmed by increasing ORAC values as well as mRNA expression of antioxidant enzymes, including *Sod*, *Gpx*1, *Cat*, and *Nqo1*. Hence, the identification of novel cytoprotective candidates such as a YD1 counter to oxidant impairment via their potential implications may help to understand better about activity against and also the several pathophysiological conditions which are associated with oxidative stress as well.

In conclusion, these results suggest that peptide YD1 from probiotics like *Bacillus amyloliquefaciens CBSYD1* could be used as natural foods and in preventing the oxidation reaction in food processing.

5. FUTURE DIRECTIONS

Antimicrobial peptides are being extensively studied for their structure, function, and mode of action. Despite these studies, there are many secure research areas from where novel information could be collected, which may provide new perspectives in the development of drugs with diverse therapeutic applications. Some of the important subjects that necessity to be addressed in near upcoming time are enlisted below.

• Recent studies show that D-amino acid containing peptides retain the same biological activity as L-amino acid containing peptides. Therefore, the design of D-amino acid containing antimicrobial peptides could make them resistant to endogenous proteases and thus more stable.

• The self-assembly of short peptides into nanostructures is a growing interest in nanobiotechnology. These self-assembled peptides exhibit striking features that may have broad applications. Core–shell nanoparticles formed by self-assembly of amphiphilic peptides show potent antimicrobial properties against a wide range of bacteria, fungi, and yeasts. More investigation is needed to design novel nanoparticles with useful therapeutic agents.

• Development of new bioinformatic tools for identifying and screening of antimicrobial peptides.

• More research is needed to design non-toxic, shorter, and potent peptidomimetic antimicrobials, which ought to be highly stable and have significant antimicrobial activity.

• Biological activity, toxicity studies, and pharmacokinetics of the antimicrobial peptides ought to be tested in animal models.

• More research is needed to recognize or identify the potential amino acids that play a significant role in the antimicrobial and cytotoxic activity of anti-microbial peptides, so that design of a greater number of antimicrobial peptides can be possible in the near future.

• Toxicity is the major barrier to converting antimicrobial peptides into active drugs. Therefore, the design of cell-selective and effective peptides must be fully explored.

• More research is needed for understanding the structure-function relationship, secondary structure, aggregation or oligomerization, and membrane interaction of antimicrobial peptides. These studies shall help in designing novel peptides in the near future.

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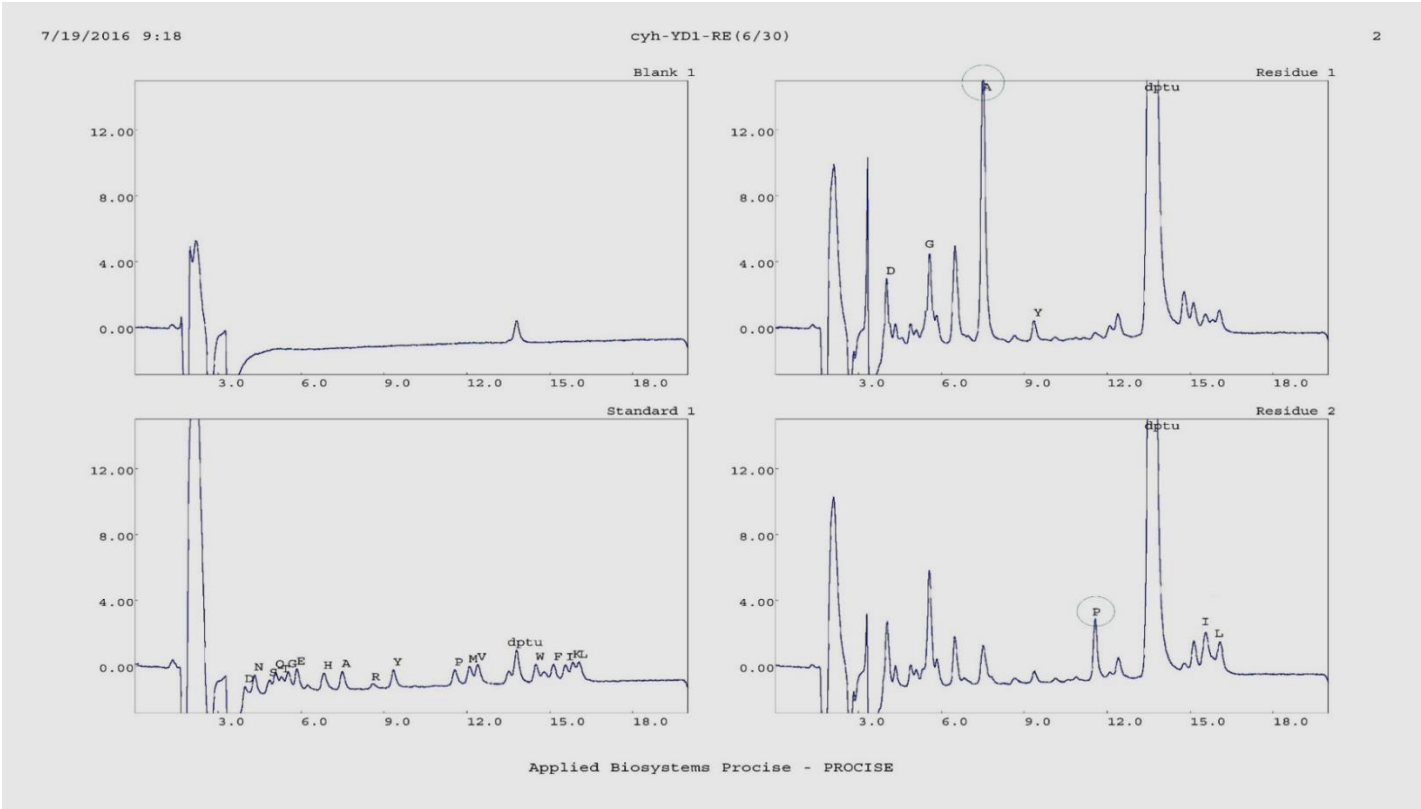
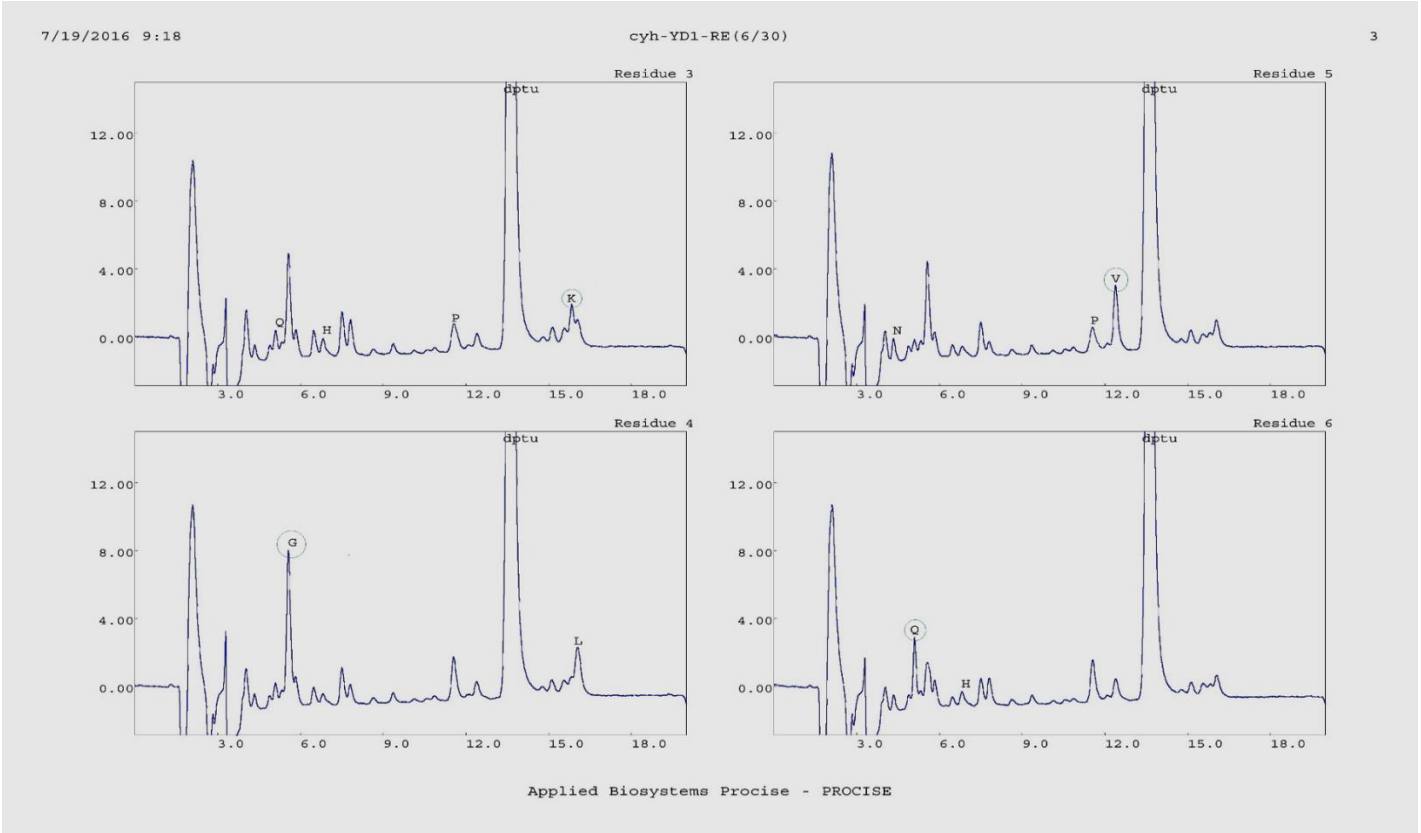
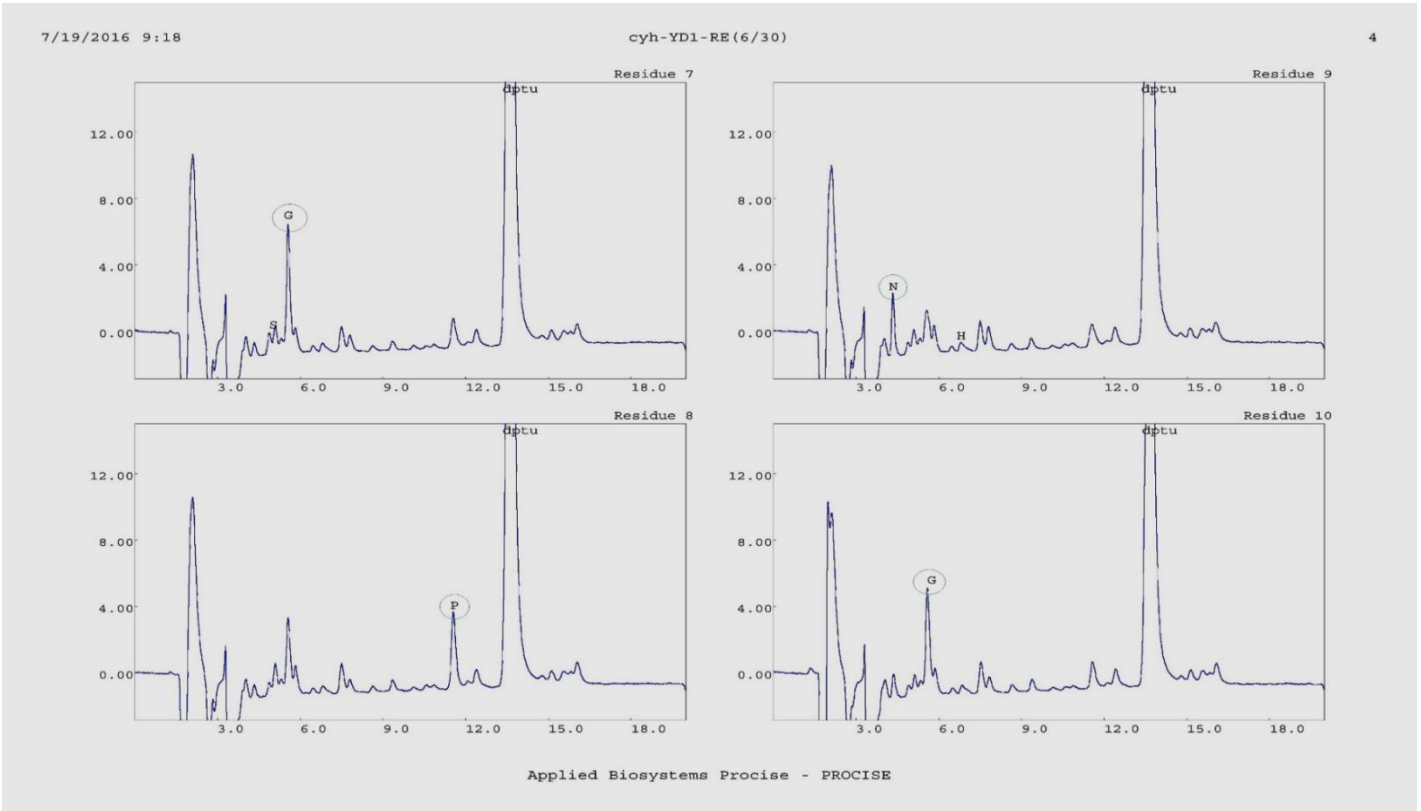
Appendix-1







Appendix-2