STUDIES ON SOME IMPORTANT PROTEINS AND PEPTIDES FROM PLANT AND MICROBIAL SOURCES

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree

of

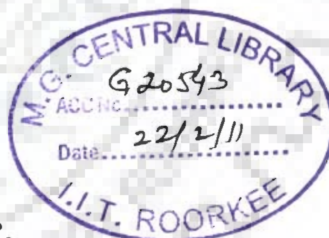
DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

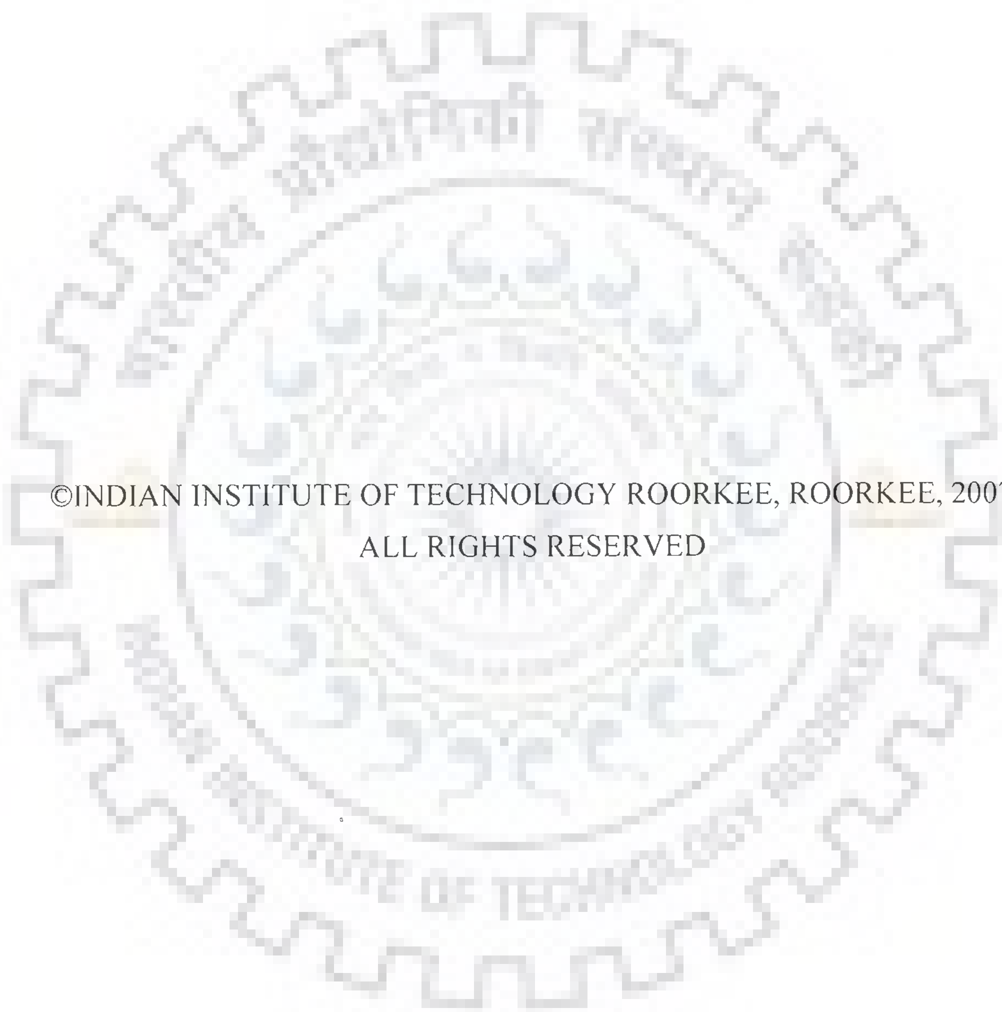
by

SAURABH AGARWAL

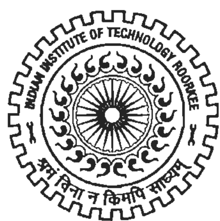


DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)

JULY, 2009



©INDIAN INSTITUTE OF TECHNOLOGY ROORKEE, ROORKEE, 2007
ALL RIGHTS RESERVED



INDIAN INSTITUTE OF TECHNOLOGY ROORKEE ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, **STUDIES ON SOME IMPORTANT PROTEINS AND PEPTIDES FROM PLANT AND MICROBIAL SOURCES** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee is an authentic record of my own work carried out during a period from January, 2004 to July 2009 under the supervision of Dr. Ashwani Kumar Sharma, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

Saurabh

(SAURABH AGARWAL)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Date: 22.7.09

(Ashwani Kumar Sharma)
Supervisor

The Ph.D. Viva-Voce Examination of **Mr. Saurabh Agarwal,** Research Scholar has been held on January 08, 2010

Signature of Supervisor

Signature of External Examiner

ABSTRACT

Proteins are important functional elements of the cell, present in all kinds of life forms ranging from viruses to eukaryotic cells. Within, they perform various biochemical functions such as hormones, enzymes, antibodies, blood coagulating factors, receptors, signaling molecules, et cetera. They are also known to perform various structural and mechanical roles as keratin of hairs, nails and actin and myosin fibre bundles in the muscles, respectively. The chief characteristic of the proteins that enables them to carry out their diverse cellular functions is their ability to bind and interact with other molecules specifically and tightly. This binding ability of the protein is greatly influenced by the three-dimensional structure of the protein molecule, it's surface topology, the nature of the amino acids exposed on the surface or involved in the interaction with the other molecules and the physical and chemical properties of the surrounding medium in which the interactions of the protein molecule with the other molecule takes place. Formation of specific complexes between antigen and antibody is a classic example of high complementarity and specific interactions of protein molecules with various other molecules. These interactions are completely dependent on the structural and functional stability of the proteins in different physicochemical conditions. The structural and functional characteristics of the proteins can be examined both *in-vitro* and *in-vivo* conditions. In order to perform *in-vitro* analysis, a protein must be purified from other cellular components. The process usually involves cell lysis, centrifugation and various chromatographic methods. Purified protein can be further characterized biochemically by various bioassays and biophysically by using various biophysical techniques like UV-Visible Spectroscopy, Circular Dichroism, Fluorescence Spectroscopy, X-ray crystallography and NMR.

This thesis is divided into four chapters and covers the studies carried out on three important classes of proteins, which are: procaryotic respiratory nitrate reductases, an antimicrobial protein and a protease inhibitory peptide.

Chapter 1 provides the literature review, briefly describing the various types of nitrate reductases, plant proteinase inhibitors and antimicrobial peptides found in the nature. It also covers briefly the various roles of protease inhibitors and antimicrobial peptides, with emphasis on their mechanism of action.

Chapter 2 describes the cloning and sequencing of the β-subunit of procaryotic respiratory nitrate reductase gene from *Bacillus circulans* NCIM 2107. PCR primers used for amplification of beta subunit of respiratory nitrate reductase were designed from different strains of Bacillus such

as *Bacillus licheniformis, Bacillus lentus, Bacillus pumilus* and *Bacillus subtilis*. The sequence obtained directly with the transformed *E. coli* DH5α cells containing the recombinant plasmid consisted of 729 bases which included the part of the nitrate reductase β-subunit gene containing an Open Reading Frame (ORF) of 678 bases. The ORF translates into a part of β-subunit of respiratory nitrate reductases containing 226 amino acids. Homology model of the part of translated β-subunit of respiratory nitrate reductase was obtained using the modeler program.

Chapter 3 covers the purification of a peptide with trypsin inhibitory activity as well as antimicrobial activity from the seeds of *Lagenaria siceraria* (Bottle Gourd) by cation exchange chromatography on CM Macroprep column and followed by reverse phase chromatography on HPLC with retention time of 3.848 minutes, eluted at 25.6% acetonitrile concentration. The molecular mass of the protein determined by MALDI-TOF analysis was found to be 678.9 daltons. The molecule purified was confirmed to be a peptide by FTIR spectroscopic studies with the peptide giving the infra-red absorbance of 1639 cm^{-1}, which is characteristic of the amide bond. The peptidic nature of the compound was further confirmed by the strong absorption peak of the molecule in spectrophotometer at 214 nm, which is the characteristic absorbance for the amide bond in ultraviolet region. It was further investigated that the purified compound completely inhibited bovine pancreatic trypsin at a molar ratio of 1:2. At the molar ratio of 1:1 the peptide inhibition of bovine pancreatic trypsin was only 57.5%. The purified peptide showed antimicrobial activity against the *Escherichia coli* with Minimum Inhibitory Concentration (MIC) of 20 μM. Antimicrobial activity was also exhibited by the seed extract of *Lagenaria siceraria* against *Escherichia coli* when 35 μl of the extract was used for inhibition of bacterial growth.

Chapter 4 describes the purification of another antimicrobial protein from the seeds of *Citrullus fistulosus* (Indian round gourd or Tinda) by two step purification method involving first anion exchange chromatography on DEAE Macroprep followed by cation exchange chromatography on CM Macroprep. The molecular weight of the purified peptide determined by SDS-PAGE analysis under reducing conditions was found to be 9 kD. Efforts for desalting of the protein fractions and to concentrate them using centricon and centriprep of low molecular weight cutoffs were futile as no protein was detected in the filtrate thus desalting and further concentration of the protein fractions was not carried out. N-terminal sequencing of the first seven amino acids of the protein was carried out by Edman degradation. The N-terminal amino acid sequence of the first seven amino acids was Phe-Asp-Asn-Ser-Phe-Thr-Asn or FDNSFTN and showed no identity or similarity whatsoever to any class of protein from the non-redundant protein sequence (nr)

database. The purified protein showed significant antimicrobial activity against *Staphylococcus aureus*.

ACKNOWLEDGEMENT

I would like to express my gratitude to my Ph.D. supervisor **Dr. Ashwani Kumar Sharma,** Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee for helping me again and again in defining my research problem, overseeing my results, constantly pushing me for more efforts, consistent guidance, sustained interest, encouragement, constructive and critical criticism, patient and humane behavior, valuable directions and for his useful suggestions throughout the Ph.D. program. Not only did he guide me, he often overlooked my mistakes and errors with a broad and mature attitude, giving me a chance to overcome the failures. It was a pleasure to work under the guidance of such a gentle and considerate supervisor.

I would also like to extend my gratitude towards **Prof. Ritu Barthwal**, Head of Department, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee for providing me all necessary and possible facilities available in the department for carrying out my research work. Her classes on Biophysics and Structural Biology were not only helpful but inspiring and refreshing too.

I would also like to thank **Prof. G. S. Randhawa**, Chairman, Departmental Research Committee, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee for his invaluable support during my PhD program.

A word of thanks also goes to people at Department of Biochemistry, South Campus, University of Delhi for the sequencing of the cloned gene. Special thanks to **Prof. T. P. Singh** from AIIMS, New Delhi for providing the MALDI-TOF facility and to people of Chemistry Department, IIT Roorkee, Roorkee for helping me with FT-IR studies and to **Prof. Dulal Panda**, School of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai for the N-Terminal sequencing of the protein.

I also thank my labmates **Dr. Chandan Shee**, **Dr Deepankar Gehloth, Girijesh Patel**, **P. Selva Kumar** and the former M.Sc. students **Kalpana Meena** and **Abhishek Acharya** for their cordial nature and helping me with a part of my thesis work as and when I required.

I would also like to thank **Shri. Raghukul Tilak**, **Prof U.C. Chobey**, **Prof M. N. Viladkar** and **Dr. Shayamal Mukerjee** for their support and encouragement.

The kind of moral support which I have received from my friends, **P. Sundramurthy**, **Dr. Mohammad Abdul Hai Zahid**, **Dr. Anindita Mukerjee, Vipin Bansal** and **Asharam Gairola** is

beyond the scope of this acknowledgement, yet I would like to express my heartfelt gratitude to them.

I am highly indebted to my parents **Mr. Anil K. Agarwal** and **Kalpana Agarwal** for supporting me in all the ways in my academic pursuits till now.

The kind of tender love and care that I have received form my younger sister and brother **Umang Agarwal** and **Parakh Agarwal** was also a major factor that motivated me for the completion of this task.

I am highly grateful and obliged to my master **Parthasarthi Rajagopalachari** for giving me strength to carry out this task and for his love, blessings and taking care of me in all the possible manner.

Finally, I express my sincere and deepest gratitude to my funding agency, Council of Scientific and Industrial Research (CSIR), Government of India, for the research fellowship that I have received during my first five years of Ph.D. program.

(SAURABH AGARWAL)

CONTENTS

Page No.

CANDIDATE'S DECLARATION

ABSTRACT i-iii

ACKNOWLEDGEMENT iv-v

CONTENTS vi-x

INTRODUCTION 1-2

CHAPTER 1

LITERATURE REVIEW 3-46

1.1. Introduction 3

1.2. Nitrate Reductase 4

1.2.1. Cytoplasmic Assimilatory Bacterial Nitrate Reductase (Nas) 5

1.2.2. Bacterial Respiratory Membrane-Bound Nitrate Reductase (Nar) 7

1.2.3. Bacterial Dissimilatory Periplasmic Nitrate Reductase (Nas) 8

1.2.4. Assimilatory Eukaryotic Nitrate Reductase (Euk-NRase) 9

1.2.5. Nitrate Reductase without Molybdenum 10

1.3. Plant protease inhibitors 11

1.3.1. Mechanism of Action of Protease Inhibitors 11

1.3.2. Types of Protease Inhibitors 12

1.3.2.1. Kunitz Soybean Trypsin Inhibitor Family 12

1.3.2.2. Bowman-Birk Protease Inhibitor Family 14

1.3.2.3. Potato I Inhibitor Family 14

1.3.2.4. Potato II Inhibitor Family 15

1.3.2.5. Carboxypeptidase Inhibitor Family 15

1.3.2.6. Squash Inhibitor Family 15

1.3.2.7. Barley Trypsin Inhibitor Family 16

1.3.2.8. Miscellaneous Inhibitors of Serine Proteases 16

1.3.2.9. Cystatins or Cysteine Protease Inhibitors 17

1.3.2.10. Other Specificities of Inhibition 17

1.3.3. Importance of plant proteinase inhibitors 17

1.3.3.1. Role of Protease Inhibitors in Nutrition 17

1.3.3.2. Role of Protease Inhibitors in Plant Protection 18
1.3.3.3. Role of Protease Inhibitors as Anticancerous Agents 19
1.3.3.4. Role of Protease Inhibitors as Anti-inflammatory Agents 21
1.3.3.5. Role of Protease Inhibitors as Anti-HIV Agents 22
1.4. Antimicrobial Proteins and Peptides 23
1.4.1. Types of Antimicrobial Peptides 24
1.4.1.1. Thionins 24
1.4.1.2. Defensins 24
1.4.1.3. Lipid transfer proteins 25
1.4.1.4. Hevein-type peptides 26
1.4.1.5. Knottin-type peptides 26
1.4.1.6. Snakins 26
1.4.1.7. Other Families of Antimicrobial Peptides 26
1.4.2. Mode of Action of Antimicrobial Peptides 27
1.4.2.1. The Barrel-Starve Model 28
1.4.2.2. The Carpet Model 29
1.4.2.3. Shai-Matsuzaki-Huang (SMH) model 30
1.4.3. Potential Applications of Antimicrobial Peptides 30

CHAPTER 2

CLONING AND SEQUENCING OF THE β-SUBUNIT OF PROCARYOTIC RESPIRATORY NITRATE REDUCTASE GENE 33-92
2.1. Introduction 33
2.2. Materials and Methods 35
2.2.1. Materials 35
2.2.2. Cloning of β-subunit of Prokaryotic Respiratory Nitrate Reductase gene 36
2.2.2.1. Primer Designing 36
2.2.2.2. Revival of lyophilized bacterial culture of *Bacillus circulans* 37
2.2.2.3. Isolation of Bacterial Genomic DNA 38
2.2.2.4. Quantification of Genomic DNA using spectrophotometer 39
2.2.2.5. Agarose Gel Electrophoresis of Genomic DNA 40

2.2.2.6. PCR Amplification 41
2.2.2.7. Gel Elution of the Desired Amplicon 42
2.2.2.8. Ligation of the Amplicon with pGEM-T cloning vector 43
2.2.2.9. Preparation of competent cells 45
2.2.2.10. Transformation of Competent *E. coli* DH5α cells 46
2.2.2.11. Screening of Recombinant Bacterial Colonies 48
2.2.3. Plasmid DNA isolation 49
2.2.4. Conformation of the screened bacterial colonies 51
2.2.5. Sequencing of the desired Amplicon 52
2.2.5.1. Translation product of the Sequenced Recombinant Plasmid 53
2.2.5.2. Identification of the translated sequence 53
2.2.6. Homology modeling of the translated gene sequence 53
2.3. Results and Discussion 56-72
2.3.1. Primer Designing 56
2.3.2. Isolation of Bacterial Genomic DNA 57
2.3.3. Quantification of DNA using spectrophotometer 57
2.3.4. Polymerase Chain Reaction 57
2.3.5. Gel Elution of Desired Amplicon 59
2.3.6. Ligation of the amplicon with pGEM-T cloning vector 59
2.3.7. Screening of the recombinant bacterial colonies 60
2.3.8. Conformation of Screened Colonies 60
2.3.9. Sequencing of desired amplicon 61
2.3.10. Translated product of the Sequenced Plasmid 64
2.3.11. BLAST result of translated DNA sequence 65
2.3.12. Multiple Sequence Alignment of Translated DNA sequence with sequences having high sequence homology 66
2.3.13. Homology modeling of the translated gene sequence 70

CHAPTER 3
PURIFICATION AND CHARACTERIZATION OF A PEPTIDE FROM LAGENARIA SICERARIA 73-84
3.1. Introduction 73
3.2. Materials and Methods 73
3.2.1. Materials 73
3.2.2. Purification of the Peptide 74
3.2.3. Molecular Weight Determination of the Purified Peptide 75
3.2.4. Determination of the Peptidic Nature of the Compound 75
3.2.5. Trypsin Inhibitory Activity of the Peptide 75
3.2.6. Antimicrobial Activity Assay of the Purified Peptide 76
3.3. Results and Discussion 77
3.3.1. Purification of the Peptide 77
3.3.2. Molecular Weight Determination of the Peptide 79
3.3.3. Determination of the Peptidic Nature of the Compound 80
3.3.4. Trypsin Inhibitory Activity of the Peptide 81
3.3.5. Antimicrobial Activity Assay of the Purified Peptide 83

CHAPTER 4
PURIFICATION AND CHARACTERIZATION OF A PROTEIN FROM CITRULLUS FISTULOSUS 85-98
4.1. Introduction 85
4.2. Materials and Methods 85
4.2.1. Materials 85
4.2.2. Purification of the Protein by Ion Exchange Chromatography 85
4.2.3. Protein Estimation 87
4.2.4. Molecular Weight Determination of the Protein by SDS-PAGE 87
4.2.5. Blotting of the Purified Protein on PVDF Membrane 87
4.2.6. N-terminal sequencing of the Protein 89
4.2.7. Antimicrobial Activity Assay of the Purified Peptide 89
4.3. Results and Discussion 90
4.3.1. Purification of the Protein 90

4.3.2. Molecular Weight determination of the Protein 96
4.3.3. Protein Concentration 96
4.3.4. N-terminal sequencing of the Protein 96
4.3.5. Antimicrobial Activity Assay of the Purified Peptide 97

CONCLUSIONS 99-101

REFERENCES 102-128

INTRODUCTION

INTRODUCTION

Nitrogen is a basic element for life being the important component of two major macromolecules: proteins and nucleic acids and being a part of other biomolecules as hormones, neurotransmitters and vitamins. Nitrogen exist in the biosphere in several oxidation states, from (+V) to (-III). Interconversions of these nitrogen species constitute the global biogeochemical cycle, which is sustained by several biological processes with bacteria playing the main role [238]. Processes such as nitrogen fixation, assimilation, nitrification and denitrification form the part of this cycle. Nitrate reduction, an important stage of nitrogen turnover in nature is taken care of by Nitrate Reductase. Nitrate reductase, the enzyme responsible for nitrate reduction has various functions as: utilization of nitrate as the source of nitrogen i.e. nitrogen assimilation as in eukaryotes [52, 53], nitrogen assimilation in prokaryotes, production of metabolic energy during nitrate utilization as terminal acceptor of electrons (nitrate respiration) and dissipation of excess of reducing energy to maintain oxidation-reduction balance i.e. nitrate dissimilation [195, 239, 240, 281].

Protease inhibitors are another important class of proteins that are widely studied. They are widely distributed in nature and have been isolated from many sources including animals, plants and microorganisms. Plant seeds are rich source of inhibitors. Many plant protease inhibitors have been purified and characterized particularly from the seeds of *Leguminosae*, *Cucurbitaceae*, *Solanaceae* and *Gramineae* family. Protease inhibitors are known to play an important role in regulating many physiological processes like inhibition of proteases, inflammation, coagulation, fibrinolysis, intracellular protein breakdown, cell cycle, transcription and apoptosis. They also act as anti-cancer and anti-HIV agents. Their role as natural plant defense proteins and their role against plant pathogens and pests are well established.

One of the classification schemes followed to put the Protease Inhibitors into different families is based on the classes of proteolytic enzymes being inhibited by them. These inhibitor families that have been found are specific for each of the four mechanistic classes of proteolytic enzymes, and based on the active amino acid in their "reaction center", are classified as serine, cysteine, aspartic and metallo-proteases [201]. Another basis for the classification of Protease Inhibitors into inhibitor families is on the basis of sequence homology, nature of the reactive or inhibitory site and interaction with the proteases according to the standard mechanism. The various types of protease inhibitors accounting to this classification are Kunitz trypsin inhibitors, Bowman-

Birk protease inhibitors, Potato I inhibitors, Potato type II inhibitor family, Carboxypeptidase inhibitors, Squash inhibitor family, Barley Trypsin inhibitor family and cystatins [150].

Antimicrobial peptides are another important category of peptides widely found in the living organisms, right from unicellular prokaryotes to human beings. They are the ancient weapons of defense against microbes. The molecular analysis of antimicrobial peptides will help in understanding the antimicrobial strategies. Studies both in the laboratory and in the clinic confirm that emergence of microbial resistance against antimicrobial peptides are less likely. Presence of such antimicrobial agents in natural sources like plant products, which are the natural foods of human beings, can be concluded to cause fewer side effects as compared to other conventional drugs. Thus antimicrobial peptides can be used as effective therapeutic agents. It is common to classify antimicrobial peptides into four groups according to their secondary structure. This classification is as follows [301]: Linear peptides with α-helical structure; conformationally more restrained peptides, predominantly consisting of β-strands connected by intramolecular disulphide bridges; linear peptides with an extended structure, characterized by overrepresentation of one or more amino acids and peptides containing a looped structure. Another way of characterizing antimicrobial peptides classify them as thionins [83], plant defensins [90], lipid transfer proteins (LTPs) [91], hevein-type peptides [90], knottin-type peptides [90], snakins [262], sephardins [215], MBP-1 [78], macrocyclic peptides [289] and Ib-AMPs [287].

This thesis contains the studies carried out on three important classes of proteins, which are: procaryotic respiratory nitrate reductases, an antimicrobial protein from *Citrullus fistulosus* and a protease inhibitory peptide with antimicrobial activity from *Lagenaria siceraria*.

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Proteins are one among the four major biomolecules found within the living organisms, the other three being carbohydrates, lipids and nucleic acids. They all are polymers of α-amino acids linked with each other with an amide bond. They serve various biochemical roles as hormones, enzymes, antibodies, blood coagulating factors, receptors, etc. They are also known to serve in many cases for their structural or mechanical functions. The chief characteristic of proteins that enables them to carry out their diverse cellular functions is their ability to bind other molecules specifically and tightly.

Nitrate reductase is one of the very important enzymes from the biological perspective. It is responsible for an important activity of nitrate reduction, which is one of the major steps in the biogeochemical cycle of nitrogen turnover. Nitrate reductase, the enzyme responsible for nitrate reduction has various functions as: utilization of nitrate as the source of nitrogen i.e. nitrogen assimilation as in eukaryotes [52, 53], nitrogen assimilation in prokaryotes, production of metabolic energy during nitrate utilization as terminal acceptor of electrons (nitrate respiration) and dissipation of excess of reducing energy to maintain oxidation-reduction balance i.e. nitrate dissimilation [195, 239, 240, 281].

Proteolysis is a key process in all living organisms and must be carefully controlled and regulated so as not to be hazardous to the organism itself. It is, therefore, not surprising that a large number of naturally occurring proteinaceous protease inhibitors have been found in animals, plants and microorganisms [150, 162, 241, 197]. Protease inhibitors are molecules that controls the activity of proteases, a catalytic enzyme that catalyzes the hydrolysis of proteins to form smaller polypeptide units. Proteases are mostly classified according to the main catalytic amino acid residue in their active site: (1) serine proteases, with a serine and a histidine; (2) cysteine proteases, with a cysteine; (3) aspartic proteases, with an aspartate group and (4) metalloproteases, with a metallic ion (Zn2+, Ca2+, or Mn2+) [201], in their active site. They have evoked tremendous interest because of their pivotal role in the regulation of various physiological and pathological processes. Due to the essential nature of proteases, protease inhibitors play a crucial role in developing therapeutics and as bio-control agents. Proteases are essential in the life cycles of organisms that cause mortal diseases such as malaria, cancer and AIDS [29, 261]. Specific inhibition of these proteases can be used as a strategy for drug design for the prevention and propagation of many causative agents of diseases and many protease inhibitors therefore, act as

anti-cancer and anti-HIV agents. Protease inhibitors can be divided into two classes: small molecules and macromolecules [282]. Small molecule inhibitors use the chemical nature of the active site and extended substrate-binding pockets to bind and inhibit the protease. Because of their small size and limited recognition determinants, these inhibitors often cannot discriminate sufficiently between closely related serine proteases [254, 159].

Antimicrobial proteins and peptides found in the various living organisms are another important class of proteins that form the part of the effective defense mechanism against the pathogens. Antimicrobial proteins have been identified long back in 1942 and more than 500 antimicrobial peptides and proteins have been isolated since then. They are cytolytic peptides that serve in the vertebrates and invertebrates for both offensive and defensive purposes. They can rapidly kill a broad range of microbes and have additional activities that impact on the quality and effectiveness of innate responses and inflammation. Furthermore, the challenge of bacterial resistance to conventional antibiotics and the unique mode of action of antimicrobial peptides have made such peptides promising candidates for the development of a new class of antibiotics. Accordingly, the molecular basis of their action is of considerable interest and requires to be elucidated. The diversity of antimicrobial peptides discovered is so great that it is difficult to categorize them except broadly on the basis of their secondary structure [81, 301]. The fundamental structural principle is the ability of a peptide to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete parts of the molecule. It is common to classify antimicrobial peptides into four groups according to their secondary structure. This classification is as follows [301]: Linear peptides with α-helical structure; conformationally more restrained peptides, predominantly consisting of β-strands connected by intramolecular disulphide bridges; linear peptides with an extended structure, characterized by overrepresentation of one or more amino acids and peptides containing a looped structure. Another way of characterizing antimicrobial peptides classify them as thionins [83], plant defensins [90], lipid transfer proteins (LTPs) [91], hevein-type peptides [90], knottin-type peptides [90], snakins [262], sephardins [215], MBP-1 [78], macrocyclic peptides [289] and Ib-AMPs [287].

1.2. Nitrate Reductase

Nitrate reduction, the most important stage of nitrogen turnover in nature, has several functions: i) utilization of nitrate as a source of nitrogen (nitrate assimilation); ii) production of

metabolic energy during nitrate utilization as terminal acceptor of electrons (nitrate respiration), and iii) dissipation of excess of reducing energy to maintain oxidation–reduction balance (nitrate dissimilation).

The first, most important stage in the chain of nitrate reduction to nitrites is catalyzed by nitrate reductase (NRase). There are four types of nitrate reductases —eukaryotic assimilatory NRase (Euk-NR) [52, 53] and three different bacterial enzymes, i.e. cytoplasmic assimilatory (Nas), membrane-bound respiratory (Nar), and periplasmic dissimilatory (Nap) NRases [195, 239, 240, 281].

With the exception of some extremophilic microorganisms, nitrate reductase of all kind generally has molybdenum and molybdenum-cofactor present in the active centre of the enzyme. Bacterial nitrate reductases carry molybdenum as Molybdopterin-Guanine Dinucleotide (MGD) in their active centre, whereas in case of eukaryotic nitrate reductase it is the Mononucleotide form of Molybdopterin that contains molybdenum and is present in the active centre of the enzyme. In bacterial MGD-binding subunit, molybdenum is generally coordinated by four thiolate ligands located in two MGD halves. Molybdenum can even be additionally coordinated by –S, -O or –Se bonds of cysteine, serine or selenocysteine residues of the polypeptide chain. It can even be accessible to oxo (-O) and hydroxo (-OH) groups or by water in different molybdenum enzymes [72, 67, 187]. Reactions catalyzed by nitrate reductase follow the oxo-transferase mechanism, in which oxo group on the oxidized Mo(VI) loses OH^-/H_2O upon reduction to Mo(IV). Nitrate is able to bind Mo in the reduced state and undergo reduction to nitrite, which regenerates the oxo-group on release of Mo(VI). Structure of catalytic subunit of Periplasmic Nitrate reductase (Nap) of *D. desulfuromonas* [72] and *Paracoccus pantotrophus* [50] has been elucidated. The number of Mo-O- Ser bond is found more conserved in respiratory nitrate reductase (Nar), compared to Mo-S-Cys bond found commonly in cytoplasmic assimilatory nitrate reductase (Nas) and periplasmic nitrate reductase (Nap).

1.2.1. Cytoplasmic Assimilatory Bacterial Nitrate Reductase (Nas)

Two types of cytoplasmic assimilatory bacterial nitrate reductases have been found in bacteria based on the structure of the cofactor. The two types of assimilatory bacterial NRases found are: ferredoxin or flavodoxin dependent Nas and NADH-dependent Nas.

Ferredoxin or Flavodoxin- dependent bacterial and cyanobacterial NRases contain in their active centre Molybdenum-cofactor and [Fe-S] clusters and are devoid of Flavin Adenine Dinucleotide (FAD) and cytochromes [244, 245]. These proteins are monomers and have molecular weight in the range of 75-105 kD. Ferredoxin and Flavodoxin acts as a physiological electron donor here. Analysis of the amino acid sequence of these enzymes has shown the presence of cysteine residues at the N-terminus, which evidently binds the [4Fe-4S] centre. Flavodoxin dependent cytoplasmic assimilatory nitrate reductase (Nas) has been isolated from *Azotobacter vinelandii* and *Plectonema boryanum* [189]. Ferredoxin-dependent NRases have been found in *Azotobacter chroococcum, Clostridium perfringens*, and *Ectothiorhodospira shaposhnikovii* [52].

NADH-dependent NRases are heterodimers consisting of the diaphorase FAD-containing subunit and a catalytic subunit with molybdenum cofactor and N-terminal [4Fe-4S] centre. Besides, NADH-dependent NRase of *Klebsiella oxytoca* (*pneumoniae*) contains an additional [2Fe-2S] center bound to a C-terminal cluster of cysteine residues, which resembles by its amino acid sequence the NifU protein, the *nifU* gene product, involved in formation of [Fe-S] clusters in the nitrogenase active centre [166]. This region is absent from the ferredoxin-dependent NRase, whereas in the NADH-dependent NRases it may play the role of the ferredoxin-similar domain carrying out electron transfer. The NADH-dependent NRase of *Bacillus subtilis* does not contain the NifU-like domain in the catalytic subunit, but contains two tandem NifU-like regions in the diaphorase subunit, which may be involved in [4Fe-4S] or [3Fe-4S] binding [208]. NADH-dependent NRase is also present in *Rhodobacter capsulatus* consisting of diphorase FAD-containing subunit of 45 kD and a catalytic subunit of 95 kD.

Regulatory and structural genes responsible for nitrate and nitrite uptake (transfer) are generally located in the same operon. Few genes code for nitrate and nitrite transfer across the periplasm and plasma membrane.

The level of *nas* gene transcription in enterobacteria is under double control, namely, it is the repression by ammonium via the general system of nitrogen regulation (Ntr) and specific induction by nitrate or nitrite [165, 315]. In the photosynthesizing bacterium *Rhodobacter capsulatus* assimilatory NRase is induced by nitrate and repressed by low C/N ratio [57]. Positive regulation by nitrate requires nitrate reduction to nitrite, evidently the real activator of the nitrate assimilation gene transcription [120, 175].

At present, no structure has been reported for any assimilatory nitrate reductase. Bacterial assimilatory cytoplasmic nitrate reductases are more closely related to the periplasmic

dissimilatory nitrate reductase compared to the membrane bound respiratory nitrate reductase [281].

1.2.2. Bacterial Respiratory Membrane-Bound Nitrate Reductase (Nar)

The main membrane-bound nitrate reductase is widespread among enterobacteria and is well studied in *Escherichia coli* [324]. The enzyme consists of three subunits (α, β, and γ) and several cofactors and participates in creation of transmembrane proton gradient, which is coupled with nitrate reduction. It has been shown that subunits α and β are localized in the cytoplasm, whereas subunit γ is localized in the membrane and performs the attachment of subunits α and β to the cytoplasmic side of the internal membrane.

The 19-28 kD subunit γ is a hydrophobic protein that forms five transmembrane α-helical domains with N-terminus located in the periplasm and C-terminus located in the cytoplasm and containing two *b*-type hemes [26, 221]. The high-potential heme is adjacent to the cytoplasmic side of the membrane, whereas the low-potential heme is localized in the periplasm. Subunit γ obtains electrons from quinone and transfers them using *b*-hemes onto subunit β via appropriate chains of histidine ligands [176]. Quinone pool oxidation at the membrane periplasmic side forms a transmembrane electrochemical gradient used for ATP formation with involvement of ATP synthase [129].

Subunit β (43-63 kD) is a globular protein incorporating [Fe-S] clusters.

Subunit α (104-150 kD) contains the [4Fe-4S] cluster and molybdenum cofactor, just on which the nitrate reduction takes place [103]. Molybdenum can exist as Mo(VI), Mo(V), or Mo(IV), and at a low and high pH levels there is pH-dependent equilibrium between forms. Structural analysis of the molybdenum center of respiratory nitrate reductase suggests that the enzyme can be a member of the same family of mononuclear molybdenum enzymes like DMSO reductase [121]. Comparison of amino acid sequence of respiratory nitrate reductase with available databases using the BLOCK algorithm [119] has revealed a number of conserved regions characteristic of all prokaryotic molybdopterin-containing oxidoreductases. Three out of four conserved cysteine residues, found in molybdopterin-guanidine-dinucleotide-binding proteins, are located at the N-terminus of the respiratory nitrate reductase α subunit.

Nitrate reduction is known to be controlled by oxygen at several levels: at the level of gene expression and at the level of nitrogen oxo-anion transport. In *Escherichia coli*, *Pseudomonas*

fluorescens, and *Pseudomonas stutzeri* the respiratory nitrate reductase operon expression is induced by anaerobiosis and by the presence of nitrate or nitrite [137, 112, 224]. The regulatory transcription factor Fnr (regulator of fumarate- and NRases) isolated from *Escherichia coli* is an anaerobic activator of respiratory nitrate reductase and in structure is a dimer containing [Fe-S] clusters. The protein is inactivated by molecular oxygen as a result of disintegration of [4Fe-4S] clusters and is converted to the monomer form. The Fnr protein monomer can be divided into three functional domains: N-terminal sensor region incorporating the [4Fe-4S] cluster (residues 1-50), central allosteric domain contacting RNA polymerase, and C-terminal regulatory domain incorporating the H–T–H region [17]. In *Pseudomonas aeruginosa* and some other *Pseudomonas* species the Anr protein (anaerobic regulation of anaerobic deiminase and NRase) was identified as a structural and functional homolog of Fnr [87, 257]. This regulator is necessary for denitrification cascade induction during growth on nitrogen oxides [87, 257, 318].

1.2.3. Bacterial Dissimilatory Periplasmic Nitrate Reductase (Nas)

It is supposed that nitrate reduction in the periplasm plays the key role in aerobic denitrification in *Pseudomonas* species [22], *Paracoccus pantotrophus* [50, 80] and *Rhodobacter sphaeroides* flavobacterium species *denitrificans* [237, 92], in adaptation to growth under anaerobic conditions in *Ralstonia eutropha* [271], in the maintenance of the oxidation– reduction equilibrium upon nitrate usage as electron acceptor for reducing energy removal in *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, and *Thiosphaera pantotropha* [23, 75] or for doubling the nitrate trace amounts in the case of *Escherichia coli* growing on a nitrate-deficient medium [47, 226, 227]. The periplasmic nitrate reductase genes were also identified in *Pseudomonas putida* [54], *Pseudomonas aeruginosa* [302], *Pseudomonas stutzeri*, *Shewanella putrefaciens*, *Haemophilus influenzae*, and quite recently in some pathogenic bacteria like *Salmonella typhimurium*, *Vibrio cholerae*, *Yersinia pestis*, and *Campylobacter jejuni*.

Periplasmic nitrate reductases localized in the periplasm are heterodimers consisting of a catalytic 90 kD subunit, incorporating molybdenum cofactor and one [4Fe-4S] center, and of a 13-19 kD subunit incorporating two-heme cytochrome *c* [28, 223, 279]. They are not involved in formation of transmembrane potential. Both the subunits are synthesized as a precursor with N-terminal signal sequence that defines their transfer through cytoplasmic membrane into the periplasmic space.

Bacterial periplasmic nitrate reductase exhibits various physiological functions and is expressed under different conditions, although usually the nitrate reductase activity is revealed under aerobic conditions, and the enzyme synthesis is not repressed by ammonium [138, 222]. In *Escherichia coli* maximal expression of nitrate reductase is observed at low nitrate concentrations under anaerobic conditions [69, 278, 304]. On the other hand, the periplasmic nitrate reductase system of *Ralstonia eutropha* is maximally expressed under aerobic conditions in the stationary growth stage and is not induced by nitrate [271]. In *Paracoccus pantotrophus* maximal periplasmic nitrate reductase expression was found in cells grown under aerobic conditions on such reduced carbon sources as butyrate or caproate, but no expression was detected in the case of growth on more oxidized substrates like succinate or malate [260]. In the bacterium *Rhodobacter sphaeroides* DSM158 the periplasmic nitrate reductase activity was stimulated, the enzyme synthesis took place both under aerobic and anaerobic conditions, but in the latter case it was more efficient [92, 69].

Also it has been shown that the export of periplasmic nitrate reductase into the periplasm from the cytoplasm occurs through a secretary independent pathway [256, 309].

1.2.4. Assimilatory Eukaryotic Nitrate Reductase (Euk-NRase)

The assimilatory NAD(P)H:nitrate reductase belongs to the NRase family of the class of molybdenum-containing enzymes, which are widespread in plants, fungi, yeasts, and algae [53, 126, 130, 275]. Recently it has been found that eukaryotic cells are able to carry out denitrification [288]. In particular, some species of yeasts and fungi are able to perform the dissimilatory reduction of nitrate to N_2O like *Cylindrocarpon tonkinense* [270, 295, 306]. Nitrate reductase has also been isolated from the fungus *Fusarium Oxysporum* [323]. It is a water-soluble enzyme consisting of a polypeptide with molecular mass of approximately 100 kD, bound to the molybdenum-molybdopterin molecule (Mo-MPT), heme iron, and FAD. It is known that dimerization of the enzyme is necessary for its activity, and thus the native enzyme is a homodimer having a tendency to further dimerization to a homotetramer (dimer of dimer). The enzyme has two active centers carrying out the internal electron transfer from FAD onto the heme iron and then to Mo-MPT. On the first active center electrons are transferred from FAD via NADH (or NADPH) onto the enzyme; in the second active center two electrons are transferred from reduced Mo(IV) to nitrate and reduce the latter to nitrite and hydroxide. Eukaryotic NRase exhibits its activity with such electron acceptors as ferricyanide and cytochrome *c* by obtaining electrons directly from FAD

and heme iron, respectively. The enzyme has only a limited similarity to prokaryotic NRase that also contains Mo and pterin cofactor. Bacterial cofactor (MGD) differs from eukaryotic MPT by an additional nucleotide. In bacteria, the Mo atom coordinates two pterins. All bacterial NRases contain the [4Fe-4S] oxidation–reduction centers as electron carriers from the electron donor to the enzyme. However, there is still no known eukaryotic NRase that contains [Fe-S] centers as components of the electron transfer chain.

Studying genes encoding NRase of the yeast methylotroph *Hansenula polymorpha* has shown that they are located in the same locus with genes *Yna1* and *Yna2*responsible for transcription [13]. A similar gene structure was also observed in different organisms, such as the fungus *Aspergillus* [3, 105] or algae *Chlamydomonas reinhardtii* [168, 322], but not in the fungus *Neurospora crassa* [209].

1.2.5. Nitrate Reductase without Molybdenum

Very recently two NRases, free of molybdenum and molybdenum cofactor, have been isolated from the cells of vanadate-reducing bacterium *Pseudomonas isachenhovii* growing under high content of vanadium (0.5 g/liter) [6]. The periplasmic NRase was a 55 kD monomer, containing vanadium, whereas the membrane-bound NRase was a heterodimer of 130 and 67 kD subunits and no metal was found in the active centre. The reductase complex isolated from the dissimilatory iron-reducing bacterium *Geobacter metallireducens* did not contain molybdenum and molybdenum cofactor in the active centre, but contained cytochrome *c* and was able to reduce both nitrate and nitrite [199]. Another highly active NRase of 130-140 kD was isolated from cells of a halophilic denitrifying bacterium of the *Halomonas* genus, strain AGJ 1-3. The enzyme was purified to homogeneity, and it contained no molybdenum or molybdo-cofactor in the active centre [7]. The enzyme was characterized by thermal stability and relative insensitivity to NaCl in high concentrations, had temperature optimum at 70-80°C, and exhibited polyreductase activity by reducing nitrates and other oxoanions.

1.3. Plant Protease Inhibitors

Protease Inhibitors are widely found in insects, other animals, plants and microbes. Protease Inhibitors are peptides and proteins that inhibit proteases specifically and competitively by binding strongly and irreversibly to the protease at the substrate binding site of the protease. In animals their main function is the regulation of endogenous proteases. In case of plants their endogenous function is not very clear. Only few inhibitors are known to inhibit the endogenous proteases of seeds. Protease Inhibitors existence in nature was first reported at the end of the nineteenth century as the anti-Trypsin activity in serum was observed [82]. Thereafter, the isolation and characterization of protein protease inhibitors was carried out and many fundamental concepts associated with protease-inhibitor interactions were studied [143, 146, 144, 145, 147, 31, 30]. The structural basis of the interaction between endoproteinase and protease inhibitors has also been studied [36]. The knowledge of the distribution of protein protease inhibitors of plant origin reveals hundreds of inhibitors distributed among different botanical families [161]. Majority of protease inhibitors in plant kingdom originates from four main families namely Leguminosae, Solanaceae, Gramineae and Cucurbitaceae [89]. Seeds, tubers and other storage organs are the most common site for protease inhibitors in case of plants [248]. Sometimes also found in abundance in tissues of leaves and stem too. The molecular weight of plant protease inhibitors is mainly in the range of 3,000 – 25,000 daltons.

1.3.1. Mechanism of Action of Protease Inhibitors

The Protease Inhibitors react with the protease by binding at the active site in a competitive and a reversible manner. The enzyme-inhibitor complexes are very stable and enzyme activity can be completely neutralized. X-ray crystallography and NMR studies revealed that in enzyme-inhibitor complexes, about 10–15 residues of the inhibitor are in contact with the enzyme. Their specific nature strongly affects both the strength and the specificity of enzyme-inhibitor interaction.

The reactive site is defined as the part of the inhibitor molecule that enters into direct molecular contact with the active center of the protease upon formation of the protease-inhibitor complex. At or near the reactive site of the inhibitor resides an amino acid residue that is specifically recognized by the primary substrate-binding site of the target protease. This amino

acid residue is termed P_1 [258]. Adjacent to P_1 resides the amino acid residue P'_1. The peptide bond joining these two residues, named the reactive site peptide bond, is susceptible to hydrolysis during complex formation between the inhibitor and its target protease [151]. Ionizable P_1 residues in the serine protease inhibitors undergo large pK shifts on complex formation [231]. Most protease inhibitors differ from the substrates for proteases in that, the reactive sites are held within disulfide bridges. After the hydrolysis of the reactive site peptide bond, the resulting modified inhibitor is still held together, practically without change in conformation [150].

Most of the well-studied inhibitors of serine proteases interact with the enzymes by this standard mechanism only. The latter has been derived from studies with inhibitors that were isolated from plants, mammalian and avian sources. Inhibitors obeying the standard mechanism are highly specific substrates for limited proteolysis by their target enzymes.

1.3.2. Types of Protease Inhibitors

One of the classification schemes followed to put the Protease Inhibitors into different families is based on the classes of proteolytic enzymes being inhibited by them. These inhibitor families that have been formed are specific for each of the four mechanistic classes of proteolytic enzymes, and based on the active amino acid in their "reaction center", are classified as serine, cysteine, aspartic and metallo-proteases.

Another basis for the classification of Protease Inhibitors into inhibitor families is on the basis of sequence homology, nature of the reactive or inhibitory site and interaction with the proteases according to the standard mechanism [150]. The various types of Protease Inhibitors categorized as per this classification are: Kunitz Trypsin Inhibitors (KTI) [145, 147], Bowman-Birk Inhibitors (BBI) [32, 34], Potato I Inhibitors, Potato II Inhibitors, Carboxypeptidase Inhibitors, Squash Inhibitors, Barley Trypsin Inhibitors, Miscellaneous Inhibitors of Serine Proteases, Cystatins (Cysteine protease inhibitors) and Other Specificities of Inhibition.

1.3.2.1. Kunitz Soybean Trypsin Inhibitor Family

The first plant protease to be isolated and characterized was Kunitz soybean trypsin inhibitor (KTI) [145,147]. KTI has played a key role in the early study of proteases, having been used as the main substrate in the biochemical and kinetic work that led to the definition of the

standard mechanism of action of Protease Inhibitors. The purification, crystallization, kinetics of interaction and complex formation of KTI with trypsin comprises a major landmark in the study of protease inhibitors. KTI consists of 181 amino acid residues and includes two disulfide bridges. The precursor for KTI has a molecular weight of ~24000 daltons and the mature KTI has a molecular weight of ~20000 daltons. Its three-dimensional structure per se and in complex with porcine trypsin was also determined [286, 8, 141, 163]. The predominant conformation found in KTI has been demonstrated to be more or less like β-sheet structure, with a small amount of regular β-sheet.

KTI is primarily an inhibitor of trypsin, but also weakly inhibits chymotrypsin. KTI inhibits trypsins of various species, e.g., human, bovine, avian and fish. Of other enzymes, human plasmin, cocoonase, plasma kallikrein and a serum trypsin-like enzyme are inhibited. It blocks the conversion of prothrombin to thrombin. Bovine and porcine kallikrein, human and bovine thrombin, liver esterase and β-subtilisin BPN′ are not inhibited by KTI, although complexes are formed with both thrombins.

Of the other sources for KTI type inhibitor other than soybeans, those from *Erythrina* seeds received the greatest amount of attention. KTIs have been extracted from *Erythrina variegata*, *Erythrina caffra* [132], etc. Determination of primary structure of a KTI type trypsin inhibitor from winged bean seeds (*Psophocarpus tetragonolobus*) [51] has already been done. KTIs have also been isolated from *Enterolobium contortisiliquum* seeds [18], *Bauhinia variegata* seeds [73], *Delonix regia* seeds [225], velvet bean (*Mucuna Pruriens*) [174], *Dimorphandra mollis* seeds [173], *Capaifera langsdorffii* [273] and from many other plants seeds.

A highly stable and potent Kunitz-type trypsin inhibitor of 34 kD was purified to homogeneity from the seeds of *Putranjiva roxburghii*. The purified inhibitor inhibited bovine trypsin competitively in 1:1 molar ratio with an equilibrium dissociation constant of 1.4×10^{-11} M. The inhibitor retained its inhibitory activity over a broad range of pH (2–12) and temperature (20–80°C) [59]. Also, 27 kD KTI type protease inhibitor was purified to homogeneity from the seeds of *Murraya koenigii* (curry leaf tree) The purified protein inhibited bovine pancreatic trypsin completely in a competitive manner in a molar ratio of 1:1.1 with an equilibrium dissociation constant of 7×10^{-9} M [267].

1.3.2.2. Bowman-Birk Protease Inhibitor Family

The Bowman-Birk Inhibitor from soybean (BBI) serves as the prototype for a family of inhibitors that are predominant in legume seeds. BBI has a molecular weight of about 8000 daltons with a high content of half cystines forming seven disulfide bridges. The inhibitor consists of two tandem homology regions on the same polypeptide chain, each with a reactive, inhibitory site [205, 204]. In aqueous solutions, the inhibitor undergoes self-association, which is concentration-dependent. BBI inhibits human, bovine, porcine, dog and avian trypsin and chymotrypsin, at a 1:1 enzyme-inhibitor ratio [33]. It inhibits carp trypsin and chymotrypsin with a 1:2 stoichiometry of inhibition, indicating the binding of two molecules of either trypsin or chymotrypsin to one molecule of inhibitor [65, 66]. BBI is also a potent inhibitor of trypsin and chymotrypsin from the digestive tracts of insects, such as *Tenebrio molitor* [160], *Locusta migratoria* [253] and *Tribolium castaneum* [319] and has shown a strong interaction with elastases from human and dog granulocytes [259].

1.3.2.3. Potato I Inhibitor Family

This Inhibitor family is also referred to as Chymotrypsin Inhibitor I family because its specificity is directed most strongly toward chymotrypsin, although it inhibits subtilisin, pronase, as well as some other alkaline microbial proteases, and it is also a weak inhibitor of trypsin. The inhibitors belonging to this family are non-covalent tetramers of four different subunits. Each of these protomers has a molecular weight of about 10000 daltons and is comprised of a single chain with a single intra chain disulfide bridge. One mole of tetramer inhibits four moles of chymotrypsin, which suggest that each of the subunit possesses a chymotrypsin binding site. The amino acid sequences of the four protomers display extensive homology with each other. The cross reactivity among the tetrameric species is identical showing that the protomer possess considerable reasons that are immunologically very similar.

1.3.2.4. Potato II Inhibitor Family

Inhibitor II has a molecular weight of 20000 daltons and is composed of dimers of four distinctly different protomers, which are not homologous to the protomers that make up Inhibitor I. Each dimer inhibits two molecules of chymotrypsin, indicating that each of the subunit has a binding site for chymotrypsin. The activity of each of the protomers toward trypsin varies. Different varieties of potato yielded different, but characteristic inhibitor patterns. Despite the varietal differences, Inhibitor II preparations from numerous potato varieties were immunochemically related [247].

1.3.2.5. Carboxypeptidase Inhibitor Family

The first report came on a polypeptide from potato tubers that specifically inhibit the pancreatic metallo-carboxypeptidases [249, 233]. The carboxypeptidase inhibitor has a molecular weight of 4300 daltons, inhibiting potently and competitively mammalian pancreatic carboxypeptidases A and B from various animals and other metallo-carboxypeptidases [250, 114]. The X-ray crystallographic structure also demonstrates that the inhibitor binds like an extended substrate and that the carboxy terminal peptide bond Val38 - Gly39 is cleaved in the enzyme inhibitor complex. The Gly39 appears to be trapped in the binding pocket of the enzyme [236], indicating that this residue is required for enzyme inhibitor interaction [113]. These finding suggest that the region from Cys18 – Cys27 and the carboxy terminal section of the inhibitor should be of particular importance in the direct interaction with enzymes. Due to the unusual heat stability and solubility in ethanol of the carboxypeptidase inhibitors, a large scale of inhibitor purification can be easily accomplished [218].

1.3.2.6. Squash Inhibitor Family

The squash (cucurbitaceae) inhibitor family consists of protein inhibitors of trypsin that were isolated from squash, melon and cucumber seeds. Many more have been isolated from bottle gourd seeds (*Lagenaria leucantha*), bitter gourd (*momordica charantia*), ridged gourd (*Luffa acutangula*) and winter squash (*Cucurbita maxima*). The striking characteristic of this family is

that its member inhibitors are very small: their molecular weight is about 3000 daltons [308]. They consist of 27 – 33 amino acid residues and are cross-linked by three disulfide bridges. The reactive site peptide bond is between residue 5 (Lys, Arg or Leu) and 6 (always Ile), and they inhibit proteinases via the standard mechanism. The inhibitors appear in numerous members of the cucurbitaceae family as isoinhibitors designated usually as CMTI, with a suffix I, II or III, assigned to the P_1 amino acid residue in the reactive site peptide bond, respectively [214].

Squash Inhibitors are highly stable and rigid proteins. They inhibit trypsin, plasmin, kallikrein, blood clotting factors (Xa and XII a) and cathepsin G.

1.3.2.7. Barley Trypsin Inhibitor Family

Numerous protease inhibitors have been found in cereal grains, such as barley, maize (corn), rye, rice, sorghum and oats. The highly homologous trypsin inhibitors from barley, rye and maize as well as the bifunctional amylase-trypsin inhibitor from seeds of ragi, contain an Arg-Leu reactive site peptide bond in positions corresponding to the Arg33-Leu34 in the sequence of the barley inhibitor [172]. The latter, a single polypeptide chain of molecular weight of about 13000 daltons with 5 disulfide bridges serves as the prototype for this group [206].

1.3.2.8. Miscellaneous Inhibitors of Serine Proteases

Miscellaneous Inhibitor of Serine Proteases have been isolated and characterized from the seeds of different plants. They all vary highly in their number and sequence of amino acids, molecular weight and number of disulfide bonds. Some of them are isolated from *Amaranthus hypochondriacus* seeds [297], *Amaranthus caudatus* seeds [117], trypsin-chymotrypsin inhibitor from *Amaranthus hypochondriacus* seeds [290], 14 amino acid trypsin inhibitor from sunflower seeds [170], cationic inhibitors of serine proteinases from *Fagopyrum esculentum* seeds [296], serine proteinases inhibitor from *Sinapis alba* seeds [188], trypsin inhibitor from *Sinapis arvensis* seeds [284], low molecular mass serine proteinases isoinhibitor for oil-rape seeds [12], Mustard Trypsin Inhibitor (MTI-2) from yeast *Pichia pastoris* [303] and a MTI from *Sinapis alba* that inhibits chymotrypsin also [246].

1.3.2.9. Cystatins or Cysteine Protease Inhibitors

Cysteine protease inhibitors (Cystatin) have been isolated from plant, animal and microbial sources. Among the early isolated Cystatin from plants are seven isoinhibitors from pineapple stem and from papaya latex [235, 14, 15]. Oryzacystatins are the major cystatins isolated from the rice till date. The inhibition of cysteine proteinases by cystatin is reversible and competitive but the precise inhibitory mechanism is unknown. Oryzacystatin binds equally well to active and inactive enzyme and it has been suggested that inhibition occurs by preventing access of the substrate to the catalytic site of the enzyme. The amino terminal 21 residues containing Gly5 and the COOH terminal 11 residues are not essential, suggesting that a portion of the polypeptide segment containing Gln53 – Gly57 is necessary to elicit its Cysteine inhibitory activity efficiently [9].

1.3.2.10. Other Specificities of Inhibition

A multifunctional protease inhibitor has been isolated from Bauhinia seeds [100]. Other inhibitors isolated and characterized are from: Kohlrabi [285], potatoes [180], cabbage seeds [55], rapeseed [58], *Cajanus cajan* seeds [99], latex of *Carica papaya* [207], squash phloem exudates [61] and *Cassia fistula* [312].

1.3.3. Importance of plant proteinase inhibitors

Protease Inhibitors play diverse roles in nutrition, plant protection, etc. They also act as anticancerous, anti-inflammatory and as anti HIV agents. Thus, their various roles are briefly investigated here.

1.3.3.1. Role of Protease Inhibitors in Nutrition

Protease inhibitors of plant origin are significant components of human food. The evidence that inhibitors constitute a hazard to health is only presumptive and should be placed in perspective

in relation to total protease inhibitors in the overall diet. Most of the in vivo research has been done with small animals that consumed large quantities of a particular food component over a relatively long period of time- a situation quite remote from the eating patterns of humans. Moreover, the association of legume seeds rich in protease inhibitors with prevention of human cancer stimulated the insight into the possible action of protease inhibitors as cancer chemopreventive agents.

1.3.3.2. Role of Protease Inhibitors in Plant Protection

The physiological significance of plant protease inhibitors has been questioned for a long time. Only a few inhibitors are known to inhibit the endogenous proteases of seeds. Plant protease inhibitors may be found as genuine components of the plant, particularly in the seeds, or may be induced in response to pathogen attack. It is known that legume seeds and cereal grains contain inhibitors of gut proteases of stored-product insects suggested the possibility that the inhibitors may have evolved as a defense mechanism against predatory insects. It has been shown that the rapid accumulation of protein protease inhibitors in potato and tomato leaves in response to a systemic signal that appears in increased levels as a direct result of the wounding of the plant leaves, either mechanically or following attack by insects. The latter became starved as a result of the inhibition of their own digestive proteases by the ingested plant protease inhibitors [43]. Attention has been recently drawn to the ability of well-adapted insect species to overcome the effects of plant protease inhibitors.

Protease inhibitors also exhibit a very broad spectrum of activity including suppression of pathogenic nematodes like *Globodera tabaccum*, *G. pallida*, and *Meloidogyne incognita* by CpTi, inhibition of spore germination and mycelium growth of *Alternaria alternata* by buckwheat trypsin/chymotrypsin and cysteine Protease Inhibitors from pearl millet inhibit growth of many pathogenic fungi including *Trichoderma reesei*. These advantages make protease inhibitors an ideal choice to be used in developing transgenic crops resistant to insect pests.

The role of serine PIs as defensive compounds against predators is particularly well established, since the major proteinases present in plants, used for processes such as protein mobilization in storage tissues, contain a cysteine residue as the catalytically active nucleophile in the enzyme active site. Serine proteinases are not used by plants in processes involving large scale protein digestion, and hence the presence of significant quantities of inhibitors with specificity

towards these enzymes in plants cannot be used for the purposes of regulating endogenous proteinase activity. In contrast, a major role for serine PIs in animals is to block the activity of endogenous proteinases in tissues where this activity would be harmful, as in case of pancreatic trypsin inhibitors found in mammals. The serine class of proteinases such as trypsin, chymotrypsin and elastase, which belong to a common protein superfamily, are responsible for the initial digestion of proteins in the gut of higher animals. Therefore it is presumed that the only role of serine proteases in the plants is to inactivate the intestinal proteases of the insects. Cystatins also are known to inhibit the gut proteases of the insects which are active at pH 5-7. Aspartate Protease Inhibitors especially from potato does not seem to have any major role in insect control except in six families of hemiptera. Metalloprotease inhibitors extracted from tomato and potato inhibits metalloproteases from animals and microbes.

The current evidence suggests that the regulation of the production of the inhibitors occurs *via.* the octadecanoid (OD) pathway, which catalyzes the break down of linolenic acid and the formation of jasmonic acid (JA) to induce protease inhibitor gene expression [139]. There are four systemic signals responsible for the translocation of the wound response, which includes systemin, abscisic acid (ABA), hydraulic signals (variation potentials) and electrical signals. These signal molecules are translocated from the wound site through the xylem or phloem as a consequence of hydraulic dispersal. The plant systemin an 18-mer peptide has been intensely studied from wounded tomato leaves which strongly induced expression of protease inhibitor (PI) genes. Thus the action of protease inhibitors is regulated by any kind of wound or injury induced to the plant.

1.3.3.3. Role of Protease Inhibitors as Anticancerous Agents

There is a strong evidence for a particularly strong relationship between the ingestion of soybeans and low incidence and mortality rates for breast, colon, and prostate cancers. Although breast, colon, and prostate cancers are major public health problems in the Western World, cancer rates in these organs are significantly lower inmost countries of the Pacific basin. When Orientals migrate to the United States, their offspring develop the common Western cancers at approximately the same rates, as do Americans. This observation has suggested that it is not a genetic difference in susceptibility to breast, colon, and prostate cancer development that results in the variation in cancer rates, but instead it is more likely due to variables such as dietary habits.

The protease inhibitors discussed in this report are extremely potent agents having the ability to prevent cancer, with some unique characteristics as anticarcinogenic agents. The anticarcinogenic protease inhibitors have the ability to irreversibly suppress the carcinogenic process, they do not have to be continuously present to suppress carcinogenesis, they can be effective when applied in both *in vivo* and *in vitro* carcinogenesis assay systems at long time periods after carcinogen exposure, and they are effective as anticarcinogenic agents at extremely low molar concentrations.

Protease inhibitors seem to regulate the anticancerous activity in bacteria by regulating the proteases involved in the process of transformation. The carcinogen can induce the release of protease responsible for the induction of an error prone DNA repair mechanism thereby causing mutations leading to transformation. A similar event is also seen in case of yeast, where the recombination events of the chromosomes due to increase in protease levels after carcinogen exposure leads to transformation. Protease Inhibitors can again block the action of proteases in this case thus inhibiting the process of transformation.

A potential model by which the anticarcinogenic protease inhibitors could be affecting c-myc expression is that, it is assumed that radiation and chemical carcinogens are inducing proteolytic activity involved in the regulation of c-myc. This postulated protease is one that is capable of destroying a regulatory protein involved in the regulation of c-myc. It is assumed that this regulatory protein would bind to the promoter region of the gene. It is hypothesized that carcinogen treatment would lead to increased levels of the protease, which would then lead to decreased levels of the regulatory protein; decreased binding of the regulatory protein to the promoter region of c-myc would then lead to increased levels of c-myc expression. Evidence in support oft his proposed model comes from experiments showing that (1) carcinogens induce elevated levels of proteolytic activity (2) radiation increases c-myc expression and (3) c-myc gene expression increases in radiation-induced tumors *in vivo* the induction of transformation *in vitro*. Another way that protease inhibitors could be affecting gene expression in carcinogen-treated cells is through an effect on gene amplification. It is known that gene amplification is induced by carcinogens in a widespread fashion in mammalian cells. It has been observed that certain anticarcinogenic protease inhibitors, including BBI, can inhibit this carcinogen-induced gene amplification. Radiation is known to induce gene amplification and the radiation induction of gene amplification can be further potentiated by tumor promoting agents such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA). The anticarcinogenic protease inhibitors have been thought to be highly

antipromotional in their effects. It is expected that the mechanism(s) by which tumor promoting agents enhance malignant transformation will be directly affected by the anticarcinogenic protease inhibitors. Also it is known that the transformation is induced by the over expression of c-fos gene and the product of the product of these genes are proteases that leads to the activation of c-myc genes. Therefore, again the Protease Inhibitors can block the action of the products of c-fos gene thus preventing the important step in the transformation process [136, 135].

1.3.3.4. Role of Protease Inhibitors as Anti-inflammatory Agents

It is now known that BBI is highly anti-inflammatory. In several of the animal carcinogenesis studies, a reduction in the level of lymphoid aggregates or lymphocytic infiltrates in the BBI/BBIC-treated animals was directly correlated with a decrease in tumor yields.

The mechanism by which BBI reduces the inflammatory response is unknown, although several different reactions could contribute to this activity. As one example, it is known that BBI is a potent inhibitor of mast cell chymase. Chymase is known to perform a number of pro-inflammatory functions, including the activation of procollagenase and the activation of the inactive cytokine, 31 kD interleukin 1b to the 18 kD biologically active species. It is thought that the conversion of the 31 kD inactive cytokine to an active cytokine has a critical role in the initiation of the inflammatory response.

The anticarcinogenic protease inhibitors have been shown to prevent the influx of polymorphonuclear leukocytes (PMN) into regions of inflammation, as well as the production of active oxygen species by these cells, which would be expected to contribute to anti-inflammatory activity. The anticarcinogenic protease inhibitors previously have been shown to inhibit inflammation specifically associated with tumor promotion, which is a stage of carcinogenesis subject to inhibition. Thus, the inhibition of promotion by protease inhibitors may be viewed as a beneficial effect. Thus far no investigations have shown effects of BBI on the normal functioning of cells of the immune system [136, 135].

1.3.3.5 Role of Protease Inhibitors as Anti-HIV Agents

Protease Inhibitors can be also used as a drug for tackling infection by HIV-I virus. The present drugs that are being used against HIV infection are a cocktail of reverse transcriptase inhibitors and Protease Inhibitors. The virus life cycle involves integration of the viral genome into the host genome and transcription of a primary transcript from the viral genome integrated into the T Helper Lymphocytes. This primary transcript is further cleaved and is translated. Virus capsid proteins are translated as a single RNA transcript called as Gag-Pol transcript. The translated Gag-Pol protein needs to be cleaved into the respective capsid proteins. Here a major role is played by one the viral proteases being translated. This viral protease is a aspartate protease and has got a aspartyl residue at its active site. This protease cleaves specifically at the carboxy terminal of the proline amino acid residue if it is flanked by hydrophobic amino acids at both the sides. The protease inhibitor acting as a drug can inhibit this protease and terminate the live cycle of the HIV-I virus in the host cell. One of the protease inhibitors that can block the action of this aspartyl protease is Pepstatin. Some already available drugs that are exploiting this mechanism to inhibit the infection of HIV-I virus by blocking the action of the protease are Saquinavir, Ritonavir and Indonavir.

1.4. Antimicrobial Proteins and Peptides

Antimicrobial peptides are one among the four major antimicrobial compounds of plant origin. The other three being phenolics, alkaloids and terpenoids [181].

During the past decades, living organisms of all types have been found to produce a large repertoire of antimicrobial peptides that play an important role in innate immunity to microbial invasion. In higher organisms they are mainly produced on epithelial surfaces and in phagocytic cells that play a crucial role in the innate as well as the adaptive defense systems [88, 274, 110, 316]. Antimicrobial peptides exhibit rapid killing, often within minutes in vitro, and a broad spectrum of activity against various targets, including Gram-positive and -negative bacteria, fungi, parasites, enveloped viruses, and tumor cells [16, 110, 59, 84, 321]. In addition, their antimicrobial activity can be enhanced by the synergy between individual cationic peptides within a host, and between the peptides and other host factors, such as lysozyme [110]. Hundreds of antimicrobial peptides have been isolated so far and irrespective of their origin, spectrum of activity, and structure, most of these peptides share several common properties. They are generally composed of < 60 amino acid residues (mostly common L-amino acids), their net charge is positive, they are amphipathic, and in most cases they are membrane active. The interaction of antimicrobial peptides with membranes alters the organization of the bilayer and makes it permeable, causing membrane depolarization [186, 140]. This has led to the general conclusion that the interaction of most antimicrobial peptides with membranes, involving electrostatic and hydrophobic interactions, is a necessary precursor to cell death. However, it should be underlined that alternative and/or coexistent antimicrobial mechanisms cannot be ruled out at this stage, and evidence is accumulating that some peptides might actually act by binding to intracellular targets, or by stimulating host defense mechanisms [109]. Indeed, certain peptides have been found to interact directly with host cells to stimulate host gene products, including specific chemokines, chemokine receptors, integrins, transcriptional factors et cetera [110]. Furthermore, it has been suggested that the cationic antimicrobial peptides have many potential roles in inflammatory responses, which represent an orchestration of the mechanisms of innate immunity [108].

1.4.1. Types of Antimicrobial Peptides

It is common to classify antimicrobial peptides into four groups according to their secondary structure. This classification is as follows [301]: Linear peptides with α-helical structure; conformationally more restrained peptides, predominantly consisting of β-strands connected by intramolecular disulphide bridges; linear peptides with an extended structure, characterized by overrepresentation of one or more amino acids and peptides containing a looped structure. Another way of characterizing antimicrobial peptides classify them as thionins [83], plant defensins [90], lipid transfer proteins (LTPs) [91], hevein-type peptides [90], knottin-type peptides [90], snakins [262], sephardins [215], MBP-1 [78], macrocyclic peptides [289] and Ib-AMPs [287].

1.4.1.1. Thionins

Thionins consists of 45-47 amino acid residues and comprising two subgroups with four disulfide bonds and three disulfide bonds respectively. They have been isolated from the leaves of *Pyrularia pubera* and *Hordeum vulgare*. The three dimensional structure of thionins has been studied in detail, both by X-ray crystallography and NMR. The studies shows that the structure of thionins is well conserved and consists of two anti parallel α-helices and one β sheet, with hydrophobic and hydrophilic residues are segregated at the outer and inner helix surfaces. Toxic activity of thionins has been attributed to its binding to the phospholipids. The toxicity of thionin to plant pathogens has been reported [83] and has been extensively investigated [287].

1.4.1.2. Defensins

This is a highly complex group of 4 kD open-ended cysteine-rich peptides arranged with different structural motifs. They have been mostly isolated from mollusc, acari, arachnids, insects, mammals and plants. Defensins are arranged in families, based on their structural differences. Invertebrates [125, 5, 74, 49, 190, 273, 200] and plant [46, 90, 263, 167] defensins are characterized by three and four disulfide bridges, respectively. They show a common structure comprising an α-helix linked to a β-sheet by two disulfide bridges, distinctive structure known as the CSab motif. In mammals, α - and β-defensins are characterized by an antiparallel -β sheet

structure, stabilized by three disulfide bridges [321]. Some of them naturally exist as cyclic molecules such as the theta-defensins [291, 154]. It has been difficult to determine whether all molecules are homologous or have independently evolved similar features, but evidences are in favour of a distant relationship. The best evidence of this relationship is structural, particularly from their overall three-dimensional structure and from the spacing of half-cystine residues involved in intra-chain disulfide bonds.

1.4.1.3. Lipid transfer proteins

A group of proteins termed nonspecific lipid transfer proteins (ns-LTPs) is found in plants. Originally these proteins were identified by their ability to catalyze the transfer of lipids between membranes *in vitro* [133]. The suggestion that they would act as intracellular transporters of lipids between organelles was later questioned because of the fact that ns-LTPs are extracellular proteins. Other functions have been ascribed to ns-LTPs, including transport of cutin monomers [277] and involvement in flowering [216]. Also ns-LTPs have been suggested to be important in several types of plant stress response. These include responses to pathogens [191], drought [294] and temperature changes [77, 293]. Despite their implication in these diverse aspects of plant biology, it is not clear which specific role ns-LTPs play here [134, 76].

ns-LTPs are small proteins of about 90 residues with high values of pI (>9). The three-dimensional structures of ns-LTPs from several plant species are known, and they all consist of four α helices held together by four conserved disulfide bonds [116]. A hydrophobic central cavity is found between the four helices. *In vitro* studies have shown that ns-LTPs can bind a variety of fatty acids [320] and lipids [76]. High-resolution structures of ns-LTPs in complex with fatty acids are available from barley [158] and maize [106]. In these structures the fatty acid occupies the central hydrophobic cavity. However, it has been difficult to draw any conclusions about the *in vivo* activity of ns-LTPs from their lipid binding properties because it is unknown which ligands, if any, are bound to ns-LTPs *in vivo*.

ns-LTPs are encoded by multigene families [10]. In a given plant, several ns-LTP genes can be found, and they are often specifically expressed in both time and tissue. Also individual ns-LTP genes are induced under a variety of conditions. It is not clear whether the various ns-LTPs have functional overlap. If this is the case, it may prove difficult to use genetic approaches in determining the function of ns-LTPs.

1.4.1.4. Hevein-type peptides

Hevein, the most abundant protein in the latex of rubber trees, is a 43-residue, cysteine-rich peptide homologous to the chitin-binding domain of different types of multidomain proteins from plants [46, 153, 127] and to other single-domain peptides that have antimicrobial properties [299, 45, 44]. The hevein-like, antimicrobial peptide from sweet pepper contains the same 8 disulfide-linked cysteines that are present in the peptide from latex [299, 44], whereas in that from amaranth, the last two cysteines are missing because of a C-terminal deletion [45]. Three β-sheet strands, as well as an α-helix turn that links the second and third strands, are the most relevant three-dimensional features of the hevein structure, as determined by proton NMR [4].

1.4.1.5. Knottin-type peptides

Knottin-type peptides, 36-37 amino acid residues long, have been isolated from *Mirabilis jalapa*, *Phytolacca americana*. These peptides have six disulfide-linked cysteines that form the so-called knottin pattern. The three dimensional structure determined by proton NMR shows the global fold involving a cystine-knotted three-stranded anti-parallel β-sheet, a flexible loop and four β-reverse turns. Knottin-type peptides inhibit a wide range of fungi and gram-positive bacteria and their activities are reduced by the presence of divalent cations.

1.4.1.6. Snakins

Snakins are recently discovered antimicrobial peptides, which are 63 amino acids long containing 12-cysteine residues. They have been isolated from potato tubers. These peptides are found to be active (EC_{50} = 1-20μM) against fungal and bacterial plant pathogens. It causes a rapid aggregation of both Gram-positive and Gram-negative bacteria.

1.4.1.7. Other Families of Antimicrobial Peptides

Other families of antimicrobial peptides isolated from plants include MBP-1 from maize [78], Ib-AMPs isolated from the seeds of *Impatiens balsamina* [287, 217], shepherdins isolated from the roots of *Capsella bursa-pastoris* and macrocyclic peptides purified from different plants

of the Rubiaceae family. The MBP-1 is active against both fungi and bacteria. Ib-AMPs inhibits fungi and Gram-positive bacteria. The structure of Ib-AMPs investigated by CD and NMR shows that the peptide has two hydrophilic patches, which are at opposite ends of the molecule separated by a large hydrophobic patch. Little is known about the mode of action of this peptide.

1.4.2. Mode of Action of Antimicrobial Peptides

Many antimicrobial peptides bind in a similar manner to negatively charged membranes and permeate them, resulting in the formation of a pathway for ions and solutes [229]. Before reaching the phospholipid membrane peptides must transverse the negatively charged outer wall of Gram-negative bacteria containing LipoPolySaccharides (LPS) or through the outer cell wall of Gram-positive bacteria containing acidic polysaccharides. Hancock and coworkers described this process as a 'self promoted uptake' with respect to Gram-negative microorganisms [111]. In this mechanism, the peptides initially interact with the surface LPS, competitively displacing the divalent polyanionic cations and partly neutralize LPS. This causes disruption of the outer membrane and peptides pass through the disrupted outer membrane and reach the negatively charged phospholipid cytoplasmic membrane. The membrane-active properties of such peptides have been extensively studied using model membranes [229]. The amphipathic peptides can partition into cytoplasmic membrane through hydrophobic and electrostatic interactions, causing stress in the lipid bilayer. When the unfavorable energy reaches a threshold, the membrane barrier property is lost, which is the basis of the antimicrobial action of these peptides.

The action of antimicrobial peptides induces membrane defects such as phase separation or membrane thinning, pore formation, promotion of non-lamellar lipid structure or bilayer disruption, depending on the molecular properties of both peptide and lipid [169]. These pathways are variously termed transmembrane pores, wormholes or toroidal pores, and channel aggregates [21, 183, 265, 110]. An interesting 'in plane diffusion' model has also been proposed, where lipid-mediated channel formation is based on the curvature stain imposed on lipid membranes in the presence of intercalated amphipathic peptides. This model is independent of peptide aggregation, which has been reported to be entropically and electrostatically disfavored even in the presence of negatively charged phospholipids [21].

Two general mechanisms were originally proposed to describe the process of phospholipid membrane permeation by membrane-active peptides, the 'barrel-stave' [79] and the 'carpet' [228] mechanisms. The third model suggested is Shai-Matsuzaki-Huang (SMH) model.

1.4.2.1. The Barrel-Stave Model

The 'barrel stave' mechanism describes the formation of transmembrane channels/pores by bundles of peptides. Progressive recruitment of additional peptide monomers leads to a steadily increasing pore size. Leakage of intracellular components through these pores subsequently causes cell death. To allow pore formation the inserted molecules should have distinct structures, such as, an amphipathic or hydrophobic α-helix, β-sheet, or both α-helix and β-sheet structures. A crucial step in the barrel stave mechanism requires peptides to recognize one another in the membrane bound state. Peptide assembly can occur on the surface or within the hydrophobic core of the membrane, since hydrophobic peptides can span membranes as monomers [24]. In contrast, it is energetically unfavorable for a single amphipathic α-helix to transverse the membrane as a monomer. The low dielectric constant and inability to establish hydrogen bonds prohibits direct contact of the hydrophobic core of the membrane with the polar face of a single amphipathic α-helix. Therefore such monomers must associate on the surface of the membrane before insertion and the inserted peptides associated in membranes such that their non-polar side chains face the hydrophobic lipid core of the membrane and the hydrophilic surfaces of peptides point inward into the water-filled pore. An amphipathic α-helical peptide with a highly homogeneously charged hydrophilic face cannot form a transmembrane pore unless its charges become neutralized. It is therefore unlikely that antimicrobial peptides with a large number of lysines or arginines spread along the peptide chain will permeate the membrane by barrel-stave mechanism.

Because these peptides insert into the hydrophobic core of the membrane, they should have two important properties [264]: (i) their interaction with the target membrane is driven predominantly by hydrophobic interactions. (ii) if they adopt amphipathic α-helical structure, their net charge along the peptide backbone should be close to neutral. Alternatively they can be composed of predominantly hydrophobic amino acids [25]. As a consequence of these properties the peptides bind to phospholipid membranes irrespective of the membrane charge, and therefore, should be toxic to both bacteria and normal mammalian cells. Indeed, functional studies revealed

that pardaxin [234], alamethicin [255] and the helix α5 of δ-endotoxin [93] kill both bacteria and erythrocytes by the barrel-stave mechanism.

1.4.2.2. The Carpet Model

Early studies suggested that antimicrobial peptides kill microorganisms by forming transmembrane pores presumably via the barrel-stave mechanism [184]. However, the interaction of certain antimicrobial and lytic peptides with membranes clearly differentiated from the barrel-stave pore formation and a new mechanism was suggested. The carpet model was proposed for the first time to describe the mode of action of dermaseptin S [228], and later on was used to describe the mode of action of other antimicrobial peptides, such as dermaseptin natural analogues [95], cecropins [94], the human antimicrobial peptide LL-37 [213], caerin 1.1 [314], trichogin GA IV [192] and diastereomers of lytic peptides [212, 266, 210]. According to the carpet model, the peptides first bind onto the surface of the target microbial cell membrane and subsequently the membrane is covered by a 'carpet-like' cluster of peptides. Initial interaction with the negatively charged target membrane is electrically driven. In the second step, after a threshold concentration has been reached, the peptides cause membrane permeation. This model further suggests that the membrane breaks into pieces and leads to lysis of the microbial cell when a threshold concentration of peptide is reached [211]. High local concentration on the surface of the membrane depends upon the type of the target membrane and can occur either after all the surface of the membrane is covered with peptide monomers, or alternatively, antimicrobial peptides that associate on the surface of the membrane can form a local carpet. At an intermediate stage wormhole formation has been suggested to occur. Pores like these may enable the passage of low molecular weight molecules prior to complete membrane lysis. The formation of so-called wormholes or toroidal pores was proposed to describe the mode of action of dermaseptin [194], magainin [171, 185, 186, 317], protegrin [118] and melittin [316]. Neutral in-plane scattering data showed that pores of magainin molecules are almost twice as large as the alamethicin pores and suggested that the lipid layer bends back on itself like the inside of a torus [171]. This requires a lateral expansion in the polar headgroup region of the bilayer, which is filled up by individual peptide molecule. The positively charged amino acids are spread along the peptide chain of magainin and continuously in contact with the phospholipid head group during the process of peptide permeation, even when the peptide oriented perpendicular to the membrane plane. A

structure of organized holes composed of lipids and peptides were recently reported [317].The presence of negatively charged lipids is important for a peptide carpet to form, as they help to reduce the repulsive electrostatic forces between positively charged peptides. In addition, high local peptide concentrations are achieved when cationic peptides phase-separate into domains rich in acidic phospholipids [152]. The carpet model describes a situation in which these peptides are in contact with the phospholipid head group throughout the entire process of membrane permeation. Furthermore, a peptide that permeates the membrane via this mechanism can adopt different secondary structures, lengths, and can be either linear or cyclic upon its binding to the membrane. The net charge of the peptides needs to be highly positive and spread along the peptide chain that they only bind very weakly, or not bind zwitterionic membranes. However, highly positively charged antimicrobial peptides can lyse erythrocytes membranes if they form oligomers in solution [264].

In brief, peptides that act via the carpet mechanism and remain in contact with the acidic phospholipid head groups should have the following properties [264]: (i) their net charge needs to be highly positive and spread along the peptide chain. (ii) they should bind very weakly, or not bind zwitterionic membranes and as a consequence not be hemolytic. It should be noted however, that highly positively charged antimicrobial peptides can lyse erythrocytes if they form oligomers in solutions (iii) no preferable structure is required, as long as a certain level of hydrophobicity and number of positive charges are preserved.

1.4.2.3. Shai-Matsuzaki-Huang (SMH) model

This is the most acceptable model [265, 183] that can explain the activity of most antimicrobial peptides. According to this model peptides interact with membrane, followed by displacement of lipids, alteration of membrane structure, and in certain cases entry of the peptide into the interior of the target cell. In general peptides operating by the SMH mechanism kill microbes at micromolar concentrations.

1.4.3. Potential Applications of Antimicrobial Peptides

The widespread increase of bacterial resistance towards many conventional antibiotics has resulted in an intensive search for alternative antimicrobial agents [37]. In this respect,

antimicrobial peptides are on the brink of a breakthrough. Due to the increasing interests of antimicrobial peptides, many companies are making efforts to introduce the antimicrobial peptide products on the market.

Natural antimicrobial peptides have potential application in food preservation as they specifically kill microbial cells by destroying their unique membranes. Interest in LAB bacteriocins has been sparked by growing consumer demands for natural and minimally-processed foods. LAB bacteriocins have well-documented lethal activity against foodborne pathogens and spoilage microorganisms [63] and can play a vital role in the design and application of food preservation technology [156, 193]. Currently, nisin is approved as a food preservative in more than 40 countries worldwide [71] and the use of pediocin PA-1 is covered by several European and US patents [300, 38]. Both nisin and pediocin PA-1 have applications in dairy and canned products. Studies of model food systems demonstrate that pediocin-like bacteriocins are better at killing pathogens in meat products, where nisin is ineffective [155, 193].

Antimicrobial peptides tend to be involved in a local response to infections and the first clinical trials thus have been directed towards topical infections. Magainin Pharmaceuticals have taken the α-helical magainin variant peptide MSI-78 into phase-III clinical trials in studies of efficacy against polymicrobic foot-ulcer infections in diabetes. It was announced that these trails demonstrated equivalency to orally administered ofloxacin, but with less side effect. Applied Microbiology has initiated a trial testing the efficacy of the bacterial lantibiotic peptide nisin against *Helicobacter pylori* stomach ulcers. The antibacterial activity of nisin may not be impressive, but its endotoxin neutralizing activity upon intravenous administration has led to a dramatically increased survival rate. Iseganan (IB-367, Intrabiotics, Mountain View, CA, USA), a protegrin-derivative [196], has passed phase II clinical trials for application against oral mucositis successfully and the company has announced plans to launch Phase II/III clinical study to investigate iseganan HCl [96] in the prevention of ventilator-associated pneumonia (VAP). Another formulation of this company, iseganan HCl solution for inhalation, has completed phase I clinical trials in cystic fibrosis patients. Other companies, for examples, Periodontix Inc. (Watertown, MA, USA) has entered phase I clinical trials for the application of a histatin-derived peptide against oral candidiasis and Trimeris (Durham, NC, USA) has successfully completed a phase II clinical trial, in which peptide T-20 [283, 64, 307] reduced the viral load of HIV-infected patients with up to 97%. Also Demegen (Pittsburgh, PA, USA) has successfully completed animal studies with peptide D2A21 [242] as therapeutic for several types of cancer and has been

developing this peptide gel formulation as a wound healing product to treat infected burns and wounds. Demegen's P113L (histatin 5 fragment) Oral Rinse exhibits significant binding to oral mucosal membranes and has an excellent human safety profile in over 400 treated patients. Another product of Demegen, P113D derived from histatins [252], had been granted orphan drug status for the treatment of cystic fibrosis infections.

Interestingly, a number of evidence has shown efficacy of some antimicrobial peptides against systemic infections, including α-helical-peptide efficacy against *P. aeruginosa* peritoneal infections, β-sheet-protegrin efficacy against methicillin-resistant *S. aureus* (MASA), vancomycin-resistant *Enterococcus faecalis* (VRE) *and P. aeruginosa* infections, and indolicidin in liposomal formulation against *Aspergillus* fungal infections [2, 276, 251]. Entomed (Illkirch, France)'s product, heliomicin [149] for systemic antifungal treatment is under preclinical stage. Human lactoferricin (AM Pharma, Bunnik, Netherland) and bactericidal/permeability-increasing protein (Xoma, Berkeley, CA, USA, [102]) have also been proved to have potential for systemic applications. This has indicated that antimicrobial peptides could be used as injectable antibiotics against serious bacterial and fungal infections that are resistant to conventional antibiotics. Indeed, Neuprex™, a systemic formulation of the recombinant BPI-derived peptide rBPI 21 (Xoma Corp., Berkeley, CA, USA, [123]), has proven to be very effective in treatment of meningococcal sepsis in phase II/III clinical trials and more than 1000 patients have received NEUPREX in clinical studies without any safety concerns.

CHAPTER 2

CLONING AND SEQUENCING OF THE β-SUBUNIT OF THE PROCARYOTIC RESPIRATORY NITRATE REDUCTASE GENE

2.1. Introduction

Purification of the proteins is a standard prerequisite to carry out any kind of functional or structural studies on them. Quite often the protein of interest is present in low amounts in the source or the source is in limited amount or available only during a certain period of the year thus limiting the yields of purified protein. Also, a lot of this protein is itself lost during the process of purification. Therefore, quite often the gene of the protein of interest is isolated from the sample along with the genomic DNA and is amplified by PCR using the primers designed specifically complementary to first and last few nucleotides of the gene for the desired protein. Thereafter the amplified gene is purified and inserted into a suitable vector. This recombinant vector is then inserted into the suitable carrier and the transformed carrier is cultured. The transformed carrier expresses large amount of the protein of interest due to the presence and expression of the foreign gene from the source organism to produce large amount of the exogenous protein of interest.

The Gram-positive family of *Bacillaceae* contains four aerobic, endospore forming genera *i.e. Thermoactinomyces, Sporosarcina, Sporolactobacillus* and *Bacillus* [230]. Compared to the three other genera, the genus *Bacillus* is quite heterogeneous: it consists of the entire aerobic spore forming Gram-positive bacteria that can not be classified as belonging to one of the three other genera within the *Bacillaceae* family. The *Bacillus* species are widely distributed in soil and water, and certain strains tolerate high temperatures and extreme pH values. Most species are harmless to humans and animals and have been used in several traditional food fermentations, including the production of natto from soybeans in Japan. Only a few pathogens are known, including *B. anthracis*, the causative agent of anthrax [292], *B. cereus*, which causes food poisoning and several insect pathogens of which *B. thuringiensis* is the most well known [11]. The genus *Bacillus* is an important source of commercial enzymes, such as cellulases, lipases, starch degrading enzymes and proteases [85]. The low level of reported incidence of pathogenicity of *B. subtilis* and the widespread use of its products and those of its close relatives in the food, beverage, and detergent industries have resulted in the granting of GRAS (generally regarded as safe) status to *B. subtilis* by the U.S. Food and Drug Administration. The ability of many *Bacillus* species to secrete high levels of proteins, both of homologous and heterologous nature has made these bacteria of considerable importance for biotechnological applications [85].

Nitrogen being a basic element for life is the important and major constituent of two major macromolecules: proteins and nucleic acids and also a part of other biomolecules as hormones, neurotransmitters and vitamins. Nitrogen exist in the biosphere in several oxidation states, from (+V) to (-III). Interconversions of these nitrogen species constitute the global biogeochemical cycle, which is sustained by several biological processes with bacteria playing the main role [238]. Processes such as nitrogen fixation, assimilation, nitrification and denitrification form the part of this cycle. Nitrate reduction, an important stage of nitrogen turnover in nature is taken care of by Nitrate Reductase. Nitrate reductase, the enzyme responsible for nitrate reduction has various functions as: utilization of nitrate as the source of nitrogen i.e. nitrogen assimilation as in eukaryotes [52, 53], nitrogen assimilation in prokaryotes, production of metabolic energy during nitrate utilization as terminal acceptor of electrons (nitrate respiration) and dissipation of excess of reducing energy to maintain oxidation-reduction balance i.e. nitrate dissimilation [195, 239, 240, 281].

Nitrate reduction plays a key role in the nitrogen cycle and has important agricultural, environmental, and public health implications. Assimilatory nitrate reduction, performed by bacteria, fungi, algae, and higher plants, is one of the most fundamental biological processes, accounting for more than 10^4 megatons of inorganic nitrogen transformed each year [104]. However, there is worldwide concern over the excessive use of fertilizers in agricultural activities, leading to nitrate accumulation in groundwater. Consumption of drinking water with high nitrate levels has been associated with methemoglobinemia and gastric cancer due to endogenous formation of genotoxic *N*-nitroso compounds by bacteria in the gastrointestinal tract [298]. The main threat to the environment comes from eutrophication of aquatic ecosystems. Nitrogen oxides generated by denitrification are also associated with the greenhouse effect and the depletion of stratospheric ozone [324]. Therefore, nitrate reduction has become an important focus for research in the last several years, generating a vast literature.

Nitrates and sulfates being the major pollutants found in many industrial wastes, the concern about removal of Nitrate pollution is on the rise. Thus, nowadays, nitrate reductase is therefore being used for biological remediation. A company named as the Nitrate Elimination Company, Inc. (NECi) was founded in 1993 in Lake Linden MI, USA. NECi develops and manufactures enzyme-based products for water testing and water treatment. NECi has developed Green Chemistry Methods for Nitrate analysis and testing. NECi markets Nitrate Analysis Reagent Kits for automated analyzer instruments and Nitrate Test Kits for all types of nitrate

testers. These Green Products are built around the sustainable NECi Nitrate Analysis Method. Their current product line focuses on superior stock nitrate reductase.

Nitrate Reductase gene has earlier been cloned from many organisms. Nitrate reductase genes has been purified from cyanobacterium *Synechococcus* PCC6301 [164], *Fusarium oxysporum* [177], Cyanobacterium *Anacystis nidulans* [142] and from few other microorganisms. The Nar operon, responsible for the transcription of respiratory nitrate reductase genes has been cloned from *Escherichia coli* [203, 243]. The β subunit of respiratory nitrate reductase has been cloned from *Thermus thermophilus* [70]. Also, all the genes encoding for the respiratory nitrate reductase system have been cloned from *Bacillus subtilis* [122].

In this work, nitrate reductase gene is cloned and sequenced from *Bacillus circulans* NCIM 2107. First the genomic DNA of *Bacillus circulans* is isolated and the gene for nitrate reductase is amplified by PCR using the appropriate forward and reverse primers. Thereafter the PCR products were run on the Agarose Gel with 100bp ladder and checked for the desired product, which was then eluted from the band obtained on the agarose gel using the spin gel extraction kit. The purified Amplicon thus obtained was then inserted or ligated into the linearized pGEM-T cloning vector. Thereafter the recombinant plasmids were transferred into the competent *Escherichia coli* DH5α cells and the transformed cells with recombinant plasmid were screened by growing in medium containing ampicillin, X-gal and IPTG. Plasmid isolation is carried out from the screened bacterial colonies and was analyzed on agarose gel for the presence of recombinant plasmid. Further conformation of the recombinant plasmids was done by performing PCR using the same set of primers and plasmid DNA as a template. Sequencing of the gene is carried out directly from the transformed E. coli DH5α cells carrying the recombinant plasmid.

2.2. Materials and Methods

2.2.1. Materials

Lyophilized culture of *Bacillus circulans* NCIM 2107 was procured from National Collection of Industrial Microorganisms (NCIM) National Chemical Laboratory (NCL), Pune. Other chemicals required were Tris-base or (Hydroxymethyl)aminomethane, Ethylene Diamine Tetra Acetic acid (EDTA), double distilled water, Milli q water, Tryptone, Yeast Extract, Sodium Chloride (NaCl), Beef Extract, Peptone, Agar, Sodium Dodecyl Sulfate (SDS), Concentrated

Hydrochloric Acid (Conc. HCl), Proteinase-K, Calcium Acetate, RNase A, Phenol, Chloroform, Isoamyl alcohol, Potassium Acetate, Ethanol, Agarose, Ethidium Bromide (EtBr), Bromophenol Blue, Glycerol, Sucrose, Standard DNA Marker, Glacial Acetic acid, Sodium Hydroxide (NaOH), Taq Buffer, deoxy Nucleotide TriPhosphates Mix or dNTPs Mix, Forward and Reverse Primers, Taq Polymerase, Millipore water, Sodium Acetate, Isopropanol, Ampicillin, Calcium Chloride ($CaCl_2$), Potassium Chloride (KCl), Magnesium Chloride ($MgCl_2$), Glucose, 5-bromo-4-chloro-3-indolyl-β-D-galactoside or X-gal, Dimethylformamide and IsoPropylThio-ß-D-Galactoside (IPTG). Spin Gel Extraction Kit of Bangalore Genei™ containing Gel Solubilizer, Wash Buffer and Elution Buffer was also used along with Promega's pGEM-T Vector Kit containing Linearized TA Vector, Rapid Ligation Buffer and T4 DNA Ligase. *E. coli* DH5α was used as the carrier for recombinant vector.

2.2.2 Cloning of β-subunit of Prokaryotic Respiratory Nitrate Reductase Gene

2.2.2.1. Primer Designing

DNA amplification requires primers, which are short oligonucleotide (around 20bp long) sequences that anneal to a single standard DNA template in a unique location, thereby functioning as the initiation site for chain extension by DNA polymerase. Efficacy and sensitivity of PCR largely depend on the efficiency of primers. The other factors being quantity of the DNA template, the mixture of chemical reagents and the time-temperature program scheme. Some of the basic things which are kept in mind for primer designing are:

Melting temperature(Tm): It is the most crucial criterion, which determines the stability of the duplex formed between the primer and the DNA template and also the specificity of the reaction.

G/C content: The G/C content should be selected for stable specific binding, yet allow efficient melting.

Primer length: The length of a primer is a trade-off between being sufficient for uniqueness, providing stability, and being as short as possible to minimize cost. A 20-mer is considered to be optimum, but the some range around this optimum is tolerated.

In addition, it has also been found that having a G or C at the 3' terminus of the primer is valuable to increase primer/template stability and encourage efficient extension (this is known as GC clamp). It is also important that the primer not be able to fold onto itself and form a hairpin

structure, thus inhibiting its ability to bind to the template. To avoid this, primers are tested for self complementarity, especially at the 3' end. Finally, primers should bind to unique location within the sequence template, therefore it is reasonable to avoid common repetitive elements or common dinucleotide and trinucleotide repeats.

Now, for primer designing all the sequences of the beta subunit of respiratory nitrate reductases (Nars) of various members of the genus *Bacillus* were retrieved from the database and were subjected to pairwise and multiple alignment using the programs BLAST and CLUSTALW respectively. The sequences were analyzed for conserved regions or domains at N and C terminals. These sets of conserved regions were used to design a set of degenerate primers with codon bias also in account.

The Primers thus designed were ordered to be synthesized from outside. Thereafter, TE buffer (10:1) was prepared by adding 0.2 ml of 0.5 M EDTA to 1 M Tris-Cl 1 ml (pH 8.0) solution with 98.8 ml of double distilled H_2O and it was used to maintain the primary stock solution of primers at a concentration of 100 pmol/μl at -20°C. Secondary and tertiary stock solutions of primers were prepared as 20 pmol/μl and 5 pmol/μl in water respectively and were stored again at -20°C.

2.2.2.2. Revival of lyophilized bacterial culture of Bacillus circulans

First Nutrient Agar was prepared by adding 3.0 g Beef Extract, 5.0 g Peptone and 15.0 g Agar to 900 ml of double distilled H_2O, then the pH of the solution was adjusted to 7.2 and the final volume was made upto 1000 ml. Thereafter, the nutrient agar was autoclaved and poured into Petri-plates under aseptic conditions inside a laminar flow hood. Next, a culture tube containing the lyophilized culture was cut near the inserted cotton plug and the cotton plug was removed. And the mouth of the vial was flamed. 400 μl of sterile 0.9% Saline was added to the opened vial and it was allowed to stand for 30 minutes. Thereafter, one loop full culture was streaked on the nutrient agar plate and the remaining suspension was transferred as (5 μl, 10 μl, 20 μl, 30 μl...) to Petri plates and was spread uniformly over the whole surface with a sterile Spreader. Finally, the inoculated plates were incubated at 37°C overnight.

2.2.2.3. Isolation of bacterial genomic DNA

Isolation of bacterial genomic DNA requires perfect lysis of the bacterial cells so that the whole bacterial genome is poured out of the cell into the solution and contamination of the bacterial genome with bacterial RNA and proteins needs to be checked. The standard way of remove proteins from nucleic acid solutions is to extract first the phenol:chloroform (optionally containing hydroxyquiniline at 0.1%) and then with chloroform. This procedure takes advantage of the fact that deproteinization is more efficient when two different organic solvents are used instead of one. Furthermore, although phenol denatures proteins efficiently, it does not completely inhibits RNase activity, and it is a solvent for RNA molecules that contains long tracts of Poly(A) [42]. These problems can be circumvented by using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1). The subsequent extraction with chloroform removes any lingering traces of phenol from the nucleic acid preparations. Additional measures are required for the removal of proteins from complex mixtures of molecules such as cell lysates. In these cases, it is usual to remove most of the protein by digestion with proteolytic enzymes such as Pronase or Proteinase K, which are active against a broad spectrum of native proteins, before extracting with organic solvents.

For isolation of bacterial genomic DNA first Nutrient Broth was prepared by adding 5.0 g peptone and 3.0 g beef extract to 900 ml of double-distilled water. The pH of the solution was adjusted to 7.2 and the final volume was made upto 1000 ml. Prepared broth was autoclaved prior to use. To this sterile broth, a loop full of *Bacillus circulans* was used for inoculation and the culture was maintained overnight at 120 rpm, 37°C.

Also, Proteinase K solution was prepared by dissolving the lyophilized powder of the enzyme in sterile 50 mM Tris-Cl (pH 8.0), 1.5 mM Calcium Acetate at a concentration of 20 mg/ml. This stock solution was divided into small aliquots and stored at -20°C. An aliquot can be thawed and refrozen several times, but it should then be discarded. The activity of proteinase K is several folds higher at 50°C then at 37°C.

Then, solution of phenol:chloroform:isoamyl alcohol (25:24:1) was prepared by taking 250 ml of distilled phenol melted at 60°C, equilibrated once with 1 M Tris-HCl, pH=8.0, then twice with 0.1 M Tris-HCl, pH 8.0 (or until the pH of the aqueous layer is 8.0) and adding 240 ml of chloroform to it with 10 ml of isoamyl alcohol and 0.8 g of 8-hydroxyquinoline.

Next, Lysis buffer was prepared having the composition of 10 mM NaCl, 20 mM Tris (pH 8.0), 0.5% SDS w/v and having 100 μg/ml of Proteinase K. First, 29.21 g of NaCl was dissolved in

70 ml of distilled water and the volume was made upto 100 ml. Then, 1 M Tris-Cl (pH 8.0) solution was prepared by adding 12.141 g of Tris-base to 80 ml of distilled water. Then pH was adjusted to 8.0 with Conc. HCl and the final volume was made upto 100 ml. The solution was autoclaved and stored at room temperature. Next, 10% SDS w/v was prepared by dissolving 5.0 g of SDS in 40 ml of distilled water by heating at 68°C. The pH of the solution was adjusted to 7.2 with Conc. HCl and the final volume was made upto 50 ml by adding water. Now, the lysis buffer was prepared by mixing 5 M NaCl 0.2 ml, 1.0 Tris (pH 8.0) 2.0 ml, 5.0 ml of 10% SDS (w/v) and Proteinase K (20 mg/ml) 0.5 ml. Final volume was made to 100 ml with distilled water. The lysis buffer, thus prepared was sterilized by autoclaving and stored at room temperature.

Then, 2 ml of overnight grown culture was taken in autoclaved microcentrifuge tube. Next, the bacterial cells were harvested by centrifugation at 5000 rpm, 3 minutes. The pellet observed was taken and suspended in 500 µl of TE buffer and was again centrifuged at 5000 rpm for 3 minutes. This step was repeated twice. Washed cells were suspended in 500 µl lysis buffer and incubated at 55°C for 1 hour. After incubation 500 µl phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently. The mixture was centrifuged at 12000 rpm, 4°C for 10 minutes. Upper aqueous layer was transferred into new microcentrifuge tube and in this equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently. The mixture was then centrifuged at 12000 rpm, 4°C for 10 minutes and the upper aqueous layer was collected. To this aqueous layer, 1/10 volume of chilled 3 M sodium acetate was added and mixed. Chilled ethanol (2.5 volume of the above solution) was added and placed for DNA precipitation at -20°C for 1 hour. The precipitated DNA was obtained as pellet after centrifugation at 12000 rpm, 4°C for 15 minutes. Finally it was dissolved in appropriate amount of TE buffer.

2.2.2.4. Quantification of Genomic DNA using spectrophotometer

Because of its rapid, simple and non-destructive nature, absorption spectroscopy has long been a method of choice to measure the measure the amount of DNA or RNA in concentrated pure solutions. For quantitating the amount of DNA or RNA, readings are taken at wavelength of 260 nm and 280 nm. The reading at 260 nm allows calculations of nucleic acid in the sample. An OD of 1 corresponds to ~50 µg/ml for double stranded DNA, 40 µg/ml for single stranded DNA and RNA, and ~33 µg/ml for single stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm (OD_{260}:OD_{280}) provides an estimate of the purity of the nucleic acid. Pure

preparations of DNA and RNA have $OD_{260}:OD_{280}$ values of 1.8 to 2.0, respectively. If there is significant contamination with protein or phenol, the $OD_{260}:OD_{280}$ will be less than the values given above and accurate quantification of the amount of nucleic acid will not be possible.

This test is useful only for highly purified preparations of nucleic acid, since it detects any compound that absorbs significantly at 260 nm, which includes, for example, DNA, RNA, EDTA and Phenol. Nucleic acids absorb so strongly at 260 nm that only a significant amount of protein contamination will cause a significant change in the ratio of the absorbance at the two wavelengths [305, 98, 179, 178, 313].

The specific absorption coefficients of both DNA and RNA are affected by the ionic strength and the pH of the solution [18, 313]. Accurate measurements of the concentration can be made only when the pH is carefully controlled and the ionic strength of the solution is low. Also, it is difficult to measure the absorbance of low volumes of solution and the method is reliable only over a fairly narrow range of concentrations (5 μg/ml to 90 μg/ml).

5μl of the DNA sample was diluted to 1 ml in nuclease fee water. Its OD was determined at 260 nm and 280 nm. The ratio of OD_{260} / OD_{280} was calculated to check the purity of the DNA.

2.2.2.5. Agarose Gel Electrophoresis of Genomic DNA

Agarose gel electrophoresis is a method used in molecular biology to separate DNA, or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate farther than longer ones. But conformation of the DNA molecule and the concentration of the gel is also a factor. The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is Ethidium Bromide, usually abbreviated as EtBr. It fluoresces under UV light when intercalated into DNA. Loading buffers are added with the DNA in order to visualize it and sediment it in the gel well. Negatively charged indicators, Xylene cyanol and Bromophenol blue, in the loading buffer keep track of the position of the DNA. They run at about 5000bp and 300bp respectively, but the precise position varies with percentage of the gel.

Firstly, TAE buffer was prepared. 0.5 M EDTA (pH 8.0) is required for the same. It was prepared by adding 18.6 g Na_2EDTA in 70 ml of distilled water. It was constantly stirred on magnetic stirrer and the pH was adjusted to 8.0 with 4 M NaOH solution. Then the volume was made upto 100 ml with distilled water and the solution was autoclaved and stored at room

temperature. Now, 50X TAE buffer was prepared by adding 24.2 g of Tris-base, 5.71 ml of Glacial Acetic Acid and 2.0 ml of 0.5 M EDTA Na_2 (pH 8.0) to distilled water and the volume was made upto 100 ml. The buffer was then autoclaved and stored at room temperature.

Next, 6X loading buffer was prepared by adding 0.25% w/v of Xylene Cyanol, 0.25% w/v of Bromophenol Blue and 40% w/v of Sucrose to sterile distilled water and the pH was adjusted to 8 with 1 M NaOH and was stored at -20°C.

Also, 0.4 g of agarose was added to 50 ml 1X TAE Buffer. The mixture was heated in a microwave oven till the agarose was completely dissolved. 2 μl of Ethidium bromide (10 mg/ml stock) was added after the solution became lukewarm. This solution was poured into the gel casting tray fitted with appropriate comb and allowed to solidify. This gel was used for the submerged electrophoresis for separating the DNA samples using 1X TAE as the running buffer.

Then, 4 μl of isolated genomic DNA was mixed with 0.20 volumes of 6X loading buffer and loaded into the gel well. Also, 2 μl of Standard DNA Marker was loaded into another well. The gel was then run at 100 volts for 20 minutes. Then, working staining solution was prepared by adding 10 μl of ethidium bromide stock (10 mg/ml) solution to 200 ml distilled water and the gel was stained for 3-5 minutes and then destained in distilled water. Finally, the gel was visualized under ultra violet.

2.2.2.6. PCR Amplification

The polymerase chain reaction (PCR) is a technique widely used to amplify a piece of DNA by in vitro enzymatic replication. Genomic DNA or any DNA material is used as template to which complementary primers bind and the region in between the primers is amplified by 5'→ 3' polymerase activity of Taq Polymerase. As PCR progresses, the DNA thus generated is itself used as template for replication. Most PCR methods typically amplify DNA fragments of up to 10kb.

Standard Reaction mixture (20 μl) was prepared according to the table below:

Reaction component	Volume added
10x Taq Buffer	2 μl
dNTP mix	4 μl
Primer 1	0.5 μl
Primer 2	0.5 μl
Taq Polymerase (5U/μl)	1 μl

Template DNA	2 µl
Water	10 µl
Total	**20 µl**

Two PCR reaction mixtures were prepared: one with template DNA and the other serving as negative control where template DNA is replaced with water.

The PCR program for amplification includes the following steps:

Lid temperature: 104°C

1) Initial Denaturation	94°C for 3 minutes
2) Denaturation	94°C for 1 minute
3) Annealing	54°C for 1 minute
4) Extension	72°C for 1 minute
5) Go to step 2	For 30 cycles
6) Final Extension	72 °C for 10 minutes
7) Hold at	4°C for 1 hour
8) End.	

Finally, the PCR products were run in 0.8% agarose gel against 100bp ladder to check for the desired product.

2.2.2.7. Gel Elution of the Desired Amplicon

Gel elution of the desired amplicon was carried out using Spin Gel Extraction Kit (Bangalore Genei™). The kit utilizes a gel solubilisation solution for melting agarose piece containing the DNA band of the desired amplicon. This mixture of gel solubilisation solution and melted agarose containing the DNA of our interest is then loaded onto small silica membrane column provided with the kit. After a brief centrifugation of the loaded column, DNA is selectively bound to the membrane while the contaminants pass through. The remaining contaminants within in the column are selectively eluted out with the wash buffer (provided in the kit). And finally the pure nucleic acid is then eluted in minimal volume of the elution buffer or water.

Firstly the amplified DNA fragments from PCR were run on a gel made up of low-melting agarose. Next, the DNA containing agarose gel slice was cut out and weighed in a microcentrifuge tube. Care was taken to cut only that region of the agarose gel that contained the DNA as excess agarose gel reduces the efficiency of DNA extraction. Three volumes of gel solubiliser solution

was then added to one volume of agarose gel (i.e. 300 μl for 100 mg gel) and incubated at 50°C for 10 minutes. The tube was periodically vortexed every 2-3 minutes to allow better mixing of the gel. The colour of the mixture was checked and 3 M Sodium Acetate was added in case the colour was red or pink to bring it back to yellow. The colour, being the indicator of the pH of the mixture. 100 μl of Isopropanol was then added for every 100 mg of agarose gel weighed. Next, the spin column was kept in a 2 ml collection tube and solution containing the solubilised gel with amplicon and isopropanol was loaded onto the spin column, containing the silica gel. Then, the tube was centrifuged for 1 minute at 10,000 rpm. The flow through collected was discarded and the column was placed back in the same collection tube. 700 μl of diluted wash buffer was then poured over the column and the collection tube was again centrifuged at 10,000 rpm for 1 minute. The flow through was discarded again and the column was placed back in the same collection tube. The empty column was then centrifuged for 2 minutes to ensure that no wash buffer remained in the column. The spin column was then placed in a new 1.5 ml tube and 50 μl of elution buffer was added to the center of the column membrane. The column was allowed to stand at room temperature for 5 minutes and then centrifuged for 1-2 minutes to elute the DNA. The elution step was repeated again using a fresh 1.5 ml microcentrifuge tube so that the first elute is not diluted.

2.2.2.8. Ligation of the amplicon with pGEM-T cloning vector

Cloning of PCR products generated by many thermostable DNA polymerases enzymes used for PCR such as *Taq* DNA Polymerase add single deoxyadenosine residues to the 3′-ends of PCR products [62, 124]. So called "T" vectors, including Promega's pGEM-T Vectors, are manufactured with 3′-T overhangs complementary to these A-overhangs. Promega's pGEM-T is prepared by cutting either the pGEM® -5Zf(+) Vector with *Eco*R V (Type II restriction endonuclease) and adding a 3′-terminal thymidine to both ends.

The high copy number pGEM®-T Vector contains T7 and SP6 RNA Polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase (Figure 2.1A). The Vector uses the gene for Ampicillin resistance for easy selection. Insertional inactivation of the alpha-peptide allows recombinant clones to be directly identified by blue/white color screening on indicator plates. The multiple cloning region (Figure 2.1B) includes restriction sites conveniently arranged for generating nested sets of deletions. The

pGEM®-T Vector also contain the origin of replication of the filamentous phage f1 for the preparation of single-stranded DNA (ssDNA).

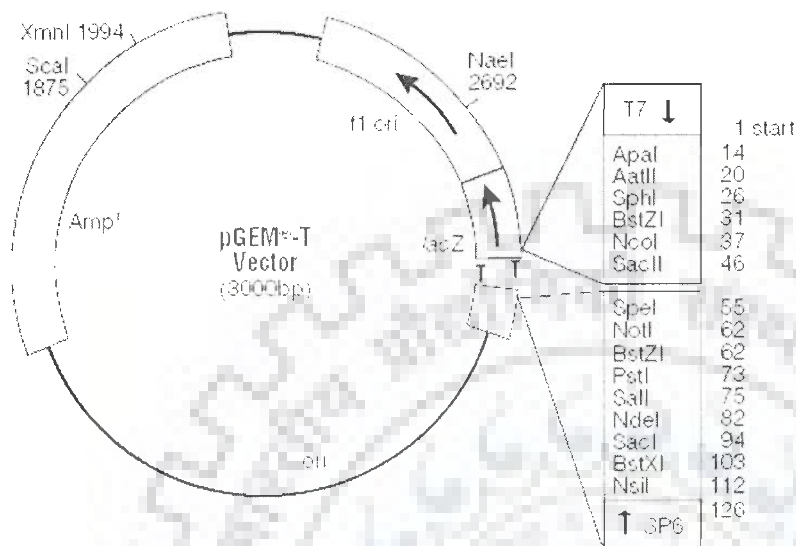


Figure 2.1A: pGEM-T vector with Multiple Cloning Sites (MCS) and selectable marker genes.

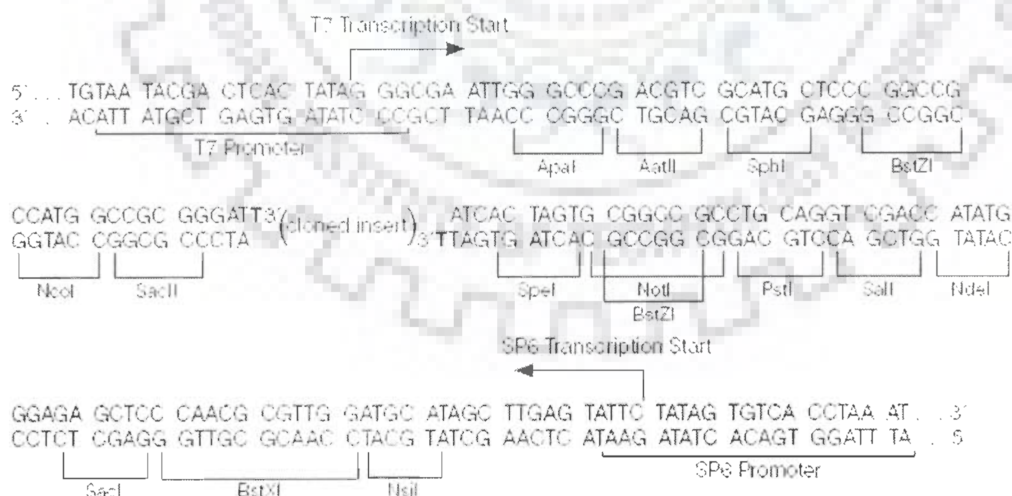


Figure 2.1B: The promoter and multiple cloning site of the pGEM®-T: The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA Polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA Polymerase.

The pGEM®-T Vectors offer a number of options for removal of the insert DNA of interest. *BstZ* I may be used in a single digest with the pGEM®-T Vector. Alternatively, *Pst* I may be combined with either *Sph* I, *Nco* I or *Sac* I in a double digest with this vector. The choice of method to be used depends upon the sequence of the insert to be cloned and the availability of the restriction enzymes.

Specialized applications of the pGEM®-T Vector include: cloning PCR products, construction of unidirectional nested deletions, ssDNA production, blue/white screening for recombinants and transcription *in vitro* from dual opposed promoters.

The enzyme used for ligating the Amplicon with pGEM-T vector was T4 DNA Ligase. Bacteriophage T4 DNA Ligase is a polypeptide of 68 kD that catalyses the formation of phosphodiester bonds between adjacent 3′-hydroxyl and 5′-phosphate termini in DNA [310]. It is used for joining DNA molecules with compatible cohesive termini. Intermolecular ligation is stimulated by low concentration of the agents such as polyethylene glycol that promote the efficient interaction of macromolecules in aqueous solutions. It is also used for joining blunt-ended double stranded DNA molecules to one another or synthetic linkers. This reaction is much slower than the ligation of cohesive termini. However, the rate of blunt end ligation is improved greatly by the addition of monovalent cations such as 150-250 mM NaCl and low concentrations of ethylene glycol [220, 115].

Firstly, 2 µl ligase buffer (2X) was taken in a fresh, sterile pcr-tube. To this 0.5 µl DNA ligase (3 Weiss/µl) was added. Then, 0.5 µl Linearized TA cloning vector was mixed with eluted PCR product (about 80 µg or 1 µl). Finally, total reaction volume was made to 5 µl by adding Millipore water and kept overnight at 4°C for ligation.

2.2.2.9. Preparation of competent cells

E. coli DH5α is used for preparation of competent cells. It is a recombination-deficient amber suppressing strain used for plating and growth of plasmids and hsdR17 recA1endA1 gyrA96 thi-1 relA1 cosmids. The o80 LacZΔM15 mutation permits α-complementation of the amino terminus of β-galactosidase encoded in pUC vectors [107].

Competence refers to the state of being able to take up exogenous DNA from the environment. Competence is not encoded in the cell's genes. Instead it is induced by laboratory procedures in which cells are passively made permeable to DNA, using conditions that do not

normally occur in nature. The bacterial cell membranes are negatively charged and so repel the negatively charged DNA from entering the cell. The positively charged Ca^{2+} ions during the $CaCl_2$ treatment mask the negative charges of the membranes and aid in the entry of the DNA into the cell during a transient heat shock. An excellent preparation of competent cells will give ~10^8 colonies per microgram of plasmid.

Firstly Luria-Bertani (LB) medium was prepared by dissolving 10 g Tryptone, 5 g Yeast extract and 10 g of NaCl in 950 ml of distilled water and the pH of the resulting solution was adjusted to 7.0 with 4 M NaOH. The final volume of the solution was then adjusted to 1000 ml by adding distilled water. The media thus prepared was autoclaved and stored at room temperature.

Ampicillin stock (50 mg/ml) was also prepared by adding 500 mg of Ampicillin Hydrochloride to 6ml of distilled water. The final volume of the solution was made upto 10 ml by adding distilled water and the solution was filter sterilized with 0.22 µm filters. Ampicillin stock thus prepared was aliquoted and stored at -20°C.

0.1 M $CaCl_2$ solution was then prepared by adding 2.19 g of $CaCl_2.6H_2O$ to 80 ml of distilled water and the final volume was made upto 100 ml. The solution thus prepared was autoclaved, aliquoted and stored at -20°C.

Firstly single colony of host strain i.e. *E. coli* DH5α was inoculated in 2 ml of LB media and incubated at 37°C in a shaker at 150 rpm. The overnight grown culture (1 ml) was inoculated in 50 ml LB media containing ampicillin at working concentration id 80 µg/ml. Culture was incubated at 37°C with 200-250 rpm for 3 to 4 hrs till OD_{600} reached 0.4 to 0.5. Culture were transferred in pre-cooled, sterile Oakridge tube and kept on ice for 10-15 minutes. Cells were then harvested by centrifugation at 5000 rpm for 10 minutes at 4°C. Supernatant obtained was discarded completely and pellet was re-suspended in 5 ml of 0.1 M $CaCl_2$. After that the cells were incubated for 30 minutes on ice. The cells were then suspended in 2 ml 0.1 M $CaCl_2$ and aliquoted in sterile microcentrifuge tubes with 200 µl in each. These tubes were then tightly covered with parafilm and stored at 4°C until use.

2.2.2.10. Transformation of Competent E. coli DH5α cells

Transformation may be described as the stable, heritable uptake of exogenous DNA into a host cell (*E. coli*). Cells that are in a state in which they can be transformed by DNA in their environment are said to be competent. In a significant number of bacteria, entry into the competent

state is encode by chromosomal genes and signaled by certain environmental conditions. Such bacteria are said to be capable of undergoing "Natural Transformation". Many other bacteria do not become competent under ordinary conditions of culture but they can be made competent by a variety of highly artificial treatments such as exposure of cells to high concentrations of divalent cations such as Ca^{++}, Mg^{++}, Mn^{++} etc. Such systems of transformation have been termed as "Artificial Transformation".

The exact mechanism of DNA uptake by cells with artificially induced competency is unknown. Transformation efficiency can be increased by hot and cold temperature treatment at different steps. Transformation is measured in terms of "transformation efficiency" which is defined as the number of host cells transformed by one microgram of DNA and is calculated as follows:

Number of transformants per μg of DNA =

$$\frac{\text{Number of transformed cells}}{\text{Microgram of DNA used}} \times \frac{\text{Final volume of cell suspension (ml)}}{\text{Volume of cell suspension used (ml)}}$$

Firstly, Luria-Bertani (LB) agar plates and SOC media is prepared.

LB was prepared by adding 15 g of agar to all the constituents of LB broth for every 1000 ml. LB agar prepared was then autoclaved and then LB agar plates were prepared with working Ampicillin concentration of 80 μg/ml.

For preparation of SOC media, first SOB media was prepared by dissolving 20 g Tryptone, 5 g yeast extract and 0.5 g of NaCl to 950 ml of deionized water. 10 ml of 250 mM solution of KCl was then added (KCl solution is prepared by 1.86 g of KCl in 100 ml of deionised water) to the above solution. pH of the solution was adjusted to 7.0 with 5.0 N NaOH. Then, the volume of the solution was made upto 1 litre by adding deionised water. The resultant solution was then sterilized by autoclaving at 15 psi (1.05 Kg/cm^2) for 20 minutes. Just before use 5 ml of 2 M $MgCl_2$ solution (This solution was made by dissolving 19 g of $MgCl_2$ in 90 ml of deionized water. The volume of the solution was adjusted to 100 ml with distilled water and the solution was autoclaved at 15 psi for 20 minutes.) was added to the SOB broth solution. About 20 ml of 1 M sterile glucose solution (This solution was made by dissolving 18 g of glucose to 90 ml of deionised water. After the sugar has dissolved, the volume of the solution was adjusted to 100 ml with distilled water and the solution was then sterilized by passing through a 0.22 μm filter.) was then added to 980 ml of autoclaved SOB medium, cooled to 60°C or less.

Next, 60 µl competent cells are taken in two fresh, sterile microcentrifuge tubes. About 2 µl of ligation mixture of plasmid was added to one of the microcentrifuge tubes, aseptically. The tubes were then incubated on ice for 30 minutes. Next, the mixture was given heat shock at 45°C for exactly 90 seconds. This transformation mix was then placed on ice for 2 minutes and then 200 µl SOC media was added to it. It was then incubated at 37°C for 45 minutes to 1 hour. Then, 10, 30, 50 and 100 µl of transformation mix was spread on Ampicillin containing LB agar media plates. These plates were then incubated at overnight at 37°C for growth.

2.2.2.11. Screening of recombinant bacterial colonies

It is generally difficult to identify the recombinants by looking the colonies after transformation but in few cases, colonies containing a recombinant plasmid may be smaller than normal because the plasmid expresses a foreign protein that retards growth of the host cells

Several methods are available to distinguish bacteria transformed by recombinant plasmids form those carrying empty wild-type plasmids. Screening of cells containing foreign DNA is done based on the selection markers carried on by this DNA. Like pGEM-T plasmid has ampicillin resistance factor that enables only transformed cells to grow on LB-Ampicillin plates. Non-transformants, which are ampicillin sensitive, do not produce colonies on the selective medium. Transformants and non-transformants are thus easily distinguishable.

Identification among the recombinants is generally done by Insertional-inactivation. With most cloning vectors, insertion of DNA fragment into the plasmid destroys the integrity of one of the genes present on the molecule. As a result, the characteristic coded by the inactivated gene is no longer displayed by the host cells and this is termed as Insertional-inactivation. For example, pGEM-T is a high copy number plasmid of size 3000bp. It carries a 103bp polycloning region and ampicillin resistance marker, along with coding information for the amino terminal of β-Galactosidase (*LacZ*) gene. Some strains of *E. coli* bear a deletion at the amino terminal end of *LacZ* gene and thus synthesize an inactive C-terminal fragment. On transforming such competent bacterial strains with pGEM-T, the host and the plasmid encoded fragments associate to form an enzymatically active protein. This type of complementation is known as α complementation. Bacterial strains or colonies with active *Lac* gene can be recognized as they form blue colour colonies in presence of X-gal or 5-bromo-4-chloro-3-indolyl-β-D-galactoside (chromogenic substrate for β-galactosidase) and IPTG or isopropylthio-ß-D-galactoside (inducer for the

expression of the enzyme). However, insertion of the fragment of foreign DNA into the polycloning site of plasmid results in production of an amino terminal fragment that is not capable of α complementation. Hence, cells carrying recombinant plasmid will form white colonies. This procedure of screening of recombinant cells is called Blue-White screening.

Firstly, X-gal and IPTG working solutions were prepared.

Stock solution of X-gal was made by dissolving X-gal in dimethyl formamide at a concentration of 20 mg/ml. For this, 200 mg of X-gal was first dissolved in 10 ml of Dimethylfom1amide (DMF). The solution was then aliquoted in microcentrifuge tubes and sterilized by filtering with 0.22 µm syringe filter. The tubes were then wrapped with aluminium foil as the solution is photo-sensitive. The aliquots were than stored at - 20°C.

Stock solution of IPTG (20% w/v, 0.8 M) was then prepared by dissolving 2 g of IPTG in approximately 8 ml of distilled water. The final volume of the solution was adjusted to 10 ml by addition of more distilled water. The solution is then sterilized by passing through 0.22 µm sterile syringe filters and was stored at -20°C.

Thereafter, LB agar plates were prepared with Ampicillin at a concentration of 80 µg/ml. Approximately 40 µl of 2% X-gal solution and 7µl of 20% IPTG solution was added onto the center of pre-made LB agar plates. Next, with the help of a sterile spreader, the X-gal and IPTG solutions were evenly spread throughout the plate and then incubated at 37°C until all of the fluid had been absorbed by the LB agar. Finally, 10 µl, 20 µl and 50 µl of the transformed bacterial suspension culture was spreaded over the surface of a LB agar plate with the help of a sterile spreader. After the inoculums have been absorbed, the plates were incubated in an inverted position for 20 hours, during which the color develops to its full extent. The plates were then removed from the incubator and kept at 4°C for 4 hours, during which the blue color develops to its full extent. The recombinants were then identified by blue/white selection screening.

2.2.3. Plasmid DNA isolation

Alkaline Lysis, in combination with the detergent SDS is generally used to isolate plasmid DNA from *E. coli* [34]. Exposure of bacterial suspensions to the strongly ionic detergents at high pH opens the cell wall, denatures chromosomal DNA and proteins and releases plasmid DNA into the supernatant. Although the alkaline solution completely disrupts the base pairing, the strands of closed circular plasmid DNA are unable to separate from each other because of being topologically

intertwined with each other. As long as the intensity and duration of exposure to OH^- is not too great, the two strands of plasmid DNA falls once again into register when the pH is returned to normal.

During lysis, bacterial proteins, broken cell walls denatured chromosomal DNA become enmeshed in large complexes that are coated with dodecyl sulphate. These complexes are efficiently precipitated from the solution when sodium ions are replaced by potassium ions [128]. After the denatured material has been removed by centrifugation, native plasmid DNA can be recovered from the supernatant.

Alkaline lysis in the presence of SDS is a flexible technique that works well with all strains of *E. coli* and with bacterial cultures ranging in size from 1ml to >500 ml. The closed circular plasmid DNA recovered from the lysate can then be purified in many different ways and to different extents, according to the need of the experiment.

Thus, isolation of the recombinant plasmid DNA was carried out, first by preparation of RNase stock solution, which was prepared by adding 10mg of Bovine Pancreatic RNase A to 10 µl of 1 M Tris-Cl (pH 7.5) and 3 µl of 5 M NaCl solution. The volume of the above solution was then made upto 1 ml with distilled water. The solution was then heated upto 100°C for 5 minutes and was cooled to room temperature. The solution was then aliquoted in sterile 1.5 ml microcentrifuge tubes and was stored at -20°C for future use.

Also, required were plasmid isolation solution I (Resuspension Buffer), Plasmid Isolation Solution II (Lysis Buffer) and plasmid isolation solution III (Neutralizing Buffer). Plasmid Isolation Solution I was prepared by dissolving 0.901 g of glucose in 90 ml of distilled water and by adding 2 µl of 0.5 M'EDTA to it, along with 2.5 ml of 1 M Tris-Cl (pH 8.0). The solution volume was made upto 100 ml by addition of distilled water. The resultant solution having a final concentration of 50 mM glucose, 25 mM tris and 10 mM EDTA was autoclaved and stored at 4°C.

Solution II was prepared by adding 2 ml of 10 N NaOH and 10 ml of 10% SDS w/v to 88 ml of distilled water. The final concentration of Plasmid Isolation Solution II was 0.2 N NaOH and 1% SDS w/v.

Plasmid Isolation Solution III was prepared by mixing 60 ml of 5 M potassium acetate, 11.5 ml of Glacial Acetic Acid and 28.5 ml of distilled water. The resultant solution had the concentration of 3 M potassium and 5 M of acetate. Finally, the Plasmid Isolation Solution III was autoclaved and was stored at 4°C and was transferred to the ice-bucket just before using.

Thereafter, single bacterial colony containing the recombinant plasmids was inoculated in 2 ml of LB medium with appropriate antibiotic overnight at 37°C with vigorous shaking. 1.5 ml of this overnight culture was then transferred to microcentrifuge tube and the remaining culture was stored at 4°C. The cells in microcentrifuge tube were then harvested by centrifugation at 5000 rpm for 5 minutes. Supernatant was removed as much as possible by inverting tubes on a tissue paper. Bacterial pellet was then resuspended in 100 µl of ice cold solution I by vortexing and was kept on ice cold for 10 minutes. 200 µl of freshly prepared solution II was added and the contents were mixed properly by inverting the tube 5 times and the solution was stored on ice for 5 minutes. Then, 150 µl of ice cold solution III was then added and the resultant solution was then stored on ice for 10 minutes after vortexing. The microcentrifuge tubes were then centrifuged at 12000 rpm for 5 minutes at 4°C and supernatant was transferred to fresh microcentrifuge tube. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was then added and mixed by vortexing. The microcentrifuge tubes were then again centrifuged at 12000 rpm for 5 minutes at 4°C and the supernatant was transferred to fresh microcentrifuge tube again. Next, the Plasmid DNA was precipitated by adding 0.6 volume of isopropanol to the mixture and the solution was kept for 30 minutes at room temperature. The solution was again centrifuged at 12000 rpm for 5 minutes at 4°C and this time the supernatant was discarded completely. Pellet was then washed with 70% ethanol and was allowed to air dry. Pellet was then dissolved in 50 µl of TE buffer (pH 8.0.) and thereafter was given RNase treatment (100 µg/ml) at 37°C for 1 hour. 10 µl of this was loaded on the agarose gel and was then visualized and analyzed for the presence of plasmid band on the agarose gel. The plasmid thus isolated was then stored at -20°C.

M.G. CENTRAL LIBRARY
G20543
ACC No.
Date
I.I.T. ROORKEE

2.2.4. Confirmation of the screened bacterial colonies

Further confirmation of the transformation of the bacterial culture with recombinant plasmid was done by isolation of desired plasmid by Minipreparation (Alkaline lysis with SDS) and its analysis by agarose gel electrophoresis. Still, further conformation of the recombinant plasmid (pGEM-T + Amplicon) was done by performing PCR using the same set of primers and plasmid DNA as the template.

2.2.5. Sequencing of the desired Amplicon

Primer walking is a sequencing method of choice for sequencing DNA fragments between 1.3 and 7kb. Such fragments are too long to be sequenced in a single sequence read using the chain termination method. This method works by dividing the long sequence into several consecutive short ones. The DNA of interest may be a plasmid insert, a PCR product or a fragment representing a gap when sequencing a genome. The term "primer walking" is used where the main aim is to sequence the genome. The term "chromosome walking" is used instead when we know the sequence but don't have a clone of a gene. For example the gene for a disease may be located near a specific marker such as an RFLP on the sequence [1].

The fragment is first sequenced as if it were a shorter fragment. Sequencing will be performed from each end using either universal primers or primers designated by the customer. This should identify the first 1000 bases (approx). In order to completely sequence the region of interest, design and synthesis of new primers complementary to the final 20 bases of the known sequence is necessary to obtain contiguous sequence information.

The basic technique is as follows:

1). A primer that matches the beginning of the DNA to sequence is used to synthesize a short DNA strand adjacent to the unknown sequence, starting with the primer.

2). The new short DNA strand is sequenced using the chain termination method.

3).The end of the sequenced strand is used as a primer for the next part of the long DNA sequence.

This way, the short part of the long DNA that is sequenced keeps "walking" along the sequence. The method can be used to sequence entire chromosomes (thus, chromosome walking). A different method with the same purpose which becomes more popular for large-scale sequencing (e.g., the Human Genome Project) is shotgun sequencing.

The amplified PCR product was directly sent to Axygen Scientific Pvt. Ltd., New Delhi for the sequencing of the amplicon. But the sequencing result and Chromatogram was not clear so the Amplicon was inserted into the pGEM-T vector and was transformed into DH5α cells and the cells with recombinant plasmids were screened out and was sent for DNA sequencing as a live plate to Department of Biochemistry, University of Delhi-South Campus, New Delhi.

2.2.5.1. Translation product of the Sequenced Recombinant Plasmid

Translation product of the sequenced DNA was determined by using various translation tool programs available as stand-alone or as web-server based programs.

Expasy Translation Tool was used to determine the translated protein sequence from the sequenced plasmid DNA.

2.2.5.2. Identification of the translated sequence

Identification of the translated gene sequence was carried out by performing the sequence blast of the translated gene sequence against the non-redundant database of sequences in the blast program. All the other parameters for performing the sequence blast were set to default and were not altered at all.

Thereafter the first ten sequences with the highest score and lowest Expect-values or E-values were taken for performing the multiple sequence alignment against the query sequence by either using the COBALT or CLUSTALW program as stand-alone or web-servers.

2.2.6. Homology modeling of the translated gene sequence

The crystal structures of the oxidized forms of α and β chain complex of respiratory nitrate reductase [130] and full protein comprising of α, β and γ chain of respiratory nitrate reductase [28] were reported at resolutions of 2.0 and 1.9Å, respectively. α, β, γ complex has a flower-like arrangement with dimensions of 90 x 128 x 70Å. The γ subunit, which is completely immersed in the membrane, is associated with the α-β dimer through a hydrophobic patch present in β. The global arrangement of the catalytic subunit α is similar to those from other enzymes belonging to the DMSO reductase family [199], having four domains with α-β type folding. In this sense, it presents high homology with Dd Nap, Ec Fdh-N, Ec Fdh-H and Dg Fdh [131, 28, 198]. As shown in Figure 2.2 [101], α subunit contains the active site, a Mo-bisMGD cofactor, and an iron–sulfur center of the [4Fe–4S] type (FS0). β subunit contains one [3Fe–4S] (FeS4) and three [4Fe–4S] clusters (FeS1–3), whereas γ subunit contains two b-type hemes responsible for the MQH2 oxidation and proton translocation. These eight redox centers are separated by 12–14Å, giving an electron transfer pathway of about 90Å.

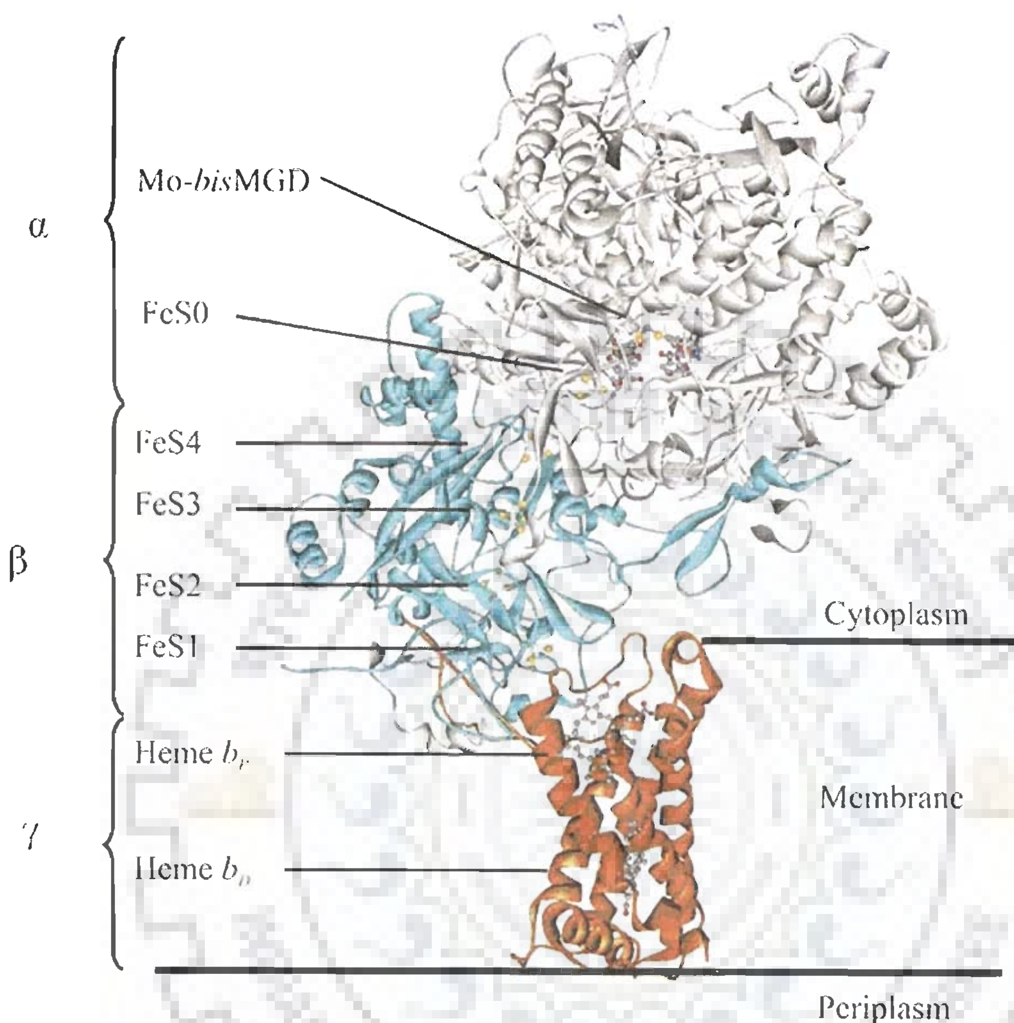


Figure 2.2: Overall three-dimensional structure of respiratory nitrate reductase from *E. coli K12*. The names of the respective subunits together with the metal cofactors are indicated.

The structure of respiratory nitrate reductase reveals that the active site is composed of a molybdenum atom coordinated to four sulfur atoms from two pterin cofactors, as found in all the members of the DMSO reductase family, and bidentate coordination with the oxygen atoms from the side chain carboxylate group of an aspartate molecule (Asp222) [28], which has not been observed so far in a mononuclear Mo-enzyme. The soluble α-β complex shows a similar active site structure with two pterin molecules but, in contrast, the coordination with aspartic acid is through only one oxygen with the sixth coordination position being occupied by an oxo group at 1.8Å [130]. These differences cannot be attributed to the redox state of the sample since both respiratory nitrate reductase and its α-β complex were crystallized in aerobic conditions and therefore

structures must represent oxidized forms of the enzymes. The coordination of metal sites by side chain carboxylates from aspartic and glutamic acid molecules has been the subject of several studies [48]. These studies showed that carboxylate groups present high flexibility, which is reflected in these two crystallized forms of Ec respiratory nitrate reductase. Another novel aspect of respiratory nitrate reductase is the unusual coordination of FeS0 by one histidine and three cysteines, which was found only once before in Dg hydrogenase [130, 28].

Also, α subunit plays the role of the catalytic subunit in respiratory nitrate reductase [103] and γ subunit helps in anchoring the whole protein to the membrane and is involved in transfer of electrons to the β subunit [176].

Homology model for the translated gene sequence was obtained by taking the PDB co-ordinates of *E. coli* β-subunit of respiratory nitrate reductase, which showed the identity of 91% with the translated gene sequence and the BLAST score of 429 with a low E-value of 9e-119. The PDB file used for taking the co-ordinates of β-subunit of respiratory nitrate reductase was 1q16 [28] and was used for modeling the translated sequence in Modeller. This generated five output files and all the five models generated were checked for the number of amino acids distributed in the most favourable region in the Ramachandran plot using Procheck program. Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% of the amino acid residues in the most favoured regions.

Thus the output file with highest percentage of amino acids (over 90%) was chosen as the final model of the translated gene sequence.

2.3. Results and Discussion

Bacillus circulans is a Gram positive bacteria requiring temperature around 30°C for its growth. The bacterium grows well on nutrient agar and nutrient broth. It is an obligate aerobic bacteria having rod shaped structure. Motility of the bacterium varies from strain to strain. It is used in industry for the production of L-glutamic acid and has been reported to produce HBL toxin. As far as pathogenicity of the bacterium to humans is concerned, it is non-pathogenic to humans but is known to cause certain infections also. Many genes have earliar been cloned from the genome of *Bacillus circulans* but not the gene for beta subunit of respiratory nitrate reductase. Therefore in this chapter cloning and sequencing of the beta subunit of nitrate reductase has been described.

2.3.1. Primer Designing

PCR primers were designed from different *Bacillus* species such as, *Bacillus licheniformis, Bacillus lentus, Bacillus pumilus* and *Bacillus subtilis*. The forward primer sequence was 5′CATGCCATGGATGGTGAGGAAG-3′, harboring the start codon region of the signal peptide of *B. circulans*. The reverse primer sequence, was 5′GAATCTCGAGTTGAGCGGCAG-3′.

Length of the forward primer for PCR amplification of β- subunit of respiratory nitrate reducatse was 22 bases and had a molecular weight of 6864.5 daltons, with 55% of GC content. 1 ml solution of the primer with O.D. of 1 at 260 nm had a concentration of 3.936 μM and contained about 27 μg of the forward primer sequence. Reverse complementary strand for the forward primer was 5′CTT CCT CAC CAT CCA TGG CAT G-3′ and the Tm value of the forward primer sequence was found to be 57°C. The forward primer sequence had two set of sites for self complementarity:

5′CATGCCATGGATGGTGAGGAAG-3′

and 5′CATGCCATGGATGGTGAGGAAG-3′

Similarly, length of the reverse primer sequence used for the PCR amplification of β-subunit of respiratory nitrate reducatse was found to be of 21 bases and a molecular weight of 6511.3 daltons, with 57% of GC content. 1 ml solution, of the reverse primer sequence with the O.D. of 1 at 260 nm had the primer concentration of 4.276 μM and contained about 27.8 μg of the reverse primer sequence. Reverse complementary sequence for the reverse primer was 5′ CTG

CCG CTC AAC TCG AGA TTC- 3′ and the Tm value of the reverse primer sequence was found to be 56°C. The primer sequence did not show any self complementarity at all.

Thus the forward and reverse primers designed for PCR amplification of the beta subunit of respiratory nitrate reductase were found to be appropriate, with respect to the GC content and Tm values only. The forward primer sequence was found to be appropriate with respect to GC, which was over 50% and a good Tm value of 57°C but was found lacking in the aspect of self complementarity, as it is shown to have two reading frames where total of eight bases are involved in self complementarity. Whereas in case of reverse primer sequence, the GC content of over 50%, and the Tm values are satisfactory (neither too high and neither too low) and also the reverse primer did not showed any region of self complementarity. Thus the reverse primer sequence acted as the ideal primer sequence for gene amplification.

2.3.2. Isolation of Bacterial Genomic DNA

Genomic DNA of *Bacillus circulans* was isolated by phenol:chloroform treatment without much difficulty.

2.3.3. Quantification of DNA using spectrophotometer

The ratio of OD_{260}/OD_{280} of 5μl/ml of isolated bacterial genomic DNA was found to be 1.775, which indicates a relatively pure preparation of DNA. These results suggest a good and quite pure yield of isolated bacterial genome after its isolation from the lysed bacterial cells as the ratio of around 1.8 and DNA isolation preparations having the OD_{260}/OD_{280} ratio of 1.8 or more are accepted as quite pure.

2.3.4. Polymerase Chain Reaction (PCR)

The PCR amplification was optimized using different parameters of primer concentration, concentration of *Taq* polymerase, $MgCl_2$, DMSO and different annealing temperature staring from 50°C. It was found that at 54°C with 0.5 μl primer of forward and reverse primers (5 pmol/μl) and 1 μl of Taq DNA polymerase (5 U/μl), the reaction showed the highest specific amplification of around 1100bp with the pair of forward and reverse primers used.

Thus, the PCR program used for the amplification of the gene, consisted of: 1 cycle of initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, 30 cycles of annealing at 54°C for 1 minute, 30 cycles of extension at 72°C for 3 min, and 1 cycle of final extension at 72°C for 8 minutes. The program ended with storage of the amplified product at 4°C for 1 hour. This program gave amplification for beta subunit of respiratory nitrate reductase. PCR products were then analyzed by running onto the 0.8% (w/v) agarose gel at 100 volts for 20 minutes.

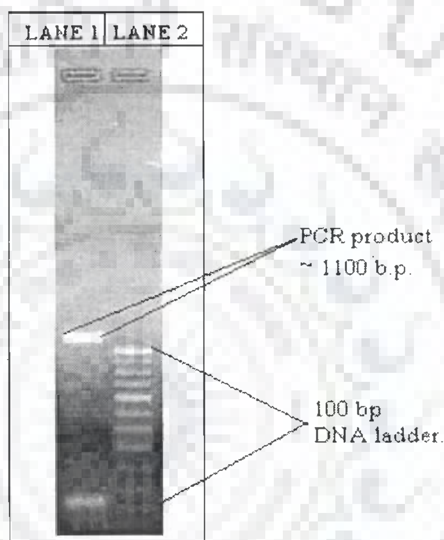


Figure 2.2: Agarose gel electrophoresis of PCR product in 0.8% agarose gel, where Lane 1 is showing amplified PCR product, 3 μl of which was loaded onto the gel. And Lane 2 is showing 100bp DNA ladder, 2 μl of which was again loaded onto the electrophoretic gel.

The PCR amplification which was optimized with the above set of primers utilized normal volumes and concentration of primers usually employed and also utilized normal and usual amount of *Taq* polymerase, $MgCl_2$ and DMSO. Normal annealing temperature in the range of 50°s (in Celsius) was used for the annealing of primer sequences to the gene of interest. All the other steps of the PCR reaction were carried out at the standard temperatures as followed in the protocol for PCR amplification, without any alterations.

Bright and intense band of the amplified PCR product on the 0.8% agarose gel indicates good amount of amplification of the gene had been achieved using the given set of primers.

2.3.5. Gel elution of desired Amplicon

The desired fragment of amplified DNA was eluted by spin gel elution kit of Bangalore GeNei. The final step of elution using elution buffer was carried out twice. First, using 30 μl elution buffer and repeated again with 20 μl of elution buffer. The quantity of DNA during the first elution slot was twice as compared to second one, which was determined by agarose gel electrophoresis with 2 μl of the eluted sample being loaded onto the separate lanes of 0.8% (w/v) agarose gel.

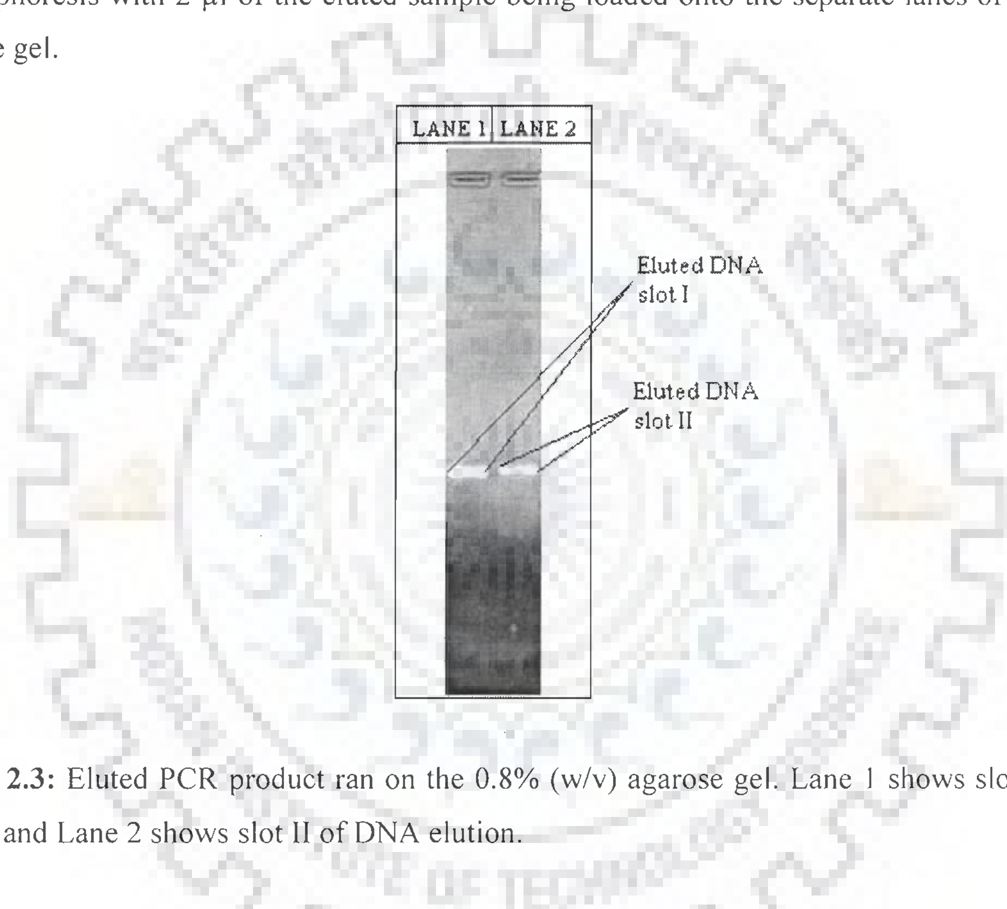


Figure 2.3: Eluted PCR product ran on the 0.8% (w/v) agarose gel. Lane 1 shows slot I of DNA elution and Lane 2 shows slot II of DNA elution.

Gel elution of the PCR products was quite effective as can be observed with the first, very intense and second, less intense band of the eluted PCR products from 0.8% agarose gels.

2.3.6. Ligation of the amplicon with pGEM-T cloning vector

The ligation of PCR Amplicon with pGEM-T cloning vector (Promega) was performed according to the user manual with overnight incubation at 4°C. The reaction was carried out using positive and negative control reactions.

2.3.7. Screening of recombinant bacterial colonies

The confirmation of recombinant plasmid was done with ampicillin (80 μg/ml), IPTG and X-gal using the blue-white selection technique.

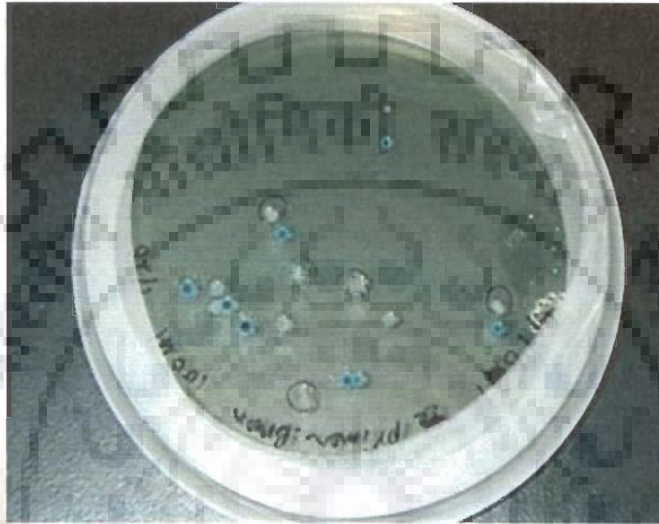


Figure 2.4: Blue-white screening of transformed colonies. The transformed colonies are seen in white.

Screening of the recombinant bacterial colonies revealed a good number of white colonies, which have picked up the recombinant plasmid within their cells. This not only demonstrates the successful transformation of the DH5α *E. coli* cells with the recombinant plasmid but also verifies the successful ligation of the amplicon with the pGEM-T plasmid.

2.3.8. Confirmation of screened colonies

The insertion of recombinant plasmid into the screened bacterial colonies was further confirmed by isolating recombinant plasmid DNA from such colonies and using it as the template for PCR amplification. The PCR product showed a distinct band with forward and reverse primers confirming the presence of transformed colonies with the recombinant plasmids.

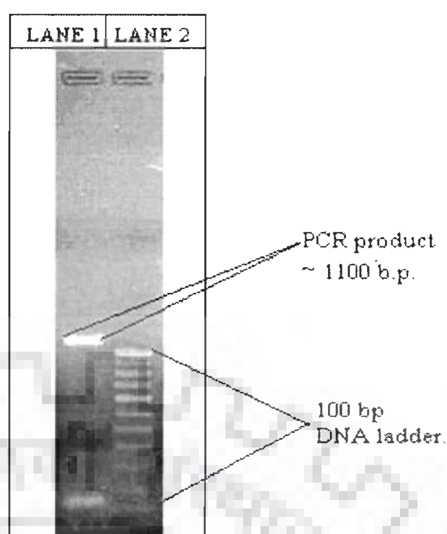


Figure 2.5: Agarose gel electrophoresis of PCR product in 0.8% agarose gel, where Lane 1 is showing amplified PCR product of isolated recombinant plasmids (obtained from the positively screened colonies) loaded onto the gel. And Lane 2 is showing 100bp DNA ladder.

The presence of the same amplified product band (as obtained after the PCR amplification of the nitrate reductase gene from the isolated bacterial DNA of *Bacillus circulans*), obtained by agarose gel electrophoresis of the amplified isolated recombinant plasmids (obtained from the positively screened colonies) further strengthens that the correct screening of the bacterial colonies was carried out.

2.3.9. Sequencing of desired Amplicon

PCR Amplicon was directly sent to Axygen Scientific Pvt. Ltd., New Delhi. But the data obtained was very poor, ambiguous and unsatisfactory. So the PCR Amplicon was ligated to the cloning vector pGEM-T, transformed into *E. coli.*DH5α strains and the transformed strains sent to University of Delhi, South Campus for sequencing. The results were:

Figure 2.7: Chromatogram of the sequenced recombinant plasmid provided by Department of Biochemistry, South Campus, University of Delhi.

1
GCCGCCATGGCCGCGGGAtTGAATCTCGAGTTGAGCGGCAGTGCGACGTGTTCCTGAATCCGCACGATCCGGCGGTCATTGAGGAAGCGG

91
TTAAACAGGGTATTCCACAGAACGTGATCGATGCGGCCCAGCGCTCCCCGGTCTACAAAATGGCGATGGACTGGAAGCTGGCCCTGCCGC

181
TGCACCCGGAATACCGCACTCTGCCGATGGTCTGGTACGTGCCGCCGCTGTCACCGATTCAGTCTTACGCTGACGCGGGCGGCCTACCGA

271
TGAGCGATGGCGTCCTGCCTGCCGTAGAGAGCCTGCGCATTCCCGTGCAGTATCTGGCGAACATGCTTAGCGCGGGCGATACCGGCCCGG

361
TGCTGCGCGGCCTGAAGCGCATGATGGCGATGCGACACTACAAACGCTCCCAGACCGTCGAAGGCGTGACCGATACCCGCGCCATCGAAG

451
AGGTCGGCCTGACCGAAGCGCAGGTTGAAGAGATGTACCGCTATCTGGCCATCGCCAACTACGAGGATCGCTTCGTGATCCCCACCAGCC

541
ACCGTGAAATGGCGCGCGATGCCTTCCCGGAACGTAACGGATGTGGTTTCACCTTTGGCGACGGCTGCCACGGCTCTGACACCAAATTTA

631
ACCTCTTCAACAGCAGCCGGATTGATGCCATCAACATCACCGAGGTGCGCGAACATGGGGAGGGAGAGTAATGAAGATCCTCAAAATCAT

721
CGCGTTGCT 729

Figure 2.6: DNA sequence with the reverse primer and T7 promoter of pGEM-T vector provided by University of Delhi, South Campus containing the ORF of β-subunit of respiratory nitrate reductase.

Sequence obtained contained 20 bases of T7 promoter region of pGEM-T vector, 678 bases of Open Reading Frame or ORF of β-subunit of respiratory nitrate reductase containing 21 bases of the reverse primer sequence and 31 bases of the pGEM-T plasmid.

2.3.10. Translated product of the Sequenced Plasmid

Translation product of the sequenced DNA sequence turns out to be:

ERQCDVFLNPHDPAVIEEAVKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPE

YRTLPMVWYVPPLSPIQSYADAGGLPMSDGVLPAVESLRIPVQYLANMLSAGDT

GPVLRGLKRMMAMRHYKRSQTVEGVTDTRAIEEVGLTEAQVEEMYRYLAIANY

EDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRIDAINITEVR

EHGEGE

Figure 2.8: Translated sequence of the β-subunit of nitrate reductase gene obtained from sequencing of the recombinant plasmid DNA provided by Department of Biochemistry, South Campus, University of Delhi.

2.3.11. BLAST result of translated DNA sequence

The top ten sequences with highest blast scores and lowest E-values for the translated gene sequence as the query were as follows:

1). Hypothetical protein ENTCAN_02391 [*Enterobacter cancerogenus ATCC 35316*] with the bit score of 446, E-value of 8e-124 and having 95% identity with the query sequence

2). Respiratory nitrate reductase beta subunit [*Enterobacter* sp. *638*] with the bit score of 437, E-value of 5e-121 and having about 93% identity with the query sequence.

3). Nitrate reductase 2, beta subunit [*Salmonella enterica* subsp. *enterica* serovar Newport str. *SL317*] with bit score 433, E-value 5e-120 and having about 92% of identity.

4). Hypothetical protein CKO_01502 [*Citrobacter koseri ATCC BAA-895*] with bit score 433, E-value 5e-120 and having about 92% of identity.

5). Nitrate reductase 2 beta subunit [*Salmonella typhimurium LT2*] with bit score 432, E-value of 7e-120 and 92% identity.

6). Nitrate reductase 2, beta subunit [*Salmonella enterica* subsp. *enterica* serovar Javiana str. *GA_MM04042433*] with bit score of 432, E-value of 1e-119 and 92% identity.

7). Nitrate reductase 2, beta subunit [*Salmonella enterica* subsp. *enterica* serovar Choleraesuis str. *SC-B67*] with bit score of 432, E-value of 1e-119 and 92% identity.

8). Nitrate reductase 2, beta subunit [*Salmonella enterica* subsp. *enterica* serovar Kentucky str. *CDC 191*] with bit score of 432, E-value of 1e-119 and 92% identity.

9). Respiratory nitrate reductase 2 beta chain [*Salmonella enterica* subsp. *enterica* serovar Typhi str. *CT18*] with bit score of 432, E-value of 1e-119 and 92% identity.

10). Respiratory nitrate reductase 2 beta chain [*Salmonella enterica* subsp. *enterica* serovar Gallinarum str. *287/91*] with bit score of 432, E-value of 2e-119 and 92% identity.

BLAST results of the translated gene sequence against the non-redundant protein database in BLAST, clearly displays high sequence homology of the translated gene product with β-subunit of respiratory nitrate reductases from different microbial sources. Identity of more than 90% between β-subunit of respiratory nitrate reductases from different microbial sources and the translated gene sequence, with high scores and low E-values clearly indicate that the translated gene sequence is of β-subunit of respiratory nitrate reductases.

2.3.12. Multiple Sequence Alignment of Translated DNA sequence with highly identical protein sequences

The multiple sequence alignment profile of the above 10 protein sequences with the translated DNA sequence as the query sequence was:

```
Nr.  --------------------------------------------------
1).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNIWTGREGMEYAWFNNVETKPGI 50
2).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
3).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
4).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
5).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
6).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
7).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
8).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
9).  MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50
10). MKIRSQVGMVLNLDKCIGCHTCSVTCKNVWTGREGMEYAWFNNVETKPGI 50

Nr   --------------------------------------------------
1).  GYPKNWEDQQEWQGGWVRDVNGKIRPRLGGKMGVISKIFANPVIPQIDDY 100
2).  GYPKNWEDQQEWQGGWVRDVNGKIRPRLGGKMGVISKIFANPVIPQIDDY 100
3).  GYPKNWEDQQEWQGGWVRDVNGKIRPRLGGKMGVISKIFANPVIPQIDDY 100
4).  GYPKNWEDQQEWQGGWVRDVNSKIRPRLGGKMGVISKIFANPVIPQIDDY 100
5).  GYPKNWEDQQEWQGGWVRDVNGKIRPRLGGKMGVISKIFANPVIPQIDDY 100
6).  GYPKNWEDQQEWQGGWVRDVNGKIRPRLGGKMGVISKIFANPVIPQIDDY 100
7).  GYPKNWEDQQEWQGGWVRDVNGKIRPRLGGKMGVISKIFANPVIPQIDDY 100
8).  GYPKNWEDQEEWQGGWVRDVNGKIRPRLGGKMGVITKIFANPVIPQIDDY 100
9).  GYPKNWEDQDEWQGGWIRGIHGKLTPRLGSKLGVLSKIFSNPVMPQIDDY 100
10). GYPKNWEDQEEWQGGWVRGITGKLTPRLGGRVGVLSKIFANPSLPGIDDY 100

Nr   --------------------------------------------------
1).  YEPFTFDYQHLHTAPESKHQPTARPRSLIDGKRMDKVIWGPNWEELLGGE 150
2).  YEPFTFDYQHLHTAPESKHQPTARPRSLIDGKRMDKVIWGPNWEELLGGE 150
3).  YEPFTFDYQHLHTAPESKHQPTARPRSLLDGKRMDKVIWGPNWEELLGGE 150
4).  YEPFTFDYQHLHNAPESKHQPTARPRSLIDGKRMDKVIWGPNWEELLGGE 150
5).  YEPFTFDYQHLHNAPESKHQPTARPRSLIDGKRMDKVIWGPNWEELLGGE 150
6).  YEPFTFDYQHLHTAPESKHQPTARPRSLIDGKRMDKVIWGPNWEELLGGE 150
7).  YEPFTFDYQHLHNAPESKHQPTARPRSLIDGKRMDKVIWGPNWEELLGGE 150
8).  YEPFTYDYQHLHSAPESKNQPTARPRSLIDGKRMDKIVWGPNWEELLGGE 150
9).  YEPFTFDYQDLHRAPEGDHLPTARPRSLIDGKRMDKIVWGPNWEELLGGE 150
10). YEPFTFDYQDLHRAPEGDHLPTARPRSLISGKRMDKIVGGPNWEELLGGE 150
```

```
Nr  --------------------------------------------------
1).  FEKRARDRNFDNIQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
2).  FEKRARDRNFDNIQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
3).  FEKRARDRNFDNIQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
4).  FEKRARDRNFDNIQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
5).  FEKRARDRNFDNIQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
6).  FEKRARDRNFDNIQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
7).  FEKRARDRNFDNIQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
8).  FSKRARDRNFEKMQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200
9).  FEKRARDRNFQKIQKEMYGQFENTFMMYLPRLCEHCLNPSCAATCPSGAI 200
10). FEKRARDRNFDNMQKEMYGQFENTFMMYLPRLCEHCLNPSCVATCPSGAI 200

Nr   --------------------------------------------------
1).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
2).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
3).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
4).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
5).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
6).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
7).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
8).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
9).  YKREEDGIVLIDQDKCRGWRLCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250
10). YKREEDGIVLIDQDKCRGWRMCISGCPYKKIYFNWKSGKSEKCIFCYPRI 250

Nr  --------------------------------------ESRVERQCDVFL 12
1).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
2).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEDAASTEHETDLYERQCDVFL 300
3).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
4).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
5).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
6).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
7).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
8).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
9).  ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
10). ESGQPTVCSETCVGRIRYLGVLLYDADRIEEAASTEHETDLYERQCDVFL 300
```

```
Nr    NPHDPAVIEEAVKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  62
1).   NPHDPAVIEEALKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
2).   NPHDPAVIEEALKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
3).   NPHDPAVIEEALKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
4).   NPHDPAVIEEALKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
5).   NPHDPAVIEEALKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
6).   NPHDPAVIEEALKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
7).   NPHDPAVIEEALKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
8).   DPNDPAVIEEALKQGIPQNVIEAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
9).   DPHDPAIIEEALKQGIPQNTIDAAQRSPVYKLAMDWKLALPLHPEYRTLP  350
10).  NPHDPAVIEEAFKQGIPQNVIDAAQRSPVYKMAMDWKLALPLHPEYRTLP  350
```

```
Nr    MVWYVPPLSPIQSYADAGGLPMSDGVLPAVESLRIPVQYLANMLSAGDTG  112
1).   MVWYVPPLSPIQSYADAGGLPHNGNILPAVETLRIPVQYLANMLSAGDTG  400
2).   MVWYVPPLSPIQSYADAGGLPHNGNILPAVETLRIPVQYLANMLSAGDTG  400
3).   MVWYVPPLSPIQSYADAGGLPHNGNILPAVETLRIPVQYLANMLSAGDTG  400
4).   MVWYVPPLSPIQSYADAGGLPHNGNILPAVETLRIPVQYLANMLSAGDTG  400
5).   MVWYVPPLSPIQSYADAGGLPHNGNILPAVETLRIPVQYLANMLSAGDTG  400
6).   MVWYVPPLSPIQSYADAGGLPHNGNILPAVETLRIPVQYLANMLSAGDTG  400
7).   MVWYVPPLSPIQSYADAGGLPHNGNILPAVETLRIPVQYLANMLSAGDTG  400
8).   MVWYVPPLSPIQSYADAGGLPQTEGVLPAIESLRIPVQYLANMLSAGDTG  400
9).   MVWYVPPLSPIQSYADAGGLPQSDGVLPAVESLRIPVQYLANMLSAGDPA  400
10).  MVWYVPPLSPIQSYADAGGLLQTDSILPAVESLRIPVQYLANMLSAGDTG  400
```

```
Nr    PVLRGLKRMMAMRHYKRSQTVEGVTDTRAIEEVGLTEAQVEEMYRYLAIA  162
1).   PVIRALKRMMAMRHYMRSQTVEGVTDTRAIDEVGLSVQQVEEMYRYLAIA  450
2).   PVIRALKRMMAMRHYMRSQTVEGVTDTRAIDEVGLSVQQVEEMYRYLAIA  450
3).   PVIRALKRMMAMRHYMRSQTVEGVTDTRAIDEVGLSVQQVEEMYRYLAIA  450
4).   PVIRALKRMMAMRHYMRSQTVEGVTDTRAIDEVGLSVQQVEEMYRYLAIA  450
5).   PVIRALKRMMAMRHYMRSQTVEGVTDTRAIDEVGLSVQQVEEMYRYLAIA  450
6).   PVIRALKRMMAMRHYMRSQTVEGVTDTRAIEEVGLSVQQVEEMYRYLAIA  450
7).   PVIRALKRMMAMRHYMRSQTVEGVTDTRAIEEVGLSIQQVEEMYRYLAIA  450
8).   PVLRALKRMMAMRHYMRSQTVEGVTDTRAIEEVGLSVEQVEEMYRYLAIA  450
9).   PVLRALKRMMAMRHYKRSQTVEGVTDTRAIEEVGLTEAQVEEMYRYLAIA  450
10).  PVLRALKRMMAMRHYKRSQTVEGVTDTRAIDEVGLSVEQVEEMYRYLAIA  450
```

```
Nr NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 212
1). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
2). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
3). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
4). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
5). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
6). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
7). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
8). NYEDRFVIPTSHREMARDAFPERNGCGFTFGDGCHGSDTKFNLFNSSRID 500
9). NYEDRFVIPTSHREMARDAFPEKNGCGFTFGDGCHGSDTKFNLFNSSRID 500
10).NYEDRFVIPTSHREMARDAFPEKNGCGFTFGDGCHGSDTKFNLFNSSRID 500
```

```
Nr AINITEVREHGEGE 226
1). AINITEVRDKAEGE 514
2). AINITEVRDKAEGE 514
3). AINITEVRDKAEGE 514
4). AINITEVRDKAEGE 514
5). AINITEVRDKAEGE 514
6). AINITEVRDKAEGE 514
7). AINITEVRDKAEGE 514
8). AINITEVRDKAEGE 514
9). AINITEVREHGEGE 514
10).AINIDEVRKHGEGE 514
```

Figure 2.9: Multiple sequence alignment of ten amino acid sequences (1-10) with highest sequence identity to the translated sequence of the cloned recombinant plasmid DNA sequence (Nr).

Further, the CLUSTALW and COBALT results for the multiple sequence alignment of the gene sequence obtained, with the various β-subunit sequences of the prokaryotic respiratory nitrate reductases also indicate that the cloned and the translated gene sequence is that of β-subunit of respiratory nitrate reductase only. But it also indicates that the translated gene sequence was partial and is from amino acid number 299 to the C-terminal of the full β-subunit sequences of the prokaryotic respiratory nitrate. Thus cloning and sequencing of the partial β-subunit sequences of the prokaryotic respiratory nitrate gene has been achieved.

2.3.13. Homology modeling of the translated gene sequence

Distribution of amino acids in the Ramachandran plot of the output file generated by Modeller with highest percentage of residues falling in the most favoured region is shown:

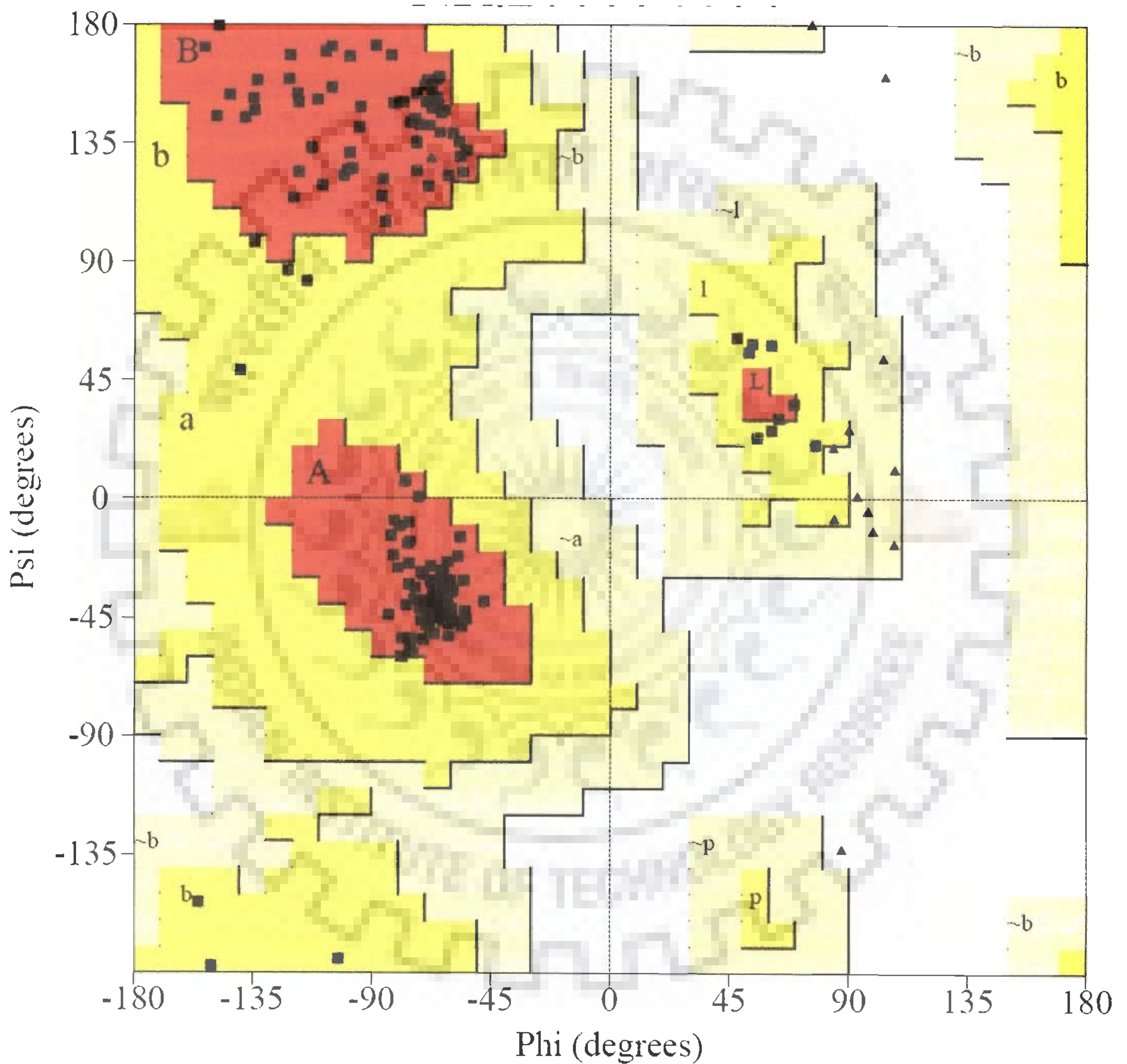


Figure 2.10: Distribution of the amino acids in the Ramachandran plot, of the output Modeller file chosen for building the homology model of the partial β-subunit of the respiratory nitrate reductase.

The plot statistics for the distribution of amino acids in the Ramachandran plot, of the output Modeller file chosen for building the homology model of the partial β-subunit of the respiratory nitrate reductase is as follows:

PLOT STATISTICS

Description	No. of Amino Acids	Percentage
Residues in most favoured regions [A, B, L]	178	92.7%
Residues in additional allowed regions [a, b, l, p]	14	7.3%
Residues in generously allowed regions [~a, ~b, ~l, ~p]	0	0.0%
Residues in disallowed regions 0 0.0%		
	----	------
Number of non-glycine and non-proline residues	192	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	16	
Number of proline residues	16	

Total number of residues	226	

Table 2.1: Distribution of the amino acids shown in the Ramachandran plot of figure 2.10.

Distribution statistics for the amino acids of the output file of Modeller program (chosen for building the homology model of the partial β-subunit of the respiratory nitrate reductase) in the Ramachandran plot clearly specifies that 92.7% of the amino acid residues are located in the most favoured region A, B and L of the Ramachandran plot and thus the selected output file is good enough for building the homology model.

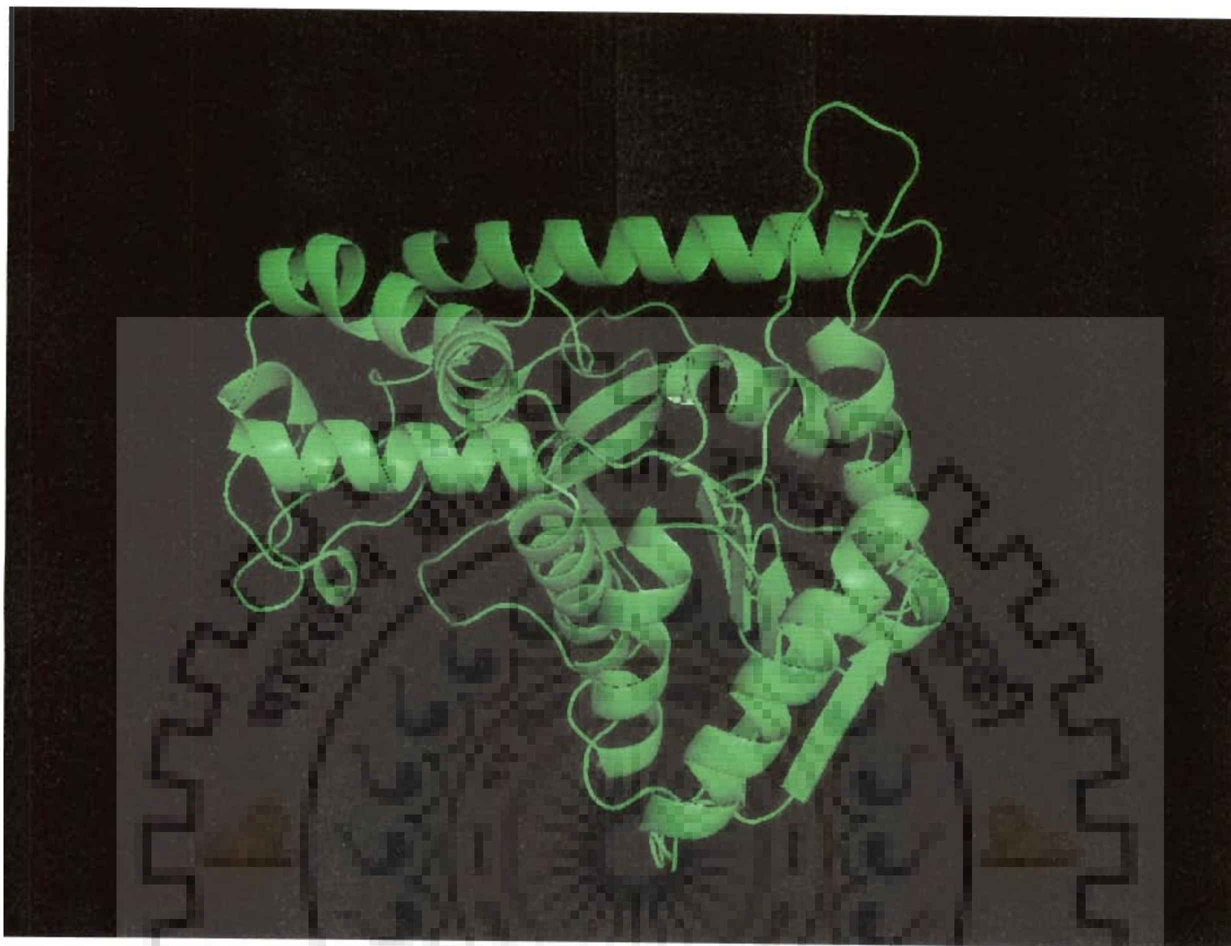
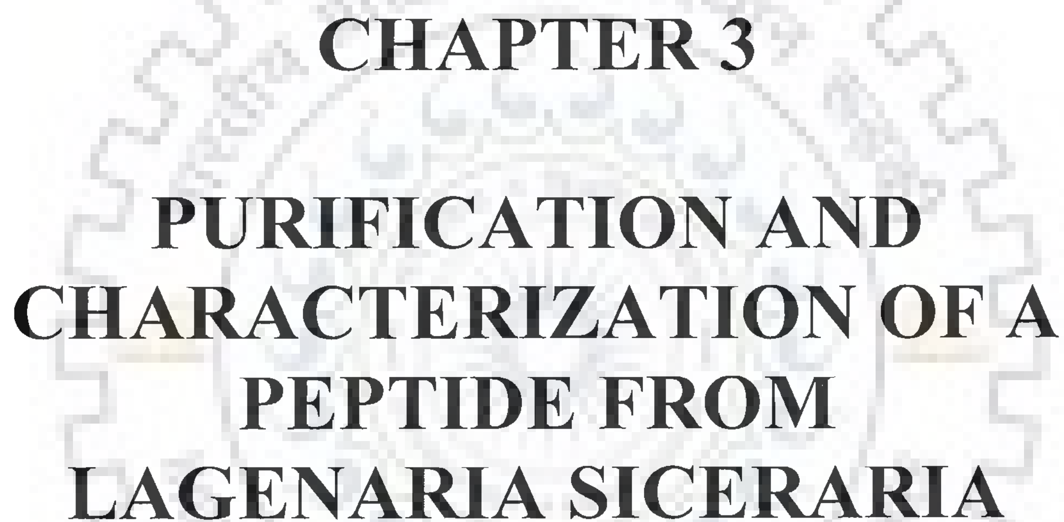


Figure 2.11: Homology model of the partial β-subunit of the respiratory nitrate reductase derived from the translated sequence of the partially cloned gene of β-subunit of the respiratory nitrate reductase from *Bacillus circulans*.

Homology model of the partial β-subunit of the respiratory nitrate reductase from amino acid number 299 to the C-terminal of the β-subunit has been done and contains the hydrophobic patch for attachment of α-β complex to the γ subunit of the protein.

Thus in this chapter, cloning and sequencing of the partial β-subunit of the respiratory nitrate reductase has been described.



CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF A PEPTIDE FROM LAGENARIA SICERARIA

3.1. Introduction

Plant defense system involves a large number of low molecular weight natural compounds. This includes various bioactive peptides having antimicrobial and protease inhibitory activity. These antimicrobial peptides play an important role in resistance to microbial pathogens [46, 90]. The antimicrobial peptides known as natural antibiotics can be an important alternative to synthetic antibiotics. Plant protease inhibitors particularly serine protease inhibitors are widespread in plant seeds and play an important role in plant defense against predators and pathogens [150, 214, 39, 268], either as an antimicrobial agent or protease inhibitor. There are not many reports of a peptide or protein having combination of both the activities. A 13 residue antimicrobial peptide has been shown to have potent protease inhibitory activity [232]. Other reports, where both activities have been reported, are of large peptides or proteins [311, 97, 312]. The peptides having both the activities can be very effective in crop protection because of their improved stability and broad spectrum resistance against pest and microbial pathogens. The combination of both activities can be helpful in enhancing their role in plant defense. Antimicrobial peptides, made of less than a dozen amino acids, have been shown to be present among all eukaryotes including plants [86]. They are rapidly produced, easily diffusable and seems ideal for fast and efficient defense against microbes [202].

Bottle gourd (*Lagenaria siceraria*) which belongs to Cucurbitaceae family is widely used as a vegetable and purgative. The chapter describes the identification of a small peptide like compound having both antimicrobial and trypsin inhibitory activity from the seeds of *Lagenaria siceraria*.

3.2. Materials and Methods

3.2.1. Materials

Seeds of *Lagenaria siceraria* procured locally, Sodium Acetate (CH_3COONa), Acetic Acid (CH_3COOH), Distilled Water, CM Macroprep (Biorad), Sodium Chloride (NaCl), Millipore Water, 0.22 μm filtered Acetonitrile, Bovine Serum Albumin (BSA), Trypsin, Concentrated Hydrochloric Acid (Conc. HCl), Calcium Chloride ($CaCl_2$). Tris Base, N-benzoyl-L-arginine p-nitroanilide (BAPA), Dimethyl Sulfoxide (DMSO), Tri-Fluoro Acetic acid (TFA), 3,5-dimethoxy-

4- hydroxycinnamic acid (Sinapinic Acid), Ethanol, Potassium Bromide (KBr), *E. coli* culture, Tryptone, Yeast Extract, Agar and Ampicillin.

3.2.2. Purification of the Peptide

Purification of the desired peptide was carried out by Cation exchange chromatography on CM Macroprep column and HPLC using 50 mM acetate buffer (pH 4.5) for ion exchange chromatography and HPLC grade water and Acetonitrile for HPLC.

Firstly, the 50 mM acetate buffer was prepared using stock solutions of 0.2 M Acetic Acid (Prepared by mixing 11.55 ml of CH_3COOH with 988.45 ml of distilled water to make up the total volume upto 1000 ml) and 0.2 M solution of CH_3COONa (Prepared by adding 16.4 g of CH_3COONa or 27.2 g of $CH_3COONa.3H_2O$ in 950 ml of distilled water and the final volume was made upto 1000 ml by the addition of more distilled water). It was prepared by adding required amount of 0.2 M solution of acetic acid to 24.5 ml solution of 0.2 M sodium acetate and 100 ml of distilled water to make the pH of the solution to 4.5 finally. Thereafter, the volume of the solution was adjusted to 200 ml. The solution was then filtered through Whatman filter paper (Grade-2) and stored at 4°C for future use.

Next, 10 g of mature *Lagenaria siceraria* seeds were procured locally and were crushed to a fine powder using mortal and pestle. The powdered seeds were then soaked overnight at 4°C in 30 ml of 50 mM acetate buffer, pH 4.5. The homogenate was centrifuged at 12,000x g for 1 hour, and subsequently the supernatant was collected and the pellet was discarded. The supernatant thus collected was then loaded onto the CM Macroprep column (1.5 x 20 cm Econo-column, Biorad) equilibrated with 50 mM of Acetate Buffer, pH 4.5. The column was then washed extensively with the same buffer and all the unbound molecules were than washed by checking the optical density of the wash periodically. The column was washed until the O.D. of the wash dropped down to 0.05 at 280 nm. Then, the bound molecules were eluted with a NaCl step gradient from 0 to 500 mM NaCl in 50 mM acetate buffer, pH 4.5. The peptide of interest was further separated from large molecules on a Centriprep with a 3 kD cutoff membrane. The filtrate was then lyophilized and finally purified using Symmetry C18 column (4.6 x 150 mm, 5 μm, Waters, USA) equilibrated with HPLC grade water. The fractions were then eluted in acetonitrile for 15 minutes with a linear gradient of 0–100%. The fractions showing trypsin inhibitory activity (retention time 3.848 minutes) were then pooled for further analysis.

3.2.3. Molecular Weight Determination of the Purified Peptide

Molecular weight determination of the purified peptide was carried out by mass spectrometric analysis. Matrix Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF) analysis was therefore used as a method of choice for the same. MALDI-TOF analysis of the peptide was carried out on KRATOS, Shimadzu Machine by first dissolving 10 mg of the lyophilized purified peptide in 1ml of millipore water and then 100 μl of the same was mixed with 100 μl of 0.2% of aqueous trifluoroacetic acid (TFA). Then 1 μl of the above acidified solution was spotted on the stainless steel slide along with 1 μl of the 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) matrix solution with the concentration of 10 mg/ml (dissolved in 50:50 ethanol/water containing 0.1% of trifluoroacetic acid). The slide was then placed into the given slot into the mass spectrometer and the spectra was then acquired.

3.2.4. Determination of the Peptidic Nature of the Compound

Determination of the peptidic nature of the compound was done by Fourier Transform-Infra Red (FT-IR) spectroscopy and by measuring the O.D. of the purified peptide at 214 nm on spectrophotometer.

FTIR spectra were measured at room temperature in KBr pellets on Perkin Elmer spectrometer. Typically, 5 μl of 10 mg/ml^{-1} sample in 50 mM Tris-Cl buffer (pH 7.5), were transferred onto a KBr pellet. Spectra were collected at ambient temperature in the spectral region of 4000 to 400 cm^{-1} with a spectral resolution of $0.5cm^{-1}$ prior to zerofilling. A spectrum representing atmospheric water and buffer composition were subtracted from the sample spectra.

3.2.5. Trypsin Inhibitory Activity of the Peptide

Trypsin inhibitory activity assay of the purified peptide was performed by dissolving trypsin in 0.001 N HCl containing 20 mM $CaCl_2$ at the concentration of 200 μg/ml. Then 100 μl of $4x10^{-7}$ M of the enzyme solution was incubated with 200 μl of different concentrations of inhibitor solutions in 50 mM Tris-Cl buffer, pH 7.5 at 30°C for 10 minutes. After that, 1 ml of 15 mM BAPA solution containing 2.5% DMSO was added in the same buffer. After incubation at 30°C

for another 10 minutes, 200 μl of 30% acetic acid was added to stop the enzyme reaction, and the absorbance was measured at 410 nm spectrophotometrically [269].

3.2.6. Antimicrobial Activity Assay of the Purified Peptide

For carrying out the antimicrobial assay of the purified peptide firstly Luria-Bertani Broth and Agar were prepared. For preparation of LB broth 10 g of Tryptone, 5 g of yeast extract and 10 g of sodium chloride were dissolved in 950 ml of distilled water pH of the solution was adjusted to 7.0 with 1 M NaOH solution and the final volume was made upto 1000 ml. The resultant solution was thus autoclaved and stored at room temperature. LB Agar comprises of all the constituents of LB media and 15 g of agar per 1000 ml of the liquid agar solution. LB agar solution was thus autoclaved and poured into the Petri plates in sterile conditions under the laminar flow.

Thus the assay for antibacterial activity was performed by incubating 10^5 bacterial (*Escherichia coli*) colony forming units (in 50 μl) on LB-Agar medium and placing sterile paper disk containing 10 μl of peptide solution, on inoculated agar plate. In the assay, antibiotic ampicillin was used as a positive control and water as negative control. The antimicrobial activity was evaluated by measuring the zone of inhibition at different concentrations of peptide against the *E. coli* using agar well diffusion method [219]. For the Minimum Inhibitory Concentration (MIC) value, *E. coli* was grown to late log phase. Approximately 10^4 bacterial cells were incubated in LB broth containing different concentrations of peptide (ranging from 20 to 0.31 μM) for 18 hours at 37°C. The lowest concentration inhibiting bacterial growth was taken as the MIC value [56].

3.3. Results and Discussion

3.3.1. Purification of the Peptide

The compound with dual activity was purified from the seeds of *Lagenaria siceraria* in two steps. In first step, two major peaks were obtained on cation-exchange column (CM Macroprep). The fractions eluted at 0.5M NaCl representing second major peak in elution profile were taken for further purification. The small molecular mass compounds were separated in second peak from large molecules by passing through a 3 kD cutoff centriprep. The small molecular mass compounds of less than 3 kD in filtrate were lyophilized and finally separated using Symmetry C_{18} column in reverse-phase HPLC in second step. The fractions having trypsin inhibitory activity was the major peak with retention time of 3.848 minutes and was eluted in 25.6% acetonitrile concentration (Figure 3.1and Figure 3.2).

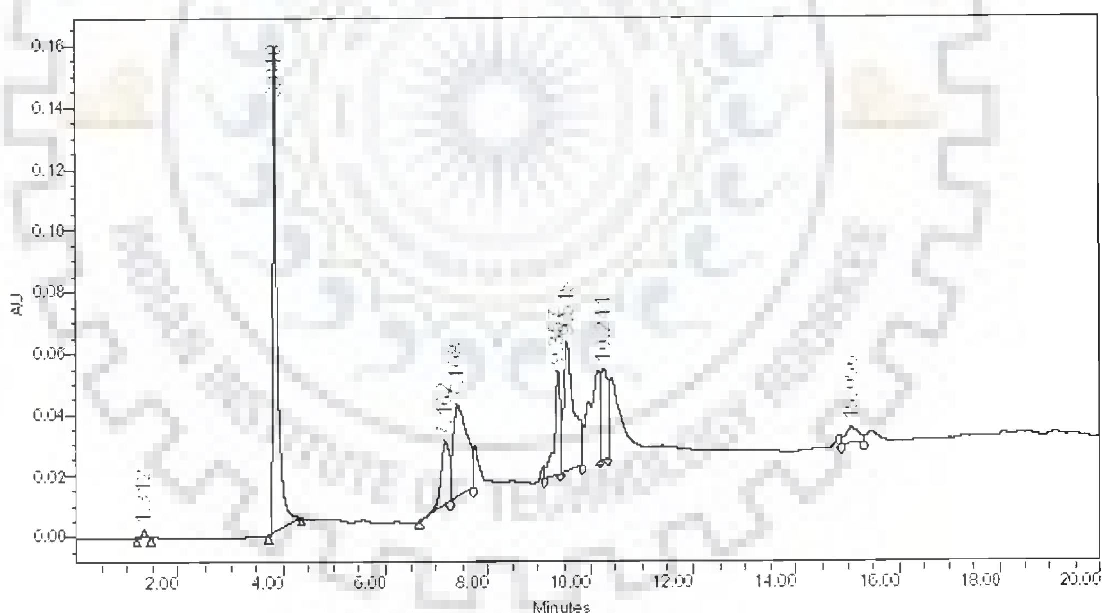
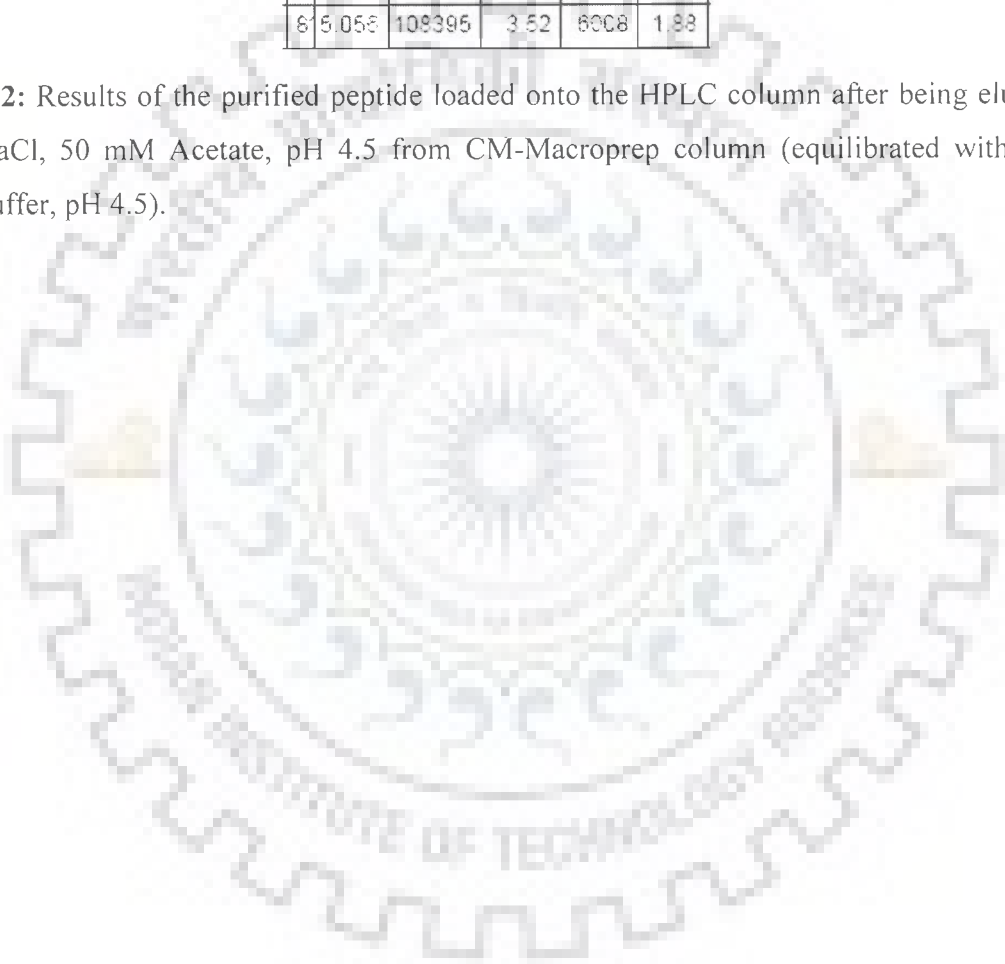


Figure 3.1: Elution profile of the compound on reverse phase HPLC Symmetry C_{18} column. Compound was eluted with retention time 3.848 minutes at 25.6% acetonitrile concentration.

	RT (min)	Area (Ʒ*sec)	% Area	Heigh (Ʒ)	% Heigh
1	1.312	17699	0.58	2788	0.87
2	3.848	938290	30.50	153033	47.84
3	7.162	252214	8.20	20797	6.50
4	7.409	589406	19.16	30408	9.51
5	9.357	287719	9.35	34458	10.77
6	9.545	600553	19.52	42395	13.25
7	10.241	282484	9.18	29983	9.37
8	15.058	108395	3.52	6008	1.88

Figure 3.2: Results of the purified peptide loaded onto the HPLC column after being eluted with 0.5 M NaCl, 50 mM Acetate, pH 4.5 from CM-Macroprep column (equilibrated with 50 mM acetate buffer, pH 4.5).



3.3.2. Molecular Weight Determination of the Peptide

The fractions having trypsin inhibitory activity (purified by HPLC) were pooled and lyophilized for further analysis. The purity of the peptide was confirmed by MALDI-TOF studies. The molecular mass of the peptide was determined to be 678.9 daltons by MALDI-TOF mass spectrometry (Figure 3.3).

Figure 3.3: MALDI-MS spectra of purified compound with M/z value 678.9.

3.3.3. Determination of the Peptidic Nature of the Compound

The FTIR spectrum at ambient temperature showed characteristic absorption bands at 1639 cm^{-1} where the C= O stretching vibrations of the protonated carbonyl groups appear, which is characteristic of amide I bands [182]. The strong positive absorption at this region is the clear indication of presence of amide bond in the molecule (Figure 3.4). The compound showed strong absorbance at 214 nm on a spectrophotometer and also on a dual wavelength (214 and 280 nm) detector while purifying on HPLC. These results clearly demonstrated the peptidic nature of this compound.

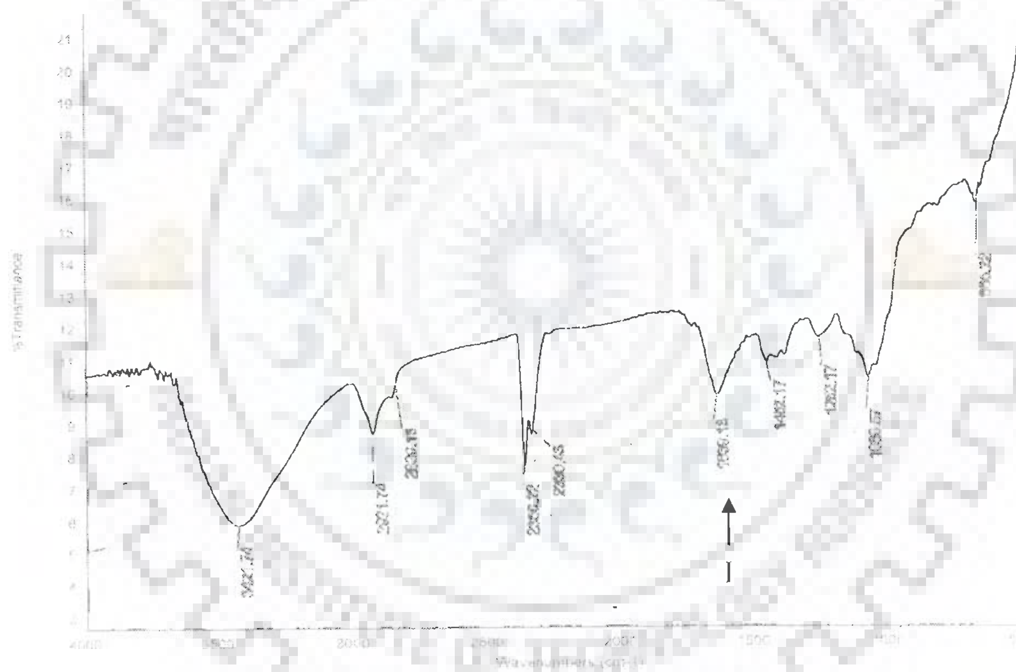


Figure 3.4: FTIR spectra of purified compound showing amide I band at 1639 cm^{-1}.

3.3.4. Trypsin Inhibitory Activity of the Peptide

The inhibitory activity of the compound against trypsin was determined by measuring the residual hydrolytic activity of trypsin towards BAPNA as a substrate. The compound completely inhibited bovine pancreatic trypsin at a molar ratio of 1:2. At the molar ratio of 1:1 the trypsin was inhibited by 57.5% (Figure 3.5 and Figure 3.6)

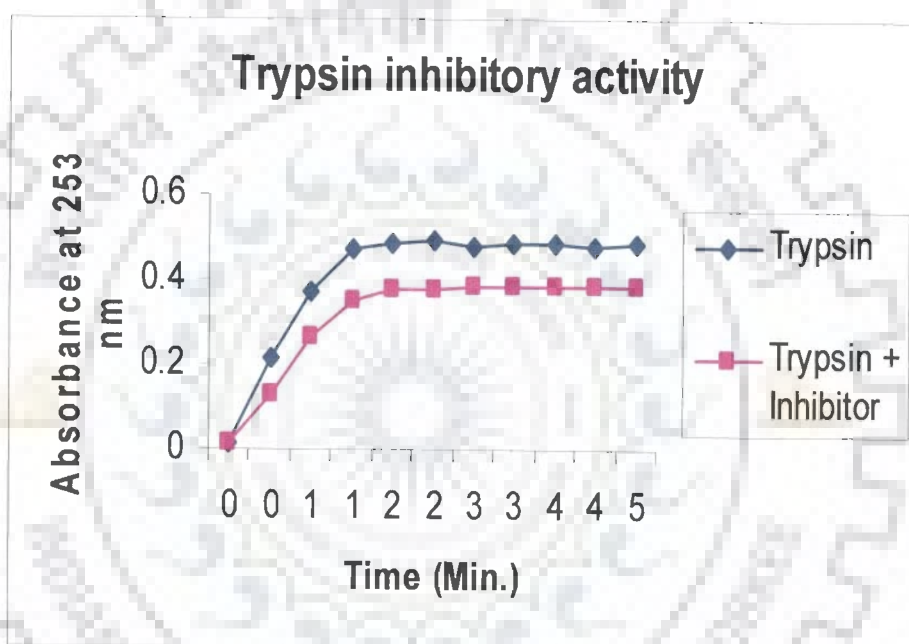


Figure 3.5: Trypsin Inhibitory activity obtained with the purified peptide eluted with 0.5 M NaCl, 50 mM acetate buffer, pH 4.5 from CM-Macroprep column.

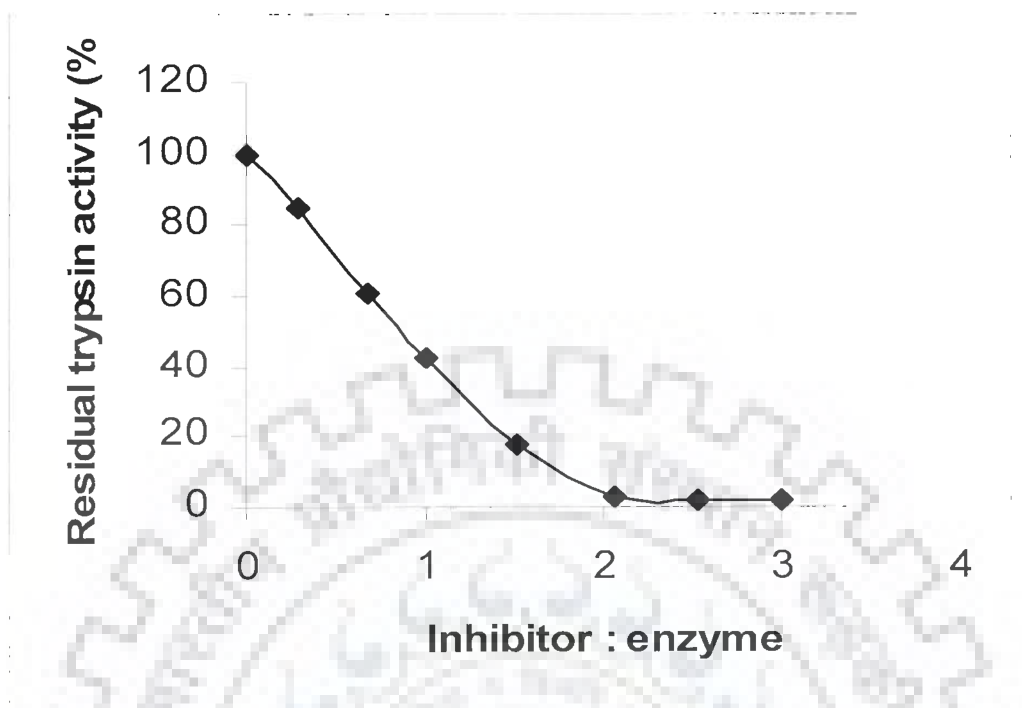


Figure 3.6: Trypsin inhibitory activity of purified compound showing residual trypsin activity (%) as function of the inhibitor concentration at a fixed trypsin concentration. All experiments were done three times and averaged.

3.3.5. Antimicrobial Activity Assay of the Purified Peptide

The antibacterial activity of the compound was demonstrated by agar diffusion method where a zone of inhibition was seen at different concentrations (Figure 3.7). The minimal inhibitory concentration of compound against *E. coli* was found to be 20 μM.

Figure 3.7: Antibacterial activity of the purified compound against *E. coli*. (A) 10 μl of 20 μM compound, (B) 10 μl of 10 μM compound, (C) Ampicillin as positive control, (D) Water as negative control (E) Supernatant.

Thus, a low molecular mass peptide like compound with antimicrobial and trypsin inhibitory activity was isolated from the seeds of *Lagenaria siceraria*. It was purified by ion-exchange and reverse-phase chromatography. The molecular weight of the compound was 678.9 daltons as determined by MALDI-MS. The infra-red absorbance at 1639 cm^{-1}, characteristic of an amide bond, by FTIR spectroscopic studies, and absorption at 214 nm on spectrophotometer indicates the peptidic nature of the compound. The compound exhibited antimicrobial activity when tested against *Escherichia coli* with minimum inhibitory concentration of 20 μM, and trypsin inhibitory activity inhibiting trypsin at a molar ratio of 1:2. As per the database of the antimicrobial and trypsin inhibitory peptides, this may be the smallest compound of peptidic nature having both the activities.

The possible role of such a small peptide with protease inhibitory and antimicrobial activity in plants such as *Lagenaria siceraria* could be many. One of the possible roles of the plant protease inhibitors can be the suppression of its own proteases to regulate various physiological processes such as germination, growth, flowering, et cetera. Generally it is observed that very few plant protease inhibitors are involved in the regulation of endogenous proteases but are rather involved in plant defense. These protease inhibitors may be induced more at the time of physiological trauma as in the case of attach by various pathogens. Many plant protease inhibitors are known to inhibit the proteases important to the pathogens. Also, the role of protease inhibitors as anti-inflammatory, anticancerous and as anti-HIV agents has already been well established and the purified peptide could be a one such potent agent against cancer, AIDS or inflammation.

The antimicrobial activity of the peptide could also be involved in the plant defense against microbial infection in case of Bottle gourd (*Lagenaria siceraria*). Antimicrobial peptides such as these could have potential application as food preservatives. Such peptides can also be utilized to treat local, topical infections. Also there is a possibility that the peptide could also act against systemic infections.

Since the peptide is very small in size and is water soluble, its delivery as a potential drug molecule upto the cellular level will not be challenging.

CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF A PROTEIN FROM CITRULLUS FISTULOSUS

4.1. Introduction

Through extensive literature survey it was found that not much work had been carried out on the plant *Citrullus fistulosus* of the cucurbitaceae family except for the NADH Dehydrogenase subunit F protein of 310 amino acids and Ycf9 protein of 46 amino acids only [68]. Therefore the given chapter rightly focuses on the purification and characterization of a novel protein from the *Citrullus fistullosus*. Infact there are very few plants of genus Citrullus that have been explored for their protein content. Only some information is available on the proteins of *Citrullus vulgaris*, extracted from the cotyledons of the plant [157, 280].

4.2. Materials and Methods

4.2.1. Materials

Seeds of *Citrullus fistulosus* (procured locally), Tris Base, Concentrated HCl, DiEthylAminoEthyl Macroprep or DEAE Macroprep, Sodium Chloride (NaCl), Sodium Azide (NaN_2), CarboxyMethyl Macroprep or CM Macroprep, Coomassie Brilliant Blue G-250, Ethanol, Phosphoric Acid (H_3PO_4), Acrylamide, Bis-acrylamide, Methanol, Glacial Acetic Acid, Coomassie Brilliant Blue R-250, Ammonium Persulphate, Glycine, Sodium DodecylSulphate (SDS), Glycerol, Bromophenol Blue, β-Mercaptoethanol, TetraMethylEthyleneDiamine (TEMED), Protein Molecular Weight Marker, Milli-Q Water, N-cyclohexyl-3-aminopropanesulfonic acid or CAPS, HPLC grade methanol, Polyvinylidine Flouride or PVDF Membrane, *Staphylococcus aureus* culture, Yeast Extract, Tryptone, Agar and Broad Range Penicillin.

4.2.2. Purification of the Protein by Ion Exchange Chromatography

First, 10 g of decoated matured seeds of *Citrullus fistulosus* were taken and crushed with mortar-pestle to a fine powder. The crushed seeds were then soaked overnight in 30 ml of 10 mM Tris-Cl, pH 7.15 buffer at 4°C. Thereafter, the soaked extract was filtered through a muslin cloth and the filtrate was centrifuged at 7000 rpm using JA-20 rotor in Beckman's High Speed Centrifuge at 4°C for 1 hour. The supernatant thus formed was collected and the pellet was

discarded. Thereafter the supernatant was loaded on the Anion exchange column (DEAE Macroprep). For this, 5 ml of DEAE Macro prep (Bio-rad) resin was taken and washed twice with distilled water as to be free of any preservative (70% ethanol or 0.05% Sodium Azide). The resin was then equilibrated with 10 mM Tris-Cl, pH 7.15 and packed by slight pouring into the Econo-column (1.5 x 20 cm, Biorad). And the matrix was allowed to settle. Thereafter 25 ml of the supernatant was collected (after the centrifugation) and loaded onto the column. Flow Through after loading was collected separately into the beaker and was stored at 4°C. The column, fitted with the flow-adaptor and attached to the Econo-pump (Bio-rad) was then washed with the same equilibration buffer, at the flow rate of 1.0 ml/minute. Washing was continued till the O.D. reached a minimum of 0.05 at 214 nm. The column was then eluted with 0-1 M NaCl, 10 mM Tris-Cl, pH 7.15 solution in step gradient manner at the flow rate of 1.0 ml/minute. 6ml fractions of the elute were then collected, while eluting with each buffer in the step gradient manner. The column was then washed with 5 bed volumes of 1.0 M NaOH. Since the OH^- ions bind strongly to the resin, therefore it was further replaced by Cl^- ions by washing the column with 5 bed volumes of 1 M NaCl solution. The column was then unpacked and the resin was then washed repeatedly with distilled water in the beaker. Finally the washed resin was stored in 0.05% Sodium Azide at 4°C.

Next, about 24 ml of the Flow Through collected by Anion Exchange Chromatography was loaded onto the Cation Exchanger packed with 5 ml of CM Macroprep (washed with distilled water and equilibrated by 10 mM Tris-Cl, pH 7.15). Flow through of the cation exchanger was then collected and stored for further analysis at 4°C. Thereafter, the column was washed with the same buffer at the flow rate of 1ml/minute until the O.D. was below 0.05 at 214 nm and the elution was carried out in step gradient manner with 0-1 M NaCl, 10 mM Tris-Cl, pH 7.15 solution. Again 6ml fractions of the elute were collected at the flow rate of 1.0 ml/minute in the step gradient manner. Finally the column was washed with 0.2 M NaOH solution at the flow rate of 1.0 ml/minute, followed by further washing with distilled water at the same flow rate. Thereafter, again to further specify the exact salt concentration at which the pure protein will elute and also to decrease the amount of salt present in the purified fraction, again 24 ml of DEAE flow-through was loaded onto the CM Macroprep column containing 5 ml of CM Macroprep. The column was again pre-equilibrated with 10 mM Tris-Cl, pH 7.15 buffer and washed with the same buffer till the O.D. of 0.05 was achieved at 214 nm. The column was then eluted with 10 mM, Tris-Cl, pH 7.15 buffer containing different NaCl concentrations ranging from 0.1 M to 0.25 M, in a step

gradient manner. The column was then washed with 0.2 M NaOH. Finally, the column was unpacked and the matrix was preserved in 0.02% NaN_2 solution at 4°C.

4.2.3. Protein Estimation

Protein concentration in crude extract and fractionated protein samples were estimated by standard dye-binding method using bovine serum albumin (Sigma) as standard. For crude extract, mature seeds (10 g) were crushed with mortal and pestle and soaked overnight at 4°C in 30 ml of 10 mM Tris-HCl buffer, pH 7.15. The homogenate was cleared by centrifugation at 7000 rpm using JA-20 rotor in Beckman's High Speed Centrifuge at 4°C for 1 hour and the supernatant was used to determine the soluble protein concentration by the method of Bradford, using BSA as standard [41].

4.2.4. Molecular Weight Determination of the Protein by SDS-PAGE

Sodium DodecylSulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out both under, reducing and non reducing conditions as described by Laemli [148]. Relative molecular weight was determined by performing SDS-PAGE of the protein with molecular weight standards under reducing condition calibrated on 15% Tris-HCl gel. The molecular weight standards used were myosin (194.2 kD), β-galactosidase (115.6 kD), bovine serum albumin (97.3 kD), ovalbumin (53.5 kD), carbonic anhydrase (37.2 kD), soyabean trypsin inhibitor (29.3 kD), lysozyme (20.4 kD) and aprotinin (6.9 kD). The proteins were detected by staining the gel with the staining solution containing 0.025% Coomassie brilliant blue R-250 w/v.

4.2.5. Blotting of the Purified Protein on PVDF Membrane

For blotting the purified protein onto the PVDF membrane, firstly 15% gel was casted 18 to 24 hours before the actual blotting process was started. Thereafter 30 μl of the purified protein sample was loaded on to the gel and the gel was run at 100 volts. Thereafter the gel was washed in distilled water for 3 hours. Next the blotting buffer was prepared using CAPS rather than the usual Tris-glycine buffer, as to prevent the interference by glycine (present in the electrophoresis buffer) in the process of N-terminal sequencing of the protein. Therefore, 100 mM CAPS buffer at pH 11

was prepared and 200 ml of HPLC grade methanol and 1600 ml of Milli-Q water was then added to the 200 ml of freshly prepared CAPS buffer, thus making the total volume of the blotting buffer to 2000 ml.

Thereafter filter papers, fiber pad and blotting membrane of the same size as that of the previously ran gel were taken. Blotting membrane was then wetted in 100% methanol for 30 seconds and then for 5 minutes in Milli-Q water, and finally for 15 minutes in the blotting buffer. Similarly, the previously ran gel, filter papers and fiber pads were also equilibrated with the same blotting buffer for 30 minutes. Then, a sandwich was prepared comprising of fiber pad, filter papers and previously equilibrated gel. The gel was then covered with a pre-wetted PVDF membrane (soaked in blotting buffer), on top of which again filter papers were placed. Finally, again a fiber pad was placed over it and a glass rod was rolled over the sandwich to ensure that no air bubble remains between the gel and the PVDF membrane, placed next to each other. Then the sandwich was placed in the gel blotting cassette and the cassette was closed. The closed cassette was then placed into the blotting unit, such that the side of the sandwich with the ran gel on the top faces the cathode and the side of the sandwich with PVDF membrane on the top faces the anode. The blotting unit was then filled up with the blotting buffer and the band of the purified protein on the gel was then blotted onto the PVDF membrane at 100 volts, 350 mÅ for 90 minutes.

After the blotting was complete, the PVDF membrane was rinsed with distilled water and stained with 0.025% w/v Coomassie Brilliant Blue R-250 solution (prepared by adding 0.25 g of Coomassie Brilliant Blue R-250 to the mixture of 500 ml Methanol, 400 ml of distilled water and 100 ml of Glacial Acetic Acid). The resultant, stained protein blot thus obtained on the PVDF membrane was cut out from the rest of the PVDF membrane. Thereafter, the stained blot was destained using the destaining solution (comprising of 500 ml of Methanol, 400 ml of Distilled Water and 100 ml of Glacial Acetic Acid). After destaining, PVDF membrane was rinsed with 100% methanol and then was soaked in 100ml of distilled water for 15 minutes to remove the excess acetic acid and methanol. Next, the PVDF membrane was air dried and placed into a fresh microcentrifuge tube and sealed with parafilm.

4.2.6. N-terminal sequencing of the Protein

The sealed microcentrifuge tube with the blotted purified protein band was then send to School of Biosciences and Bioengineering, IIT Mumbai for N-terminal sequencing of the purified protein. Seven amino acids of the N-terminal sequence of the polypeptide were thus determined.

4.2.7. Antimicrobial Activity Assay of the Purified Peptide

Antibacterial activity of collected fractions after the elution from CM Macroprep and Cibacron Blue 3GA Agarose were assayed using Radial Diffusion Method. Petri plates were first autoclaved and poured with 20 ml of growth medium (Luria-Bertani Agar) into each of the autoclaved Petri plates. They were then allowed to solidify at room temperature and seeded with 24 hours old culture of *Staphylococcus aureus* bacterial culture grown in LB Broth. Thereafter wells were made with the help of sterile punter and loaded with different amount of protein (5 µl, 10 µl, 15 µl, 20 µl, 25 µl) in different wells. Phosphate buffer served as the negative control and the wells with broad spectrum Penicillin was used as the positive control. Incubation was done at 37^0C for 12 hours. The assessment of antibacterial activity was based on the measurement of diameter of Zone of Inhibition formed around the wells. Five independent trials were conducted for each elution fractions [19].

4.3. Results and Discussion

4.3.1. Purification of the Protein

The purification of the protein was carried out by first loading the extract onto the DEAE Macroprep column and by analyzing the flow through, wash, and elute by SDS-PAGE (Figure 4.1). Elution profile for the same was also observed (Figure 4.2 and Table 4.1).

Figure 4.1: 15% SDS-PAGE of the various fractions during the purification of the protein: L1- Molecular weight marker, L2- *Citrullus fistulosus* Extract, L3- DEAE flow through, L4- CM Macroprep Flow Through, L5- 0.05 M NaCl fraction of elution on CM Macroprep, L6- 0.1 M NaCl fraction of CM elute, L7- 0.2 M NaCl fraction of CM elute, L8- 0.4 M NaCl fraction of elute, L9- 0.5 M fraction of CM elute and L10- 1.0 M fraction of CM elute.

Fraction Number	**O. D. at 0.25 M NaCl**	Fraction Number	**O.D. at 0.40 M NaCl**	Fraction Number	
1	-1.4150	19	-0.0865	35	0.1368
2	1.2034	20	0.3873	36	0.0990
3	1.5766	21	0.7034	37	0.1050
4	1.0989	22	0.6227	38	0.0798
5	0.8506	23	0.5206	39	0.0621
6	0.7276	24	0.4361		**O.D. at 0.9 M NaCl**
7	0.6639		**O.D. at 0.50 M NaCl**	40	0.1206
8	0.5993	25	1.7980	41	0.0110
9	0.5201	26	1.3283	42	0.2967
10	0.4329	27	0.3541	43	0.1828
11	0.3648	28	0.1742	44	0.0357
12	0.3220	29	0.1210	45	0.0139
13	0.3823	30	0.0724	46	-2.4707
14	0.3232		**O.D. at 0.75 M NaCl**		**O.D. at 1 M NaCl**
15	0.2559	31	-0.6868	47	2.4971
16	0.1388	32	0.4236	48	2.2883
17	0.0243	33	0.2331	49	0.4229
18	-0.0714	34	0.1646	50	0.1695
				51	0.0482

Table 4.1: O.D. of the various fractions collected (at 214 nm) during the elution of DEAE column loaded with the seed extract of *Citrullus fistulosus*. 10 mM Tris-Cl, pH 7.15 buffer, with different concentrations of NaCl, was used as the elution buffer.

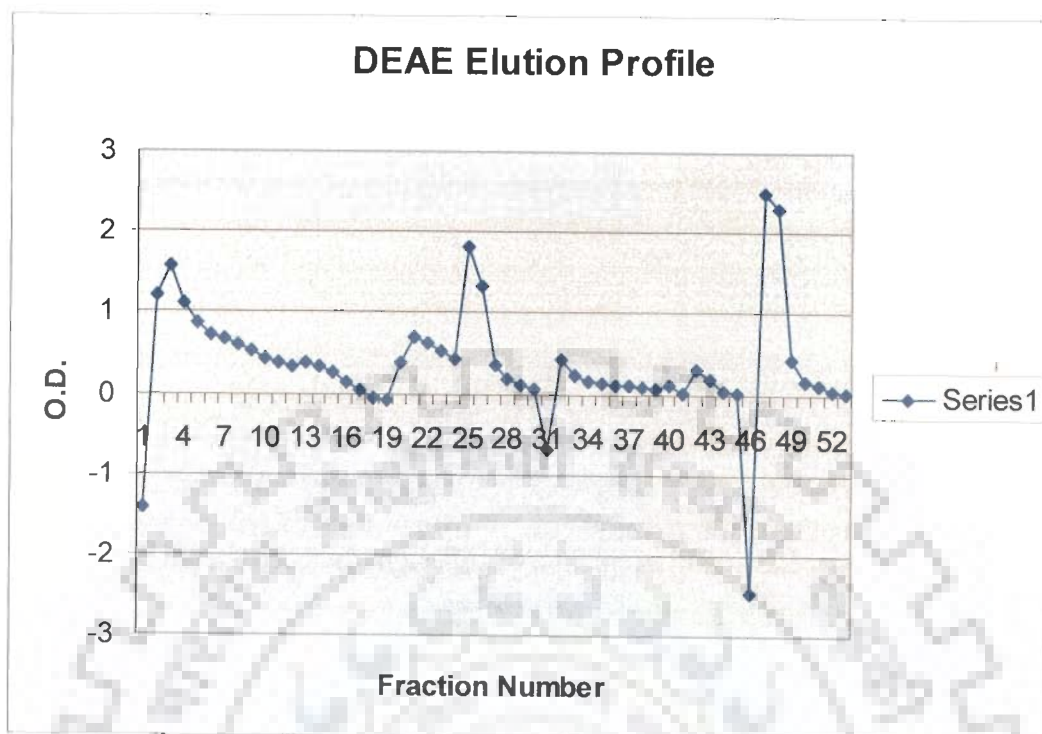


Figure 4.2: Elution profile of the extract loaded onto the DEAE column and eluted with 0.25 M, 0.4 M, 0.5 M, 0.75 M, 0.9 M and 1.0 M NaCl in 10 mM, Tris-Cl, pH 7.15 buffer.

Thereafter the flow through of DEAE Macroprep column was loaded onto the CM Macroprep column equilibrated with the 10 mM Tris-Cl, pH 7.15 buffer and the elution was carried out with the elution buffer containing various concentrations of NaCl in the equilibration buffer. The elution of the CM Macroprep column was followed by SDS-PAGE analysis (Figure 4.1) and also the elution profile of the DEAE flow through loaded onto the column was observed (Table 4.2 and 4.3 and Figure 4.3 and Figure 4.4).

Fraction Number	O.D. at 0.25 M NaCl	Fraction Number	O.D. at 0.5 M NaCl	Fraction Number	O.D. at 1 M NaCl
1	0.0240	17	-0.0561	32	-0.0804
2	2.8557	18	-0.0350	33	-0.1195
3	2.8015	19	-0.0340	34	-0.1270
4	2.4522	20	-0.0466	35	-0.1372
5	1.4343	21	-0.0560	36	-0.1381
6	0.8114		**O.D. at 0.75 M NaCl**		
7	0.5108	22	-0.1080		
8	0.2452	23	-0.0976		
9	0.1106	24	-0.0841		
10	0.0640	25	-0.0895		
11	0.4890	26	-0.1131		
	O.D. at 0.4 M NaCl		**O.D. at 0.9 M NaCl**		
12	-0.0014	27	-0.2600		
13	0.1435	28	-0.2423		
14	0.1166	29	-0.2209		
15	0.0557	30	-0.1112		
16	0.0168	31	-0.2209		

Table 4.2: O.D. (at 214 nm) of various fractions collected during the elution of CM Macroprep column with 10 mM Tris-Cl, pH 7.15 elution buffer of different salt concentrations.

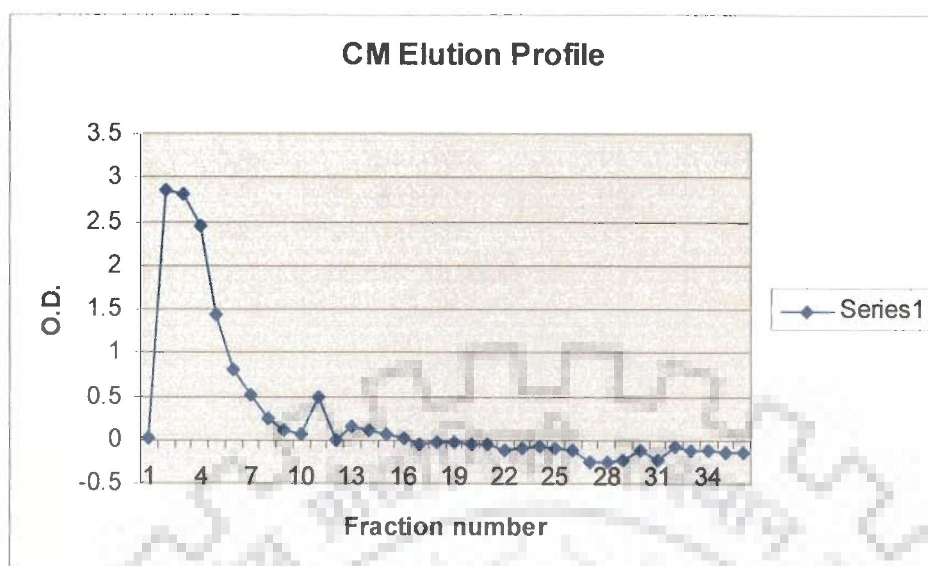


Figure 4.3: Elution profile of the DEAE Flow through loaded onto the CM column and eluted with 0.25 M, 0.4 M, 0.5 M, 0.75 M, 0.9 M and 1.0 M NaCl in 10 mM, Tris-Cl, pH 7.15 buffer.

Fraction Number	**O.D at 0.1 M NaCl**	Fraction Number	**O.D. at 0.15 M NaCl**	Fraction Number	**O.D. at 0.2 M NaCl**
1	0.0516	17	0.0448	23	-0.0132
2	0.8034	18	0.0189	24	-0.0165
3	2.2797	19	0.0331	25	0.0552
4	1.8481	20	0.0241	26	0.0846
5	1.8464	21	0.0115	27	0.0052
6	1.7142	22	-0.0048	28	-0.0726
7	1.2274				
8	0.7760				
9	0.4444				
10	0.2344				
11	0.1414				
12	0.0921				
13	0.0494				
14	0.0517				
15	0.0315				
16	0.0203				

Table 4.3: O.D. (at 214 nm) of various fractions collected during the elution of CM column with 10 mM Tris-Cl, pH 7.15 elution buffer of different salt concentrations.

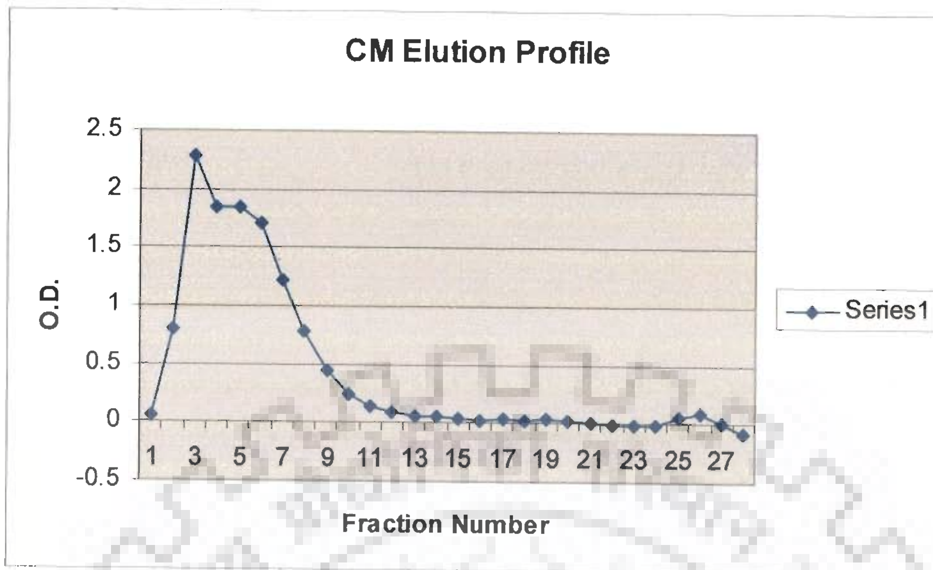


Figure 4.4: Elution profile of the DEAE flow through loaded onto the CM column and eluted with 0.1 M, 0.15 M and 0.2 M NaCl in 10 mM, Tris-Cl, pH 7.15 buffer.

4.3.2 Molecular Weight determination of the Protein

Molecular weight determination of the protein was done by SDS-PAGE analysis. And the molecular weight of the protein was determined to be 9 kD.

4.3.3. Protein Concentration

Protein concentration found in the seed extract of *Citrullus fistulosus* using the Bradford Assay was determined to be 2.5 mg/ml of the extract. The protein concentration of the purified protein fraction obtained after elution with CM Macroprep column was found to be 1 mg/ ml, by Bradford assay.

4.3.4. N-terminal sequencing of the Protein

The N-terminal sequence of the first 7 amino acids of the purified protein was found to be as Phe-Asp-Asn-Ser-Phe-Thr-Asn or FDNSFTN.

4.3.5. Antimicrobial Activity Assay of the Purified Peptide

The assessment of antibacterial activity was based on the measurement of diameter of Zone of Inhibition formed around the wells. Five independent trials were conducted for each elution fractions [19].

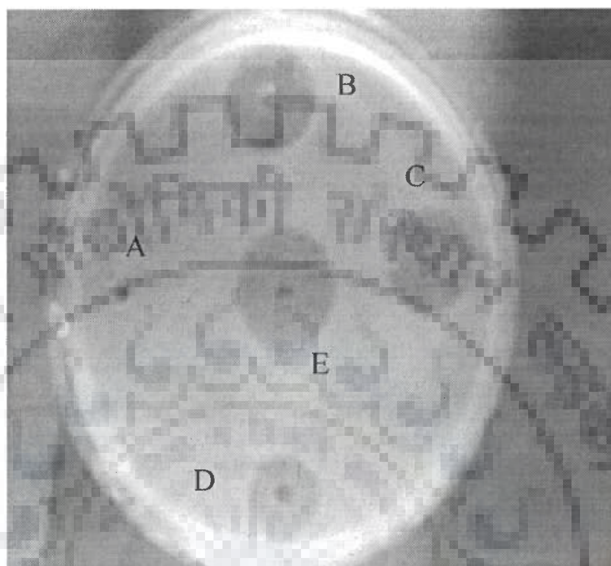


Figure 4.5: Antibacterial activity of the purified protein against *Staphylococcus aureus*. (A) 0.1 M NaCl in 10 mM, Tris-Cl, pH 7.15 buffer used as the negative control, (B) 30 μl of the purified protein at the concentration of 1 mg/ml, (C) 25 μl of the purified protein at the concentration of 1mg/ml, (D) 20 μl of the purified protein at the concentration of 1 mg/ml and (E) Broad range penicillin used as positive control.

Thus, a protein of 9 kD was purified to homogeneity by the two step purification procedure involving anion and cation exchange chromatography on DEAE and CM Macroprep. No purified protein fraction was obtained by elution of the seed extract on the DEAE column but a purified 9 kD protein was obtained by running the flow through of DEAE Macroprep column onto the CM column.

Molecular weight determination of the polypeptide was done by SDS-PAGE and was found to be 9 kD. Bradford dye binding assay was used for determining the amount of the protein present in the extract and the purified protein fraction.

The N-terminal sequencing report of the polypeptide showed that there was no significant match for the provided N-terminal sequence with that of antimicrobial peptides or with any other protein for that matter.

The purified protein also showed good antibacterial activity against the *Staphylococcus aureus* bacterium, with 0.1 M NaCl in 10 mM Tris-Cl, pH 7.15 used as the negative control and broad range penicillin being used as the positive control.

CONCLUSIONS

CONCLUSIONS

Cloning and Sequencing of the β-subunit of the Procaryotic Respiratory Nitrate Reductase Gene

The forward and reverse primers designed for PCR amplification of the beta subunit of respiratory nitrate reducatse were found to be appropriate, with respect to the GC content and Tm values only. The forward primer sequence was found to be appropriate with respect to GC, which was over 50% and a good Tm value of 57°C but was found lacking in the aspect of self complementarity, as it showed two regions each of eight base pairs in total having high self complementarity. Whereas in case of reverse primer sequence, the GC content (over 50%), and the Tm values of 56°C was found to be satisfactory (neither too high and neither too low) and also the reverse primer did not showed any region of self complementarity. Thus the reverse primer sequence was found to be alright in all respects.

The quantity or the amount of the isolated bacterial genome determined spectroscopically was found to be having the OD_{260}/OD_{280} ratio of 1.775. These results suggest a good and quite pure yield of isolated bacterial genome after its isolation from the lysed bacterial cells.

The PCR amplification which was optimized with the primers designed for the amplification of the β subunit of prokaryotic respiratory nitrate reductase gene utilized normal volumes and concentration of primers usually employed and also utilized normal and usual amount of *Taq* polymerase, $MgCl_2$ and DMSO. Normal annealing temperature in the range of 50-60°C was used for the annealing of primer sequences to the gene of interest. All the other steps of the PCR reaction were carried out at the standard temperatures as followed in the protocol for PCR amplification, without any alterations.

The bright and intense band of the amplified PCR product on the 0.8% agarose gel indicates good amount of amplification of the gene had been achieved using the given set of primers.

Gel elution of the PCR products was quite effective as can be observed with the first, very intense and second, less intense band of the eluted PCR products on 0.8% agarose gels.

Screening of the recombinant bacterial colonies using IPTG, X-gal and Amplicillin was carried out and a good number of white colonies were picked up having the recombinant plasmid within their cells.

The presence of the same amplified product band, obtained by agarose gel electrophoresis of the amplified isolated recombinant plasmids (obtained from the positively screened colonies) further strengthens that the correct screening of the bacterial colonies was carried out.

Blast results of the translated gene sequence obtained by the direct sequencing of the plasmid showed high identity with the β-subunit of respiratory nitrate reductase of the prokaryotes.

Further, the CLUSTALW and COBALT results for the multiple sequence alignment of the gene sequence obtained, with the various β-subunit sequences of the prokaryotic respiratory nitrate reductases also indicate that the cloned and the translated gene sequence is that of partial β-subunit of respiratory nitrate reductase only.

Homology model of the partial β-subunit of respiratory nitrate reductase was prepared using the Modeller program and the model with highest percentage of amono acids in the most favourable region in the Ramachandran plot was chosen as the representative model for the translated gene sequence.

Purification and Characterization of a Peptide from *Lagenaria* siceraria

A low molecular mass peptide like compound with antimicrobial and trypsin inhibitory activity was isolated from the seeds of *Lagenaria siceraria.* It was purified by ion-exchange and reverse-phase chromatography. The molecular weight of the compound was 678.9 daltons as determined by MALDI-MS. The infra-red absorbance at 1639 cm^{-1}, characteristic of an amide bond, by FTIR spectroscopic studies, and absorption at 214nm on spectrophotometer indicates the peptidic nature of the compound. The compound exhibited antimicrobial activity when tested against *Escherichia coli* with minimum inhibitory concentration of 20 μM, and trypsin inhibitory activity inhibiting trypsin at a molar ratio of 1:2. As per the database of the antimicrobial and trypsin inhibitory peptides, this may be the smallest compound of peptidic nature having both the activities.

Purification and Characterization of a Protein from *Citrullus Fistulosus*

A protein of 9 kD was purified to homogeneity by the two step purification procedure involving anion and cation exchange chromatography on DEAE and CM Macroprep. No purified protein fraction was obtained by elution of the seed extract on the DEAE column but a purified 9

kD protein was obtained by running the flow through of DEAE Macroprep column onto the CM column.

Molecular weight determination of the polypeptide was done by SDS-PAGE and was found to be 9 kD. Bradford dye binding assay was used for determining the amount of the protein present in the extract and the purified protein fraction.

The N-terminal sequencing report of the polypeptide showed that there was no significant match for the provided N-terminal sequence with that of antimicrobial peptides or with any other protein for that matter.

The purified protein also showed good antibacterial activity against the *Staphylococcus aureus* bacterium, with 0.1 M NaCl in 10 mM Tris-Cl, pH 7.15 used as the negative control and broad range penicillin being used as the positive control.

REFERENCES

REFERENCES

(1). ^ Cann A.DNA viruses: A Practical Approach. *Oxford University Press* 1999, ISBN0199637180.

(2). Ahmad I., Perkins W. R., Lupan D. M., Selsted M. E. and Janoff A. S. Liposomal entrapment of the neutrophil-derived peptide indolicidin endows it with in vivo antifungal activity. *Biochim. Biophys. Acta* 1995. 1237: 109-114.

(3). Amaar Y. G. and Moore M. M. Mapping of the nitrate-assimilation gene cluster (crnA-niiA-niaD) and characterization of the nitrite reductase gene (niiA) in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Curr. Genet.*1998, 33: 206-215.

(4). Andersen N. H., Cao B., Rodriguez-Romero A. and Arreguin B. Hevein-NMR and assessment for solution state folding for the agglutinin-toxin motif. *Biochemistry* 1993, 32: 1407–1422.

(5). Andreu D. and Rivas L. Animal antimicrobial peptides: an overview. *Biopolymers* 1998, 47: 415-433.

(6). Antipov A. N., Lyalikova N. N., Khijniak T. V. and L'vov N. P. Molybdenum free nitrate reductases from vandate reducing bacteria. *FEBS Lett.*1998, 441, 257-260.

(7). Antipov A. N., Morozkina E. V., Sorokin D. Yu., Golubeva L. B., Zvyagilskaya R. A. and L'vov N. P. Characterization of molybdenum-free nitrate reductase from haloalkalophilic bacterium Halomonas sp. strain AGJ 1-3. *Biochemistry (Moscow)* 2005, 70: 799-803.

(8) Antuch W, Berndt K. D., Chávez M. A., Delfín J. and Wüthrich K. The NMR solution structure of a Kunitz-type proteinase inhibitor from the sea anemone *Stichodactyla helianthus*. *Eur J Biochem* 1993, 212: 675-684.

(9). Aria S., Watanabe H., Kondo H., Emori Y and Abe K. Papain-inhibitory activity of oryzacystatin, a rice seed cysteine proteinase inhibitor, depends on the central Gln-Val-Ala-Gly region conserved among cystatins superfamily members. *J. Biochem (Tokyo)* 1991, 109: 294-298.

(10). Arondel V., Vergnolle C., Cantrel C. and Kader J. C. Lipid transfer proteins are encoded by a small multigene family in *Arabidopsis thaliana*. *Plant Sci.*(*Shannon*) 2000, 157: 1–12.

(11). Aronson A. I. Insecticidal toxins. In: *Bacillus subtilis* and other Gram-positive bacteria. *American Society for Microbiology* 1993, Washington D.C.: 953-964.

(12). Ascenzi P., Ruoppolo M., Amoresano A., Pucci P., Consonni R., Zetta L., Pascarella S., Bortolotti F. and Menegatti E. Characterization of low-molecular-mass trypsin isoinhibitors of oil-rape (*Brassica napus* var. oleifera) seed. *Eur. J. Biochem* 1999, 261: 111-118.

(13). Avila J., Gonzalez C., Brito N., Machin F., Perez M. D. and Siverio J. M. A second Zn(II)(2)Cys(6) transcriptional factor encoded by the YNA2 gene is indispensable for the transcriptional activation of the genes involved in nitrate assimilation in the yeast *Hansenula polymorpha*. *Yeast* 2002, 19: 537-544.

(14). Baines B. S. and Brocklehurst K. Characterization of papaya peptidase A as a cysteine proteinase of *Carica papaya* L. with active centre properties that differ from those of papain by using 2,2′-dipyridyl disulphide and 4-chloro-7-nitrobenzofurazan as reactivity probes. Use of two-protonic-state electrophiles in the identification of the catalytic site thiol groups. *Biochem J* 1982, 205: 205-211.

(15). Baines B. S., Kierstan M. P. J. and Brocklehurst K. Polypeptide inhibitor of papain and bromelain. *Biochem. Soc. Trans* 1982, 10: 171-172.

(16). Baker M. A., Maloy W. L., Zasloff M. and Jacob L. S. Anticancer efficacy of magainin 2 and analogue peptides. *Cancer Res* 1993, 53: 3052-3057.

(17). Bates D. M., Lazazzera B. A. and Kiley P. J. Characterization of FNR* mutant proteins indicates two distinct mechanisms for altering oxygen regulation of the Escherichia coli transcription factor FNR *J. Bacteriol* 1995, 177: 3972-3978.

(18). Batista I. F. C., Oliva M. L. V., Araujo M. S., Sampaio M. U., Richardson M., Fritz H. and Sampaio C. A. M. Primary structure of the Kunitz-type trypsin inhibitor from *Enterolobium contortisiliquum* seeds. *Phytochemistry* 1996, 41: 1017-1022.

(19). Bauer A. W., Kirby W. M., Sherris J. C. and Turck M. Antibiotic susceptibility testing by a standardised single disc method. *American Journal of Clinical Pathology* 1966, 45: 493-496.

(20). Beaven G. H., Holiday E. R. and Johnson E. A. Optical properties of nucleic acids and their components. *In The nucleic acids (ed. E. Chargaff and J.N. Davidson), Academic Press, New York* 1955, 1: 493-553.

(21). Bechinger B. The structure, dynamics, and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy. *Biochim. Biophys. Acta* 1999, 1462: 157-183.

(22). Bedzyk L., Wang T. and Ye R. W. The Periplasmic Nitrate Reductase in *Pseudomonas* sp. Strain G-179 Catalyzes the First Step of Denitrification *J. Bacteriol* 1999, 181: 2802-2806.

(23). Bell L. C., Richardson D. J. and Ferguson S. J. Periplasmic and membrane-bound respiratory nitrate reductases in *Thiosphaera pantotropha*: The periplasmic enzyme catalyzes the first step in aerobic denitrification. *FEBS Lett* 1990, 265: 85-87.

(24). Ben Efraim I. and Shai Y. The structure and organization of synthetic putative membranous segments of ROMK1 channel in phospholipid membranes. *Biophys. J.* 1997, 72: 85-96.

(25). Ben Efraim I., Bach D. and Shai Y. Spectroscopic and functional characterization of the putative transmembrane segment of the minK potassium channel. *Biochemistry* 1993, 32: 2371-2377.

(26). Berks B. C., Ferguson S. J., Moir J. W., and Richardson D. J. Enzymes and associated electron transport systems that catalyse the respiratory reduction of nitrogen oxides and oxyanions. *Biochim. Biophys. Acta* 1995, 1232: 97-173.

(27). Berks B. C., Richardson D. J., Reilly A., Willis A. C. and Ferguson S. J. The napEDABC gene cluster encoding the periplasmic nitrate reductase system of *Thiosphaera pantotropha*. *Biochem. J* 1995, 309: 983-992.

(28). Bertero M. G., Rothery R. A., Boroumand N., Palak M., Huo C., Lim D., Blasco F., Weiner J. H. and Strynadka N. C. J. Insights into the respiratory electron transport pathway from the structure of nitrate reductase A. *Nat. Struct. Biol* 2003, 10: 681-687.

(29). Billings P. C., Morrow A. R., Ryan C. A. and Kennedy A. R. Inhibition of radiation-induced transformation of C3H/10T1/2 cells by carboxypeptidase inhibitor I and inhibitor II from potatoes. *Carcinogenesis* 1987, 10: 687-691.

(30). Birk Y. Protein proteinase inhibitors of plant origin and their significance in nutrition. *Huisman J, van der Poel TFB, Liener IE (eds) Proceedings, workshop on antinutritional factors in legume seeds* 1989. TNO, Agricultural University Wageningen, Pudoc Wageningen, Netherlands: 83-94.

(31). Birk Y. Proteinase inhibitors. *Neuberger A, Brocklehurst K (eds) Hydrolytic enzymes* 1987. Elsevier, Amsterdan: 257-305.

(32). Birk Y. Purification and some properties of a highly active inhibitor of trypsin and α-chymotrypsin from soybeans. *Biochim. Biophys. Acta* 1961. 54: 378-381.

(33). Birk Y. The Bowman-Birk inhibitor: trypsin- and chymotrypsin-inhibitor from soybeans. *Int. J. Pept. Protein Res* 1985, 25: 113-131.

(34). Birk Y., Gertler A. and Khalef S. A pure trypsin inhibitor from soybeans. *Biochem. J* 1963, 87: 281-284.

(35).. Birnboim H. C. and Dolly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 1979, 7: 1513-1523.

(36). Bode W. and Huber R. Structural basis of the endoproteinase-protein inhibitor interaction. *Biochim. Biophys. Acta* 2000, 1477: 241-252.

(37). Bonomo R. A. Multiple antibiotic-resistant bacteria in long-term-care facilities: an emerging problem in the practice of infectious diseases. *Clin. Infect. Dis.* 2000, 31: 1414-1422.

(38). Boudreaux D. P. and Matrozza M. A. Composition for extending the shelf life of processed meat. *U.S. Patent* 1993, 5,219,603.

(39). Bowles D. J. Defense-related proteins in higher plants. *Annu. Rev. Biochem* 1990, 59: 873-907.

(40). Boyington J. C., Gladyshev V. N., Khangulov S. V., Stadtman T. C. and Sun P. D. Crystal Structure of Formate Dehydrogenase H: Catalysis Involving Mo, Molybdopterin, Selenocysteine, and an Fe_4S_4 Cluster. *Science* 1997, 275: 1305-1308.

(41). Bradford M. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding. *Anal Biochem* 1976, 72: 248-254.

(42). Brawerman G., Mendecki J. and Lee S.Y. A procedure for the isolation of mammalian messenger ribonucleic acid. *Biochemistry* 1972, 11: 637-641.

(43). Broadway R. M. and Duffey S. S. Plant proteinase inhibitors: mechanism of action and effect on the growth and digestive physiology of larval *Heleothis zea* and *Spodoptera exiqua. J. Insect Physiol* 1986, 32: 827-833.

(44). Broekaert W. F., Cammue B. P. A., Osborn R. W. and Rees S. B. This invention relates to antimicrobial proteins, processes for their manufacture and use, and DNA sequences coding for them. *International Patent Application* 1994, WO94/11511.

(45). Broekaert W. F., Mariën W., Terras F. R. G., De Bolle M. F. C., Proost P., Van Damme J., Dillen L., Claeys M., Rees S. B. Vanderleyden J. and Cammue B. P. A. Antimicrobial peptides from *Amamrtthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. *Biochemistry* 1992, 31: 4308–4314.

(46). Broekaert W., Cammue B., De Bolle M., Thevissen K., De Samblanx G. and Osborn R. Antimicrobial peptides from plants. *Critical Reviews in Plant Sciences* 1997, 16: 297-323.

(47). Brondijk T. H. C., Fiegen D., Richardson D. J., and Cole J. A. Roles of NapF, NapG and NapH, subunits of the *Escherichia coli* periplasmic nitrate reductase, in ubiquinol oxidation. *Mol. Microbiol* 2002, 44: 245-255.

(48). Brondino C. D., Calvo R., Atria A. M., Spodine E., Nascimento O. R. and Pena O. Magnetic Interactions in the Copper Complex (L-Aspartato)(1,10-phenanthroline)copper(II) Hydrate. An

Exchange-Coupled Extended System with Two Dissimilar Copper Ions *Inorg. Chem* 1997, 36: 3183–3189.

(49). Bulet P., Hetru C., Dimarcq J. and Hoffmann D. Antimicrobial peptides in insects; structure and function. *Developmental Comparative Immunology* 1999, 23: 329-344.

(50). Butler C. S., Charnock J. M., Bennett B., Sears H. J., Reilly A. J., Ferguson S. J., Garner, C. D., Lowe D. J., Thomson A. J., Berks B. C., and Richardson D. J. Models for Molybdenum Coordination during the Catalytic Cycle of Periplasmic Nitrate Reductase from *Paracoccus denitrificans* Derived from EPR and EXAFS Spectroscopy. *Biochemistry* 1999, 38: 9000-9012.

(51). Caldwell J. B., Strike P. M. and Kortt A. A. Amino acid sequence of the acidic Kunitz-type trypsin inhibitor from winged-bean seed (*Psophocarpus tetragonolobus* L. DC.). *J Prot. Chem* 1990, 9: 493-500.

(52). Campbell W. H. Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annu. Rev. Plant Physiol. Plant Mol. Biol* 1999, 50: 277-303.

(53). Campbell W. H. Structure and Function of Eukaryotic NAD(P)H:Nitrate Reductase. *Cell. Mol. Life Sci* 2001, 58: 194-204.

(54). Carter J. P., Richardson D. J. and Spiro S. Isolation and characterisation of a strain of *Pseudomonas putida* that can express a periplasmic nitrate reductase. *Arch. Microbiol.* 1995, 163, 159-166.

(55). Carter T. H., Everson B. A. and Ratnoff O. D. Cabbage seed protease inhibitor- a slow tight-binding inhibitor of trypsin with activity toward thrombin, activated Stuart factor (factor Xa), activated Hageman factor (factor XIIa) and plasmin. *Blood* 1990, 75: 108-111.

(56). Casteels P., Ampe C., Jacobs F. and Tempst P. Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *J. Biol. Chem* 1993, 268: 7044-7054.

(57). Castillo F., Dobao M. M., Reyes F., Blasco R., Roldan M. D., Gavira M., Caballero F. J., Moreno-Vivian C. and Martinez-Luque M. Molecular and regulatory properties of the nitrate reducing systems of Rhodobacter. *Curr. Microbiol* 1996, 33: 341-346.

(58). Cecilliani F., Bortolotti F., Menegatti E., Ronchi S, Ascenzi P. and Palmieri S. Purification, inhibitory properties, amino acid sequence and identification of the reactive site of a new serine proteinase inhibitor from oilrape (*Brassica napus*) seed. *FEBS Lett* 1994, 342: 221-224.

(59). Chaudhary N. S., Shee C., Islam A., Ahmad F., Yernool D., Kumar P. and Sharma A. K. Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds. *Phytochemistry* 2008, 69: 2120-2126.

(60). Chen Y., Xu X., Hong S., Chen J., Liu N., Underhill C. B., Creswell K. and Zhang L. RGD-tachyplesin inhibits tumor growth. *Cancer Res* 2001, 61: 2434-2438.

(61). Christeller J. T., Farley P. C., Ramsay R. J., Sullivan P. A. and Laing W. A. Purification, characterization and cloning of a aspartic proteinase inhibitor from squash phloem exudates. *Eur. J. Biochem* 1998, 254: 160-167.

(62). Clark J. M. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucl. Acids Res* 1988. 16: 9677-9686.

(63). Cleveland J., Montville T. J., Nes I. F. and Chikindas M. L. Bacteriocins: safe, natural antimicrobials for food preservation. *J. Food Microbiol* 2001, 71: 1-20.

(64). Cohen C. J., Dusek A., Green J., Johns E. L., Nelson E. and Recny M. A. Longterm treatment with subcutaneous T-20, a fusion inhibitor, in HIV-infected patients: patient satisfaction and impact on activities of daily living. *AIDS Patient Care Stds* 2002, 16: 327-335.

(65). Cohen T., Gertler A. and Birk Y. Pancreatic proteolytic enzymes from carp (*Cyprinus carpio*). I. Purification and physical properties of trypsin, chymotrypsin, elastase and carboxypeptidase B. *Comp. Biochem Physiol* 1981a, 69B: 639-646.

(66). Cohen T., Gertler A. and Birk Y. Pancreatic proteolytic enzymes from carp (*Cyprinus carpio*). II. Kinetic Properties and Inhibition studies of trypsin chymotrypsin and elastase. *Comp. Biochem Physiol* 1981b, 647-653.

(67). Czjzek M., Dos Santos J. P., Pommier J., Giordano G., Mejean V. and Haser R. Crystal structure of oxidized trimethylamine N-oxide reductase from *Shewanella massilia* at 2.5 Å resolution. *J. Mol. Biol* 1998, 284: 435-447.

(68). Dane F. and Lang P. Sequence variation at cpDNA regions of watermelon and related wild species: implications for the evolution of Citrullus haplotypes. *Am. J. Bot* 2004, 91: 1922-1929.

(69). Darwin A. J., Ziegelhoffer E. C., Kiley P. J., and Stewart V. *J. Bacteriol* 1998, 180: 4192-4198.

(70). de Grado M., Castán P. and Berenguer J. A High-Transformation-Efficiency Cloning Vector for *Thermus thermophilus*. *Plasmid* 1999, 42: 241-245.

(71). Delves-Broughton J. Nisin and its uses as a food preservative. *Food Technol* 1990, 44: 100-117.

(72). Dias J. M., Than M. E., Humm A., Huber R., Bourenkov G. P., Bartunik H. D., Bursakov S., Calvete J., Caldeira J., Carneiro C., Moura J. J. G., Moura, I., and Romao M. J. Crystal Structure of the first dissimilatory nitrate reductase at 1.9 Å solved by MAD method. *Structure Fold Des* 1999, 7: 65-79.

(73). Di-Ciero L., Oliva M. L., Torquato R, Kohler P., Weder J.K.P., Camillo-Novello J., Sampaio C.A., Oliveira B. and Marangoni S. The complete amino acid sequence of a trypsin inhibitor from *Bauhinia variegates* var. *candida* seeds. *J. Protein Chem* 1998, 17: 827-834.

(74). Dimarcq J. L., Bulet P., Hebru C. H. and Hoffmann J. Cysteine-rich antimicrobial peptides in invertebrates. *Biopolymers* 1998, 47: 465-477.

(75). Dobao M. M., Martinez-Luque M., Moreno-Vivian C. and Castillo F. Effect of carbon and nitrogen metabolism on nitrate reductase activity of *Rhodobacter capsulatus* E1F1. *Can. J. Microbio* 1994, 40: 645-650.

(76). Douliez J. P., Michon T., Elmorjani K. and Marion D. Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J. Cereal Sci* 2000, 32: 1–20.

(77). Dunn M. A., Hughes M. A., Zhang L., Pearce R. S., Quigley A. S. and Jack P. L. Nucleotide sequence and molecular analysis of the low temperature induced cereal gene BLT4. *Mol. Gen. Genet* 1991, 229: 389–394.

(78). Duvick J. P., Rood T., Rao A. G. and Marshak D. R. Purification and characterization of a novel antimicrobial peptide from maize (*Zea mays* L.) kernels. *J. Biol. Chem* 1992, 267: 18814-18820.

(79). Ehrenstein G. and Lecar H. Electrically gated ionic channels in lipid bilayers. *Q. Rev. Biophys* 1977, 10:1–34.

(80). Ellington M. J. K., Sawers G., Sears H. J., Spiro S., Richardson D. J. and Ferguson S. J. Characterization of the expression and activity of the periplasmic nitrate reductase of *Paracoccus pantotrophus* in chemostat cultures. *Microbiology* 2003, 149: 1533-1540.

(81). Epand R. M. and Vogel H. J. Diversity of antimicrobial peptides and their mechanisms of action. *Biochim. Biophys. Acta* 1999, 1462: 11-28.

(82). Fermi C. and Pernossi L. Untersuchungen űber die Enzymes. *Vergleichende Studie. Z Hyg Infektionskr* 1894, 18: 83-89.

(83). Fernandez de Caleya R., Gonzalez-Pascual B., Garcia-Olmedo F. and Carbonero P. Susceptibility of phytopathogenic bacteria to wheat puro-thionins in vitro. *Appl Microbiol* 1972, 23: 998–1000.

(84). Fernandez-Lopez S., Kim H. S., Choi E. C., Delgado M., Granja J. R., Khasanov A., Kraehenbuehl K., Long G., Weinberger D. A., Wilcoxen K. M., and Ghadiri M. R. Antibacterial agents based on the cyclic D,L-alpha-peptide architecture. *Nature* 2001, 412: 452-455.

(85). Ferrari E., Jarnagin A. S. and Schmidt B. F. Commercial production of extracellular enzymes. In: Bacillus subtilis and other Gram positive bacteria. *American Society for Microbiology* 1993, Washington D.C.: 917-938.

(86). Gabay J. E. Ubiquitous natural antibiotics. *Science* 1994, 264: 373-374.

(87). Galimand M., Gamper M., Zimmermann A. and Haas D. Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. *J. Bacteriol* 1991, 173: 1598-1606.

(88). Ganz T. and Lehrer R. I. Defensins. *Pharmacol. Ther* 1995, 66: 191-205.

(89). Garcia-Olmeda F., Sakedo G., Sanchez-Monge R., Gomez L., Royo J., Carbonero P. Plant proteinaceous inhibitors of proteinases. *Oxf. Surv. Plant Cell Mol. Biol* 1987, 4: 275-334.

(90). Garcia-Olmedo F., Molina A., Alamillo J.M. and Rodriguez Palenzuela P. Plant defense peptides. Biopolym. Pept. Sci 1998, 47: 479-491.

(91). García-Olmedo F., Molina A., Segura A. and Moreno M. The defensive role of nonspecific lipid-transfer proteins in plants. *Trends Microbiol* 1995, 3: 72-74.

(92). Gavira M., Roldan M. D., Castillo F. and Moreno-Vivian C. Regulation of *nap* Gene Expression and Periplasmic Nitrate Reductase Activity in the Phototrophic Bacterium *Rhodobacter sphaeroides* DSM158. *J. Bacteriol.* 2002, 184: 1693-1702.

(93). Gazit E., Bach D., Kerr I. D., Sansom M. S., Chejanovsky N., and Shai Y. The α-5 segment of *Bacillus thuringiensis* δ-endotoxin: in vitro activity, ion channel formation and molecular modeling. *Biochem. J.* 1994, 304: 895-902.

(94). Gazit E., Boman A., Boman H. G. and Shai Y Interaction of the mammalian antibacterial peptide cecropin P1 with phospholipid vesicles. *Biochemistry* 1995, 34: 11479-11488.

(95). Ghosh J. K., Shaool D., Guillaud P., Ciceron L., Mazier D., Kustanovich I., Shai Y. and Mor A. Selective cytotoxicity of dermaseptin S3 toward intraerythrocytic *Plasmodium falciparum* and the underlying molecular basis. *J. Biol. Chem.* 1997, 272: 31609-31616.

(96). Giles F. J., Redman R., Yazji S. and Bellm L. Iseganan HCl: a novel antimicrobial agent. *Expert Opin. Investig. Drugs* 2002, 11: 1161-1170.

(97). Giudici A. M., Regente M. C. and De la Canal L. A potent antifungal protein from *Helianthus annuus* flowers is a trypsin inhibitor. *Plant Physiol. Biochem* 2003, 38: 881-888.

(98). Glasel J. Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *BioTechniques* 1995, 18: 62-63.

(99). Godbole A., Krishna T.G. and Bhatia C.R. Purification and characterization of protease inhibitors from pigeon pea (*Cajanus cajan* (L) Millsp.) seeds. *J. Sci. Food Agric* 1994, 64: 87-93.

(100). Goldstein Z., Trop M. and Birk Y. Multifunctional proteinase inhibitor from *Bauhinia* seeds. *Nature* 1973, 246: 29-31.

(101). Gonza´lez P. J., Correia C., Moura I., Brondino C. D. and Moura J. J. G. Bacterial nitrate reductases: Molecular and biological aspects of nitrate reduction. *Journal of Inorganic Biochemistry* 2006, 100: 1015–1023.

(102). Gray P. W., Flaggs G., Leong S. R. and Gumina R. Cloning of the cDNA of a human neutrophil bactericidal protein. *J. Biol. Chem* 1989, 264: 9505-9509.

(103). Gregory L. G., Bond P. L., Richardson D. J. and Spiro S. Characterisation of a nitrate-respiring bacterial community using the nitrate reductase gene (NarG) as a functional marker. *Microbiology* 2003, 149: 229-237.

(104). Guerrero M. G., Vega J.M. and Losada M. The assimilatory nitrate-reducing system and its regulation. *Annu. Rev. Plant Physiol* 1981, 32: 169–204.

(105). Hall N. and Tomsett A. B. Structure–function analysis of NADPH:nitrate reductase from *Aspergillus nidulans*: analysis of altered pyridine nucleotide specificity *in vivo*. *Microbiology* 2000, 146: 1399-1406.

(106). Han G. W., Lee J. Y., Song H. K., Chang C., Min K., Moon J., Shin D. H., Kopka M. L., Sawaya M. R., Yuan H. S., Kim T. D., Choe J., Lim D., Moon H. J. and Suh S. W. Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution X-ray crystallography.. *J. Mol. Biol* 2001, 308: 263–278.

(107). Hanahan D. Studies on transformation with *Escherichia coli* with plasmids. *J. Mol. Biol* 1983, 166: 557-580.

(108). Hancock R. E. W. and Diamond G. The role of cationic antimicrobial peptide in innate host defences. *Trends Microbiol* 2000, 8: 402-410.

(109). Hancock R. E. W. and Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. *FEMS Microbiol. Lett* 2002, 206: 143-149.

(110). Hancock R. E. W. and Scott M. G. The role of antimicrobial peptides in animal defences. *Proc. Natl. Acad. Sci. USA* 2000, 97: 8856-8861.

(111). Hancock R. E. W. Peptide antibiotics. *Lancet* 1997, 349: 418-422.

(112). Hartig E., Schiek U., Vollack K. U. and Zumft, W. G. Nitrate and nitrite control of respiratory nitrate reduction in denitrifying *Pseudomonas stutzeri* by a two-component regulatory system homologous to NarXL of *Escherichia coli*. *J. Bacteriol* 1999, 181: 3658-3665.

(113). Hass G. M. and Ryan C. A. Carboxypeptidase inhibitor from potatoes. *Methods Enzymol* 1981. 80: 778-779.

(114). Hass G. M., Agar S. P. and Makus D. J. Specificity of the carboxypeptidase inhibition from potatoes. *Proc. Fed. Am. Soc. Exp. Biol* 1980, 39: 1689-1710.

(115). Hayashi K., Nakazawa M., Ishizaki Y., Hiraoka N. and Obayashi A. Regulation of inter- and intramolecular ligation with T4 DNA ligase in the presence of polyethylene glycol. *Nucleic Acids Res* 1986, 14: 7617-7631.

(116). Heinemann B., Andersen K. V., Nielsen P. R., Bech L. M. and Poulsen F. M. Structure in solution of a four-helix lipid binding protein. *Protein Sci* 1996, 5: 13–23.

(117). Hejgaard J., Dam J., Petersen L. C. and Bjorn S.E. Primary structure and specificity of the major serine proteinase inhibitor of amaranth (*Amaranthus caudatus* L.) seeds. *Biochim. Biophys. Acta* 1994, 1204: 68-74.

(118). Heller W. T., Waring A. J., Lehrer R. I. and Huang H. W. Multiple states of beta-sheet peptide protegrin in lipid bilayers. *Biochemistry* 1998, 37: 17331-17338.

(119). Henikoff S. and Henikoff J. G. Automated assembly of protein blocks for database searching. *Nucleic Acids Res.* 1991, 19: 6565-6572.

(120). Herrero A., Muro-Pastor A. M. and Flores E. Nitrogen Control in Cyanobacteria. *J. Bacteriol* 2001, 183: 411-425.

(121). Hille R., Retey J., Bartlewski-Hof V., Reichenbechen W., and Schink B. Mechanistic aspects of molybdenum containing enzymes. *FEMS Microbiol. Rev* 1999, 22: 489-501.

(122). Hoffmann T., Troup B., Szabo A., Hungerer C. and Jahn D. The anaerobic life of Bacillus subtilis: cloning of the genes encoding the respiratory nitrate reductase system. *FEMS Microbiol. Lett* 1995, 131: 219-225.

(123). Horwitz A. H., Leugh S. D., Abrahamson S., Gazzano-Santoro H., Liu P. S., William R. E., Carroll S. F. and Theofan G. Expression and characterization of cysteine-modified variants of an amino-terminal fragment of bactericidal/permeability-increasing protein. *Protein Expr. Putif* 1996: 8: 28-40.

(124). Hu G. DNA polymerase-catalyzed addition of nontemplated extra nucleotides to the 3' end of a DNA fragment. *DNA Cell Biol* 1993, 12: 763-770.

(125). Hubert F., Nel T. and Roch P. A member of the arthropod defensin family from edible mediterranean mussels (*Mytilus galloprovincialis*). *Eur. J. of Biochem* 1996, 240: 302-306.

(126). Inokuchi R., Kuma K., Miyata T. and Okada M. Nitrogen-assimilating enzymes in land plants and algae: phylogenic and physiological perspectives. *Physiol. Plant* 2002, 116: 1-11.

(127). Iseli B., Boller T. and Neuhaus J. M. The N-terminal cysteine-rich domain of tobacco class I chitinase is essential for chitin binding but not for catalytic or antifungal activity. *Plant Physiol* 1993, 103: 221–226.

(128). Ish-Horowicz D. and Burke J.F. Rapid and efficient cosmid cloning, *Nucleic Acids Res* 1981, 9: 2989-2999.

(129). Jormakka M., Byrne B. and Iwata S. Protomotive force generation by a redox loop mechanism. *FEBS Lett* 2003, 545: 25-30.

(130). Jormakka M., Richardson D., Byrne B. Iwata S. Architecture of NarGH reveals a structural classification of Mo-bisMGD enzymes. *Structure (Camb)* 2004, 12:95-104.

(131). Joseph-Horne T., Hollomon D. W. and Wood P. M. Fungal respiration: a fusion of standard and alternative components. *Biochim. Biophys. Acta* 2001, 1504: 179-195.

(132). Joubert F. J., Merrifield E. H. and Dowdle E. B. D. The reactive sites of proteinase inhibitors from *Erythrina* seeds. *J. Biol. Chem* 1987, 19: 601-606.

(133). Kader J. C. Proteins and the intracellular exchange of lipids. I. Stimulation of phospholipid exchange between mitochondria and microsomal fractions by proteins isolated from potato tuber. *Biochim. Biophys. Acta* 1975, 380: 31–44.

(134). Kader J. C. Lipid-transfer proteins: a puzzling family of plant proteins. *Trends Plant Sci* 1997, 2: 66-70.

(135). Kennedy A. R. Chemopreventive agents: protease inhibitors. *Pharmacol. Ther* 1998b, 78: 167-209.

(136). Kennedy A. R. The Bowman-Birk inhibitor from soybean as an anticancerous agent. *Am J. Clin. Nutr* 1998a, 68: 1406-1412.

(137). Khoroshilova N., Popescu C., Munck E., Beinert H. and Kiley P. J. Iron-sulfur cluster disassembly in the FNR protein of Escherichia coli by O2: [4Fe-4S] to [2Fe-2S] conversion with loss of biological activity. *Proc. Natl. Acad. Sci. USA* 1997, 94: 6087-6092.

(138). Kiley P. J. and Beinert H. Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiol. Rev* 1998, 22: 341-352.

(139). Koiwa H., Bressan R.A. and Hasegawa P.M. Regulation of protease inhibitors and plant defense. *Trends Plant Sci* 1997, 2: 379-384.

(140). Kourie J. I. and Shorthouse A. A. Properties of cytotoxic peptide-formed ion channels. *Am. J. Physiol-Cell Physiol* 2000, 278: C1063-C1087.

(141). Krauchenco S, Pando S. C., Marangoni S. and Polikarpov I. Crystal structure of the Kunitz (STI)-type inhibitor from Delonix regia seeds. *Biochem Biophys Res Commun* 2003, 312: 1303-1308.

(142). Kuhlemeier C. J., Logtenberg T., Stoorvogel W., Van Heugten H. A. A., Borrias W. E. and Van Arkel G. A. Cloning of Nitrate Reductase Genes from the Cyanobacterium *Anacystis nidulans*. *J. Bacteriol* 1984, 159: 36-41.

(143). Kunitz M. and Northrop J. H. Isolation from beef pancreas of crystalline trypsinogen, trypsin, a trypsin inhibitor and an inhibitor-trypsin compound. *J. Gen. Physiol* 1936, 19: 991-1007.

(144). Kunitz M. Crystalline soybean trypsin inhibitor I. *J. Gen. Physiol* 1946, 29: 149-154.

(145). Kunitz M. Crystalline soybean trypsin inhibitor II. General properties. *J. Gen. Physiol* 1947a, 30: 291-310.

(146). Kunitz M. Crystallization of a trypsin inhibitor from soybean. *Science* 1945, 101: 668-669.

(147). Kunitz M. Isolation of a crystalline protein compound of trypsin and of soybean trypsin-inhibitor. *J. Gen. Physiol* 1947b, 30: 311-320.

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

(149). Lamberty M., Caille A., Landon C., Tassin-Moindrot S., Hetru C., Bulet P. and Vovelle F. Solution structures of the antifungal heliomicin and a selected variant with both antibacterial and antifungal activities. *Biochemistry* 2001, 40: 11995-112003.

(150). Laskowski M. Jr and Kato I. Protein Inhibitors of proteinases. *Annu. Rev. Biochem* 1980, 49: 593-626.

(151). Laskowski M. Jr. Protein inhibitors of serine proteinases-mechanism and classification. *Freidman M (ed) Nutritional and toxological significance of enzyme inhibitors in foods* 1986,

Plenum, New York: 1-17.

(152). Latal A., Degovics G., Epand R. F., Epand R. M. and Lohner K. Structural aspects of the interaction of peptidyl-glycylleucine-carboxyamide, a highly potent antimicrobial peptide from frog skin, with lipids. *Eur. J. Biochem* 1997, 248: 938-946.

(153). Lee H.-L., Broekaert W. F. and Raikhel N. V. Co- and post-translational processing of the hevein preproprotein of latex of the rubber tree (*Hevea brasiliensis*). *J. Biol. Chem* 1991, 266: 15944–15948.

(154). Lehrer R. I. and Ganz T. Defensin of vertebrate animals. *Current Opinion in Immunology* 2002, 14: 96-102.

(155). Leisner J. J., Greer G. G. and Stile M. E. Control of beef spoilage by a sul®de-producing Lactobacilus sake strain with bacteriocinogenic *Leuconostoc gelidum* UAL 187 during anaerobic storage at 2 °C. *Appl. Environ. Microbiol* 1996, 62: 2610-2614.

(156). Leistner L. and Gorris L. G. M. Food preservation by hurdle technology. *Trends Food Sci. Technol* 1995, 6: 41-46.

(157). Leo D. F., Volpicella M., Licciulli F., Liuni S., Gallerani R. and Ceci R.L. Plant-PIs: a database for plant protease inhibitors and their genes. *Nucleic Acids Research* 2002, 30: 347–348.

(158). Lerche M. H. and Poulsen F. M. Solution structure of barley lipid transfer protein complexed with palmitate. Two different binding modes of palmitate in the homologous maize and barley nonspecific lipid transfer proteins. *Protein Sci* 1998, 7: 2490–2498.

(159). Leung D., Abbenante G.. and Fairlie D .P. Protease inhibitors: current status and future prospects. *J Med Chem* 2000, 43: 305-341.

(160). Levinsky H., Birk Y. and Applebaum S. W. Isolation and characterization of a new trypsin-like enzyme from *Tenebrio molitor* L. larvae. *Int. J. Pept. Protein Res* 1977, 10: 252-264.

(161). Liener I .E. and Kakade M. L. Protease inhibitors. *Liener IE (ed) Toxic constituents of plant foodstuffs* 1980, 2nd edn. Academic Press, New York: 7-71.

(162). Liener I. E. Protease inhibitors and lectins. *Int Rev Biochem* 1979, 27: 97-122.

(163). Liepinsh E, Nagy A, Trexler M, Patthy L and Otting G. Second Kunitz-type protease inhibitor domain of the human WFIKKN1 protein. *J Biomol NMR* 2006 35: 73-88.

(164). Lightfoot D. A., Baron A. J., Cock J. M. and Wootton J. C. A nitrate reductase gene of the cyanobacterium *Synechococcus* PCC6301 inferred by heterologous hybridization, cloning and targeted mutagenesis. *Genetica* 1992, 85: 107-112.

(165). Lin J. T., and Stewart V. Nitrate and Nitrite-mediated Transcription Antitermination Control of *nasF* (Nitrate Assimilation) Operon Expression in *Klebsiella pneumoniae* M5al. *J. Mol. Biol* 1996, 256: 423-435.

(166). Lin J. T. and Stewart V. Nitrate Assimilation by Bacteria. *Adv. Microb. Physiol* 1998, 38: 1-30.

(167). Liu Y., Luo J., Xu C., Ren F., Peng C., Wu G. and Zhao J. Purification, characterization, and molecular cloning of the gene of a seed-specific antimicrobial protein from pokeweed. *Plant Physiology* 2000, 122: 1015-1024.

(168). Llamas A., Igeno M., Galvan A. and Fernandez E. Nitrate signaling on the nitrate reductase gene promoter depends directly on the activity of the nitrate transport systems in *Chlamydomonas*. *Plant J.* 2002, 30: 261-271.

(169). Lohner K. and Prenner E. J. Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimic systems. *Biochim. Biophys. Acta* 1999, 1462: 141-156.

(170). Luckett S., Santiago- Garcia R., Barker J. J., Konarev A .L .V., Shewry P. R., Clarke A. R. and Brady R. L. High-resolution structure of a potent cyclic proteinase inhibitor from sunflower seeds. *J. Mol. Biol* 1999, 290: 525-533.

(171). Ludtke S. J., He K., Heller W. T., Harroun T. A., Yang L., and Huang H. W. Membrane pores induced by magainin. *Biochemistry* 1996, 35: 13723-13728.

(172). Lyons A., Richardson M., Tatham A. S. and Shewry P. R. Characterization of homologous inhibitors of trypsin and alpha-amylase from seeds of rye (*Secale cereale* L.). *Biochim. Biophys. Acta* 1987, 915: 305-313.

(173). Macedo M. L. R., de Matos D. G. G., Machado O. L. T., Marangoni S. and Novello J. C. Trypsin inhibitor from *Dimorphandra mollis* seeds: purification and properties. *Phytochemistry (Oxford)* 2000, 54: 553-558.

(174). Machuka J. Characterization of the seed proteins of velvet bean (*Mucuna pruriens*) from Nigeria. *Food Chem* 2000, 68: 421-427.

(175). Maeda S. and Omata T. A Novel Gene (narM) Required for Expression of Nitrate Reductase Activity in the Cyanobacterium *Synechococcus elongatus* Strain PCC7942.4) *J. Bacteriol* 2004, 186: 2107-2114.

(176). Magalon A., Rothery R. A., Giordano G., Blasco F. and Weiner J. H. Characterization by electron paramagnetic resonance of the role of the Escherichia coli nitrate reductase (NarGHI)

iron-sulfur clusters in electron transfer to nitrate and identification of a semiquinone radical intermediate. *J. Bacteriol* 1997, 179: 5037-5045.
(177). Malardier L., Daboussi M. J., Julien J., Roussel F., Scazzocchio C. and Brygoo Y. Cloning of the nitrate reductase gene (niaD) of *Aspergillus nidulans* and its use for transformation of *Fusarium oxysporum*. *Gene* 1989, 78: 147-156.
(178). Manchester K. I. Use of UV methods for the measurement of protein and nucleic acid concentrations. *BioTechniques* 1996, 20: 968-970.
(179). Manchester K. I. Value of $A_{260/280}$ ratios for measurement of purity of nucleic acids. *BioTechniques* 1995, 19: 208-219.
(180). Mares M., Meloun B., Pavlik M., Kostka V. and Baudys M. Primary structure of cathepsin D inhibitor from potatoes and it's structure relationship to soybean trypsin inhibitor family. *FEBS Lett.* 1989, 251: 94-98.
(181). Marjorie Murphy Cowan. Plant products as antimicrobial agents. *Clinical Microbiology Reviews* 1999, 12: 564-582.
(182). Martinez G. V. and Millhauser G. L. FTIR spectroscopy of alanine-based peptides: Assignment of the amide I' modes for random coil and helix. *J. Struct. Biology* 1995, 114: 23-27.
(183). Matsuzaki K. Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochim. Biophys. Acta* 1999, 1462: 1-10.
(184). Matsuzaki K., Harada M., Funakoshi S., Fujii N. and Miyajima, M. Physicochemical determinants for the interactions of magainins 1 and 2 with acidic lipid bilayers. *Biochim. Biophys. Acta* 1991, 1063: 162-170.
(185). Matsuzaki K., Murase O. and Miyajima M. Kinetics of pore formation by an antimicrobial peptide, magainin 2, in phospholipid bilayers. *Biochemistry 1995b*, 34: 12553-12559.
(186). Matsuzaki K., Yoneyama S., Fujii N., Miyajima K., Yamada K., Kirino Y. and Anzai K. Membrane permeabilization mechanisms of a cyclic antimicrobial peptide, tachyplesin I, and its linear analog. *Biochemistry* 1997, 36: 9799-9806.
(187). McAlpine A. S., McEwan A. G., and Bailey S. The high resolution crystal structure of DMSO reductase in complex with DMSO. *J. Mol. Biol* 1998, 275: 613-623.
(188). Menegatti E., Tedeschi G., Ronchi S., Bortolotti F., Ascenzi P., Thomas R. M., Bolognesi M. and Palmieri S. Purification, inhibitory properties and amino acid sequence of a new serine proteinase inhibitor from white mustard (*Sinapis alba* L.) seeds. *FEBS Lett* 1992, 301: 10-14.

(189). Mikami B. and Ida S. Purification and properties of ferredoxin—nitrate reductase from the cyanobacterium *Plectonema boryanum. Biochim. Biophys. Acta-Protein Structure and Molecular Enzymology* 1984, 791: 294-304.

(190). Mitta G., Vandenbulcke F., Hubert F. and Roch P. Mussel defensins are synthesised and processed in granulocytes then released into the plasma after bacterial challenge. *Journal of Cell Sciences* 1999, 112: 4233-4242.

(191). Molina A., Segura A. and García-Olmedo F. Developmental and pathogen-induced expression of three barley genes encoding lipid transfer proteins. *FEBS Lett* 1993, 316: 119–122.

(192). Monaco V., Formaggio F., Crisma M., Toniolo C., Hanson P. and Millhauser G. L. Orientation and immersion depth of a helical lipopeptaibol in membranes using TOAC as an ESR probe. *Biopolymers* 1999, 50: 239-253.

(193). Montville T. J. and Winkowski K. Biologically-based preservation systems and probiotic bacteria. *In: Doyle MP, Beuchat LR, Montville TJ (eds) Food microbiology: fundamentals and frontiers*. 1997, American Society for Microbiology Press, Washington D.C.: 557-577.

(194). Mor A. and Nicolas, P. The NH2-terminal α-helical domain 1-18 of dermaseptin is responsible for antimicrobial activity. *J. Biol. Chem* 1994, 269: 1934-1939.

(195). Moreno-Vivián C., Cabello P., Martínez-Luque M., Blasco R. and Castillo F. Prokaryotic Nitrate Reduction: Molecular Properties and Functional Distinction among Bacterial Nitrate Reductases. *J. Bacteriol* 1999, 181: 6573-6584.

(196). Mosca D. A., Hurst M. A., So W., Viajar B. S. C., Fujii C. A. and Falla T. J. IB-367, a protegrin peptide with in vitro and in vivo activities against the microflora associated with oral mucositis. *Antimicrob. Agents Chemother* 2000, 44: 1803-1808.

(197). Mosolov V. V. and Valueva T. A. Plant Protein Inhibitors of Proteolytic Enzymes [in Russian], *Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow*, 1993.

(198). Moura J. J., Brondino C. D., Trincao J. and Romao M. J. Mo and W bis-MGD enzymes: nitrate reductases and formate dehydrogenases. *J. Biol. Inorg.* Chem 2004, 9: 791–799.

(199). Murillo M. F., Gugliuzza T., Senko J., Basu P. and Stolz J. F. A heme-C-containing enzyme complex that exhibits nitrate and nitrite reductase activity from the dissimilatory iron-reducing bacterium *Geobacter metallireducens. Arch. Microbiol.* 1999, 172: 313-320.

(200). Nakajima Y., Van der Goes Van Natersyasui A., Taylor D. and Yamakawa M. Two isoforms of a member of the arthropod defensin family from the soft tick, *Ornithodoros moubata* (Acari: Argasidae*). Insect Biochemistry and Molecular Biology* 2001, 31: 747-751.

(201). Neurath H. Evolution of Proteolytic Enzymes. *Science* 1984, 224: 350-357.

(202). Nissen-Meyer J. and Nes I. F. Ribosomally synthesized antimicrobial peptides: their function, structure, biogenesis and mechanism of action. *Arch. Microbiol* 1997, 167: 67-77.

(203). Nohno T., Kasai Y. and Saito T. Cloning and Sequencing of the Escherichia coli chlEN Operon Involved in Molybdopterin Biosynthesis. *J. Bacteriol* 1988, 170: 4097-4102.

(204). Odani S. and Ikenaka T. Scission of soybean Bowman-Birk proteinase inhibitor into two small fragments having either trypsin or chymotrypsin inhibitory activity. *J. Biochem (Tokyo)* 1973b, 74: 857-860.

(205). Odani S. and Ikenaka T. Studies on soybean trypsin inhibitors. VIII. Disulphide bridges in soybean Bowman-Birk proteinase inhibitor. *J. Biochem (Tokyo)* 1973a, 74: 697-715.

(206). Odani S., Koide T. and Ono T. The complete amino acid sequence of barley trypsin inhibitor. *J. Biol. Chem* 1983, 258: 7998-8003.

(207). Odani S., Yokokawa Y., Takeda H., Abe S. and Odani S. The primary structure and characterization of carbohydrate chains of the extracellular glycoprotein proteinase inhibitor from latex of *Carica papaya*. *Eur. J. Biochem* 1996, 241: 77-82.

(208). Ogawa K. I., Akagawa E., Yamane K., Sun Z. W., LaCelle M., Zuber P. and Nakano M. M. The nasB operon and nasA gene are required for nitrate/nitrite assimilation in *Bacillus subtilis*. *J. Bacteriol* 1995, 177: 1409-1413.

(209). Okamoto P. M. and Marzluf G. A. Nitrate reductase of *Neurospora crassa*: the functional role of individual amino acids in the heme domain as examined by site-directed mutagenesis. *Mol. Gen. Genet* 1993, 240: 221-230.

(210). Oren Z. and Shai Y. Cyclization of a cytolytic amphipathic alpha-helical peptide and its diastereomer: effect on structure, interaction with model membranes, and biological function. *Biochemistry* 2000, 39: 6103-6114.

(211). Oren Z. and Shai Y. Mode of action of linear amphipathic α-helical antimicrobial peptides. *Biopolymers (Peptide Sci.)* 1998, 47: 451-463.

(212). Oren Z., Hong J. and Shai Y. A comparative study on the structure and function of a cytolytic alpha-helical peptide and its antimicrobial beta-sheet diastereomer. *Eur. J. Biochem* 1999b, 259: 360-369.

(213). Oren Z., Lerman J. C., Gudmundsson G. H., Agerberth B. and Shai Y. Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. *Biochem. J.* 1999a, 341: 501-513.

(214). Otlewski J. and Krowarsch D. Squash inhibitor family of serine proteinases. *Acta Biochim. Pol* 1996, 43: 431-444.

(215). Park C. J., Park C. B., Hong S.-S., Lee H.-S., Lee S. Y. and Kim C. Characterization and cDNA cloning of two glycine- and histidine-rich antimicrobial peptides from the roots of shepherd's purse, Capsella bursa-pastoris. *Plant Mol. Biol* 2000, 44: 187-197.

(216). Park S. Y., Jauh G. Y., Mollet J. C., Eckard K. J., Nothnagel E. A., Walling L. L. and Lord E. M. A lipid transfer-like protein is necessary for lily pollen tube adhesion to an in vitro stylar matrix. *Plant Cell* 2000, 12: 151–164.

(217). Patel S. U., Osborn R., Rees S. and Thornton J. M. Structural studies of Impatiens balsamina antimicrobial protein (Ib-AMP1). *Biochemistry* 1998, 37: 983-990.

(218). Pearce G. and Ryan C. A. A rapid, large scale method for purification of metallo-carboxypeptidase inhibitor from potato tubers. *Anal. Biochem* 1983, 130: 223-225.

(219). Perez C, Pauli M and Bazerque P. An antibiotic assay by the agar-well diffusion method. *Acta Biol. Med. Exper* 1990, 15: 113-115.

(220). Pheiffer B. H. and Zimmerman S. B. Polymer-stimulated ligation: Enhanced blunt- or cohesive-end ligation of DNA or deoxyribooligonucleotides by T4 DNA ligase in polymer solutions. *Nucleic Acids Res* 1983, 11: 7853-7871.

(221). Philippot L. and Hojberg O. Dissimilatory nitrate reductases in bacteria. *Biochim. Biophys. Acta* 1999, 1446: 1-23.

(222). Philippot L. Denitrifying genes in bacterial and archaeal genomes. *Biochim. Biophys. Acta* 2002, 1577: 355-376.

(223). Philippot L., Clays-Josserand A., Lensi R., Trinsoutrot I., Normand P., and Potier P. Purification of the dissimilative nitrate reductase of *Pseudomonas fluorescens* and cloning and sequencing of its corresponding genes. *Biochim. Biophys. Acta* 1997, 1350: 272-276.

(224). Philippot L., Mirleau P., Mazurier S., Siblot S., Hartmann A., Lemanceau P., and Germon J. C. Characterization and Transcriptional analysis of *pseudomonas fluorescens* denitrifying clusters containing the nar, nir, nor and nos genes. *Biochim. Biophys. Acta* 2001, 1517: 436-440.

(225). Polikarpov I., Golubev A. M., Perles L. A., Pando S. C., Novello J. C. and Marangoni S. Purification, crystallization and preliminary crystallographic study of a Kunitz-type trypsin inhibitor form of *Delonix regia* seeds. *Acta crystallogr* 1999. 55: 1611-1613.

(226). Potter L. C., Millington P. D., Thomas G. H., Rothery R. A., Giordano G., and Cole J. Novel growth characteristics and high rates of nitrate reduction of an *Escherichia coli* strain,

LCB2048, that expresses only a periplasmic nitrate reductase. *FEMS Microbiol. Lett* 2000, 185: 51-57.

(227). Potter L., Angove H., Richardson D. and Cole J. A. Nitrate reduction in the periplasm of Gram-negative bacteria. *Adv. Microb. Physiol 2001*, 45: 51-112.

(228). Pouny Y., Rapaport D., Mor A., Nicolas P. and Shai Y. Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. *Biochemistry* 1992, 31: 12416-12423.

(229). Prenner E. J., Lewis R. N. and McElhaney R. N. The interaction of the antimicrobial peptide gramicidin S with lipid bilayer model and biological membranes. *Biochim. Biophys. Acta* 1999, 1462: 201-221.

(230). Priest F. G. Systematics and ecology of *Bacillus*. In: *Bacillus subtilis* and other Gram-positive bacteria. *American Society for Microbiology* 1993, Washington D.C.: 3-16.

(231). Qasim M. A., Ranjbar M. R., Wynn R., Anderson S. and Laskowski M. Jr. Ionizable P_1 residues in serine proteinase inhibitors undergo large pK shifts on complex formation. *J. Biol. Chem* 1995, 270: 27419-27422.

(232). Quigshun Li, Christopher B., Lawrence H., Maelor D. and Nicholas P. E. A tridecapeptide possess both antimicrobial and protease-inhibitory activities. *Peptides* 2002, 23: 1-6.

(233). Rancour J. M. and Ryan C. A. Isolation of a carboxypeptidase B inhibitor from potatoes. *Arch Biochem. Biophys* 1968, 125: 380-383.

(234). Rapaport D. and Shai Y. Interaction of fluorescently labeled pardaxin and its analogues with lipid bilayers. *J. Biol. Chem* 1991, 266: 23769-23775.

(235). Reddy M. N., Keim P .S., Heinrikson R. L. and Kezdy F. J. primary structural analysis of sulfhydral protease inhibitors from pineapple stem. *J. Biol. Chem* 1975, 250: 1741-1750.

(236). Ress D. C. and Lipscomb W. N. Structure of the protein inhibitor complex of carboxypeptidase A at 2.5-Å resolution. *Proc. Natl. Acad. Sci. USA* 1980, 77: 4633-4637.

(237). Reyes F., Gavira M., Castillo F. and Moreno-Vivian C. Periplasmic nitrate-reducing system of the phototrophic bacterium *Rhodobacter sphaeroides* DSM 158: transcriptional and mutational analysis of the *napKEFDABC* gene cluster .*Biochem. J* 1998, 331: 897-904.

(238). Richardson D. J. and Watmough N. J. Inorganic nitrogen metabolism in bacteria. *Curr. Opin. Chem. Biol* 1999, 3: 207–219.

(239). Richardson D. J. Bacterial respiration: a flexible process for a changing environment. *Microbiology* 2000, 146: 551-571.

(240). Richardson D. J., Berks B. C., Russell D. A., Spiro S. and Taylor C. J. Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell. Mol. Life Sci* 2001, 58: 165-178.

(241). Richardson M. The Proteinase Inhibitors of plants and microorganisms. *Phytochemistry* 1977, 16:159-169.

(242). Robertson C. N., Roberson K. M., Pinero A., Jaynes J. M. and Paulson D. F. Peptidyl membrane-interactive molecules are cytotoxic to prostatic cancer cells in vitro. *World J. Urol* 1998, 16: 405-409.

(243). Rondeau S. S., Hsu P. Y. and DeMoss J. A. Construction in vitro of a cloned nar operon from Escherichia coli. *J Bacteriol* 1984, 159: 159-166.

(244). Rubio L. M., Flores E. and Herrero A. Molybdopterin guanine dinucleotide molybdenum cofactor in *Synechococcus* sp. nitrate reductase: identification of mobA and isolation of a putative moeB gene. *FEBS Lett* 1999, 462: 358-362.

(245). Rubio L. M., Flores E., and Herrero A. Purification, cofactor analysis, and site-directed Mutagenesis of Synechococcus ferredoxin-nitrate reductase. *Photosynth. Res*. 2002, 72: 13-26.

(246). Ruoppolo M., Amoresano A., Pucci P., Pascarella S., Polticella F., Trovato M., Menegatti E. and Ascenzi P. Characterization of five low-molecular mass trypsin inhibitors from white mustard (*Sinapis alba* L.) seed. *Eur. J. Biochem* 2000, 267: 6486-6492.

(247). Ryan C. A. and Santarius K. Immunological similarities of proteinase inhibitors from potatoes. *Plant Physiology* 1976, 58: 683-685.

(248). Ryan C. A. Proteolytic enzymes and their inhibitors in plant. *Ann Rev Plant Physiol* 1977, 24: 173-196.

(249). Ryan C. A. Synthesis of chymotrypsin inhibitor I protein in potato leaflets induced by detachment. *Plant Physiol.* 1968, 43: 1859-1865.

(250). Ryan C. A., Hass G. M. and Kuhn R. W. Purification and properties of a carboxypeptidase inhibitor from potatoes. *J. Biol. Chem* 1974, 249: 5495-5499.

(251). Saiman L., Tabibi S., Starner T. D., San Gabriel P., Winokur P. L., Jia H. P., McCray P. B. Jr., Tack B. F. Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis. *Antimicrob. Agents Chemother* 2001, 45: 2838-2844.

(252). Sajjan U. S., Tran L. T., Sole N., Rovaldi C., Akiyama A., Friden P. M., Forstner J. F., and Rothstein D. M. P-113D, an antimicrobial peptide active against *Pseudomonas aeruginosa*, retains

activity in the presence of sputum from cystic fibrosis patients. *Antimicrob. Agents Chemother* 2001, 45: 3437-3444.

(253). Sakal E., Applebaum S. W. and Birk Y. Purification and characterization of *Locusta migratoria* chymotrypsin. *Int. J. Pept. Protein Res* 1988, 32: 590-598.

(254). Sanderson P. E. Small, noncovalent serine protease inhibitors. *Med Res Rev* 1999, 19: 179-197.

(255). Sansom M. S. P. Alamethicin and related peptaibols-model ion channels. *Eur. Biophys. J* 1993, 22: 105-124.

(256). Sargent F., Bogsch E. G., Stanley N., Wexler M., Robinson C., Berks B. C. and Palmer T. Overlapping functions of components of a bacterial Sec-independent protein export pathway. *EMBO J.* 1998, 17: 3640-3650.

(257). Sawers R. G. Identification and molecular characterization of a transcriptional regulator from Pseudomonas aeruginosa PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli*. *Mol. Microbiol* 1991, 5: 1469-1481.

(258). Schechter I. and Berger A. On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun* 1967, 27: 157-162.

(259). Schiessler H., Ohlsson K., Olsson I., Arnhold M., Birk Y. and Fritz H. Elastases from human and canine granulocytes. II. Interaction with protease inhibitors of animal, plant and microbial origin. *Hoppe Seylers Z Physiol. Chem* 1977, 358: 53-58.

(260). Sears H. J., Sawers G., Reilly A., Berks B. C., Ferguson S. J. and Richardson D. J. Control of periplasmic nitrate reductase gene expression (*napEDABC*) from *Paracoccus pantotrophus* in response to oxygen and carbon substrates. *Microbiology* 2000, 146: 2977-2985.

(261). Seelmeir S., Schmidt H., Turk V., Vonder Helm K. Human immunodeficiency virus has an aspartic-type protease that can be inhibited by pepstatin A. *Proc Natl Acad Sci* 1988, 85: 6612–6616.

(262). Segura A., Moreno M., Madueno F., Molina A. and Garcia-Olmedo F. Snakin-1, a peptide from potato that is active against plant pathogens. *Mol. Plant Microbe Interact* 1999, 12: 16-23.

(263). Segura A., Moreno M., Molina A. and Garcia-Olmedo F. Novel defensin subfamily from spinach (*Spinacia oleracea*). *FEBS Letters* 1998, 435: 159-162.

(264). Shai Y. and Oren Z. From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides* 2001, 22: 1629-1641.

(265). Shai Y. Mechanism of the binding, insertion, and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim. Biophys. Acta* 1999, 1462: 55-70.

(266). Sharon M., Oren Z., Shai Y. and Anglister J. 2D-NMR and ATR-FTIR study of the structure of a cell-selective diastereomer of melittin and its orientation in phospholipids. *Biochemistry* 1999, 38: 15305-15316.

(267). Shee C. and Sharma A. K. Purification and characterization of a trypsin inhibitor from seeds of *Murraya koenigii*. *Journal of Enzyme Inhibition and Medicinal Chemistry* 2007, 22: 115–120.

(268). Shewry P. R. and Lucas J. A. Plant proteins that confer resistance to pest and pathogens. *Adv. Bot. Res* 1997, 26: 135-192.

(269). Shibata H., Hara S., Ikenaka T. and Vabe J. Purification and characterization of proteinase inhibitors from winged bean (Psophocarpus tetragonolobus (L.) DC.) seeds. *J Biochem* 1986, 99: 1147-1155.

(270). Shoun H., Kim D.-H., Uchiyama H. and Sugiyama J. Denitrification by fungi. *FEMS Microbiol. Lett*. 1992, 94: 277-282.

(271). Siddiqui R. A., Warnecke-Eberz U., Hengsberger A., Schneider B., and Friedrich B. Structure and function of a periplasmic nitrate reductase in *Alcaligenes eutrophus* H16. *J. Bacteriol* 1993, 175: 5867-5876.

(272). Silva J. A., Macedo M. L. R., Novello J. C. and Marangoni S. Biochemical characterization and N-terminal sequences of two new trypsin inhibitors from *Copaifera langsdorffii* seeds. *J. Prot. Chem* 2001, 20: 1-7.

(273). Silva Jr. P. I., Daffre S. and Bulet P. Isolation and characterization of gomesin, an 18-residue cysteine-rich defense peptide from the spider *Acanthoscurria gomesiana* hemocytes with sequence similarities to horseshoe crab antimicrobial peptides of the tachyplesin family. *Journal of Biological Chemistry* 2000, 275: 33464-33470.

(274). Simmaco M., Mignogna G. and Barra D. Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers* 1998, 47: 435-450.

(275). Siverio J. M. Assimilation of nitrate by yeasts. *FEMS Microbiol. Rev* 2002, 26: 277-284.

(276). Steinberg D. A., Hurst M. A., Fukii C. A., Kung A. H. C., Ho J. F., Cheng F.-C., Loury D. J. and Fiddes J. C. Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity. *Antimicrob. Agents Chemother* 1997, 41: 1738-1742.

(277). Sterk P., Booij H., Schellekens G. A., Van Kammen A. and De Vries S. C. Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* 1991, 3: 907–921.

(278). Stewart V., Bledsoe P. J. and Williams S. B. Dual Overlapping Promoters Control napF (Periplasmic Nitrate Reductase) Operon Expression in Escherichia coli K-12. *J. Bacteriol.* 2003, 185: 5862-5870.

(279). Stewart V., Lu Y. and Darwin A. J. Periplasmic Nitrate Reductase (NapABC Enzyme) Supports Anaerobic Respiration by *Escherichia coli* K-12. *J. Bacteriol* 2002, 184: 1314-1323.

(280). Stirpe F and Barbieri L. Ribosome-inactivating proteins up to date. *FEBS* 1986, 195: 1-8.

(281). Stolz J. F. and Basu P. Evolution of nitrate reductase: molecular and structural variations on a common function. *ChemBioChem* 2002, 3: 198-206.

(282). Stoop A. A. and Craik C. S. Engineering of a macromolecular scaffold to develop specific protease inhibitors. *Nature Biotechnology* 2003, 21: 1063-1068.

(283). Su S. B., Gong W. H., Gao J. L., Shen W. P., Grimm M. C., Deng X., Murphy P. M., Oppenheim J. J. and Wang J. M. T20/DP178, an ectodomain peptide of human immunodeficiency virus type-1 gp41, is an activator of human phagocyte N-formyl peptide receptor. *Blood* 1999, 93: 3885-3892.

(284). Svendsen J., Nicolova D., Goshev I. and Genov N. Primary structure, spectroscopic and inhibitory properties of a two-chain trypsin inhibitor from the seeds of charlock (*Sinapis arvensis* L.), a member of the napin protein family. *J. Pept. Protein Res* 1994, 43: 425-430.

(285). Svendson I. B., Nicolova D., Goshev I and Genov N. Isolation and characterization of a trypsin inhibitor from the seeds of kohlrabi (*Brassica napus* var. rapifera) belonging to the napin family of storage proteins. *Carlsberg Res. Commun* 1989, 54: 231-239.

(286). Sweet R. M., Wright H. T., Janin J., Chothia C. H. and Blow D. M. Crystal structure of the complex of porcine trypsin with soybean trypsin inhibitor (Kunitz) at 2.6-Å resolution. *Biochemistry* 1974, 13: 4212-4228.

(287). Tailor R., Acland D. P., Attenborough S., Cammue B .P. A., Evans I. J., Osborn R. W., Ray J., Rees S. B. and Broekaert W. F. A novel family of small cysteine-rich antimicrobial peptides from seeds of *Impatiens balsamina* is derived from a single precursor protein. *J. Biol. Chem* 1997, 272: 24480-24487.

(288). Takaya N. Dissimilatory nitrate reduction metabolisms and their control in fungi. *J. Biosci. Bioeng* 2002, 94: 506-510.

(289). Tam, J.P., Yi-An L., Jin-Long Y. and Koiu-Wei C. An unusual structural motif of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. *Proc. Natl. Acad. Sci. USA* 1999, 96: 8913-8918.

(290). Tamir S., Bell J., Finlay T. H., Sakal E., Smirnoff P., Gaur S. and Birk Y. Isolation, characterization and properties of a trypsin-chymotrypsin inhibitor from Amaranth seeds. *J. Protein Chem* 1996, 15: 219-229.

(291). Tang Y. Q., Yuang J., Osapay G. D., Osapay K., Tran D., Miller C. J., Ouellette A. J. and Selsted M. E. A cyclic antimicrobial peptide produces in primate leukocytes by the ligation of two truncated alpha-defensins. *Science* 1999, 286: 498-502.

(292). Thorne C.B. *Bacillus anthracis*. In: *Bacillus subtilis* and other Gram-positive bacteria. *American Society for Microbiology* 1993, Washington D.C.: 113-124.

(293). Torres-Schumann S., Godoy J. A. and Pintor-Toro J. A. A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol. Biol* 1992, 18: 749–757.

(294). Treviño M. B. and O'Connell M. A. Three Drought-Responsive Members of the Nonspecific Lipid-Transfer Protein Gene Family in *Lycopersicon pennellii* Show Different Developmental Patterns of Expression. *Plant Physiol* 1998, 116: 1461–1468.

(295). Tsuruta S., Takaya N., Zhang L., Shoun H., Kimura K., Hamamoto M. and Nakase T. Denitrification by yeasts and occurrence of cytochrome P450nor in *Trichosporon cutaneum*. *FEMS Microbiol. Lett* 1998, 168, 105-110.

(296). Tsybina T. A., Dunaevsky Y. E., Musolyamov A., Egorov T. A. and Belozersky M. A. Cationic inhibitors of serine proteinases from buckwheat seeds. *Biochemistry (Moscow)* 2001, 66: 941-947.

(297). Valdes-Rodriguez S., Segura-Nieto M., Chagolla-Lopez A., Verver-y-Vargas C.A., Martinez-Gallardo N. and Blanco-Labra A. Purification, characterization and complete amino acid sequence of the trypsin inhibitor from amaranth (*Amaranthus hypochondriacus*) seeds. *Plant Physiol* 1993, 103: 1407-1412.

(298). Van Maanen J. M. S., Welle I. J., Hageman G., Dallinga J. W., Mertens P. L. J. M. and Kleinjans J. C. S. Nitrate contamination of drinking water: relationship with *HPTR* variant frequency in lymphocyte DNA and urinary excretion of *N*-nitrosamines. *Environ. Health Perspect* 1996, 104: 522–528.

(299). Van Parijs J., Broekaert W. F., Goldstein I. J. and Peumans W. J. Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. *Planta* 1991, 183: 258–264.

(300). Vandenbergh P. A., Pucci M. J., Kunka B. S. and Vedamuthu E. R. Method for inhibiting Listeria monocytogenes using a bacteriocin. *European Patent Application* 1989, 89101125.6.

(301). van't Hof W., Veerman E. C. I., Helmerhorst E. J. and Amerongen A. V. N. Antimicrobial peptides: properties and applicability. *Biol. Chem* 2001, 382: 597-619.

(302). Vollack K., Xie J., Hartig E., Romling U. and Zumft W. G. Localization of denitrification genes on the chromosomal map of *Pseudomonas aeruginosa*. *Microbiology* 1998, 144: 441-448.

(303). Volpicella M., Schipper A., Jongsma M.A., Spoto N., Gallerani R. and Ceci L.R. Characterization of recombinant mustard trypsin inhibitor 2 (MTI2) expressed in *Pichia pastoris*. *FEBS Lett* 2000, 468: 137-141.

(304). Wang H., Tseng C.-P. and Gunsalus R. P. The napF and narG Nitrate Reductase Operons in Escherichia coli are Differentially Expressed in Response to Submicromolar Concentrations of Nitrate but Not Nitrite. *J. Bacteriol* 1999, 181: 5303-5308.

(305). Warburg O. and Christian W. Isolierung und kristallisation des gärungsferments enolase. *Biochem. Z* 1942, 310: 384-421.

(306). Watsuji T., Takaya N., Nakamura A. and Shoun H. Denitrification of nitrate by the fungus *Cylindrocarpon tonkinense*. *Biosci. Biotech. Biochem* 2003, 67: 1115-1120.

(307). Wei X., Decker J. M., Liu H., Zhang Z., Arani R. B., Kilby J. M., Saag M. S., Wu X., Shaw G. M. and Kappes J. C. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother* 2002, 46: 1896-1905.

(308). Weiczorek M., Otlewski J., Cook J., Parks K., Leluk J., Willimowska-Pelc A., Polanowski A., Wilusz T. and Laskowski M. Jr. The squash family of serine proteinase inhibitors. Amino acid sequence and associated equilibrium constants of inhibitors from squash, summer squash, zucchini and cucumber seeds. *Biochem. Biophys. Res. Commun* 1985, 126: 646-652.

(309). Weiner J. H., Bilous P. T., Shaw G. M., Lubitz S. P., Frost L., Thomas G. H., Cole J. A. and Turner R. J. A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* 1998, 93: 93-101.

(310). Weiss B., Jacquemin-Sablon A., Live T. R., Fareed G. C. and Richardson C. C. Enzymatic breakage and joining of deoxyribonucleic acid. IV. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. *J. Biol. Chem* 1968, 243: 4543-4555.

(311). Wiedow O., Harder J., Bartels J., Streit V. and Christophers E. Antileukoprotease in human skin: An antibiotic peptide constitutively produced by keratinocytes. *Biochem. Biophys. Res. Commun* 1998, 248: 904-909.

(312). Wijaya R., Neumann G. M., Condron R., Hughes A. B and Polya G. M. Defense proteins from seeds of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. *Plant Sci (Shannon)* 2000, 159: 243-255.

(313). Wilfinger W. W., Mackey K. and Chomczynski P. Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques* 1997, 22: 474-481.

(314). Wong H., Bowie J. H. and Carver J. A. The solution structure and activity of caerin 1.1, an antimicrobial peptide from the Australian green tree frog, *Litoria splendida*. *Eur. J. Biochem.* 1997, 247: 545-557.

(315). Wu Q. and Stewart V. NasFED Proteins Mediate Assimilatory Nitrate and Nitrite Transport in *Klebsiella oxytoca* (*pneumoniae*) M5al. *J. Bacteriol* 1998, 180: 1311-1322.

(316). Yang L., Harroun T. A., Weiss T. M., Ding L. and Huang H. W. Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys. J* 2001, 81: 1475-1485.

(317). Yang L., Weiss T. M., Lehrer R. I. and Huang H. W. Crystallization of antimicrobial pores in membranes: magainin and protegrin. *Biophys. J* 2000, 79: 2002-2009.

(318). Ye R. W., Haas D., Ka J. O., Krishnapillai V., Zimmermann A., Baird C. and Tiedje J. M. Anaerobic activation of the entire denitrification pathway in Pseudomonas aeruginosa requires Anr, an analog of Fnr *J. Bacteriol* 1995, 177: 3606-3609.

(319). Yonah N. Isolation and characterization of proteolytic digestive enzymes of the larval red flour beetle (*Tribolium castaneum*) specifically inhibited by inhibitors from soybean and wheat. *PhD Thesis, Hebrew University of Jerusalem* 1991, Jerusalem, Israel.

(320). Zachowski A., Guerbette F., Grosbois M., Jolliot-Croquin A., and Kader J. C. Characterisation of acyl binding by a plant lipid-transfer protein. *Eur. J. Biochem* 1998, 257: 443–448.

(321). Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002, 415: 389-395.

(322). Zhou J.-J., Fernandez E., Galvan A. and Miller A. J. A high affinity nitrate transport system from Chlamydomonas requires two gene products. *FEBS Lett* 2000, 466: 225-227.

(323). Zhou Z., Takaya N., Nakamura A., Yamaguchi M., Takeo K. and Shoun H. Ammonia fermentation, a novel anoxic metabolism of nitrate by fungi. *J. Biol. Chem.* 2002, 277: 1892-1896.

(324). Zumft W. G. Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev* 1997, 61: 533-616.