STUDY OF MECHANISM OF ACTION OF ANTIVIRAL PROTEIN FROM *Bougainvillea spectabilis* ON *E. coli* AND RICE rRNAs

Thesis submitted in part fulfillment of requirements for
the degree of ***Master of Science*** *in Biotechnology to the*
Tamil Nadu Agricultural University, Coimbatore -3.

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CERTIFICATE

This is to certify that the thesis entitled **STUDY OF MECHANISM OF ACTION OF ANTIVIRAL PROTEIN FROM *Bougainvillea spectabilis* ON *E. coli* AND RICE rRNAs"** submitted in part fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology to the Tamil Nadu Agricultural University, Coimbatore, is a record of bonafide research work carried out by **Mr. V. JAYANTHU** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree/diploma/fellowship or other similar titles or prizes and that the work has not been published in part / full in any scientific or popular journal or magazine.

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Abstract

ABSTRACT

STUDY OF THE MECHANISM OF ACTION OF ANTIVIRAL PROTEIN FROM *Bougainvillea spectabilis* ON *E. coli* AND RICE rRNAs

By

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Degree	:	Master of Science in Biotechnology
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The present study was undertaken to "Study of the mechanism of action of antiviral protein from *Bougainvillea spectabilis* on *E. coli* and rice rRNAs".

The antiviral protein was purified from the root extract of *Bougainvillea* by $(NH_4)_2SO_4$ and CM-Sepharose cation exchange chromatography following the standardised protocol developed in our lab. The purified BAP was found to have a single polypeptide chain of molecular size 28 kDa. The total RNA was isolated from *E.coli* and rice leaves by using TRIzol reagent of GIBCO BRL Pvt. Ltd, USA. The presence of conserved region in 25 S rRNA of rice was confirmed by RT-PCR using two 25S rRNA conserved region specific primers that are specific to conserved regions of 25S rRNA of plants. This conserved region houses the adenine residue susceptible to RIPs. The BAP was found to have N-glycosidase activity and cleaved the rRNA of *E. coli*. This was confirmed by primer extension analysis involving a primer complimentary to 44-60 bases down stream from the susceptible adenine residue. This study inferred that the mechanism of action of BAP was like other ribosome inhibiting proteins (RIPs).

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LIST OF ABBREVIATIONS

%	- Percentage
° C	- Degree Centigrade
μg	- microgram
$(NH_4)_2SO4$	- Ammonium sulphate
2- ME.	-2-Mercapto ethanol
AIDS	- Auto Immuno Deficiency Syndrome
AVP	- Antiviral protein.
BAP	- *Bougainvillea* antiviral protein
bp	- base pairs
CaMV	- Cowpea aphid borne mosaic virus.
cDNA	- Complementry DNA
CM-Sepharose	- Carboxy Methyl- Sepharose
DEAE-Sepharose	- Diethyl aminoethyl - Sepharose
DEPC	- Diethyl pyrocarbonate
DNA	- Deoxy ribonucleic acid
ds	- Double Stranded
DTT	- Dithiothreitol
DW	- Distilled water
EDTA	- Ethylenediaminetetra cetic acid
Fig	- Figure
g	- gram
h	- hour
HCl	- Hydrochloric acid
HIV	- Human Immuno deficiency Virus
Kbp	- Kilo basepairs

KCl	- Potassium chloride
kDa	- kilo Daltons
L	- lit
MAP	- Mirabilis antiviral protein
mg	- milligram
$MgCl_2$	- Magnesium chloride
min.	- minute(s)
ml	- millilitre
Na_2HPO_4	- Di sodium hydrogen phosphate
NaCl	- Sodium chloride
NaH_2PO_4	-Sodium Di Hydrogen Phosphate
NaOH	- Sodium hydroxide
ng	- nanogram
O/N	- Overnight
PAGE	- Poly Acrylamide Gel Electrophoresis
PAP	- Phytoaleca antiviral principle
PCR	- Polymerase Chain Reaction
RNA	- Ribonucleic acid
rpm	- Revolutions per minute
RT	- Reverse transcriptase
SDS	- Sodium Dodecyl Sulfate
sec	- Seconds
ss	- Single stranded
TMV	- Tobacco Mosaic Virus
TSWV	- Tomato Spotted Wilt Virus
U	- Units
UV	- Ultraviolet

Introduction

CHAPTER I

INTRODUCTION

A large number of higher plants is reported to contain plant viral inhibitors which can be effectively used to control plant viruses. These inhibitors are referred to as plant antiviral principles.

Duggar and Armstrong reported for the first time in 1925, that the crude extract of pokeweed inhibited the infectivity of tobacco mosaic virus. Following this, the occurrence of viral inhibitory substances were noticed in a number of plants belonging to different families of angiosperms, of the order Centrospermae. Most of the viral inhibitors were found to be proteins and some of the antiviral proteins have been characterized. (Irvin *et al.*, 1980; Takanami *et al.*, 1990; Balasaraswathi *et al.*, 1998; Balasubrahmanyam *et al.*, 2000). Among the different antiviral proteins so far identified, *Mirabilis* and pokeweed antiviral proteins (MAP & PAPs) are fully characterized and some are partially characterized like *Bougainvillea* antiviral protein (BAP). There are several mechanisms that exist regarding the action of these antiviral proteins. Out of these, the widely accepted mechanism is that antiviral proteins (AVP) inactivate the ribosomes by RNA N-glycosidase activity cleaving the N-glycosidic bond at A4324 of 25S rRNA of eukaryotes (Endo *et al.*, 1988) and A 2660 of 23S rRNA of prokaryotes(Habuka *et al.*, 1991). But recently it was found that AVP cleave RNA not only at the adenine residue but also at guanine residue and they may depurinate the capped RNA template rather than ribosome depurination (Hudak *et al.*, 2000). However, ribosome inactivation was found to be the key way of action of AVPs.

Bougainvillea spectabilis Willd. is an ornamental shrub belonging to the family Nyctaginaceae. The crude leaf extract of *B. spectabilis* has been reported to inhibit plant viruses like tobacco mosaic virus, cowpea aphid borne mosaic virus and tomato spotted wilt virus (Sadasivam *et al.*, 1991). The *Bougainvillea* antiviral protein (BAP) has been characterized by Balasaraswathi *et al.*, 1998 and found to inactivate the eukaryotic ribosomes (yeast ribosomes) by RNA N-glycosidase activitiy (Murugan, 1999).

The present study on *Bougainvllea* antiviral protein was taken up with the following objectives

1. Purification of *Bougainvillea* antiviral protein (BAP) from *Bougainvillea spectabilis* Willd.
2. Studying the mechanism of action of BAP on prokaryotic (*E. coli*) rRNA.
3. Studying the mechanism of action of BAP on eukaryotic (rice) rRNA.

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

Plant protection against pathogen has gained utmost importance in modern agriculture. Each year, there are yield losses in crop production due to the attack of different pathogens. Among the plant pathogens, viruses are the most important that cause much damage to crops. There is no direct way to control viruses. Though pesticides are effective in control of vectors carrying viruses, but they cause environmental pollution, hazardous to the life of human beings. So it is necessary to develop eco-friendly measures to ensure safer environment to maintain the bio-diversity. Many plant extracts are reported to contain viral inhibitory substances, called antiviral principles (AVP). They include proteins, tannins, nucleic acid and polysaccharides that constitute plants array of natural defense strategies to combat plant pathogens. The first report on AVP`s came as early as 1925 when Duggar and Armstrong demonstrated that the ability of the sap of the poke weed *Phytolacca decandra* L. to prevent infection of tobacco mosaic virus (TMV) in tobacco. Later this discovery paved way for scientific community to identify plants possessing antiviral property.

The literature on AVPs have been comprehensively reviewed (Ragetli and Wientraub, 1962; Francki, 1964; Sabitha, 1969; Wyatt and Shepherd, 1969; Moraes *et al.*, 1974; Tomlinson *et al.* 1974; Fukaya and Taniguchi, 1979; Verma and Kumar, 1980; Awasthi *et al.* 1985; Narayanasamy and Ramiah, 1983; Verma *et al.*, 1985; Yoshii and Sako, 1987; Kuruchev, 1988; Sadasivam *et al.*, 1991; Balasaraswathi *et al.*, 1998).

2.1. Anti viral principles in different plant species

A plethora of plant species exhibit antiviral activity against many plant viruses. Zaitlin and Siegel (1963) reported that the homogenates of tobacco leaf tissues inhibited TMV infection in several hosts. Sabitha (1969) reported the presence of potent viral inhibitor in *Bougainvillea spectabilis* after screening 422 plant species for the presence of viral inhibitors. Thakur and Sastry (1971) reported that the aqueous extracts of *Boerhaavia difffusa, Bougainvillea spectabilis* and *Mirabilis jalapa* inhibited the petunia distortion strain of TMV on *Datura innoxia* and potato virus on *Chenopodium amaranticolor* even at 10^{-4} times dilution. These extracts were found to be effective when sprayed on host 24 h before or 5 minutes after inoculation.

Apablaza and Bernier (1972) reported the inhibitory property of leaf extracts of pepper, geraniums and Jimson on TMV. The antiviral activity of the leaf extracts of *Phytolacca americana* on cucumber mosaic virus and influenza virus was reported by Tomlinson *et al.*, (1974). Verma and Mukerjee (1975) reported that the brinjal leaf extract induced local and systemic resistance in *Nicotiana glutinosa* against TMV and in *Nicotiana tabacum* against tobacco ring spot virus when applied 24 h before virus inoculation.

Rao *et al.*, (1984) have reported the antiviral activity of coralloid root extracts of *Cycas revoluta* on potato virus-X (PVX) , potato virus Y(PVY), TMV, Tomato aspermy virus(TAV) and tomato ring spot virus (TRSY) on tomato, when applied 24 hrs before virus inoculation or when mixed with different virus inocula and applied on hypersensitive and systemic hosts. Verma *et al.* (1985) reported the presence of antiviral principles in the leaf extracts of *Pseuderanthemum atropurpureum* and *Bougainvillea spectabilis* against TMV and sunnhemp rosette viruses.

Kubo *et al.* (1990) reported the ability of leaf extracts of *Mirabilis jalapa* on controlling Cucumber mosaic virus (CMV) and potato virus (PVY). The aqueous extracts of *Coronus capitata, Apium graveolans, Gardenia gummifera*, the alcoholic extract of *Lowsonia alba, Costus speciosus* and *Hexaneethyloacetate* extract of *Artemisia annua* reduced virus infection when applied 24 h prior to virus challenge, as reported by Khan *et al.* (1991).

Sadasivam *et al.* (1991) have reported the antiviral property of *Boerhaavia diffusa, Bougainvillea spectabilis, Clerodendrum aculeatum and Sorghum* sp against TSWV on groundnut and capsicum, CAMV on cowpea and TMV on tobacco. Chen *et al.* (1991) found that pokeweed antiviral protein (PAP) from *Phytolacca americana* completely inhibited tobacco mosaic virus infection of tobacco. The effect of PAP on CMV, alfalfa mosaic virus (AMV), PVX, PVY, African cassava mosaic virus (ACMV) and cauliflower mosaic virus was also reviewed.

Patil and Joi (1992) reported the control of TMV infection by the leaf extract of *Capsicum annum, Accacia arabica, Datura metel* and *Chenopodium amaranticolor.* They showed more than 80% inhibition of virus by the application of these leaf extracts. The control of sunnhemp rosette virus and TMV by the leaf extracts of *Pseuderanthemum atropurpureum* and *Bougainvillea spectabilis* was reported by Verma *et al.* (1985) . Patel and Patel (1993) reported the complete inhibition of chlorotic mottle virus infection in tobacco by extracts of *Clerodendrum inermi*, *Parkinsonia aculeata* and *Ipomea carnea.* The occurrence of antiviral principle in *Basella rubra* and *Bougainvillea spectabilis* which inhibited the infection of *Nicotiana benthomiana* by artichoke mottle crinkle virus (AMCV) was reported by Bolognesi *et al..* (1997)

Other plant source possessing the antiviral principle are *Sambucus nigra* (Cilores *et al.*, 1996), *Cucurbit pepo* (Yoshinari *et al.*, 1996), *Cinnamomum porrectum* (Li *et al.*, 1996). *Momordica charantia* (Pu *et al.*, 1996), *Bryonica diocia* (Gawlak *et al.*, 1997). *Soponora officinalis* (Di-cole *et al.*,1997). *Trichosanthes kirlowii* (Shih *et al.*, 1998) and edible mushroom *Volvariella volvacea* was also found to contain antiviral principle (Yao *et al.*, 1998). The AVPs in various plant species is given in the Table 1.

Table 1. Distribution of AVPs in plant kingdom

Family	Species	Reference
Amaranthaceae	*Amaranthus caudatus*	Verma *et al.*, 1987
	Amaranthus tricolor	Baranwal and Verma, 1993
	Gomphrena flobosa	Grasso and Shepherd, 1978
Basellaceae	*Basella alba*	Nagarajan and Murthy, 1988
	Basella rubra	Bolognesi *et al.*, 1997
Caryophyllaceae	*Dianthus caryophyllus*	Grasso and Sheperd, 1978
	Stellaria media	Grasso and Shepherd, 1978
Chenopodiaceae	*Chenopodium album*	Grasso and Shepherd, 1978
	C. amaranticolor	
	C. quinoa	
	C. ambrosiodes	
Nyctaginaceae	*Boerhaavia diffusa*	Thakur and Sastry, 1971, Verma and Awasthi, 1979
	Bougaivillea spectabilis	Verma *et al.*, 1985, Sadasivam *et al.*, 1991
		Balasaraswathi *et al.*, 1995; Bolognesi *et al.*, 1997
	Mirabilis jalapa	Kubo *et al.*, 1990
Solanaceae	*Capsicum annum*	Neema *et al.*, 1981
	Datura metel	Grasso and Shepherd, 1988
	D. stramonium	
Cucurbitaceae	*Cucurbita pepo*	Yoshinari *et al.*, 1998
	Momordica charantia	Stevens *et al.*, 1987
Phytoloccaceae	*Phytolacca americana*	Molina *et al.*, 1999
Poaceae	*Sorghum spp*	Narayanasamy and Ramiah, 1983
Punicaeae	*Punica granatum*	Sabitha, 1969
Euphorbiaceae	*Ricinus communis*	Stevens *et al.*, 1981
	Gelonium multiflorum	Stevens *et al.*, 1981

2.2. Taxonomic distribution of antiviral principles

Smookler (1971) pointed out that plant from different taxonomic families of angiosperms posses potent inhibitor of plant virus infection. More than 180 plants from different taxonomic families of angiosperm have been found to contain potent inhibitors of viruses. The major families include Chenopodiaceae, Amaranthaceae, Nyctaginaceae, and Basellaceae of the order Centrospermae.

Grasso and Shepherd (1978) proposed that species which have inhibitors of plant virus infection are concentrated in some taxonomic groups and the production of viral inhibitors might be controlled by genes that conferred some special advantage in disease resistance, which led to retention in the course of evolution of plants under Centrospermae, which is believed to have its phylogenetic origin in Phytoloccaceae Baranwal and Varma (1993) have identified 10 plants showing antiviral activities belonging to different families *viz.*, Acantahaceae, Araliaceae, Leguminosae.

2.3. Chemical nature and characteristics of AVP

2.3.1. Chemical nature

Most of the AVP`s are proteinaceous in nature. However glycoproteins, polysaccharides, phenols, flavanoids, alkaloids and tannins have also been reported to exhibit antiviral activity. The chemical nature of different AVP`s are listed in Table 2.

Proteins

Fukaya and Taniguchi (1979) reported the proteinaceous nature of Phytolacca inhibitor. The antiviral principle from sorghum leaves was indicated to be a protein (Narayanasamy and Ramiah, 1983). The antiviral principle present in *Spinacea oleracea* was also a protein (Straub *et al.*, 1986) . A potent plant virus inhibitor in *Mirabilis jalapa* was found to be a protein. (Kubo *et al.*, 1990). Sadasivam *et al.*, (1991) found that the active principles in *Sorghum* sp and *Bougainvillea spectabilis* were proteinaceous in nature based on the heat inactivation above 60°C. The antiviral principles namely saporin,

Table 2.Chemical nature of AVPs from different plant species

Plant species	Chemical nature	References
Phytolacca americana	Protein/Glycoprotein	Wyatt and Shepherd, 1969
Punica granatum	Glycoprotein	Sabitha, 1969
Chenapodium album	Protein	Smookler, 1971
C. amaranticolor	Protein	Grasso and Shepherd. 1978
Brassica chinensis	Protein	Grasso and Shepherd, 1978
Datura stramonium	Protein	Grosso and Shepherd, 1978
Beta vulgaris	Polysaccharide	Ebrahim *et al.*, 1972
Capsicum annuum	Phenolics	Fischer and Nienhaus, 1973
Abutilon striatum	Polysaccharides	Moraes *et al.*, 1974
Solanum sp.	Glycoalkaloids	Roychoudhary and Basu, 1983
Bougainvillea spectabilis	Protein	Sadasivam *et al.*, 1991 and Balasaraswathi, 1995
Boerhaavia diffusa	Glycoprotein	Awasti *et al.*, 1985
Basella alba	Glycoprotein	Ushashri *et al.*, 1982
Mirabilis jalapa	Protein	Kung *et al.*, 1990
Sorghum sp.	Protein	Sadasivam *et al.*, 1991
Dianthus caryophyllus	Protein	Stripe *et al.*, 1986
Celosia cristata	Glycoprotein	Balasubrahmanyam *et al.*, 2000

pokeweed antiviral principle, *Mirabilis* antiviral principles, trichosanthin, volvarin and pepocin etc. are all proteins (Stripe *et al.*, 1980; Stripe *et al.*, 1981; Barbieri *et al.*, 1982; Jimenez and Vasquez, 1985; Stripe and Barbieri, 1986; Maraganore *et al.*, 1987; Kamenosono *et al.*, 1988; Takanami *et al.*, 1990; Yao *et al.*, 1998; Yoshinari *et al.*, 1996).

Glycoproteins

Some of the AVPs have been reported to be glycoproteins. The AVP from the dried roots of *Boerhaavia diffusa* has been found to be a glycoprotein capable of inhibiting several viruses affecting vegetable crops (Awasthi *et al.*, 1985). The AVP in *Punica granatum* was found to be a glycoprotein inhibiting TMV infection (Sabitha, 1969). Gelonin Modecin, dianthin-30, dianthin 32, the A-chain of ricin contained the sugar. It has been found that the Phytolacca antiviral protein from the seeds (PAP-S) is a glycoprotein. The plant viral inhibitors from *Basella alba* (Ushashri *et al.*, 1982), *Nicotiana glutinosa* (Palm, 1967) and *Boerhaavia diffusa* (Verma *et al* ., 1979) were found to be glycoproteins.

Polysaccharides

Lipids, glucose, galactose, and xylose present in the flower extracts of red clover exhibited the antiviral activity (Elkandelgy and Wilcoxon, 1966). The polysaccharide of sugarbeet leaves showed the inhibitory property to TMV (Ebrahim and Nieahaus, 1972). The inhibitors of TMV from the leaves of *Abutilon striatum* showed positive reactions with molish and anthrone tests (Moraes *et al.*, 1974) indicating the polysaccharide nature of the antiviral compound. The polysaccharide fraction of *Brassica oleracea* was capable of reducing virus infection (Verma, 1974).

Alkaloids

The extracts from *Solanum* sp. inhibiting the TMV and sunnhemp rosette virus infection was found to be steroidal glycoalkaloids. Solanine and solamargine were identified in the active fraction (Roychoudhury and Basu, 1983).

Phenolics

The inhibitory activity of *Capsicum annum* extract was found to be related to a protein and a phenolic substance. The phenolic substance was classified as a flavone, isoflavone or flavanone compound (Fischer and Nienhans, 1973).

2.3.2. Characteristics of Antiviral Proteins

Plant AVPs are basic proteins of around 30 kDa . Though the antiviral activity has been attributed to various chemicals, detailed study was restricted to the following plant species.

Phytolacca antiviral proteins (PAPs)

Wyatt and Shepherd (1969) isolated the viral inhibitor from *Phytolacca americana* and reported it as a basic protein with the molecular weight of 23 kDa. The protein contained about 12% lysine by weight and its biological activity was destroyed by succinylation of its free amino groups. The antiviral proteins PAP-I, PAP-II and PAP-S were isolated from spring, summer leaves and from the seeds, respectively (Barbieri *et al.*, 1982). The molecular weight of PAP-II, PAP-S is 27 kDa and 30 kDa, respectively (Irvin, 1980; Barbieri, 1982).

Kung *et al.*, 1990 reported the complete amino acid sequence of PAP-S, which showed homology to ricin A chain at 76 positions, particularly the amino acids at positions 170-183 are conserved.

Mirabilis antiviral protein (MAP)

The complete amino acid sequence of MAP was reported by Habuka *et al.*, 1989. It consisted of 250 amino acids having a molecular weight of 24 kDa and an isoelectric point of 9.8 with no sugar moiety. Habuka *et al.* (1991) found that MAP had an intermolecular disulphide bond and elimination of this increased its antiviral activity.

Bougainvillea antiviral protein (BAP)

Balasaraswathi *et al.* (1995) purified and partially characterized an antiviral protein from *Bougainvillea spectabilis* and found to be a basic protein with a molecular weight of 28 kDa. The pI of this protein was greater than 8.6. The amino acid composition of BAP was revealed and the sequence of first thirty amino acids were determined from the N-terminal end. The sequence of BAP showed 52% identity with PAP-S and 30.4% identity with MAP (Balasaraswathi *et al.*, 1998).

Other antiviral proteins

Pu *et al.* (1996) isolated an AVP, named momorcharin from the seeds of *Momordica charantia* and has a molecular weight of 11.5 kDa. Yoshinari *et al.* (1996) isolated and characterised an AVP, Peposin from *Cucurbita pepo* which is a 26 kDa protein with pI value of 9.9. Purification and characterization of gelonin, a RIP from seeds of *Gelonium multiflorum* with molecular weight of 30 kDa was reported by Singh *et al.*, (1999). Balasubrahmanyam *et al.*, (2000) isolated and characterized two AVPs CCP-25 and CCP 27 from *Celosia cristata* with molecular weight of 25 and 27 kDa respectively. Ye *et al.*, 2000 reported the proteinaceous nature of structurally dissimilar proteins with antiviral and anti fungal potency from cowpea.

2.4. Purification of antiviral proteins

Most of the antiviral proteins can be purified like other proteins, which involves different purification procedures depending upon the chemical and physical nature of the inhibitor present in plant extract. Purification steps involve dialysis, chromatography techniques like cation, anion exchange chromatography, HPLC, FPLC etc. Chromatography is the best technique to purify the protein to homogeneity. PAP from *Phytolacca americana* was purified by CM - Sephadex column chromatography by Wyatt and Shepherd (1969). Irvin (1975) purified PAP by DEAE-cellulose chromatography followed by phosphocellulose chromatography. Finally the PAP was eluted with the KCl gradient.

The purification of second antiviral protein, PAP II from the summer leaves of *Phytolacca* was reported by Irvin *et al.* (1980) and PAP-S was isolated from the seeds of *Phytolacca americana*. They included an additional step in purification of PAPII in addition to purification of PAPI *viz.*, Sephadex G-75 column chromatography. Wang *et al.*, 1992 purified *Mirabilis* antiviral proteins from the root tubers of *Mirabilis jalapa* by using CM-Sepharose, column chromatography followed by FPLC.

Gelonin from *Gelonium multiflorum* was purified in a single step by chromatography on CM-cellulose column (Stripe *et al.*, 1980). Vinod Singh *et al.* 1999 purified the gelonin by following conventional method involving CM-cellulose chromatography and gel filtration chromatography followed by protein isolation and a second method involving S-Sepharose (fast flow) chromatography.

Dianthin 30 and Dianthin 32 were also purified using CM-cellulose column (Stripe *et al.*, 1981). Luffin a and b were purified by gel filtration through sephadex G-75 column followed by CM-cellulose column and FPLC (Kamenosono *et al.*, 1988). Edelbsum *et al.*, (1990) reported the purification and characterization of two antiviral proteins from tobacco using monoclonal antibodies to human beta interferon. *Mirabilis* antiviral protein (MAP) was purified to homogeneity, from *Mirabilis jalapa* by ammonium sulphate fractionation followed by ion exchange chromatography using CM-Sepharose and DEAE-Sepharose columns (Takanami *et al.*, 1990). An antiviral protein from *Bougainvillea spectabilis* was purified by ammonium sulphate fractionation followed by ion exchange chromatography and RP-HPLC techniques (Balasaraswathi and Sadasivam, 1994).

2.5. Specificity of antiviral proteins

The antiviral proteins identified so far were highly specific to the plant species from which they are isolated. This was tested by using immunological techniques. According to Kubo *et al.*, (1990) the *Mirabilis* antiviral protein antiserum cross reacted only with MAP and not with any other extract of plants containing AVPs.

Irvin *et al.*,(1980) reported that anti-PAP serum did not cross react with PAP-II and gave a weak reaction with PAP-S which indicates that no precursor relationship exists between the two proteins, PAPI and PAPII. This suggests the differential expression of these genes during the growth of pokeweed (*Phytolacca americana*) plant. Similarly Balasaraswathi, (1995) reported that anti-BAP serum did not cross react with any other plant extracts containing antiviral proteins.

2.6. Mechanism of action of AVPs

A definite understanding of the mechanism of action for any of the antiviral proteins from higher plants has not yet been determined. The most widely accepted mechanism of action of AVP is that they inactive the ribosome thereby inhibiting the protein synthesis. They inactivate the host plant ribosomes rather than virus directly (Stevens *et al* ., 1981). But Hudak *et al* ., (2000) reported that PAP acts on the viral RNA directly. The ability of both PAP and PAP II to inhibit polyphenylalanine synthesis on *Artemisia salina* was reported by Irvin (1975) and Irvin *et al.,* (1980). Momorcharin, modecin and gelonin were also found to inhibit protein synthesis in rabbit reticulocyte system (Stevens *et al.*, 1981). Dianthin 30 and Dianthin 32 were found to inhibit protein synthesis in rabbit reticulocyte lysate and wheat germ systems by inactivating the 60S ribosomal subunit (Reisbig and Brula, 1983).

2.6.1. Ribosome inactivating proteins (RIPs)

Many plant antiviral proteins posses the ribosome inactivating property. Therefore, these proteins are also referred as ribosome inactivating proteins (RIPs) (Habuka *et al.*, 1989). Bolognesi *et al.* (1977) reported the presence of new single chain (type I) ribosomes inactivating proteins from the seeds of *Basella rubra* L. and *Bougainvillea spectabilis* wild. Ready *et al.* (1984) reported a new ribosome inhibiting protein from *Phytolacca dodecandra* namely dodecandrin. RIPs exhibit N-glycosidase activity which depurinate an adenine residue of the 60S subunit of eukaryotic ribosome making them unable to bind to elongation factor II and inhibit protein synthesis (Roberts and Selitrennikoff, 1986; Endo and Tsurugi, 1987; Endo *et al.*, 1987; Barbieri and Stripe, 1982; Olsnes and Pihl, 1982; Stripe and Barbieri, 1986). Similar RIPs were isolated from *Cinnamomum porrectum* (Li *et al.*, 1996), *Clerodendrum inermi* (Olivieri *et al.*, 1996), from soapwort (Barbieri *et al.*, 1996). A list of RIPs were given in Table 3.

2.6.2. AVPs as RIPs

AVPs specifically cleaves the N-glycosidic bond at specific adenine residue A4324 of rat liver 28S rRNA and A2660 of prokaryotic (*E. coli*) 23S rRNA . These susceptible adenine residue lie in the conserved regions. The *E.coli* A 2660 in *E.coli* corresponds to A 4324 in the former. This A 2660 in *E. coli* rRNA directly interacts with elongation factors EF-G and EF-TU. MAP was found to cleave the N-glycosidic bond at positions A 1014 of 16S rRNA and A 2660 of 23S rRNA of *E. coli* in both naked state and in the ribosomes of *E. coli* (Habuka *et al.*, 1991).

BAP was found to inhibit *in vitro* protein translation (Balasaraswathi *et al.*, 1998) and inactivate rice ribosome (Chitra, 1997), Sieboldin-b, a heterodimeric protein and a type II RIP possessing carbohydrate binding specificity and strong *in vitro* activity was isolated and biological properties were studied in *Sambucus sieboldiana* (Rojo *et al.*, 1997). Sieboldin-b has structural homology with ricin and abrin.

Balasaraswathi *et al.*, (1999) has reported the RNA N-glycosidase activity of BAP on yeast rRNA. Vivanco *et al.*, (1999) found two novel type I RIPs from storage roots of *Mirabilis expansa* that depurinate 26S rRNA of yeast. The RNA N-glycosidase activity of four, type I RIPs from leaves of *Phytolacca dioica* was reported by Dimaro *et al.*, (1999). Cho *et al.*, (2000) reported the ribosome inactivating property of an AVP from *Dianthus sinensis* L. Kwon *et al.*, (2000) isolated a RIP from *Amaranthus viridis* and molecular

Table 3. List of Ribosome inactivating proteins in plants

Plant species	Nature of RIP	References
Sambucus ebulus	Type I	Girbes *et al.*, 1993
S. elbus	Type II	De Benito *et al.*, 1995
S. sieboldiana	Type II	Rojo *et al.*, 1997
Saponaria oeymoides	Type II	Bolognesi *et al.*, 1995
Momordica charantia	Type I	Pu *et al.*, 1996
Clerodendron inermi	Type I	Olivieri *et al.*, 1996
Cucurbita pepo	Type I	Yoshinari *et al.*, 1996
Basella rubra	Type I	Bolognesi *et al.*, 1997
Bougainvillea spectabilis	Type I	Bolognesi *et al.*, 1997
Gyrophilla elegans	Type I	Yoshinari *et al.*, 1996
Amaranthus viridis	Type I	Kwon *et al.*, 1997
Sechium edule	Type I	Wu *et al.*, 1998
Mirakilis jalapa	Type I	Vivanco *et al.*, 1999
Zea mays	Type I	Gao, 2000
Iris hollandica	Type I	Hao *et al.*, 2001

cloning and expression studies were conducted in *E. coli.* Two closely related lectins (agglutinin b and agglutinin r) were isolated from bulbs of Dutch Iris (*Iris hollandica* and its N-glycosidase activity has also been reported (Hao *et al.*, 2001).

Recently Rajmohan *et al.*, (2000) reported that in addition to N-glycosidase activity at A4324, PAP also removes another adenine (A 4321) and a guanine residue (G 4323) from eukaryotic rRNA. But the deguanylation was found to be 20-fold less efficient compared to the deadenylation. The structural basis of the interaction of PAP with guanine residue of rRNA was analysed through X-ray crystallography and protein modelling studies by Kurinov *et al* ., (1999). They found that PAP accommodated a guanine base in the active site pocket without large conformational changes. Hudak *et al.,* (2000) reported that PAP mutants like PAPx (active site mutant containing point mutation E176V, PAP-n (N-terminal mutant containing point mutation at G 750) are not able to depurinate tobacco ribosomes. But they are able to inhibit translation of bromo mosaic virus and potato virus X (PVX). Furthermore, translation of only capped, but not uncapped luciferase transcripts are inhibited. So they proposed that the PAP inactivate the virus by recognising the cap structure and depurinating the capped RNA rather than depurinating ribosomes.

On the basis of structural diversity, plants RIPs have been classified into 2 major groups (Stripe *et al.*, 1986;Mundy *et al.*,1994). Type-I RIPs, the most numerous group containing pokeweed antiviral protein (PAP), trichosanthin, Barley RIP-30 and gelonin, are all synthesized as single chain enzymes of about 30 kDa (Maraganore *et al.*, 1987). They may accumulate in starchy endosperm storage cells (RIP-30) (Leah *et al.*, 1991) or be secreted from vegetative or seed cells (PAP) (Lodge *et al.,* 1993). In contrast, trichosanthin and L-trichosanthin accumulate in root cells.

Type-II RIPs such as prototypical ricin from castor bean endosperm are synthesized as large precursors which accumulate in protein bodies and are processed to a 30 kDa, RIP-A chain, linked to a 30 kDa lectin-B-chain through a single disulfide bond.

(Lord *et al.*, 1985). Type-I RIPs, which do not posses the B-chain binding domain do not bind readily to cells and consequently having relatively low mammalian cytotoxicity compared to type-II RIPs. However, type-I RIPs can enter cells by fluid-phase endocytosis (Stripe *et al.*, 1982)

A third RIP type has been characterized which apparently contains two cytoplasmically localised but quite different members from maize and barley. (Bass *et al.*, 1992 Chaudhary *et al* ., 1994). The maize type-III RIP accumulates in the Kernel as a 34 kDa inactive precursor which, upon germination may be processed into a two-chain active RIP by removal of an internal peptide from within the catalytic domain (Walsh *et al.*, 1991). Similarly the barley JIP-60 protein has been shown to posses ribosome inactivating activity and is synthesized as a precursor that requires removal of similar peptide for activation. (Choudhry *et al.*, 1994). Therefore, the primary distinction between type-III and type-I or II RIPs is that the former requires removal of a peptide from catalytic domain for activation (Endo *et al.*, 1987).

Most RIPs show developmentally regulated organ specific expression. However, several examples are known for inducible expressed RIPs including the jasmonate responsive JIP-60 of barley and beetins which are induced by viral infection and show response to salicylic acid and hydrogen peroxide (Gibes *et al.*, 1996). Most RIPs may have roles in programmed cell death (Greenberg *et al.*, 1997). The only evidence suggesting most RIPs have roles in suicide are of corrective: Most organs, which accumulate high levels of RIPs are either storage organs which do not develop further, or organs which senesce rapidly. The barley seed RIP30, which accumulate to high levels in endosperm storage cells late in embryo development, could be involved in the programmed senescence, of this storage organ at seed . (Leah *et al.*, 1990)

Single chain RIPs such as pokeweed antiviral protein, PAP, have been shown to posses antiviral activities towards viruses of both plant and animal origin. (Hartley *et al.,* 1993). Three distinct isoforms of PAP accumulate in the cell wall matrix of leaves and seeds and can readily be obtained from water-macerated leaf tissue. Exogenous application of small amounts of PAP to the surface of plant leaves completely prevents mechanical transmission of unrelated viruses to several different host plants. Expression of PAP cDNA in transgenic tobacco and potato plants, which are thought to lack endogenously applied PAP showed resistance to a range of viruses. Although the mechanisms of PAP-mediated resistance are not yet understood, one possibility is that PAP enters the host cells along the virus and prevents the translation of viral RNA by inactivating conspecific ribosome. PAP may be a secreted suicide factor whose reentry into host cells is potentiated by cellular permeabilization by the pathogen. (Taylor *et al.*, 1994). In contrast, tritin and barley RIP showed no depurination activities on tobacco ribosomes and they were ineffective in inhibiting virus spread. This supports the hypothesis that the antiviral activity of some RIPs, like PAP, work via inactivation of conspecific ribosomes.

Some RIP showed synergistic action with pathogenesis related proteins. For example, barley single-Chain RIP along with chitinase CHI-26 and (1-3)-β-glucanase inhibited growth of *Trichoderma* more than 95%, when all three proteins are added at 0.25 µg/well in microtiter plate assay. Similar results were found in inhibition of Fusarium, *Rhizoctonia solani, Botrytis cinera* (Jach *et al.,* 1995). Barley RIP 30 gene was expressed in transgenic tobacco under the control of wound-inducible promoter of the potato wun I gene. These plants accumulated RIP mRNA and protein upon infection by *Rhizoctonia solani* (Logemann *et al* ., 1993).

The type-I RIP efficiency can be increased by constructing chimeric molecules. Chimeric constructs were made by fusing PCR amplified chitinase binding domains from virus genes to the N-terminal of the barley RIP30. These are found to inhibit the protein synthesis of reticulocyte lysates under *in vitro* conditions (Boller *et al* ., 1990).

The sequence similarities between RIP catalytic RNA-N-glycosidase domains are remarkably high. This is not only recognized by alignment of the primary sequences but also reflected in the super-imposition of the three dimensional model structures based on the known 3D structures. All RIPs share conserved 3D structures of the active cleft centred around the conserved catalytic amino acid residue E_{177} (numbering according to ricin A) along with the invariant Y80, Y123, and W211 which have been shown to be essential for substrate binding (Chen *et al.*, 1997). Although RIP from monocotyledons species like barley, wheat & maize share a high similarity to all other RIPs , they seem to form a distinct group, which diversified significantly earlier during evolution.

2.7. RIPs against human diseases

In addition to inhibiting plant viruses, many RIPs were extensively studied as the toxic moiety of immunotoxins, which are conjugated with monoclonal antibodies targeted to tumour cells in human systems. (Frankel, 1988). RIPs have been clinically assessed as anti-HIV agents in AIDS patients (Barbieri *et al.*, 1993) and as toxic components of immuno toxins targeted at malignant cells (Avadia *et al.*, 1990). Particularly PAP has been used on the design of therapeutic immunotoxins, which may act as " magic bullets" aimed at cancer of HIV affected cells. It is also shown to effectively inhibit the replication of polio virus, influenza virus and herpes simplex virus (Zarling *et al.*, 1990; Irvin & Uckin 1992). Momocharin from bitter melon, is capable of inhibiting HIV-infection in T-lymphocytes and monocytes as well as replication of HIV-1 in infected cells, yet is not toxic to normal uninfected cells. (Lee-Hung *et al.*, 1995).

Dianthin from *Dianthus caryophyllus,* and gelonin are reported to be having anti tumour properties. Similarly trichosanthin is found to have anti HIV properties (Huang *et al.,* 1995).

Hypericin, a polycyclic anthrone, from St.John Wort (*Hypericum perforetum)* was found to be inhibiting the replication of HIV, cytomegalovirus, Epstein-Barr virus, Sindbus virus (James *et al.,* 1991).It disrupts the uncoating of lipid envelope of both DNA and RNA viruses, thus preventing infected cells from releasing HIV copies. The antiviral properties of hypericin were increased by exposure to fluorescent light. It will disrupt assembly budding and shedding and possibly protein synthesis, all of and which depend on the integrity of the viral membrane. (Wood *et al.,* 1990). Hayashi *et al.,* (1992) reported that the antiviral property of Yucca leaf protein against herpes simplex virus-1. Luffin and saporin were reported to be the potent inhibitors of HIV integrase (Collins *et al.,* 2000).

2.8.Studies on Bougainvillea AVP

Water extracts from fresh or air dried leaves of *B. spectabilis* inhibited virus infection in several plants when sprayed prior to virus inoculation by systemic nature and was not associated with any phytotoxicity symptoms. The difference of resistance induced however, slightly varied in different host-virus combinations (Verma *et al.,* 1985).

B. spectabilis leaf extract when mixed with the viral inoculum inhibited TSWV multiplication on cowpea plants by more than 90% (Kurchev, 1988). Antiviral properties in the leaf extracts of *B. spectabilis* against tomato spotted wilt virus, and TMV have been identified and suggested to be proteinaceous in nature by Sadasivam *et al.,* (1991) and Balasaraswathi and Sadasivam (1994).

The biophysical properties of the AVP present in *B. spectabilis* extract have been reported by Verma *et al.,* (1985). The viral inhibitors present in the extracts were quite stable to dilutions, temperature and storage. The inhibitors could be precipitated by ammonium sulphate and ethanol, which suggested the proteinaceous nature of the inhibitors of *B. spectabilis.*

Balasaraswathi *et al.,* (1998) have purified an anti viral protein from *Bougainvillea* roots (BAP) to homogeneity and sequenced the first 30 N- terminal amino acids. The structural and functional homology of BAP with other antiviral proteins like PAP and MAP were also studied. BAP inhibited *in vitro* protein translation and exhibited RNA, N-glycosidase activity on yeast rRNA. Hence BAP may be a RIP and may act similar to other RIPs (Balasaraswathi, 1999; Balasaraswathi *et al.,* 2000).

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

Bougainvillea antiviral protein (BAP) required for the study was purified from the roots of *Bougainvillea spectabilis* wild following the protocol developed in our lab.

3.1. Preparation of root extracts of *Bougainvillea* (Balasaraswathi, 1995)

The roots of the *Bougainvillea spectabilis* wild were selected for the extraction of antiviral protein since it has been reported to contain more amount of AVP compared to other plant parts.

Materials

1. 10 mM phosphate buffer (pH 7.2): 28 ml of 0.2 M NaH_2PO_4 was mixed with 72 ml of 0.2 M Na_2HPO4 and the total volume was made up to 2 l. To this β- mercaptoethanol was added to 0.1%.
2. Sample: Roots (collected from the collected from the pink coloured flower trait of *Bougainvillea spectabilis* wild from TNAU campus).

Method

1. The roots were scrapped into small pieces using a knife. Then it was ground to a fine powder in the presence of liquid nitrogen.
2. Root extract was prepared by grinding 50 g root powder with 500 ml of pre chilled phosphate buffer containing β- mercaptoethanol in a waring blender.
3. The homogenate was filtered through muslin cloth.
4. The filtrate was centrifuged at 6000 rpm for 20 min at 4^0 C. The supernatant was collected and labeled as crude extract.

3.2. Purification of *Bougainvillea* antiviral protein (BAP) from the root extract

Sample extraction

BAP was purified by $(NH_4)_2SO_4$ precipitation followed by ion exchange chromatography.

3.2.1. Ammonium sulfate precipitation

Materials

1. Crude root extract
2. Ammonium sulfate
3. Magnetic stirrer
4. Dialysis bag (Sigma chemical Co., USA)

Method

1. To the crude root extract, ammonium sulfate was added slowly with constant stirring to bring up to 90% saturation. The contents were centrifuged at 5000xg for 30 min at 4^0 C.
2. The pellets were collected and redissolved in 5 ml of extraction buffer.
3. The solution was then transferred to pretreated dialysis bags and dialysed against DW with 3 changes of DW for 24 h. The dialysates were then centrifuged, and the supernatants pooled and lyophilized.

3.2.2. CM-Sepharose cation exchange chromatography

Materials

1. Lyophilised root proteins obtained from above
2. CM-Sepharose, fast flow (Sigma chemical Co., USA)
3. 10 mM phosphate buffer pH 6.0.
4. 10 mM phosphate buffer pH 6.0 containing 25mM NaCl, 0.1% 2-ME
5. 10 mM phosphate buffer pH 6.0 containing 300mM NaCl, 0.1% 2-ME

6. Pharmacia column (25x30mm)
7. Fraction collector (2112 Redirac, LKB, Sweden)
8. UV-Visible spectrophotometer (Beckman DU 64)
9. Gradient mixer
10. Magnetic stirrer

Method

1. CM-Sepharose was packed in a column of 25 X 300 mm size and equilibrated first with 10mM PB followed by 10 mM PB containing 25 mM NaCl.
2. 0.75 g lyophilised protein sample, dissolved in 10 ml of 10 mM phosphate buffer containing 0.1% 2-ME and 25 mM NaCl, was centrifuged at 10,000 X g for 15 min.
3. The supernatant obtained was applied on to the pre equilibrated column.
4. The column was washed with the start buffer and then eluted with a linear gradient of NaCl (25-300 mM) in the same buffer using gradient mixture and magnetic stirrer.
5. 5ml fractions were collected using LKB fraction collector at the rate of 0.32 ml/min.
6. The absorbance of the fractions collected were measured using Beckman spectrophotometer at 280 nm.

The above steps were carried out in a cold room maintained at 2^0 C.

Fractions showing the major peak were pooled and dialysed against 10 mM phosphate buffer pH 7.2 with three changes of buffer and lyophilised.

3.3. Protein estimation (Bradford, 1976)

Protein contents were estimated in crude root extract and purified BAP.

Materials

1. Protein stock standard: 50 mg BSA in 50 ml DW
2. Working standard: 10 ml of stock was diluted to 100ml with DW in a standard flask, the solution contains 100 μg/ml protein.

3. Bradford dye concentrate: 100 mg of CBBG 250 was dissolved in 50 ml of 50% ethanol and to this 100ml of ortho phosphoric acid was added and final volume was made up to 200 ml.
4. Working dye solution: The dye concentrate was diluted 5 times with distilled water.

Method

1. Aliquots of samples were taken in test tubes and final volume was made up to 1ml with DW.
2. To each sample, 5 ml of working dye solution was added and allowed for 5 min. for color development.
3. The absorbance was read at 595 nm using a suitable blank.
4. The protein standards were prepared by taking different aliquots of working standard solutions ranging from 10- 100μg.
5. From the standard graph, the unknown protein concentration was calculated.

3.4. SDS - PAGE (Laemmli, 1970)

Crude protein from the Bougainvillea roots, Purified BAP obtained from CM-Sepharose was analyzed by SDS -PAGE to check the purity and molecular weight.

Materials

1. Acrylamide solution: 29.2g acrylamide and 0.8g Bis -acrylamide were dissolved in 100ml of distilled water, filtered and stored at 4^0 C in a brown bottle.
2. Resolving gel buffer: 18.15 g Trizma base was dissolved in 200 ml DW and the pH adjusted to 8.8 with HCl.
3. Stacking gel buffer:3 g Trizma base was dissloved in 50 ml DW and the pH adjusted to 6.8 with HCl.
4. 10 % SDS: 10 g SDS was dissolved in DW and made up to 100 ml.

5. 10 %Ammonium persulfate: 0.1 g Ammonium persulfate was dissolved in 1 ml DW.
6. TEMED
7. Sample loading buffer(2X): 2.5 ml stacking gel buffer, 4 ml of 10%SDS, 2 ml glycerol and 1 ml 2-mercaptoethanol were mixed and 40 mg bromophenol blue added. The final volume was made up to 10 ml.
8. Tank buffer: 1 g Trizma base and 57.6 glycine were dissolved in 1 l DW and to this 40 ml 10% SDS was added and the final volume made up to 4 l.
9. Staining solution:200 mg Commassie Brilliant blue R was dissolved in 80 ml methanol and 20 ml glacial acetic acid was added and the volume made up to 200 ml with DW. The solution is filtered and used.
10. Destaining solution: 200 ml methanol and 50ml acetic acid were mixed and the volume made upto 500 ml.
11. Electrophoreis apparatus (Broviga, India)
12. Power pack (Hoefer PS 500XT)

Sample preparation

Samples were prepared by mixing equal volume of 2X sample loading buffer and heated in a boiling water bath for 3-5min, just before loading.

Method

1. Electrophoresis was carried out in 180 x 160 x 1 mm gel in a discontinuous buffer system using 12% or 14% acrylamide gels.
2. Gel recipe for 1-mm thick slab gel was given in the following table.
3. The contents of separating gel were degassed for 10 - 15 min. Ammonium persulphate and TEMED were added just before pouring the gel. The gel was poured between glass plates sealed at the bottom and allowed to polymerize.
4. Then the gel was over laid with a film of DW to accelerate polymerization.

Table 4. Gel recipe for 1mm thick slab gel

Contents	Separating gel (ml)		Stacking Gel (ml)
	12%	14%	4%
Acrylamide	12	14	1.33
Resolving gel buffer	7.05	7.5	-
Stacking gel buffer	-	-	2.5
10% SDS	0.3	0.3	0.1
Water	10.04	8.04	6.015
Ammonium persulfate	0.15	0.15	0.05
TEMED	0.01	0.01	0.005
Total	30	30	10

5. After polymerization, the water layer was removed and stacking gel poured, after placing the comb on the top of the sandwich. The comb was carefully removed after polymerization of the stacking gel and rinsed with tank buffer before loading the samples.
6. The samples were then loaded carefully onto the gel wells and run at a constant current of 10 mA till the samples completely entered the stacking gel and then at 20 mA for 4-6 hr at room temperature until the dye front reached the lower edges of the plates.
7. After the electrophoretic run , the gel was removed and left in the staining solution overnight and then destained in destaining solution until a clear back ground was got. The gel was then photographed.

3.5. Nondenaturing- PAGE at low pH

Materials

1. Resolving gel buffer (10X): 3.37 g KOH was dissolved in water, 21.52 ml of acetic acid was added and the pH adjusted to 4.3 and the vol. made up to 100ml.
2. Stacking gel buffer (10X): 3.36 g of KOH was dissolved in water. 3.606 ml of acetic acid was added, the pH was adjusted to 6.8 and made up to 100 ml.
3. Running buffer (10X): 12.47 g of β-alanine was dissolved in water, 20.04 ml of acetic acid and pH was adjusted to 6.8 and made up to 100 ml.
4. 10% APS
5. TEMED
6. Sample buffer (5X): 3.1ml of 1M Tris pH 6.8, 5 ml Glycerol and 0.002 g of methyl green were added and made up to 10 ml.
7. Staining solution: As given under 3.4
8. Destaining Solution: As given under 3.4
9. Acrylamide stock solution:

   a) Stacking solution: 23.4 g of acrylamide and 7.8 g bisacrylamide were dissolved in 100 ml of DW.

   b) Resolving solution: As given under 3.4

Method

1. Electrophoresis was carried out in 180 x 160 x 1 mm gel in a discontinuous buffer system.
2. Separating gel and stocking gel was prepared as per the table given below and the protocol followed was as in 3.4.

Gel recipe for a 1 mm thick slab gel.

Contents	Resolving gel (7.7%)	Stacking gel (3.125 %)
Resolving gel buffer	1.5 ml	-
Stacking gel buffer	-	0.5 ml
Acrylamide		
a) Resolving	3.7 ml	-
b) Stacking	-	-
10 % APS	0.150 ml	0.150 ml
TEMED	0.009 ml	0.006 ml
Water	9.65 ml	3.85 ml

3. After polymerisation samples were loaded on to the gel.
4. The polarity was reversed and electrophoresis was carried out at 20 mA for 5-6 h at 4^{O}C.
5. The gel was stained, destained and photographed and documented.

3.6. Isolation of total RNA from rice tissues

Total RNA was isolated from rice leaf tissues and *E.coli* cells following TRIzol method. All glasswares used for RNA isolation should be sterilised in hot air oven at the temperature of 200^0 C for 1 hr. Only the DEPC treated water was used for RNA extraction.

3.6.1. Isolation of total RNA from rice by TRIzol method

Total RNA was isolated from rice leaves by TRIzol method, an improved method of single-step RNA isolation from tissues (Chomczynski and Sacchi, 1987).

Materials

1. Sample : Matured leaves of rice
2. TRIzol reagent (GIBCO BRL Pvt. Ltd., USA)
3. Chloroform

4. Isopropyl alcohol
5. Ice cold 75% ethanol
6. RNase free water.

Method

1. One gram of the sample was ground to a fine powder with liquid nitrogen, in a pestle and mortar.
2. The ground tissue was transferred to 50 ml centrifuge tube containing 10 ml of TRIzol reagent and mixed thoroughly.
3. The homogenised samples were incubated at room temperature for 5 min to dissociate nucleoprotein complexes.
4. To the above, 2 ml of chloroform was added and shook vigorously for 15 sec and incubated at room temperature for 2-3 min.
4. The contents were centrifuged at 12000 rpm for 15 min at 4^0 C.
5. The aqueous phase was carefully transformed into a fresh tube and 5 ml of isopropanol was added and was incubated at 20° C for atleast 30 min.
6. It was then centrifuged at 12000 rpm at 4° C for 10 min.
7. The supernatant was discarded and the RNA pellet was resuspended in 10 ml of 75% ethanol.
8. The samples were vortexed and centrifuged at 7500 rpm for 5 min at 4^0 C.
9. The RNA pellet was dried briefly and resuspended in 50-100μl sterile water and incubated for 10 min at 55-60° C. The RNA was stored at –70^0C until further use.
10. The quality of RNA prepared was analysed through 1% agarose gel.

3.6.2. Isolation of total RNA from *E.coli*

Total RNA was extracted from *E.coli* by TRIzol method.

The bacterial culture was prepared as given below

Bacterial cell culture preparation

Materials

1. *E. coli* culture (DH5α strain)
2. LB broth: 1.25g of yeast extract, 2.5g tryptone, 2.5g NaCl were dissolved in 250 ml DW and autoclaved (5 ml of LB broth was autoclaved separately).
3. Inoculation needle
4. Laminar flow .

Method

1. A loop of *E.coli* cells was taken from the petri plate and aseptically transformed to 5 ml LB broth under laminar flow.
2. The LB broth was kept in a shaker (180rpm) at 37^{O} C for 15 h.
3. Then the broth was aseptically transferred to 250 ml of autoclaved LB broth and kept in incubator at 37^{O}C at 180 rpm for 15 h.

RNA isolation

Materials

Same as described in 3.6.1 except the sample used was bacterial cells from above.

Method

1. Bacterial cells were pelleted from LB broth by centrifugation at 9000 rpm for 15 min.
2. Remaining steps were followed as described in 3.6.1.

3.7. Agarose gel electrophoresis

RNA, cDNA, amplified DNA were analysed by agarose gel electrophoresis.

Materials

1. Agarose
2. TBE buffer (10x) pH 8.3; 10.78 g of tris base, 5.5 g of boric acid and 0.819 g of EDTA were dissolved in 80 ml of water pH adjusted and made upto 100ml.
3. Samle :Denatured RNA / cDNA /amplified DNA
4. Ethidium bromide stock : 10 mg/ml solution
5. Sample loading buffer (5x) : 5ml of Glycerol, 0.02ml of 0.5M EDTA, 40 mg each of bromophenol blue and xylene cyanol were mixed and made upto 10ml with DEPC treated water.

Method

1. 0.3 g of agarose was added to 30 ml of 1 X TBE buffer and melted
2. After cooling to 50^0 C, 1 µl of ethidium bromide was added, mixed thoroughly and poured on to the gel casting tray fixed with a comb and allowed to solidify for 30-45 min.
3. The gel was kept in the electrophoretic apparatus and filled with TBE buffer
4. The samples were mixed with sample loading buffer to a final concentration 1 X and loaded on to the gel.
5. Electrophoresis was carried out at 50 volts for 3-4 h
6. The gel was then viewed on a UV transilluminator and documented

3.8. Quantification of RNA

The total RNA from leaf sample of rice , *E.coli* were quantified

Materials

1. RNA samples
2. UV spectrophotometer
3. Quartz cuvette

Method

1. 1 μl of RNA sample was diluted to 1ml with water
2. The absorbance was measured at 260nm and the RNA concentration was calculated using the following formula.

   1 OD at 260 nm = 40 μg RNA

3.9. Agarose acrylamide composite gel electrophoresis of RNA (Peacock *et al.,* 1968)

Materials

1. TBE buffer
2. Acrylamide stock solution: 36 g acrylamide and 1.8 bisacrylamide were dissolved in 100 ml distilled water.
3. 10 %Ammonium persulphate
4. TEMED
5. Agarose

Method

1. 0.25 g of Agarose was added to 40 ml TBE buffer and melted at 80 °C and kept for cooling.
2. To 4.16 ml of acrylamide stock, 400 μl APS and 5.4 ml of TBE buffer were added, mixed and kept ready.
3. When the melted agarose reached 52 °C the acrylamide - TBE -APS mixture was added to the agarose. 40μl of TEMED was also added to the contents and mixed thoroughly and poured into the gel casting tray fixed with a comb.
4. The gel was then allowed to polymerize for 3 -4 h.
5. The comb was removed carefully and kept in electrophoresis apparatus.
6. The samples are loaded as described in 3.7 and the electrophoresis was carried out at 50 volts for 3-4h.
7. The gel was then stained, destained, visualized under UV trans illuminator and photographed.

3.10. Confirmation for the presence of conserved sequences in 25S rRNA of rice

Materials

1. Total RNA from rice leaves
2. RT-PCR kit supplied by Bangalore Genei pvt ltd., Bangalore.
3. Synthetic oligonucleotide primers, 'a' and 'b' corresponding to the 5' and 3' conserved regions in 25S rRNA of plants.

Method

First strand cDNA synthesis

1. 5µl of total RNA sample was taken in an eppendorf tube.
2. RNAse free water was added to bring the vol. to 9µl.
3. 1µl primer 'b' was added to the above
4. The vial was placed at 65 ^{0}C for 10 min. and then at room temperature for 2 min.
5. It was then spun briefly and the following reagents were added in order

    1.0 µl RNase inhibitor

    1.0 µl 0.1M DTT

    4.0 µl RT buffer (5X)

    2.0 µl 30mM dNTP mix

    0.5 µl Reverse transcriptase

    11.5 µl DEPC treated sterile water

6. The contents were mixed well and incubated at 42 ^{0}C for 1 h.
7. Then it was kept at 95°C for 2 min. Spun briefly and quickly placed in ice.

This was immediately used for the second strand synthesis.

PCR amplification

1. PCR mix was prepared by adding the following in order

First strand cDNA	-	2 µl
10X PCR buffer	-	5 µl

10mM dNTP mix - 1 µl

primer 'a' - 1 µl

Primer 'b' - 1 µl

Taq DNA polymerase (3 U/µl) - 1 µl

DEPC - treated sterile DW - To make up to50 µl

2. The contents were mixed and spun briefly
3. PCR was performed in eppendorf Thermal cycler.
4. The PCR was performed and the product was then analysed using 1.5% agaraose gel.

3.11. Mechanism of action of BAP

RNA N- glycosidase activity, of purified BAP on *E. coli* and rice rRNA was tested to find out the mechanism of action of BAP. Total RNA was isolated from both bacteria and rice. The cleavage of *E.coli* and rice rRNA by BAP was studied after treatment with acidic aniline on agarose and agarose-acrylamide composite gels.

N-glycosidase activity of BAP

N-glycosidase activity of BAP was tested using 2 methods *viz.*,

1. Modified Habuka *et al* ., 1991 method
2. Hudak *et al* ., 2000 method

Materials

1. TMK buffer: 25mM Tris-HCl (0.197g Trizma base is dissolved in 50 ml water and pH adjusted to 7.6 with HCl). To this KCl and $MgCl_2$ were added to bring the concentrations to 25mM and 5mM respectively.
2. 1M aniline: To 1ml aniline, 28.6 ml DEPC treated DW was added and pH was adjusted to 4.5 using glacial acetic acid.
3. Purified BAP: The BAP purified by CM-Sepharose column chromatography was used.

4. *E. coli* RNA , rice RNA
5. Incubator

3.11.1. Modified Habuka et al ., 1991 method

1. A quantity of 5μg of RNA was treated with the different concentrations of the BAP *viz*., 0.5 μg, 1 μg, 2 μg 2.5 μg, 5 μg in 100 ml of TMK buffer.
2. The above treated RNA are incubated at 37^{O}C for 30 min.
3. The reaction was terminated by adding 100μl of 2 % SDS.
4. RNA was precipitated with 3 vol of absolute ethanol and 1/10th vol of 2M sodium acetate keeping at -20^{O}C 0/N.
5. Centrifugation was done at 15, 000 X g for 15 min at 4^{O} C.
6. The pellet was washed with 70% ethanol, dried and dissolved in minimal amount of water.
7. To this 1M aniline was added and heated to 60^{O}C for 20 minutes under dark.
8. Then the samples were loaded in agarose and agarose-acrylamide composite gels and electrophoresis was carried out at 50 V.

3.11.2.Hudak *et al* . 2000 Method

1. 500 ng RNA was incubated with different concentrations of BAP (5ng, 100ng, 1 μg, 2 μg)
2. Incubation of treated RNA was done at 37^{O}C for 30 min
3. Then the BAP is removed by phenol and phenol: chloroform (24:1) extraction.
4. The RNA was precipitated at -80^{O}C by adding ethanol.
5. RNA was taken and dissolved in DEPC treated water.
6. To the RNA samples, 1M aniline was added and incubated at 60^{O}C for 10 minutes.
7. Then the samples were loaded in agarose and agarose-acrylamide composite gel and electrophoresis was carried at 50 V.

3.12. Detection of depurination of adenine of 25S rRNA by primer extension analysis

1. Total RNA
2. RT-PCR Kit supplied by Bangalore Genei pvt ltd., Bangalore.
3. Synthetic oligonucleotide primer 'c' complementary to the nucleotide sequence 44-60 bases downstream from the susceptible adenine residue (4324) of the universally conserved region in 25S-like rRNA of prokaryotes and eukaryotes.

Method

1. To a sterile RNase-free eppendorf tube, 5μg of total RNA sample was added.
2. To this, 1 μl primer 'c' was added.
3. The vial was placed at 65^{O}C for 10 min. and then at room temperature for 2 min.
4. It was then spun briefly and the following were added sequentially

    1 μl RNase inhibitor

    1 μl 0.1 M DTT

    4 μl RT buffer(5X)

    2 μl 30m M dNTP mix

    DEPC-treated sterile water - make up to 20 μl

5. The contents were mixed well and incubated at 42^{O} C for 1 h for cDNA synthesis.
6. Then, it was kept at 95^{O} C for 2 min, spun briefly and quickly placed on ice.
7. The product was analysed using 1.5% agarose gel as described previously.

Results

CHAPTER IV

RESULTS

Bougainvillea antiviral protein was purified from *B. spectabilis* and was used for the study of its mechanism of action on prokaryotic and eukaryotic rRNAs viz., *E. coli* and rice rRNAs.

4.1. Extraction of BAP from *Bougainvillea* roots

Bougainvillea antiviral protein (BAP) was extracted from the roots of *Bougainvillea spectabilis* using 10 mM Sodium phosphate buffer pH 7.2 following the standardised protocol developed in our lab. The total proteins in the extract were precipitated with 90% saturation of ammonium sulphate.

4.2. Purification of BAP

The BAP was separated from the $(NH_4)_2SO_4$ precipitated proteins using cation exchange chromatography following the protocol already standardised in our lab. The BAP was eluted from the column by using 10 mM phosphate buffer, pH 6.0 and with a linear gradient of NaCl (25-300 mM). The CM - Sepharose profile is shown in Fig 1.

4.3 Characterisation of BAP

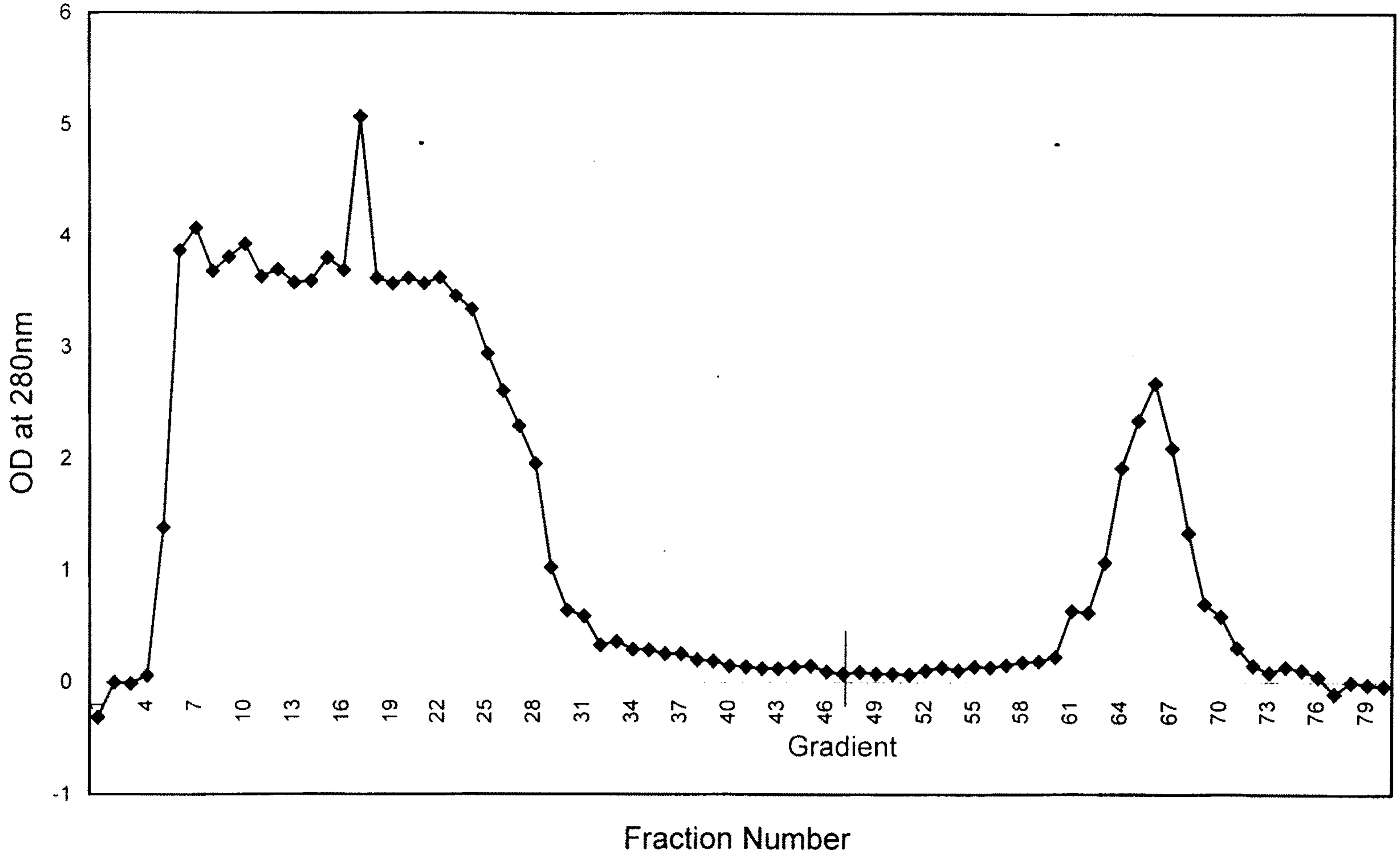
4.3.1. Protein estimation by Braford method

The crude protein extract and BAP obtained after CM-Sepharose chromatography were estimated by the Bradford's method (Table 4). 1μl of purified BAP contained 3.8μg protein.

4.3.2. BAP on SDS-PAGE

Both crude protein extract and purified BAP were electrophoresed on SDS- PAGE. The results are shown in Plate 1. The purified BAP was seen as a single band of ~28 kDa.

Fig. 1. Elution profile of *B. Spectabilis* root proteins by CM Sepharose Column chromatography



PURIFICATION FLOW CHART OF BAP

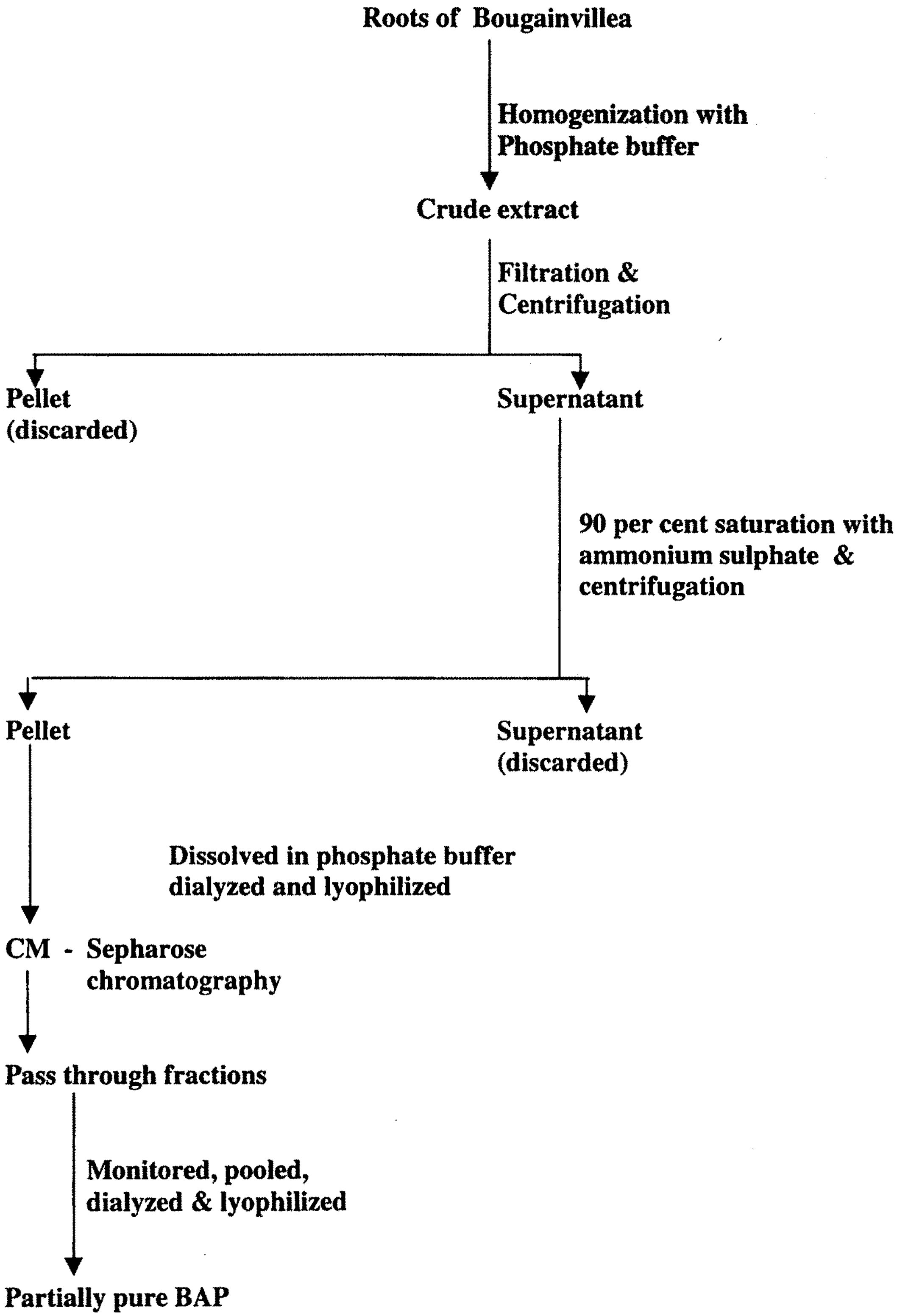


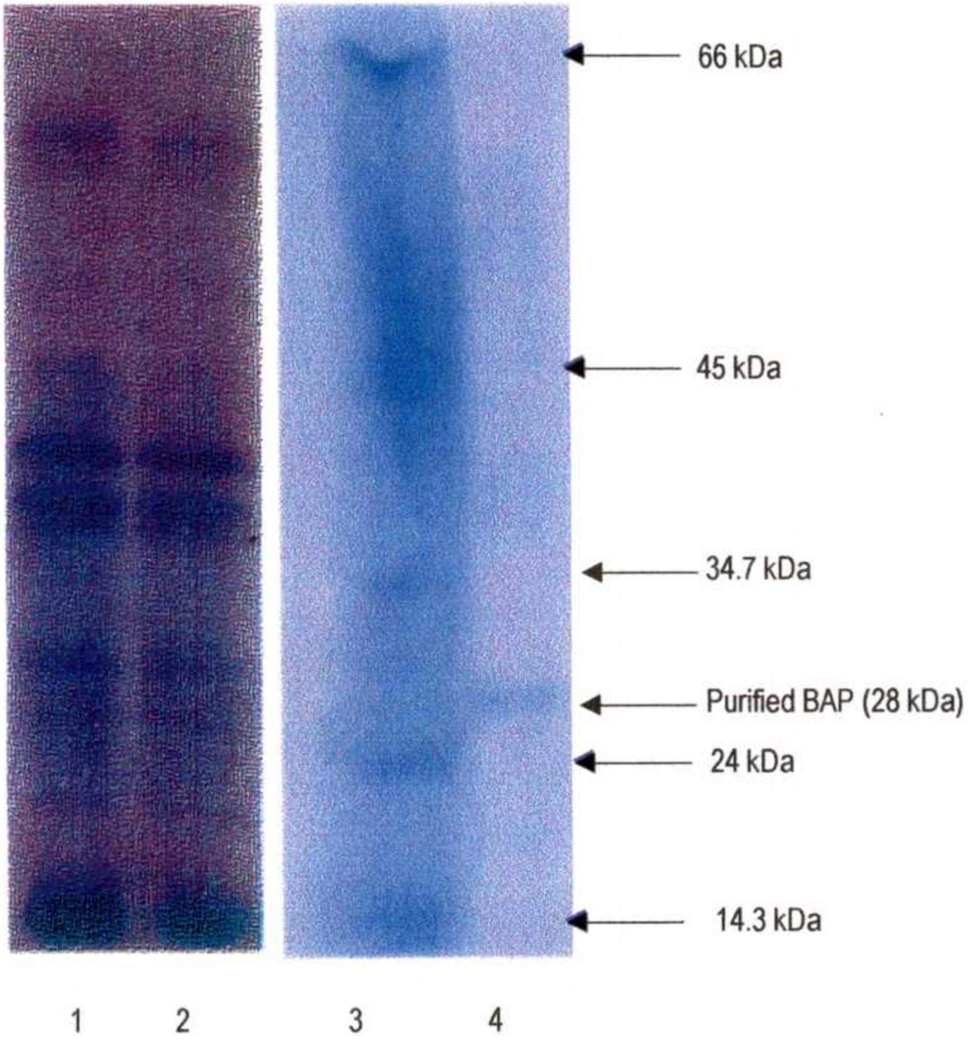
Table 4. Protein estimation in crude and purified BAP - Bradfords method

Volume of standard BSA (µg)	Absorbance reading at 595 nm
2	0.201
4	0.427
6	0.621
8	0.835
10	1.074
crude protein	0.81
purified BAP	0.421

Protein quantity present in crude BAP = 7.8 µg / µl

Protein quantity present in purified BAP = 3.8 µg / µl

Plate 1. SDS – PAGE profile of crude protein extract and purified BAP



Lanes 1, 2 - Crude protein extract
3 - SDS – PAGE marker
4 - Purified BAP

4.3.3. BAP on native gel

The BAP was seen as a single band in acidic native gel as seen in Plate 2.

4.4. Mechanism of action of BAP

The inhibitory activity of many antiviral proteins have been reported to be due to their RNA N- glycosidase activity on rRNAs of host plants. In the present study the viral inhibitory activity of purified BAP was studied on prokaryotic (*E. coli*) rRNA and eukaryotic (rice) rRNA.

4.4.1.Isolation of total RNA from *E. coli*

DH5α strain of *E. coli* was used as the source material for RNA isolation. Total RNA was isolated by using TRIzol method. The quality of RNA was checked on 1% agarose gel electrophoresis.The *E.coli* RNA profile is shown in Plate 3.

4.4.2. Isolation of total RNA from Rice leaves

The total RNA was isolated from the rice leaves by Trizol method. The quality of RNA was checked by 1% agarose gel electrophoresis. The rice RNA profile is shown in Plate 4.

4.4.3. Quantification of RNA

Total RNA isolated from the *E.coli* and rice were quantified by measuring the absorbance at 260 nm. The O.D values and quantity of RNA are shown in the table 5.

4.4.4. Analysis for the presence of conserved region in 25S rRNA of rice

Ribosome inactivating proteins exert their effect by cleaving the N-glycoside bond at A 2660 in the conserved region of 23S rRNA of prokaryotes and A 4324 in the conserved region of 28S rRNA of eukaryotes. Hence checking for the presence of the conserved regions may give hint regarding susceptibility of its beholder rRNA to RIP activity. Earlier studies in our lab showed that BAP is also a RIP. Hence analysis were

Table 5. RNA quantification of *E. coli* and rice

RNA type	O.D at 260 nm	Quantity (μg/μl)
Rice RNA	0.3646	7.2
E. coli RNA	0.513	10.26

Table 6 . PCR programme

Programme	**Temperature (°C)**	**Time (Min.)**
Initial denaturation	94	3
Denaturation	94	1
Annealing 35 cycles	59	1
Extension	70	1.30
Final extension	72	8
Holding temperature	4	∞

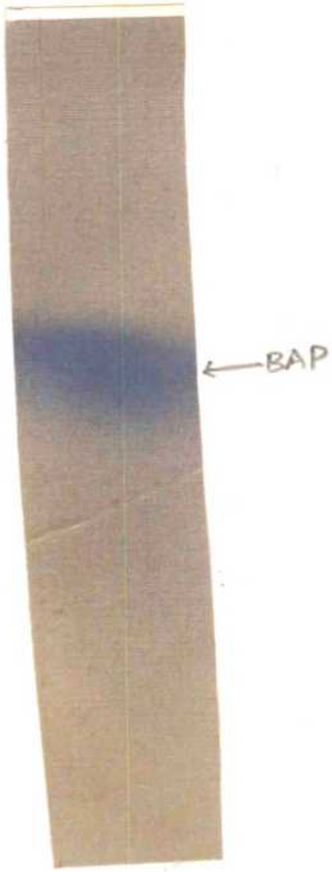


Plate 2. BAP on acidic native PAGE

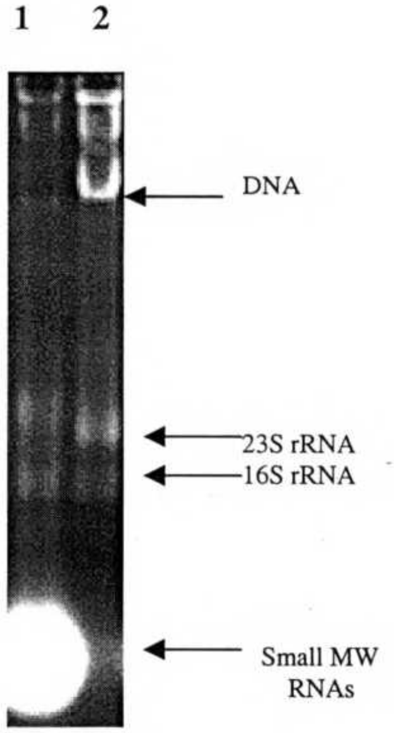


Plate 3. Total RNA Profile of *E.coli* on 1% agarose gel.

Lanes 1,2- *E coli* RNA (5μg)

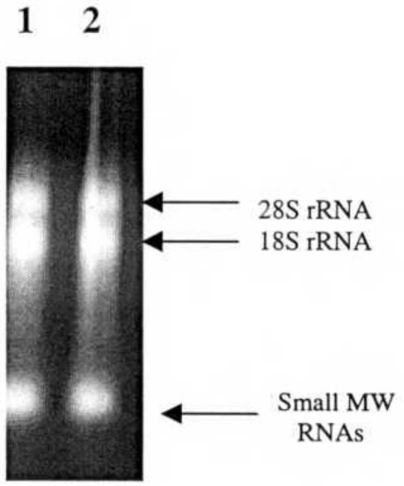


Plate 4. Total RNA profile of rice on 1% agarose gel.

Lanes 1,2 -Rice RNA (5μg)

carried out to detect the presence of conserved region in the 25S rRNA of rice. A conserved region of 12 nucleotides in 25S like rRNA is shown in Fig 2.Total RNA was reverse transcribed with primer 'b'. RT-PCR was carried with the PCR programme shown in Table 6, with the primers 'a' and 'b' to get ds cDNA. (primer 'a': 5' AAC GTA GTA CGA GAG GAA C 3'; primer 'b': 5' AAG TCG TCT GCA AAG GAT T 3'). The primers 'a' & 'b' were constructed based on the sequences in the conserved regions at 5' and 3^1 ends of 25S rRNA in plants (Fig 3). A band at about 250 bp level was seen in case of rice (Plate 5) indicating the presence of the conserved region in the 25S rRNA of rice.

With *E.coli* no such product was seen as primer 'b' is specific only to plants.

4.4.5. RNA N- glycosidase activity of BAP on *E.coli* and rice rRNA

5μg of each of *E. coli* RNA and rice RNA were treated with 30 nM of BAP alone, aniline alone and with BAP and aniline. The products were analysed by agarose-acrylamide composite gel. A new diagnostic band appeared between 16S and 5S rRNA in case of *E.coli* with BAP and aniline treatment (Plate 6) indicating the presence of RNA N-glycosidase activity of BAP on *E. coli* rRNA. Treatment of RNAs with aniline alone did not show presence of any extra band. Though the conserved region was found to be present, no new diagnostic band appeared after treatment with BAP and aniline in case of rice. (Plate 7).

4.4.6. Primer extension analysis

The depurination of 50S subunit of *E.coli* by BAP was studied by primer extension analysis, using the primer 'c' (5' TTC GCG CCA CTG GCT TT 3'). This primer is complementary to 44 -60 bases downstream from the susceptible adenine residue of the conserved region (Fig. 4). The product of primer extension was of low molecular weight i.e, approximately 45 bases (as against the larger fragment in the absence of depurination of 23 S rRNA by BAP) *in E.coli* (Plate 8). The susceptible adenine residue located in the conserved region is shown in Fig 4.

Fig. 2. The universally conserved region of 12 nucleotides in 25S like rRNA of prokaryotes and eukaryotes

		*	
Mirabilis jalapa	AAUGGUAAUUCAACCU	AGUACGAGAGGA	ACCGUUGUUC
Nicotiana tabacum	----------A------------------	------------------------	------------------
Oryza sativa	G----------A-----------------	------------------------	------------------
Triticcum aestivum	G--------------A-------------	------------------------	------------------
Rat	C---------C------UG-----UC	------------------------	-CA--------G---
Yeast	------A------------G-------U--	------------------------	-A----------C-----
E.coli	G-------G-----GGG------GCU	------------------ -----	-C---G—GGA—

* Indicates cleavable adenine residue in conserved region of 25S like rRNA

Fig. 3. Consensus motif found in 25S rRNA of plants

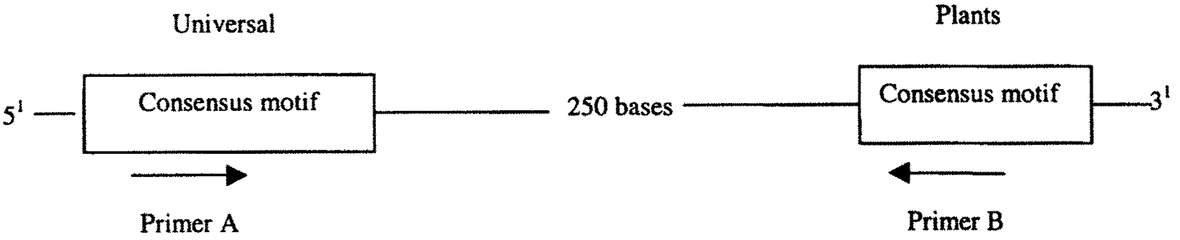


Fig. 4. Nucleotide sequence of the 25S r RNA surrounding the adenine susceptible to depurination and the position of primer 'c'

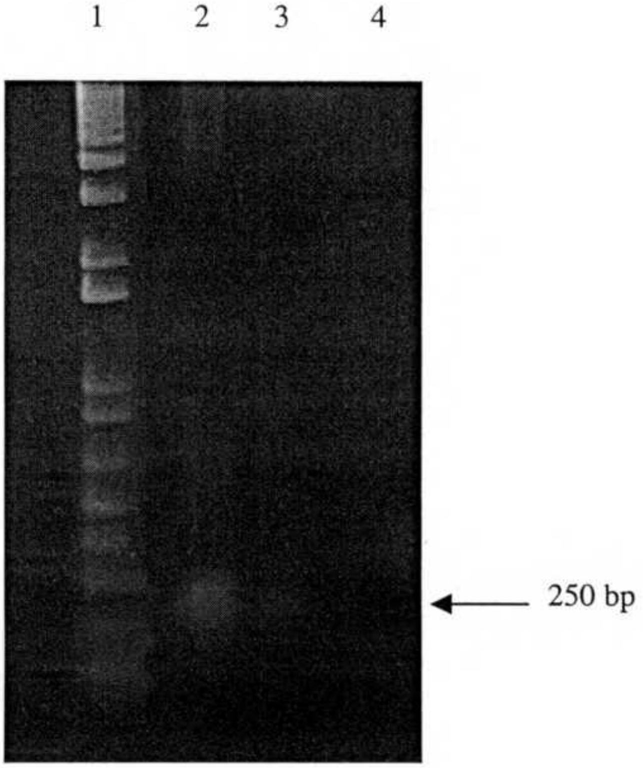


Plate 5. Analysis for the presence of conserved region in 25S rRNA of rice

Lane	**1.**	**1Kb plus ladder**
	2, 3.	**PCR product obtained with rice RNA**
	4.	**PCR product obtained with *E.coli* RNA**

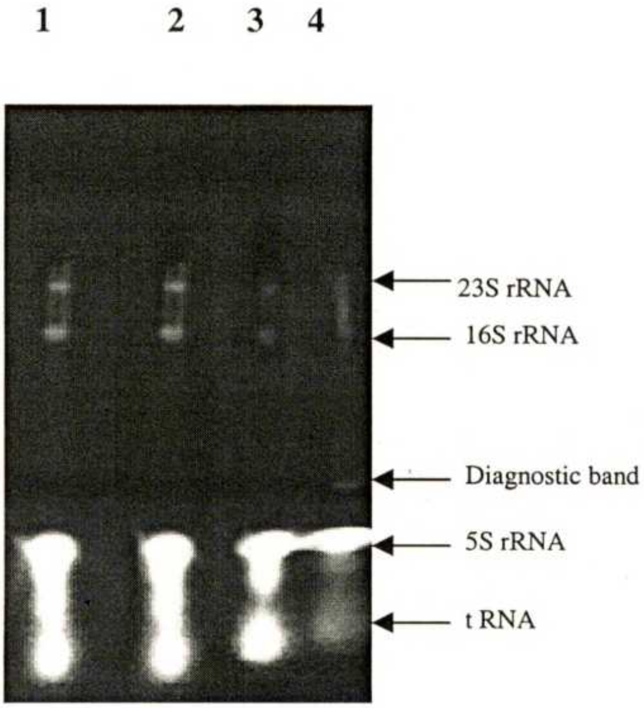


Plate 6. RNA N- glycosidase activity of BAP on *E.coli* rRNA in composite gel

Lanes **1 - *E coli* RNA (5µg)**
2 - *E coli* RNA (5µg) + BAP (30 nM)
3 - *E coli* RNA (5µg) + 1M aniline
4 - *E coli* RNA (5µg) +BAP (30 nM)+ 1 M aniline

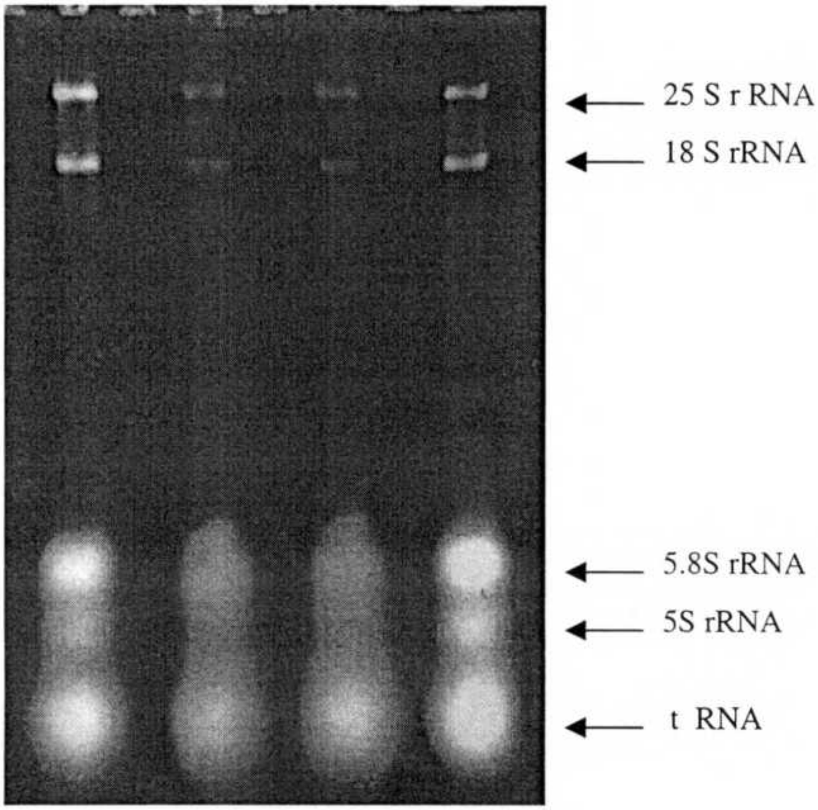


Plate 7: Test for RNA N-glycosidase activity of BAP on rice rRNA in composite gel

Lane 1. Rice RNA (5μg)
2. Rice RNA (5μg) +BAP(30 nM) + 1M aniline
3. Rice RNA (5μg) +1M aniline
4. Rice RNA (5μg) + BAP (10μg)

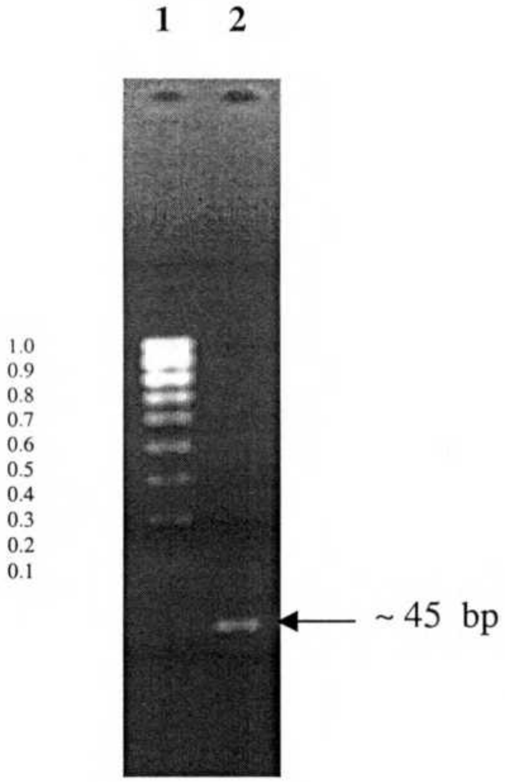


Plate 8: Confirmation of *E.coli* rRNA cleavage by primer extension analysis

Lane 1. 1Kb DNA marker

2. cDNA of E.coli RNA treated with BAP

Discussion

CHAPTER V

DISCUSSION

5.1. Purification of BAP

The study of mechanism of action of BAP required purified protein. Hence BAP was purified and used in this study.

5.1.1. Ammonium sulphate precipitation

Purification of proteins by ammonium sulfate is one of the most widely used preliminary purification procedures. It is based on the differential solubility of different proteins in salt solution. Ammonium sulphate precipitation increases the specific activity of the protein from 2 to 5 fold.

5.1.2. CM- Sepharose column chromatography

It is based on the concept that at a particular pH, most proteins have either positive or negative charge depending on the pI of the protein and hence are attracted to an opposite charge. Different proteins have differing amounts of charge and hence bind more tightly or less tightly to the oppositely charged column material. Since BAP is a basic protein, it binds firmly to the negatively charged CM-Sepharose column, whereas other contaminant proteins having negative charge (acidic proteins) will be eluted first. The elution of the bound basic proteins from the column can be achieved by raising the ionic strength of the elution buffer. In this study the proteins were eluted by increasing the NaCl concentration gradually from 25 mM to 300 mM in the elution buffer. The purified BAP thus obtained showed a single band on SDS - PAGE.

5.2. Characterization of BAP

The purity of BAP was ascertained by SDS - PAGE and native PAGE.

5.2.1. Molecular size

Appearance of BAP as a single band of 28 kDa size confirmed the presence of a single protein in the preparation obtained. This protein cross reacted with anti - BAP serum confirming the protein to be BAP. BAP cross reacted only with anti - BAP serum showing its specificity to *B.spectabilis* (Balasaraswathi, 1995). The molecular weight of BAP as determined by SDS-PAGE was about 28 kDa (Balasaraswathi *et al.*, 1998). This molecular mass of BAP is found close to other reported anti viral proteins namely MAP, PAP, PAPII, PAP-S, Dianthin 30 and Dianthin 32 whose molecular weights range from 24 to 32 kDa (Takanami *et al.*, 1990; Irvin *et al.*, 1980; Stripe *et al.*, 1981 and Barbieri *et al.*, 1982).

5.2.2. BAP on native polyacrylamide gel

The movement of proteins through native gel is based on charge and molecular weight and shape of the protein. BAP moved as a single band in acid native- PAGE confirming the presence of a single basic protein in the sample analysed. This result was in agreement with many of the AVPs *viz, Phytolacca americana, Dianthus caryophyllus, Yucca recurvifolia* (Irvin, 1975; Ragetli,1962; Verma *et al.*, 1979) that are all basic proteins.

5.3.Mechanism of action of BAP

Plant antiviral proteins have been reported to have ribosome inactivating property in addition to their antiviral activity and the antiviral property has been found to be due to their ribosome inhibiting capacity. Many AVPs like ricin A chain, modeccin A chain and Dodecandrin inhibit protein synthesis in eukaryotes by inactivating the 60S ribosomes. Hence these proteins are termed as RIPs (Habuka *et al.*, 1989) and the RIPs inactivate ribosomes by their RNA N-glycosidase activity which cleaves the N-glycosidic bond at A 4324 of 28 S rRNA of eukaryotes.

Studies on PAP mutants revealed that an intact active site of RNA N - glycosidase of a PAP is necessary for antiviral activity (Turner *et al.*, 1997). *Mirabilis* antiviral protein (MAP) has been reported to inactivate both eukaryotic and prokaryotic ribosomes by means of site specific RNA N- glycosidase activity (Habuka *et al.*, 1992). *Phytolacca* antiviral proteins have been found to inactivate both eukaryotic and prokaryotic ribosomes (Stirpe *et al.*, 1988 and Hartley *et al.*, 1991). Similarly other antiviral proteins like Dianthin 30 and 32 also inhibit translation (Hartley *et al.*, 1991).

As BAP is functionally (antiviral activity) similar to MAP, PAPs and Dianthin, the mechanism of action of BAP is expected to be similar to these antiviral proteins. BAP has been found to inhibit *in vitro* protein translation on rabbit reticulocyte lysate system (Balasaraswathi *et al.*, 1998). This suggests that BAP should also act by the mechanism of inactivating ribosomes through RNA N- glycosidase activity like any other antiviral protein. So in present study, the BAP was tested for the presence of RNA N - glycosidase activity on *E. coli*, a prokaryotic RNA and on rice, a eukaryotic RNA.

5.3.1. Isolation of RNA from *E. coli* and rice

RNA was isolated from *E.coli* and rice leaves by TRIzol method, the RNA preparations made for this study were of good quality as shown in the corresponding plates. However, there was small amount of DNA contamination.

Certain properties of cellular structure and metabolic activity in plants like presence of a durable cell wall, the relatively high abundance of polysaccharides and the accumalation of phenolics, terpenes, tannins and other secondary metabolites, which are distinctly distant from mammals, present obstacles to the purification of RNA from plant tissues. Thea *et al.*, (1996) have given a comprehensive list of published plant RNA isolation procedures. Of these, TRIzol method is followed in which DNA contamination was found to be less.

5.3.2. RNA N- glycosidase activity of BAP

In this study, when *E.coli* RNA was incubated with BAP, a diagnostic RNA band appeared on the gel after rRNAs were treated with acid aniline which indicated the presence of RNA N- glycosidase activity. The cleavage due to N- glycosidase activity could be diagnosed only after treating with 1M aniline pH 4.5. Since aniline is a electrophilic reagent, it will attack the region where depurination has already occurred. The mechanism of action of aniline may be via SE 1 mechanism. Thus the whole rRNA will be cleaved into fragments only in the presence of aniline, which then will give a diagnostic band with lower molecular weight (Habuka *et al.* 1991; Hartley *et al.*, 1991). Hence there was no separate band seen when RNA treated with BAP alone.

However with rice rRNA, such diagnostic band was not seen.

The presence of N- glycosidase activity *in B.spectabilis* has also been reported by Bolognesi *et al.* (1997). They isolated a single chain RIP from the leaves of *Bougainvillea spectabilis*. This RIP released adenine from the eukaryotic and prokaryotic ribosomes and genome RNA of artichoke mottled crinkle virus indicating the presence of poly nucleotide adenine glycosidase activity. This RIP also showed antiviral activity.

5.3.3. Presence of conserved region in 25S rRNA of rice

The adenine residue cleaved by RIPs is present in a region that is conserved through evolution being present in ribosomes from bacteria to mammals (Stirpe *et al.*, 1992). Therefore detection of presence of conserved region which has susceptible adenine residue will help to establish the susceptibility of such rRNA through the action of RIPs.

In addition to conserved region that house the cleavable adenine residue, 25S rRNA of plants were reported to contain another conserved region of approximately 250 bases downstream from the universally conserved region. Therefore PCR can be performed using primers specific to these conserved regions, wherein the product of amplification will indicate the presence of conserved region of rRNA in the study. In this study two primers 'a' and 'b' constructed based on the nucleotide sequence of the conserved region of 25S rRNA were used. When the RT-PCR reactions were carried out, a product of around 250 bp level was seen. This confirms the existence of conserved region in the 25S rRNA of rice like other plants *viz.*, *Mirabilis*, wheat, citrus etc indicating the possible susceptibility of rice ribosomes by BAP. The amplified product was not seen in *E. coli* since the primers were constructed based on the conserved 5' and 3' region of 25S rRNA in plants.

5.3.4. Confirmation of RNA N-glycosidase activity of BAP through primer extension analysis

Primer extension analysis of RNA is a useful method for quantitating specific RNA species and for mapping the location of transcriptional start sites. This technique is being widely used to identify the cleavage site of restriction enzymes and other nucleases. The cleavage site of new type II restriction endonuclease, *Str*I has been identified by primer extension analysis (Nagaraja *et al.*, 1999).

The basic protocol involves the annealing of a specific, complementary oligo nucleotide to the RNA followed by the synthesis of a cDNA copy of the RNA with reverse transcriptase. Kataoka *et al.* (1992a) have reported the RNA N-glycosidase activity of PAP through primer extension analysis. Similarly, the cleavage site of MAP was found to be A4324 of 25S rRNA through primer extension analysis (Kataoka *et al.*, 1992b). They have reported the use of a oligo nucleotide sequence complementary to the sequence 44 to 60 bases downstream from the adenine corresponding to the A4324 in rat liver 28S rRNA (which is found to be conserved) for detection of cleavage site.

In this study the same oligo nucleotide sequence was used for primer extension because BAP showed N-glycosidase activity on *E. coli* rRNA (Kataoka *et al.*, 1992a). When *E. coli* RNA was reverse transcribed with primer 'c', a low molecular weight product was obtained indicating the depurination of the specific adenine residue (A 2660) of 23S rRNA of *E.coli.*

Summary

CHAPTER VI

SUMMARY

1. An antiviral protein from the roots of *Bougainvillea spectabilis* was purified by $(NH_4)_2SO_4$ precipitation followed by CM-Sepharose cation exchange chromatography.
2. The BAP was found to be a single polypeptide chain as judged by denaturing SDS-PAGE and non - denaturing acid native gel electrophoresis.
3. BAP was a basic protein of molecular mass of about 28 kDa.
4. Total RNA isolated from *E.coli* and rice using TRIzol method was found to be of good quality compared to other RNA isolation methods.
5. The presence of conserved region in 25S rRNA, which is essentially the region of attack by ribosomal inactivating proteins was confirmed in rice.
6. BAP had RNA N-glycosidase activity on *E.coli* rRNA cleaved 23S,16S rRNAs. However, BAP could not show a specific diagnostic band with rice rRNA.
7. The inactivation of ribosomes by adenine depurination of *E. coli* rRNA by BAP was confirmed through primer extension analysis.

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