

**RIBONUCLEASE R<sub>s</sub> FROM *RHIZOPUS*  
*STOLONIFER* : STRUCTURAL  
CHARACTERISTICS AND IMMOBILIZATION**

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**UNIVERSITY OF PUNE**  
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BY

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***I salute that Madhava, the source of supreme  
bliss, whose grace makes the dumb  
eloquent and the cripple cross mountains***  
*(Gita Dhyanam, Stanza 6)*

DEDICATED TO MY PARENTS  
***Mrs. SUHASINI***  
AND  
***ARUN DESHPANDE***

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## DECLARATION

Certified that the work incorporated in the thesis entitled “ **Ribonuclease from *Rhizopus stolonifer* : Structural Characteristics and Immobilization**” submitted by **Miss. Rajashree Arun Deshpande** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. V. Shankar  
Research Guide

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*Rajashree A. Deshpande*

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## SUMMARY

Biological information is stored by DNA and manifested by proteins. RNA serves as the channel: DNA → RNA → Protein. This flow of information through RNA is controlled by enzymes catalyzing the synthesis of RNA, its modification and degradation. RNA degradation is catalyzed by RNA depolymerases, often called as “Ribonucleases (RNases)”. Besides the salvage of cellular or extracellular RNAs, RNases participate in vital cellular functions such as DNA replication, transcription and RNA processing, splicing and editing and control of translation by determining the turnover of mRNA. A few RNases exhibiting special biological actions (RISBASEs) are responsible for angiogenesis, antitumor activity, infertility and immunosuppression in animals and gametophytic self-incompatibility, fruit development and starvation rescue in plants. RNases are important analytical enzymes and have found extensive application in the determination of RNA structure. They are also used for the reduction of RNA in single cell protein preparations. RNase A, RNase T1 and barnase have served as model proteins for structural studies of globular proteins. Due to extensive applications of RNases, numerous attempts have been made to obtain highly active and stable immobilized preparations suitable for various biotechnological applications. RNase Rs, an extracellular RNase from *Rhizopus stolonifer*, is an atypical member of RNase T2 family of cyclizing RNases, which produces 2', 3'cyclic nucleotides as the major end product of RNA hydrolysis. In addition, RNase Rs is highly stable at ambient temperature and to changes in pH.

Specific biological functions of proteins and their stability is dependent on their unique and highly individualistic three dimensional structure. Quantitative studies on folding and stability of proteins reveal the magnitude and balance between different non-covalent forces and their contributions to stability. Stability of proteins is usually studied by chemical and thermal denaturation over a range of pH values. Since RNase Rs exhibited high stability, studies were carried out to (1) evaluate the influence of protein denaturants on the structure and function of RNase

Rs and (2) prepare an active and stable immobilized preparation suitable for the preparation of 2',3' cyclic nucleotides.

### **Chapter 1: General introduction**

This part comprises of literature survey with reference to RNases of T2 family, their occurrence, properties and applications.

### **Chapter 2 : Partial N-terminal sequence and crystallization of RNase Rs**

Comparison of the N-terminal sequence of the first 15 residues of RNase Rs with RNases of T2 family showed maximum homology (87%) with the N-terminal sequence of RNase Rh from *Rhizopus niveus*, 60% homology with RNase Le2 from *Lentinus edodes* and 50% homology with others. Cys10 is one of the conserved cysteines in fungal RNases from T2 family and it is also conserved in RNase Rs. Crystals of RNase Rs were obtained by hanging drop vapour diffusion method, in 30mM sodium acetate buffer pH 5.5, using 18% PEG 8000 as the precipitant. The crystals belonged to the orthorhombic space group  $P2_12_12_1$  with the unit cell dimensions of  $a = 44.27 \text{ \AA}$ ,  $b = 67.65 \text{ \AA}$  and  $c = 72.51 \text{ \AA}$ . The crystals diffracted to less than  $1.95 \text{ \AA}$ .

### **Chapter 3: Activation of RNase Rs in presence of low concentrations of urea**

RNase Rs showed approximately 2 fold increase in its activity when incubated in presence of 2M urea at  $37^\circ\text{C}$ . The increase in its activity, in presence of urea, was comparable to the activity at its optimum temperature i.e.  $45^\circ\text{C}$ . Compared to the native enzyme at  $37^\circ\text{C}$ , the  $K_m$  and  $V_{max}$  of RNase Rs at  $45^\circ\text{C}$  and in presence of 2 M urea at  $37^\circ\text{C}$  showed an increase while  $k_{cat}/K_m$  decreased. Arrhenius plots in presence and absence of urea showed a decrease in the activation energy in presence of urea. Though there was no change in the secondary structure of the protein in presence of urea, minor changes were observed in the tertiary structure. Hence, the increase in the activity of RNase Rs, in presence of 2 M urea

at 37°C, is due to the lowering of the activation energy as a result of changes in the microenvironment of the active site.

#### **Chapter 4: Chemical and thermal denaturation of RNase Rs**

The conformational stability of RNase Rs was determined by following its chemical and thermal unfolding. The denaturation of RNase Rs with urea and temperature was monitored over the pH range of 1-10, following the change in intrinsic fluorescence of the protein on excitation at 295 nm. The native protein exhibited emission  $\lambda_{\text{max}}$  around 336 nm which shifted to 355 nm on denaturation. Equilibrium unfolding with urea showed that values of  $D_{1/2}$  (5.7 M) and  $G_2^{\text{H}_2\text{O}}$  (12.8 kcal/mol) were highest at pH 5.0 and hence the maximum conformational stability of RNase Rs was observed near pH5.0, its isoelectric pH. The curves of fraction unfolded ( $f_U$ ) obtained with fluorescence and CD measurements overlapped at pH 5.0. Denaturation with guanidine hydrochloride, at pH5.0, gave similar values of  $G_2^{\text{H}_2\text{O}}$  but the  $D_{1/2}$  was 3.1 M, approximately half that of urea. Thermal denaturation of RNase Rs resulted in aggregation and precipitation of the protein above 55°C between pH 4.0 - 5.5, i.e. near the pI where the repulsive forces due to charge are minimum thus allowing the hydrophobic interaction to mediate aggregation. In the pH ranges i.e. pH 1.0 - 3.0 and pH 6.0 - 10.0 where aggregation did not take place, the  $T_m$  values of thermal denaturation showed a similar trend in comparison to  $D_{1/2}$  of urea denaturation. Thermal denaturation in presence of 2 - 5 M urea, at pH 5.0, showed a gradual decrease in  $T_m$  as well as in aggregation with increase in urea concentration. Base denaturation of RNase Rs was observed above pH 11.0, where emission  $\lambda_{\text{max}}$  shifted to 355 nm. Based on the activity regained on dilution, the unfolding in urea was reversible at all pH studied and the emission  $\lambda_{\text{max}}$  shifted back to 336 nm on dilution of the 8 M urea denatured protein, at pH 5.0. In case of thermal unfolding, based on both the spectrum and activity, >60 % reversibility was seen between pH 2.0 - 3.0 in the acidic range and pH 6.0- 9.0 on the alkaline side of the pI of RNase Rs. Both urea and temperature induced denaturation studies showed that RNase Rs unfolds through a two-state F U

mechanism. The pH dependence of stability described by  $G_2^H O(\text{urea})$  and  $G(25^\circ\text{C})$  suggested that electrostatic interactions among the charged groups make a significant contribution to the conformational stability of RNase Rs.

### **Chapter 5: Immobilization of RNase Rs**

Purified RNase Rs when covalently coupled to aminoethyl (AE) Bio-Gel P-2, via its carbohydrate moiety, retained 35-40% activity of the soluble enzyme. Optimization of coupling conditions showed that the most active immobilized preparations are obtained when 400 units of 100  $\mu\text{M}$  periodate oxidized enzyme are allowed to react with 1 ml (packed volume) of AE-Bio-Gel P-2 at  $6\pm 1^\circ\text{C}$  for 15 h. Immobilization did not change the pH and temperature optima of the enzyme but it increased the temperature stability. Immobilization did not bring about a change in the  $K_m$  but resulted in a 2.5-fold decrease in the  $V_{\text{max}}$ . Substrate concentrations as high as 25 mg of RNA could be converted to more than 80% 2', 3'cyclic nucleotides, in 14 h, at pH 5.5 and  $37^\circ\text{C}$ . On repeated use, the bound enzyme retained 70% of its initial activity after 6 cycles of use. The bound enzyme could be stored, in wet state, for 60 days without any significant loss in its initial activity. The ability of AE-Bio-Gel P-2 bound RNase to convert high concentrations of RNA to 2',3'cyclic nucleotides shows that it has the potential for the commercial production of cyclic nucleotides.

### **Publications :**

1. **Deshpande, R. A.** and Shankar, V. (1998) Immobilization of RNase Rs via its carbohydrate moiety to aminoethyl-Bio-Gel P-2 and its application for the hydrolysis of RNA to 2',3' cyclic nucleotides. *Process Biochemistry* **8**, 819-824.
2. **Deshpande, R. A.**, Kumar, A. R., Khan, M. I. and Shankar, V. (2001) Ribonuclease Rs from *Rhizopus stolonifer*: lowering of optimum temperature in the presence of urea. *Biochim. Biophys. Acta* **1545**, 13-19.

3. **Deshpande R. A.**, Khan M. I. and Shankar V. (2001) Equilibrium unfolding of RNase Rs from *Rhizopus stolonifer*: pH dependence of chemical and thermal denaturation. (manuscript in preparation).



# ***Chapter 1***

*General introduction*



## 1.1 Ribonucleases

Biological information is stored by DNA and manifested by proteins. RNA serves as the channel: DNA → RNA → Protein. This crucial flow of information is controlled by enzymes which catalyze the synthesis and modification of RNA and its degradation. RNA synthesis is catalyzed by RNA polymerases whereas its degradation by RNA depolymerases, often called as “Ribonucleases (RNases)”. Studies on RNases began when Jones (1920) discovered a thermostable enzyme in the extracts of bovine pancreas which hydrolysed RNA. Subsequently Noguchi (1924) found nucleic acid degrading enzymes in Taka-diastrase, a commercial digest prepared from *Aspergillus oryzae*. Although Otani (1935) reported the presence of RNA degrading enzymes from other fungi, the major discovery in the history of RNases was the crystallization of bovine pancreatic RNase by Kunitz (1940). The enzyme withstood several criteria of purity available at that time and became the first tool to distinguish between RNA and DNA. The discovery of Schmidt and coworkers (1951) that pancreatic RNase cleaves after pyrimidine residues extended the use of this enzyme to more detailed studies of RNA structure. Sato and Egami (1957) purified RNase T1 and T2 from Taka-diastrase and showed that the former preferentially attacked the bonds next to guanylic acid residues in RNA liberating 3’GMP via 2’,3’cyclic GMP while the latter preferentially attacked the adenylic acid linkages. Thus, for the first time, RNases with different specificities from pancreatic RNase were demonstrated in a microorganism. Since then many RNases with varying specificities have been reported from microbes, plants and animals. RNases, which cleave phosphodiester bonds in RNA to 3’mononucleotides via 2’,3’cyclic nucleotides can be classified into two groups based on their molecular masses, namely RNase T1 and RNase T2 families (Egami and Nakamura, 1969). RNase T1 family consists of low Mr enzymes of 11-12 kDa. Majority of enzymes belonging to this group are guanylic acid specific though some are purine specific or base non-specific. RNase T2 family consists of enzymes with Mr in the range of 24-36 kDa and are either non-specific or adenylic acid preferential. McClure *et al.* (1989) gave a new dimension

to the studies of T2 family RNases when they showed that the amino acid sequence of S-glycoproteins of *Nicotiana glauca*, responsible for self-incompatibility in plants, exhibit significant homology with fungal RNases like RNase T2 and RNase Rh from *Rhizopus niveus*. The two longest stretches of homology contained histidine residues implicated in the catalytic activity of RNase T2. This prompted the authors (McClure *et al.*, 1989) to investigate the RNase activity in S-glycoproteins and noted that they indeed have inherent RNase activity. Since then RNases from a wide variety of sources were shown to possess two conserved active site segments (CAS I and II) and were classified under RNase T2 family. Subsequently Irie (1999) suggested the classification of RNases based on their sequence homology and optimum pH (Table 1.1). The present compilation gives a comprehensive account of RNases of T2 family with respect to their occurrence, purification, physicochemical properties, biological role and applications.

## 1.2 Assay

RNases are usually assayed by measuring the release of acid soluble nucleotides, at 260 nm, following the hydrolysis of RNA. Unit of the enzyme is defined either on the basis of change in OD (Anfinsen *et al.*, 1954) or  $\mu$ moles nucleotides liberated (Fujimoto *et al.*, 1974). The sensitivity of the assay can be enhanced using RNA complexed with methylene blue (Greiner-Stoeffele *et al.*, 1996) ethidium bromide (Kamm *et al.*, 1970) and acridine orange (Chaplinski and Webster, 1973) or radiolabelled RNA (Mendelsohn and Young, 1978).

In the past few years, substrates facilitating sensitive assay and kinetic analyses of RNase activity have been synthesized by coupling either mono or oligonucleotides to a chromogen such that the optical absorption or fluorescence of the chromogen is enhanced several fold on cleavage of the substrate. For example, Kelemen *et al.* (1999) developed a hypersensitive fluorogenic substrate, 6-FAM-dArUdAdA-6-TAMRA, comprising of a tetranucleotide with a 6-carboxyfluorescein label (6-FAM) at the 5' end and 6-carboxy-tetramethylrhodamine (6-TAMRA) label at the 3' end. The fluorescence of

**Table 1.1 Classification of transferase type RNases**

---

**I. Alkaline RNases**

- A. *RNase T1 family*: Microbial origin; optimum pH ~7.0 - 8.0, Mr ~12 kDa, guanylic acid specific  
*e.g.* RNase T1, RNase F1, RNase N1, barnase, biRNase
- B. *RNase A family*: Vertebrate origin; Optimum pH ~7.0 - 8.0, Mr ~14 kDa, pyrimidine specific  
*e.g.* RNase A, onconase, bovine seminal RNase, non-secretory RNases from vertebrate spleen, liver, monocytes, etc.

**II. Acid RNases**

- RNase T2 family*: Mixed origin- microbes, plants, animals; optimum pH ~4.0 - 5.0, Mr >20000, base non-specific, base preference observed  
*e.g.* RNase T2, RNase Rh, S-RNases
- 

Based on Irie (1999).

fluorescein, quenched by the proximal rhodamine in the substrate, is enhanced 180 fold on cleavage of the tetranucleotide at the uridine site. In addition, these substrates can indicate the base specificity or preference of the enzyme depending on the ribonucleotide used in synthesis of the substrate.

In case of cyclizing type of RNases, the activity is determined by measuring the increase in  $A_{284}$  and  $A_{280}$  following the hydrolysis of cytidine 2',3' cyclic phosphate (Crook *et al.*, 1960) and uridine 2',3' cyclic phosphate (Richards, 1955) respectively. Unit is defined on the basis of increase in OD. Moreover, cleavage of

the ring which results in proton release formed the basis of the titrimetric assay (Stark and Stein, 1964).

### 1.3 Detection

In situ detection of the RNase activity on polyacrylamide gels is based on activity staining, which utilizes the ability of RNA to bind to dyes such as acridine orange, crystal violet, methylene blue and toluidine blue. In this method, RNA is either incorporated in the gel (Blank *et al.*, 1982) or diffused into the gel after electrophoresis (Wilson, 1969). The presence of the enzyme is indicated by a clear band against coloured RNA-dye complex background.

Schill and Schumacher (1972) developed a radial diffusion method for the quantitation of RNase activity on agarose gels, where the hydrolytic zones produced on diffusion of RNase through RNA-agar were visualized by staining with ethidium bromide. This method was employed for the qualitative detection of cultures producing RNases using Luria broth-RNA agar medium (Favre *et al.*, 1993).

### 1.4 Occurrence and localization

It is well known that ribonucleases play an important role in functioning of the cell and hence every living organism must produce one or the other type of ribonucleases. RNases from T2 family are wide spread in distribution and have been isolated from microbes, plants and animals (Table 1.2). In *A. hydrophila* and *E. coli*, the periplasmic and cytoplasmic RNases exhibit similar physical properties although some differences were observed in their sensitivity to temperature and metal ion. The cytoplasmic RNase is regarded as a form of periplasmic RNase since the *ma* insertion mutation of *E. coli* resulted in complete loss of both the cytoplasmic and periplasmic RNases (Cannistraro and Kennell, 1991). The classification of *P. polycephalum* and *D. discoideum* is ambiguous and these are classified under Myxomycetes in the plant kingdom (slime molds) and Mycetozoa in the animal kingdom (Hiramaru *et al.*, 1969). RNases Phy a and Phy b

**Table 1.2: T2 family RNases**

Enzyme	Source	Location	Reference
<b>Virus</b>			
E-ms	Classical swine fever virus (CSFV)	capsid	Schneider <i>et al.</i> , 1993
Gp44/48	Hog cholera virus (HCV)	nucleo-capsid	Hime <i>et al.</i> , 1995
Gp53	Bovine viral diarrhea virus (BVDV)	nucleo-capsid	Hime <i>et al.</i> , 1995
<b>Bacteria</b>			
RNase AH	<i>Aeromonas hydrophila</i>	periplasm & cytoplasm	Favre <i>et al.</i> , 1993
RNase I	<i>Escherichia coli</i>	periplasm	Zhu <i>et al.</i> , 1990
RNase I*	<i>Escherichia coli</i>	cytoplasm	Cannistraro and Kennell, 1991
<b>Protzoa</b>			
RNase Phy a & Phy b	<i>Physarum polycephalum</i>	extracellular	Inokuchi <i>et al.</i> , 1993
RNase I & II	<i>Tetrahymena pyriformis</i>	intracellular	Maouri and Georgatsos, 1987
RNase DdI	<i>Dictyostelium discoideum</i>	myxo-amoebae	Inokuchi <i>et al.</i> , 1998
<b>Fungi</b>			
RNase YI*	<i>Saccharomyces cerevisiae</i>	cytoplasm	Cannistraro and Kennell, 1997
RNase Rh	<i>Rhizopus niveus</i> (Gluczyme <sup>a</sup> )	extracellular	Tomoyeda <i>et al.</i> , 1969 Horiuchi <i>et al.</i> , 1988

Enzyme	Source	Location	Reference
RNase T2	<i>Aspergillus oryzae</i> (Taka-diaxase <sup>a</sup> )		Sato and Egami, 1957
RNase M	<i>Aspergillus saitoi</i> (Molsin <sup>a</sup> )		Irie, 1967
RNase Trv	<i>Trichoderma viride</i> (Cellulase T-AP <sup>a</sup> )		Inada <i>et al.</i> , 1991
RNase Irp1 & Irp2	<i>Irpex lacteus</i> (Driselase <sup>a</sup> )		Watanabe <i>et al.</i> , 1995
RNase Le2	<i>Lentinus edodes</i>	fruiting bodies	Shimada <i>et al.</i> , 1991
RNase Le37 & Le45	<i>Lentinus edodes</i>	extracellular	Kobayashi <i>et al.</i> , 1998
RNase Rs	<i>Rhizopus stolonifer</i>	extracellular	Chacko <i>et al.</i> , 1996
<i>A. niger</i> RNase	<i>Aspergillus niger</i>	extracellular	Horitsu <i>et al.</i> , 1974
RNase Ru	<i>Rhizopus niveus</i> (Gluczyme <sup>a</sup> )	extracellular	Horitsu <i>et al.</i> , 1980 Horiuchi <i>et al.</i> , 1988
<b>Plant</b>			
NaS <sub>2</sub> -RNase	<i>Nicotiana glauca</i> (Tobacco)	style	McClure <i>et al.</i> , 1989
PiS-RNase	<i>Petunia inflata</i>	style	Singh <i>et al.</i> , 1991
LpS-RNase	<i>Lycopersicon peruvianum</i> (Wild tomato)	style	Parry <i>et al.</i> , 1997a
RNase LE	<i>Lycopersicon esculentum</i> (Tomato)	extracellular	Nürnberg <i>et al.</i> , 1990
RNase LX	<i>Lycopersicon esculentum</i>	intracellular extravacuolar	Löffler <i>et al.</i> , 1992

Enzyme	Source	Location	Reference
RNase LV1, LV2 & LV3	<i>Lycopersicon esculentum</i>	vacuoles	Löffler <i>et al.</i> , 1992
RNase Tf1 & Tf2	<i>Lycopersicon esculentum</i>	developing fruit	McKeon <i>et al.</i> , 1991
RNase MC1	<i>Momordica charantia</i> (Bitter gourd)	seed	Ide <i>et al.</i> , 1991
Melonin	<i>Cucumis melo</i> (Watermelon)	seed	Rojo <i>et al.</i> , 1994a
Cusativin	<i>Cucumis sativus</i> (Cucumber)	seed	Rojo <i>et al.</i> , 1994b
RNase LC1	<i>Luffa cylindrical</i> (Sponge gourd)	seed	Watanabe <i>et al.</i> , 1990a
RNase NE	<i>Nicotiana alata</i>	style	Dodds <i>et al.</i> , 1996
RNase RNS1, RNS2 & RNS3	<i>Arabidopsis thaliana</i>		Taylor and Green, 1991
ZRNase I	<i>Zinnia elegans</i>	differentia- ting TE	Ye and Droste, 1996
ZRNase II	<i>Zinnia elegans</i>	extracellular	Ye and Droste, 1996
<b>Animal</b>			
RNase Tp	<i>Todarodes pacificus</i> (Squid)	liver caecal fluid	Kusano <i>et al.</i> , 1998 Edmonds and Roth, 1960
RNase Oy	<i>Crusdstrea grigus</i> (Oyster)		Watanabe <i>et al.</i> , 1993
RNase RCL2	<i>Rana catesbeiana</i> (Bullfrog)	liver	Yagi <i>et al.</i> , 1995
RNase CL1	Chicken	liver	Uchida <i>et al.</i> , 1996

Enzyme	Source	Location	Reference
RNase CL II	Chicken	liver	Miura <i>et al.</i> , 1984
RNase Bsp1	bovine	spleen	Ohgi <i>et al.</i> , 1988
RNase Pm	<i>Psammechinus miliaris</i> (sea urchin)	unfertilized eggs	Fernlund and Josefson. 1968
RNase Dm	<i>Drosophila melanogaster</i> (fruit fly)		Hime <i>et al.</i> , 1995
RNase Hsp1	<i>Homo sapiens</i> (human)		Trubia <i>et al.</i> , 1997

<sup>a</sup> Trade name of the commercial digest of the fungus.

were extracellular. RNase DdI was isolated from lysosomes of myxoamoebae whereas RNase I and II were isolated from the soluble fraction of detergent lysed cells. Many of the fungal RNases are isolated from commercial digests but *A. niger* RNase, RNase Rs, RNase Rh, RNase Le37 and Le45 are extracellular whereas RNase Le2 is located in the fruiting bodies of *L. edodes*.

In plants, stilar S-RNases associated with gametophytic self-incompatibility have been isolated from members of families Solanaceae *e.g.* *Nicotiana glauca* (tobacco), *Lycopersicon peruvianum* (tomato) and *Solanum tuberosum* (potato) and Rosaceae *e.g.* *Pyrus pyrifolia* (Japanese pear) and *Malus x domestica* (apple). The presence of many S-RNases is indicated on the basis of sequence homology. These extracellular S-RNases are localized to the surface of the stigmatic papillae, the extracellular matrix in the transmitting tract of the style and are also found in the walls of cells that comprise the inner epidermis of the ovary (Anderson *et al.*, 1989). Since S-RNases are controlled by a single, multiallelic S-locus, multiple



forms of S-RNases (e.g. RNases S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, S<sub>6</sub>, S<sub>7</sub> and S<sub>F11</sub> from *N. alata*) are present and it is beyond the scope of this review to discuss all of them. Hence only well characterized S-RNases from *N. alata* (McClure *et al.*, 1989), *L. peruvianum* (Parry *et al.*, 1997a) and *Petunia inflata* (Singh *et al.*, 1991), are considered here. Several extracellular and intracellular RNases, e.g. RNase LE, RNase MC1, etc. have been isolated from other tissues of self-compatible plants. These are called S-like RNases since they show sequence homology to S-RNases but are not involved in self-incompatibility.

In animals, RNases Tp, RNase RCL2 and RNase CL I and II were isolated from liver whereas RNase Bsp1 was from spleen. RNase Oy was isolated from homogenized oysters. RNases have also been reported from squid caecal fluid and mature unfertilized eggs of sea urchin.

Based on the comparison of gene sequence, protein encoded by DmRNase66B from *Drosophila melanogaster*, nucleocapsid glycoproteins gp 44/48 and gp 53 in hog cholera virus and bovine viral diarrhea virus, respectively (Hime *et al.*, 1995) and protein encoded by chromosome 6 (6q27) of human genome (Trubia *et al.*, 1997) are predicted to be RNases from T2 family.

## **1.5 Medium optimization and induction**

Most of the well characterized T2 family RNases have been purified from commercially available digests of various microorganisms and hence very few reports exist on medium optimization studies for enzyme production. Horitsu *et al.* (1974) optimized the culture conditions for extracellular RNase production by *A. niger* and noted that inorganic nitrogen sources like ammonium nitrate and ammonium chloride to be better than organic nitrogen sources. Among the inorganic nitrogen sources, ammonium nitrate gave better yield. Maximum enzyme levels (800 U/ml) were obtained in 7 days when the culture was grown in 500 ml flasks containing 50 ml synthetic medium (15% sucrose, 0.2% ammonium nitrate, 0.1% KH<sub>2</sub>PO<sub>4</sub> and 0.025% MgSO<sub>4</sub>·7H<sub>2</sub>O, pH 2.2) under shaking. Chacko *et al.* (1996) studied the influence of various medium components on the production of

extracellular RNase by *R. stolonifer* and showed that divalent metal ions had a marked effect on the growth and enzyme production. Maximum activity (3000 U/ml) was obtained in 5 days when the culture was grown in YPG medium containing  $Mg^{2+}$  (12 mM) and  $Mn^{2+}$  and  $Fe^{2+}$  (2 ppm). However, inorganic phosphate repressed enzyme production.

In plants, different physiological conditions were seen to influence the expression of RNases. The extracellular RNase LE and intracellular RNases LX, LV1, LV2 and LV3, from the suspension cultured tomato cells, were induced in response to phosphate limiting conditions created by either transfer of cells grown in phosphate rich medium to phosphate deficient medium (Nümberger *et al.*, 1990; Löffler *et al.*, 1992) or by the addition of phosphate sequestering agents such as D-mannose, 2-deoxy-D-glucose, D-galactose and glycerol, to a phosphate rich medium (Köck *et al.*, 1998). In response to phosphate limitation, RNase NE from *N. alata* was expressed in root tissue, which was not observed under the normal growth conditions (Dodds *et al.*, 1996). The expression of RNase LX gene in *L. esculentum* was induced in leaves during advanced stage of senescence (Lers *et al.*, 1998). In *A. thaliana*, RNase RNS2 was induced during senescence in leaves and petals and in response to phosphate starvation, as detected by increase in RNase RNS2 mRNA levels (Taylor *et al.*, 1993). The tomato fruit RNases, Tf1 and Tf2 displayed a developmental profile associated closely with promotion of cell growth and development (McKeon *et al.*, 1991). ZRNase I from *Z. elegans* was expressed during xylogenesis whereas ZRNase II was induced in response to wounding (Ye and Droste, 1996).

## 1.6 Purification

Many of the fungal RNases are isolated from the commercial digests of different fungi and hence the initial steps involve extraction and concentration of the crude extract followed by various purification steps. However, in case of intracellular RNases, the steps involved are, lysis of cells, isolation of organelles by differential centrifugation and concentration of the crude extract by salt or solvent

precipitation followed by conventional methods like ion exchange chromatography and gel filtration. In case of thermostable enzyme like RNase T2, heat treatment was used as one of the purification steps (Sato and Egami, 1957; Uchida, 1966). Although, ion-exchangers like DEAE- and CM-cellulose are widely used, phosphocellulose has also been used for the purification of RNase M (Irie, 1967), RNase Le2 (Shimada *et al.*, 1991) and RNase Bsp1 (Ohgi *et al.*, 1988). Moreover, inorganic supports like acid clay was employed for the purification of RNase Rh (Tomoyeda *et al.*, 1969) and *A. niger* RNase (Horitsu *et al.*, 1974) whereas calcium phosphate was used for RNase T2 (Naoi-Tada *et al.*, 1959). Hydrophobic interaction chromatography (HIC) was used for purification of S-RNase LpS3 from *L. peruvianum* (Parry *et al.*, 1997a). RNase I and I\* from *E. coli* (Cannistraro and Kennell, 1991), RNase AH (Favre *et al.*, 1993), RNase YI\* (Cannistraro and Kennell, 1997), PiS-RNases (Singh *et al.*, 1991) and cusativin (Rojo *et al.*, 1994b) were purified using FPLC as one of the steps.

Affinity chromatography has been used extensively for the purification of T2 family RNases. RNase Irp1 and Irp2 (Watanabe *et al.*, 1995), RNase Le37 (Kobayashi *et al.*, 1998), RNase Tp (Kusano *et al.*, 1998), RNase Oy (Watanabe *et al.*, 1993), RNase RCL2 (Yagi *et al.*, 1995), RNase Bsp1 (Ohgi *et al.*, 1988) and RNase DdI (Inokuchi *et al.*, 1998) were purified on Heparin-Sepharose. Other affinity adsorbents used include 2',5'-ADP-Sepharose and agarose for RNase Trv (Inada *et al.*, 1991) and RNase RCL2 (Yagi *et al.*, 1995), ApUp-agarose for RNase LE (Nürnbergger *et al.*, 1990) and RNase Phy a and Phy b (Inokuchi *et al.*, 1993), poly G- Sepharose for RNase Bsp1 (Ohgi *et al.*, 1988), UMP-Sepharose and Blue-agarose for RNase CL-II (Miura *et al.*, 1984), DNA-ECTEOA-cellulose for RNase I and II from *T. pyriformis* (Maouri and Georgatsos, 1987) and 5'-adenylate-aminohexyl-Sepharose for RNase T2 (Kanaya and Uchida, 1981). Immunoaffinity chromatography was used in case of E-ms from CSFV (Schneider *et al.*, 1993).

Crystallization was used as the final step of purification of RNase Rh. Tomoyeda *et al.* (1969) crystallized RNase Rh by dialyzing the concentrated solution of the partially purified enzyme against 100 mM sodium acetate buffer pH

5.0 containing 0.5-0.6 saturated ammonium sulfate whereas, Komiyama and Irie (1971) could crystallize the enzyme by dialysing the partially purified enzyme against distilled water.

## **1.7 Physical properties**

### *1.7.1 Molecular weight and subunit structure*

Mr of T2 family RNases fall in the range of 19 - 97 kDa but majority of them are between 20 and 40 kDa (Table 1.3). The structural protein E-ms from CSFV (Schneider *et al.*, 1993) and RNase YI\* from *S. cerevisiae* (Cannistraro and Kennell, 1997) are high molecular mass proteins with a Mr of 97 kDa and 75 kDa respectively. RNases Phy a and Phy b from *P. polycephalum* are comparatively low Mr proteins of approximately 19.7 kDa (Inokuchi *et al.*, 1993).

Most of the T2 family RNases consist of a single polypeptide chain but E-ms from CSFV (Schneider *et al.*, 1993), RNase Tf1 from developing tomato fruit (McKeon *et al.*, 1991) and RNase CL1 from Chicken liver are multimeric proteins. E-ms is a homodimer of 97 kDa and approximately half of the mature protein consists of carbohydrate (Schneider *et al.*, 1993). RNase Tf1 is a dimer of 30 kDa and 29 kDa subunits of which only 30 kDa subunit exhibits activity on renaturation after SDS-PAGE (McKeon *et al.*, 1991). RNase CL1 is a trimer made up of three peptides of 17, 19 and 163 amino acids and the Mr of the unreduced protein on SDS-PAGE is 31 kDa (Uchida *et al.*, 1996). Similarly, RNase Tp is a dimer made up of two peptides of 198 and 23 amino acids and the Mr of the unreduced protein on SDS-PAGE is 28 kDa (Kusano *et al.*, 1998).

RNase T2 separated into two fractions namely, RNase T2 and T2-L, on Sephadex G-75 but both fractions showed similar properties (Kanaya and Uchida, 1981). Moreover, RNase T2-L separated into 5 fractions, on Sephadex G-150, with all the fractions yielding single bands, on SDS-PAGE, corresponding to 145 kDa, 110 kDa, 78 kDa, 66 kDa and 43 kDa, suggesting that these are not oligomers of T2. Although the amino acid composition of these different species was found to be

**Table 1.3: Physicochemical properties of T2 family RNases**

Name	Molecular Mass (kDa)	Optimum pH	Optimum Temperature	pI	Reference
E-ms	97	6.0-6.5	55		Schneider <i>et al.</i> , 1993
RNase AH	24				Favre <i>et al.</i> , 1993
RNase I	27			8.23	Meador and Kennell, 1990
RNase I*	27				Cannistraro and Kennell, 1991
RNase YI*	75	6.2			Cannistraro and Kennell, 1997
RNase Rh	24	5.0	50-55		Tomoyeda <i>et al.</i> , 1969 Komiyama and Irie, 1971
RNase T2	36.6	4.5		5.0	Sato and Egami, 1957 Uchida, 1966
RNase M	38	4.0	50	4.7	Irie, 1967; Irie <i>et al.</i> , 1971
RNase Trv	34.5/30/27.5 <sup>a</sup>	4.5	55	3.9	Inada <i>et al.</i> , 1991
RNase Irp1	25	4.5			Watanabe <i>et al.</i> , 1995
RNase Irp2	20	4.5			Watanabe <i>et al.</i> , 1995
RNase Le2	21	4.25		4.5	Shimada <i>et al.</i> , 1991

Name	Molecular Mass (kDa)	Optimum pH	Optimum Temperature	pI	Reference
RNase Le37	37/28.14 <sup>a</sup>	3.5			Kobayashi <i>et al.</i> , 1998
RNase Le45	45	3.5			Kobayashi <i>et al.</i> , 1998
RNase Rs	28.2	5.5	45	5.0	Chacko and Shankar, 1998
<i>A. niger</i> RNase	28.5	3.5	50	2.8	Horitsu <i>et al.</i> , 1974
NaS-RNase	28-34	7.0	50	>9.0	McClure <i>et al.</i> , 1989; Jahnen <i>et al.</i> , 1989
PiS-RNase	25	7.0	50		Singh <i>et al.</i> , 1991
LpS-RNase	30.4				Royo <i>et al.</i> , 1994
RNase LE	22	5.5		3.9	Nürberger <i>et al.</i> , 1990
RNase MC1	21.2	5.5			Ide <i>et al.</i> , 1991 Numata <i>et al.</i> , 2001
Melonin		5.5			Rojo <i>et al.</i> , 1994a
Cusativin	22.9	5-7			Rojo <i>et al.</i> , 1994b
RNase LC1	19				Watanabe <i>et al.</i> , 1990a
RNase Tf1	59	6.9			McKeon <i>et al.</i> , 1991
RNase Tf2	29	6.9			McKeon <i>et al.</i> , 1991
RNase LX	26	5.9		4.3	Löffler <i>et al.</i> , 1992

Name	Molecular Mass (kDa)	Optimum pH	Optimum Temperature	pI	Reference
RNase LV1	24	5.6		4.8	Löffler <i>et al.</i> , 1992
RNase LV2	25	5.6		4.0	Löffler <i>et al.</i> , 1992
RNase LV3	23	5.6		4.1	Löffler <i>et al.</i> , 1992
ZRNase I	24				Ye and Droste, 1996
ZRNase II	22.4				Ye and Droste, 1996
RNase Tp	28	5.0			Kusano <i>et al.</i> , 1998
RNase Oy	28	5.0			Watanabe <i>et al.</i> , 1993
RNase RCL2	24.8	5.0	50-55		Yagi <i>et al.</i> , 1995
RNase CL1	31				Uchida <i>et al.</i> , 1996
RNase CL II	41	6.0			Miura <i>et al.</i> , 1984
RNase Bsp1	27/20/17 <sup>a</sup>	6.5			Ohgi <i>et al.</i> , 1988
RNase Pm	37	5.3-5.5			Ferlund and Josefson, 1968
RNase Phy a	20	4.5-4.75			Inokuchi <i>et al.</i> , 1993
RNase Phy b	20	4.5-4.75	50		Inokuchi <i>et al.</i> , 1993; 1999
RNase I & II		5.5-6.0			Maouri and Georgatsos, 1987
RNase DdI	24.8	5.0	60		Inokuchi <i>et al.</i> , 1998; 1999

<sup>a</sup> Different molecular mass species observed were due to differential glycosylation.

identical to that of RNase T2, the carbohydrate composition was different suggesting that the multiple species are due to differential glycosylation. RNase T2 separated into RNase T2-A and RNase T2-B on DEAE-cellulose and even though differentially glycosylated, these fractions exhibited the same Mr on SDS-PAGE (Uchida, 1966). The purified preparation of RNase Trv gave three closely migrating bands of Mr 34.5 kDa, 30 kDa and 27.5 kDa, on SDS-PAGE (Inada *et al.*, 1991). However, endoglycosidase F treated RNase Trv yielded a single band of 27.5 kDa suggesting that, the multiple bands observed are due to differential glycosylation. Similarly, RNase Bsp1 from bovine spleen gave two bands on SDS-PAGE corresponding to 20 kDa and 27 kDa which after deglycosylation with endoglycosidase F migrated as a single species of 17-18 kDa (Ohgi *et al.*, 1988).

### 1.7.2 Isoelectric point

The isoelectric point of majority of T2 family RNases have not been reported but in the case of some of the well characterized fungal and plant enzymes, they are in the range of 3.9 - 9.0 (Table 1.4). Most of the T2 family RNases are acidic proteins with pI in the range of 4.0-6.0. However, *A. niger* RNase is a highly acidic protein with a pI of 2.8 (Horitsu *et al.*, 1974) whereas the NaS-RNases were shown to be basic proteins although their precise pI could not be determined (Jahnen *et al.*, 1989).

### 1.7.3 Glycoprotein nature

Majority of the T2 family RNases except RNase Rh (Komiyama and Irie, 1971), RNase Le2 (Shimada *et al.*, 1991; Kobayashi *et al.*, 1998) and RNase LE (Jost *et al.*, 1991) are glycoproteins and show a wide variation in their carbohydrate content (Table 1.4). Highest carbohydrate content, nearly 50% of the molecule, was observed with E-ms from CSFV and the carbohydrate moiety is probably important for the pathogenicity of CSFV (Schneider *et al.*, 1993). The N-glycosylation is seen at the consensus sequence Asn-X-Thr/Ser whereas O-glycosylation was observed only in case of RNase Le 37 and Le 45 from *L. edodes* which exhibited



**Table 1.4: Glycoprotein Nature of T2 family RNases**

Name	Type of Glycosylation	Amount and type of sugars present	Reference
E-ms	N	50%	Schneider <i>et al.</i> , 1993
RNase Rh		non-glycosylated	Komiyama and Irie, 1971
RNase T2	N	12-15%, T2-A:mannose, glucose, galactose and xylose T2-B: mannose, galactose, glucose, xylose	Uchida, 1966.
RNase M	N	7% neutral sugars (mannose and glucose) 1.6% amino sugars (glucosamine)	Irie <i>et al.</i> , 1971
RNase Trv	N	differential, 2.1% neutral sugars, glucosamine (2.1)	Inada <i>et al.</i> , 1991
RNase Irp1	neither N nor O	2.5% neutral sugar	Watanabe <i>et al.</i> , 1995
RNase Irp2		non-glycosylated	Watanabe <i>et al.</i> , 1995
RNase Le2		non-glycosylated	Kobayashi <i>et al.</i> , 1998,
RNase Le37	N O	hexosamine (2.1), mannose (2.3) mannose (~28) at C-terminus	Kobayashi <i>et al.</i> , 1998; Inokuchi <i>et al.</i> , 2000
RNase Le45	N and O	mannose (73), glucosamine (4.3)	Kobayashi <i>et al.</i> , 1998

Name	Type of Glycosylation	Amount and type of sugars present	Reference
RNase Rs		10.5 % neutral sugar	Chacko and Shankar, 1998
<i>NaS</i> -RNase	N	differential glycosylation	Woodwind <i>et al.</i> , 1989
<i>LpS</i> <sub>3</sub> -RNase	N	differential glycosylation	Parry <i>et al.</i> , 1998
RNase LE		non-glycosylated	Jost <i>et al.</i> , 1991
RNase MC1		non glycosylated	Ide <i>et al.</i> , 1991
Cusativin		non-glycosylated	Rojo <i>et al.</i> , 1994b
RNase Tp	N	glucosamine (4), galactosamine (4)	Kusano <i>et al.</i> , 1998
RNase Oy	N	1.3-1.7 mol of glucosamine <sup>a</sup>	Watanabe <i>et al.</i> , 1993
RNase RCL2	N	hexosamine (2) <sup>a</sup>	Yagi <i>et al.</i> , 1995
RNase CL1	N	hexosamine (6) and neutral sugars(4.5)	Uchida <i>et al.</i> , 1996
RNase Bsp1		differential glycosylation	Ohgi <i>et al.</i> , 1988
RNase Phy b	N	1.5-1.8mol glucosamine <sup>a</sup>	Inokuchi <i>et al.</i> , 1993
RNase DdI	N	mannose (13), xylose (1), Glucose (1), glucosamine (3), galactosamine (1)	Inokuchi <i>et al.</i> , 1998

Numbers in parentheses correspond to the number of residues of the sugar.

<sup>a</sup> as detected from amino acid composition or sequence analysis

heavy O-mannosylation at the C-terminus rich in Ser and Thr residues (Inokuchi *et al.*, 2000). In most cases, the carbohydrate moiety is made up of glucose, mannose and glucosamine but in case of RNase T2, the carbohydrate moiety also contained galactose and xylose (Uchida, 1966).

The glycans from S-RNases were characterized extensively in view of the importance of glycans for recognition in pathology and physiology. The S-RNases from Solanaceae and Rosaceae are controlled by a single but multiallelic S-locus. S-RNases from each allele is a glycoprotein with 1 - 5 potential sites of glycosylation, as deduced from amino acid sequence based on the consensus sequence Asn-Xaa-Ser/Thr. The structural analysis of the N-glycans from S-RNases of *N. alata* showed microheterogeneity *i.e.* presence of different types of N-glycans attached to a single site (Oxley and Bacic, 1995; Woodward *et al.*, 1992; Oxley *et al.*, 1996; 1998). Microheterogeneity was also reported in case of *L. peruvianum* S<sub>3</sub>-RNase (Parry *et al.*, 1998). Since the N-glycosylation consensus sequence at site I is conserved in all S-RNases but is absent in S-like RNases, glycosylation at this site was considered important for its role in self-incompatibility. However, transgenic *P. inflata* plants of S<sub>1</sub>S<sub>2</sub> genotype, which produced normal level of non-glycosylated S<sub>3</sub> protein, acquired the ability to reject S<sub>3</sub> pollen completely, suggesting that the carbohydrate moiety does not have a role in recognition or rejection of self-pollen (Karunanandaa *et al.*, 1994).

## 1.8 Catalytic properties

### 1.8.1 Optimum pH and pH stability

The pH optima of majority of T2 family RNases are in the range of 4.0 - 6.0. The lowest optimum pH of 3.5 was exhibited by RNases Le37 and Le45 (Kobayashi *et al.*, 1998) and *A. niger* RNase (Horitsu *et al.*, 1974) whereas, the pH optima of NaS-RNases (McClure *et al.*, 1989) and PiS-RNases (Singh *et al.*, 1991) was 7.0 (Table 1.3).

The T2 family RNases, in general, exhibit low pH stability. For example, RNases Trv (Inada *et al.*, 1991), Le2 (Shimada *et al.*, 1991), DdI (Inokuchi *et al.*,

1999) and Phy a and b (Inokuchi *et al.*, 1993) were stable between pH 4.0 - 10.0, pH 3.0 - 7.0, pH 2.0 - 11.0 and pH 3.0 - 6.5 respectively, for 1 h at 37°C. RNase RCL2 was highly stable between pH 5.0 - 11.0 although it was unstable below pH 4.0 and above pH 11.0 (Yagi *et al.*, 1995). RNase T2 is most stable around neutral pH at room temperature (Uchida, 1966) whereas RNase Rh was stable above pH 5.0 at 5°C for 48 h (Tomoyeda *et al.*, 1969). RNase Rs showed high pH stability and retained significant amount of its initial activity (>85%) between pH 6.0 - 10.0 at 37°C for 12 h. RNases I and II from *T. pyriformis* were stable in 10 mM HCl for 30 min at 0°C in the presence of albumin (Maouri and Georgatsos, 1987).

### 1.8.2 Optimum temperature and temperature stability

The temperature optima of majority of T2 family RNases have not been reported but in the case of some of the well characterized enzymes, they are in the range of 45°C -60°C (Table 1.3). RNase Rs (Chacko and Shankar, 1998) exhibited the lowest optimum temperature of 45°C while the highest optimum temperature of 60°C was shown by RNase DdI (Inokuchi *et al.*, 1999).

Majority of the T2 family RNases namely, RNase Rh (Tomoyeda *et al.*, 1969), RNase Trv (Inada *et al.*, 1991), RNase Rs (Chacko and Shankar, 1998), RNase Le2 (Shimada *et al.*, 1991), RNase BsP1 (Ohgi *et al.*, 1988) and RNase DdI (Inokuchi *et al.*, 1998) were stable upto 45°C -50°C without much loss of activity. RNase M (Irie, 1967) and PiS-RNase (Singh *et al.*, 1991) were stable upto 70°C for 10 min. However, RNase YI\* from *S. cerevisiae* was the most unstable with 50 % loss of activity in 8 min and 24 seconds at 37°C and at 60°C, respectively (Cannistraro and Kennell, 1997). In contrast, RNase T2 tolerated heating at 90°C for 5 min at pH 6.0 without any loss of activity but above 90°C rapidly lost the activity (Uchida, 1966).

### 1.8.3 Effect of salt and protein denaturants

Sodium chloride >100 mM inhibited RNase T2 (Uchida, 1966) whereas the activity of *PiS*-RNases in presence of 200 mM sodium phosphate was only 20% of that with 10 mM sodium phosphate (Singh *et al.*, 1991). RNases Tf1 and TF2 retained only 50% of its activity in presence of 50 mM NaCl and total loss of activity occurred in presence of 200 mM NaCl (McKeon *et al.*, 1991). RNases I and II from *T. pyriformis* were inhibited at 200 mM and >160 mM KCl with 90% and 50% loss of activity, respectively (Maouri and Georgatsos, 1987).

RNase M from *A. saitoi* was highly resistant to urea and showed slight increase in its activity in presence of 8M urea. In contrast, 6M guanidine-HCl inactivated the enzyme (Irie *et al.*, 1971). RNase II from *T. pyriformis* showed 2 fold increase in its activity in 3 M urea whereas RNase I gradually lost its activity >1 M urea (Maouri and Georgatsos, 1987). RNase I\* from *E. coli* could degrade 5S rRNA only in presence of 1 M urea (Cannistraro and Kennell, 1991). Cusativin (Rojo *et al.*, 1994b) and melonin (Rojo *et al.*, 1994a) were active in presence of 7 M urea.

### 1.8.4 Inhibitors

Since T2 family RNases are neither metalloenzymes or metal requiring enzymes, they are not inhibited by metal chelators like EDTA. However, divalent cations like  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Hg}^{2+}$  are potent inhibitors and in a few cases  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Cd}^{2+}$  were also found to be inhibitory. Interestingly,  $\text{Zn}^{2+}$  had no effect on the activity of *NaS*<sub>2</sub>-RNase (McClure *et al.*, 1989) and RNase I from *E. coli* (Cannistraro and Kennell, 1991).

RNase T2 (Uchida and Egami, 1971), RNase Rh and RNase M (Irie, 1969) were inhibited competitively by various mononucleotides. Inhibitory effect of various nucleotides was in the order of 2'AMP>2'GMP and 3'AMP>2'(3')UMP for RNase T2 (Uchida and Egami, 1971) whereas for RNase Rh it was 2'AMP>2'GMP>5'GMP>3'GMP>3'AMP>5'CMP>2'3'cCMP>5'AMP

>2',3'cUMP>5'UMP. Additionally, the inhibitory effect of adenosine and guanosine nucleotides on RNase Rh was in the order 2'AMP>3'AMP>5'AMP and 2'GMP>3'GMP >5'GMP. The inhibition of RNase M by different nucleotides was in the order 2'AMP>2'CMP>2'GMP>2'IMP>2',3'cUMP. Furthermore, a pH dependent difference in the inhibition levels were observed with adenosine nucleotides and at pH 4.5 it was 2'AMP>3'AMP>5'AMP whereas at pH 6.5 it was in the order of 2'AMP>5'AMP>3'AMP (Komiyama and Irie, 1972). In case of RNase Rs nucleotides viz. 2'AMP, 3'AMP and 5'GMP showed only 25 - 30% inhibition under substrate limiting conditions (Chacko and Shankar, 1998).

Human placental RNase inhibitor, a potent inhibitor of pancreatic RNase, had no effect on animal acid RNases such as bovine spleen RNase Bsp1 and squid RNase Tp (Irie, 1999).

#### 1.8.5 Mode of action and substrate specificity

The T2 family RNases fall in the category of transferase type RNases and cleave RNA endonucleolytically liberating oligonucleotides and/or mononucleotides with terminal 3' phosphate via 2',3' cyclic phosphate intermediate. Majority of these enzymes hydrolyse RNA producing 3' mononucleotides as the major end-products. In contrast, RNase Rs (Chacko and Shankar, 1998) and RNase LE (Nürnbergger *et al.*, 1990) produced 2',3'cyclic nucleotides as the major end-products of RNA hydrolysis (Table 1.5).

T2 family RNases are essentially non base-specific, though they show some base preference during the initial stages of hydrolysis (Table 1.5). Base specificity or preference is determined based on (a) the rate of release of four nucleotides (2',3' cyclic and 3' mononucleotides) (b) the rate of cleavage of homopolymers and RNA and (c) hydrolysis of different dinucleoside monophosphates. Majority of the enzymes are either adenylic or guanylic acid preferential. However, E-ms (Schneider *et al.*, 1993), RCL2 (Yagi *et al.*, 1995), RNase Bsp1 (Ohgi *et al.*, 1988) are uridylic acid preferential whereas PiS-RNases (Singh *et al.*, 1991), cusativin

**Table 1.5: Reaction end products and base specificity**

Name	Base specificity and preference	Reaction end products or homopolymers hydrolysed	Reference
E-ms	U specific	<i>b</i> poly U>>>RNA>>>poly A	Schneider <i>et al.</i> , 1993
RNase AH		<i>c</i> UpA>CpA>GpA=ApA	Favre <i>et al.</i> , 1993
RNase I	C preferential	<i>c</i> CpA>>UpA	Favre <i>et al.</i> , 1993
RNase YI*		<i>b</i> polyA=polyC=polyU <i>c</i> 5'GpX	Cannistraro and Kennell, 1997
RNase Rh	A preferential	<i>a</i> AMP>GMP>>CMP>UMP <i>b</i> polyA, polyG>polyC, polyU <i>c</i> ApY, GpY>CpY, UpY	Tomoyeda <i>et al.</i> , 1969 Sanda <i>et al.</i> , 1979
RNase T2	A preferential	<i>a</i> AMP>UMP=CMP, GMP	Sato and Egami, 1957
RNase M	A preferential	<i>a</i> AMP>CMP>UMP>GMP	Irie, 1967
RNase Trv		<i>a</i> AMP>GMP>UMP>CMP	Inada <i>et al.</i> , 1991
RNase Irp1 and Irp2	A preferential	<i>a</i> AMP>GMP>>CMP>UMP	Watanabe <i>et al.</i> , 1995
RNase Le2	A preferential	<i>a</i> AMP>GMP>CMP>UMP	Shimada <i>et al.</i> , 1991
RNase Le37	A preferential	<i>a</i> AMP>GMP>CMP>UMP	Kobayashi <i>et al.</i> , 1998
RNase Le45	A preferential	<i>a</i> AMP>GMP>CMP>UMP	Kobayashi <i>et al.</i> , 1998

Name	Base specificity and preference		Reaction end products or homopolymers hydrolysed	Reference
RNase Rs	G preferential	<i>a</i>	2',3'NMP>>>3'NMP cGMP>cAMP>cUMP >cCMP	Chacko and Shankar, 1998
		<i>b</i>	polyU>RNA>polyA>polyC >polyG	
RNase An	A preferential	<i>a</i>	AMP>CMP>GMP>UMP	Horitsu <i>et al.</i> , 1974
RNase LE	G preferential	<i>a</i>	cGMP>>>cAMP>cUMP >cCMP	Ohgi <i>et al.</i> , 1997b
		<i>b</i>	polyU>polyI>polyA> polyC	
		<i>b'</i>	polyI>>>polyU> polyA>>>polyC	
		<i>c</i>	XpY, X=G>A>U>C	
RNase MC1 <sup>†</sup>	U specific	<i>a</i>	UMP>GMP>AMP>CMP	Irie <i>et al.</i> , 1993
		<i>b</i>	polyU>>>>RNA>>>polyA =polyC>polyI	
		<i>c</i>	CpU>>>UpU, ApU	
Melonin	pyrimidine specific C preferential	<i>b</i>	polyC>>>polyU	Rojo <i>et al.</i> , 1994a
Cusativin	C Preferential	<i>b</i>	polyC>>>polyA, polyU=polyG=0	Rojo <i>et al.</i> , 1994b
RNase LC1 <sup>†</sup>	U specific	<i>a</i>	UMP>GMP>AMP>CMP	Irie <i>et al.</i> , 1993



Name	Base specificity and preference	Reaction end products or homopolymers hydrolysed	Reference
		<i>b</i> polyU>>>RNA>>polyA =polyC>polyI	
		<i>c</i> CpU>>UpU	
RNase Tf1 and Tf2	pyrimidine specific, C preferential	<i>b</i> polyC>polyU>>polyA, polyG	McKeon <i>et al.</i> , 1991
RNase LV1-3	G preferential	<i>a</i> GMP>AMP>UMP>CMP <i>c</i> GpG>GpU>GpC>ApG>	Abel <i>et al.</i> , 1989
RNase Tp	G preferential	<i>a</i> GMP>AMP>UMP>CMP	Kusano <i>et al.</i> , 1998
RNase Oy	G preferential	<i>a</i> GMP>AMP>UMP	Watanabe <i>et al.</i> , 1993
RNase RCL2		UMP>GMP>AMP	Yagi <i>et al.</i> , 1995
RNase CL1	G preferential		Uchida <i>et al.</i> , 1996
RNase CL II	U specific	<i>b</i> polyU>>RNA>>polyA>polyI>polyC=0	Miura <i>et al.</i> , 1984
RNase Bsp1	pyrimidine specific, U preferential		Ohgi <i>et al.</i> , 1988
RNase Pm	G preferential		Fernlund and Josefson, 1968
RNase Phy b	G preferential	<i>a</i> GMP AMP>UMP, CMP	Inokuchi <i>et al.</i> , 1993
RNase I	pyrimidine preferential	<i>b</i> polyU>RNA>poly C>polyA >polyG=0	Maouri and Georgatsos, 1987
RNase II	purine preferential	<i>b</i> polyU>polyA>RNA>polyC =polyG=0	Maouri and Georgatsos, 1987

Name	Base specificity and preference		Reaction end products or homopolymers hydrolysed	Reference
RNase DdI	G preferential	<i>a</i>	GMP>UMP>AMP>CMP	Inokuchi <i>et al.</i> , 1998

<sup>†</sup>specific cleavage at 5' side of uridine, specificity for B2 site.

*a* order of nucleotide released

*b* order of homopolymers hydrolysed by measuring acid soluble nucleotides or hyperchromicity

*b'* order of homopolymers hydrolysed as monitored by hyperchromicity

*c* order of dinucleotides hydrolysed

(Rojo *et al.*, 1994b), melonin (Rojo *et al.*, 1994a) and RNases Tf1 and Tf2 (McKeon *et al.*, 1991) showed preference for cytidylic acid linkages. Evaluation of base specificity / preference based on the hydrolysis of homopolymers can at times be misleading because the pattern obtained with RNA can be different. For example, RNase Rs hydrolysed homopolymers and RNA in the order of poly U>RNA>poly A>>poly C and poly G was resistant to cleavage. However, with RNA, it preferentially liberated 2',3'cyclic GMP indicating its preference for guanylic acid linkages (Chacko and Shankar, 1998). The inability of the enzyme to cleave poly G was correlated to the secondary structure of the substrate under the assay conditions. In case of RNase LE, a guanylic acid preferential enzyme, the method used for quantitation based on homopolymer hydrolysis also influenced the preference e.g. values expressed in terms of acid soluble nucleotides showed higher activity on polyU whereas hyperchromicity measurements showed higher activity on poly I (Ohgi *et al.*, 1997b). RNase MC1 from seeds of *M. charantia* exhibited

absolute specificity for uridine and cleaved 5' to the phosphoribose whereas other T2 family RNases cleave 3' to the phosphoribose. (Irie *et al.*, 1993). The cytoplasmic RNases I\* from *E. coli* (Cannistraro and Kennell, 1991) and YI\* from *S. cerevisiae* (Cannistraro and Kennell, 1997) did not degrade natural RNA and in addition, 5S rRNA was cleaved only in presence of 1 M urea suggesting that these enzymes apparently have difficulty with the structured forms assumed by longer complex molecules.

## 1.9 Structure and Function

### 1.9.1 Primary Structure

The primary structure of typical RNases from RNase T2 family are shown in Fig. 1.1. One of the characteristic features of these RNases is the existence of two common sequences (CAS I and CAS II) which are conserved from viruses to animals. Amino acid residues important for the catalytic activity, such as His 46, His 104, His 109 and Glu 105 (RNase Rh numbering) are located in these segments (Irie, 1999).

In animal and plant RNases, common sequences of -WP- and -Y(F)P- are observed. In addition, in all RNases, several sequences with hydrophobic amino acids such as Leu, Ile, Phe and Tyr are conserved and probably are required to maintain the active conformation via their mutual interaction in the interior of the enzyme molecule. In general, fungal RNases have 10, bacterial RNases 6 while animal and plant RNases have 8 cysteine residues. The 4 cysteine residues forming disulfide bonds, 8C-9C and 11C-14C are common to all RNases of T2 family and hence, might have fundamental importance for maintaining the active conformation (Irie, 1999).

An important feature of the primary structure of S-RNases is the presence of hypervariable and conserved sequences, similar to those of immunoglobulins. These conserved sequences are considered important for the activity and framework of the molecule whereas the hypervariable regions are important for the

allele specificity, which is associated with hydrophilic regions. The latter are predicted to be on the surface of molecule and would be accessible for binding to





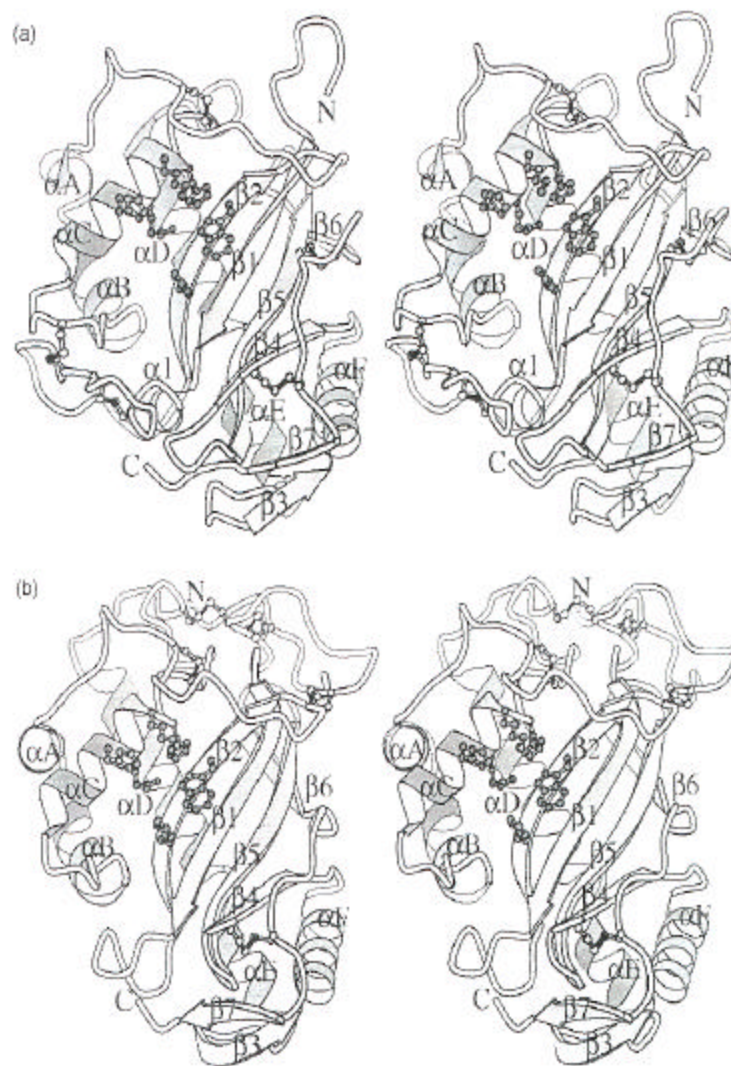


appropriate receptors in the pollen or pollen tube (Anderson *et al.*, 1989).

### 1.9.2 Three dimensional structure

RNase Rh from *R. niveus* was the first member of RNase T2 family to be crystallized. RNase Rh crystals were originally obtained by dialysis against 0.5-0.6 saturated ammonium sulfate in 100 mM sodium acetate buffer, pH 5.0 (Tomoyeda *et al.*, 1969) or cold water (Komiya and Irie, 1971). However, these crystals were rectangular plates and very thin needles, respectively, and unfit for crystallography. Kurihara *et al.* (1989) crystallized RNase Rh by vapour diffusion technique in hanging drop mode, using polyethylene glycol as the precipitant. Crystallization of RNase LE from *L. esculentum* cultured cells (Tanaka *et al.*, 2000), RNase MC1 from seeds of *M. charantia* (De and Funatsu, 1992) and S-RNase S<sub>F11</sub> from *N. alata* (Ida *et al.*, 2001) was achieved by the same technique whereas, crystals of RNase I from *E. coli* were obtained with phosphate as the precipitant (Lim *et al.*, 1993). Crystals of RNase Rh (Kurihara *et al.* 1989), RNase LE (Tanaka *et al.*, 2000) and RNase MC1 (De and Funatsu, 1992; Nakagawa *et al.*, 1999) belonged to orthorhombic space group  $P2_12_12_1$  while that of RNase I and RNase S<sub>F11</sub> belonged to the tetragonal space group  $P4_32_12$  and monoclinic space group  $P2_1$  respectively. Structure of RNase Rh (Kurihara *et al.*, 1992; 1996) and RNase LE (Tanaka *et al.*, 2000) was solved by multiple isomorphous replacement method at 2.5 Å and 1.65 Å resolution respectively, whereas the structure of RNase MC1 was solved using single isomorphous replacement method at 1.75 Å (Nakagawa *et al.*, 1999). The size of RNase Rh and RNase LE molecules were roughly 50 x 40 x 25 Å whereas that of RNase MC1 was 50 x 40 x 35 Å. A stereo view of the three dimensional structure of RNase Rh and RNase LE is shown in Fig. 1.2. The crystal structure revealed that all these enzymes belonged to the ( $\alpha$ + $\beta$ )-type class of proteins with six  $\alpha$ -helices and seven  $\beta$ -strands for RNase Rh, seven  $\alpha$ -helices and seven  $\beta$ -strands for RNase LE and ten helices (six  $\alpha$ -helices and four





**Fig. 1.2: Stereo diagram showing the overall structures of T2 family RNases.** Stereoribbon drawing of (a) RNase LE and (b) RNase Rh (Tanaka *et al.*, 2000).

$3_{10}$ -helices) and eight  $\beta$ -strands for RNase MC1. Four  $\beta$ -strands viz  $\beta 1$ ,  $\beta 2$ ,  $\beta 4$  and  $\beta 5$  in the center of the molecule form an anti-parallel  $\beta$ -sheet and the  $\alpha D$ -helix runs across this sheet, whereas  $\alpha C$ -helix runs parallel to the central sheet. The hydrophobic core in the interior of the molecule is built by Phe 43, Val 146, Phe 150, Met 166, Ile 170, Phe 174, Ile 180, Val 190 and Ile 192 (RNase Rh numbering). The overall structure of RNase LE (Tanaka *et al.*, 2000) and RNase MC1 (Nakagawa *et al.*, 1999) is similar to that of RNase Rh except for the exposed loop regions. The root mean square deviation for 142 and 151 structurally equivalent  $\alpha$ -carbon atoms of RNase LE and MC1 respectively was 1.20  $\text{\AA}$  and 1.76  $\text{\AA}$ . This showed that the structural cores of these molecules are well conserved in plant and fungal RNases. The solvent content of RNase Rh crystals was about 47% with approximately 852 water molecules in a single asymmetric unit. Seventy one water molecules out of 102 were found to have direct interactions with protein polar atoms.

### 1.9.3 Active site

#### 1.9.3.1 Chemical modification

Chemical modification studies revealed the involvement of histidine in the catalytic activity of RNase M (Harada and Irie, 1973; Irie *et al.*, 1986), RNase Rh (Komiyama and Irie, 1974; Sanda *et al.*, 1985a), RNase T2 (Kawata *et al.*, 1990), *LpS3*-RNase (Parry *et al.*, 1997a), *NaS<sub>6</sub>*-RNase (Ishimizu *et al.*, 1995) and RNase Rs (Rangarajan *et al.*, 1999). In addition, carboxylate groups have been implicated in the catalytic activity of RNase Rh (Sanda *et al.*, 1985b), RNase M (Watanabe *et al.*, 1983) and RNase Rs (Rangarajan *et al.*, 1999). Above studies also showed the involvement of lysine in the catalytic activity of RNase Rs (Rangarajan *et al.*, 1999). The presence of tryptophan at or near the active site was shown in case of RNase M (Irie *et al.*, 1972, Ohgi and Irie, 1977) and RNase Rh (Sanda and Irie, 1980).

### 1.9.3.2 Site directed mutagenesis

Site directed mutagenesis offers replacement of a single residue without affecting the overall conformation of the molecule, whereas in chemical modification the loss of activity can be due to blocking of the active site by the modification of a residue at or near the active site. Ohgi *et al.* (1992) prepared mutants of the amino acid residues viz. H46F, H104F, H109F, presumed to constitute the active site and commonly conserved amino acid residues among several T2 family RNases viz. Asp 51 and Glu 105 (Ohgi *et al.*, 1993; 1996a) of RNase Rh. Since crystallographic analysis of RNase Rh-ligand complex showed the presence of Lys 108 near the active site, Ohgi *et al.* (1995; 1996b) prepared several Lys mutants. The enzymatic activity of these mutants towards different substrates is given in Table 1.6. The replacement of His with Phe viz. H46F, H104F and H109F or Ala viz. H46A, H104A and H109A, resulted in the inactivation of the enzyme and the degree of inactivation was more pronounced for H46F and H109F (<0.02% of native RNase Rh activity) than H104F (approximately 1% activity of the native enzyme). The  $K_m$  of the H104F mutant for several substrates and  $K_i$  for 2'AMP increased, while those for other His mutants were not markedly different from the native enzyme. The substitution of either His 46 or His 109 markedly affected the catalytic activity with less effect on nucleotide binding whereas, substitution of His 104 affected both enzymatic activity as well as the binding ability to the phosphate moiety of the substrate. Hence, it was concluded that His 46 and His 109 are more important for the catalytic activity and His 104 could be the binding site for the negatively charged phosphate group of the substrate. Based on the [<sup>1</sup>H] NMR spectra of the three His mutants, the three His residues were assigned and from the titration curves for each peak in various pH ranges, pK<sub>a</sub> values of these residues were found to be 6.7, 5.9 and 6.3 for His 46, His 104 and His 109 respectively (Ohgi *et al.*, 1992). The enzymatic activities of His 46 and His 109 in the second step of the reaction were monitored using 2',3'cyclic nucleotides as substrates. H46F did not hydrolyse 2',3'cyclic nucleotides to 3'nucleotides while H109F exhibited 0.7 - 4.5% activity of native RNase Rh (Irie *et al.*, 1997).

**Table 1.6: Enzymatic activities of various mutants of RNase Rh**

Mutant enzyme	Relative activity (%) on			References
	RNA	ApU	UpU	
RNase Rh	100	100	100	Ohgi <i>et al.</i> , 1992
H46F	<0.02	0.19		Ohgi <i>et al.</i> , 1992
H104F	0.30	0.88		Ohgi <i>et al.</i> , 1992
H109F	<0.02	0.24		Ohgi <i>et al.</i> , 1992
E105Q	0.96	1.84		Ohgi <i>et al.</i> , 1993
D51N	66	0.19	31	Ohgi <i>et al.</i> , 1993
K108R	34			Ohgi <i>et al.</i> , 1995; 1996
K108L	3.1			Ohgi <i>et al.</i> , 1995; 1996

Adapted from Irie, 1999.

In presence of 13-fold excess uridine, H109F but not H46F catalyzed the synthesis of ApU from 2',3'cyclic AMP. These results showed that His 46 plays a major role in the catalytic activity of RNase Rh. The enzymatic activities of E105Q, E105D and E105A mutant enzymes decreased markedly but those of D51N, D51E and D51A decreased only slightly when RNA was used as the substrate. Since the  $K_m$  values of E105Q were very similar to those of native RNase Rh, Glu 105 was assumed to be important for catalysis. The D51N mutant had about 30% activity of the native enzyme toward UpU but it showed only 0.2% activity on ApU and in addition, this substitution caused the enzyme to be more guanylic acid preferential. Hence, Asp 51 was suggested to be one of the residues forming the base recognition site and responsible for base preference (Ohgi *et al.*, 1993). The role of Asp 51 in adenylic acid preference of RNase Rh was supported by increased activity of D51N, D51S and D51T towards GpG and poly I (Ohgi *et al.*, 1996a). Mutants of Lys 108 of RNase Rh viz K108R, K108T and K108L

showed 34%, 5% and 3% activity respectively, of the wild-type enzyme (Ohgi *et al.*, 1995; 1996b). The  $K_m$  values and rate of release of all four nucleotides from RNA by these mutant enzymes were similar to that of the wild type enzyme. Hence, it was concluded that Lys 108 participates in catalysis and not in substrate binding and the positive charge of Lys 108 is indispensable for the catalytic activity. These observations also revealed that the positively charged residue at this position is preferred and that the side-chain with the hydroxyl group, which donates protons via hydrogen binding, is better than the aliphatic side-chain (Ohgi *et al.*, 1995; 1996b). The mutants of Trp 49 such as, W49Y, W49A and W49I, showed very less activity towards both RNA and dinucleoside phosphates whereas W49F exhibited only 16% activity of the native enzyme (Ohgi *et al.*, 1996c). Therefore it was suggested that Trp 49 is very important for the enzyme activity, in particular for maintaining the active conformation of the enzyme, as it is located near the central part of the active site. In addition, the increased pyrimidine base specificity of W49F mutant indicated either direct or indirect interaction between Trp 49 and pyrimidine bases. The Tyr 57 mutants of RNase Rh, *viz.* Y57G, Y57A, Y57V, Y57I, Y57M and Y57K, showed different level of activity on RNA and dinucleoside phosphates (XpGs) whereas, the activity of the mutant enzymes Y57F and Y57W on both RNA and dinucleoside phosphates was similar to that of the wild-type enzyme. Hence, the authors concluded that an aromatic residue at the 57th position is necessary for the higher activity against dinucleoside phosphates and the stacking with base is favorable for the activity (Ohgi *et al.*, 1996c). Subsequently Ohgi *et al.* (1997a) prepared double mutants of RNase Rh and noted that D51SY57W and D51TY57W were more guanylic preferential whereas D51NW49F was more guanylic and cytidylic acid preferential. These results indicated that Asp 51, Trp 49 and Tyr 57 do not interact very strongly with each other and therefore the effect of double mutation is additive and this inturn supported the importance of Asp 51 in the adenylic acid preference of RNase Rh.

Site directed mutagenesis was employed for studying the importance of His residues in RNase activity and self-incompatibility and role of Asn residue in base

preference of S-RNases. The S<sub>3</sub>-RNase gene from *P. inflata* was mutagenized by replacing the codon for His 93 with a codon for asparagine and the mutant gene was introduced into *P. inflata* plants of S<sub>1</sub>S<sub>2</sub> genotype (Huang *et al.*, 1994). Although the level of mutant S<sub>3</sub> protein produced by transgenic plants was comparable to that of the wild-type protein, the plants failed to reject the S<sub>3</sub> pollen and the mutant S<sub>3</sub> protein did not exhibit RNase activity. These results clearly showed that gametophytic self-incompatibility in *P. inflata* involves the ribonucleolytic activity of S-glycoproteins and His 93, one of the conserved His (corresponding to His 109 of RNase Rh) is essential for the ribonucleolytic activity of S-RNases. In the natural mutant of S-RNase from *L. peruvianum*, presence of Asp in place of His 33 (the conserved His corresponding to His 46 of RNase Rh) not only resulted in the loss of RNase activity but also rendered the plant self-compatible, thus confirming the importance of His 33 for the RNase activity of S-RNases and the role of RNase activity for self-incompatibility (Royo *et al.*, 1994). Ohgi *et al.* (1997b) noted that the guanylic acid preference of the wild-type RNase LE could be changed to more adenylic preference by substitution of Asp for Asn 44 (corresponding to Asp 51 of RNase Rh), further confirming the role of Asn 44 (and Asp 51 of RNase Rh) in base recognition. RNase MC1 from seeds of *M. charantia* is characterized by its ability to specifically cleave at the 5' side of uridine (Irie *et al.*, 1993). Site directed mutagenesis was used to evaluate the contribution of the four amino acids viz. Asn 71, Val 72, Leu 73 and Arg 74, for base specificity as predicted by sequence comparison and crystallographic data (Numata *et al.*, 2001). The kinetic analyses of the mutants N71T, V72L, L73A and R74S with a dinucleoside monophosphate, CpU, showed that, compared to the wild type enzyme, N71T exhibited 7.0 fold increase in K<sub>m</sub> and 2.3fold decrease in k<sub>cat</sub> while L73A showed 14.4 fold increase in K<sub>m</sub> but with no change in k<sub>cat</sub>, indicating that Asn 71 and Leu 73 have an essential role in recognition of the uracil base. In contrast, replacements of Val 72 and Arg 74 by Leu and Ser respectively, had little effect on the enzyme activity. Moreover, mutants N71T and N71S showed a remarkable shift in its specificity i.e. from uridylic acid to guanylic acid, as evidenced by the cleavage of CpG, although they

did exhibit specificity for uridylic acid linkages in yeast RNA and homopolynucleotides. Based on these observations, Asn 71 was suggested to have at least two roles namely (a) base recognition by hydrogen bonding and (b) stabilizing the conformation of the binding site and thereby orienting the active site residues.

### 1.9.3.1 Crystallographic analysis

#### 1.9.3.1.1 Active site

Chemical modification and site-directed mutagenesis revealed the involvement His 46, His 104, His 109, Glu 105 and Lys 108 in the catalytic activity of RNase Rh. The crystal structure of RNase Rh showed that His 46 is located on the  $\beta$ 2-strand whereas His 104, Glu 105, Lys 108 are located on and His 109 just at the end of  $\alpha$ C-helix, respectively (Kurihara *et al.*, 1996) (Fig. 1.2). Thus, this centrally placed  $\alpha$ C-helix and  $\beta$ 2-strand formed the active site of RNase Rh. The same geometric arrangement was observed in RNase MC1 (Nakagawa *et al.*, 1999) and RNase LE (Tanaka *et al.*, 2000). Based on the crystallographic analysis of RNase Rh, these predicted functional residues with close proximity on  $\alpha$ C-helix and  $\beta$ 2-strand were shown to interact with each other directly (Glu 105O<sup>ε1</sup>-Trp 49N<sup>ε1</sup> = 2.93 Å, Glu 105O<sup>ε1</sup>-His 109N<sup>ε2</sup> = 3.20 Å) or via a bridging water molecule (Glu 105O<sup>ε2</sup>-Wat = 2.67 Å, His 109N<sup>ε2</sup>-Wat = 2.91 Å, His 104N<sup>ε2</sup>-Wat = 2.78 Å) (Kurihara *et al.*, 1996).

#### 1.9.3.1.2 Binding site

Crystallographic analysis of the RNase Rh-2'AMP complex indicated that Asp 51 is hydrogen bonded and the side-chains of Tyr 57 and Trp 49 are stacked with the base moiety of the nucleotide (Tanaka *et al.*, 2000). Thus, the B1 site of base recognition is formed by Trp 49, Asp 51 and Tyr 57. The indole ring of Trp 49 formed a hydrogen bond with the carboxyl group of Glu 105 and showed partial stacking interaction with the imidazole ring of His 109 (Kurihara *et al.*, 1996).

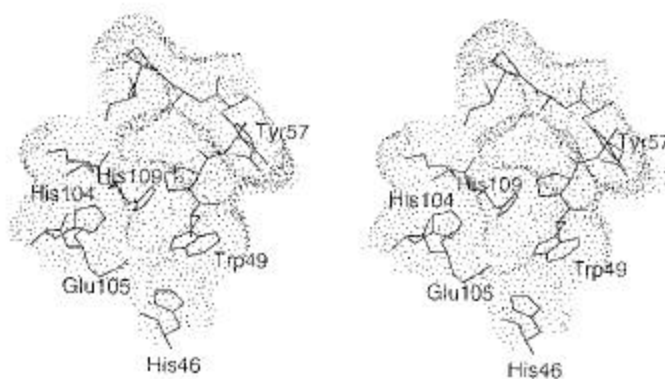
Hence, the function of Trp 49 was suggested to be fixing the orientation of the side-chains of these catalytically important residues. Another base recognition site, B2 site, adjacent to the 3' side of the B1 site, was identified by X-ray crystallography of the d(ApC)-RNase Rh complex (Tanaka *et al.*, 2000). The B2 site was shown to consist of Gln 32, Pro 92, Ser 93, Asn 94, Gln 95 and Phe 101. Moreover, Phe 101 stacked with cytosine of the dinucleoside phosphate d(ApC). Among these residues, Phe 101 and Pro 92 are essentially conserved in all T2 family RNases studied so far. The crystallographic structure of RNase Rh given by Kurihara *et al.* (1996) (Fig. 1.2) revealed that Gln 32 is located on the  $\beta$ 1-strand and Trp 49 and Asp 51 on the  $\beta$ 2-strand with Tyr 57 on the exposed loop connecting  $\beta$ 2-strand and  $\alpha$ A-helix. However, Pro 92, Ser 93, Asn 94, Gln 95 are located on the loop connecting  $\alpha$ B- and  $\alpha$ C-helices and Phe101 is located on  $\alpha$ C-helix. Thus, it can be seen that  $\alpha$ C-helix,  $\beta$ 2-strand and the exposed loops in their vicinity together form the active cleft of RNase Rh.

Kurihara *et al.* (1996) suggested that one of the two water molecules bound at active site, forming hydrogen bonds with the side-chains of His 109, Glu 105 and His 104, might indicate the binding position of the oxygen atom of the substrate phosphate group. Comparing the active site of RNase LE with that of recombinant RNase Rh, two water molecules were found to be geometrically conserved. In RNase LE, one of the two bound water molecules forms hydrogen bonds with the side chains of His 92 and Lys 96 (His 104 and Lys 108 of RNase Rh) while the other with the side-chains of Glu 93 and His 97 (Glu 105 and His 109 of RNase Rh), indicating the binding position of the oxygen atom of the substrate phosphate group. X-ray crystallographic data for the RNase Rh-2'AMP complex showed that the conserved water molecule binding site of the free enzyme is occupied by the 2'-phosphate of the inhibitor molecule. Hence, it was concluded that this conserved water molecule binding site is the P<sub>1</sub> site of RNase Rh as well as that of RNase LE (Tanaka *et al.*, 2000).



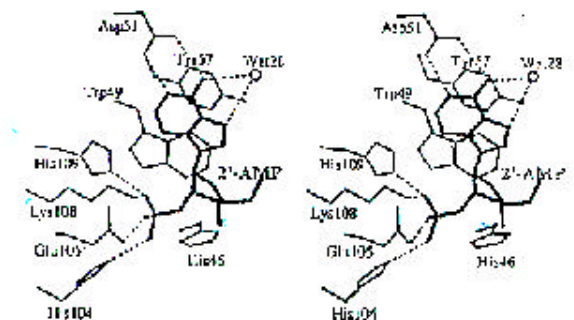
### 1.9.3.1.3 Enzyme-RNA interaction

The base recognition at the B1 site of RNase T2 family enzymes appeared to be performed mainly by hydrophobic (double-sided stacking) interactions and subsidiarily controlled by hydrogen bonds between the base and the side-chain atoms (Kurihara *et al.*, 1996). The authors proposed a model for binding of adenine to RNase Rh (Fig. 1.3; 1.4), where the base is stabilized by hydrogen bonds formed with the side-chain of Asp 51 and by sandwich-stacking interactions with the aromatic side-chains of Trp 49 and Tyr 57. Crystal structure of the d(ApC)-RNase Rh complex indicated that the base moiety of cytosine is stacked with Phe101 on one side while its other side has van der Waals contact with the side-chains of Asn94 and Gln95. Thus, the binding at the B2 site was assumed to be one-sided stacking as observed in RNase Rh (Tanaka *et al.*, 2000). In case of RNase LE, a double- sided stacking was suggested as the positions of Asn 94 and Gln 95 of



**Fig. 1.3: The molecular surface around the active site** (Kurihara *et al.*, 1996)

RNase Rh are structurally equivalent to Tyr 17 of RNase LE (Tanaka *et al.*, 2000). X-ray crystallographic studies on RNase MC1 complexed with 2'UMP and 3'UMP indicated that the side chains of Gln 9 and Asn 71 interact with O4 and N3 respectively, of uracil, by hydrogen bonding (Suzuki *et al.*, 2000). Additionally, the



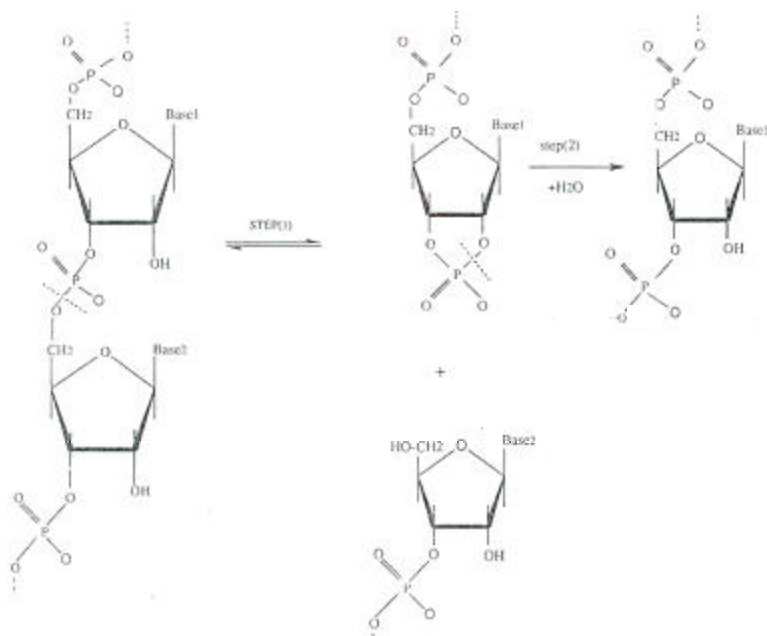
**Fig. 1.4: Stereo view of the RNase Rh-2'-AMP complex .** Adapted from Irie (1999).

side chains of Leu 73 and Phe 80 formed a hydrophobic pocket, which bound uracil by sandwich-like stacking interactions thus enhancing hydrogen bonding between the enzyme and the base.

#### 1.9.4 Mechanism of action

##### 1.9.4.1 Reaction mechanism

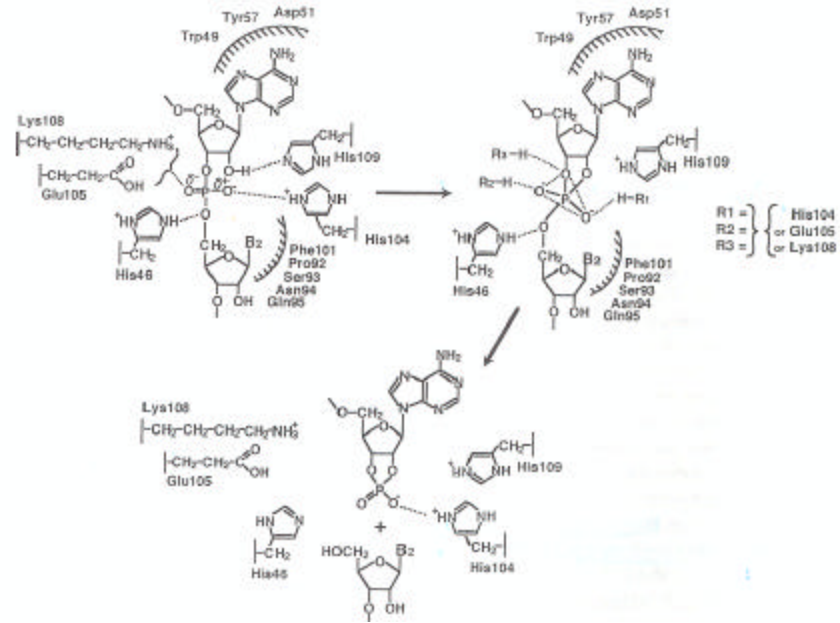
The catalytic activity of cyclizing RNases consists of two steps namely (a) transphosphorylation, resulting in formation of 2',3'cyclic phosphate and (b) hydrolysis, leading to the formation of 3'mononucleotides (Fig. 1.5). Based on chemical modification, site-directed mutagenesis and crystallographic studies, Irie *et al.* (1994; 1997) proposed the role of individual residues and the probable general acid-base mechanism in the transphosphorylation and hydrolytic steps for RNase Rh (Fig. 1.6).



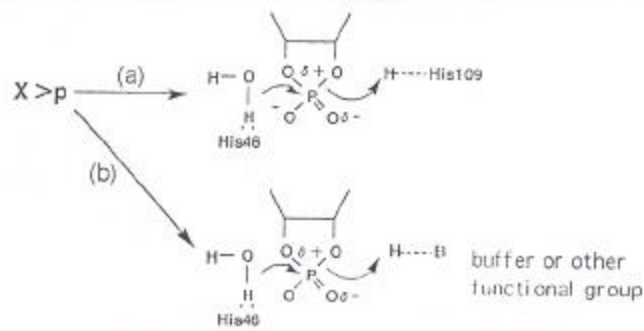
**Fig. 1.5: Catalytic mechanism of 2',3' cyclizing RNases.** The catalytic activity consists of two steps (1) transphosphorylation and (2) hydrolysis. (Adapted from Kurihara *et al.*, 1996).

In the transphosphorylation step, His 46 and His 109 act as the general acid and base respectively. His 46 which has a higher  $pK_a$  value (6.70) than His 109 ( $pK_a$  6.30) may act as a general acid catalyst which interacts with the 5' oxygen atom of the scissile phosphodiester bond and donates a proton to the departing nucleoside or nucleotide. His 109 participates as a general base catalyst which removes the hydrogen of the 2'-OH of the ribose moiety. His 104 interacts with the phosphate anion P-O, thus partially acting to polarize the P-O bond. Such an interaction will result in the positively polarized P atom being attacked by the nucleophilic 2'-oxygen, forming a pentacovalent intermediate. The nucleoside will be released from the intermediate and a cyclic nucleotide will be formed. Glu 105 may stabilize the intermediate or may interact with the oxygen atom of P=O and polarize the phosphorus atom. The only positively charged residue located around the active site is Lys 108 and therefore is expected to form a salt bridge with the phosphate group of the substrate and stabilize the transition state intermediate. In the successive step

A



B



**Fig. 1.6: Proposed mechanism of action of RNase Rh**

**A** Transphosphorylation step (Adapted from Irie, 1999)

**B** Hydrolytic step (Adapted from Irie *et al.*, 1997)

of hydrolysis, His 46 acts as a base catalyst where the unprotonated His 46 attacks or activates water and HOCH<sub>2</sub><sup>-</sup> of ribose. The activated hydroxyl group then attacks the P-O group (polarized by Glu 105 or Lys 108) and the proton may be donated by His 109 (protonated fraction of H109), now acting as a general acid, thereby resulting in formation of 3' nucleotide.

The three-dimensional disposition of the active site residues is extremely well conserved in RNase Rh and RNase LE. This geometrical conservation strongly indicates that the two enzymes share a common catalytic mechanism (Tanaka *et al.*, 2000). Since the two segments CAS I and CAS II, containing the catalytic amino acid residues, are common to all acid RNases (RNase T2 family), it is assumed that same mechanism prevails for other members of this family (Irie, 1999).

#### 1.9.4.2 Origin of base specificity

The X-ray crystallographic structure of RNase Rh, showed that the active site is rather flat without any deep clefts (Kurihara *et al.*, 1996). A small hydrophobic pocket containing the side-chains of Trp 49 and Tyr 57 around the active site is probably the base-recognition site (B1-site) as no other pocket corresponding to "sub-sites" was found. Hence, the above authors concluded that the flat interface of the enzyme with the substrate and the existence of only one base-recognition pocket may be responsible for the lack of rigid base-specificity. Based on simple modeling studies, it was postulated that this hydrophobic pocket can accommodate the purine ring of adenine and guanine but it is a little too large for the pyrimidine ring of cytosine and uracil and hence the preferential base-specificity (A>G>C>U) of the enzyme. In addition, the point mutation of Asp 51 to Asn resulted in a dramatic decrease in the affinity of the inhibitor, 2'-AMP, indicating that Asp 51 probably has direct interactions with the base of the substrate. The D51N mutant of RNase Rh showed preference for guanylic acid linkages rather than adenylic acid linkages (Ohgi *et al.*, 1993) whereas, N44D mutant of RNase LE became more adenylic acid preferential compared to wild-type

enzyme (Ohgi *et al.*, 1997b). These observations suggested that Asp 51 of RNase Rh and Asn44 of RNase LE are crucial for base-recognition and preference.

Tanaka *et al.* (2000) observed a cluster of hydrophobic residues in RNase LE which did not exist in the active site of the base-specific ribonucleases such as RNase A (pyrimidine-specific) and RNase T1 (guanine-specific). The base recognition of RNase LE appeared to depend mainly on the cluster of hydrophobic residues whereas in case of base-specific RNases, a very tight hydrogen-binding network functions in base recognition in addition to stacking with the side-chain of aromatic amino acid residues. Hence, the above authors suggested that a tight hydrogen bonding network, rather than a hydrophobic interaction (stacking of base and aromatic side-chains) is crucial for strict base specificity of RNases such as RNase A and RNase T1 and its absence for the base non-specificity of RNase LE.

The X-ray crystallographic and site directed mutagenesis studies on the uridine specific RNase MC1 suggested that amino acids at the B2 site play a more essential role in base recognition in plant RNases than the amino acid residues at the B1 site (Numata *et al.*, 2001). The rate of hydrolysis of CpU by N71S and N71D mutants increased suggesting that Ser and Asp at Asn 71 position (as observed in case of fungal and animal RNases) might be stabilizing the B2 site strongly than Asn, resulting in enhanced rate of hydrolysis. In plant RNases such as RNase LE, where unfavorable residues such as Thr are present at the position equivalent to Asn 71, an extra disulfide bond in the neighborhood of Thr was observed and was assumed to have a role equivalent to Asn 71 in stabilizing the loop. The replacement of Asn 71 by Thr or Ser, in RNase MC1, changed the specificity of the enzyme from uridine to guanosine. This shift was comparable to that of guanine specific RNases, LE and LX which have Thr and Ser at the corresponding position. Hence it was suggested that the presence of Thr or Ser in plant RNases, at positions equivalent to Asn 71 in RNase MC1, is one of the determinants responsible for the base preference of these enzymes.

## 1.10 Applications

Base non-specific RNases are mainly used for the base composition analysis of RNA. For this purpose, enzymatic digestion of RNA is preferred to alkaline hydrolysis as the latter leads to slight degradation of nucleotide bases, in particular the minor components. Moreover, alkaline hydrolysis of RNA gives rise to a mixture of 2' and 3' nucleotides, thus complicating the procedure.

The advent of immobilized enzyme technology has led to increasing efforts to replace the conventional enzymatic process with immobilized systems as immobilization offers advantages like reusability, enhanced stability, greater control of the catalytic process and the development of continuous process. Ohgi *et al.* (1974) bound RNase M to cyanogen bromide activated Sepharose and the bound enzyme showed <10% retention of activity. Efforts to increase the efficiency of the immobilized preparation by varying different parameters such as coupling pH, buffers and matrix to enzyme ratio had no effect. The bound enzyme could effectively hydrolyze RNA, suggesting that it has the potential for base analysis of RNAs.

RNase T2 has played an important role in the discovery and identification of new minor constituents of tRNA viz. 5,6 dihydrouridylic acid, uridine-5-oxyacetic acid, *N*<sup>6</sup>-methyl adenosine and *N*-9-( $\beta$ -D-ribofuranosyl)purin-6-ylcarbamoyl threonine and the syntheses of certain nucleoside-3'-phosphates such as coenzyme A (Uchida and Egami, 1971). Reddy and Shankar (1989) bound partially purified RNase T2 to ConA Sepharose through its carbohydrate moiety and the bound enzyme showed retention of activity varying from 70 % at low enzyme load to 9% at high enzyme load. Immobilization did not alter the pH and temperature optima of the enzyme but the pH stability and *K*<sub>m</sub> decreased. However, the bound enzyme exhibited enhanced stability against temperature, toxic metal ions and urea. Ease of preparation coupled with the high activity and stability of the immobilized preparation suggested that it can be employed for the base composition analysis of RNA.

RNase YI\* shows little activity against stable duplexes but can recognize and cleave short single-stranded regions of the 5S rRNA as well as base pair mismatches. Hence RNase YI\* can be used for probing RNA structure and detecting base pair mismatches in RNA as well as RNA-DNA hybrid (Cannistraro and Kennell, 1997).

## **1.11 Biological Role**

RNases play a vital role in cellular functions and in addition, T2 family RNases are reported to have a role in nutrition in fungi and plants, gametophytic self-incompatibility and defence in plants and pathogenesis in viruses.

### *1.11.1 RNA metabolism*

RNases are considered to play a prominent role in RNA metabolism by participating in vital cellular functions such as DNA replication, transcription, processing, splicing and editing of RNA and controlling translation by determining the turnover of mRNA (Deutscher, 1988). The base non-specific RNases assist the highly specific enzymes responsible for the interconversions that make up RNA metabolism. Hence, high RNase levels are observed during cell growth, organ differentiation and development. For example, RNase Tf1 and Tf2 from developing tomato fruit, display a developmental profile closely associated with promotion of cell growth and development (McKeon *et al.*, 1991). ZRNase I from *Z. elegans* was reported to be associated with differentiating tracheal elements (TE) in *Zinnia* stems (Ye and Droste, 1996).

### *1.11.2 Nutritional*

In fungi, the extracellular RNases are reported to have a role in nutrition by scavenging nucleotides and phosphate for growth and metabolism.



### 1.11.3 Phosphate remobilization

In plants, S-like RNases viz. extracellular RNase LE (Nürnberger *et al.*, 1990), vacuolar RNase LV1-LV3 and extravacuolar RNase LX from tomato cells (Löffler *et al.*, 1992), RNase from *A. thaliana* (Taylor *et al.*, 1993; Bariola *et al.*, 1994) and RNase NE from *N. alata* (Dodds *et al.*, 1996) were induced in response to phosphate limiting conditions. The induction of RNase activity was accompanied by a parallel increase in cyclic nucleotide phosphodiesterases (Abel *et al.*, 2000). The aforementioned RNases in tomato cells were induced on artificial depletion of cytoplasmic Pi pools by administering phosphate sequestering metabolites such as D-mannose, D-galactose, 2-deoxy-D-glucose and glycerol to a phosphate containing medium (Köck *et al.*, 1998). The induction of S-like RNases under Pi limiting conditions indicated that Pi remobilization may be the functional role of S-like RNases in higher plants and these RNases operate as a component of the phosphate starvation rescue system. Phosphorus, an essential structural component of many biomolecules, is one of the most important yet least available mineral nutrients required by plants (Abel *et al.*, 2000). To accomplish and maintain Pi homeostasis inside a plant, a complex system is required which includes processes of uptake, intracellular partitioning, storage, translocation and phosphate rescue from diverse substrates (Köck *et al.*, 1998). Plants often exist under Pi limiting conditions and hence it seems logical that they have RNases, inducible on phosphate deprivation, which could help scavenge Pi from the surroundings. Goldstein *et al.* (1988a; 1988b) proposed the existence of a phosphate-starvation inducible rescue system in plants, similar to the multigene emergency rescue system i.e. the *pho* regulon of *E. coli* and *S. cerevisiae*, which is turned on under conditions of phosphate deficiency.

### 1.11.4 Senescence

Senescence is a sequence of events occurring in tissues after their maturation, during which macromolecules are degraded and important nutrients transported away from the dying tissues. Thus superficially, the biochemical events

characteristic of senescence resemble those of phosphate starvation and thus it is not surprising that some hydrolytic enzymes are common to both events. RNase activity is invariably higher in infected plants than in healthy plants although this increase may be more related to physical stresses and premature senescence than to expression of pathogen-specific RNases (Green, 1994; Parry *et al.*, 1997b). RNases that are secreted could help degrade the RNA from senescing cells that have been damaged or lysed whereas, vacuolar RNases might function in the uptake of phosphate by digesting RNA present in the vacuoles, taken up via autophagy, or from cytoplasmic RNA if the vacuoles lyse. During senescence of tomato leaves, RNase LE and RNase LX genes are expressed with the expression of the latter being more pronounced (Lers *et al.*, 1998). It was shown that RNase LX is related to RNA metabolism in the final stage of senescence.

#### *1.11.5 Self-incompatibility*

Self-incompatibility is a mechanism that prevents the pollen of one plant from fertilizing other flowers of the same plant. Self-incompatibility is often observed in plants belonging to Solanaceae and Rosaceae. In self-incompatible plants, the growth of pollen tube is retarded or stopped, thereby inhibiting the delivery of male gametes to the ovary. Huang *et al.* (1994) rendered *P. inflata* plant self-compatible by mutating the catalytic His 93 of S3-RNase to Asn whereas, an inactive S-RNase from a naturally occurring self-compatible accession of *L. peruvianum* had Asn replacing a His, corresponding to the catalytically active His 46 of RNase Rh (Royo *et al.*, 1994). These observations confirmed the role of S-RNases in self-incompatibility although their precise mode of action is still not clear. McClure *et al.* (1989) suggested that S-RNases degrade rRNA, thus acting like a cytotoxin in the pollen tube.

#### *1.11.6 Defence against pathogens*

RNases produced in the pistil may function to defend the plant against pathogen attack, *e.g.* RNase RNS2 from *A. thaliana*. Eventhough the pistil is a rich

source of nutrients it is rarely colonized. This can be correlated to the combined action of RNases, proteinase inhibitors and other plant defence proteins in the pistil (Green, 1994). S-like RNases found in plant seeds *e.g.* RNase MC1 from *M. charantia* (Ide *et al.*, 1991) and RNase LC1 and LC2 from *L. cylindrica* (Irie *et al.*, 1993) are thought to protect the seeds from pathogens. Galiana *et al.* (1997) during their studies on induction of resistance of tobacco plant to leaf infection by *Phytophthora parasitica* var. *nicotianae*, noted that the cellular gene encoding extracellular S-like RNase NE is expressed rapidly after inoculation with the pathogenic fungi and this activity could control the fungal invasion. Ye and Droste (1996) observed induction of acid RNases ZRNase II on wounding in *Zinnia elegans*. This was correlated to the rapid death of the cells around the wound, with protection of the remaining tissues.

#### *1.11.7 Pathogenesis*

The envelope glycoprotein (E-ms) of classical swine fever virus (CSFV) was shown to have RNase activity (Schneider *et al.*, 1993). The E-ms inhibited the infection of porcine kidney cells with CSFV and completely inhibited concanavalin A-induced proliferation of porcine, bovine, ovine and human lymphocyte cells *in vitro*. It also inhibited protein synthesis of lymphocytes of various species, without damaging the cell membrane, leading to apoptosis. These observations suggested that this glycoprotein plays an important role in the pathogenesis of CSFV (Irie, 1999).

## Present investigation

Ribonucleases play an important role in vital cellular functions like RNA processing, splicing and editing and control of translation by determining the turnover of mRNA. Some of them, with special biological actions, have been implicated in angiogenesis, antitumor activity, infertility and immunosuppression in animals and gametophytic self-incompatibility in plants. Moreover, RNase A, RNase T1 and barnase have served as model proteins for the structural studies of globular proteins. RNases are important analytical enzymes and have found extensive application in the determination of RNA structure and also for the reduction of RNA in single cell protein preparations. Due to their extensive applications numerous attempts have been made to obtain highly active and stable immobilized preparations suitable for various biotechnological applications.

Specific biological functions of proteins and their stability is dependent on their unique and highly individualistic three dimensional structure. Quantitative studies on folding and stability of proteins reveal the magnitude and balance between different non-covalent forces and their contributions to stability. RNases from T2 family have been extensively characterized for their physicochemical and catalytic properties as well as structure and role of individual amino acids in RNA hydrolysis. RNase Rs, an extracellular RNase from *Rhizopus stolonifer*, is an atypical member of RNase T2 family of cyclizing RNases which produces 2', 3'cyclic nucleotides as the major end product of RNA hydrolysis. In addition, RNase Rs is highly stable at ambient temperature and to changes in pH. Although RNase Rs has been purified and characterized for its physicochemical and catalytic properties, no information is available regarding its structure and the conformational stability. Hence, studies were carried out to (1) evaluate the influence of protein denaturants on the structure and function of RNase Rs and (2) prepare an active and stable immobilized preparation suitable for the production of 2',3'cyclic nucleotides.



	80															100															120																					
	αB															αC															C9																					
Rh	K	S	K	D	S	S	L	Y	N	S	M	L	T	Y	W	P	S	N	Q	G	N	N	N	V	F	W	S	H	E	W	S	K	H	G	T	C	V	S	T	Y	D	P	D	C	Y	D	N	Y				
T2	Q	E	Q	G	R	T	E	L	L	S	Y	M	K	K	Y	W	P	N	Y	E	G	G	A	D	D	D	E	E	F	W	E	H	E	W	N	K	H	G	T	C	I	N	T	I	D	P	S	C	Y	K	D	Y
M	E	A	Q	D	R	T	E	L	L	S	Y	M	K	E	Y	W	P	D	Y	E	G	A	D	E	D	E	S	F	W	E	H	E	W	N	K	H	G	T	C	I	N	T	I	D	P	S	C	Y	T	D	Y	
Trv	T	A	N	G	A	D	D	T	L	Q	Y	M	Q	T	Y	W	K	D	Y	E	G	G	A	D	E	D	E	S	F	W	E	H	E	W	N	K	H	G	T	C	I	N	T	L	D	P	S	C	Y	T	D	Y
Irp	T	A	Q	G	A	S	D	T	L	D	F	M	N	Q	Y	W	V	D	I	N	G	G	A	D	Q	N	E	Q	F	S	E	H	E	W	S	K	H	G	T	C	Y	S	T	L	E	T	S	C	L	P	S	G
Le2	T	A	Q	G	A	S	D	T	L	Q	F	M	N	Q	Y	W	L	N	D	I	N	G	G	A	S	N	E	E	L	W	E	H	E	W	A	T	H	G	T	C	Y	S	T	L	Q	T	S	C	L	P	S	G
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Tp	K	P	I	E	A	E	.	.	.	.	M	R	K	Y	W	P	N	N	L	F	A	.	.	.	D	D	S	P	D	L	S	F	W	K	H	E	W	K	K	H	G	T	C	.	S	L	S	D	K	L	T	P
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CL1	K	D	.	.	.	.	L	M	S	D	M	R	R	Y	W	P	D	V	I	H	.	.	.	S	S	L	N	R	T	Q	F	W	K	H	E	W	E	K	H	G	T	C	.	A	A	T	L	P	I	L	N	
Bsp1	K	D	.	.	.	.	L	L	P	D	M	K	M	Y	W	P	D	L	L	H	.	.	.	P	S	N	S	S	L	Q	F	W	S	H	E	W	K	K	H	G	T	C	.	A	A	Q	L	.	.	N		
NaS2	G	K	.	.	.	.	K	K	N	D	L	D	E	R	W	P	D	L	T	K	.	T	K	F	D	S	L	D	K	Q	A	F	W	K	D	E	Y	V	K	H	G	T	C	.	C	C	N	D	K	F		
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αC ¾ ¾ ¾ ¾ ¾ C A S II ¾ ¾ ¾

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Irp	L	K	G	A	E	A	V	A	F	F	Q	Q	V	V	T	L	F	K	T	L	P	T	Y	Q	W	L	A	K	A	G	I	T	P	D	S	S	K	T	F	T	L	S	E	I	T	S	A	L	K	S	A	A	.	G	V	T	P			
Le2	L	K	G	A	E	A	V	A	F	F	E	Q	V	V	T	L	F	K	T	L	P	T	Y	E	W	L	T	N	Q	G	I	K	P	S	S	S	T	H	T	Y	S	A	L	T	A	A	L	E	.	A	E	A	G	V	I	P				
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I	R	.	.	D	P	.	D	A	Y	F	G	T	M	V	R	L	N	Q	E	I	K	E	S	E	A	G	K	F	L	A	D	N	Y	G	K	T	V	S	R	R	D	F	D	A	F	A	K	S	W	G	K	E	N	V	K	A				
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Bsp1	I	.	.	S	Q	R	K	Y	F	G	K	S	L	D	L	Y	K	A	L	A	L	T	S	M	L	Q	K	L	G	I	E	P	S	T	D	H	Y	Y	Q	V	S	D	I	R	D	A	L	V	.	T	V	Y	K	V	V	P				
NaS2	D	.	.	D	R	E	Q	Y	F	D	L	A	M	T	L	R	D	K	F	D	L	L	S	S	L	R	N	H	G	I	S	.	R	G	F	.	S	Y	T	V	Q	N	L	N	K	T	I	K	A	I	T	G	N	G	.	G	V	P		
PlS2	E	.	.	D	Q	E	A	Y	F	L	L	A	I	R	L	K	D	K	L	D	L	L	T	T	L	R	N	T	H	G	I	T	P	.	G	T	K	H	T	F	G	.	E	I	Q	K	A	I	K	T	V	T	N	N	K	D	P			
LpS3	D	.	.	Y	N	Q	E	Q	Y	F	D	L	A	I	E	L	K	E	K	F	D	L	L	K	T	L	K	N	H	G	I	T	P	S	K	.	.	T	N	T	V	I	D	V	E	E	A	I	K	A	V	T	K	.	E	V	.	P		
MC1	I	.	.	N	Q	A	A	Y	F	K	L	A	V	D	M	R	N	N	Y	D	I	I	G	A	L	R	P	H	A	A	G	P	N	G	.	R	T	K	S	R	Q	A	I	K	G	F	L	K	A	K	F	.	G	K	F	P				
LE	I	.	.	N	Q	H	A	Y	F	K	K	A	L	D	L	K	N	Q	I	D	L	L	S	I	L	Q	G	A	D	I	H	P	D	G	.	E	S	Y	D	L	V	N	I	R	N	A	I	K	S	A	I	.	G	Y	T	P				

αD αE β3 αF



Rh	.	.	.
T2	.	.	.
M	.	.	.
Trv	S	.	.
Irp	G	.	.
Le2	.	.	.
Phy b	S	F	.
AH	Q	K	R
I	Q	P	.
Tp	S	E	.
Oy	V	S	.
RCL2	Y	V	.
CL1	.	E	.
Bsp1	.	.	.
NaS2	.	.	.
PiS2	.	.	.
LpS3	W	.	.
MC1	.	.	.
LE	.	.	.

Rh  
T2  
M  
Trv  
Irp  
Le2  
Phy b  
AH  
I  
Tp  
Oy  
RCL2  
CL1  
Bsp1  
NaS2  
PiS2  
LpS3  
MC1  
LE

and



## ***Chapter 2***

*Partial N-terminal sequence  
and  
crystallization of RNase Rs*

## 2.1 SUMMARY

Comparison of the N-terminal sequence of the first 15 residues of RNase Rs with RNases of T2 family showed maximum (87%) homology with the N-terminal sequence of RNase Rh from *Rhizopus niveus*, 60% homology with RNase Le2 from *Lentinus edodes* and 50% homology with the others. Cys10 is one of the conserved cysteines in fungal RNases from T2 family and it is also conserved in RNase Rs. Crystals of RNase Rs were obtained by hanging drop vapour diffusion method, in 30mM sodium acetate buffer pH 5.5, using 18% PEG 8000 as the precipitant. The crystals belonged to the orthorhombic space group  $P2_12_12_1$  with the unit cell dimensions of  $a = 44.27 \text{ \AA}$ ,  $b = 67.65 \text{ \AA}$  and  $c = 72.51 \text{ \AA}$ . The crystals diffracted to less than  $1.95 \text{ \AA}$ .

## 2.2 INTRODUCTION

Ribonucleases belonging to T2 family are ubiquitous in distribution. These enzymes have Mr 20000 Da and except for self-incompatibility RNases exhibit an acidic optimum pH between 4.0 – 5.0. They are base non-specific but adenylic acid preferential and hydrolyse RNA to 3' mononucleotides via the intermediate formation of 2',3' cyclic nucleotides. McClure et al. (1989), for the first time, demonstrated that two short stretches in the amino acid sequence of S-glycoproteins of *Nicotiana glauca*, responsible for self-incompatibility in plants, show significant homology with fungal RNases like RNase T2 and RNase Rh. These segments (CAS I and CAS II), which also contain the active site residues, were shown in RNases from a wide variety of sources and have become the hallmark of T2 family RNases. Besides the conserved active site residues a few hydrophobic residues, cysteines and disulfide bridges are also conserved (Irie, 1999). These are believed to be important for maintaining the active conformation of the molecule. The crystal structure of only a few T2 family RNases viz. RNase Rh from *R. niveus* (Kurihara et al., 1996), RNase MC1 from *M. charantia* (Nakagawa et al., 1999) and RNase LE from *L. esculentum* (Tanaka et al., 2000)

has been solved. These studies revealed that the overall structure of fungal and plant RNases from T2 family is similar, despite low sequence homology and different cysteine and disulfide content, suggesting that the structural cores in the T2 family are conserved. However, the structure of T2 family RNases was found to be considerably different from the cyclizing RNases belonging to RNase A and RNase T1 families.

RNase Rs, the extracellular enzyme from *Rhizopus stolonifer* is an atypical member of T2 family because it shows preference for guanylic acid linkages and produces 2',3' cyclic nucleotides as the major end product of RNA hydrolysis (Chacko and Shankar, 1998). Although majority of cyclizing RNases show the involvement of more than two histidine residues in the catalytic activity, the active site characterization of RNase Rs revealed the involvement of a single histidine residue (Rangarajan et al, 1999). Hence, an investigation of the primary and three dimensional structure of RNase Rs will not only help in comparing the sequence and structural homology between RNase Rs and other members of RNase T2 family but also in understanding its atypical nature. This Chapter describes the partial N-terminal sequence and crystallization of RNase Rs.

## **2.3 MATERIALS**

DEAE-Cellulose (DE-52) and CM-cellulose (CM-52) (Whatman Paper Ltd., Maidstone, U.K.); polyethylene glycol (PEG) 8000, CAPS, poly (vinylidene difluoride) (PVDF) membrane and Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO, USA); uranyl acetate (Loba Chemie Pvt Ltd, Mumbai, India) and perchloric acid (Qualigens, Mumbai, India) were used. All other chemicals used were of analytical grade. Commercial yeast RNA (Sisco Research Laboratories, Mumbai, India) was purified by repeated precipitation with absolute ethanol.

## 2.4 METHODS

### 2.4.1 Determination of RNase activity

This was carried out essentially according to Chacko *et al.* (1996). The total reaction mixture of 0.75 ml contained 1.25 mg RNA in 200 mM sodium acetate buffer pH 5.5 and appropriately diluted enzyme. The reaction was initiated by the addition of RNA followed by incubation at 37°C for 15 min. The reaction was then arrested by the addition of 0.25 ml of MacFadyen's reagent (0.75 % w/v uranyl acetate in 25% v/v perchloric acid). The mixture was left on ice for 10 min and the precipitate was removed by centrifugation (2000 g, 20 min). Subsequently, 0.1 ml of the supernatant was diluted with 2.5 ml of distilled water and the acid soluble ribonucleotides were measured at 260 nm. The amount of acid soluble ribonucleotides was calculated by assuming a molar absorption coefficient of 10,600 M<sup>-1</sup>cm<sup>-1</sup> (Curtis *et al.*, 1966). One unit of RNase activity is defined as the amount of enzyme required to liberate 1µmol of acid soluble nucleotides/min under the assay conditions.

### 2.4.2 Microorganism and growth

*Rhizopus stolonifer* obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune (NCIM 880) was routinely maintained on PDA slants (potato 20 % w/v, dextrose 2 % w/v and agar 2 % w/v). Enzyme production was carried out by inoculating 100 ml of the YPG medium (yeast extract 0.1 % w/v, peptone 0.5 % w/v, glucose 2 % w/v, Mg<sup>2+</sup> 12 mM, Mn<sup>2+</sup> and Fe<sup>2+</sup> 2 ppm each) in 500 ml Erlenmeyer flask with a seven day old well sporulated slant followed by incubation at 30°C for 120 h, on a rotary shaker (150 rpm). The extracellular broth was collected by decantation and used as the source of enzyme.

### 2.4.3 Purification of RNase Rs

RNase Rs was purified to homogeneity according to Chacko and Shankar (1998). Unless otherwise stated, all the purification steps were carried out at 6±1°C.

#### 2.4.3.1 DEAE-Cellulose chromatography

The crude broth after extensive dialysis against 30 mM potassium phosphate buffer, pH 7.0, was adsorbed onto a DEAE-cellulose column (2.5 x 12 cm), pre-equilibrated at pH 7.0 with the same buffer, at a flow rate of 15 ml/h. The column was then washed with the same buffer till the flow through fractions showed no RNase activity. Subsequently, the elution of the bound enzyme was affected with a linear gradient, 300 ml total volume, of NaCl (0-500 mM) in 30 mM potassium phosphate buffer, pH 7.0. Fractions of 5 ml were collected at a flow rate of 15 ml/h and those having specific activity more than 10,000 U/mg were pooled, dialysed extensively against 30 mM sodium acetate buffer pH 5.5 and used for CM-cellulose chromatography.

#### 2.4.3.2 CM-Cellulose chromatography

The partially purified enzyme obtained from the above step was adsorbed onto a CM-cellulose column (2 x 25 cm), pre-equilibrated at pH 5.5 with 30 mM sodium acetate buffer, at a flow rate of 15 ml/h. The column was washed with the same buffer till the effluent fractions showed no RNase activity. The bound enzyme was then eluted with a linear gradient, 400 ml total volume, of NaCl (0-500 mM) in 30 mM sodium acetate buffer, pH 5.5. Fractions of 4 ml were collected and those having specific activity above 25,000 U/mg were pooled, dialysed extensively against 30 mM sodium acetate buffer, pH 5.5 and stored at -20°C. No loss of activity was observed when the enzyme was stored under this condition. The specific activity of the purified enzyme which ranged from 55,000 - 60,000 U/mg was used for further studies.

#### 2.4.4 *N-terminal analysis*

Purified RNase Rs was extensively dialysed against Milli Q water and subjected to SDS-PAGE at pH 8.3 [10% (w/v) polyacrylamide gel] according to Laemmli (1970). Subsequently, the protein was electroblotted onto a PVDF membrane, in 10 mM CAPs buffer pH 11.0 [containing 10% (v/v) methanol], under

a constant current of 250 mA for 40 min as described by LeGendre *et al.* (1993). After the electrotransfer, the PVDF membrane was washed several times with Milli Q water and stained with Coomassie Brilliant Blue R-250. The N-terminal amino-acid sequence was determined by subjecting the blot to Edman degradation on an automated protein sequencer (Applied Biosystems Procise Sequencer, USA).

#### 2.4.5 *Crystallization and characterization*

Crystallization conditions were systematically scanned using a variety of buffers and precipitants in hanging drop mode of vapour diffusion technique. Hanging drops of 10  $\mu$ l containing 5  $\mu$ l RNase Rs (5 mg/ml) and 5  $\mu$ l of varying concentrations of the precipitant were set up in 24-wells tissue culture plates (Nunc, Denmark), maintained at room temperature (20-25°C). RNase Rs crystals were obtained using 18 % (w/v) PEG 8000 as the precipitant in 30 mM sodium acetate buffer pH 5.5. Crystals were observed in 10-15 days and diffracted to about 1.95 Å. Characterization of the crystals was carried out using the X-ray intensity data collected on an Image plate detector (Marresearch, Germany) installed on a rotating anode X-ray source (Rigaku, Japan) operated at 40 kV and 70 mA (CuK $\alpha$  radiation) with a Ni monochromator and a 0.3 mm collimator.

## 2.5 RESULTS

### 2.5.1 *N-terminal sequence*

The partial N-terminal sequence of RNase Rs alongwith the partial N-terminal sequence of T2 family RNases is shown in Fig. 2.1. It was observed that out of 15 residues of RNase Rs 13 were identical to that of RNase Rh, indicating 87% homology. RNase Rs showed 60% homology with RNase Le2 from *Lentinus edodes* and 50% homology with the other T2 family RNases. Cys10 is one of the conserved cysteines in fungal RNases from T2 family and it is also conserved in RNase Rs.

	1						10						%homology						
Rs	S	Y	T	S	S	T	A	L	S	C	S	N	S	A	N				
Rh	S	S	C	S	S	T	A	L	S	C	S	N	S	A	N	87			
T2	E	F	P	S	C	P	K	D	I	P	F	S	C	Q	N	S	T	A	33
M	T	I	D	T	C	S	S	D	S	P	L	S	C	Q	T	D	N	E	40
Trv	A	S	K	T	C	P	S	N	T	P	L	S	C	H	N	T	T	V	40
Irp	V	N	S	G	C	G	T	S	G	A	E	S	C	S	N	S	D	D	53
Le2	I	S	S	G	C	G	T	T	G	A	L	S	C	S	S	N	A	K	60

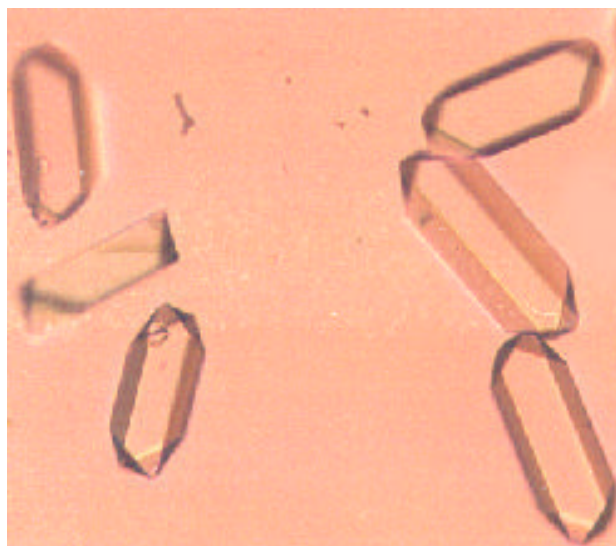
**Fig. 2.1: Comparison of N-terminal sequence of RNase Rs with other fungal RNases from T2 family.** Rs, *Rhizopus stolonifer*, Rh, *Rhizopus niveus*, T2, *Aspergillus oryzae*, M, *Aspergillus saitoi*, Trv, *Trichoderma viride*, Irp, *Irpex lacteus*, Le2, *Lentinus edodes*. Sequences of RNases Rh to Le2 and their alignment were adapted from Irie (1999). Amino acid numbering is done according to RNase Rh.

### 2.5.2 Crystallization

The successful crystallization of RNase Rs was achieved by vapour diffusion method using PEG 8000 as the precipitant. The crystals belonged to the orthorhombic space group  $P2_12_12_1$  (Fig. 2.2). The unit cell dimensions were  $a = 44.27 \text{ \AA}$ ,  $b = 67.65 \text{ \AA}$  and  $c = 72.51 \text{ \AA}$ , giving volume of the unit cell as  $2.17 \times 10^5 \text{ \AA}^3$ .

## 2.6 DISCUSSION

The partial N-terminal sequence of RNase Rs showed significant homology with RNase Rh, which is not surprising since both RNase Rs and Rh are from the same genus viz. *Rhizopus*. Both proteins exhibit similar physicochemical properties but different base preferences and reaction endproducts. Earlier Chacko and Shankar (1998) had reported a blocked N-terminus of RNase Rs since Edman degradation with



**Fig. 2.2: Crystals of RNase Rs**

a reduced pyridylethylated RNase Rs did not yield any residue till four cycles. However, in the present case the untreated enzyme did not show blocked N-terminus

suggesting that the earlier observation could be due to the pretreatment of the protein.

Crystallization of proteins has been used for either purification of the protein or preparing a product suitable for structural studies. The methods of crystallization are based on decreasing the solubility of the protein as a function of temperature, pH, ionic strength and buffer composition. Proteins and nucleic acids vary dramatically in solubility as a function of pH due to their zwitterionic nature. In general, macromolecules are most insoluble at their isoionic point and hence this becomes the most probable condition for their crystallization (McPherson, 1976). Change in pH and/or salt concentration can be achieved by various means and among them dialysis offers the easiest and gentle method. RNase Rs crystallized on dialysis of a concentrated protein solution in 30 mM potassium phosphate buffer pH 6.0 against either 30 mM sodium acetate buffer pH 5.5 or Milli Q water, with



the characteristic silky sheen of crystalline proteins. Similarly, RNase Rs crystallized when concentrated above 1 mg/ml in 30 mM sodium acetate buffer pH 5.5. These crystals were very thin rods. Similar observations have also been made in case of RNase Rh. Crystallization was used as the last step in the purification of RNase Rh and this was achieved by dialysis of the concentrated solution of partially purified enzyme against 0.5- 0.6 saturated ammonium sulfate in 100 mM sodium acetate buffer pH 5.0 (Tomoyeda *et al.*, 1969) or dialysis against cold water (Komiya and Irie, 1971). The crystals obtained by the former method were rectangular plates whereas those with the latter were thin long rods. Thus, both RNase Rs and RNase Rh were found to crystallize on dialysis at or near pH 5.0, their isoelectric point.

Crystals suitable for structural studies by either X-ray or neutron diffraction should possess a certain minimal size (linear dimensions of at least 0.2 mm), a high degree of order and a certain mechanical strength (Zeppezauer, 1971). The crystals of RNase Rs obtained on dialysis did not fulfil these criteria and hence conditions for appropriate crystal formation were determined. Subsequently, crystallization of RNase Rs was achieved by vapour diffusion method with 18 % PEG 8000 as the precipitant in 30mM sodium acetate buffer pH 5.5. The crystallization using hanging drop vapour diffusion method with PEG as the precipitant was reported for other T2 family RNases namely RNase Rh from *R. niveus* (Kurihara *et al.*, 1989), RNase LE from *L. esculentum* (Tanaka *et al.*, 2000), RNase MC1 from *M. charantia* (De and Funatsu, 1992) and S<sub>F11</sub>-RNase from *N. alata* (Ida *et al.*, 2001). The crystallization of RNase Rh and RNase MC1 was achieved using 10% PEG 8000 as the precipitant in 50 mM sodium cacodylate buffer pH6.7 and 30% PEG 8000 in sodium acetate-sodium cacodylate mixture of pH 6.6-6.8, respectively. The crystallization of RNase LE and S<sub>F11</sub>-RNase was achieved with 36% PEG1540 in citrate buffer pH 5.0 and 28-34% PEG 6000 plus 800 mM NaCl in 100 mM cacodylate buffer pH 6.5, respectively. The crystals of RNase I from *E. coli* were obtained with 2M phosphate as the precipitant (Lim *et al.*, 1993).

Crystals of RNase Rs belonged to orthorhombic space group  $P2_12_12_1$  similar to that of RNase Rh (Kurihara *et al.* 1989), RNase LE (Tanaka *et al.*, 2000) and RNase MC1 (De and Funatsu, 1992). On the contrary, crystals of RNase I (Lim *et al.*, 1993) and  $S_{F11}$ -RNase (Ida *et al.*, 2001) belonged to the tetragonal space group  $P4_32_12$  and monoclinic space group  $P2_1$  respectively.

The unit cell dimensions of RNase Rs crystals were comparable to that of RNase Rh Type II crystals. In case of RNase Rh two types of crystals were obtained which were morphologically similar but differed only in their unit cell dimensions. Type I had  $a = 68.3 \text{ \AA}$ ,  $b = 73.0 \text{ \AA}$  and  $c = 50.0 \text{ \AA}$  whereas Type II had  $a = 67.5 \text{ \AA}$ ,  $b = 72.3 \text{ \AA}$  and  $c = 44.2 \text{ \AA}$  (Kurihara *et al.*, 1989). Type I crystals were suitable for X-ray diffraction studies whereas Type II crystals could not withstand long exposure to X-rays. Kurihara *et al.* (1996) found Type I crystals to be twinned with Type II showing both types of diffraction patterns and the yield of pure Type I crystals was low. Hence Type II' crystals were obtained by modifying the earlier conditions by addition of divalent cations such as  $Mg^{2+}$ ,  $Ca^{2+}$ , and  $Ba^{2+}$  at a concentration of 10mM. The crystal morphology and cell dimensions were identical to Type II crystals but the Type II' crystals could withstand exposure to X-rays better than Type II. Although from the same space group, the cell dimensions of RNase LE and RNase MC1 crystals were slightly different. The unit cell dimensions for RNase LE crystals were  $a = 74.10 \text{ \AA}$ ,  $b = 78.72 \text{ \AA}$  and  $c = 33.00 \text{ \AA}$  (Tanaka *et al.*, 2000) whereas those for RNase MC1 crystals were  $a = 67.69 \text{ \AA}$ ,  $b = 75.42 \text{ \AA}$ ,  $c = 38.76 \text{ \AA}$  (Nakagawa *et al.*, 1999) The size of molecule of RNase Rh and RNase LE was roughly  $50 \times 40 \times 25 \text{ \AA}$  whereas that of RNase MC1 was  $50 \times 40 \times 35 \text{ \AA}$ .

## ***Chapter 3***

*Activation of RNase Rs  
in presence of low  
concentrations of urea*

### 3.1 SUMMARY

RNase Rs showed approximately 2 fold increase in its activity when incubated in presence of 2M urea at 37°C. The increase in its activity, in presence of urea, was comparable to the activity at its optimum temperature i.e. 45°C. Compared to the native enzyme at 37°C, the  $K_m$  and  $V_{max}$  of RNase Rs at 45°C and in presence of 2 M urea at 37°C showed an increase while  $k_{cat}/K_m$  decreased. Arrhenius plots in presence and absence of urea showed a decrease in the activation energy in presence of urea. Though there was no change in the secondary structure of the protein in presence of urea, minor changes were observed in the tertiary structure. Hence, the increase in the activity of RNase Rs, in presence of 2 M urea at 37°C, is due to the lowering of the activation energy as a result of changes in the microenvironment of the active site.

### 3.2 INTRODUCTION

Protein denaturants like urea and guanidine hydrochloride are reported to activate (i.e. increase the catalytic activity) enzymes such as dihydrofolate reductase (Kaufman, 1968), adenylate kinase (Zhang *et al.*, 1997), lysozyme (Chang and Carr, 1972), *Pseudomonas aeruginosa* exotoxin (Beattie and Merrill, 1996), nitrate reductase (Rigano and Violante, 1972), phosphoglucomutase (Bocchini *et al.*, 1967) and alkaline protease from white croaker fish muscle (Toyohara *et al.*, 1987). The degree of activation varies depending upon the enzyme and the denaturant used. Generally, activation is observed in presence of low concentrations of the denaturants and a subtle conformational change at the active site, preceding any conspicuous changes in the overall enzyme conformation, was attributed for the increased catalytic activity (Zhang *et al.*, 1997; Fan *et al.*, 1996).

A few nucleases are reported to be resistant to high concentrations of denaturants. SaRD, a thermostable ribonuclease from *Sulfolobus acidocaldarius* was active in presence of 7 M urea (Kulms *et al.*, 1995) while S1 nuclease from *Aspergillus oryzae*, actively degraded single stranded DNA, in cell lysates,

resulting from the action of 9 M urea in presence of SDS (Zechel and Weber, 1977). RNase II from *T. pyriformis* showed 2 fold increase in its activity with 3 M urea whereas RNase I gradually lost its activity >1 M urea (Maouri and Georgatsos, 1987). RNase I\* from *E. coli* could degrade 5S rRNA only in presence of 1 M urea (Cannistraro and Kennell, 1991). Cusativin (Rojo *et al.*, 1994b) and melonin (Rojo *et al.*, 1994a) were active in presence of 7 M urea, used for RNA sequencing. RNase Rs, from *Rhizopus stolonifer*, is an atypical member of the RNase T2 family of cyclizing RNases which produces 2',3'cyclic nucleotides as the major end products of RNA hydrolysis (Chacko and Shankar, 1998). Extensive investigations have been carried out on the influence of protein denaturants on the structure-function correlations of T1 family RNases. Although the aforementioned T2 family RNases are active in presence of denaturants, studies on their structure-function correlations in presence of denaturants do not exist. This Chapter details the influence of protein denaturants on the catalytic activity of RNase Rs.

### **3.3 MATERIALS**

Urea, guanidine hydrochloride (Gu-HCl) and poly U (Sigma Chemical Co., St. Louis, MO, USA); guanidine thiocyanate (Gu-SCN) and potassium thiocyanate (KSCN) (Merck, Darmstadt, Germany); uranyl acetate (Loba Chemie Pvt Ltd, Mumbai, India) and perchloric acid (Qualigens, Mumbai, India) were used. All other chemicals used were of analytical grade. Commercial yeast RNA (Sisco Research Laboratories, Mumbai, India) was purified by repeated precipitation with absolute ethanol.

### **3.4 METHODS**

#### *3.4.1 Determination of RNase activity*

RNase Rs was assayed at pH 5.5 and 37°C by measuring the increase in acid soluble ribonucleotides at 260 nm. The amount of acid soluble ribonucleotides was calculated by assuming a molar absorption coefficient of 10,600 M<sup>-1</sup>cm<sup>-1</sup> (Curtis *et al.*, 1966) (Chapter 2). One unit of RNase activity is defined as the

amount of enzyme required to liberate 1  $\mu$ mol of acid soluble nucleotides/min under the assay conditions.

### 3.4.2 *Purification of RNase Rs*

Cultivation of *R. stolonifer* and purification of RNase Rs was carried out as described in Chapter 2.

### 3.4.3 *Protein determination*

Protein concentrations were determined according to Lowry *et al.* (1951) using BSA as standard.

### 3.4.4 *Circular dichroism (CD) measurements*

CD spectra of RNase Rs, under various conditions, were recorded on a computer interfaced JASCO J-715 Spectropolarimeter using a cylindrical quartz cell of 1 mm and 10 mm pathlength for far-UV and near-UV spectra respectively.

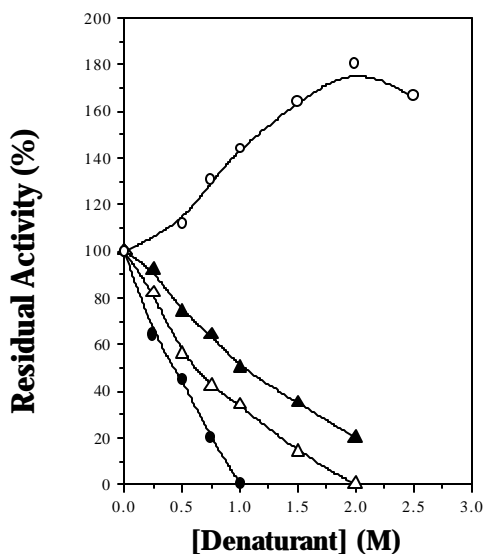
### 3.4.5 *Second derivative absorption spectra*

The second derivative absorption spectra were derived from the UV absorption spectra of RNase Rs recorded on a JASCO V-530 UV-visible Spectrophotometer under various conditions.

## 3.5 RESULTS

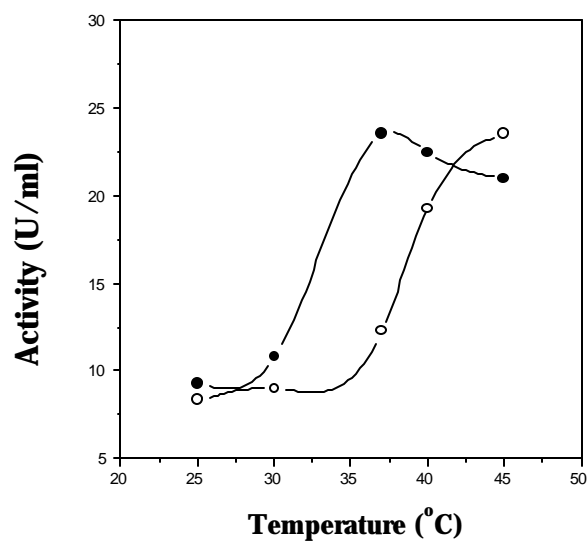
### 3.5.1 *Effect of denaturants on the catalytic activity of RNase Rs*

RNase Rs lost significant amount of its initial activity (>80%) when assayed in presence of 2 M Gu-HCl, 750 mM Gu-SCN and 1.5 M of KSCN. On the contrary, the enzyme activity increased with increasing concentration of urea and maximum activity (~1.9 fold) was observed in presence of 2 M urea (Fig 3.1).



**Fig. 3.1: Effects of urea (○), Gu-HCl (▲), Gu-SCN (△) and KSCN (●) on the activity of RNase Rs.** The activity of RNase Rs was determined under standard assay conditions (pH 5.5 and 37°C) in presence of various concentrations of the denaturants.

Influence of temperature showed that, increase in enzyme activity in presence of 2 M urea was maximum (~1.9 fold) at 37°C. Above this temperature and beyond this concentration of urea, the enzyme activity decreased (Fig 3.2). The activity of RNase Rs at its optimum temperature (45°C) was approximately 2 fold that observed at 37°C (Fig 3.2) whereas, in presence of 2 M urea at 37°C it was almost equal to that observed at 45°C without urea. Additionally, the activation at 37°C was seen only in presence of urea and the enzyme reverted to its original level of activity on dilution of urea. Similar observations were made when the enzyme activity was monitored using poly U as substrate where, the activity was 1.6 and 1.8 fold higher at 45°C and at 37°C in presence of 2 M urea respectively, compared to its activity in the absence of urea at 37°C (Table 3.1).



**Fig. 3.2: Influence of temperature on the activity of RNase Rs in the absence (○) and presence (●) of urea.** The activity of RNase Rs was determined at various temperatures in absence and presence of 2 M urea as described under Methods.

**Table 3.1: Hydrolysis of poly U with RNase Rs.**

Experimental Conditions	Relative activity (%)
Native, at 37°C	100
Native, at 45°C	158
2 M urea, at 37°C	182
2 M Gu-HCl, at 37°C	30



### 3.5.2 Kinetic studies

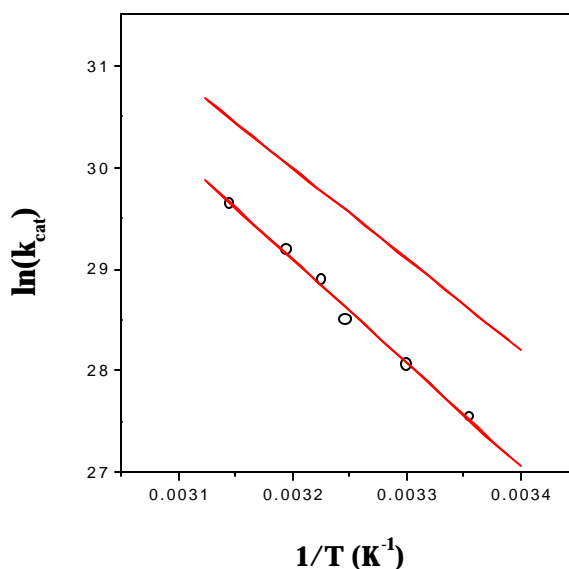
The kinetic analyses of the enzyme, in the presence and absence of 2 M urea, revealed that both  $K_m$  and  $V_{max}$  were markedly affected by the presence of urea (Table 3.2). The apparent  $K_m$  and  $V_{max}$  increased 10 fold and 3.6 fold, respectively in presence of 2 M urea at 37°C. However, at 45°C, only 3.9 fold and 2.4 fold increase in the  $K_m$  and  $V_{max}$ , respectively were observed. The catalytic efficiency ( $k_{cat}/K_m$ ), showed approximately 2 fold decrease at 45°C and in presence of 2 M urea at 37°C.

**Table 3.2: Kinetic parameters of RNase Rs in presence and absence of 2 M urea**

Experimental conditions	$K_m$ mg/ml	$V_{max}$ $\mu\text{mole}/\text{min}/\text{mg}$	$k_{cat}$ $\text{min}^{-1}$	$k_{cat}/K_m$ $\text{min}^{-1}\text{mg}^{-1}$
Native, at 37°C	0.255	97164	$2.74 \times 10^{12}$	$10.74 \times 10^{12}$
Native, at 45°C	0.993	236500	$6.67 \times 10^{12}$	$6.72 \times 10^{12}$
2 M urea, at 37°C	2.157	353000	$9.95 \times 10^{12}$	$4.6 \times 10^{12}$

Values calculated by Michaelis Menten kinetics with Enzfitter (version 1.0) program. The values given are the mean of three independent sets of experiments with S. D. less than 10 %.

The effect of temperature on the activity of RNase Rs, in presence and absence of 2 M urea, is shown in Fig 3.3. In the temperature range studied (25°C-45°C) the Arrhenius plots were linear and a decrease in the slope, corresponding to the lowering of the activation energy, in presence of urea was observed. Activation energies calculated from these plots were 20.2 Kcal/mol for native RNase Rs and 17.8 Kcal /mol in presence of 2 M urea.

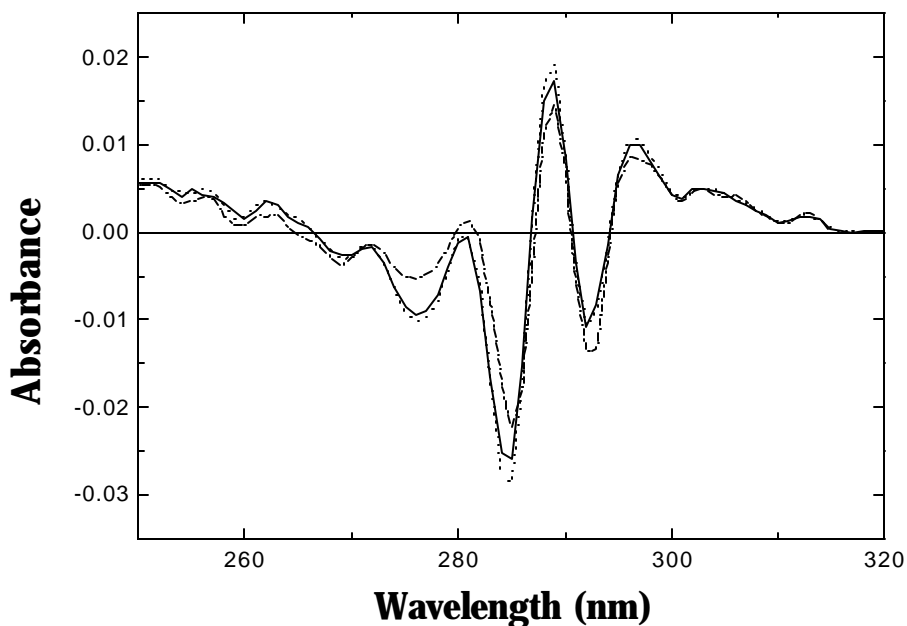


**Fig. 3.3: Arrhenius plot for RNase Rs.** The reaction rate constant ( $V_{\max}$ ) for RNase Rs was determined at different temperatures (25-45°C) in the absence ( ) and presence ( ) of 2 M urea. The graph was plotted using Microcal Origin (version 4.1). The values plotted are the mean of three independent sets of experiments with S. D. less than 10 %.

### 3.5.3 Effect of urea on the secondary and tertiary structure

The conformational changes of RNase Rs, induced by temperature and urea, were monitored by second derivative absorption spectra and circular dichroism. The second derivative absorption spectra between 250 nm and 320 nm (Fig 3.4) showed characteristic minima at 254 nm, 260 nm and 269 nm corresponding to phenylalanine (Phe), minima at 276 nm and 285 nm and maxima at 281 nm and 289 nm corresponding to tyrosine (Tyr) and minima at 285 nm and 292 nm and maxima at 289 nm and 297 nm corresponding to tryptophan (Trp). A marked increase in the minima at 276 nm was observed at 45°C and at 37°C in presence of

2 M urea. As Tyr and Trp signals overlap in the region of 280 to 292 nm, the effect of urea and temperature on the microenvironment of tyrosine was calculated from the peak-to-peak distance (Table 3.3) by the method of Ragone *et al.* (1984). The ratio increased at 45°C and in presence of 2 M urea at 37°C. The fraction of Tyr residues exposed to the solvent was calculated using the equation  $\alpha = (r_n - r_a) / (r_u - r_a)$  where,  $r_n$  and  $r_u$  are the numerical values of the ratio  $a/b$  determined for the native and unfolded (in 6 M Gu-HCl) protein, respectively while  $r_a$  is the  $a/b$  value of a mixture containing the same molar ratio of aromatic amino acids dissolved in ethylene glycol (Ragone *et al.*, 1984). Table 3 shows that 78 % and 94 % of the total Tyr residues are exposed to the solvent in the native protein at 37°C and 45°C, respectively whereas, in 2 M urea at 37°C all the Tyr residues are exposed.



**Fig. 3.4: Second derivative absorption spectrum.** RNase Rs (2 mg/ml) in 50 mM sodium acetate buffer, pH 5.5, in absence of urea at 37°C (– · –) and at 45°C (—), in presence of 2 M urea at 37°C (....).

**Table 3.3: Relative exposure of Tyrosine residues of RNase Rs in presence and absence of 2 M urea**

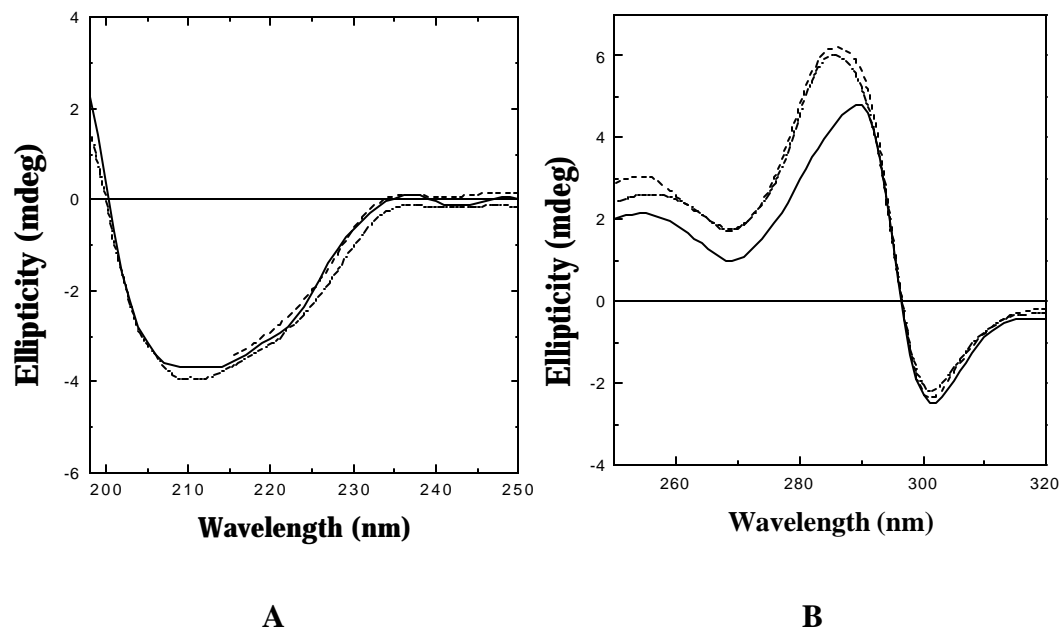
Experimental conditions	a <sup>*</sup>	b <sup>+</sup>	r = a/b	α <sup>**</sup>
Native, at 37°C	0.03689	0.02203	1.674	0.78
Native, at 45°C	0.04096	0.02092	1.96	0.94
2 M urea, at 37°C	0.04782	0.02159	2.215	1.06

\* a = distance between peaks at 287nm and 283nm

+ b = distance between peaks at 295nm and 291nm

\*\* α is the fraction of Tyr residues exposed to solvent in the native state, using coefficient relative to ethylene glycol (r<sub>a</sub>).  $\alpha = (r_n - r_a)/(r_u - r_a)$ , r<sub>u</sub> =2.019, r<sub>a</sub> =0.07.

The CD spectra of RNase Rs under various experimental conditions are shown in Fig 3.5 and Fig 3.6. The far-UV spectrum of native RNase Rs (Fig 3.5), at 37°C, showed a spectrum corresponding mainly to β sheeted structure. The spectra in presence of urea overlapped the native spectrum in the region 250-215 nm, below which the spectra could not be recorded due to interference of urea. The characteristic folded conformation of the peptide backbone did not change under any of the experimental conditions studied. However, in the near-UV range of 290-250 nm, the amplitude of the signal showed a change at 45°C and at 37°C in presence of 2 M urea (Fig 3.6).



**Fig. 3.5: CD spectra of RNase Rs.** CD spectra of RNase Rs in absence of urea at 37°C (—) and at 45°C (- · -) and in presence of 2 M urea at 37°C (----).

**A.** Far-UV CD spectra of RNase Rs (0.1 mg/ml) in 50 mM sodium acetate buffer pH 5.5. The CD spectra in presence of 2 M urea at 37°C could not be recorded below 215 nm due to increase in signal/noise ratio.

**B.** Near-UV CD spectra of RNase Rs (2 mg/ml) in 50 mM sodium acetate buffer, pH 5.5.

The spectra shown are the average of five continuous scans, corrected by subtracting the appropriate blank runs on RNase Rs free solutions and subjected to a moderate degree of noise-reduction analysis.

### 3.6 DISCUSSION

Denaturants viz. Gu-HCl, Gu-SCN and KSCN inactivated RNase Rs at 37°C but surprisingly the enzyme activity increased in presence of urea and maximum activation (~ 1.9 fold) was observed with 2 M urea. The increase in the activity was comparable to the activity at its optimum temperature i.e. 45°C. Artificial activation in presence of urea has also been reported for white croaker fish muscle alkaline protease where, an increase in the activity at 37°C in presence of 5 M urea was observed except that the level of activation was not comparable to the activity at its optimum temperature i.e. 60°C (Toyohara *et al.*, 1987).

Urea and guanidine hydrochloride are known to denature nucleic acids due to their hydrogen-bond-breaking properties (Cox and Kanagalingam, 1968). To ascertain whether the activation of RNase Rs, at 37°C, was due to changes in the protein or in the polymeric substrate RNA, the activity was also monitored using poly U since the latter exhibits little secondary structure above 15°C (Sarkar and Yang, 1965). RNase Rs exhibited a comparable increase in the activity, for hydrolysis of poly U, in presence of 2M urea at 37°C and loss of activity in presence of Gu-HCl suggesting changes in the protein conformation. Hence, the activation in presence of 2 M urea at 37°C, when assayed with RNA or poly U, clearly indicates that substrate conformation is not involved in activation.

The increase in the enzyme activity was accompanied by changes in the kinetic constants of RNase Rs. The  $K_m$  of the enzyme increased at 45°C and in presence of 2 M urea at 37°C with a concomitant increase in  $k_{cat}$ . It is catalytically advantageous for an enzyme to bind the substrate weakly i.e. maximization of the rate requires high  $K_m$  values (Fersht, 1985). The increase in the  $K_m$  at 45°C and in presence of 2 M urea at 37°C suggest minimum residence time between RNase Rs and RNA resulting in an increase in the turnover number. According to the induced fit model of mechanism of enzyme action, the complementarity of the distorted enzyme to the undistorted transition state results in an increase in the  $K_m$  leading to lowering of the  $k_{cat}/K_m$ . The lowering of  $k_{cat}/K_m$  at 45°C and in presence of 2 M urea at 37°C can be as a result of the induced-fit mechanism of RNase Rs action. This

catalytically advantageous increase in  $K_m$  and  $k_{cat}$  has also been observed in case of activation of chicken liver dihydrofolate reductase with urea (Kaufman, 1968) and thermophilic xylose isomerase from *Thermotoga neapolitana* (Vieille *et al.*, 1995). In case of dihydrofolate reductase, increase in the  $K_m$  corresponded to those observed for the enzyme isolated from other sources which do not respond to these reagents (Kaufman, 1968). However, with thermophilic xylose isomerase, decrease in the catalytic efficiency was attributed either to temperature induced conformational changes at the active site of the enzyme or to the thermal sensitivity of the substrate leading to interference in the substrate binding (Vieille *et al.*, 1995). The Arrhenius plots for RNase Rs obtained in presence and absence of urea indicated a lowering of the activation energy. In case of dihydrofolate reductase, a slight increase in the activation energy was observed in presence of urea and the increase in values corresponded to those observed for the enzyme isolated from other sources, which do not respond to this reagent (Kaufman, 1968). The activation of RNase Rs with urea accompanied by changes in kinetic constants point towards the possibility of urea induced change(s) in the conformation at the active site, yielding a catalytically more favorable enzyme structure in presence of 2 M urea at 37°C, similar to that observed at 45°C.

The general effect of urea, Gu-HCl and temperature on protein is to alter the tertiary structure through rupture of the stabilizing bonds and unfolding of the molecule. As a result, changes in the conformation and microenvironment of the side chain residues occur during unfolding of proteins. These changes can be detected by a number of physico-chemical techniques such as, size exclusion chromatography, second derivative spectroscopy, circular dichroism, spectrofluorometry and trypsin digestion. CD spectra in the far-UV region reflect principally the peptide bond transitions i.e. the secondary structure of a protein whereas, the near-UV spectra show changes in the side chain conformation and its microenvironment. Second derivative absorption spectroscopy, in the UV region, has proved to be an effective analytical tool because of its ability to resolve the complex protein absorption spectrum into individual contributions of Phe, Tyr and

Trp residues (Ragone *et al.*, 1984). Since the positions of the peaks do not change after prolonged exposure to perturbing agents, the microenvironmental changes are followed by monitoring the changes in the ratio of peak-to-peak distances calculated under various experimental conditions where, an increase in the ratio indicates a more polar microenvironment. In the present studies, although the secondary structure of RNase Rs remained unaltered, the microenvironment of the aromatic side chains showed a change with respect to Tyr residues as seen from the second derivative absorption spectra. The increase in the ratio of peak-to-peak distance in the second derivative absorption spectra suggested the microenvironment becoming more polar and nearly all the Tyr residues were exposed to the solvent at 45°C and in presence of urea at 37°C.

The mechanism of action of urea in the denaturation of proteins is still not clear. Urea is reported to stabilize both the native and denatured protein conformations. The large number of binding sites exposed in the unfolded state favors denaturation. Both Gu-HCl and urea bind to the surface of the folded protein whereas, the urea molecules (not the charged Gu-HCl) also permeate to the interior of the protein occupying small cavities and somewhat perturbing the close-packed interior (Creighton, 1993). A recent molecular dynamics simulation study also suggests that the preferential adsorption of urea molecules onto the charged hydrophilic residues on the protein surface results in a swelling of the protein and exposure of the hydrophobic residues. The onset of water into the interior leads to destabilization of the native state resulting in the denaturation of the protein (Wallquist *et al.*, 1998). Urea can bind, even at submolar concentrations, to micromolar concentrations of protein leading to structural alterations in the compact folded structure (Liepinsh and Otting, 1994). In presence of the denaturant the conformation of enzyme active site, which is held together by relatively weak forces, can be easily perturbed resulting in either inactivation or activation of the enzyme (Tsou, 1993). A subtle conformational change at the active site, responsible for the altered enzyme activity, has been shown to precede any change in the conformation of the enzyme molecule in presence of denaturants, as observed in



the activation of dihydrofolate reductase (Fan *et al.*, 1996) and adenylate kinase (Zhang *et al.*, 1997) as well as in the inactivation of RNase A (Xiao and Zhou, 1996). Hence in the present study, the increase in the activity of RNase Rs in presence of urea at 37°C, can be correlated to urea induced conformational changes in the active site of the enzyme resulting in a conformation similar to that at 45°C, ultimately resulting in similar activity levels at 45°C and in presence of 2 M urea at 37°C. In conclusion, in presence of urea, the optimum temperature of RNase Rs is lowered due to a decrease in the activation energy of the enzyme-substrate complex, as a result of changes in the enzyme active site.

***Chapter 4***  
*Chemical and thermal  
denaturation of RNase Rs*

## 4.1 SUMMARY

The conformational stability of RNase Rs was determined by following its chemical and thermal unfolding. The denaturation of RNase Rs with urea and temperature was monitored over the pH range of 1-10, following the change in intrinsic fluorescence of the protein on excitation at 295 nm. The native protein exhibited emission  $\lambda_{\max}$  around 336 nm which shifted to 355 nm on denaturation.

Equilibrium unfolding with urea showed that values of  $D_{1/2}$  (5.7 M) and  $G_2^{\text{H}_2\text{O}}$  (12.8 kcal/mol) were highest at pH 5.0 and hence the maximum conformational stability of RNase Rs was observed near pH5.0, its isoelectric pH. The curves of fraction unfolded ( $f_U$ ) obtained with fluorescence and CD measurements overlapped at pH 5.0.

Denaturation with guanidine hydrochloride, at pH5.0, gave similar values of  $G_2^{\text{H}_2\text{O}}$  but the  $D_{1/2}$  was 3.1 M, approximately half that of urea. Thermal denaturation of RNase Rs resulted in aggregation and precipitation of the protein above 55°C between pH 4.0 - 5.5, i.e. near the pI where the repulsive forces due to charge are minimum thus allowing the hydrophobic interaction to mediate aggregation. In the pH ranges i.e. pH 1.0 - 3.0 and pH 6.0 - 10.0 where aggregation did not take place, the  $T_m$  values of thermal denaturation showed a similar trend in comparison to  $D_{1/2}$  of urea denaturation. Thermal denaturation in presence of 2 - 5 M urea, at pH 5.0, showed a gradual decrease in  $T_m$  as well as in aggregation with increase in urea concentration. Base denaturation of RNase Rs was observed above pH 11.0, where emission  $\lambda_{\max}$  shifted to 355 nm. Based on the activity regained on dilution, the unfolding in urea was reversible at all pH studied and the emission  $\lambda_{\max}$  shifted back to 336 nm on dilution of the 8 M urea denatured protein, at pH 5.0. In case of thermal unfolding, based on both the spectrum and activity, >60 % reversibility was seen between pH 2.0 - 3.0 in the acidic range and pH 6.0- 9.0 on the alkaline side of the pI of RNase Rs. Both urea and temperature induced denaturation studies showed that RNase Rs unfolds

through a two-state F U mechanism. The pH dependence of stability described by  $G^{H_2O}(\text{urea})$  and  $G(25^\circ\text{C})$  suggested that electrostatic interactions among the charged groups make a significant contribution to the conformational stability of RNase Rs.

## 4.2 INTRODUCTION

Specific biological functions of proteins emerge directly from the details of their unique and highly individualistic three-dimensional structure, attained in milliseconds after protein synthesis. The three dimensional structure of a protein is held together by non-covalent interactions such as hydrogen bonds, ionic interactions, hydrophobic interactions, van-der Waals forces and covalently, by disulfide bridges. Conditions that disturb these stabilizing forces affect the native conformation of the protein thereby changing most of its physical properties in addition to its biological activity. The magnitude and balance between different stabilizing forces is studied under various denaturing conditions and interpreted in terms of conformational stability. RNase A, RNase T1 and barnase have proved to be excellent models for investigating the conformational stability of globular proteins. The T2 family RNases, even though extensively characterized for their physico-chemical properties and biological role, have not been characterized for their structural stability, with the exception of RNase M and RNase Rh. RNase M from *A. saitoi* was shown to be resistant to denaturation in presence of 8 M urea whereas, 6 M guanidine-HCl denatured the protein completely (Irie *et al.*, 1971;1972). Thermal denaturation was used to determine the effect of amino acid replacements, through site directed mutagenesis, on the conformational stability of RNase Rh from *R. niveus* (Ohgi *et al.*, 1992). RNase Rs exhibits high stability to changes in pH at ambient temperature (Chacko and Shankar, 1998). Hence studies on conformational stability of RNase Rs

through chemical and thermal denaturation and their pH dependence were carried out and the details are given in this Chapter.

### **4.3 MATERIALS**

Urea, guanidine hydrochloride (Gu-HCl) and buffer salts (Sigma Chemical Co., St. Louis, MO, USA); uranyl acetate (Loba Chemie Pvt Ltd, Mumbai, India) and perchloric acid (Qualigens, Mumbai, India) were used. All other chemicals used were of analytical grade. Commercial yeast RNA (Sisco Research Laboratories, Mumbai, India) was purified by repeated precipitation with absolute ethanol.

### **4.4 METHODS**

#### *4.4.1 Determination of RNase activity*

RNase Rs was assayed at pH 5.5 and 37°C by measuring the increase in acid soluble ribonucleotides at 260 nm. The amount of acid soluble ribonucleotides was calculated by assuming a molar absorption coefficient of 10,600 M<sup>-1</sup>cm<sup>-1</sup> (Curtis *et al.*, 1966). One unit of RNase activity is defined as the amount of enzyme required to liberate 1 µmol of acid soluble nucleotides/min under the assay conditions (Chapter 2).

#### *4.4.2 Protein determination*

Protein concentrations were determined according to Lowry *et al.* (1951) using BSA as standard.

#### *4.4.3 Purification of RNase Rs*

Cultivation of *R. stolonifer* and purification of RNase Rs was carried out as described in Chapter 2. RNase Rs crystallized when concentrated above 1mg/ml in 30 mM sodium acetate buffer, pH 5.5. The crystalline protein was dissolved in Milli Q water containing 200 mM NaCl (effective concentration) so as to obtain 5 mg/ml solution and was used for further studies.

#### 4.4.4 Buffers and solutions

The buffers used at different pH values were KCl-NaOH (pH 1.0 - 1.5), glycine-HCl (pH 2.0 - 3.0), sodium acetate (pH 4.0 - 5.5), sodium phosphate (pH 6.0 - 7.0), Tris-HCl (pH 8.0 - 9.0), glycine-NaOH (pH 9.0 - 10.0) and Na<sub>2</sub>HPO<sub>4</sub>-NaOH (pH 11.0 - 12.0). The urea and Gu-HCl stock solutions were prepared fresh on the day of use as described by Pace *et al.* (1989).

#### 4.4.5 Equilibrium unfolding studies

Equilibrium unfolding as a function of urea, Gu-HCl and temperature was monitored by fluorescence. For chemical denaturation, the protein ( $A_{280} = 0.05$  or  $50 \mu\text{g/ml}$ ) was incubated in presence of various concentrations of the denaturant in 50 mM buffers of various pH, at 25°C for 24 h. In the thermal unfolding experiments, the spectra were recorded 5 min after the desired temperature was reached. The intrinsic tryptophan fluorescence spectra of the protein were recorded on a Perkin Elmer LS50B Spectrofluorometer, equipped with Julabo F25 waterbath, by exciting the protein at 295 nm using a cell of 1.0 cm pathlength and both excitation and emission slit widths were set at 5 nm. Rayleigh (light) scattering was measured at 240 nm with both the excitation and emission slit widths set at 5 nm. CD spectra of RNase Rs were recorded on a computer interfaced JASCO J-715 Spectropolarimeter using a cylindrical quartz cell of 1 mm for far-UV spectra.

#### 4.4.6 Data analysis

The denaturation curves were plotted, with ratio of fluorescence intensities at the emission  $\lambda_{\text{max}}$  of the native protein and the denatured protein, against the denaturant molarity or temperature and further analysis of the data was performed as described by Pace *et al.* (1989). From the denaturation curves, a two state F U

unfolding mechanism was assumed and consequently for any of the points only the folded and unfolded conformations were present at significant concentrations. Thus, if  $f_F$  and  $f_U$  represent the fraction of protein present in the folded and unfolded conformations respectively,  $f_F + f_U = 1$ . The observed value of Y at any point will be  $Y = Y_F f_F + Y_U f_U$  and  $f_U$  was calculated using following equation

$$f_U = \frac{(Y - (Y_F + m_F[D]))}{(Y_U + m_U[D]) - (Y_F + m_F[D])} \quad (1)$$

where, Y is the value of the spectroscopic property measured at a concentration [D],  $Y_F$  and  $Y_U$  represent the intercepts,  $m_F$  and  $m_U$  the slopes of the folded and unfolded baselines of the data respectively, which were obtained from linear least-squares fits to the baseline. For a two state F U unfolding mechanism, the equilibrium constant K and  $G_U$ , the free energy of unfolding by denaturant at concentration [D] was calculated using Equation 2 and 3 respectively.

$$K = f_U / (1 - f_U) \quad (2)$$

$$G_U = -RT \ln K \quad (3)$$

where, R is the gas constant (1.987 cal/deg/mol) and T is the absolute temperature. It is assumed that the free energy of unfolding,  $G_U$ , has a linear dependence on the concentration of the denaturant [D]

$$G_U = G_2^{H_2O} + m[D] \quad (4)$$

$G_2^{H_2O}$  and m are therefore the intercept and the slope respectively, of the plot of  $G_U$  versus [D].  $G_2^{H_2O}$  corresponds to the free energy difference between the folded and unfolded states in the absence of any denaturant and m is a measure of the cooperativity of the unfolding reaction. The concentration of denaturant at which the

protein is half unfolded (when  $G_U = 0$ ) is given by  $D_{1/2}$  and from Equation 4,  $G_2^{H_2O} = -mD_{1/2}$ .

The data from the thermal unfolding curves were obtained under the same conditions as those for urea unfolding curves. Values of  $f_U$ ,  $K$  and  $G_U$  were calculated using equations 1, 2 and 3.  $T_m$ , the midpoint of thermal denaturation, was obtained as the temperature at which  $G_U = 0$  from the plot of  $G_U$  versus  $T$ . The slope of such a plot at  $T_m$  yielded  $S_m$ , the change in entropy. The enthalpy change for unfolding at  $T_m$ ,  $H_m$ , was calculated using the equation

$$H_m = T_m S_m \quad (5)$$

$C_p$ , the change in heat capacity that accompanies protein unfolding was obtained from the slope of the plot of  $H_m$  versus  $T_m$ , where  $T_m$  was varied as a function of pH or urea concentration.  $G_U$  at 25°C was calculated using equation

$$G(T) = H_m(1 - T/T_m) - C_p[(T_m - T) + T \ln(T/T_m)] \quad (6)$$

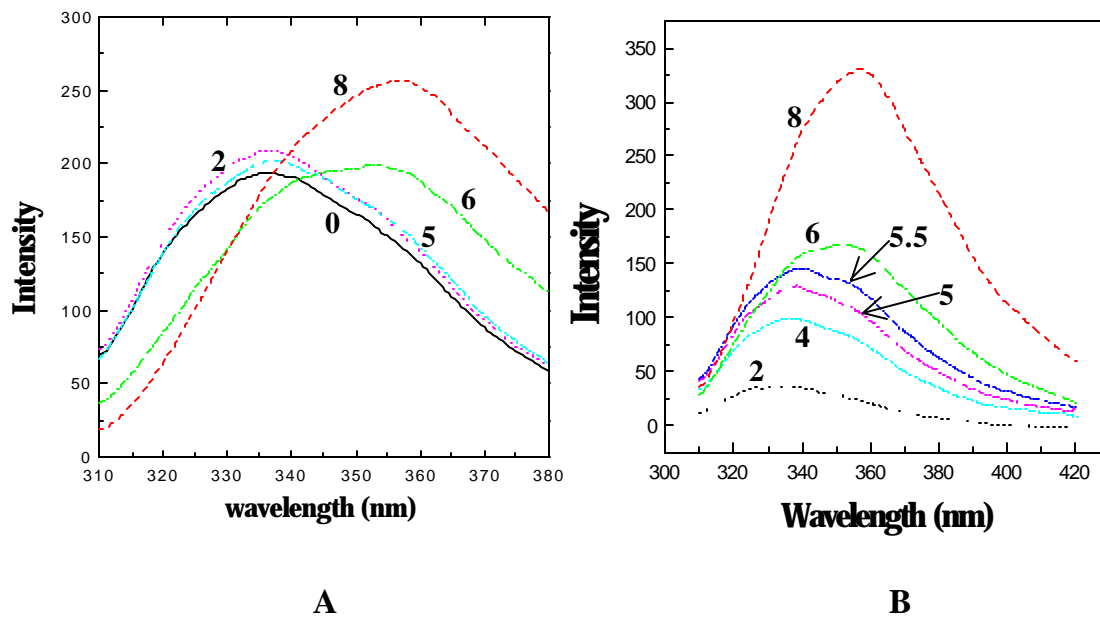
## 4.5 RESULTS

### 4.5.1 Chemical denaturation

Denaturation of RNase Rs with urea at various pH was monitored by following the change in intrinsic fluorescence of the protein on excitation at 295 nm. At acidic pH (1.0 - 2.0) the fluorescence intensity decreased whereas at pH 9.0 the intensity increased as compared to that at pH 5.0. The native protein exhibited emission  $\lambda_{max}$  around 336 nm which shifted to 355 nm on denaturation (Fig. 4.1 A). On dilution of urea, >80% activity was regained between pH 1.0 - 9.0 whereas only 60% recovery was observed at pH 10.0 (Table 4.1). The fluorescence spectra of the denatured protein on dilution of urea were recorded at pH 5.0. The emission  $\lambda_{max}$  shifted back to 336 nm at and below 5.5 M urea, although the intensity was low on account of dilution of the



protein (Fig. 4.1B). The fluorescence data was used to prepare the denaturation curves (Fig. 4.2) which were then analysed to obtain  $G_U$  using Equations 1, 2 and 3. The  $G_U$  was plotted as a function of concentration of urea (Fig. 4.3) and to obtain  $D_{1/2}$  and  $G_2^{H_2O}$  (Equation 4). The plots of  $D_{1/2}$  and  $G_2^{H_2O}$  as a function of pH (Table 4.2, Fig. 4.4) showed that the values of  $D_{1/2}$  (5.7 M) and  $G_2^{H_2O}$  (12.5 kcal/mol) were highest at pH 5.0 and decreased on both acidic and alkaline range.



**Fig. 4.1: Fluorescence spectra of RNase Rs.** Fluorescence of RNase Rs was measured at 25°C, pH 5.0 and in presence of urea concentrations. Numbers indicate the concentration of urea (M).

- A** Denaturation of RNase Rs after 24 h incubation at 25°C and pH 5.0.
- B** Renaturation of RNase Rs after denaturation with 8 M urea on dilution of urea.

The denaturation at the extremes of pH was achieved at low urea concentrations as seen with the  $D_{1/2}$  value of  $\sim 3.5$  M and  $G_2^{H_2O}$  value of  $\sim 7$  kcal/mol. At pH 1.0 and 1.5, the  $D_{1/2}$  and  $G_2^{H_2O}$  were slightly higher than that at pH 2.5. RNase Rs was denatured using Gu-HCl at pH 5.0 and the data was treated in a similar manner as that of urea denaturation (Fig. 4.5). The  $D_{1/2}$  and  $G_2^{H_2O}$  values were 3.1 M and 13.7 kcal/mol, respectively. The denaturation with urea at pH 1.0, 2.5, 5.0 and 9.0 was monitored by

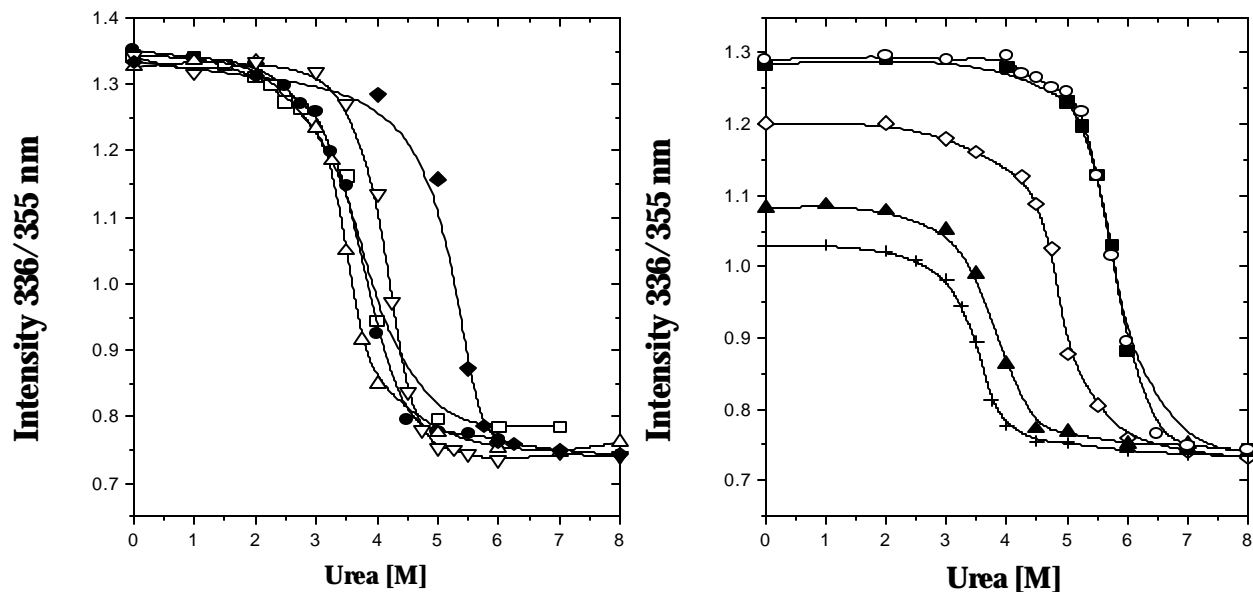
CD and the denaturation curves were prepared by plotting  $\theta$  at 220 nm (Fig. 4.6) and the  $f_U$  was calculated using Equation 1. The  $f_U$  plots obtained from CD and fluorescence data at pH5.0 overlapped perfectly and the values of  $D_{1/2}$  (5.66 M) and  $G_2^{H_2O}$  (12.3 kcal/mol) obtained by CD measurements were similar to that obtained from fluorescence measurements (Table 4.2, Fig. 4.7). However,  $f_U$  plot from CD measurements could not be obtained at pH 9.0 since the denaturation did not seem to be complete till 7 M urea.

**Table 4.1: Activity on renaturation after pH, urea and temperature denaturation.**

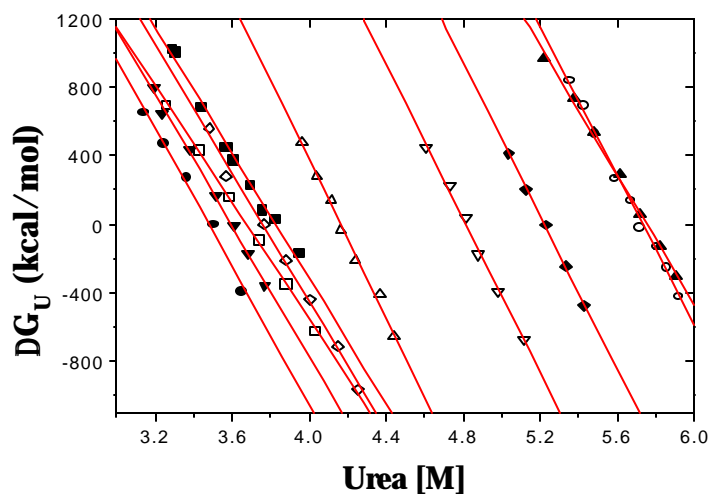
pH	Recovery of activity (%)		
	pH	Urea	Temperature
1	90	100	30
1.5	84	81	30
2.0	ND	ND	80
2.5	87	80	71
3.0	80	88	60
4.0	ND	ND	0
4.5	100	96	0
5.0	100	85	0
5.5	93	90	0
7.0	82	77	83
8.0	ND	ND	62
9.0	77	92	64
10.0	71	60	29
11.0	6	ND	ND

The activity was measured after 24h incubation at 25°C at various pH and was expressed as % of enzyme activity at 0h for pH denaturation. For urea denaturation activity was measured after ~1h on dilution of urea and was expressed as % of the activity at 0M urea concentration after 24h incubation at

25°C. In case of thermal denaturation, activity is expressed as % of activity at 25°C.



**Fig. 4.2: Urea denaturation curves.** The ratio of the fluorescence intensity at wavelengths 336 and 355 nm as a function of urea concentration at pH 1.0 ( ), 1.5 ( ), 2.5 ( ), 3.0 ( ), 4.5 ( ),

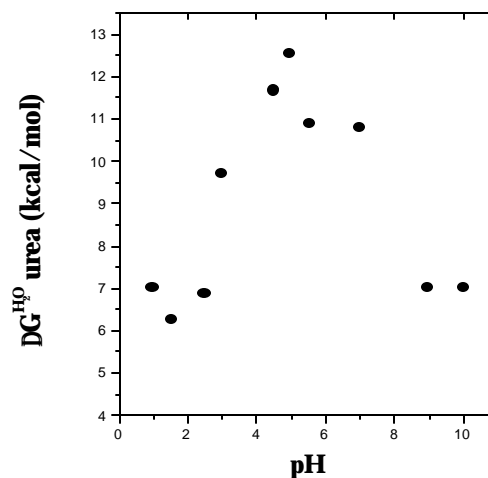
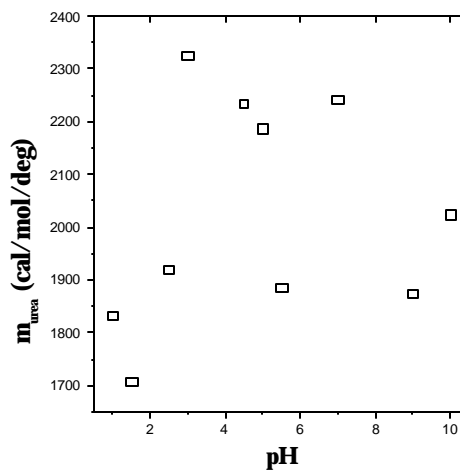
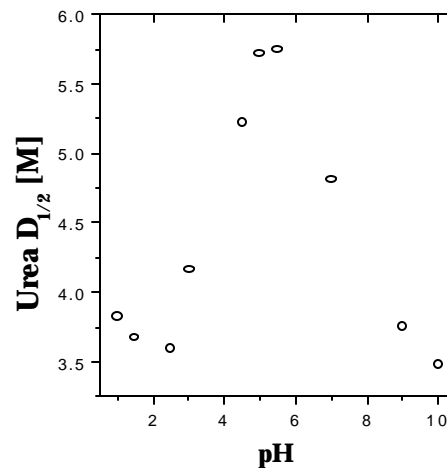


5.0 ( ), 5.5 ( ), 7.0 ( ), 9.0 ( ) and 10.0 (+).

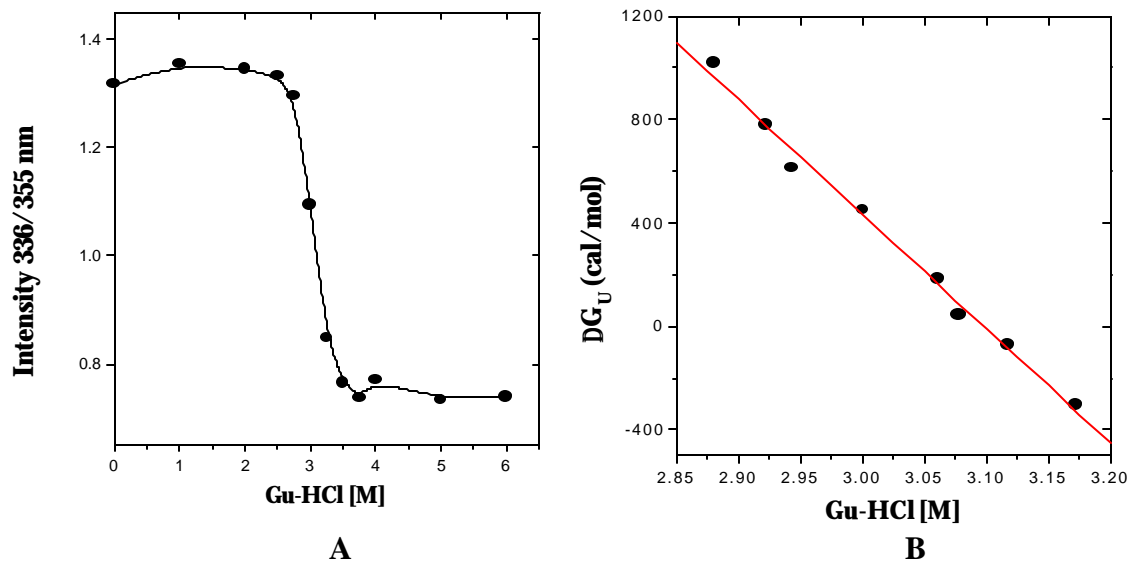
**Fig. 4.3:**  $G_U$  as a function of urea concentration.  $G_U$  was calculated using equations 1, 2, and 3 from the urea denaturation curves obtained at pH 1.0 ( ), 1.5 ( ), 2.5 ( ), 3.0 ( ), 4.5 ( ), 5.0 ( ), 5.5 ( ), 7.0 ( ), 9.0 ( ) and 10.0 ( ).

**Table 4.2 : Parameters for urea denaturation**

pH	$G_2^{H_2O}$ (kcal/mol)	m (cal/mol/deg)	$D_{1/2}$ M
1	7.012	1833.4	3.825
1.5	6.267	1706.02	3.674
2.5	6.898	1918.7	3.595
3	9.675	2324.4	4.162
4.5	11.668	2234.6	5.222
5	12.526	2186.4	5.72
5.5	10.855	1887.7	5.75
7	10.799	2243.23	4.81
9	7.044	1874.5	3.758
10	7.04	2024.35	3.478

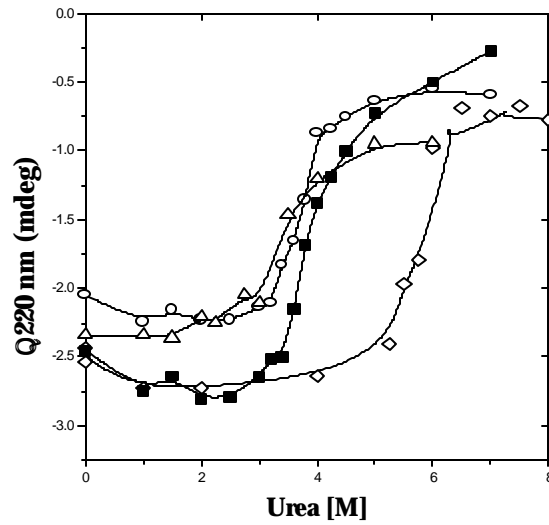


**Fig. 4.4:  $D_{1/2}$ ,  $m$  and  $G_2^{H_2O}$  as a function of pH for the urea denaturation of RNase Rs.**  $D_{1/2}$  ( ),  $m$  ( ) and  $G_2^{H_2O}$  ( ) values were obtained with linear extrapolation method from plots of  $G_U$  as a function of urea concentration using equation 4 as described in Methods.



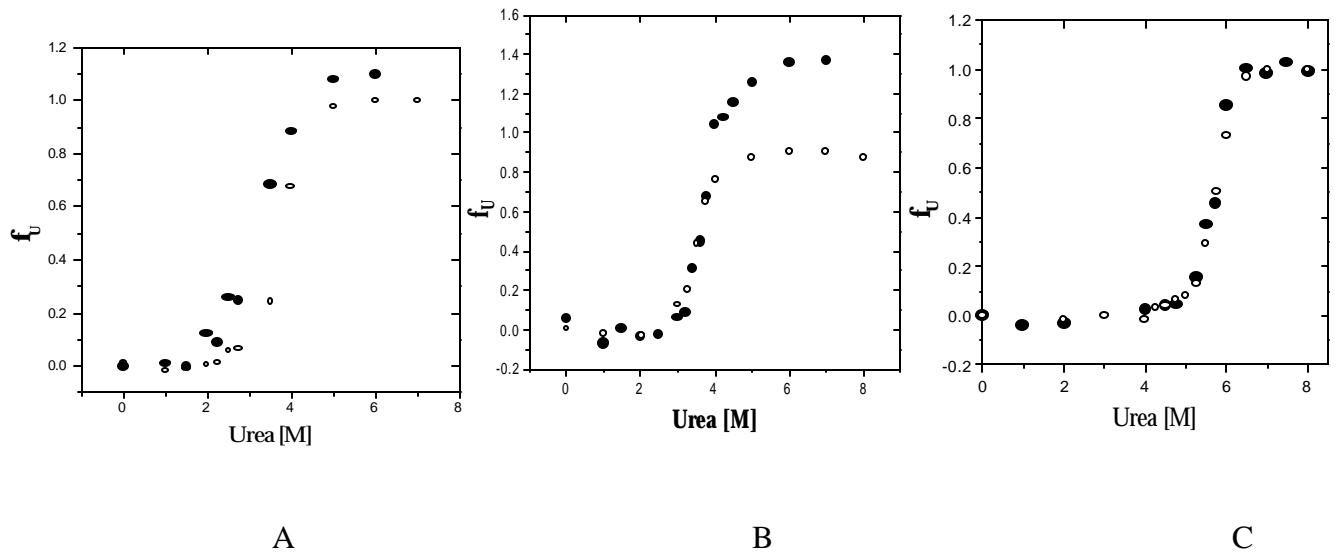
**Fig. 4.5 : Gu-HCl denaturation of RNase Rs at pH 5.0.**

- A Denaturation curve obtained by plotting ratio of fluorescence intensities at wavelengths 336 and 355 nm as a function of Gu-HCl concentration. Fluorescence spectra of RNase Rs were recorded after 24h incubation of the enzyme in different concentrations of Gu-HCl at 25°C.
- B  $G_U$  as a function of Gu-HCl concentration.  $G_U$  was obtained as described for urea denaturation.



**Fig. 4.6: Urea denaturation curves obtained by CD measurements.**

Denaturation curve obtained by CD measurements at pH 1.0 (○), 2.5 (△), 5.0 (□) and 9.0 (◇).

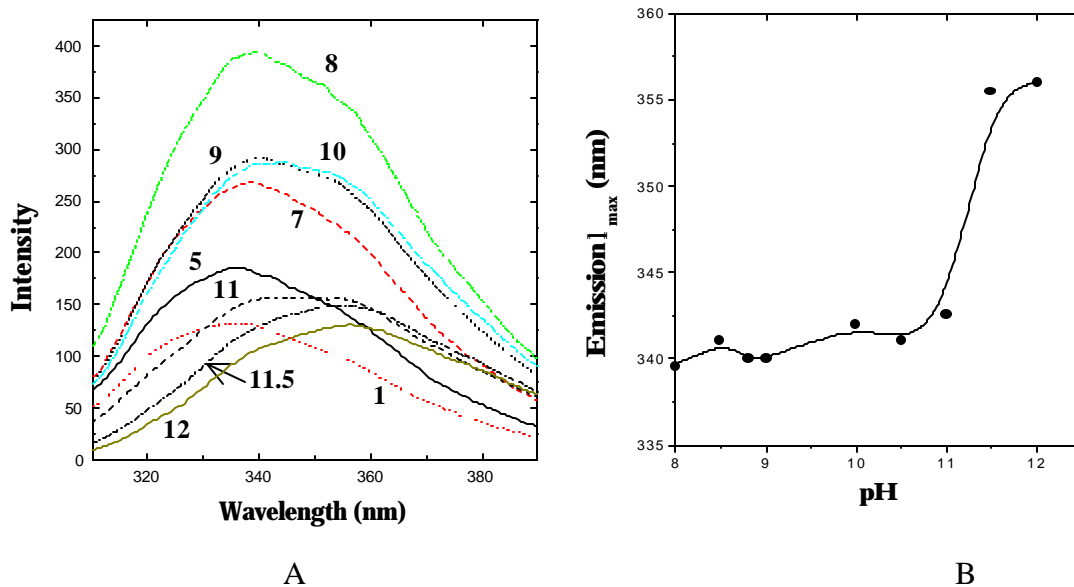


**Fig. 4.7:  $f_U$  curves for urea denaturation.**  $f_U$  from CD (●) and fluorescence (○) denaturation curves was calculated using equation 1 and was plotted as a function of urea concentration.

A at pH 1.0    B at pH 2.5    C at pH 5.0

#### 4.5.2 pH dependent denaturation

The fluorescence spectra of RNase Rs were recorded at pH 1.0 – 12.0. The emission  $\lambda_{\max}$  in the acidic range did not shift although the fluorescence intensity was reduced drastically (Fig. 4.8A). In contrast, the emission  $\lambda_{\max}$  slowly increased from 336 nm, at pH values between 4.0 - 5.5, to ~342 nm till pH11.0 and then shifted to 355 nm at pH 11.5 and above (Fig. 4.8B). At the extreme alkaline pH, the protein displayed a fluorescence spectrum similar to that in presence of 8M urea or 6M Gu-HCl although the fluorescence intensity reduced drastically (Fig. 4.8A).



**Fig. 4.8: Base induced denaturation of RNase Rs.**

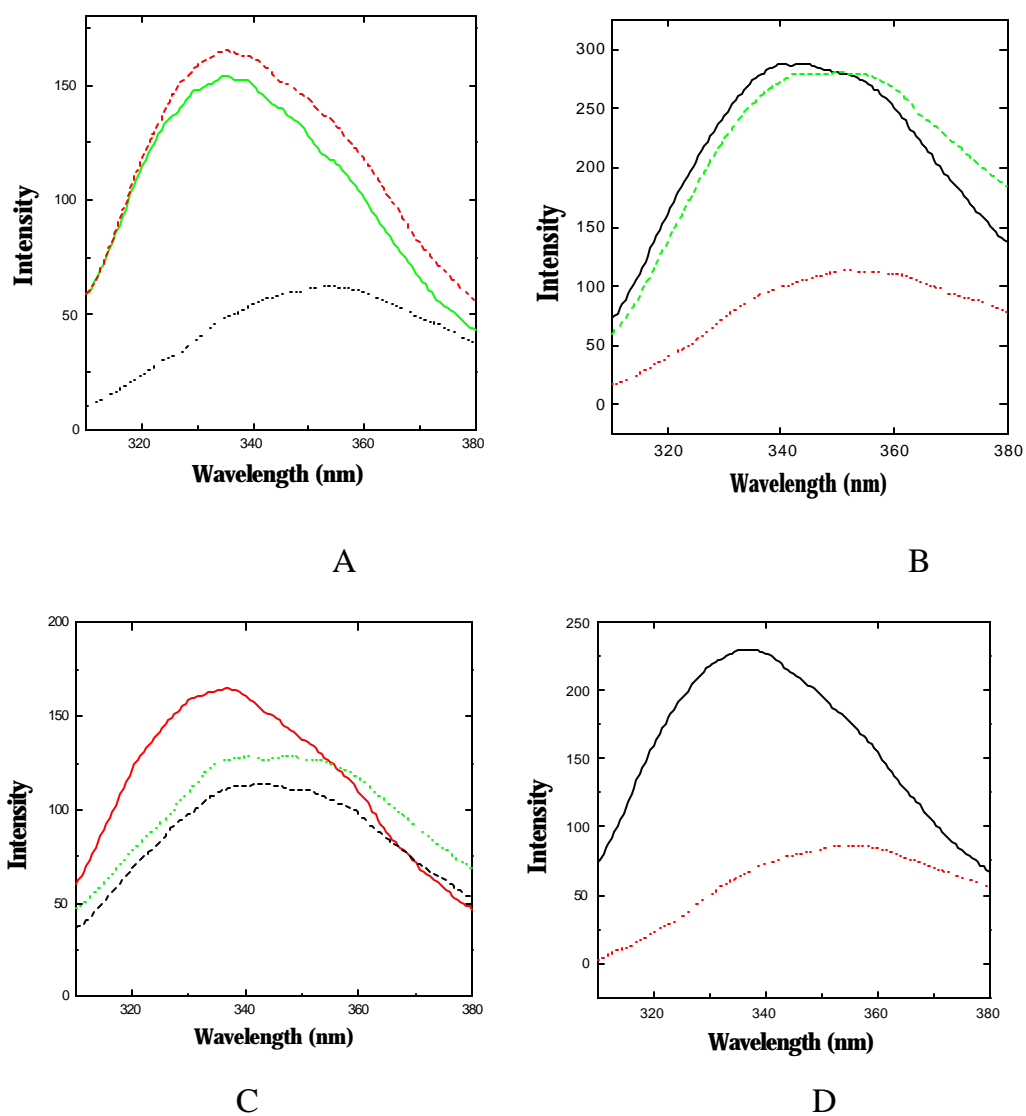
- A Fluorescence spectra of RNase Rs incubated at 25°C for 24h at different pH (as indicated by the numbers)
- B The emission  $\lambda_{\max}$  plotted as a function of pH.



### 4.5.3 Thermal denaturation

The thermal denaturation of RNase Rs at various pH was monitored using the intrinsic fluorescence of the protein. The fluorescence intensity decreased at high temperatures and the shift in emission  $\lambda_{\max}$  to 355 nm was observed. At pH 2.0 - 3.0 and pH 6.0 - 9.0 the spectra reverted to the original and >60% activity could be recovered on cooling to 4°C (Fig. 4.9A, Table 4.1). However, at pH 1.0, 1.5 and 10.0 the recovery of activity was ~30% and the spectra were not restored back to original (Fig. 4.9B, Table 4.1). In contrast, in pH range 4.0 – 5.5, RNase Rs was seen to precipitate above 50°C as indicated by the increase in light scattering (Fig. 4.10). Light scattering measurements did not show the formation of precipitates at pH 3.0 and 6.0. In pH range 4.0 – 5.5, the emission  $\lambda_{\max}$  at high temperature did not correspond to that of the denatured protein and the spectrum as well as activity were not restored to original on cooling (Fig. 4.9C, Table 4.1). Thermal denaturation of RNase Rs at pH 5.0 was followed in presence of different concentrations of urea and the precipitation of protein could be prevented at >2 M urea (Fig. 4.10). The emission  $\lambda_{\max}$  shifted to 355 nm on denaturation and fluorescence spectra corresponded to that of denatured protein and the typical denaturation spectra were observed (Fig. 4.9D).

The thermal denaturation data from the denaturation curves (Fig. 4.11) between pH 1.0 – 3.0 and 6.0 – 10.0 and at pH 5.0 in presence urea were analysed using Equation 1, 2 and 3 and  $G_U$  was plotted against T to obtain  $T_m$  and  $S_m$  (Fig. 4.12, Table 4.3).  $T_m$  increased with the increasing pH in the acidic range i.e. from 44°C at pH 1.0 to 63°C at pH 3.0, whereas  $T_m$  decreased with the increasing pH from 61.5°C at pH 6.0 to 54.8°C at pH 10.0 (Fig. 4.14 A). In case of thermal denaturation, at pH 5.0, in presence of urea,  $T_m$  was seen to decrease with the increasing concentration of urea from 55.7°C at 2 M urea to 32.3°C at 5.5 M urea (Table 4.4).

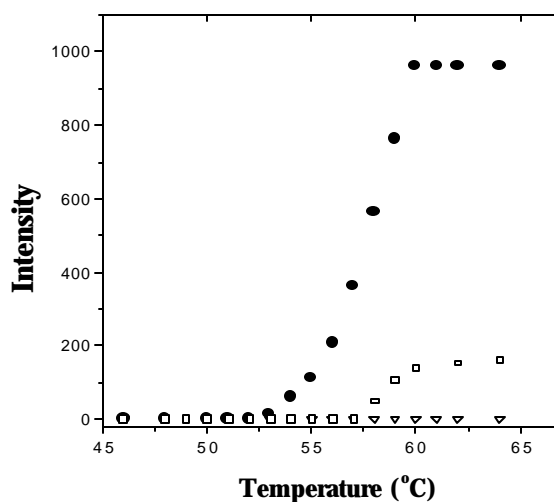


**Fig. 4.9: Fluorescence spectra on thermal denaturation of RNase Rs.**

Fluorescence spectra of native enzyme at 25°C (—), denatured enzyme (---) and renatured enzyme after overnight cooling at 4°C (· · ·).

- A at pH 2.0 – 3.0 and 6.0 – 9.0
- B at pH 1.0, 1.5 and 10.0
- C at pH 4.0 – 5.5
- D at pH 5.0 in presence 3 M urea.

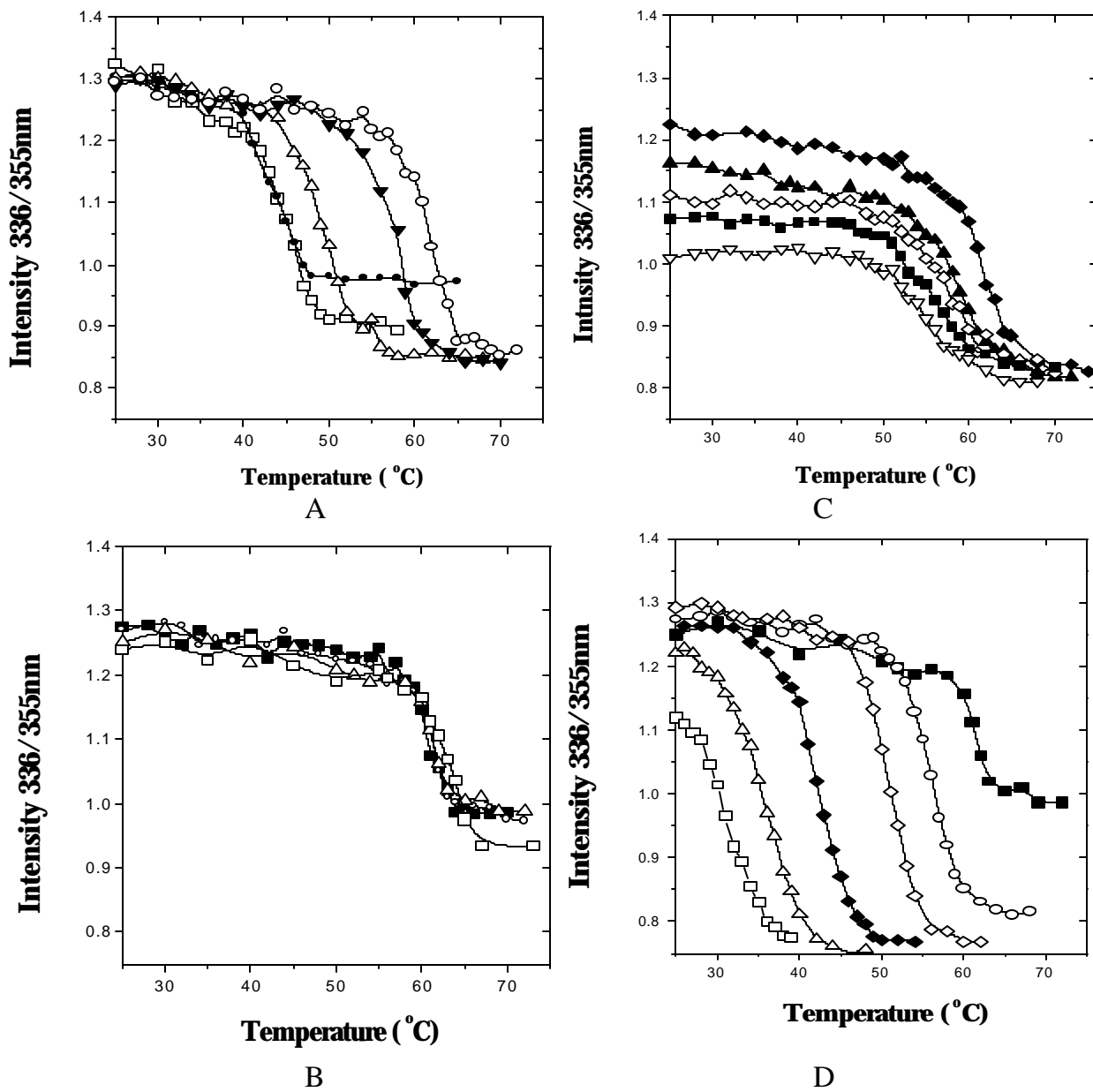




**Fig. 4.10: Light scattering by RNase Rs.** Increase in the light scattering was plotted as a function of temperature for thermal denaturation between pH 4.0 – 5.5 (●), thermal denaturation at pH 3.0, 6.0 and in presence of 3 M urea at pH 5.0 (□) and thermal denaturation in presence of 2 M urea (△).

The change in enthalpy,  $H_m$ , was determined at  $T_m$  for the different urea concentrations used and were plotted as a function of  $T_m$  (Fig. 4.13). The slope of this plot yielded  $C_p$  (997.8 cal/mol/deg) which was then used to calculate  $G(25^\circ\text{C})$  from the thermal denaturation curves, in absence of urea, determined at different pH. The highest  $G(25^\circ\text{C})$  values (9.8 kcal/mol) were observed at pH 6.0 which decreased to ~ 5 kcal/mol at pH 1.5 on the acidic side and pH 8.0 on the alkaline side (Fig. 4.14 B). However, the  $G(25^\circ\text{C})$  values increased to ~7 kcal/mol at the extremes of pH on the acidic as well as alkaline side.





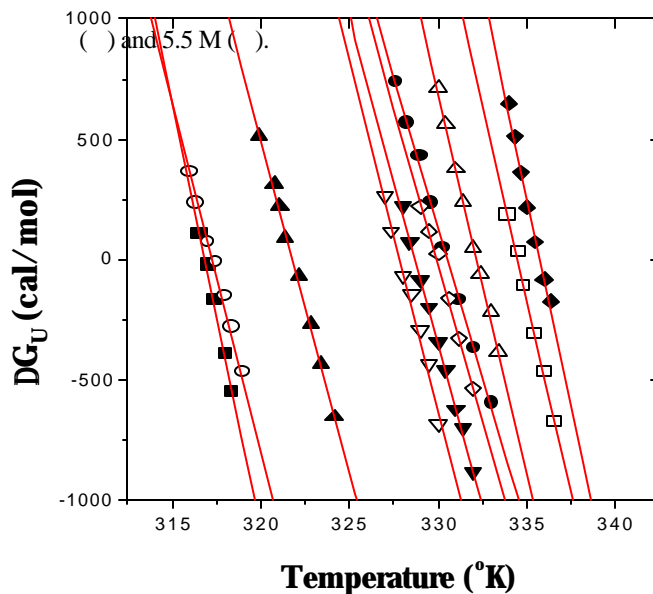
**Fig. 4.11: Thermal denaturation curves for RNase Rs.** The ratio of fluorescence intensities at 336 and 355 nm was plotted as a function of temperature

A at pH 1.0 ( ), 1.5 ( ), 2.0 ( ), 2.5 ( ), 3.0 ( ),

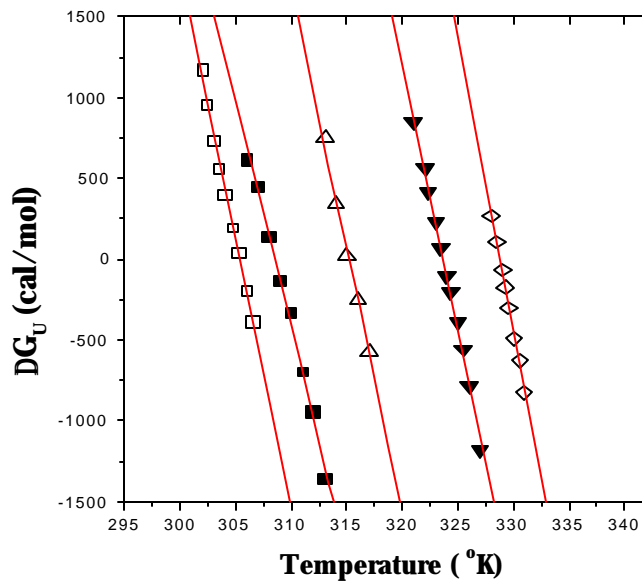
B at pH 4.0 ( ), 4.5 ( ), 5.0 ( ), 5.5 ( ),

C at pH 6.0 ( ), 7.0 ( ), 8.0 ( ), 9.0 ( ) and 10.0 ( ).

D at pH 5.0 in presence of urea 0M ( ), 2 M ( ), 3 M ( ), 4M ( ), 5 M



A

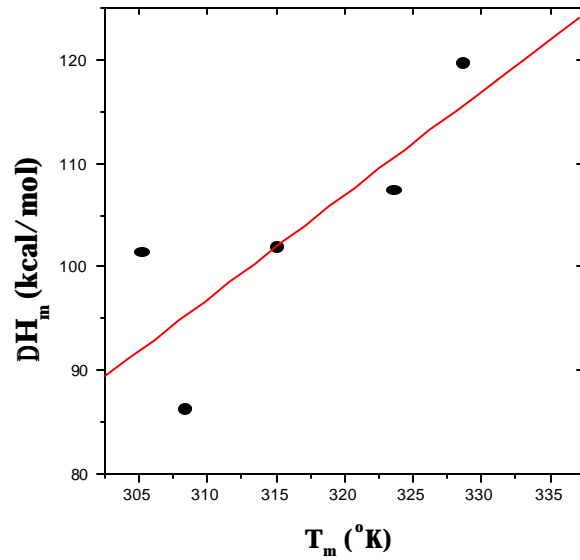


B

**Fig. 4.12:  $G_U$  as a function of temperature.**

A  $G_U$  was calculated using equations 1, 2, and 3 from the thermal denaturation curves obtained at pH 1.0 ( ), 1.5 ( ), 2.0 ( ), 2.5 ( ), 3.0 ( ), 6.0 ( ), 7.0 ( ), 8.0 ( ), 9.0 ( ) and 10.0 ( ).

B  $G_U$  was calculated using equations 1, 2, and 3 from the thermal denaturation curves obtained at pH 5.0 in presence of urea 2 M ( ), 3 M ( ), 4M ( ), 5 M ( ) and 5.5 M ( ).



**Fig. 4.13: Determination of  $C_p$ .** Thermal denaturation experiments were performed in presence of different concentrations of urea at pH 5.0 to vary  $T_m$ .  $S_m$  was obtained as the slope of the plots of  $G_U$  versus  $T$ .  $H_m$  was determined at each value of  $T_m$  using equation 5.



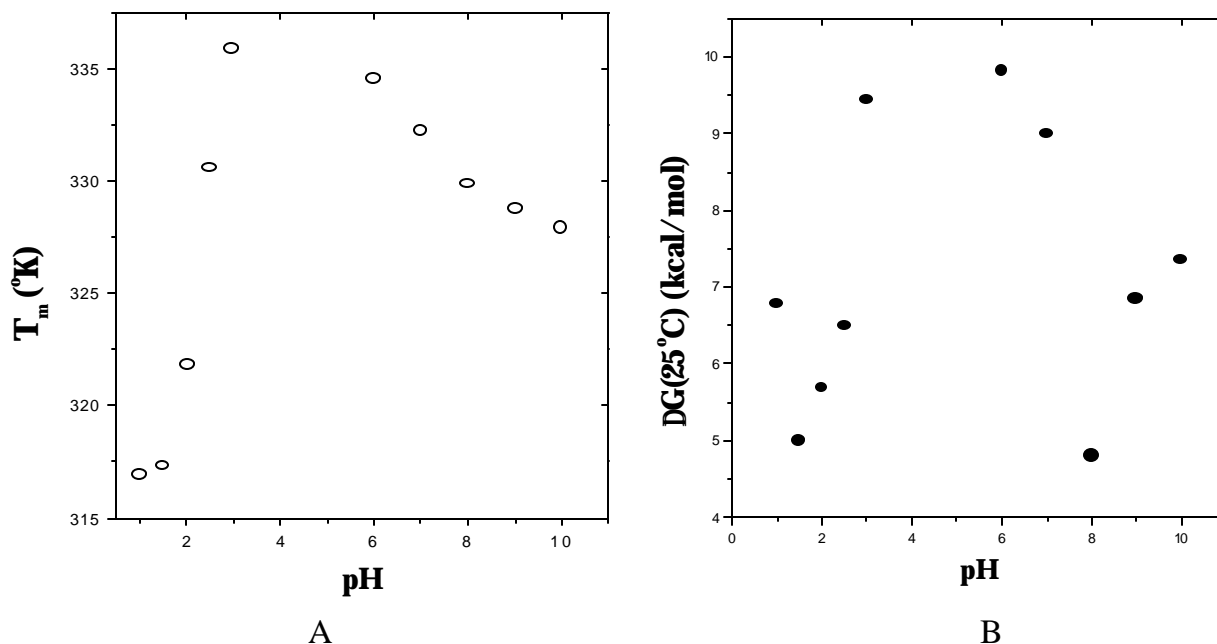
**Table4.3: Parameters for thermal denaturation**

pH	$S_m$ (cal/mol/deg)	$T_m$ (°C)	$T_m$ (°K)	$H_m$ (kcal/mol)	$G(25^\circ\text{C})$ (kcal/mol)
1	389.15	43.9	316.94	123.34	6.79
1.5	290.18	44.29	317.29	92.072	5.00
2	276.67	48.82	321.82	89.04	5.69
2.5	250.00	57.56	330.56	82.64	6.48
3	308.22	62.88	335.88	103.52	9.46
6	325.55	61.51	334.51	108.9	9.82
7	316.04	59.20	332.20	104.99	8.99
8	200.44	56.88	329.88	66.12	4.80
9	271.03	55.73	328.73	89.09	6.85
10	293.077	54.87	327.87	96.09	7.35

**Table 4.4 : Parameters for thermal denaturation in presence of urea at pH 5.0**

Urea [M]	$S_m$ (cal/mol/deg)	$T_m$ (°C)	$T_m$ (°K)	$H_m$ (kcal/mol)	$G(25^\circ\text{C})$ (kcal/mol)
2	363.86	55.74	328.74	119.61	9.70
3	331.52	50.66	323.66	107.3	7.46
4	322.87	42.17	315.17	101.76	5.07
5	279.37	35.49	308.49	86.18	2.75
5.5	331.5	32.33	305.33	101.21	2.34

---



**Fig. 4.14:  $T_m$  and  $G(25^\circ\text{C})$  as a function of pH for thermal denaturation of RNase Rs.**  $T_m$  ( ) was calculated from plots of  $G_U$  against  $T$  using equation 4.  $G(25^\circ\text{C})$  ( ) was calculated using equation 6.  $C_p$  value used was obtained from the slope of the plot of  $H_m$  versus  $T_m$  varied as a function of urea.

## 4.6 DISCUSSION

The native conformation of a protein molecule, which determines its biological activity, is stabilized by various non-covalent and covalent forces and perturbation of these forces results in denaturation of the protein molecule. Although biologically inactive, the non-native or denatured conformations are important for phenomena such as protein folding and stability, transport across membranes and proteolysis and

protein turnover (Dill and Shortle, 1991). The protein denaturation studies have proved important not only for the conformational stability studies by revealing the magnitude and balance between stabilizing forces but also in understanding the phenomenon of protein folding. Denaturation studies are useful especially for measuring the differences in conformational stability among proteins differing slightly in chemical structure because of differences in amino acid sequence or alterations resulting from chemical modification. Understanding of the folding reaction is essentially derived from the knowledge of the molecular origins of stability and structure in compact denatured states of proteins.

The conformational stability studies are carried out by denaturation of proteins using different agents such as temperature, pH, chaotropic agents and pressure, either individually or in combination. Denaturation is followed by monitoring the change in any measurable physical property of the protein viz. intrinsic fluorescence, optical rotation (CD), UV absorbance etc. Intrinsic fluorescence of the protein is used as a probe for its tertiary structure and is usually monitored by specifically exciting tryptophan at 295 nm. The fluorescence of tryptophan is greatly influenced by factors such as polarity, pH, temperature and composition of the solvent (Freifelder, 1982). Tryptophan in non-polar solvents exhibits maximum emission at 320 nm whereas in aqueous environment it is at 355 nm. The fluorescence intensity decreases on protonation of tryptophan and is also quenched by neighbouring protonated acidic groups. In addition the fluorescence is also quenched at high temperatures. RNase Rs exhibited  $\lambda_{\text{max}}$  at 336 nm indicating partially buried tryptophans and the shift in  $\lambda_{\text{max}}$  to 355 nm suggested that they are completely exposed to solvent as a result of conformational changes due to denaturation of the enzyme in presence of denaturants. The intensity of fluorescence reduced at extremes of pH and high temperature although high concentrations of urea and Gu-HCl enhanced it. The denaturation curves exhibited the characteristic pattern of three regions namely, pre-transition, transition and post-transition (Tanford 1968; Pace *et al.*, 1989). The steep transition regions were

an indication of the cooperative conformational changes occurring within a narrow range of denaturant concentration or temperature.

The transition region of denaturation curves is characterized by two parameters, the transition mid-point ( $D_{1/2}$  and  $T_m$ ) and the steepness of the transition region ( $m$ ). The transition region also yields the free energy of unfolding ( $G_2^{H_2O}$ ) which is the difference in free energy between the folded and unfolded conformations under physiological conditions and is used as a measure of the stability of the protein to denaturants. Moreover, the behaviour of  $G_2^{H_2O}$  obtained with chemical and thermal denaturation over a range of pH offers a measure of the contributions of electrostatic interactions to the conformational stability of proteins. Majority of proteins exhibit denaturation curves in a single step and are considered to follow a two-state F U mechanism (Pace, 1975; 1989). Since, the chemical and thermal denaturation curves obtained for RNase Rs exhibited a single step, a two-state unfolding mechanism was assumed for further analysis and the aforementioned parameters were determined using the linear extrapolation model (Pace, 1986). Urea denaturation of RNase Rs over a range of pH showed highest  $D_{1/2}$ (urea) and  $G_2^{H_2O}$  at pH 5.0, the pI of RNase Rs, indicating that the protein is most stable at its pI. In general, proteins are most stable at their isoelectric pH since the electrostatic interactions among the charged groups will tend to favor the maximum conformational stability occurring near the pI (Pace, 1990). RNase A and RNaseT1 were stable at pH 9.0 and 5.0 respectively, which was near to their pI (pH 9.6 and 4.0 respectively) (Pace *et al.*, 1990). However, barnase exhibited maximum stability between pH 5.0 - 6.0, much below its pI (pH 9.0) (Pace *et al.*, 1992). The  $G_2^{H_2O}$  values obtained from analysis of the denaturation curves with different denaturants for the same protein should generally be similar (Pace, 1986) and in case of RNase Rs the denaturation with Gu-HCl at pH 5.0 yielded  $G_2^{H_2O}$  similar to that of urea denaturation. The  $D_{1/2}$ (Gu-HCl) was nearly half that of  $D_{1/2}$ (urea) at pH5.0,

indicating Gu-HCl to be a more effective denaturant. Gu-HCl is known to be a more potent denaturant than urea (Pace, 1986). The conformational stability of almost all naturally occurring globular proteins is between 5 - 15 kcal/mol and the  $G_2^{\text{H}_2\text{O}}$  (~13 kcal/mol) value obtained for RNase Rs falls in this range (Pace, 1990).

The mechanism by which proteins fold and unfold has always been an important study. It is obvious that a protein molecule will assume various partially folded conformations in the course of folding and unfolding. For denaturation studies, the concentration of these intermediates at equilibrium in the transition region is of considerable importance. Although many proteins show denaturation curves with a single step, this is no guarantee that intermediates do not exist (Pace, 1975). The presence of intermediates, at significant concentrations, is detected with non-coincidence of the denaturation curves obtained by using different techniques. Analysis in terms of two-state mechanism can still be useful even in cases where denaturation deviates substantially from a two-state mechanism since intermediates present in low concentrations will not lead to large errors in equilibrium analysis of unfolding (Pace, 1975). In the present case, the coincidence of the urea denaturation curves obtained with fluorescence and CD at pH 5.0 supports a two-state unfolding mechanism. The tryptophan fluorescence is known to be affected by pH of the solvent and is seen to decrease in the acidic pH. This might be the cause of the non-coincidence of the denaturation curves at pH 1.0 and 2.5.

Although RNase Rs exhibited maximum stability at pI for urea denaturation, it was seen to precipitate above 50°C in the range of one pH unit on either side of pI. The precipitation occurred much before the protein was completely denatured since the fluorescence spectrum never reached that for denatured protein. Hence, the precipitation was probably because at pI the intermolecular repulsive forces due to charge are minimum, allowing the hydrophobic interaction to mediate aggregation. Aggregation in thermal denaturation is a common phenomenon (Tanford, 1968). In case of RNase T1, slow formation of precipitates was sometimes observed in protein

solutions at high concentrations on prolonged incubation above the denaturation temperature (Oobatake *et al.*, 1979). In the present case, aggregation could be prevented with urea, since urea and Gu-HCl are known to increase the solubility of almost all of the component parts of a protein (Pace, 1975). The denaturation was achieved at lower temperatures with increasing concentration of urea. The  $T_m$  and  $G(25^\circ\text{C})$  showed a similar pattern to urea denaturation, in the pH range where reversible denaturation was seen.

Chemical denaturation is usually reversible whereas thermal denaturation may not always be reversible (Pace, 1975). The denaturation with urea (pH 1.0 - 10.0), Gu-HCl (pH 5.0) and temperature (in the pH ranges 2.0 – 3.0 and 6.0 – 9.0) was reversible as evidenced by the restoration of fluorescence spectra and >60% recovery of enzymatic activity. On the contrary, irreversible denaturation (only 30% of recovery of activity) was observed at the extreme pH i.e. pH 1.0, 1.5 and 10.0 with thermal denaturation of RNase Rs. The changes in pH alone are sufficient to induce conformational changes in a protein and many proteins have been shown to denature at extremes of pH and in some cases are reported to attain a partially folded molten-globule structure (Goto *et al.*, 1990). RNase Rs was not denatured at acidic extremes although the protein was irreversibly denatured at extreme alkaline pH even at  $25^\circ\text{C}$ . Covalent changes in the structure of proteins are known to occur at high pH which probably cause the irreversibility in most cases (Pace, 1975). Similar base-induced denaturation was observed in case of barstar (Khurana *et al.*, 1995) whereas its acid denaturation lead to formation of molten globule (Khurana and Udgaonkar, 1994).

In conclusion, RNase Rs followed a two state F U unfolding mechanism during urea, Gu-HCl and thermal denaturation. The pH dependence of  $G_2^{\text{H}_2\text{O}}$  suggests that the electrostatic interactions among the charged groups make a significant contribution to the conformational stability of the enzyme.

# ***Chapter 5***

## *Immobilization of RNase Rs*



## 5.1 SUMMARY

Purified RNase Rs when covalently coupled to aminoethyl (AE) Bio-Gel P-2, via its carbohydrate moiety, retained 35-40 % activity of the soluble enzyme. Optimization of coupling conditions showed that the most active immobilized preparations are obtained when 400 units of 100  $\mu$ M periodate oxidized enzyme are allowed to react with 1 ml (packed volume) of AE-Bio-Gel P-2 at  $6\pm 1^\circ\text{C}$  for 15 h. Immobilization did not change the pH and temperature optima of the enzyme but it increased the temperature stability. Immobilization did not bring about a change in the  $K_m$  but resulted in a 2.5-fold decrease in the  $V_{max}$ . Substrate concentrations as high as 25 mg of RNA could be converted to more than 80 % 2',3'cyclic nucleotides, in 14 h, at pH 5.5 and  $37^\circ\text{C}$ . On repeated use, the bound enzyme retained 70 % of its initial activity after 6 cycles of use. The bound enzyme could be stored, in wet state, for 60 days without any significant loss in its initial activity.

## 5.2 INTRODUCTION

The advent of immobilized enzyme technology has led to increasing efforts to replace the conventional enzymatic reactions with immobilized enzymes as immobilization affords advantages like, reusability, enhanced stability, greater control of the catalytic process and the development of continuous process. RNases are important analytical enzymes and have found extensive application in the determination of RNA structure. They are also used for the reduction of RNA in single cell protein preparations. The synthetic activity associated with enzymes like RNase T1 and *Aspergillus clavatus* RNase have been exploited for the preparation of tri-, tetra- and penta- nucleotides, having guanylic acid at the 5' end (Reddy and Shankar, 1993). Due to extensive applications of RNases, numerous attempts have been made to obtain highly active and stable immobilized preparations suitable for various biotechnological applications. RNase Rs from *Rhizopus stolonifer* produces 2',3'cyclic nucleotides as the major end products of RNA hydrolysis. It is a glycoprotein and contains 10.5 % neutral sugar (Chacko and Shankar, 1998). It has been reported that coupling of glycoproteins to solid supports poses considerable difficulties due to the shielding of the reactive groups of amino acid side chains by carbohydrates.

To overcome this problem, several glycoprotein conjugates have been prepared, where the carbohydrate side chains provide the point of attachment between the enzyme and the matrix (Zaborsky and Ogletree, 1974; Hsiao and Royer, 1979; Marek *et al.*, 1984; O'Shannessy and Wilchek, 1990). Such methods of binding, either by adsorption or covalent coupling, to solid supports afford high retention of enzyme activity, probably due to the fact that the carbohydrate moiety of the enzyme is not essential for its catalytic activity and hence the protein moiety is free of the restrictions imposed upon it as a result of binding. Moreover, studies on the active site nature of RNase Rs suggested the involvement of histidine, lysine and carboxylate in the catalytic activity of the enzyme (Rangarajan *et al.*, 1999). In view of this, attempts were made to bind RNase Rs covalently via its carbohydrate moiety and assess its potential for the production of 2',3' cyclic nucleotides.

### **5.3 MATERIALS**

DEAE-Cellulose (DE-52) and CM-cellulose (CM-52) (Whatman Paper Ltd., Maidstone, U.K.); Bio-Gel P-2 (40-80  $\mu$ m; Bio-Rad, Richmond, CA, USA); all 3', 5'-mononucleotides, 2',3'-cyclic nucleotides and 2,4,6-trinitrobenzenesulphonic acid (TNBS) (Sigma Chemical Co., St. Louis, MO, USA); ethylenediamine and sodium periodate (Sisco Research Laboratories, Mumbai, India); uranyl acetate (Loba Chemie Pvt. Ltd., Mumbai, India); HPLC grade acetonitrile (E. Merck India Ltd., Mumbai, India); sodium cyanoborohydride (Aldrich Chemical Co., Milwaukee, WI, USA) and ethylene glycol (SD Fine Chemicals Pvt. Ltd., Boisar, India) were used. All other chemicals used were of analytical grade.

### **5.4 METHODS**

#### *5.4.1 Purification of RNA*

The commercial RNA (Sisco Research Laboratories, Bombay, India) gave high blanks and hence it was purified according to Lindahl and Fresco (1967) with minor modifications. Five g of crude RNA was dissolved in 1 l of 10 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl. The insoluble material was removed by centrifugation (6800 g, 10 min) and after the

addition of 1.5 l of cold ethanol to the supernatant, the mixture was left overnight at -20°C. The precipitated RNA was collected by centrifugation (6800 g, 10 min) and lyophilized. The dried residue was dissolved in 200 ml of 1 mM EDTA, pH 7.0 and deproteinized using phenol : chloroform : isoamylalcohol (25:24:1, v/v). Subsequently the mixture was centrifuged (6800 g, 10 min) and from the aqueous layer, RNA was precipitated with 1.5 volumes of cold ethanol. The precipitated RNA was collected by centrifugation (6800 g, 10 min), lyophilized and stored at 4°C till further use.

#### 5.4.2 *Determination of RNase activity*

RNase Rs was assayed at pH 5.5 and 37°C by measuring the increase in acid soluble ribonucleotides at 260 nm. The amount of acid soluble ribonucleotides was calculated by assuming a molar absorption coefficient of 10,600 M<sup>-1</sup>cm<sup>-1</sup> (Curtis *et al.*, 1966) (Chapter 2).

The immobilized enzyme was assayed by incubating appropriate amounts of the bound enzyme with 3 ml of the standard reaction mixture at 37°C, in a thermostated shaker water bath (125 rpm) for 15 min, followed by estimating the acid soluble nucleotides liberated at 260 nm.

One unit of RNase activity is defined as the amount of enzyme required to liberate 1 μmol of acid soluble nucleotides/min under the assay conditions.

#### 5.4.3 *Protein determination*

Protein concentrations were determined using the formula  $1.55 A_{280} - 0.76 A_{260} = \text{protein (mg/ml)}$  (Stoscheck, 1990).

#### 5.4.4 *Purification of RNase Rs*

Cultivation of *R. stolonifer* and purification of RNase Rs was carried out as described in Chapter 2.

#### 5.4.5 *Preparation of amino ethyl Bio-Gel P-2 (AE-Bio-Gel P-2)*

Amination of Bio-Gel P-2 was carried out essentially according to the procedure of Inman and Dintzis (1969). In a typical experiment, 50 ml of ethylenediamine was preheated, under mild stirring, in an oil bath maintained at 90°C kept in a fume hood. Subsequently, 5 g of dry Bio-Gel P-2 beads were slowly added and the reaction was allowed to proceed at 90°C for 5 h. At the end of the reaction, the reaction mixture was cooled in ice and mixed with an equal volume of crushed ice. The aminated gel was then washed extensively with 100 mM NaCl to remove the excess free amine (as indicated by a negative colour test with TNBS) followed by 50 mM sodium acetate buffer, pH 5.5 and stored in the same buffer, at 4°C, till further use.

#### 5.4.6 *Determination of amino group content of AE-Bio-Gel P-2*

This was carried out according to Antoni *et al.* (1983). AE-Bio-Gel P-2 (0.1 ml packed volume) in 10 ml of 100 mM sodium tetraborate was mixed with 1 ml of 10 mM TNBS and incubated at 37°C for 2h under mild agitation (~100 rpm). The blank consisted of 10 ml of 100mM sodium tetraborate and 1 ml of 10 mM TNBS, without the gel. After the incubation period, the gel was removed by centrifugation and 1 ml of the supernatant was diluted with 5 ml of 100 mM sodium tetraborate and mixed with 0.5 ml of 30 mM glycine. The blank was made up of 6 ml of diluted supernatant and 0.5 ml of water instead of glycine. The reaction mixture was incubated at 25°C for 25 min and then mixed with 10 ml of cold methanol and the absorbance was measured at 340 nm. The amino group content was calculated by assuming a molar absorption coefficient  $12400 \text{ M}^{-1}\text{cm}^{-1}$ . The amino group content of the matrix was 545  $\mu\text{moles/ml}$  matrix (packed volume).

#### 5.4.7 *Periodate oxidation of RNase Rs*

Two ml of the purified enzyme (2600 U) in 50 mM sodium acetate buffer, pH 5.5, was incubated with 100  $\mu\text{M}$  sodium periodate, in the presence of 500  $\mu\text{g}$  of RNA, at 4°C for 30 min, in dark. Subsequently, the reaction was arrested by the addition of 100  $\mu\text{l}$  of ethylene glycol and

incubated for an additional 30 min. The reaction mixture was then dialysed extensively against 50 mM sodium acetate buffer, pH 5.5 and used for immobilization studies.

#### 5.4.8 *Immobilization technique*

In a typical experiment, 400 U (17 µg protein) of oxidized RNase Rs, in 2 ml of 50 mM sodium acetate buffer, pH 5.5, was incubated with 1 ml (packed volume) AE-Bio-Gel P-2, at 4°C for 15 h, under mild agitation (~120 rpm). Subsequently, 5 mg of solid sodium cyanoborohydride was added to the above mixture and incubated for an additional 2 h at 4°C, under mild agitation. The supernatant was decanted and the matrix was washed successively with the coupling buffer, 1 M NaCl in the coupling buffer followed by coupling buffer, till the washings showed no RNase activity. The amount of enzyme and protein bound to the matrix was determined by estimating the difference in the enzyme activity before loading and after coupling. The immobilized enzyme was stored in the coupling buffer at  $4\pm 1^\circ\text{C}$ .

#### 5.4.9 *Determination of efficiency*

Efficiency of the immobilized RNase Rs preparation was determined by assaying appropriate amounts of the bound enzyme, under standard assay conditions, followed by calculating the ratio of measured activity to bound activity. The specific activity of the immobilized enzyme is defined as measured activity/mg protein/ml matrix.

#### 5.4.10 *Hydrolysis of RNA*

The total reaction mixture of 5 ml containing 25 mg of RNA, in 200 mM sodium acetate buffer pH 5.5, was incubated with 10 U (expressed activity) of immobilized RNase Rs in a thermostated shaker waterbath (125 rpm) at 37°C. Samples (375 µl) were removed at fixed time intervals and the reaction was terminated by the addition of 1.125 ml of chilled absolute ethanol. The mixture was left overnight at -20°C and the undigested RNA was removed by centrifugation. The supernatant was lyophilized, reconstituted in 100 µl of Milli Q water and subjected to HPLC.

#### 5.4.11 Separation of the reaction products

High Performance Liquid Chromatography (Perkin Elmer Model 410 solvent delivery system, Rheodyne Model 7125 Injector with 20  $\mu$ l loop) of the reaction products was carried out on a LiChroCART LiChrospher 100 RP18 column (250 x 4 mm, 5  $\mu$ m, Merck, Germany) as described by Chacko and Shankar (1998). The mobile phase comprising of a linear gradient of 0-3 % (v/v) acetonitrile in 100 mM triethylammonium acetate, pH 6.2, was used at 25°C and at a flow rate of 1.5 ml/min. Ten  $\mu$ l of sample or the standard solution was injected onto the column and the nucleotides were detected, at 254 nm, using a Hewlett Packard Model 1050 MW Detector. The amount occupied by each peak was computed on the basis of total area occupied by each peak of the sample and standard. The nucleotides eluted in the order of 2',3'-cCMP, 2',3'-cUMP, 3'-CMP, 3'-UMP, 3'-GMP, 2',3'-cGMP, 3'-AMP and 2',3'-cAMP with retention times of ca. 6.440, 8.945, 11.412, 15.700, 19.077, 20.383, 29.378 and 33.080 min respectively (data not shown).

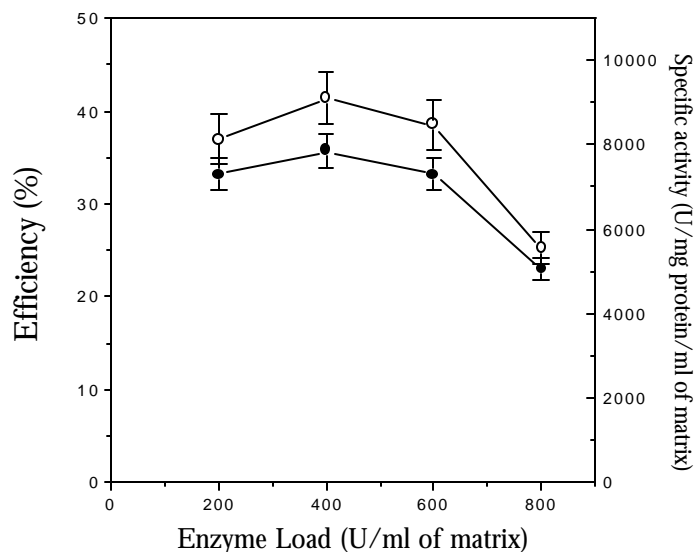
## 5.5 RESULTS

### 5.5.1 Optimization of coupling conditions

When 200 U of periodate oxidized RNase Rs were reacted with 1 ml (packed volume) of AE-Bio-Gel P-2, 176 U were bound. The effectiveness factor ( $\eta$ ) of the immobilized preparation was approximately 0.32, indicating the efficiency of the immobilized preparation to be 32%. RNase Rs lost significant amount of its activity (~60%) when subjected to oxidation. However, the inactivation could be reduced to a significant extent (<20%) by oxidizing in presence of RNA. Effect of matrix to enzyme ratio, on the activity of immobilized preparation, revealed that after an initial increase the efficiency decreased with increase in the enzyme load and maximum efficiency was obtained when approximately 400 U of the enzyme were reacted with 1 ml (packed volume) of AE-Bio-Gel P-2 at pH 5.5. The decrease in the efficiency at higher enzyme load (>400 U) can be attributed to overcrowding of the enzyme on the matrix as there was a decrease in the specific activity of the bound enzyme (Fig. 5.1). The results of a

typical procedure for the immobilization of periodate oxidized RNase Rs on AE-Bio-Gel P-2, under optimized conditions, are given in Table 5.1.

When 400 U of periodate oxidized RNase Rs were reacted with 1 ml (packed volume) of AE-Bio-Gel P-2, at pH 5.5 for 15 h, 306 U were bound. The effectiveness factor ( $\eta$ ) of the immobilized preparation was 0.363 indicating the efficiency of the immobilized system to be approximately 37 % (Table 5.1).



**Fig. 5.1: Effect of matrix to enzyme ratio on the efficiency of immobilized**

**RNase Rs. Efficiency ( ) and specific activity ( ).** One ml (packed volume) of AE-Bio-Gel P-2 was incubated with varying amounts (200 - 800 U) of periodate oxidized enzyme at pH 5.5 and 4°C for 15 h and the efficiency of the immobilized preparations was determined as described under Methods.

**Table 5.1: Immobilization of periodate oxidized RNase Rs on AE-Bio-Gel P-2**

Enzyme loaded		Enzyme bound		Activity of the	Efficiency*
Activity (U)	Protein (µg)	Activity (U)	Protein (µg)	complex (U expressed)	
400.0	15.1	306.0	11.6	111.0	36.28

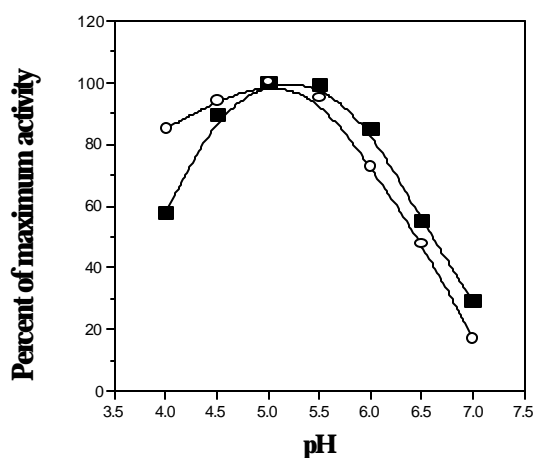
$$*Efficiency = \frac{\text{Activity of the complex}}{\text{Bound activity}} \times 100$$



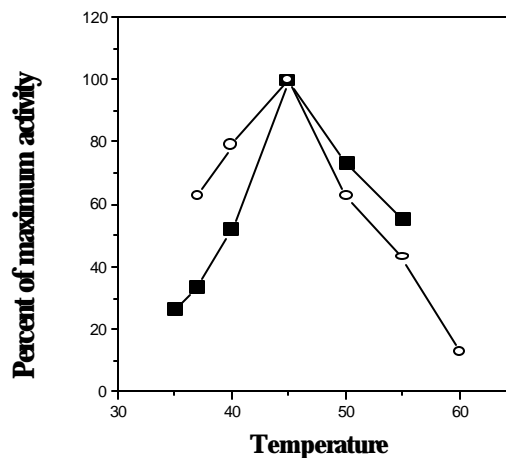
### 5.5.2 Optimum pH and temperature and kinetic parameters

Comparison of the pH activity profiles of soluble and immobilized RNase Rs revealed no change in the optimum pH (5.5) of the enzyme as a result of immobilization (Fig. 5.2). Similarly, no change was observed in the optimum temperature (45°C) of the enzyme due to immobilization (Fig. 5.3). Determination of the kinetic parameters of soluble and immobilized RNase Rs showed practically no change in the  $K_m$  but approximately 2.5-fold decrease in the  $V_{max}$  (Table 5.2).

**Fig. 5.2**



**Fig.5.3**



**Fig. 5.2:** pH activity profiles of soluble (○) and immobilized (■) RNase

**Rs.** Both soluble and immobilized enzymes (1 U) were assayed in a series of pH (4.0 - 7.0) at 37°C as described under Methods.

**Fig. 5.3:** Temperature activity profiles of soluble (○) and immobilized (■)

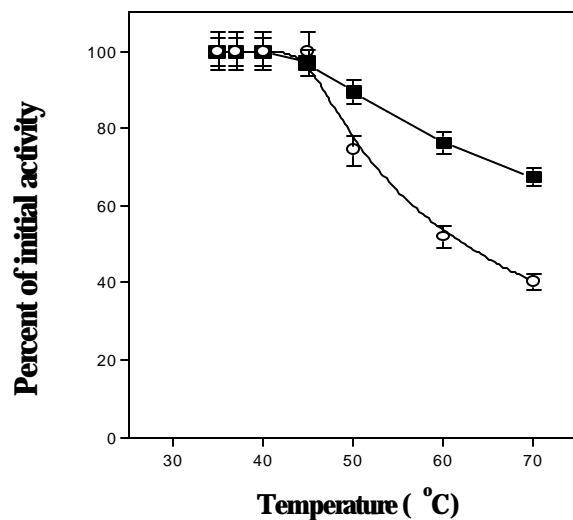
**RNase Rs.** Both soluble and immobilized enzymes (1 U) were assayed in a series of temperatures (35 - 70°C) at pH 5.5 as described under Methods.

**Table 5.2: Kinetic data of soluble and immobilized RNase Rs**

State of the enzyme	K <sub>m</sub> (mg/ml)	V <sub>max</sub> ( $\mu$ mol/mg protein/ml matrix)
Soluble	439.70 $\pm$ 50	98892.30 $\pm$ 25000
Immobilized	443.70 $\pm$ 50	44227.38 $\pm$ 2300

### 5.5.3 Stability of the enzyme

Temperature stability studies on soluble and immobilized RNase Rs showed the bound enzyme to be more stable, as indicated by its inactivation pattern. While the soluble enzyme lost more than 65% of its initial activity, in 15 min at 70°C, the immobilized enzyme retained 60% of its initial activity (Fig. 5.4).

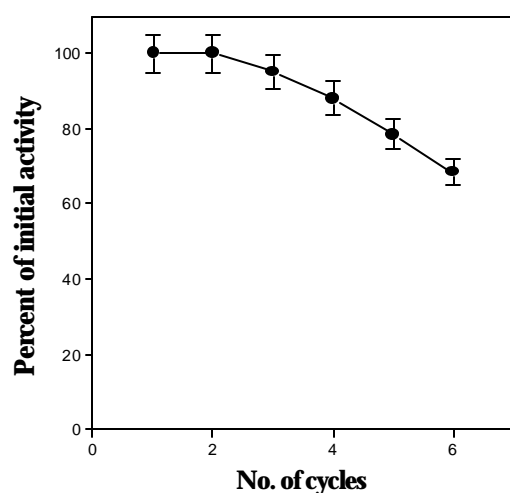


**Fig. 5.4: Temperature stability of soluble (○) and immobilized (■) RNase**

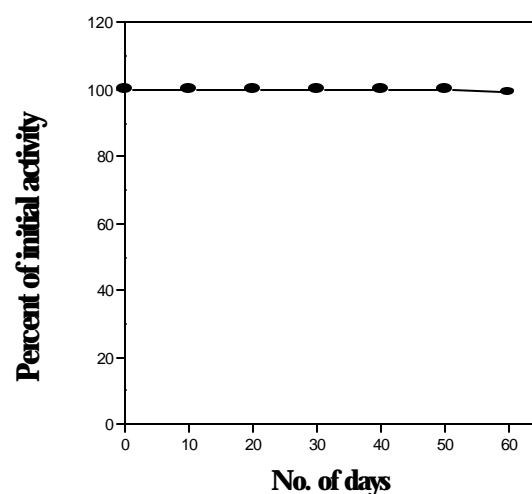
**Rs.** Both soluble (75 U) and immobilized (2 U) enzymes were preincubated at different temperatures, ranging from 35 - 70°C, in 50 mM sodium acetate buffer pH 5.5 and their residual activities were determined under standard assay conditions.

Studies on repeated usability showed that the bound enzyme retained more than 70% of its initial activity after 6 cycles (Fig. 5.5). The bound enzyme showed good storage stability and could be stored in 50 mM sodium acetate buffer pH 5.5, at 4°C, for 60 days without any apparent loss in its initial activity (Fig. 5.6). In batch operations, AE-Bio-Gel P-2 bound enzyme could hydrolyse high concentrations of RNA (25 mg) quantitatively to 2',3'cyclic nucleotides (>80 %), in 14 h, at pH 5.5 and 37°C (Fig. 5.7, Table 5.3).

**Fig. 5.5**



**Fig. 5.6**

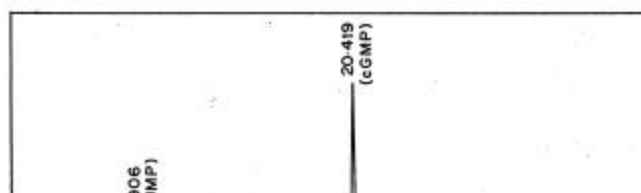


**Fig. 5.5: Effect of number of assay cycles on the activity of immobilized**

**RNase Rs.** 1 U of AE-Bio-Gel P-2 bound enzyme was assayed under standard assay conditions. After every use, the immobilized enzyme was washed free of substrate and products and used for the next assay.

**Fig. 5.6: Storage stability of AE-Bio-Gel P2 bound RNase Rs.** The

immobilized enzyme was stored at 4°C in 50 mM sodium acetate buffer pH 5.5. Aliquots were removed at fixed time intervals and assayed under standard assay conditions as described under Methods.



**Fig. 5.7: Liquid chromatogram of RNA hydrolysate.** Hydrolysis of RNA and separation of reaction products was carried out as described under Methods.

**TABLE 5.3: Composition of the hydrolytic products of RNA by immobilized RNase Rs after 14 h of incubation**

<b>Product</b>	<b>(mmol)</b>
2',3' cGMP	668.0
2',3' cAMP	339.0
2',3' cUMP	325.0
2',3' cCMP	420.0
3'GMP	55.20
3'AMP	84.41
3'UMP	125.60
3'CMP	82.50

## 5.6 DISCUSSION

In any immobilized enzyme system, the important components taken into consideration are the enzyme, the matrix and the mode of attachment of the enzyme to the matrix. Apart from the enzyme, the other most important contributing component, which determines the performance of the immobilized system, is the carrier. Hence, a judicious selection of the carrier is essential, as it not only affects the operational stability but also the performance of the immobilized enzyme. In the present investigation Bio-Gel P-2 was selected as the carrier since, polyacrylamide based matrices are more resistant to chemical and microbial degradation, have a lower tendency to physically adsorb proteins, can be easily derivatized and are commercially available. The low porosity of Bio-Gel P-2 (exclusion limit 1800 Da) though limits the surface area available for enzyme binding, it reduces the diffusional constraints with respect to high molecular weight substrates (RNA in the present case) since the enzyme is bound only on the external surface of the carrier.

Optimization of coupling conditions showed that the most active preparations are obtained when 400 U of 100  $\mu$ M periodate oxidized enzyme are reacted with 1 ml (packed volume) of AE-Bio-Gel P-2 at pH 5.5 and  $6\pm 1^\circ\text{C}$  for 15 h. The AE-Bio-Gel P-2 bound enzyme showed 37% activity of the soluble enzyme. Our efforts to increase the efficiency of the immobilized preparation by increasing the stirring speed or incubation period failed to improve the retention of activity, indicating that the low activity observed can not be correlated to diffusional limitations or to the lack of exposure of the bound enzyme to the substrate.

Comparison of pH activity profiles of soluble and immobilized RNase Rs showed no change in the optimum pH of the enzyme as a result of immobilization, indicating the absence of partitioning effects in the microenvironment of the bound enzyme. Similarly, there was no change in the optimum temperature of the enzyme after immobilization. Evaluation of kinetic parameters of soluble and immobilized RNase Rs showed that the bound enzyme followed Michaelis-Menten kinetics and there was practically no change in the  $K_m$ , suggesting the absence of diffusional barriers. This view is supported by the observation that increase in the stirring speed

had no effect on the efficiency of the bound enzyme. However, the decrease in the  $V_{\max}$  can be correlated to the masking of some of the active sites during coupling.

AE-Bio-Gel P-2 bound RNase Rs showed higher temperature stability compared to its soluble counterpart. Increase in the temperature stability of the bound enzyme can be correlated to its rigid conformation in the bound form. The bound enzyme showed comparatively high stability to repeated use. In the absence of any detectable leaching (as evidenced by the absence of enzyme activity in the supernatant), the decrease in activity can be due to slight inactivation of the enzyme after every use. The stability of the bound enzyme to storage is also suggestive of its high stability. The ability of AE-Bio-Gel P-2 bound RNase Rs to convert high concentrations of RNA to 2', 3'cyclic nucleotides shows that it has the potential for the commercial production of cyclic nucleotides.

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