... to my parents

PURIFICATION AND CHARACTERISTICS OF EXTRACELLULAR NUCLEASE FROM *RHIZOPUS STOLONIFER*

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DECLARATION

Certified that the work incorporated in the thesis entitled **"Purification and characteristics of extracellular nuclease from *Rhizopus stolonifer*"** submitted by ***Mr. E. S. Rangarajan*** 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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E. S. Rangarajan

SUMMARY

Nucleic acids, the vital biomolecules present in the cell, act as carriers of genetic information. In order that the genetic information is transferred from one generation to another they have to undergo processes viz. replication, recombination and repair. All living organisms contain a set of enzymes namely nucleases, which hydrolyze the phophodiester linkages in nucleic acids. Among them, conformation non-specific endo and exonucleases have been implicated in cellular functions like replication, recombination and repair. Their ability to recognize different DNA structures have made them important analytical tools for the determination of nucleic acid structure, mapping mutations and studying interaction of DNA with various intercalating agents. Though they are wide spread in distribution, only a few enzymes namely S1 nuclease from *Aspergillus oryzae*, P1 nuclease from *Penicillium citrinum*, BAL 31 nuclease from *Alteromonas espejiana* and nucleases from *Neurospora crassa, Staphylococcus aureus*, *Serratia marcescens* and *Saccharomyces cerevisiae* have been well characterized. Majority of analytically important nucleases have an acid optimum pH and show an obligate requirement of metal ions for their activity, which in turn prevents their use in chelating buffers or in presence of metal chelators. Hence, there is a need to look for a different type of nuclease, which has a pH optimum at or around neutrality and which does not require metal ions for its activity. Screening of several fungal cultures revealed that a strain of *Rhizopus stolonifer* (NCIM 880) produces high activity of an extracellular nuclease when grown on yeast extract - peptone - glucose medium. The crude enzyme showed higher activity on sonicated and heat denatured DNA than on native DNA, at pH 7.0. Hence the present investigation was carried out to purify and extensively characterize this extracellular nuclease to understand its structure function correlation and potential applications.

Chapter 1: General introduction

This part comprises of literature survey with reference to sugar non-specific endonucleases, their occurrence, purification, properties and applications.

Chapter 2: Purification and characterization of the deoxyribonuclease activity

An extracellular nuclease from *Rhizopus stolonifer* (designated as nuclease Rsn) was purified to homogeneity by chromatography on DEAE-cellulose followed by Blue Sepharose. The Mr of the purified enzyme, determined by native PAGE, was 67,000 and it is a tetramer and each protomer consists of two unidentical subunits of Mr 21,000 and 13,000. It is an acidic protein with a pI of 4.2 and is not a glycoprotein. The purified enzyme showed an obligate requirement of divalent cations like Mg^{2+}, Mn^{2+} and Co^{2+} for its activity but is not a metalloprotein. The optimum pH of the enzyme was 7.0 and it was not influenced by the type of metal ion used. Although, the optimum temperature of the enzyme for single stranded DNA hydrolysis in presence of all three metal ions and for double stranded DNA hydrolysis in presence of Mg^{2+} was 40 °C, it showed higher optimum temperature (45 °C) for double stranded DNA hydrolysis in presence of Mn^{2+} and Co^{2+}. Nuclease Rsn was inhibited by divalent cations like Zn^{2+}, Cu^{2+} and Hg^{2+}, inorganic phosphate and pyrophosphate, NaCl and KCl but not by 3' and 5' nucleotides. It was susceptible to low concentrations of SDS, guanidine hydrochloride and urea and organic solvents like dimethyl sulphoxide, dimethyl formamide and formamide. The studies on mode and mechanism of action showed that nuclease Rsn is an endonuclease and cleaves double stranded DNA through a single hit mechanism. The end products of both single stranded DNA and double stranded DNA hydrolysis were predominantly oligonucleotides ending in 3' hydroxyl and 5' phosphoryl termini. Moreover, the type of metal ion used did not influence the mode and mechanism of action of the enzyme.

Chapter 3: Characterization of the associated ribonuclease activity

Nuclease Rsn from *Rhizopus stolonifer* is a multifunctional enzyme and hydrolyzes both DNA and RNA. The RNase activity of the enzyme also showed an obligate requirement of divalent cations like Mg^{2+}, Mn^{2+} or Co^{2+} for its activity. The optimum pH and temperature, for RNA hydrolysis, were 7.0 and 35 °C

respectively and it was not influenced by the type of metal ion used. RNase activity of nuclease Rsn was inhibited by Zn^{2+}, inorganic phosphate and pyrophosphate but not by 2', 3' or 5' mononucleotides. Action on homopolyribonucleotides revealed that, nuclease Rsn hydrolyzed poly A rapidly whereas poly(U), poly(C) and poly(G) were resistant to cleavage. Nuclease Rsn hydrolyzed RNA endonucleolytically, liberating oligonucleotides ending in 3' hydroxyl and 5' phosphoryl termini as the end product of hydrolysis indicating that it cleaves at sites 5' to the phosphoribose. Additionally, the type of metal ions used did not influence the mode of action of the enzyme. The 3' and 5' terminal base analysis revealed that, RNase activity of nuclease Rsn is adenine specific and cleaves internucleotide bonds in the order of ApA >> UpA > CpA. However, GpX and XpG were resistant to hydrolysis.

Chapter 4: Active site characterization

Chemical modification studies on purified nuclease Rsn revealed the involvement of two histidine, single tryptophan and two carboxylate residues in the catalytic activity of the enzyme. Substrates of nuclease Rsn viz. DNA and RNA could not protect the enzyme against DEP and EDAC mediated inactivation whereas, substrate protection was observed in case of NBS-mediated inactivation of the enzyme. K_m and k_{cat} values of the partially inactivated enzyme samples suggested that while histidine and carboxylate are involved in catalysis, tryptophan is involved in substrate binding. Furthermore, fluorescence quenching studies on native and modified nuclease Rsn, using metal ions, indicated the involvement of carboxylate in metal binding. The involvement of carboxylate in catalysis coupled with its role in metal binding suggests that, cleavage of DNA and RNA, by nuclease Rsn, probably follows a mechanism of metal ion-mediated catalysis.

Publications

1. Rangarajan, S. and Shankar, V. (1999) Extracellular nuclease from *Rhizopus stolonifer*: characteristics of - single strand preferential - deoxyribonuclease activity. *Biochim. Biophys. Acta* **1473**, 293-304.

2. Rangarajan, E. S. and Shankar, V. (2000) Nuclease Rsn from *Rhizopus stolonifer*: characteristics of associated - adenine specific - ribonuclease activity. *J. Biochem. Mol. Biol. Biophys.* (In press)
3. Rangarajan, E. S. and Shankar, V. (2000) Active site characterization of nuclease Rsn from *Rhizopus stolonifer*: involvement of histidine in catalysis, tryptophan in substrate binding and carboxylate in metal binding. *J. Biochem. Mol. Biol. Biophys.* (In press).

CHAPTER - 1

GENERAL INTRODUCTION

SUGAR NON-SPECIFIC ENDONUCLEASES

Nucleic acids, the vital biomolecules present in the cell, act as carriers of genetic information. For the effective transfer of genetic information from one generation to another, they have to undergo processes such as replication, recombination and repair. All living organisms contain a set of enzymes, namely nucleases, which hydrolyze phosphodiester linkages in nucleic acids. The involvement of enzymes in the break down of nucleic acids was first observed by Araki (1903) and the term "nucleases" was coined by Iwanoff (1903). Kunitz, in 1940, described two classes of nucleases based on their sugar specificity. Subsequently, different schemes of classification were proposed in an attempt to overcome the shortcomings of the earlier ones (Kunitz, 1950; Laskowski, 1959, 1967). However, with the discovery of newer nucleases and multifunctional enzymes like micrococcal nuclease (Cunningham *et al*., 1956) and snake venom phosphodiesterase (Schmidt, 1955) the classification of Kunitz (1940,1950) was found to be inadequate. In order to overcome these inadequacies, Bernard (1969) and Laskowski (1959, 1982) came up with consensus criteria for the classification of nucleases on the basis of:

(i) The nature of substrate hydrolyzed (DNA, RNA)

(ii) The type of nucleolytic attack (exonuclease and endonuclease)

(iii) The nature of the hydrolytic products formed *i.e.* mono or oligonucleotides terminating in a 3'- or a 5'- phosphate and

(iv) The nature of the bond hydrolyzed.

Though the above classification could accommodate enzymes with exceptional properties into a particular group, discrepancies still existed as the complex nature of the catalytic activities of different nucleases got unraveled. Properties related to strand specificity, site specificity and sequence specificity did not find a place in the above classification scheme. Additionally, nucleases from *Neurospora crassa* (Linn and Lehman, 1966) and BAL 31 nuclease from *Alteromonas espejiana* (Gray *et al*., 1975) exhibited mixed activities, depending on the substrate, making it difficult for them to be classified under a particular scheme. Taking all these

aspects into consideration, Linn (1982) formulated the most acceptable system of classification of nucleases (Table 1.1).

Table 1.1: Classification of nucleases

Sugar specific nucleases		Sugar nonspecific nucleases
Ribonuclease	Deoxyribonuclease	
	I. General deoxyribonucleases	
Exoribonuclease	Exodeoxyribonuclease	Exonucleases
3' phosphate termini	3' phosphate termini	3' phosphate termini
5' phosphate termini	5' phosphate termini	5' phosphate termini
Endoribonuclease	Endodeoxyribonuclease	Endonucleases
3' phosphate termini	3' phosphate termini	3' phosphate termini
5' phosphate termini	5' phosphate termini	5' phosphate termini
	II. Restriction endonucleases Type I, Type II & Type III	
	III. Damage specific deoxyribonucleases	
	IV. Topoisomerases Type I & II	
	V. Recombinase	

Adapted from Mishra (1995).

Nucleases are ubiquitous in distribution and among them non-specific endo and exonucleases have been implicated in cellular functions like replication, recombination and repair. Their ability to recognize different DNA structures have made them important analytical tools for the determination of nucleic acid structure (Kacian and Spiegelman, 1974; Drew, 1984; Przykorska *et al*., 1989), mapping

mutations (Legerski *et al.*, 1978) and studying interaction of DNA with various intercalating agents (Lau and Gray, 1979; Fox and Waring, 1987). These enzymes are characterized by their ability to hydrolyze both DNA and RNA Fraser and Low (1993) further subdivided the major sugar non-specific nucleases into three distinct but distantly related groups viz.

(i) secreted fungal single-strand-specific endonucleases,

(ii) protease-sensitive multifunctional endo-exonucleases and

(iii) mitochondrial nucleases that are closely related to bacterial *Serratia marcescens* nuclease.

Although more than 30 non-specific endonucleases have been isolated till now (Table 1.2), only Staphylococcal nuclease (Tucker *et al.*, 1978, 1979a,b) and *S. marcescens* nuclease (Benedik and Strych, 1998) have been extensively characterized. However, past few years have witnessed significant progress in the molecular enzymology of some of these enzymes. The present review gives a comprehensive account of non-specific endonucleases with respect to their occurrence, purification, physicochemical properties, biological role and applications.

Occurrence and localization

It is well known that nucleases play an important role in four R's i.e. replication, recombination, restriction and repair. Hence every living organism must produce one or the other type of nuclease. Non-specific endonucleases have been isolated from a wide variety of sources (Table 1.2). Majority of these enzymes are intracellular but enzymes from *S. aureus* (Cunningham *et al.*, 1956), *S. marcescens* (Eaves and Jeffries, 1963), *B. subtilis* (Kanamori *et al*., 1973a), *A. espejiana* (Gray *et al*., 1975), *Vibrio* sp. (Maeda and Taga, 1976), *L. enzymogens* (von Tigerstrom, 1980), *S. cerevisiae* (Chow and Resnick, 1987), *Anabaena* sp. PCC 7120 (Muro-Pastor *et al.*, 1992), *S. racemosum* (Chen *et al*., 1993), *C. echinulata* (Ho *et al.*, 1998) and *S. antibioticus* (Nicieza *et al*., 1999) are extracellular in nature. Endonucleases of mitochondrial origin have been reported from *N. crassa* (Linn and Lehman, 1966; Chow and Fraser, 1983), *S. cerevisiae* (Morosoli and

Table 1.2: Sugar non-specific endonucleases

Enzyme	Source	Reference
Microbes		
Staphylococcal nuclease	*Staphylococcus aureus*	Cunningham *et al.*, 1956
Azotobacter nuclease	*Azotobacter agilis*	Stevens and Hilmoe, 1960a
Sm nuclease	*Serratia marcescens*	Eaves and Jeffries, 1963
Neurospora nuclease	*Neurospora crassa* mitochondria	Linn and Lehman, 1966 Chow and Fraser, 1983
Bacillus nucleases	*Bacillus subtilis*	Kanamori *et al.*, 1973a
BAL 31 nuclease	*Altermonas espejiana*	Gray *et al.*, 1975
Marine bacterium nuclease	*Vibrio* sp.	Maeda and Taga, 1976
Lysobacter nuclease	*Lysobacter enzymogens*	von Tigerstrom, 1980
Yeast nuclease	*Saccharomyces cerevisiae* mitochondria	Morosoli and Lusena, 1980 von Tigerstrom, 1982 Chow and Resnick, 1987 Dake *et al.*, 1988
Nucleases SM1 & SM2	*Serratia marcescens kums* 3958	Yonemura *et al.*, 1983
Aspergillus nuclease	*Aspergillus nidulans*	Koa *et al.*, 1990
Anabaena nuclease	*Anabaena* sp. PCC 7120	Muro-Pastor *et al.*, 1992
Sr nuclease	*Syncephalastrum racemosum*	Chen *et al.*, 1993
Schizosaccharomyces nuclease	*Schizosaccharomyces pombe* mitochondria	Ikeda *et al.*, 1996
Streptococcus MF	*Streptococcus pyogenes*	Iwasaki *et al.*, 1997
Leishmania nuclease	*Leishmania* sp.	Mittra *et al.*, 1998
Streptomyces nucleases	*Streptomyces antibioticus*	Nicieza *et al.*, 1999
C1 nuclease	*Cunninghamella echinulata*	Ho *et al.*, 1998

Enzyme	Source	Reference
Plants		
Potato nuclease	Potato tubers	Nomura *et al.*, 1971
Tea leaves nucleases	Tea leaves	Imagawa *et al.*, 1982
Pollen nuclease	Tobacco pollen	Matousek and Tupy, 1984
Barley nuclease	Barley aleurone layers	Brown and Ho, 1986
Pollen nuclease	*Petunia hybrida* pollen	van der Westhuizen *et al.*, 1987
Wheat nuclease	Wheat chloroplasts	Kuligowska *et al.*, 1988
Rye germ nuclease - I	Rye germ ribosomes	Siwecka *et al.*, 1989
Rye germ nuclease - II	Rye germ ribosomes	Siwecka, 1997
Barley microspores nuclease	*Hordeum vulgare L.* uninucleate microspores	Vischi and Marchetti, 1997
Animals		
Rat liver nuclease	Rat liver nuclei	Cordis *et al.*, 1975
Endonuclease G	*Bos taurus* mitochondria	Ruiz-Carrilo and Renaud, 1987
Shrimp nuclease	Shrimp hepatopancreas	Chou and Liao, 1990
Drosophila nuclease	*Drosophila melanogaster*	Shuai *et al.*, 1992
Drosophila nuclease	*Drosophila melanogaster* embryos	Harosh *et al.*, 1992

Lusena, 1980; von Tigerstrom, 1982; Dake *et al*., 1988) and *A. nidulans* (Koa *et al*., 1990). In case of *N. crassa* (Chow and Fraser, 1983) and *A. nidulans* (Koa *et al*., 1990) their presence have been shown in vacuoles, conidia, mycelia and nuclei. Additionally, in *N. crassa*, the mitochondrial nuclease has been shown to occur as membrane-bound as well as soluble forms in approximately equal proportions (Martin and Wagner, 1975).

In plants, non-specific endonucleases have been isolated from rye germ ribosomes (Siwecka *et al*., 1989; Siwecka, 1997), tea leaves (Imagawa *et al*., 1982), wheat chloroplasts and its organelles (Kuligowska *et al*., 1988; Monko *et al*., 1994), barley microspores (Vischi and Marchetti, 1997), pollen grains of tobacco (Matousek and Tupy, 1984) and *P. hybrida* (van der Westhuizen *et al.*, 1987). Presence of these enzymes have also been shown in the lumen of endoplasmic reticulum, golgi apparatus, protein bodies and vacuoles of barley aleurone layers (Holstein *et al.*, 1991).

In the kinetoplastid parasite, *Leishmania* sp., the localization of the endonuclease has been tentatively assigned to nuclei and kinetoplasts (Mittra *et al*., 1998). In animals, they have been isolated from various organelles viz. hepatopancreas of shrimp (Chou and Liao, 1990), rat liver nuclei (Cordis *et al*., 1975), *B. taurus* mitochondria (Côtè and Ruiz-Carrillo, 1993) and embryos of *D. melanogaster* (Harosh *et al*., 1992).

Assay

A. ***Viscosimetry*:** This method is based on the measurement of decrease in viscosity of the nucleic acid samples following the action of nucleases (Laskowski, and Seidel, 1945).

B. ***Spectrophotometric methods*:** The increase in the amount of the acid soluble nucleotides (ribo or deoxyribonucleotides) produced following the hydrolysis of RNA / DNA is measured at 260 nm (Kunitz, 1950; Privat *de* Garhille and Laskowski, 1956). Unit of the enzyme is defined on the basis of µmoles of acid-soluble nucleotides liberated (Fujimoto *et al*., 1974) or µg of RNA or DNA digested (Vogt, 1973).

Viscometric and hyperchromicity measurements can also provide an insight into the mode of action of the enzyme. A sudden drop in the viscosity of the DNA solution without a significant increase in the hyperchromicity suggests an endonucleolytic cleavage whereas, a gradual decrease indicates an exo-mode of action. However, an endoexonuclease can produce significant changes in the hyperchromicity as well as a drop in the viscosity.

C. ***Radioactive measurements:*** The sensitivity of the assay can be enhanced by the use of radiolabelled substrates where, the increase in the acid soluble radioactivity or decrease in the acid insoluble radioactivity following the action of nuclease is measured. Unit of the enzyme is defined as the amount of enzyme required to render 1 nmol of labeled substrate acid soluble, under the assay conditions (Roth and Milstein, 1952).

Another rapid and sensitive assay that measures endonucleolytic activity on DNA utilizes the fact that nitrocellulose membrane can retain only large fragments of denatured DNA. In this procedure, following enzyme action, radiolabelled denatured DNA is passed through nitrocellulose filters and the decrease in the retention of radioactivity on the nitrocellulose membrane is measured (Geiduschek and Daniel, 1965).

D. ***Gel electrophoresis:*** Agarose gel electrophoresis is a convenient and rapid technique for studying the extent and nature of single- or double-strand breaks, the frequency of damage and the pattern of distribution of breaks in the substrate (Kohen *et al*., 1986). Although developed initially as a qualitative technique, it can be augmented and used as a quantitative method by end-labeling the substrate (Pollack *et al*., 1984) or by densitometric scanning following electrophoresis in agarose gels (Doetsch *et al*., 1989). Unit of the enzyme is defined as the amount of enzyme required to produce 1 fmol of nicks in the plasmid DNA under the assay conditions. Separation of the cleavage products on PAGE followed by autoradiography can provide information regarding the cleavage site (Baumann *et al*., 1986).

E. ***Phosphomonoesterase activity:*** Phosphomonoesterase activity, associated with some of the enzymes, is assayed by measuring the inorganic phosphate liberated following the hydrolysis of either 3'AMP or 5'AMP. Unit of the enzyme is defined on the basis of µmoles of inorganic phosphate liberated (Oleson and Sasakuma, 1980).

Detection

A. ***Agar plate method*:** For qualitative detection of nucleases, the nucleic acid (DNA / RNA) is incorporated into the growth medium, in agar plates, along with a dye such as Methyl-Green. The culture to be tested, for nuclease production, is spotted onto the plate and / or the sample is added in wells on the plates. A clear zone around the growth or the well, after precipitation of the unhydrolyzed Methyl-Green nucleic acid complex with HCl, indicates the presence of nuclease activity (Jeferies *et al.*, 1957; Horney and Webster, 1971).

B. ***Zymogram analysis*:** The feasibility of detecting nuclease activity in gels, containing nucleic acids, was first demonstrated by Boyd and Mitchell (1965). Following electrophoresis / isoelectric focussing, the gels are incubated in appropriate buffers for enzyme action and then stained with suitable dyes. A clear band against coloured background indicates the presence of nuclease. Alternatively, nucleases that renature after SDS treatment can be separated on SDS-PAGE containing the nucleic acid. After electrophoresis, the digested regions in the gel are detected as clear bands against the fluorescent background of ethidium bromide bound to nucleic acids (Rosenthal and Lacks, 1977).

Purification

Since majority of non-specific endonucleases are intracellular most of the purification procedures, irrespective of the source, involve steps like lysis of cells, isolation of organelles (by differential centrifugation), concentration of the crude extract by salt precipitation followed by conventional purification methods like ion-exchange chromatography and gel filtration. During the initial purification steps, one of the primary aims is to get rid of the coloured impurities contributed by pigments of the organelles and this is achieved either by extraction with acetone-water mixture (4:1 v/v) (Imagawa *et al.*, 1982) or by a simple wash with ammonium chloride followed by high speed centrifugation (Siwecka *et al.*, 1989; Siwecka, 1997). In most cases apart from sonication (Chou and Liao, 1990), grinding with glass beads or sand (Stevens and Hilmoe, 1960a; Linn and Lehman, 1966; Nomura *et al.*, 1971; Imagawa *et al.*, 1982) have also been used for

disrupting the cells. In general, ammonium sulfate precipitation apart from concentration of the crude extract is also used as a preliminary purification step. In case of membrane bound enzymes, solubilization is achieved using non-ionic detergents like Triton X-100 or Nonidet P-40 and / or high salt wash (Dake *et al*., 1988; Siwecka *et al*., 1989; Côtè *et al*., 1989; Ikeda *et al*., 1996). Moreover, in certain cases, inhibitors like phenylmethylsulfonyl fluoride, pepstatin A and leupeptin are used to overcome the proteolytic activity (Chow and Resnick, 1987; Dake *et al.*, 1988; Côtè *et al*., 1989).

Although, ion-exchangers like DEAE- and CM-cellulose are widely used for the purification of these enzymes, phosphocellulose has also been used in certain cases. For example, potato tuber nuclease, despite its net negative charge at pH 7.5, binds to phosphocellulose due to its affinity towards phosphate groups in phosphocellulose (Nomura *et al*., 1971). In this manner, this support not only acts as a cation exchanger but also as an affinity matrix.

Hyrdoxylapatite has been widely used for the purification of several enzymes including nucleases (Bernardi, 1973). This adsorbent has been used successfully for the purification of endonucleases from *S. cerevisiae* (Morosoli and Lusena, 1980; Chow and Resnick, 1987), shrimp hepatopancreas (Chou and Liao, 1990), *S. racemosum* (Chen *et al*., 1993), *Leishmania* sp. (Mittra *et al*., 1998) and *B. taurus* (Côtè *et al*., 1989). Moreover endonucleases from shrimp hepatopancreas (Chou and Liao, 1990), rye germ ribosomes (Siwecka, 1997), *S. racemosum* (Chen *et al*., 1993) and *C. echinulata* (Ho *et al*., 1998) have been purified on hydrophobic matrices like phenyl- and octyl-Sepharose.

Affinity chromatography has been used extensively for the purification of non-specific endonucleases. Endonucleases from barley aleurone layers (Brown and Ho, 1986), *S. cerevisiae* mitochondria (Dake *et al*., 1988) and *C. echinulata* (Ho *et al.*, 1998) were purified on Heparin-agarose and reactive blue 2-agarose / Cibacron Blue-agarose. Other affinity adsorbents include pdTp-aminophenyl-Sepharose (Taniuchi and Bohnert, 1975), NADP-agarose (Janski and Oleson, 1976) and 5'AMP-agarose (Gray *et al*., 1981). The affinity of non-specific endonucleases for single-stranded nucleic acids has been utilized for the purification of

endonucleases of *S. cerevisiae* (Chow and Resnick, 1987), *A. nidulans* (Koa *et al*., 1990), *D. melanogaster* (Shuai *et al*., 1992) and *Leishmania* sp. (Mittra *et al*., 1998) on ssDNA bound to cellulose. In this case, the chromotographic operation is generally carried out under conditions where the enzyme is either not active or shows very little activity.

Modern purification techniques like FPLC has been utilized successfully for the purification of non-specific endonucleases from *B. taurus* (Côtè *et al.*, 1989), *D. melanogaster* embryos (Harosh *et al*., 1992) and *S. antibioticus* (Nicieza *et al*., 1999).

Molecular mass and subunit structure

Mr of non-specific nucleases fall in the range of 14 - 140 kDa but majority of them are between 28 and 44 kDa (Table 1.3). The enzymes from marine bacterium *Vibrio* sp. (Maeda and Taga, 1976), yeast (von Tigerstrom, 1982) and *A. espejiana* F (fast) form (Wei *et al*., 1983) are high molecular mass proteins with a Mr of 100, 140 and 109 kDa respectively. Nucleases from *S. aureus* (Taniuchi *et al*., 1967), yeast mitochondria (Morosoli and Lusena, 1980), rye germ ribosomes (Siwecka *et al*., 1989) and *S. antibioticus* (Nicieza *et al*., 1999) are comparatively low Mr proteins in the range of 14 - 20 kDa.

Most of the non-specific nucleases consist of a single polypeptide chain but the enzymes from *N. crassa* mitochondria (Chow and Fraser, 1983), *S. racemosum* (Chen *et al*., 1993), *B. taurus* (Côtè *et al*., 1989) and *S. marcescens* (Friedhoff *et al*., 1994b; Miller and Krause, 1996; Franke *et al*., 1998) are dimers made up of two identical subunits of 33, 28, 26 and 26 kDa respectively. Moreover, the dimeric form of non-specific endonucleases from *S. racemosum* (Chen *et al*., 1993) and *S. marcescens* (Ball *et al*., 1992) are held together purely by non-covalent interaction and not by disulfide linkages. A genetically engineered monomeric variant H184R of *S. marcescens* nuclease was shown to exist as a monomer even at high protein concentrations. This monomeric variant exhibited a similar secondary structure, stability towards chemical denaturants and activity, as that of the wild-type enzyme (Franke *et al*., 1998). Although the monomeric

Table 1.3: Properties of sugar non-specific endonucleases

Enzyme	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	Metal ion requirement	Reference
Azotobacter nuclease	-	7.7	-	Mg^{2+}, Mn^{2+}, Co^{2+}	Stevens and Hilmoe, 1960a
Nucleases SM1 & SM2	35	8.0	-	Mg^{2+}, Mn^{2+}	Yonemura *et al.*, 1983
Sm nuclease	52	8.0-8.5	35-44	Mg^{2+}, Mn^{2+}, Co^{2+}	Benedik and Strych, 1998
Neurospora nuclease	-	6.0-7.5	37	Mg^{2+}, Mn^{2+}, Co^{2+}	Linn and Lehman, 1966
Neurospora nuclease	66	8.0	-	Mg^{2+}, Mn^{2+}	Chow and Fraser, 1983
Bacillus nucleases	-	9.0	-	Ca^{2+}	Kanamori *et al.*, 1973a
Streptomyces nucleases	18,34	8.0-8.5	-	Mg^{2+} + Ca^{2+}	Nicieza *et al.*, 1999
C1 nuclease	30	7.0-8.5	50-55	Mg^{2+}, Mn^{2+}	Ho *et al.*, 1998
Aspergillus nuclease	28	6.8-8.0	-	Mg^{2+}, Mn^{2+}, Zn^{2+}	Koa *et al.*, 1990
Yeast nuclease	76	7.0-7.5	35	Mg^{2+}, Mn^{2+}, Co^{2+} Ca^{2+}, Zn^{2+}	Dake *et al.*, 1988
Yeast nuclease	140	6.5-7.0	-	Mg^{2+}, Mn^{2+}	von Tigerstrom, 1982
Yeast nuclease	14	7.0	30-40	Mg^{2+}, Mn^{2+}	Morosoli and Lusena, 1980

Enzyme	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	Metal ion requirement	Reference
Leishmania nuclease	52	7.5	50	Mg^{2+}, Mn^{2+}	Mittra *et al*, 1998
Staphylococcal nuclease	16.8	9.0-10.0	-	Ca^{2+}	Cunningham *et al*., 1956
BAL 31 nuclease					Wei *et al*, 1983
F (fast) form	109	8.0-8.8	-	Mg^{2+}, Ca^{2+}	
S (slow) form	85	8.0-8.8	-	Mg^{2+}, Ca^{2+}	
Anabaena nuclease	29	7.5	35	Mg^{2+}, Mn^{2+}, Co^{2+}	Muro-Pastor *et al*., 1992
Yeast nuclease (influenced by *RAD52)*	72	7.5	-	Mg^{2+}, Mn^{2+}	Chow and Resnick, 1987
Lysobacter nuclease	22-28	8.0	-	Mg^{2+}, Mn^{2+}	von Tigerstrom, 1980
Vibrio nuclease	100	8.0	30-40	None	Maeda and Taga, 1976
Schizosaccharomyces nuclease	32	7.0-8.0	-	Mg^{2+}, Mn^{2+}	Ikeda *et al*, 1996
Rye germ nuclease - I	20	5.0, 6.5	-	None	Siwecka *et al*, 1989
Rye germ nuclease - II	57/62	5.0, 7.8	-	None	Siwecka, 1997

Enzyme	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	Metal ion requirement	Reference
Petunia pollen nuclease	34	5.0	-	None	van der Westhuizen *et al.*, 1987
Barley nuclease	36	6.0	55	None	Brown and Ho, 1986
Wheat nuclease	29	6.8-7.8	50	None	Kuligowska *et al.*, 1988
Potato nuclease	33	6.5-8.0	-	None	Nomura *et al.*, 1971
Tobacco pollen nuclease	60	5.2	-	*None*	Matousek and Tupy, 1984
Barley mircospore nuclease	30	-	-	Mg^{2+}	Vischi and Marchetti, 1997
Tea leaves					Imagawa *et al.*, 1982
nuclease A	35	5.5-6.5	70	None	
nuclease B	33	5.5-6.5	60-70	None	
Drosophila nuclease	32	7.0-8.5	-	Mg^{2+}, Mn^{2+}	Shuai *et al.*, 1992
Drosophilia embryo nuclease	44	6.5-7.4	-	Mg^{2+}, Mn^{2+}	Harosh *et al.*, 1992

Enzyme	Molecular mass (kDa)	Optimum pH	Optimum temperature (°C)	Metal ion requirement	Reference
Shrimp nuclease	45	7.5	-	Mg^{2+}, Mn^{2+}, Ca^{2+}	Chou and Liao, 1990
Sr nuclease	56	7.0-7.2	-	Mg^{2+}, Mn^{2+}	Chen *et al*, 1993
Rat liver nuclease	50	6-7	-	Mg^{2+}, Mn^{2+}, Co^{2+}	Cordis *et al*., 1975
Endonuclease G	50	7.5-8.0	-	Mg^{2+}, Mn^{2+}	Côtè *et al*, 1989

variant was functionally independent, at low enzyme and substrate concentrations, the native dimeric form of *S. marcescens* nuclease was relatively more active than the monomeric form or the heterodimer with one inactive subunit (Franke *et al*., 1999).

In *S. cerevisiae* and *N. crassa*, multiple nucleases having similar catalytic properties with subtle differences in the physical properties have been reported. Three endonucleases with Mr of 14, 38 and 57 have been isolated from yeast mitochondria (Morosoli and Lusena, 1980; von Tigerstrom, 1982; Dake *et al*., 1988). Among them the 38 and 57 kDa enzymes exist as dimers under natural conditions. The majority of the nuclease activity in yeast mitochondria, attributed to the 38 kDa enzyme, is located in the mitochondrial inner membrane. Additionally, the presence of multiple forms of the enzyme was correlated to proteolytic cleavage (Dake *et al*., 1988). *S. antibioticus* produces two extracellular nucleases of Mr 18 and 34 kDa, exhibiting similar properties. The 18 kDa protein was reported to be formed from a 74 kDa precursor, as evidenced by the cross-reactivity against the anti-18kDa antibodies. It was also shown that the 34 kDa protein is not formed from the 74 kDa protein and the former is not a precursor of the 18 kDa enzyme (Nicieza *et al*., 1999). In *A. nidulans*, the 28 kDa endo-exonuclease is formed due to the proteolytic digestion of the 38 kDa polypeptide via the formation of a 33 kDa intermediate. Moreover crude extracts, obtained from cells grown in presence of PMSF, showed the presence of an immunoreactive 90 kDa polypeptide which is a putative precursor of the 28 kDa endo-exonuclease (Koa *et al*., 1990). In *N. crassa*, 90 % of the intracellular neutral ssDNase activity is associated with endo-exonucleases localized in mitochondria, vacuoles and nuclei. In addition, an unstable and trypsin-activatable form of endo-exonuclease found in cytosol, nuclei and mitochondria, gave rise to two nucleases similar to endo-exonucleases reported by Linn and Lehman (1965a,b) and Fraser *et al.* (1976). From mitochondria, a strand non-specific endonuclease (Linn and Lehman, 1966) and a ssDNA-binding endo-exonuclease not inhibited by ATP (Chow and Fraser, 1983) have also been isolated. Fraser *et al*. (1986), by immunochemical techniques, demonstrated that all the above enzymes from *N. crassa* are derived

from one or more related large inactive (precursor) polypeptides which undergo proteolysis to produce various active forms of nucleases. This inactive precursor was shown to have a Mr of 93-95 kDa (Kwong and Fraser, 1978). Additionally, Fraser *et al.* (1986) showed that endo-exonucleases from *A. nidulans* and *S. cerevisiae* are immunologically related to *N. crassa* endo-exonuclease but not to single-strand-specific nucleases like S1, P1 and mung bean nuclease. Similarly, isoforms of the nucleases were observed in *S. aureus* (Chesbro *et al.*, 1966), *B. subtilis* (Kanamori *et al*., 1973a), *A. espejiana* (Gray *et al.*, 1975), *S. marcescens kums* 3958 (Yonemura *et al*., 1983), tobacco pollen (Matousek and Tupy, 1984), *P. hybrida* (van der Westhuizen *et al.*, 1987) and *S. marcescens* (Filiminova *et al*., 1991; Pedersen *et al.*, 1993a,b). In case of Staphylococcal nuclease Taniuchi and Anfinsen (1968) showed that limited proteolysis of the enzyme, in presence of Ca^{2+} and deoxythymidine 3',5'-diphosphate, produces an active non-covalently bonded derivative termed nuclease -T, which shows a similar level of activity against DNA and RNA as that of the native enzyme.

In contrast to protease mediated formation of multiple forms, shrimp nuclease exhibited multiple forms in presence of SDS / β-mercaptoethanol (Lin *et al*., 1994). The enzyme is a monomer of Mr 45 kDa cross-linked with a large number of disulfide bridges. It exhibits four different forms, on SDS-PAGE, depending on the treatment of the sample (Fig. 1.1). When the protein was subjected to heat treatment with β-mercaptoethanol, in presence and absence of SDS, it changed into an inactive Form-III (45 kDa). Moreover, Form-III could not refold and re-oxidize to form active Form-I even after the removal of SDS and β-mercaptoethanol. However, when Form-III was subjected to heat treatment with SDS in the absence of β-mercaptoethanol, the resulting Form-II (39 kDa) became catalytically active only after the removal of SDS. Based on these observations the authors opined that this inside-out incorrect structure with an exposed hydrophobic domain has a tendency to aggregate and cannot revert back into a catalytically active form (Lin *et al*., 1994).

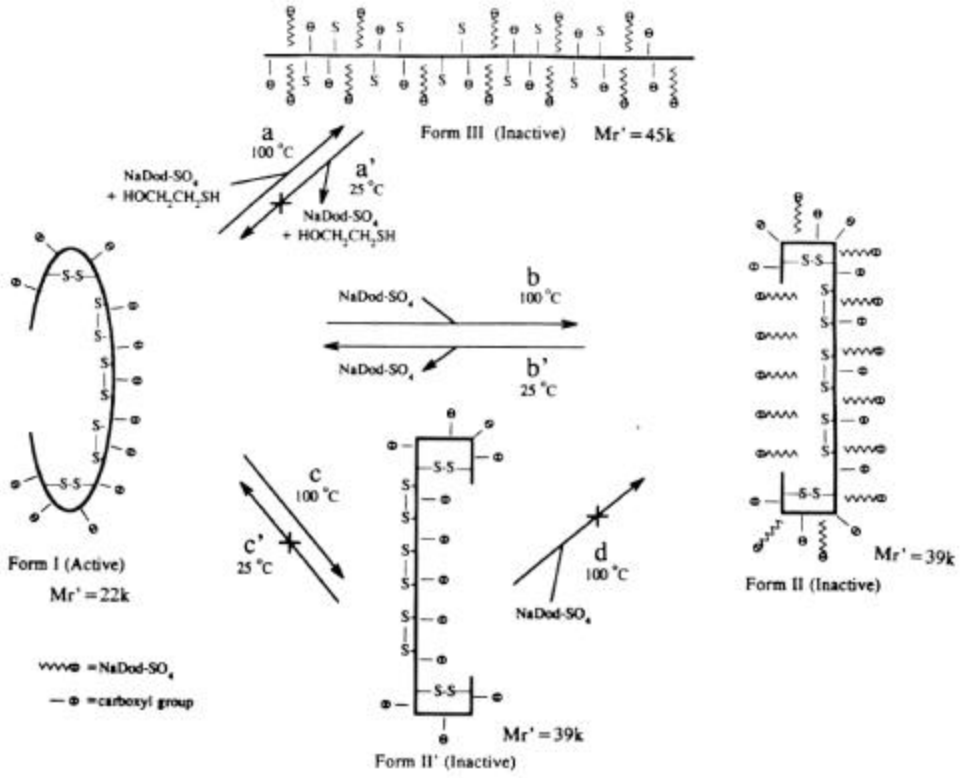


Fig. 1.1: A scheme for folding and unfolding pathways of shrimp nuclease in presence and absence of SDS and b-mercaptoethanol. (Adapted from Lin *et al*., 1994)

Isoelectric point and glycoprotein nature

The isoelectric point of majority of non-specific endonucleases have not been reported but in the case of some of the well characterized enzymes, they are in the range of 4.0-9.6. Nucleases from shrimp hepatopancreas (Chou and Liao, 1990), *A. espejiana* (Wei *et al*., 1983), rye germ ribosomes (Siwecka *et al*., 1989), *D. melanogaster* embryos (Harosh *et al*., 1992) and *S. racemosum* (Chen *et al*., 1993) are acidic proteins with a pI of 4.06, 4.2, 4.8, 4.9 and 5.0, respectively. *S. marcescens* nuclease and its isoforms are neutral proteins with a pI of 6.8, 7.3, 7.4 and 7.5 (Yonemura *et al*., 1983; Pedersen *et al*., 1993a). However, the endonuclease SM1 from *S. marcescens kums* 3958 (Yonemura *et al*., 1983) and *S. aureus* nuclease (Heins *et al*., 1967) are basic proteins with a pI of 8.1 and 9.6 respectively.

Nucleases from barley aleurone layers (Brown and Ho, 1987), *S. racemosum* (Chou and Liao, 1990) and rye germ ribosomes (Siwecka *et al*., 1989) are glycoproteins. Rye germ ribosomes nuclease I contains 28 % carbohydrate and it consists of fucose, mannose and glucosamine (Siwecka *et al.*, 1989).

Optimum pH

In general the pH optima of non-specific endonucleases are in the range of 5.0-10.0 (Table 1.3) and most of the enzymes show the same optimum pH for the hydrolysis of both polymeric and monomeric substrates. However, nucleases from *A. espejiana* (Gray *et al*., 1981), *N. crassa* (mitochondria) (Linn and Lehman, 1966) and tea leaves (Imagawa *et al*., 1982) show different pH optima for the hydrolysis of ssDNA (8.8, 6.5-7.5 and 6.0) and dsDNA (8.0, 5.5-6.5 and 5.5), respectively. On the other hand, 3'-nucleotidase-nuclease from potato tubers showed different pH optima for the nucleotidase (pH 8.0) and nuclease (pH 6.5 - 7.5) activities (Nomura *et al*., 1971). Similarly, nuclease I from rye germ ribosomes exhibited a pH optima of 6.0 and 6.5 for the nuclease and 3'-nucleotidase activities, respectively. Nucleases from wheat chloroplasts (Kuligowska *et al*., 1988) and rye germ ribosomes (Siwecka, 1997) showed an optimum pH of 7.8 and 5.0 for the hydrolysis of denatured DNA and 6.8 and 7.8 for the hydrolysis of RNA, respectively. However, rye germ ribosomes nuclease II degraded poly(I).poly(C) optimally at pH 8.5 (Siwecka, 1997). Interestingly, nucleases (A and B) from tea leaves exhibited different pH optima for the hydrolysis of different 3' mononucleotides (i.e. pH 6.0 for 3'GMP and 3'CMP and pH 6.5 for 3'AMP and 3'UMP), a property associated with the 3'-nucleotidase activity of single-strand-specific nucleases like Le1 and Le3 from *Lentinus edodes* (Shimada *et al*., 1991, Kobayashi *et al*., 1995) and P1 from *Penicillium citrinum* (Fujimoto *et al*., 1974a).

In certain cases the optimum pH of non-specific endonucleases is influenced by the type of metal ions used. For example, *S. racemosum* nuclease exhibited an optimum pH of 7.0 and 7.2 in presence of 5 mM of Mg^{2+} and Mn^{2+} respectively (Chen *et al*., 1993). However, the DNase activity of Staphylococcal nuclease showed an optimum pH of 10.0 and 9.5 in presence of 1 and 10 mM Ca^{2+}

respectively but under similar conditions the RNase activity was optimal at pH 9.5 and 9.0 (Cuatrecasas *et al.*, 1967b).

Optimum temperature and temperature stability

The temperature optima of majority of non-specific endonucleases have not been reported but in case of some of the well characterized enzymes, they are in the range of 30 °C to 70 °C (Table 1.3). Most of them exhibit the same optimum temperature for all the substrates. However, *C. echinulata* nuclease showed an optimum temperature of 50 °C and 55 °C for DNA hydrolysis in presence of Mg^{2+} and Mn^{2+}, respectively (Ho *et al.*, 1998).

Majority of non-specific endonucleases are thermolabile enzymes. However, Staphylococcal nuclease showed high stability and retained significant amount of its activity in presence of 1 N HCl for 6 h or at 100 °C for 20 min. Similarly, tea leaves nucleases were stable at 60 °C for 15 min (Imagawa *et al*., 1982) whereas, nucleases SM1 and SM2 from *S. marcescens kums* 3958 retained their activity for 24 h at 25 °C (Yonemura *et al*., 1983). Interestingly, a mitogenic factor of *S. pyogenes* exhibiting nuclease activity, was resistant to inactivation at 100 °C for 10 min and showed a biphasic temperature stability. The enzyme was stable upto 50 °C (10 min) but lost more than 90 % of its activity at 60 °C. However, it regained approximately 50 % of its original activity at 80 °C (Iwasaki *et al.*, 1997).

Metal ion requirement

Most of the non-specific endonucleases, with the exception of nucleases from potato tubers (Nomura *et al*., 1971), *Vibrio* sp. (Maeda and Taga, 1976), tea leaves (Imagawa *et al*., 1982), barley aleurone layers (Brown and Ho, 1986), *P. hybrida* (van der Westhuizen *et al*., 1987), wheat chloroplasts (Kuligowska *et al*., 1988) and rye germ ribosomes (Siwecka *et al*., 1989; Siwecka, 1997) are metal requiring enzymes. Although wheat chloroplast nuclease (Kuligowska *et al*., 1988) did not require metal ions for its activity, the ssDNase activity showed slight

stimulation (20 %) in presence of Mg^{2+}. Similarly, the activity of endonuclease from *Vibrio* sp. was stimulated by Mg^{2+} and Ca^{2+} (Maeda and Taga, 1976). In contrast, nuclease I of rye germ ribosomes (Siwecka *et al.*, 1989), pollen nucleases of tobacco (Matousek and Tupy, 1984) and *P. hybrida* (van der Westhuizen *et al*., 1987) were stimulated by Zn^{2+} while nuclease II from rye germ ribosomes was stimulated by Mn^{2+} (Siwecka, 1997). Extracellular nucleases (Bs-IA, Bs-IB and Bs-II) from *B. subtilis* required Ca^{2+} for the hydrolysis of native DNA. However, low rate of hydrolysis of denatured DNA and rRNA were observed in the absence of added metal ion (Kanamori *et al*., 1973a). The action of *N. crassa* nuclease on dsDNA is dependent on Mg^{2+} concentration but its activity on ssDNA is independent of Mg^{2+} concentration, though it is stimulated to some extent (Fraser, 1980). Addition of 10 mM of Mg^{2+}, Ca^{2+} or Fe^{2+} resulted in 2.5 fold stimulation of the ssDNase activity of *N. crassa* enzyme but it also brought about approximately 40 % inhibition of the RNase activity. The selective inhibition of the RNase activity, in presence of metal ions, was attributed to the induction of secondary structures in RNA by these metal ions.

Many of the metal requiring endonucleases show synergism when metal ions are used in combination. For example, nucleases from *S. aureus* (Cuatrecasas *et al.*, 1967b), *A. espejiana* (Gray *et al*., 1975), *A. nidulans* (Koa *et al*., 1990), shrimp hepatopancreas (Chou and Liao, 1990), mitogenic factor of *S. pyogenes* (Iwasaki *et al.*, 1997) and *S. antibioticus* (Nicieza *et al*., 1999) exhibited maximum activity in presence of Ca^{2+} and Mg^{2+}.

Effect of salt concentration

It has been reported that salt concentration in the reaction mixture can influence the activity of non-specific endonucleases. For example, the activity of BAL 31 nuclease is maximum in the range of 0 to 2 M NaCl and the enzyme shows only 40 % of its ssDNase activity in presence of 4.4 M NaCl (Gray *et al*., 1975). In case of endonucleases from *N. crassa* (Fraser, 1980) and yeast mitochondria (Chow and Resnick, 1987), 200 mM NaCl completely inhibited the dsDNase activity but it had only marginal effect on the ssDNase activity. Similarly,

D. melanogaster endonuclease showed 50 % inhibition of its dsDNase activity in presence of 30 mM NaCl but it required 100 mM NaCl to bring about the same level of inhibition of the ssDNase activity (Shuai *et al*., 1992). The inhibition of dsDNase activity, in presence of high salt concentration, was attributed to the stabilization of the AT-rich regions in dsDNA (von Hippel and Felsenfeld, 1964; Wingert and von Hippel, 1968). The ssDNase and RNase activities of yeast mitochondrial nuclease are maximal between 1 and 300 mM KCl while KCl (> 150 mM) inhibited the dsDNase activity. However, NaCl up to 100 mM did not have any effect on the enzyme activities (Dake *et al*., 1988). Similarly, Staphylococcal nuclease (Tucker *et al*., 1978) and endonuclease G from *B. taurus* (Ruiz-Carrillo and Renaud, 1987) were inhibited at NaCl concentrations above 100 mM. On the contrary, *Leishmania* endonuclease showed optimal activity in presence of 100 - 150 mM NaCl/KCl but subsequent increase in salt concentration progressively inhibited the enzyme activity (Mittra *et al*., 1998). Nucleases from *S. antibioticus* (Nicieza *et al*., 1999) and yeast mitochondria (Morosoli and Lusena, 1980), however, showed maximum activity in presence of 20 - 30 mM NaCl and 50 mM KCl, respectively. The dsDNase activity of tobacco pollen nuclease was inhibited in presence of 200 mM NaCl (Matousek and Tupy, 1984) while *Serratia* nuclease retained 25 % of its activity in presence of 1 M NaCl (Nestle and Roberts, 1969a). In case of mitogenic factor of *S. pyogenes*, concentrations of NaCl / KCl greater than 60 mM brought about 90 % inhibition of the nuclease activity (Iwasaki *et al*., 1997). Inhibition in presence of high concentrations of NaCl/KCl was also observed with endonucleases from rat liver nuclei (Cordis *et al*., 1975), *Anabaena* sp. (Muro-Pastor *et al.*, 1992) and *S. pombe* (Ikeda *et al*., 1996).

Effect of denaturants

In presence of 7 M urea, *S. marcescens* endonuclease showed approximately 2, 1.7 and 1.5 fold increase in its dsDNase, ssDNase and RNase activities, respectively (Filiminova *et al*., 1980). However, *A. agilis* endonuclease showed 5 to 10 fold stimulation of its activity towards poly(A) in presence of 2 M urea (Stevens and Hilmoe, 1960a). Gray *et al*. (1975) showed that the S (slow) form of

BAL 31 nuclease is active in presence of 5 % (w/v) SDS and can be incubated with the detergent without loss of activity if Ca^{2+} and Mg^{2+} are present at a concentration of 12.5 mM before the addition of the detergent. Purified S form of BAL 31 nuclease retained 60 % of its optimal activity in presence of 4 M urea. In case of endonuclease from barley aleurone layers, complete inactivation of the DNase and RNase activities was observed in presence of 1 % (w/v) SDS (Brown and Ho, 1987).

Activators and inhibitors

Polyamines such as spermine and spermidine, which bind to double-stranded nucleic acids also inhibit the ssDNase activity of nucleases. Spermine stimulated the exonuclease activity of BAL 31 nuclease but the cleavage specificity was considerably reduced in its presence (Shishido, 1985). The DNase and RNase activities of yeast mitochondrial endonuclease (Dake *et al*., 1988) and the RNase activity of rye germ ribosomes nuclease I (Siwecka *et al*., 1989) were stimulated in presence of 2 and 2.5 mM spermidine, respectively. Moreover, low concentration (0.1 mM) of polyamines such as putrescine and spermidine inhibited the RNase activity of rye germ ribosomes nuclease I (Siwecka *et al*., 1989). On the contrary, the DNase and RNase activities of *S. aureus* nuclease were stimulated by low amounts of putrescine, spermine and spermidine whereas, at high concentrations they inhibited both the activities (Frank *et al*., 1975). However, endonuclease from barley aleurone layers was not affected by putrescine, spermidine and spermine at 1 mM concentration (Brown and Ho, 1987). The ability of polyamines to either activate or inhibit the nuclease activity, in a concentration dependent manner, was correlated to the regulation of nucleic acid levels within the cells by controlling the nuclease activity (Levy *et al.*, 1974).

Low concentrations of ethidium bromide (EtBr) (1-10 µg/ml) brought about marginal stimulation of the dsDNase activity of yeast mitochondrial endonuclease but it had no effect on the RNase activity (Dake *et al*., 1988). Although, 10 µM of EtBr did not affect ssDNase activity of yeast mitochondrial endonuclease, slight

inhibition (30 %) of the dsDNase activity was observed with 2 μM EtBr (Morosoli and Lusena, 1980).

Endonucleases from *D. melanogaster* (Shuai *et al*., 1992) and *S. antibioticus* (Nicieza *et al*., 1999) showed optimal activity in presence of either DTT or β-mercaptoethanol. Although, *N. crassa* nuclease (Linn and Lehman, 1966) and rye germ ribosomes nuclease I (Siwecka *et al*., 1989) did not show an obligate requirement of thiol reagents for their activity, considerable stimulation of the activity was observed in presence of DTT and β-mercaptoethanol. In contrast, DTT and β-mercaptoethanol brought about significant inhibition of both the DNase and RNase activities of wheat chloroplast nuclease (Kuligowska *et al*., 1988). Ten mM DTT inhibited barley aleurone layers nuclease whereas β-mercaptoethanol, at the same concentration, had no effect on the enzyme activity (Brown and Ho, 1987). However, sulfhydryl reagents had no effect on endonucleases from *S. marcescens kums* 3958 (Yonemura *et al*., 1983), yeast mitochondria (Dake *et al*., 1988) and shrimp hepatopancreas (Chou and Liao, 1990). Interestingly, β-mercaptoethanol had no effect on the activity of *S. marcescens* endonuclease (Nestle and Roberts, 1969a), though disulfide bonds are essential for the enzyme activity (Ball *et al*., 1992). Subsequently, Filiminova *et al.* (1980, 1981) demonstrated that substantial inactivation of the enzyme activity occurred when high concentration of β-mercaptoethanol (640 mM) was used in presence of 2 M urea. The inability of β-mercaptoethanol to inactivate the native enzyme was correlated to the masking of the sulfhydryl groups in the native conformation.

Majority of the sugar non-specific endonucleases are metal requiring enzymes and hence they are strongly inhibited by metal chelators like EDTA and EGTA. In most cases, the inactivation can be readily reversed by the addition of divalent cations. The ssDNase activity of wheat chloroplast nuclease was strongly inhibited by EDTA but it had no significant effect on its RNase activity (Kuligowska *et al*., 1988). Endonucleases from rat liver nuclei (Cordis *et al*., 1975) and *S. marcescens kums* 3958 (Yonemura *et al*., 1983) were inhibited by pyrophosphate whereas, the enzyme from tobacco pollen was inhibited by

inorganic phosphate (Matousek and Tupy, 1984). While inorganic phosphate significantly inhibited the DNase and RNase activities of *B. subtilis* nuclease (Kanamori *et al*., 1973a), it only inhibited the RNase activity of rye germ ribosomes nuclease I (Siwecka *et al*., 1989). On the other hand, inorganic phosphate had no effect on Staphylococcal nuclease (Cuatrecasas *et al.*, 1967b).

Divalent cations like Mn^{2+}, Co^{2+} and Zn^{2+} inhibited nucleases from potato tubers (Nomura *et al*., 1971) and *B. subtilis* (Kanamori *et al*., 1973a) while wheat chloroplast nuclease was inhibited by Cu^{2+}, Co^{2+} and Zn^{2+} (Kuligowska *et al*., 1988). Nucleases from *S. marcescens kums* 3958 (Yonemura *et al*., 1983), shrimp hepatopancreas (Chou and Liao, 1990) and *D. melanogaster* (Shuai *et al*., 1992) were inhibited by Ca^{2+}. Though Zn^{2+} preferentially inhibited the exonuclease activity, on dsDNA, of *N. crassa* mitochondrial nuclease (Fraser, 1980) and the RNase activity of rye germ ribosomes nuclease I (Siwecka *et al*., 1989), it inhibited both the DNase and RNase activities of *S. aureus* (Cuatrecasas *et al*., 1967b) and *S. antibioticus* (Nicieza *et al*., 1999) nucleases. Additionally, Hg^{2+} and Cd^{2+} inhibited the DNase and RNase activities of Staphylococcal nuclease whereas, Ba^{2+} and Sr^{2+} inhibited only its RNase activity (Cuatrecasas *et al*., 1967b). The inhibition of the RNase activity, in presence of Mg^{2+} and Ca^{2+}, was also observed with rye germ ribosomes nuclease I (Siwecka *et al.*, 1989). While Mn^{2+} inhibited the endonuclease activity of barley microspores nuclease (Vischi and Marchetti, 1997), tea leaves nucleases (A and B) were inhibited by Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+} and Fe^{3+} (Imagawa *et al*., 1982). Interestingly, in case of tea leaves nucleases, Zn^{2+} did not inhibit the dsDNase activity but showed considerable inhibition of the ssDNase, RNase and 3'-nucleotidase activities. However, Mg^{2+} selectively inhibited the DNase and RNase activities with no effect on the 3'-nucleotidase activity (Matousek and Tupy, 1984).

Nucleotides and its analogues are potent inhibitors of non-specific endonucleases. For example, Staphylococcal nuclease was inhibited by deoxythymidine 3',5'-diphosphate, 5'dAMP and 5'dTMP. While 5'dCMP and 5'dGMP inhibited the RNase activity, the DNase activity was not affected. Moreover, among ribonucleotides, only 5'AMP could strongly inhibit both

activities of the enzyme (Cuatrecasas *et al*., 1967b). Deoxythymidine 3',5'-diphosphate and ATP also inhibited nucleases SM1 and SM2 from *S. marcescens kums* 3958 whereas, adenosine had no effect (Yonemura *et al*., 1983). In contrast, ATP did not have any effect on the activity of yeast mitochondrial endonucleases (Chow and Resnick, 1987; Dake *et al*., 1988) and endonuclease G from *B. taurus* (Ruiz-Carrillo and Renaud, 1987). In case of potato tubers nuclease, the 3'-nucleotidase activity was inhibited competitively by 5' mononucleotides, RNA and poly(A) (Suno *et al*., 1973). *S. antibioticus* nucleases were inhibited by aurin tricarboxylic acid (Nicieza *et al*., 1999) while heparin, Cibacron-Blue and aurin tricarboxylic acid inhibited yeast mitochondrial endonuclease (Dake *et al*., 1988). Rat liver endonuclease showed inhibition in presence of polydextran sulphate (Cordis *et al*., 1975).

Endo-exonuclease from *N. crassa* was inhibited by a heat-stable, trypsin-sensitive, cytosolic 24 kDa polypeptide. The protein inhibited the ssDNase activity non-competitively but the dsDNase activity was inhibited competitively. In addition, the inhibitor blocked the formation of site-specific double-strand breaks and nicking of linearized pBR322 DNA. It also inhibited the RNase activity of *N. crassa* nuclease as well as the immunochemically related nuclease from *A. nidulans* (Hatahet and Fraser, 1989). Moreover, the antibodies raised against *N. crassa* endo-exonuclease inhibited all the activities of yeast mitochondrial nucleases (Chow and Resnick, 1987; Dake *et al*., 1988). The endonuclease NucA from *Anabaena* sp. was inhibited by its polypeptide inhibitor NuiA, while the related *Serratia* nuclease was not inhibited with 10 fold excess of the inhibitor (Meiss *et al*., 1998). Cleavage of the monomeric substrate deoxythymidine 3',5'-bis-(*p*-nitrophenyl phosphate) by NucA, however, was not inhibited by NuiA suggesting that small molecules gain access to the active site of NucA in the enzyme-inhibitor complex under conditions where cleavage of DNA is completely inhibited (Meiss *et al*., 2000). Similarly, the extracellular nuclease (Nuc) from *S. marcescens* was inhibited by the signal peptide obtained from the N-terminal portion of Nuc (Chen *et al.*, 1996).

Unlike single-strand-specific nucleases namely S1 nuclease from *Aspergillus oryzae* (Oleson and Hoganson, 1981), nuclease β from *Ustilago maydis* (Rusche *et al*., 1980) and nuclease Bh1 from *Basidiobolus haptosporus* (Desai and Shankar, 2000), autoretardation due to end-product inhibition has not been reported in non-specific endonucleases. On the contrary, Staphylococcal nuclease showed autoacceleration during the hydrolysis of denatured DNA, native DNA and RNA. The phenomenon, best observed with polynucleotides intermediate between nucleic acids and short oligonucleotides, was attributed to the presence of substantial "breathing spaces" in the intermediate size substrates (Sulkowski and Laskowski, 1968).

Substrate specificity

Sugar non-specific endonucleases are multifunctional enzymes and exhibit both endo and exonuclease activities (Table 1.4). Moreover, endonucleases from potato tubers (Nomura *et al*., 1971), tea leaves (Imagawa *et al*., 1982), barley aleurone layers (Brown and Ho, 1986) and rye germ ribosomes (Siwecka *et al*., 1989) exhibit 3' phosphomonoesterase activity.

Non-specific endonucleases act on DNA, RNA and 3' mononucleotides but the rate of hydrolysis of these substrates varies depending on the source of the enzyme. Thus, potato tubers nuclease showed higher activity on 3'AMP and RNA (Nomura *et al*., 1971) whereas, rye germ ribosomes nuclease I preferred DNA to RNA and 3' mononucleotides (Siwecka *et al*., 1989). Nucleases from tea leaves (Imagawa *et al*., 1982) and barley aleurone layers (Brown and Ho, 1986) showed preference for RNA in comparison to DNA and 3' mononucleotides. The substrate specificity of potato tubers nuclease falls in the order of 3'AMP = RNA > ssDNA > dsDNA (Nomura *et al.,* 1971) while that of rye germ ribosomes nuclease I is ssDNA > dsDNA > RNA > 3'AMP (Siwecka *et al*., 1989). Tea leaves nucleases (Imagawa *et al*., 1982) and barley aleurone layers nuclease (Brown and Ho, 1987) showed substrate preference in the order of RNA > ssDNA = dsDNA > 3'AMP.

The endonucleases from *S. marcescens* (Nestle and Roberts, 1969 a,b), *Anabaena* sp. (Muro-Pastor *et al*., 1992), *N. crassa* (Linn and Lehman, 1966) and

Table 1.4: Substrate specificity and mode of action of non-specific endonucleases

Enzyme	Substrate	ssDNAse dsDNase/ ratio	Products	Mode of action	Reference
Azotobacter nuclease	DNA, RNA	ss ≥ ds	5' oligonucleotides	Endo	Stevens and Hilmoe, 1960b
Nucleases SM1 & SM2	DNA, RNA	ss ≥ ds	---	Endo	Yonemura *et al.*, 1983
Sm nuclease	DNA, RNA	ss = ds	5' mono, di, tri and tetranucleotides	Endo	Nestle and Roberts, 1969b
Neurospora nuclease	DNA, RNA	ss ≥ ds	5' mono, di, tri and tetranucleotides	Endo	Linn and Lehman, 1966
Neurospora nuclease	DNA, RNA	ss ≥ ds	5' di, tri, tetra and pentanucleotides	Endo-exo	Chow and Fraser, 1983
Bacillus nucleases	DNA, RNA	1.3	3' mononucleotides	Endo-exo	Kanamori *et al.*, 1973a
Streptomyces nucleases	DNA, RNA	ds > ss	5' mono and dinucleotides	Endo / exo	Nicieza *et al.*, 1999

Enzyme	Substrate	ssDNAse dsDNase/ ratio	Products	Mode of action	Reference
C1 nuclease	DNA, RNA	ss = ds	5' oligonucleotides	Endo	Ho *et al.*, 1998
Aspergillus nuclease	DNA, RNA	5	5' di, tri, tetranucleotides	Endo	Koa *et al.*, 1990
Yeast nuclease	DNA, RNA	2	5' products	Endo-exo	Dake *et al.*, 1988
Yeast nuclease	DNA, RNA	ss ≥ ds	5' products	Endo	von Tigerstrom, 1982
Yeast nuclease	DNA, RNA	ss ≥ ds	5' mono, di and trinucleotides	Endo	Morosoli and Lusena, 1980
Leishmania nuclease	DNA, RNA	2	5' mononucleotides and oligonucleotides	Endo	Mittra *et al.*, 1998
Staphylococcal nuclease	DNA, RNA	2	3' mononucleotides	Endo	Cunningham *et al.*, 1956
BAL 31 nuclease					Wei *et al.*, 1983
F (fast) form	DNA, RNA	3	5' mononucleotides	Endo-exo	
S (slow) form	DNA, RNA	33	5' mononucleotides	Endo-exo	
Anabaena nuclease	DNA, RNA	ss = ds	----	Endo	Muro-Pastor *et al.*, 1992

Enzyme	Substrate	ssDNAse dsDNase/ ratio	Products	Mode of action	Reference
Yeast nuclease (influenced by *RAD52)*	DNA, RNA	8	5' mononucleotides and oligonucleotides	Endo	Chow and Resnick, 1987
Lysobacter nuclease	DNA, RNA	ss ≥ ds	5' products	Endo	von Tigerstrom, 1980
Vibrio nuclease	DNA, RNA	ss ≥ ds	5' products	Endo	Maeda and Taga, 1976
Schizosaccharomyces nuclease	DNA, RNA	ss ≥ ds	5' products	Endo	Ikeda *et al*, 1996
Rye germ nuclease - I	DNA, RNA 3' monoribo-nucleotides	1.14	5' oligoribonucleotides 3' oligodeoxyribonucleotides	Endo	Siwecka *et al*, 1989
Petunia pollen nuclease	DNA, RNA	4	5' oligonucleotides	Endo	van der Westhuizen *et al.*, 1987
Wheat nuclease	DNA, RNA	15	3' oligoribonucleotides 5' oligodeoxyribonucleotides	Endo	Kuligowska *et al*, 1988

Enzyme	Substrate	ssDNAse dsDNase/ ratio	Products	Mode of action	Reference
Rye germ nuclease - II	DNA, RNA	1.14	5' oligonucleotides	Endo	Siwecka, 1997
Barley nuclease	DNA, RNA 3' monoribo-nucleotides	ss ≥ ds	5' oligonucleotides	Endo	Brown and Ho, 1987
Potato nuclease	DNA, RNA 3' monoribo-nucleotides	3	5' mononucleotides	Endo	Suno *et al*, 1971
Tea leaves					Imagawa *et al*, 1982
nuclease A	DNA, RNA 3' monoribo-nucleotides	1	5' mononucleotides and oligonucleotides	Endo	
nuclease B	DNA, RNA 3' monoribo-nucleotides	1	5' mononucleotides and oligonucleotides	Endo	

Enzyme	Substrate	ssDNAse dsDNase/ ratio	Products	Mode of action	Reference
Tobacco pollen nuclease	DNA, RNA	3	5' mono and oligonucleotides	Endo-exo	Matousek and Tupy, 1984
Barley mircospore nuclease	DNA, RNA	ss ≥ ds	5' products	Endo	Vischi and Marchetti, 1997
Drosophila nuclease	DNA, RNA	ss = ds	5' mono and oligonucleotides	Endo-exo	Shuai *et al.*, 1992
Drosophilia embryo nuclease	DNA, RNA	ds > ss	5' products	Endo	Harosh *et al.*, 1992
Shrimp nuclease	DNA, RNA	ds > ss	----	Endo	Chou and Liao, 1990
Sr nuclease	DNA, RNA	ds > ss	----	Endo	Chen *et al.*, 1993
Rat liver nuclease	DNA, RNA	7	> 5' tetranucleotides	Endo	Cordis *et al.*, 1975
Endonuclease G	DNA, RNA (DNA:RNA)	ds ≥ ss	5' products	Endo	Côtè *et al.*, 1989

S. racemosum (Chen *et al*., 1993) hydrolyzed ssDNA, dsDNA and RNA at a similar rate while Staphylococcal nuclease hydrolyzed both DNA and RNA with slight preference for ssDNA (Cuatrecasas *et al*., 1967b). Similar preference for ssDNA was also shown by endonuclease M from *Leishmania* sp. (Mittra *et al*., 1998) and yeast mitochondrial endonuclease (Dake *et al*., 1988). In addition, endonuclease M degraded ssRNA rapidly but RNA:DNA hybrid was resistant to cleavage. With increasing concentrations of Endo M, the unlabelled single-stranded overhang of DNA from the RNA:DNA hybrid was cleaved to give the perfect duplex RNA:DNA hybrid. However, in presence of 10 fold excess enzyme, the resulting RNA:DNA hybrid was also cleaved (Mittra *et al*., 1998). Similarly, endonuclease G from *B. taurus* showed RNase H activity in addition to DNase and RNase activities (Côtè and Ruiz-Carrillo, 1993). Interestingly, nucleases from *L. enzymogens* (von Tigerstrom, 1980), shrimp hepatopancreas (Chou and Liao, 1990), *S. racemosum* (Chen *et al*., 1993) and *S. antibioticus* (Nicieza *et al*., 1999) showed preference for the dsDNA.

Synthetic substrate like deoxythymidine 3',5'-bis-(*p*-nitrophenyl phosphate) has been employed for the elucidation of kinetics and mechanisms of a number of phosphodiesterases (Khorana, 1961; Razzell, 1963). Exonucleases like snake venom phosphodiesterase (Razzell and Khorana, 1959) and spleen phosphodiesterase (Bernardi and Bernardi, 1968) and endonuclease like pancreatic DNase (Liao, 1975) could cleave the synthetic substrate. Cuatrecasas *et al*. (1969b) studied the hydrolysis of various *p*-nitrophenyl ester derivatives of deoxythymidine 5' phosphate by Staphylococcal nuclease and demonstrated that *p*-aminophenyl, *p*-nitrophenyl or methyl derivatives are hydrolyzed rapidly. Based on these experiments it was proposed that Staphylococcal nuclease requires R-pdT-R' as the basic structural unit to be recognized as a substrate and the R' group at C-3' significantly influences the binding of the substrate or the inhibitor. Additionally, in the absence of R (i.e., free 5' phosphate), activity is completely inhibited as evidenced by the strong inhibition with deoxythymidine 3',5'-diphosphate (Cuatrecasas *et al*., 1967b). Friedhoff *et al*. (1996b) demonstrated that *Serratia* nuclease hydrolyzes the artificial minimal substrate, deoxythymidine 3',5'-bis-(*p*-

nitrophenyl phosphate), at a lower rate compared to DNA and RNA. The cleavage sites of Staphylococcal nuclease, *Serratia* nuclease and pancreatic DNase I are shown in Fig. 1.2. Interestingly, in *Serratia* nuclease, the presence of phosphate 3' to the bond to be cleaved is essential for hydrolysis (Friedhoff *et al*., 1996b; Kolmes *et al*., 1996). Similar observation was made incase of *Anabaena* nuclease (Meiss *et al.*, 2000).

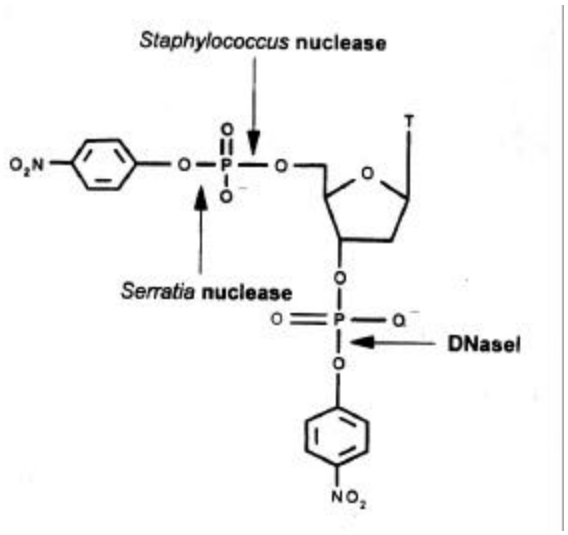


Fig. 1.2: Cleavage specificities of different nucleases.

(Adapted from Friedhoff *et al.*, 1996b)

Mode of action

Although non-specific endonucleases recognize and hydrolyze a wide spectrum of substrates, they primarily cleave the internucleotide phosphodiester linkage. Based on the requirement of a free terminus, these enzymes can be classified as -

A. ***Endonucleases*:** This class of enzymes cleave the internal phosphodiester bonds of nucleic acids with or without free termini. Their ability to convert covalently closed circular DNA to a linear form, either directly or through a nicked circular DNA intermediate, is still considered as one of the criteria for inclusion of these enzymes in this group. They show a distributive mode of action and the products of hydrolysis are mononucleotides and / or

oligonucleotides. Restriction endonucleases, structure specific enzymes like flap endonuclease, holiday junction resolvase, sequence specific homing endonucleases and nucleases from *S. marcescens*, *N. crassa* and *S. aureus* belong to this group. However, except *S. marcescens*, *N. crassa* and Staphylococcal nucleases, all the other enzymes mentioned above act mainly on DNA (especially dsDNA) and hence they will not be elaborated in this compilation.

B. ***Exonucleases*:** These enzymes generally require a free terminus for their action and are incapable of hydrolyzing covalently closed circular substrates. However, exonuclease like snake venom phosphodiesterase (Pritchard *et al*., 1977) is capable of nicking supercoiled DNA. The products of hydrolysis are predominantly mononucleotides and the mode of attack is processive. Spleen phosphodiesterase and *A. sydowii* nuclease belong to this category of enzymes. Though these enzymes are sugar non-specific, they are strict exonucleases and hence will not be discussed in detail.

C. ***Endo-exonucleases*:** The enzymes belonging to this category exhibit both exo and endo mode of action.

Although non-specific nucleases hydrolyze both DNA and RNA, either endonucleolytically or exonucleolytically, some enzymes exhibit different mode of action on these substrates (Table 1.4). Majority of these enzymes degrade nucleic acids to oligonucleotides and some mononucleotides predominantly by their endonucleolytic action. Nucleases from *N. crassa* (Chow and Fraser, 1983), tobacco pollen (Matousek and Tupy, 1984), *S. cerevisiae* (Chow and Resnick, 1987; Dake *et al*., 1988), *A. nidulans* (Koa *et al.*, 1990) and *D. melanogaster* (Shuai *et al*., 1992) cleave nucleic acids endo-exonucleolytically with the formation of oligonucleotides and a small amount of mononucleotides whereas, the nucleases from *A. espejiana* (Gray *et al*., 1975) and *B. subtilis* (Kanamori *et al*., 1973a) degrade nucleic acids in an exo-endo fashion with mononucleotides as the main product of hydrolysis. BAL 31 nuclease hydrolyzes ssDNA endonucleolytically and shortens the linear duplex DNA from both 3' and 5' ends. However, the enzyme isolated from *N. crassa* mitochondria showed distributive endonuclease

activity towards ssDNA but processive exonuclease activity towards dsDNA (Chow and Fraser, 1983). In addition, an endo-exonuclease from *S. cerevisiae*, influenced by *RAD*52 gene, showed a non-processive endo mode of action on ssDNA and weakly processive exonucleolytic activity on dsDNA resulting in the formation of oligonucleotides of varying chain length (Chow and Resnick, 1987). In contrast, *A. nidulans* nuclease showed endo and exo mode of action on both ss and dsDNA (Koa *et al*., 1990). Tobacco pollen nuclease, apart from exhibiting an endo mode of action on ssDNA and exo mode with dsDNA, also showed endonucleolytic activity on dsDNA leading to the formation of a typical 58 bp long fragment (Matousek and Tupy, 1984). The exo mode of action of *N. crassa* endo-exonuclease (Chow and Fraser. 1983) is different from those exhibited by typical exonucleases like snake venom phosphodiesterase (Williams *et al.*, 1961), spleen phosphodiesterase (Razzell and Khorana, 1961) and *A. sydowii* nuclease (Ito *et al*., 1994) in that, the hydrolytic products of *N. crassa* enzyme consists of di, tri and tetranucleotides.

The end products of hydrolysis of DNA and RNA, by non-specific endonucleases, are oligonucleotides and / or mononucleotides with 5' or 3'-phosphoryl termini. With the exception of *S. aureus* (Cunningham *et al.*, 1956) and *B. subtilis* nucleases (Kanamori *et al*., 1973a) which produce 3' mononucleotides, all the other nucleases produce 5' mononucleotides and / or oligonucleotides with 5'-PO_4 and 3'-OH termini. However, wheat chloroplast nuclease hydrolyzes ssDNA endonucleolytically, liberating oligonucleotides with 3'-OH and 5'-PO_4 termini while oligonucleotides liberated from RNA hydrolysis have 3'- PO_4 and 5'-OH termini (Kuligowska *et al*., 1988). Rye germ ribosomes nuclease I, on the other hand, liberates oligonucleotides ending in 3'-OH and 5'-PO_4 from RNA and 3'-PO_4 and 5'-OH from ssDNA and dsDNA (Siwecka *et al.*, 1989).

Mechanism of action

Mechanism of hydrolysis of dsDNA, by endonucleases, have been studied by light-scattering, viscometry and sedimentation analysis (Thomas, 1956; Schumaker *et al.*, 1956; Bernardi and Cordonnier, 1965). Two different

mechanisms for the cleavage of dsDNA by DNases are depicted in Fig. 1.3. In "double-hit" mechanism, the nicks are made at random sites in each strand at points away from each other and the complete fragmentation of duplex DNA does not occur until the two nicks are opposite to each other (multiple encounter) (Fig. 1.3 A). However, in "single-hit" mechanism, the nicks on either strand are made at points close or opposite to each other, resulting in the complete scission of the duplex DNA in a single encounter (Fig. 1.3 B) (Weir, 1993).

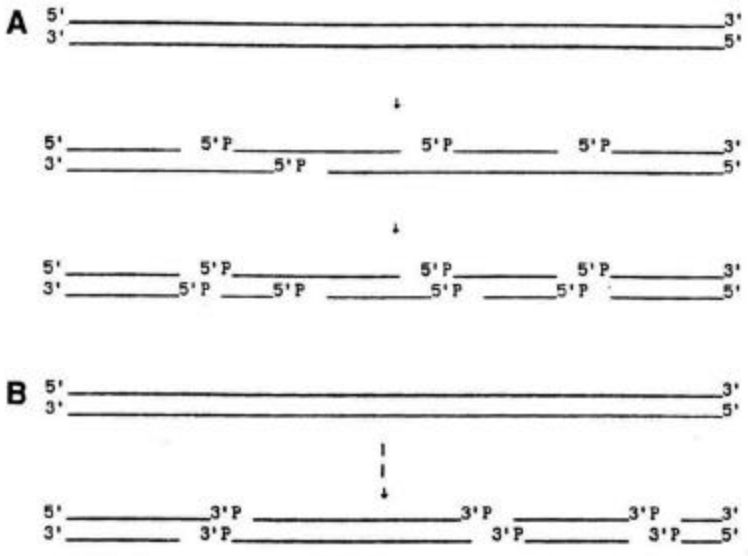


Fig. 1.3: Cleavage mechanism of duplex DNA by DNase.

A. Double-hit mechanism of DNase I in presence of Mg^{2+} ions

B. Single-hit mechanism of DNase II

(Adapted from Weir, 1993)

Melgar and Goldthwait (1968) studied the effect of metal ions on the hydrolysis of DNA by pancreatic DNase I and demonstrated that, in presence of Mg^{2+}, the DNA is cleaved predominantly by a double-hit mechanism whereas, in presence of Mn^{2+}, Ca^{2+} or Co^{2+} the mechanism of hydrolysis shifts to single-hit mode. In case of nucleases from yeast mitochondria (Morosoli and Lusena, 1980) and *S. marcescens* (Meiss *et al*., 1995), it has been suggested that the cleavage of phosphodiester linkage of native DNA occurs at the same or nearby sites, suggesting a single-hit mechanism.

In general, non-specific endonucleases show exo and endonucleolytic activities on both DNA and RNA (Table 1.4). BAL 31 nuclease (both S and F forms) degrades ssDNA exonucleolytically, whereas dsDNA degradation occurs in a terminally directed manner, in which the removal of nucleotides take place from both the ends of dsDNA. The ratio of the turnover number for the exonuclease activity of the F form, to shorten the duplex DNA, is approximately 27 ± 5 times higher compared with the S form. Apart from terminally directed exonuclease activity, some endonuclease activity was also found to be associated with both F and S species against 5'-terminated single-stranded tails generated by the exonuclease action (Wei *et al*., 1983; Zhou and Gray, 1990). The exonulcease activity of the S form, on duplex DNA, decreased with increasing G + C content, whereas the action of F form was not very much dependent on the base composition (Gray and Lu, 1993). BAL 31 nuclease F species degraded linear duplex RNA in a terminally directed manner, resulting in the removal of nucleotides from both 3' and 5' ends. In comparison, S species showed very little activity against duplex RNA (Bencen *et al*., 1984). Subsequently, Lu and Gray (1995) showed that both forms degrade ssDNA in a processive manner from the 5' end as against the 3' termini for the duplex DNA. Endo-exonucleases from *B. subtilis* (Kanamori *et al*., 1973b), *N. crassa* mitochondria (Chow and Fraser, 1983), *S. cerevisiae* (Dake *et al*., 1988), A. *nidulans* (Koa *et al*., 1990), and *D. melanogaster* (Shuai *et al*., 1992) also showed 5'→3' directed exonuclease action on duplex DNA.

Action on polynucleotides

Action of non-specific nucleases on synthetic polynucleotides revealed that the rate of hydrolysis varies with the source of the enzyme. Wheat chloroplast nuclease hydrolyzed various synthetic polynucleotides in the order of poly(A) > poly(U) > poly(C) and poly(dA) > poly(dT) > poly(dC) but poly(G) and poly(dG) were resistant to cleavage (Kuligowska *et al*., 1988). Staphylococcal nuclease also exhibited a similar preference for the homopolyribonucleotides as that of wheat chloroplast nuclease (Cuatrecasas *et al*., 1967b). However, nuclease I from rye

germ ribosomes showed high specificity for poly(C) while, other ribopolynucleotides were hydrolyzed in the order of poly(A) ≥ poly(U) = poly(G). Although rye germ ribosomes nuclease I hydrolyzed the double-stranded deoxyriboheteropolymer poly(dT).poly(rA) at a very slow rate, it failed to hydrolyze the riboheteropolymer poly(A).poly(U), suggesting its preference for single-stranded nucleic acids (Siwecka *et al*., 1989). Similarly, barley nuclease hydrolyzed the polynucleotides in the order of poly (C) > poly (U) > poly (A) > poly(A).poly(U) > poly (G) = poly(G).poly(C) (Brown and Ho, 1987). In contrast, rye germ ribosomes nuclease II hydrolyzed the double-stranded polymer poly(I).poly(C) at a higher rate than poly(A).poly(U) followed by the single-stranded polymers in the order of poly (U) > poly (A) > poly (C) > poly (I). However, poly(G) and poly(dI).poly(dC) were resistant to cleavage (Siwecka, 1997). *S. cerevisiae* nuclease could degrade poly(dT), poly(U), poly(A) and poly(C) but not poly(G), poly(A).poly(U), poly(AU) and poly(A).poly(dT) (Dake *et al*., 1988). Endonuclease from potato tubers showed preference for polynucleotides in the order of poly(U) > poly(A) > poly(I) > poly(C) whereas, the enzyme from rat liver nuclei (Cordis *et al*., 1975) hydrolyzed these substrates in the order of poly(U) > poly(C) > poly(A). *A. agilis* nuclease could hydrolyze only poly(A) and poly(U), poly(C) and poly(G) were resistant to cleavage. While 10-fold and 30-fold excess enzyme was required for significant hydrolysis of poly(U) and poly(C) respectively, poly(G) was resistant to cleavage even at higher enzyme concentrations (Stevens and Hilmoe, 1960a). *S. marcescens kums* 3958 nucleases hydrolyzed poly(A) slowly but poly(I).poly(C) was degraded at a higher rate followed by poly(A).poly(U) and poly(dG).poly(dC). However, poly(G), poly(C), poly(U), poly(dT), poly(dA) and poly(dA).poly(dT) were resistant to hydrolysis (Yomemura *et al*., 1983). On the contrary, *Serratia* nuclease cleaved the duplex form in the order of poly(dG).poly(dC) > poly(A). poly(U) > poly(dI).poly(dC) > poly(I).poly(C) but poly(dA).poly(dT) was resistant to hydrolysis (Nestle and Roberts, 1969b). Similarly, *Anabaena* nuclease could readily hydrolyze poly(dG).poly(dC) but poly(dA).poly(dT) was resistant to cleavage, suggesting the preference of the enzyme for the former (Meiss *et al*., 1998).

Action on plasmid DNA

Cleavage of closed circular DNA is considered as one of the main characteristics of endonucleases. In plasmid and phage, closed circular duplex DNA exists in a supercoiled form as a consequence of torsional strain, which at sufficiently high negative superhelical density promotes unwinding of helical twists (Bauer and Vinograd, 1974). Non-specific endonucleases nick the supercoiled DNA (Form I) to give rise to linear duplex DNA (Form III) via the formation of nicked circular DNA (Form II). Thus, *N. crassa* (mitochondria and vacuoles) nucleases could readily convert Form I DNA to Form II DNA but its subsequent conversion to linear duplex DNA (Form III) occurred at a slow rate (Chow and Fraser, 1983). Similar observations were made with nucleases from *A. espejiana* (Gray *et al*., 1975), barley aleurone layers (Brown and Ho, 1987), *S. cerevisiae* (Chow and Resnick, 1987), *A. nidulans* (Koa *et al*., 1990), *Leishmania* sp. (Mittra *et al*., 1998) and *S. antibioticus* (Nicieza *et al*., 1999). In case of *N. crassa* mitochondrial nuclease, the enzyme action can be controlled by adjusting the concentrations of Mg^{2+} in the reaction mixture. At low enzyme concentrations, in presence of 0.1 mM Mg^{2+}, it exhibits strict endonuclease activity and high specificity for Form I DNA. However 4-8 fold excess enzyme, in presence of 10 mM Mg^{2+}, accelerated the conversion of Form II DNA to Form III DNA followed by exonucleolytic degradation of Form III DNA (Chow and Fraser, 1983). *D. melanogaster* endonuclease converted Form I DNA rapidly to Form III DNA with very little accumulation of Form II DNA. The appearance of low amounts of Form II DNA was attributed to the preferential nicking on one strand before it is linearized (Shuai *et al*., 1992).

Leishmania nuclease, at low enzyme concentrations, generates single base nicks that can be ligated by T4 DNA ligase to yield covalently closed circular DNA (Mittra *et al*., 1998). Similarly, *S. pombe* endonuclease also produces nicks which are resealable by T4 DNA ligase (Ikeda *et al*., 1996). In contrast, the nicks generated in supercoiled DNA by BAL 31 nuclease (F and S forms) could not be ligated back to covalently closed DNA, since they were extended into gaps by the exonuclease action of these enzymes. However, the ligation of the gaps could be

achieved by carrying out the reaction using T4 DNA ligase in combination with T4 DNA polymerase (Przykorska *et al*., 1988). BAL 31 nuclease exhibited enhanced endonucleolytic activity on netropsin bound supercoiled plasmid DNA with no detectable exonucleolytic activity (Sakaguchi *et al.*, 1985).

It is known that intercalating agents change the superhelical density of plasmid DNA in the order of less negatively supercoiled → relaxed → positively supercoiled. Moreover, negatively supercoiled DNA is known to form stably unwound DNA conformations including Z-DNA, cruciform and homopurine-homopyrimidine structures. BAL 31 nuclease cleaves very highly supercoiled DNA prepared from covalently closed relaxed DNA (Form I°) with ethidium bromide. Initial nicking rates of PM2 Form I DNA by BAL 31 nuclease are readily measurable at superhelical densities as low as -0.02 and the nicking activity on positively supercoiled DNA becomes detectable at superhelical densities between 0.15 and 0.19 (Gray *et al*., 1981). It was also noted that non-stressed (i.e. non-supercoiled), non-modified duplex DNAs were resistant to endonucleolytic action (Wei *et al*., 1984).

Cleavage preference

Staphylococcal nuclease hydrolyzed denatured DNA at a faster rate than native DNA suggesting that, the rate of cleavage depends on the conformation of the substrate (Dirksen and Dekker, 1960). Based on the findings of Felsenfeld and Sandeen (1962) that dA-dT rich regions of native DNA undergo denaturation at somewhat lower temperatures (i.e. below T_m) than regions containing higher proportions of dG-dC nucleotide pairs, von Hippel and Felsenfeld (1964) postulated that at low temperatures dA-dT regions undergo local opening and closing or strand separation reaction ('breathing') to a greater extent than dG-dC rich regions of DNA. This 'breathing' phenomenon facilitates the formation of functional enzyme-substrate complexes and account for the relative predominance of deoxyadenylic and thymidylic acid residues in the early digestion products of DNA by Staphylococcal nuclease (von Hippel and Felsenfeld, 1964). Additionally, Cuatrecasas *et al*. (1967b) observed that 5' mononucleotides dA and dT are strong

inhibitors of Staphylococcal nuclease and suggested it to be the basis of strong binding of the enzyme to dA-dT rich regions thereby accounting for the release of deoxyadenylic and thymidylic acid residues in the early part of hydrolysis. This led Wingert and von Hippel (1968) to suggest that the specificity of the enzymatic attack, on the native DNA, depends on the conformational motility ('breathing' or 'dynamic' structure) of DNA molecule and that denatured DNA is preferentially attacked when both denatured and native DNA are present. This is supported by the observation that Staphylococcal nuclease binds to an exposed single-stranded region and nicks transiently melted base-pairs in duplex DNA (Drew, 1984). Moreover, the formation of single-stranded regions, during the course of digestion of native DNA, was correlated to ss-cut-triggered local unwinding of the double helix. The above phenomenon has been observed in DNA-degrading enzymes capable of producing single-strand nicks (Galcheva-Gargova *et al.*, 1985). The different pathways for the formation of ssDNA regions are depicted in Fig. 1.4.

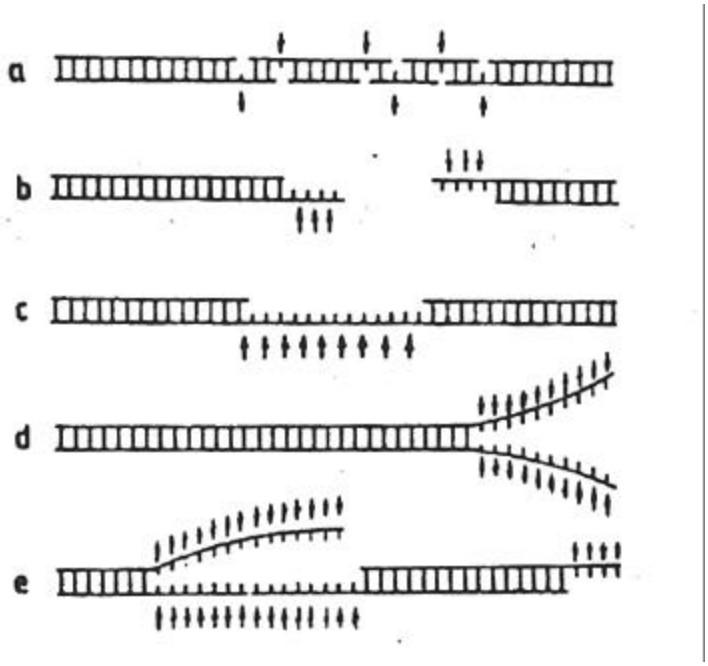


Fig. 1.4: Mechanisms of generation of ssDNA regions. The arrow indicates the cleavage site of single-strand-specific enzymes. (Adapted from Galcheva-Gargova *et al.*, 1985)

The occurrence of gaps in plasmid DNA, by the action of BAL 31 nuclease, also supports the formation of single-strand regions in native DNA by DNA degrading enzymes. Similar observation was also made with *N. crassa* endo-exonuclease (Chow and Fraser, 1983).

Meiss *et al*. (1995) studied the cleavage of PCR-generated oligonucleotides by *Serratia* nuclease and demonstrated that the enzyme cleaves preferentially the GC-rich regions [particularly d(G).d(C) tracts] in dsDNA and avoids d(A).d(T) tracts. It was also shown that compared to DNase I, *Serratia* nuclease is more non-specific and attacks a particular substrate more evenly under standard reaction conditions. Though the non-specific nature of cleavage is maintained in presence of high ionic strength or DMSO, addition of urea made the enzyme more selective and this led the authors to suggest that *Serratia* nuclease could be sensitive to global features like width of the minor groove (Meiss *et al*., 1995). Additionally, experiments with synthetic model substrates (oligonucleotides) showed that *Serratia* nuclease prefers purine rich oligonucleotides probably because it adopts a helical structure with pronounced base stacking. However, cleavage specificities with homo and heteroduplexes indicated that the A-form of nucleic acid is preferred over the B-form (Meiss *et al*., 1999). Similarly, endonuclease from *Anabaena* sp. also preferred A-form DNA (Meiss *et al*., 1998). In case of endonuclease G from *B. taurus*, it was shown that the enzyme preferentially cleaves $(dG)_n.(dC)_n$ tracts in DNA (Ruiz-Carrillo and Renaud, 1987; Côtè *et al*., 1989). Synthetic polynucleotides namely, $poly(dC)_n.poly(dG)_n$ and the oligomer d(GGGGCCCC) adopts a right handed structure similar to classical A-form DNA (Arnott and Selsing, 1974; McCall *et al*., 1985). The susceptibility of these oligonucleotides to endonuclease G led Ruiz-Carrillo and Renaud (1987) to conclude that the enzyme recognizes A-type DNA. Like Serratia (Meiss *et al*., 1999) and *Anabaena* (Meiss *et al*., 1998) nucleases, endonuclease G too does not cleave d(A).d(T) and $(dG.dA)_{34}.(dT.dC)_{34}$. However, endonuclease G hydrolyzes $(dG)_{24}.(dC)_{24}$ tracts in supercoiled DNA, in a bimodal way every 9-11 nucleotides, with the maxima in one strand corresponding to minima on the opposite strand, suggesting that the enzyme binds preferentially to one side of the double helix

(Ruiz-Carillo and Renaud, 1987). The segments of (dC-dG) in DNA restriction fragments and in recombinant plasmids adopt a left-handed conformation in high salt solution while, the neighbouring regions of natural sequences remain in right handed helices (Zimmerman, 1982). Kilpatrick *et al*. (1983) showed that BAL 31 nuclease cleaves the B-Z junction in presence of high salt concentration but it does not cleave DNA under conditions where $(dC\text{-}dG)_n$ blocks exist in B conformation.

Base specificity

Though majority of non-specific endonucleases reported so far are base non-specific, the enzymes (Sm1 and Sm2) from *S. marcescens* (Filiminova *et al*., 1996) and tea leaves (Imagawa *et al*., 1982) showed some base specificity during the initial stages of hydrolysis. Nucleases Sm1 and Sm2 from *S. marcescens* preferred uracil and guanosine residues respectively in the polynucleotide chain (Filiminova *et al*., 1996). This preferential specificity was correlated to the difference in the N-terminal tripeptide fragment (Pedersen *et al*., 1995a). Tea leaves nucleases hydrolyzed RNA with preferential liberation of 5'AMP and 5'GMP but 5'UMP and 5'CMP was liberated after a considerable lag period. Though hydrolysis of DNA gave similar results, 5'dCMP was detected only after exhaustive digestion of the substrate (Imagawa *et al*., 1982). These observations indicated the preference of the enzyme for purine nucleotides. Action of barley aleurone layers nuclease, on dinucleoside monophosphate, revealed a strong preference for purine nucleosides as the 5' residue followed by uridine as the 3' residue. Similarly, Staphylococcal nuclease hydrolyzed various dinucleoside monophosphates in the order of d-TpT > d-CpC > d-GpG. It was observed that presence of 5' phosphate in the dinucleotides brought about 25 fold increase in the activity compared to 5-fold when the phosphate was present at the 3' position (Mikulski *et al*., 1969).

Associated phosphomonoesterase activity

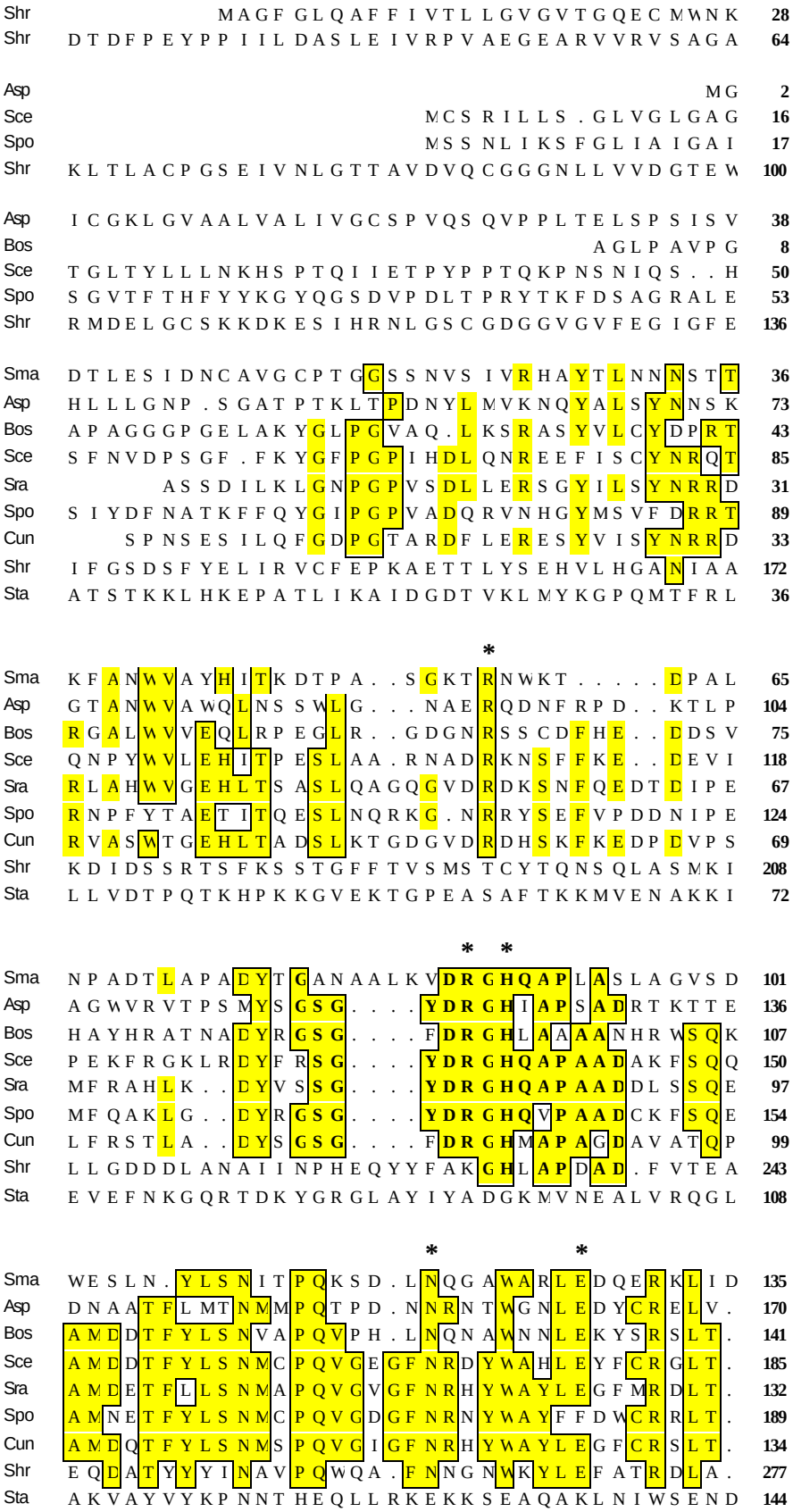
Among the non-specific endonucleases, only those from potato tubers (Nomura *et al*., 1971), barley aleurone layers (Brown and Ho, 1987), tea leaves (Imagawa *et al*., 1982) and rye germ ribosomes (Siwecka *et al*., 1989) exhibit 3'

phosphomonoesterase activity. Interestingly, all the aforementioned enzymes, except rye germ ribosomes nuclease I (Siwecka *et al*., 1989), are 3' monoribonucleotides specific. In addition, some enzymes exhibit a base preference for the hydrolysis of various 3' monoribonucleotides. Thus, tea leaves nucleases (A and B) hydrolyzed various 3' monoribonucleotides in the order of A > G > U > C (Imagawa *et al*., 1982) whereas, potato tubers nuclease hydrolyzed them in the order of A > U > G > C (Nomura *et al*., 1971). In contrast, barley aleurone layers nuclease readily hydrolyzed 3'AMP and 3'GMP while 3'CMP and 3'UMP were resistant to cleavage (Brown and Ho, 1987).

Structure and function

The amino acid sequence of nine non-specific endonucleases, derived from their gene sequence, showed that most of them are made up of a single polypeptide chain (Fig. 1.5). Based on the structural and functional similarity, endonucleases from *Anabaena* sp., *S. cerevisiae*, *S. racemosum, C. echinulata*, *S. pombe* and *B. taurus*, were shown to belong to *Serratia* family of non-specific endonucleases. In all the enzymes, except Staphylococcal nuclease, the active site residues are conserved (Fig. 1.5), including the DRGH-motif which contains the active site histidine (Friedhoff *et al*., 1994a, 1996a; Meiss *et al*., 2000). Among them, C1 nuclease from *C. echinulata* showed 44 % and 42 % sequence homology with *S. cerevisiae* and *S. pombe* nucleases respectively (Ho *et al*., 1998).

Staphylococcal nuclease consists of a single polypeptide chain of 149 amino acids with no disulfide bonds or free cysteine (Tanuichi *et al*., 1967). Shrimp nuclease is also a single polypeptide containing an open reading frame encoding a putative 21-residue signal peptide and a 381-residue mature protein. The polypeptide is cross-linked by five disulfide bonds. The disulfide (S-S) linkages were shown to occur between Cys3-Cys129, Cys49-Cys66, Cys86-Cys175 with remaining two occurring between Cys333-Cys357 and Cys337-Cys358 or Cys333-Cys358 and Cys337-Cys357 (Wang *et al*., 2000). C1 nuclease from *C. echinulata* is a monomeric protein containing 252 amino acids cross-linked by a single


Shr MAGFGLQAFFIVTLLGVGVTGQECMWNK 28
Shr DTDFPEYPPIILDASLEIVRPVAEGEARVVRVSAGA 64
Asp MG 2
Sce MCSRILLS.GLVGLGAG 16
Spo MSSNLIKSFGLIAIGAI 17
Shr KLTLACPGSEIVNLGTTAVDVQCGGGNLLVVDGTEW 100
Asp ICGKLGVAALVALIVGCSPVQSQVPPLTELSPSISV 38
Bos AGLPAVPG 8
Sce TGLTYLLLNKHSPTQIIETPYPPTQKPNSNIQS..H 50
Spo SGVTFTHFYYKGYQGSDVPDLTPRYTKFDSAGRALE 53
Shr RMDELGCSKKDKESIHRNLGSCGDGGVGVFEGIGFE 136
Sma DTLESIDNCAVGCPTGGSSNVSIVRHAYTLNNNSTT 36
Asp HLLLGNP.SGATPTKLTPDNYLMVKNQYALSYNNSK 73
Bos APAGGGPGELAKYGLPGVAQ.LKSRASYVLCYDPRT 43
Sce SFNVDPSGF.FKYGFPGPIHDLQNREEFISCYNRQT 85
Sra ASSDILKLGNPGPVSDLLERSGYILSYNRRD 31
Spo SIYDFNATKFFQYGIPGPVADQRVNHGYMSVFDRRT 89
Cun SPNSESILQFGDPGTARDFLERESYVISYNRRD 33
Shr IFGSDSFYELIRVCFEPKAETTLYSEHVLHGANIAA 172
Sta ATSTKKLHKEPATLIKAIDGDTVKLMYKGPQMTFRL 36
Sma KFANWVAYHITKDTPA..SGKTRNWKT.....DPAL 65
Asp GTANWVAWQLNSSWLG...NAERQDNFRPD..KTLP 104
Bos RGALWVVEQLRPEGLR..GDGNRSSCDFHE..DDSV 75
Sce QNPYWVLEHITPESLAA.RNADRKNSFFKE..DEVI 118
Sra RLAHWVGEHLTSASLQAGQGVDRDKSNFQEDTDIPE 67
Spo RNPFYTAETITQESLNQRKG.NRRYSEFVPDDNIPE 124
Cun RVASWTGEHLTADSLKTGDGVDRDHSKFKEDPDVPS 69
Shr KDIDSSRTSFKSSTGFFTVSMSTCYTQNSQLASMKI 208
Sta LLVDTPQTKHPKKGVEKTGPEASAFTKKMVENAKKI 72
Sma NPADTLAPADYTGANAALKVDRGHQAPLASLAGVSD 101
Asp AGWVRVTPSMYSGSG....YDRGHIAPSADRTKTTE 136
Bos HAYHRATNADYRGSG....FDRGHLAAAANHRWSQK 107
Sce PEKFRGKLRDYFRSG....YDRGHQAPAADAKFSQQ 150
Sra MFRAHLK..DYVSSG....YDRGHQAPAADDLSSQE 97
Spo MFQAKLG..DYRGSG....YDRGHQVPAADCKFSQE 154
Cun LFRSTLA..DYSGSG....FDRGHMAPAGDAVATQP 99
Shr LLGDDDLANAIINPHEQYYFAKGHLAPDADF.VTEA 243
Sta EVEFNKGQRTDKYGRGLAYIYADGKMVNEALVRQGL 108
Sma WESLN.YLSNITPQKSD.LNQGAWARLEDQERKLID 135
Asp DNAATFLMTNMMPQTPD.NNRNTWGNLEDYCRELV. 170
Bos AMDDTFYLSNVAPQVPH.LNQNAWNNLEKYSRSLT. 141
Sce AMDDTFYLSNMCPQVGEGFNRDYWAHLEYFCRGLT. 185
Sra AMDETFLLSNMAPQVGVGFNRHYWAYLEGFMRDLT. 132
Spo AMNETFYLSNMCPQVGDGFNRNYWAYFFDWCRRLT. 189
Cun AMDQTFYLSNMSPQVGIGFNRHYWAYLEGFCRSLT. 134
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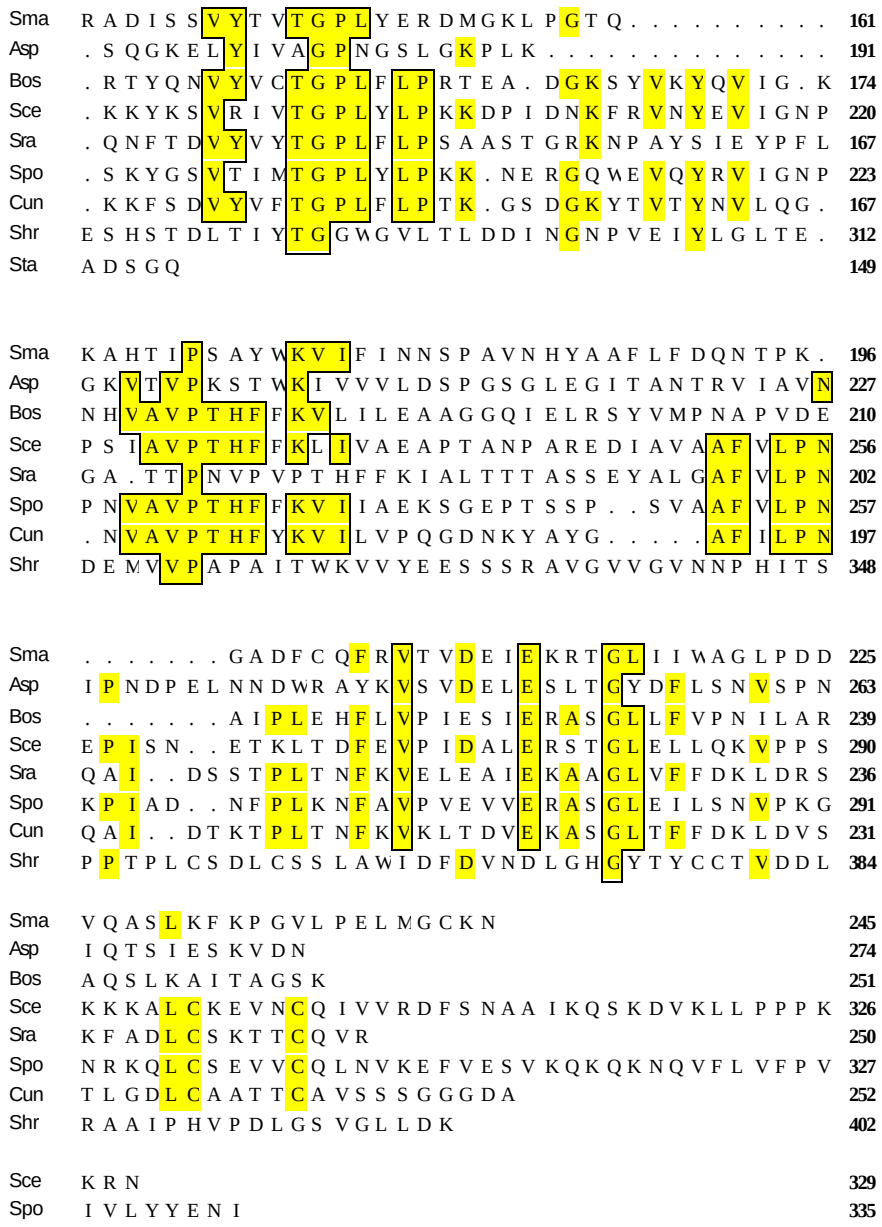

Fig. 1.5: Sequence alignment of non-specific endonucleases. Sma - *S. marcescens*, Asp - *Anabaena* sp., Bos - *Bos taurus*, Sce - *S. cerevisiae*, Sra - *S. racemosum*, Spo - *S. pombe*, Cun - *C. echinuclata*, Shr - shrimp hepatopancreas and Sta - *S. aureus*. The numbers on the right margin is the last residue of each line. Identical amino acid residues (excluding that of *S. aureus*) having ≥ 50 % sequence similarities are shaded and regions exhibiting substantial homology are boxed. The DRGH motif is shown in bold face. The conserved active site residues are marked with asterisk. The sequence alignments were made based on the data from Friedhoff *et al.* (1994a, 1996a), Ho *et al.* (1998) and Wang *et al.* (2000).

disulfide bond between Cys237-Cys242 (Ho *et al*., 1998). Similarly *S. racemosum* nuclease, a glycoprotein, is made up of a single polypeptide chain of 250 amino acids and the carbohydrate moiety is attached to Asn134. In addition, it has a single disulfide linkage between Cys242-Cys247 (Ho and Liao, 1999). *Serratia* nuclease is produced as a 266 amino acids pre-protein with a signal peptide consisting of first 21 residues (Ball *et al.*, 1987; Biedermann *et al.*, 1989). Among the two major isoforms produced by *S. marcescens* (Yonemura *et al*., 1983; Filiminova *et al*., 1991), with near identical biochemical properties, the 245 amino acid mature nuclease (Sm2), with a Mr of 26.7 kDa, is the result of a typical signal sequence processing. The second isoform (Sm1) lacks the first three N-terminal amino acids (Filiminova *et al.*, 1991; Bannikova *et al*., 1991; Pedersen *et al*., 1995a,b). In addition, two disulfide bonds were shown to occur between Cys9-Cys13 and Cys201-Cys243 (Biedermann *et al*., 1989).

Based on circular dichroism (CD) studies, Meiss *et al.* (1998, 2000) showed that *Anabaena* nuclease consists of 13-17 % α-helix and 20-27 % β-sheet whereas, *S. aureus* nuclease consists of 13-18 % α-helix (Taniuchi and Anfinsen, 1968).

Although a large number of non-specific endonucleases have been reported till date, structural studies have been limited to Staphylococcal nuclease (Arnone *et al*., 1971; Cotton *et al*., 1979; Loll and Lattman, 1989) and *Serratia* nuclease (Friedhoff *et al*., 1994a; Miller *et al*., 1994; Miller and Krause, 1996; Lunin *et al*., 1997). The crystal structure of Staphylococcal nuclease, in presence of Ca^{2+} and deoxythymidine 3',5'-diphosphate at 1.5-1.6 Å resolution, showed the involvement of 30 residues in three separate sections of the helix and in addition, about 24 residues form a three-stranded section of antiparallel β-sheet. Residues 44-53 were found to be somewhat disordered and formed a highly solvent exposed large loop (omega-loop). Moreover, the structure showed the presence of an inhibitor pocket which was predominantly neutral or hydrophobic. In addition, the inhibitor pocket also contained several residues, which specifically participate in binding of calcium ion and the nucleoside diphosphate (Arnone *et al*., 1971; Cotton *et al*., 1979; Loll and Lattman, 1989). The crystal structure of *Serratia* nuclease, a dimeric enzyme,

was first solved at 2.1 Å by Miller *et al*. (1994), which was then refined at 1.7 Å and 1.1 Å resolution by Lunin *et al*. (1997) Shlyapnikov *et al*. (2000) respectively. The crystal structure revealed that the dimer consists of two identical monomers and each monomer contains the active site. Moreover, each monomer consists of a central six-stranded anti-parallel β-sheet flanked on one side by a helical domain and on the opposite side by a dominant helix and a very long coiled loop. Crystal structure also showed that the cleft between the long helix and loop, near His89, may contain the active site (Miller *et al*., 1994).

Active site

Though considerable work has been done on the substrate specificity and mode of action, very little information is available regarding the active site nature of non-specific endonucleases**.** *S. aureus* nuclease bound 5' mononucleotides in a ratio of 1:1 and was completely dependent on the presence of Ca^{2+}. Moreover, binding of 5' mononucleotides and deoxythymidine 3',5'-diphosphate (pdTp), to the enzyme was affected by low amounts of DNA and RNA suggesting the presence of common catalytic site for the hydrolysis of both the substrates (Cuatrecasas *et al*. 1967c). Studies using amino and nitrotyrosyl derivatives of Tyr85 and Tyr113 indicated that they are in stereochemical proximity and act in a concerted manner (Cuatrecasas *et al*., 1969a). Through site directed mutagenesis it was established that, Tyr85 has a more direct role in the active site compared to Tyr113 and Tyr115 (Uhlmann and Smith, 1987). Enzyme crystals, obtained in presence of Ca^{2+} and pdTp, revealed the involvement of Lys84 and Tyr85 in the catalytic activity and they form hydrogen bonds with 3' phosphate of the inhibitor whereas, Arg35 and Arg87 form hydrogen bonds with the 5' phosphate. Carboxylate ions of Glu43, Asp21 and Asp40 serve as ligands for the binding of Ca^{2+} (Arnone *et al*., 1971). Cotton *et al*. (1979) suggested that pdTp, in particular its 5' phosphate and the activating Ca^{2+} are precisely and rigidly locked into the position at the hydrolytic site. It was also proposed that while Glu43 acts as a general base, Arg35 and Arg87 are involved in the neutralization of the charges on the phosphates and Ca^{2+} performs the dual role of aiding catalysis and stabilization of the trigonal bipyramid

transition state. Crystal structure studies of Staphylococcal nuclease at 1.6 Å resolution also confirmed the above observations (Loll and Lattman, 1989).

Based on the sequence homology of related nucleases (Fraser and Low, 1993; Friedhoff *et al.*, 1994a), mutational analysis of the conserved amino acid residues (Friedhoff *et al.*, 1994a) and crystal structure (Miller *et al.*, 1994; Miller and Krause, 1996), Friedhoff *et al*. (1996a) proposed that, the active site of *Serratia* nuclease comprises of at least four residues namely Arg57, His89, Asn119 and Glu127. Since the mutants of active site residues showed a similar effect on the hydrolysis of both DNA and RNA the authors suggested the existence of a common catalytic site for the hydrolysis of both substrates. Furthermore, it was observed that among all mutants of His89, only H89N showed measurable enzyme activity, in addition to a decrease in the k_{cat} suggesting the probable role of His89 in catalysis. However, the similar substrate binding efficiency of the wild type enzyme and mutant H89A, of the non-cleavable modified substrate (decamer in which phosphates are replaced by phosphorothioates), suggested that His89 is not involved in substrate binding. It was also demonstrated that, His89 has to be deprotonated to function as a general base in the catalytic activity (Friedhoff *et al*., 1996a). The close proximity of Arg57 and Arg87 to the phosphate backbone in the substrate (Miller *et al*., 1994) coupled with the decrease in the k_{cat} of the Arg57 mutants, with no change in the K_m suggested the involvement of Arg57 in catalysis. Moreover, the significant decrease in the k_{cat} of Arg57 mutant was correlated to its involvement in positioning and polarizing the phosphate of the scissile phosphodiester bond and / or stabilization of the transition state. Asn119 was implicated in catalysis since mutants of Asn119 showed a decrease in their k_{cat} and not in the K_m (Friedhoff *et al*., 1994a, 1996a). Subsequently, Miller *et al*. (1999) established the involvement of Asn119 in metal binding. Compared to the native enzyme, the alanine mutants of Glu127 showed reduced activity while the mutants E127D and E127Q showed similar activities as that of the native enzyme indicating that, Glu127 might be participating indirectly in the hydrolysis of the substrates (Friedhoff *et al*., 1996a). Non-specific endonuclease from *Anabaena* sp. showed nearly 30 % sequence homology with *Serratia* nuclease and this was restricted

mainly to the active site regions and the central six-stranded β-sheet of the enzyme. Based on the three dimensional structure of *S. marcescens* nuclease and mutational analysis of the catalytically active residues, a structural model for *Anabaena* nuclease was proposed (Meiss *et al*., 2000). Accordingly, it was suggested that His124, Asn155 and Glu163 (corresponding to His89, Asn119 and Glu127 of *S. marcescens* nuclease) are involved in the catalytic activity of the enzyme. Additionally, through mutational analysis it was demonstrated that Asn155 and Asp121 are involved in the coordination of the metal ion cofactor. Subsequently, cleavage studies with deoxythymidine 3',5'-bis-(*p*-nitrophenyl phosphate) revealed the role of His124 as a general base and that of Glu163 in assisting the general acid (Meiss *et al*., 2000).

Based on the sequence similarity with *S. marcescens* and site directed mutagenesis, the involvement of His87, His85 and His211 in the catalytic activity of endonucleases from *C. echinulata* (Ho *et al*., 1998), *S. racemosum* (Ho and Liao, 1999) and shrimp hepatopancreas (Wang *et al*., 2000) respectively, was demonstrated. Moreover, the probable involvement of Asn241 in catalysis and Lys210 and Arg253 in substrate binding was postulated for shrimp hepatopancreas nuclease (Wang *et al*., 2000). In case of mitogenic factor secreted by *S. pyogenes*, His122 was shown to be the residue essential for the nuclease activity (Iwasaki *et al*., 1997).

Coordination and function of metal ions

Staphylococcal nuclease is a Ca^{2+} dependent non-specific enzyme while, *S. marcescens* nuclease requires Mg^{2+} for its activity. Extensive studies on these enzymes have provided insights into the substrate binding, metal interaction and catalytic mechanism. In case of Staphylococcal nuclease, Ca^{2+} is coordinated directly to Asp21 and Asp40 while, Glu43 is coordinated to calcium ion through a water molecule. Additionally, the calcium ion was found to coordinate with two additional water molecules (Fig. 1.6) (Cotton and Hazen, 1971; Arnone *et al*., 1971; Loll and Lattman, 1990). The role of Asp21 and Asp40 were further delineated by Electron-spin Paramagnetic Resonance studies where, Asp21 was

found to influence the binding of Ca^{2+} more strongly than Asp40 (Serpersu *et al*., 1987). Through the crystal structure at 1.95-Å resolution of the D21E ternary complex where, D21E is bound to both Ca^{2+} and deoxythymidine 3',5'-diphosphate (transition-state analogue), it was demonstrated that Ca^{2+}, in the active site, binds to Glu21 via bidentate coordination and to Glu43 through an inner-sphere coordination. The cooperativity of the metal ion and the inhibitor (pdTp), in the ternary complex, was also demonstrated (Libson *et al.*, 1994).

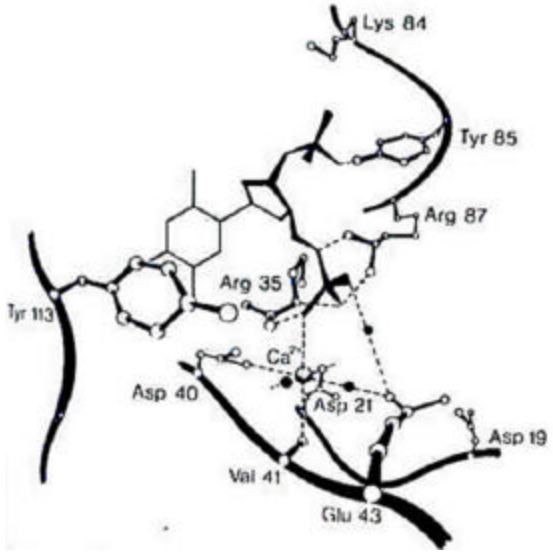

Figure 1.6: Schematic view of the binding site of Staphylococcal nuclease in enzyme-pdTp-Ca^{2+} complex. The heavy dots represent regions of electron density assigned as water molecules. Hydrogen bond and ionic interactions are shown as dotted lines. The interaction between Ca^{2+} and Val41 is through the carbonyl oxygen of the peptide bond. (Adapted from Cotton *et al.*, 1979).

On the other hand, in *Serratia* nuclease, the catalytic magnesium binding site is located between the long helix (residues 116-135) and the loop extending from residues 50-114 which contains the catalytically essential His89. It was shown that, Mg^{2+} bound to Asn119 (only protein ligand) is associated with five water molecules to complete an octahedral coordination complex (Fig. 1.7). Glu127 and His89 are located nearby and each is hydrogen bonded to water molecules in the coordination sphere. Kinetic and site-specific mutagenesis studies

suggested that this metal-water cluster contained the catalytic metal ion. The residues involved in the formation of metal-water cluster coordination are conserved in enzymes homologous to *Serratia* nuclease. Additionally, it was

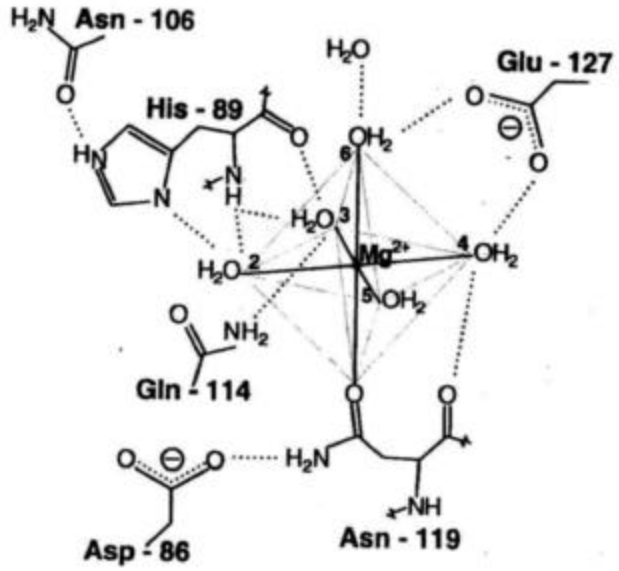

Figure 1.7: Schematic view of the magnesium binding site of *Serratia* nuclease.
(Adapted from Miller *et al*., 1999)

demonstrated that *Serratia* nuclease is an unique enzyme of the endonuclease family where, the amide side-chain acts as the sole protein ligand for magnesium coordination (Miller *et al*., 1999). Though Lunin *et al.* (1997) proposed that Asp86 is involved in Mg^{2+} coordination, Miller *et al.* (1999) demonstrated that Asp86 interacts indirectly with the metal binding site by forming hydrogen bonds with the amide nitrogen of Asn119 (Fig. 1.7) and Gln120. Similarly, Meiss *et al*. (2000) showed that the metal cofactor is bound to Asp121 in addition to Asn155, which is the primary ligand for binding the cofactor.

Enzyme-substrate interactions

In Staphylococcal nuclease, binding of either Ca^{2+} or the substrate/inhibitor to the enzyme is not mutually exclusive (Cuatrecasas *et al*., 1967a,c). Cuatrecasas *et al*. (1967c) showed that Ca^{2+} does not bind, to the enzyme, in the absence of

nucleotide or substrate and hence suggested that Ca^{2+} and nucleotide bind to the enzyme in an unique complex formed only when all three components are present. Before the crystal structures of Staphylococcal nuclease were solved, Cuatrecasas *et al.* (1968) postulated that the active site of the enzyme consists of three phosphate binding "subsites" (namely, P1, P2 and P3) and the binding of oligonucleotides occurs predominantly by ionic interactions. The contributions of subsites in the binding of substrates / inhibitors to form a complex was shown to be in the order of P1 > P2 > P3. Also, the cleavage specificity of the hydrolytic site, visualized as an integral part of the P1 subsite, was shown to be influenced by the presence of nucleoside on the 5'-side rather than the 3'-side (Cuatrecasas *et al*., 1968). Weber *et al*. (1991) postulated that the binary enzyme-dTdA complex is one of the intermediate leading to the active ternary enzyme-metal-substrate complex. Based on the conformational changes between the binary and ternary complexes it was demonstrated that the metal induced changes, both at the attacked phosphorus and at the leaving group of the enzyme-bound substrate, may contribute to catalysis.

Stanczyk and Bolton (1992), using crystal structures of the ternary complexes of Staphylococcal nuclease with Ca^{2+} and pdTp, showed that the 5' phosphate of pdTp interacts with the solvent inaccessible Arg35 and Arg87 whereas, the 3' phosphate and Ca^{2+} interact with the solvent accessible ε-ammonium group of Lys84 and phenolic hydroxyl group of Tyr85. It was also observed that the interaction of a terminal 3' phosphate for exonucleolytic activity differs slightly form the interaction with an internal phosphodiester linkage required for the endonucleolytic cleavage. Subsequently, using the ternary complexes of the enzyme with pdTp, pdGp and *p*-nitrophenyl-pdTp, the above authors (Stanczyk and Bolton, 1992) demonstrated that the conformational features of the bound nucleic acid determines the differences in the observed catalysis rather than the nucleotide itself. Similar observations were also reported by Grissom and Markley (1989).

In case of *Serratia* nuclease, the minimum substrate recognized is a pentanucleotide containing phosphate at the 5' position (L'vova *et al.*, 1976).

Friedhoff *et al*. (1996b) demonstrated that the 5'-phosphorylated pentamers are cleaved predominantly at the second phosphodiester bond and to a lesser extent (5 %) at the third phosphodiester position. Additionally, at high enzyme concentrations, cleavage of the tetramer was observed at the first and second phosphodiester position with no detectable cleavage at the third phosphodiester bond. Based on the above observations, Friedhoff *et al*. (1996b) concluded that a phosphate 3' to the scissile phosphodiester bond is important for the cleavage. This conclusion supports the cleavage of the artificial substrate deoxythymidine 3',5'-bis-(*p*-nitrophenyl phosphate) by *S. marcescens* nuclease (Kolmes *et al*., 1996). Furthermore, *Serratia* nuclease is not active against methylphosphonate substituted pentanucleotides suggesting that, the negative charges and/or the hydrogen bond acceptors on the phosphate to be attacked as well as on the neighboring phosphates are required for binding and correct orientation of the substrate in the active site of the enzyme (Friedhoff *et al*., 1996b; Srivastava *et al*., 1999). The requirement of phosphate groups for the effective binding of the substrate, to the active site of the enzyme, was found to be similar for both *S. aureus* and *S. marcescens* nucleases. Moreover, cleavage experiments with polydeoxyadenylates, containing a single phosphorothioate at different positions, indicated that *Serratia* nuclease hydrolyzed R_P-diastereomer at a higher rate than S_P-diastereomer. Hence the authors opined that, the S_P orientation prevents cleavage at the phosphorothioate group and retards the cleavage at a position 5' to the substitution whereas the R_P-phosphorothioate group enhances the cleavage at the 5' position adjacent to the site of substitution (Friedhoff *et al*., 1996b). Interestingly, the role of Trp123 in substrate binding was established by kinetic studies involving mutant and wild type enzymes (Meiss *et al*., 1999). Similarly, in case of *Anabaena* nuclease, Meiss *et al*. (2000) demonstrated the involvement of Trp159 in substrate binding. Moreover, studies on the mechanism of inhibition of *Anabaena* nuclease (NucA) by its inhibitor (NuiA), using several active site mutants of NucA, suggested that, Glu163 of NucA might be participating in cofactor binding or protonation of the leaving group as the target amino acid involved in the inhibition of the enzyme. Based on these observations, it was concluded that Glu163 of the enzyme interacts with positively

charged amino acids of the inhibitor, while Arg93, Arg122 and Arg167 of the enzyme interacts with the negatively charged amino acids of the inhibitor (Meiss *et al.*, 2000).

Water assisted metal ion catalysis

The crystal structures of Staphylococcal nuclease (Arnone *et al*., 1971; Cotton *et al*., 1979; Loll and Lattman, 1989) and *Serratia* nuclease (Lunin *et al*., 1996; Miller *et al*., 1999) revealed the presence of water molecules adjacent to the metal ions. In both cases, the reaction is triggered by the water molecule which acts as the nucleophile and attacks the scissile phosphodiester bond. The reaction mechanism, for Staphylococcal nuclease, based on the crystal structure (Cotton *et al*., 1979; Loll and Lattman, 1989), kinetic studies (Anfinsen *et al*., 1971; Chaiken and Sanchez, 1972) and site directed mutagenesis (Serpersu *et al*., 1986, 1987, 1988, 1989; Hibler *et al*., 1987; Weber *et al*., 1990) is depicted in Fig. 1.8.

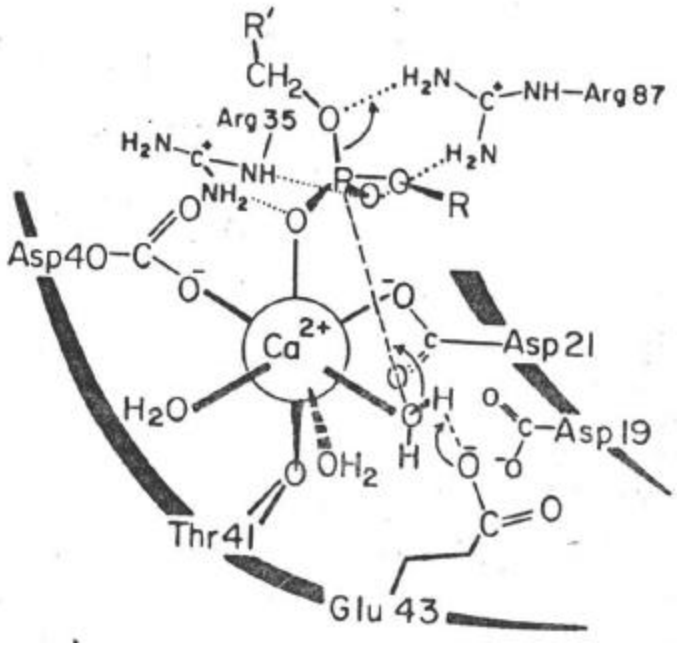

Figure 1. 8: Reaction mechanism of Staphylococcal nuclease.
(Adapted from Weber *et al*., 1991)

The reaction mechanism involves the Ca^{2+} activator bound in a septa-coordinate complex (Loll and Lattman, 1989) which are coordinated to Asp21, Asp40 and the amide carbonyl group of Thr41 as well as the 5' phosphate of the competitive inhibitor (pdTp) with the remaining three ligands probably being the water molecules. The attacking water molecule is presumed to be near the Ca^{2+}, either in direct coordination or in the second sphere of Glu43, to permit its carboxylate group to function as a general base (Hibler *et al*., 1987; Serpersu *et al*., 1989). Upon nucleophilic attack, the phosphodiester linkage of the substrate is converted in to a trigonal bipyramid intermediate stabilized by Arg87 (Serpersu *et al*., 1987; Weber *et al*., 1990), which also acts as the general acid to protonate the 5' oxygen of the leaving nucleoside.

Carboxylate groups are known to be effective nucleophilic catalysts in the intramolecular hydrolysis of phosphate esters (Steffens *et al.*, 1975). The mechanism of Staphylococcal nuclease suggested by Cotton *et al.* (1979) requires that the γ-carboxylate group of Glu43 act as a general base facilitating the attack of water on the scissile phosphodiester bond. Judice *et al*. (1993) while probing the mechanism of Staphylococcal nuclease, with unnatural amino acids, suggested that Glu43 plays a more complex structural role in catalysis rather than act as a general base. To ascertain the role of Glu43 as general base, Loll *et al*. (1995) solved the crystal structure of the ternary complex of Staphylococcal nuclease in presence of Co^{2+} as the structure is very similar to the structure obtained with Ca^{2+} with little or no distortions of the groups involved in the catalytic activity. It was observed that, Co^{2+} occupies the site identical to the Ca^{2+} binding site and both the metals show a similar interaction with the inner sphere of Glu43 indicating that, Glu43 is still fully capable of activating the water molecule to act as a general base. Additionally, it was noted that the inner sphere interaction of Ca^{2+} with Asp21 was missing in Co^{2+} structure thus establishing the fact that the loss of catalytic activity of Co^{2+} substituted nuclease is probably due to its inability to bind the inner sphere water (Loll *et al*., 1995). As mentioned earlier Glu43, in Staphylococcal nuclease, is situated at the base of the solvent-exposed and conformationally mobile omega-loop in the active site (Arnone *et al*., 1971). High resolution X-ray structures

revealed that the substitution of Glu43 by aspartic acid significantly changes the structure of the omega-loop and reduces the interaction of Ca^{2+} with its active site ligands resulting in diminished hydrogen bonded network of the water molecules (Loll and Lattman, 1990). Mehdi and Gerlt (1982) showed that the hydrolysis of substrates, by Staphylococcal nuclease, proceeds with the inversion of configuration at phosphorus aided by Ca^{2+}, which assist in proper positioning of the carboxylate group via an intervening water molecule and neutralize the phosphate ester charge by a direct ionic interaction. Similar observation was made by Potter *et al*. (1983) in case of P1 nuclease. The rate determining step for the hydrolysis of DNA/RNA, by Staphylococcal nuclease at pH 9.5, was shown to be substrate binding and product dissociation rather than cleavage of the phosphodiester bond. However, under similar conditions the E43D mutant, in which the putative active site general base catalyst Glu43 is replaced by aspartic acid, cleavage of the phosphodiester bond becomes the rate determining step (Hale *et al.*, 1993).

As mentioned earlier, *Serratia* nuclease also catalyzes the hydrolysis of phosphodiester bonds through metal ion mediated mechanism with water molecule acting as the nucleophile. Using synthetic substrates (Kolmes *et al.*, 1996), site directed mutagenesis (Friedhoff *et al*., 1994a, 1996a) and crystal structures (Miller *et al*., 1994; Miller *et al*., 1999), Friedhoff *et al*., (1999a) proposed a 'general base model' for the reaction mechanism of *Serratia* nuclease. Similar reaction mechanism has also been postulated by Meiss *et al*. (2000) for the endonuclease from *Anabaena* sp (Fig. 1.9).

In the 'general base mechanism' postulated for *Serratia* nuclease (Fig. 1.9A) His89 acts as a general base by abstracting a proton from the water molecule thereby activating it for a nucleophilic attack on the phosphorous atom adjacent to the scissile bond followed by cleavage of the 3' O-P bond (Miller *et al*., 1994; Kolmes *et al*., 1996). Magnesium ion acts as the Lewis acid to stabilize the negative charge on the penta-coordinate phosphate transition state and the leaving group. It has been postulated that Asn119 is probably involved in the binding and correct positioning of the metal cofactor. Arg57 has been shown to be involved in the transition state stabilization (Friedhoff *et al*., 1999a). Interestingly, a sequence

specific nuclease I-*Ppo*I from *Physarum polycephalum* too shows an identical active site and has been suggested to have a similar mechanism (Friedhoff *et al*., 1999a,b). *Anabaena* nuclease too follows a similar reaction mechanism where His124 acts as the general base, Asn155 in binding and correct positioning of the cofactor and Glu163 in protonation of the leaving group (Fig. 1.9B). In contrast to Arg57 in *Serratia* nuclease, the transition state stabilization in *Anabaena* nuclease is effected by Asp95 either by hydrogen bonding or by binding to a second metal ion (Meiss *et al*., 2000).

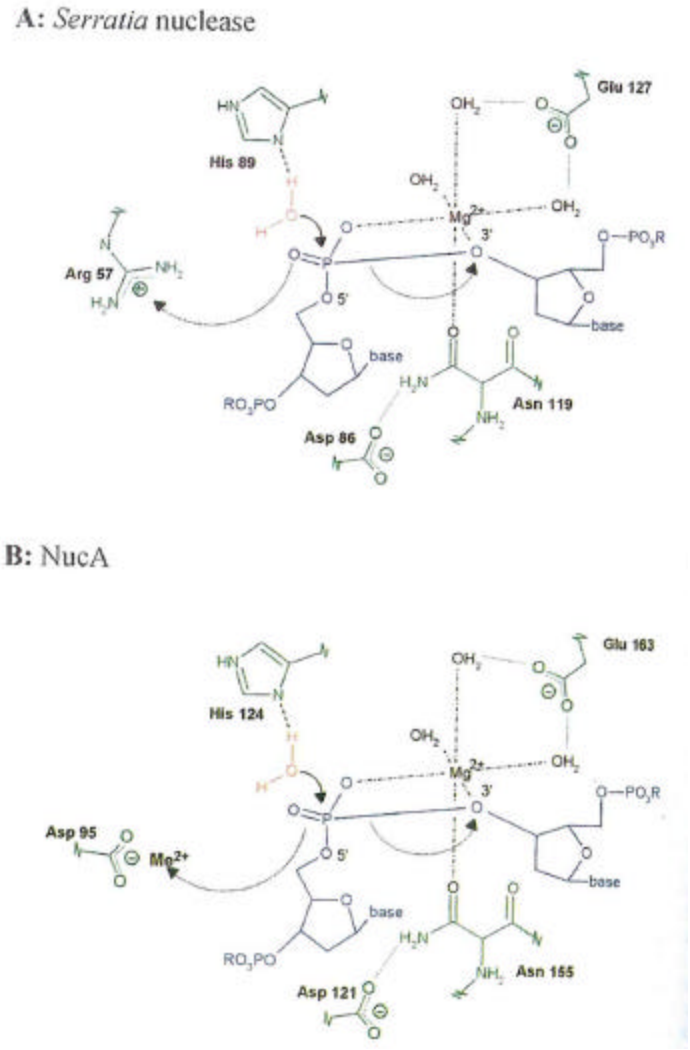

Fig. 1.9: Reaction mechanism of *Serratia* (A) and *Anabaena* (B) nucleases. (Adapted from Meiss *et al*., 2000)

Additionally, Miller *et al*. (1999) postulated two schemes for the hydrolysis of phosphodiester bonds by *Serratia* nuclease where, an unligated water molecule may be directly activated by His89 or the magnesium bound water is activated by His89 wherein the metal ion may alter the pKa of the bound water to produce a more nucleophilic hydroxide ligand and this activated water molecule may mediate the cleavage of the phosphodiester bond. In the former, the cluster polarizes the phosphate atom, stabilizes the phosphorane and helps protonate the leaving group (Fig. 1.10a) whereas in the latter, the cluster polarizes the phosphate atom, stabilizes the phosphorane and helps activate the attacking hydroxide (Fig. 1.10b).

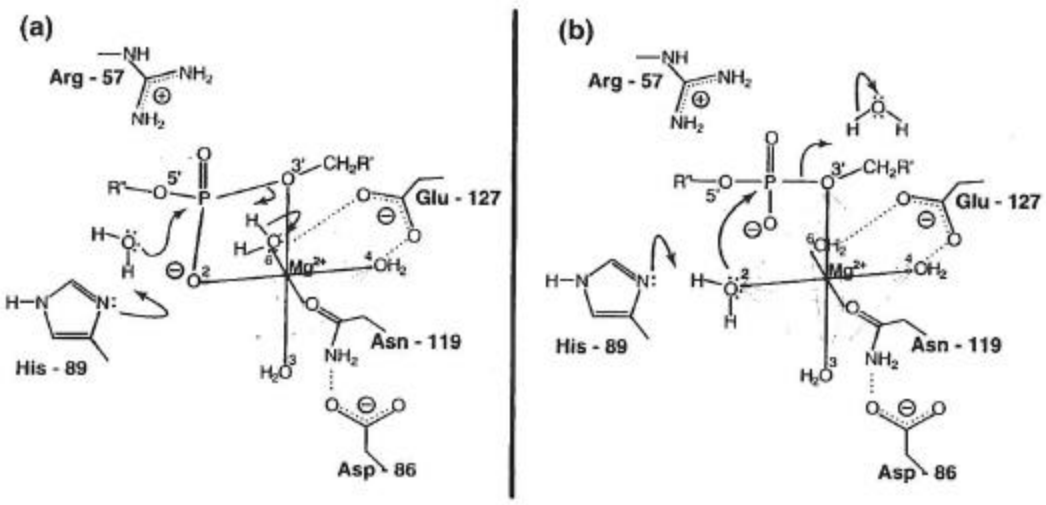

Fig. 1.10: Mechanistic schemes for nucleotide cleavage by *Serratia* nuclease. (Adapted from Miller *et al.*, 1999)

However, in the 'general acid model' of Lunin *et al.* (1997) (Fig. 1.11) Glu127 acts as the general base and abstracts a proton from the attacking water opposite the O'3-atom. The subsequent attack on phosphorus with inversion of its configuration gives rise to a penta-covalent transition state. The penta-coordinated phosphorous is then stabilized by Arg87, Arg131, Asp86 and Mg^{2+} bound to the scissile phosphate group. His89 functions as a general acid by protonating the leaving O3' (Lunin *et al*., 1997). In case of *Serratia* nuclease, the rate determining step for the hydrolysis of oligonucleotides is substrate binding and / or

phosphodiester bond cleavage but not product dissociation (Friedhoff *et al.*, 1996b).

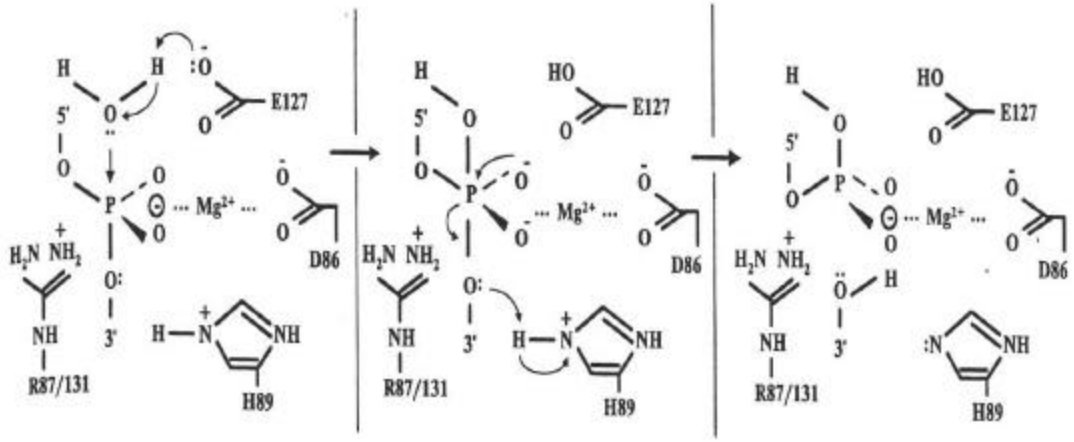

Fig. 1.11: **A general mechanism of *Serratia* nuclease-catalyzed cleavage of phosphodiester bonds** (adapted from Lunin *et al.*, 1997).

The reaction mechanisms of Staphylococcal and *Serratia* nucleases suggest that the hydrolysis of the phosphodiester bond is very much dependent on the proper orientation of the residues so as to initiate an inline attack of water molecule on the scissile phosphodiester bond. Also, the proper orientation is achieved by the concerted action of Asn21, Asn40 and Ca^{2+} in case of Staphylococcal nuclease (Cotton *et al*., 1979; Loll and Lattman, 1989) and Asn119 and Mg^{2+} in *Serratia* nuclease (Miller *et al*., 1994, 1999). It is interesting to note that, both the enzymes could perform the dual role of proper orientation and transition state stabilization of the intermediate or the leaving group efficiently by a single metal atom whereas P1 nuclease from *Penicillium citrinum*, a zinc metalloprotein, requires three metal ions for performing a similar role (Romier *et al*., 1998) (Fig. 1.12). Accordingly, the scissile phosphate of the substrate binds close to ZnII with its free oxygens replacing the two water molecules and the base, 5' to the bond to be cleaved, stacks against Phe61 and forms hydrogen bonds with Asp63. The water molecule bridging ZnI and ZnIII which, like in other binuclear metallohydrolases is presumably present as a hydroxide ion due to lowering of its pKa by the metal ions,

acts as the nucleophile attacking the phosphate in-line with P-O3' bond (Wilcox, 1996). Asp45, which also serves as a ligand of ZnI, helps to properly orient the hydroxide for the attack. Arg48 stabilizes the resulting penta-coordinate transition state and the attacking hydroxide ion along with the leaving oxyanion (O3') occupy apical positions in this transition state. ZnII plays a crucial role in activating the phosphate and stabilizing the leaving O3'-oxyanion (Fig. 1.12). Thus all the three zinc ions are important for catalysis (Romier *et al.*, 1998).

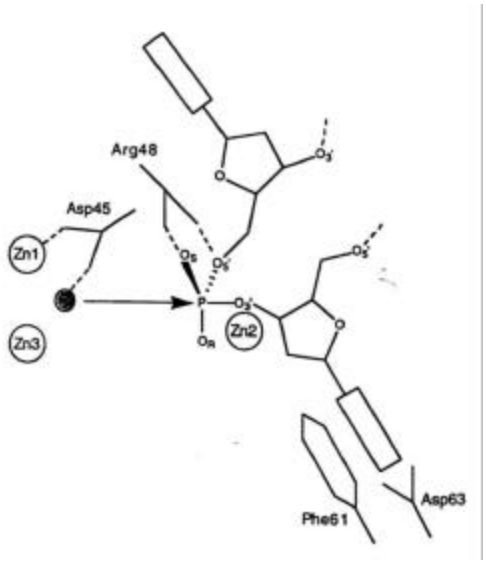

Fig. 1.12: Catalytic mechanism of P1 nuclease. (Adapted from Romier *et al.*, 1998).

Chemical Nucleases

In biological systems, RNases and DNases accomplish the scission of the phosphodiester backbone of RNA and DNA by catalyzing nucleophilic attack on the scissile bond. On the other hand, chemical nucleases are metal-ligand complexes that nick nucleic acids under physiological conditions either by oxidative attack on the ribose or deoxyribose moiety (Sigman *et al*., 1979) or by nucleophilic attack on the phosphodiester linkage (Komiyama *et al.*, 1993). Recently, efforts to develop effective artificial nucleic acid hydrolyzing agents have resulted in new systems based on metal ion complexes capable of polarizing the P=O bond and activating a coordinated water molecule to allow nucleophilic attack.

The non-enzymatic hydrolysis of DNA, under physiological conditions, have been accomplished by the use of lanthanide metal ions and their complexes (Matsumoto *et al*., 1992; Komiyama *et al*., 1993; Shiiba *et al*., 1993). Irisawa and Komiyama (1995) showed that, phosphodiester linkages in DNA and RNA can be efficiently hydrolyzed through a concerted action of La^{3+} with Fe^{3+} or Sn^{3+}. Additionally, the metal ions in combination are capable of hydrolyzing thymidylyl(3'-5')thymidine and adenyly(3'-5')adenosine. Furthermore, Cu^{2+} and Co^{2+} complexes could efficiently hydrolyze nucleic acids (Hegg *et al*., 1998; Krämer, 1999). Recently, Sissi *et al.* (2000) showed that Cu^{2+} or Zn^{2+} complexes of 1,3,5-triamino cyclohexane derivatives could convert supercoiled DNA (Form I) to linear duplex DNA (Form III) via nicked circular DNA (Form II) and proposed that with Zn^{2+}, which lacks the important redox chemistry, pure hydrolytic mechanism of the ester bond cleavage occurs. The Ce^{4+} complexes of DNA oligomers have been used as artificial restriction enzymes to hydrolyze DNA at the target site (Komiyama *et al*., 1994a,b; Komiyama, 1995; Hall *et al*., 1996). Recently, Igawa *et al.* (2000) demonstrated that Ce^{4+} can hydrolyze the phosphodiester linkages of oligonucleotides at a rate similar to that of a dinucleotide. Moreover at low Ce^{4+} concentrations, single-stranded oligonucleotides were hydrolyzed faster than dsDNA while, at high Ce^{4+} concentrations, this difference was not observed (Igawa *et al*., 2000). Chemical nucleases have been used for the preparation of site-specific nucleases, also known as targeted nucleases, wherein a specific ligand will target the cleavage site and the chemical nuclease will cleave the nucleic acid (Sigman *et al*., 1993). The ligands include DNA binding drugs, oligonucleotides (Chen and Sigman, 1986,1988), proteins (Chen and Sigman, 1987; Bruice *et al*., 1991) and peptides (Sluka *et al.*, 1987).

Biological role

As mentioned earlier, nucleases play an important role in four R's, i.e. replication, recombination, restriction and repair. Nucleases have been shown to have a role in the formation of nicks during meiotic recombination. The reduced amounts of endo-exonuclease in *rad52* mutants of *S. cerevisiae* was correlated to

its probable involvement in repair and recombination in both mitotic and meiotic cells (Chow and Resnick, 1987). The ability of *N. crassa* endo-exonuclease to act on supercoiled DNA and its ability to initiate single-strand and double-strand breaks in DNA along with the processive exonucleolytic action on circular DNA in the presence of ssDNA-binding proteins led Chow and Fraser (1983) to suggest that it might be involved in the production of stable lengths of ssDNA needed for exchanges during recombination and recombinational repair of mitochondrial DNA. In the case of *N. crassa*, compared to the wild strain, the repair deficient and UV-sensitive mutants, viz. uvs-2, uvs-3, uvs-6 and nuh-4, could not secrete endo-exonucleases. In addition, these mutants had a higher level of endo-exonuclease precursor than the wild type, indicating that these mutants may have some defect either in the protease(s) that control the nuclease level or in the regulation of protease(s). The above mutants were also sensitive to various mutagens and mitomycin C and exhibited high frequency of spontaneous, recessive lethal mutations and deletions, indicating the involvement of *N. crassa* nuclease in repair (Fraser, 1979). Moreover it has been suggested that, *N. crassa* mitochondrial endonuclease may have a role in replication (Linn and Lehman, 1966). Siwecka *et al.* (1989) postulated that the interaction between rye germ nuclease I and ribosomes to be a part of the regulatory mechanism of cell metabolism. Owing to the striking correlation between genetic instability and presence of poly(dG).poly(dC) tracts in DNA (preferred substrate of endonuclease G), Ruiz-Carrillo and Renaud (1987) suggested a probable role for endonuclease G in recombination. Pollen has been used as a promising host for introducing DNA into higher plants (Hess, 1975, 1987). The pollen nucleases from *P. hybrida* (van der Westhuizen *et al*., 1987) and tobacco (Matousek and Tupy, 1984) prefer ssDNA thereby enhancing the chances of native DNA uptake and their expression.

Recently, Nicieza *et al.* (1999) isolated two extracellular nucleases from *S. antibioticus*, with Mr of 18 and 34 kDa, which are nutritionally regulated and reach their maximum activity during aerial mycelium formation and sporulation. Their role appeared to be DNA degradation in the substrate mycelium and supply of building blocks for macromolecular biosynthesis in the aerial mycelium and they

acted in concert with the periplasmic nuclease. Of the two extracellular nucleases, the 18kDa nuclease appeared to be reminiscent of NUC-18, a thymocyte nuclease assumed to have a key role in glucocorticoid stimulated apoptosis (Compton and Cidlowski, 1987; Gaido and Cidlowski, 1991). Interestingly, the N-terminal sequence of the 18kDa protein showed striking similarity to proteins of the cyclophilin family which degrade DNA in a Ca^{2+} / Mg^{2+} dependent manner and their role in apoptosis has been reviewed (Montague *et al*., 1994, 1997). The mitogenic factor of *S. pyogenes* has been implicated in pathogenesis similar to other pyrogenic exotoxins but the role of the nuclease activity exhibited by the mitogenic factor is still not clear (Iwasaki *et al.*, 1997).

DNA / RNA non-specific nucleases from *S. aureus* and *S. epidermidis* are found in a variety of clinical and food infections (Sachs *et al*., 1978; Gudding, 1980a, b; Sundaram *et al.*, 1982; Stersky *et al*., 1986; Nunez *et al*., 1988). Similarly, nucleases from *Vibrio cholerae* (Shiebel *et al*., 1989) and *S. marcescens* (Hejazi and Falkiner, 1997) have been postulated to play a role during invasion or establishment of an infection. Matousek *et al.* (1996) showed that pollen RNases, owing to their ability to degrade dsRNA may function as, defense proteins against viral infection as components of a degradation complex which participates in the apoptosis of tapetal cell layer and for nucleoside re-utilization by the developing pollen.

The main role of the associated nucleotidase activity of nucleases is to scavenge nucleotides and phosphates for growth and survival of the organism under environmentally stressed conditions. Brown and Ho (1987) opined that barley nuclease, in concert with other acid phosphatases, might be involved in the hydrolysis of remnant nucleic acid in the endosperm for providing the valuable nutrients for heterotrophic embryonic growth. A similar role has been suggested for potato tubers nuclease (Nomura *et al.*, 1971).

Applications

Since their discovery, non-specific endonucleases, owing to their ability to recognize single-stranded nucleic acids and a wide variety of structures as well as

structural variations, have been extensively used as analytical tools for the determination of nucleic acid structure. The *N. crassa* nuclease was employed for the isolation of pure Lac operon (Shapiro *et al*., 1969), isolation of tRNA and rRNA gene hybrids (Marks and Spencer, 1970; Joseph and Stafford; 1976) and detection of sequence heterology (Bartok *et al*., 1974). Staphylococcal nuclease has been used to monitor hybridization reaction with DNA (Kacian and Spiegelman, 1974) and as a probe for drug binding sites on DNA (Fox and Waring, 1987). The ability of *S. aureus* and *N. crassa* nucleases to discriminate between uridine and cytidine, under defined conditions, have been exploited for rapid RNA sequencing (Krupp and Gross, 1979). Wheat chloroplast nuclease, due to its preference for pyrimidines in non-base paired regions, has been used as a tool for the structural analysis of RNA (Przykorska *et al*., 1989). The ssDNase activity of BAL 31 nuclease was used for probing regions of altered secondary structures in negatively and positively supercoiled closed circular DNA (Lau and Gray, 1979). Additionally, the dsDNase activity of BAL 31 nuclease which progressively shortens the duplex DNA, from both ends, was exploited for ordering restriction endonuclease generated DNA fragments (Legerski *et al*., 1978). *Serratia* nuclease, under the tradename of 'Benzonase', is used to eliminate nucleic acid contamination from purified recombinant proteins and single cell protein preparations. The ability of *Serratia* nuclease to degrade DNA has paved way for its utilization as a killer gene for the self-destruction of microorganisms released into the environment in addition to its use as an anti-viral agent (Benedik and Strych, 1998).

Present Investigation

Nucleic acids, the vital biomolecules present in cells, act as carriers of genetic information from one generation to another. In order that the genetic information is effectively transferred to the next generation, they have to undergo processes such as replication, recombination and repair. All living organism contain a set of enzymes, namely nucleases, which interact with nucleic acids and hydrolyze the phosphodiester linkages. Among them, conformation non-specific endo and exonucleases by their ability to recognize different DNA structures have been used as analytical tools for the determination of nucleic acid structure. Though non-specific endonucleases are ubiquitous in distribution, the enzymes of analytical interest originate mainly from fungal sources. However, majority of the analytically important non-specific endonucleases have an acid optimum pH and / or show an obligate requirement of metal ions for its activity which inturn prevents their use in chelating buffers or in presence of metal chelators. Hence, there is a need to look for a different type of nuclease which has a pH optimum at or around neutrality and does not require metal ion for its activity. Screening of several fungal cultures revealed that a strain of *Rhizopus stolonifer* produced high activity of extracellular nuclease when grown on YPG (yeast extract - peptone - glucose) medium. The crude enzyme showed higher activity on sonicated and heat denatured DNA than on native DNA, at pH 7.0. Hence the present investigation was carried out to purify and extensively characterize this extracellular nuclease to understand its structure-function relationship and potential applications.

CHAPTER - 2

PURIFICATION AND CHARACTERIZATION OF THE DEOXYRIBONUCLEASE ACTIVITY

SUMMARY

An extracellular nuclease from *Rhizopus stolonifer* (designated as nuclease Rsn) was purified to homogeneity by chromatography on DEAE-cellulose followed by Blue Sepharose. The Mr of the purified enzyme determined by native PAGE was 67,000 and it is a tetramer and each protomer consists of two unidentical subunits of Mr 21,000 and 13,000. It is an acidic protein with a pI of 4.2 and is not a glycoprotein. The purified enzyme showed an obligate requirement of divalent cations like Mg^{2+}, Mn^{2+} and Co^{2+} for its activity but is not a metalloprotein. The optimum pH of the enzyme, for DNA hydrolysis, was 7.0 and was not influenced by the type of metal ion used. Although, the optimum temperature of the enzyme for single stranded (ss) DNA hydrolysis in presence of all three metal ions and for double stranded (ds) DNA hydrolysis in presence of Mg^{2+} was 40 °C, it showed higher optimum temperature (45 °C) for dsDNA hydrolysis in presence of Mn^{2+} and Co^{2+}. Nuclease Rsn was inhibited by divalent cations like Zn^{2+}, Cu^{2+} and Hg^{2+}, inorganic phosphate and pyrophosphate, low concentrations of SDS, guanidine hydrochloride and urea, organic solvents like dimethyl sulphoxide, dimethyl formamide and formamide but not by 3' or 5'-mononucleotides. The studies on mode and mechanism of action showed that nuclease Rsn is an endonuclease and cleaves dsDNA through a single hit mechanism. The end products of both ssDNA and dsDNA hydrolysis were predominantly tri and tetranucleotides ending in 3'-hydroxyl and 5'-phosphoryl termini. Moreover, the type of metal ion used did not influence the mode and mechanism of action of the enzyme. Time course of 3' and 5' base analysis of the hydrolytic products of DNA suggested that nuclease Rsn is a non-specific endonuclease.

INTRODUCTION

Nucleases, especially single-strand-specific nucleases, are important analytical enzymes and have found extensive application as analytical tools for the determination of nucleic acid structure. They have also been implicated in recombination, repair (Shishido and Ando, 1985) and replication (Brown *et al.*,

1985). Though they are widely distributed, the enzymes of analytical interest originate mainly from fungal sources. However, only few enzymes such as S1 nuclease from *Aspergillus oryzae*, P1 nuclease from *Penicillium citrinum*, BAL 31 nuclease from *Alteromonas espejiana* and nucleases from *Neurospora crassa* and *Ustilago maydis* have been sufficiently characterized (Gite and Shankar, 1995). Majority of analytically important nucleases have an acid optimum pH and show an obligate requirement of metal ions for their activity, which in turn prevents their use in chelating buffers or in presence of metal chelators. Hence, there is a need to look for a different type of nuclease, which has a pH optimum at or around 7.0 and does not require metal ions for activity. Screening of several fungal cultures revealed that a strain of *Rhizopus stolonifer* (NCIM 880) produces high activity of an extracellular nuclease when grown on YPG medium. The crude enzyme showed higher activity on sonicated and heat denatured DNA than on native DNA, at pH 7.0 (Apte *et al.*, 1993). The purification and extensive characterization of this single strand preferential enzyme is described in this Chapter.

MATERIALS

DEAE-cellulose (DE-52) (Whatman Paper Ltd., Maidstone, UK); Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden); Pharmalytes, bovine serum albumin (BSA), SDS molecular weight markers, sodium bis(*p*-nitrophenyl)phosphate, 3' and 5'-mononucleotides, dithiothreitol (DTT), β-mercaptoethanol, SDS, urea, guanidine hydrochloride, Blue Sepharose, snake venom phosphodiesterase (SVPD) and spleen phosphodiesterase (SPD) (Sigma, St. Louis, MO, USA); calf intestinal alkaline phosphate and pUC 18 DNA (Bangalore Genei, Bangalore, India) and *p*-nitrophenyl phosphate (Loba-Chemie Pvt. Ltd., Mumbai, India) were used. All other chemicals used were of analytical grade. Commercial yeast RNA (Sisco Research Laboratories, Mumbai, India) was purified by ethanol precipitation.

METHODS

DNA isolation

High Mr DNA from buffalo liver was isolated according to the method of Mehra and Ranjekar (1979) with some modifications. Unless otherwise stated, all operations were carried out at 4 - 8 °C. Fresh buffalo liver, obtained from local slaughter house, was stored frozen in liquid nitrogen. Subsequently, 500 g of liver was chopped in to small pieces and suspended in minimum volume of 30 mM Tris-maleate buffer, pH 6.0 (containing 500 mM sucrose, 3 mM $CaCl_2$ and 0.1 % v/v Triton X-100) and homogenized in a blender. The homogenate was filtered through two layers of muslin cloth and the filtrate was centrifuged (9000 g, 20 min). The crude nuclear pellet obtained after centrifugation was washed 2-3 times with the homogenization buffer (till the supernatant was colourless) and then with saline-EDTA (150 mM NaCl, 100 mM EDTA, pH 8.0). The washed pellet was suspended in minimum volume of saline-EDTA, mixed with sodium dodecylsulphate to a final concentration of 2 % (w/v) and incubated at 60 °C for 30 min to facilitate the lysis of nuclei. The lysate was then brought to room temperature and 5 M sodium perchlorate was added to a final concentration of 1 M. The mixture was incubated under agitation for 30 min and then deproteinized using a mixture of chloroform and isoamyl alcohol (24 : 1). Subsequently, the mixture was centrifuged (9000 g, 10 min) and from the aqueous layer, DNA was precipitated with 2 volumes of chilled absolute ethanol. The precipitated DNA was collected by spooling with a glass rod, dried free of ethanol and dissolved in minimum volume of 300 mM sodium acetate buffer, pH 5.5. From the DNA preparation, RNA contamination was removed by incubation with RNase Rs from *Rhizopus stolonifer* (5-6 U/ml) at 37 °C for 1 h. After RNase treatment, the mixture was again deproteinized and the DNA was precipitated as described above. The precipitate was collected with a glass rod, dried free of ethanol, dissolved in sterile distilled water and stored at -20 °C.

The UV absorption of DNA was determined in the range of 220-320 nm and only those preparations with A_{300} less than 0.1 and ratios of A_{230}:A_{260} and A_{280}:A_{260} corresponding to 0.45 and 0.55 respectively, were used. High Mr nature of the

DNA preparation was also checked electrophoretically using 1 % (w/v) agarose gels.

Enzyme assays

Determination of DNase activity: This was carried out according to Apte *et al.* (1993). The standard reaction mixture of 1 ml contained 50 µg of native or sonicated and heat denatured buffalo liver DNA, in 30 mM Tris-HCl buffer pH 7.0, containing 2 mM Mg^{2+} and appropriately diluted enzyme. The reaction was initiated by the addition of DNA followed by incubation at 37 °C for 15 min. The reaction was then terminated by the addition of 1 ml of 10 % (v/v) chilled perchloric acid and 1 ml of 0.2 % BSA. The mixture was left on ice for 10 min and then centrifuged (2500 g, 15 min) to sediment the precipitate. The acid soluble deoxyribonucleotides in the supernatant were measured at 260 nm. The amount of acid soluble deoxyribonucleotides liberated were determined by assuming a molar absorption coefficient of 10,000 $M^{-1}cm^{-1}$ (Curtis *et al.*, 1966). One unit of DNase activity is defined as the amount of enzyme required to liberate 1 µmol of acid soluble nucleotides/min under the assay conditions.

Determination of RNase activity: This was done as described by Chacko *et al.* (1996). The total reaction mixture of 0.75 ml contained 1.25 mg RNA in 200 mM Tris-HCl buffer pH 7.0 (containing 2 mM Mg^{2+}) 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 (2500 g, 15 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 nucleotides liberated was determined by assuming a molar absorption coefficient of 10,600 $M^{-1}cm^{-1}$ for the ribonucleotides mixture (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.

Determination of phosphodiesterase activity: This was performed essentially according to Tomoyeda *et al.* (1969). The total reaction mixture of 3 ml contained 0.2 % (w/v) bis-*p*-nitrophenyl phosphate in 100 mM Tris-HCl buffer, pH 7.0, containing 2 mM Mg^{2+} and appropriately diluted enzyme. The reaction was initiated by the addition of the enzyme followed by incubation at 37 °C for 30 min. The reaction was then terminated by the addition of 1 ml of 10 % (w/v) trichloroacetic acid and the precipitate, if any, was removed by centrifugation (2500 g, 10 min). Subsequently, 1 ml of the supernatant was mixed with 0.5 ml of 1 N NaOH and made upto 4.5 ml with distilled water. The *p*-nitrophenol liberated was measured at 420 nm. One unit of phosphodiesterase activity is defined as the amount of enzyme required to liberate 1 µmol of *p*-nitrophenol / min under the assay conditions.

Determination of phosphomonoesterase activity: This was carried out according to Gite *et al.* (1992a). The total reaction mixture of 2 ml contained 1 mM of either 3'AMP or 5'AMP in 30 mM Tris-HCl buffer, pH 7.0, containing 2 mM Mg^{2+} and appropriately diluted enzyme. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 2 ml of freshly prepared Chen's reagent, made up of 1 volume of 12 M H_2SO_4, 2 volumes of distilled water, 1 volume of 2.5 % (w/v) ammonium molybdate and 1 volume of 10 % (w/v) ascorbic acid (Chen *et al.*, 1956). The blue color developed after 30 min incubation at 37 °C was read at 660 nm. One unit of phosphomonoesterase activity is defined as the amount of enzyme required to liberate 1 µmol of inorganic phosphate/min under the assay conditions.

Determination of phosphatase activity: The total reaction mixture of 3 ml contained 1ml of 0.2 % (w/v) sodium *p*-nitrophenyl phosphate in 30 mM Tris-HCl buffer, pH 7.0, containing 2 mM Mg^{2+} and appropriately diluted enzyme. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of 1 ml

of 10 % (w/v) trichloroacetic acid. Subsequently, 1 ml of the reaction mixture was mixed with 0.5 ml of 1N NaOH and made upto 4.5 ml with distilled water. The amount of *p*-nitrophenol liberated was measured at 420 nm. One unit of phosphatase activity is defined as the amount of enzyme required to liberate 1µmol of *p*-nitrophenol / min under the assay conditions

Protein determination

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

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^{2+} 12 mM, Mn^{2+} and Fe^{2+} 2 ppm each) with a seven day old well sporulated slant followed by incubation at 30 °C for 120 h, with shaking. The extracellular broth was collected by decantation and used as the source of enzyme.

Purification of nuclease Rsn

Unless otherwise stated, all operations were carried out at 7 ± 1 °C. During enzyme purification steps, DNase activity was monitored using sonicated and heat denatured DNA in presence of 2 mM Mg^{2+}.

***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 20 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 above buffer till the flow through fractions showed no DNase activity. Subsequently, the bound enzyme was eluted with a linear gradient of NaCl (0 - 500 mM) in 30 mM

potassium phosphate buffer pH 7.0. Fractions of 3 ml were collected at a flow rate of 15 ml/h and those having specific activity more than 200 U/mg were pooled, dialyzed extensively against 30 mM potassium phosphate buffer pH 7.0 and used for the next step.

***Chromatography on Blue Sepharose*:** The enzyme obtained from the above step was adsorbed onto a Blue Sepharose column (1 x 5 cm), pre-equilibrated with 30 mM potassium phosphate buffer, pH 7.0 (containing 2 mM Mg^{2+}), at a flow rate of 5 ml/h . The column was washed with the same buffer till the flow through fractions showed no DNase activity. The bound enzyme was then eluted with a linear gradient of NaCl (0 - 1 M) in the above buffer. Fractions of 1 ml were collected at a flow rate of 5 ml/h and those having specific activity above 10,000 U/mg were pooled, dialyzed extensively against 30 mM potassium phosphate buffer pH 7.0, concentrated by ultra filtration and stored at -20 °C.

Electrophoresis

Native and SDS-PAGE were carried out, at pH 8.3, according to Laemmli (1970) and the gels silver stained according to Blum *et al.* (1987). The pI of the enzyme was determined by density gradient single column isoelectricfocussing (Chinnathambi *et al.*, 1995) over the pH range 3.0 - 10.0.

Electroblotting and glycoprotein staining

Electroblotting of nuclease Rsn onto nitrocellulose membrane was carried out according to Towbin *et al.* (1979). The purified enzyme (25 µg) was electrophoresed at pH 8.3 on 10 % (w/v) non-denaturing polyacrylamide gel according to Laemmli (1970). After electrophoresis, the gel and the nitrocellulose membrane were sandwiched between 3 MM Whatman paper and placed in the blotting cassette. The tank was filled with Tris-glycine buffer (25 mM Tris and 192 mM glycine) pH 8.3 and the electrotransfer was carried out under a constant current of 50 mA for 3 h at 6 °C. Subsequently, the nitrocellulose membrane was subjected to periodic acid - schiff staining according to Doerner and White (1990).

Immediately after the electrotransfer, the blot was kept in a fixer (3 % v/v acetic acid) for 1h. It was then washed briefly with water and treated with periodic acid (1.0 % w/v in 3 % v/v acetic acid) for 30 min in dark. After the incubation period, the excess periodic acid was removed by washing with water (6 - 7 changes) and the blot was stained with schiff's reagent (consisting of 1 % w/v basic fuchsin, in 0.25 N HCl, containing 4 % w/v sodium metabisulfite) in dark for 30 min. The excess stain was then removed by washing the blot with 0.5 % (w/v) sodium metabisulfite solution followed by water.

Mr determination

The relative molecular mass of the enzyme was determined by native PAGE on varying concentrations (8, 10, 12, 14 and 16 % w/v) of acrylamide, as described by Hedrick and Smith (1968), using bovine serum albumin (Mr 66,000), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 29,000), soybean trypsin inhibitor (Mr 20,100) and α-lactalbumin (Mr 14,200) as reference proteins. Mr was also determined on 12 % (w/v) SDS-polyacrylamide gel (pH 8.3) according to Laemmli (1970) with bovine serum albumin (Mr 66,000), ovalbumin (Mr 45,000), carbonic anhydrase (Mr 29,000), soybean trypsin inhibitor (Mr 20,100) and α-lactalbumin (Mr 14,200) as reference proteins.

Amino acid analysis

Salt-free lyophilized enzyme sample (85 µg) was hydrolyzed in gas phase 6N HCl for 20 h at 110 °C. Following hydrolysis, the sample was again lyophilized, dissolved in 100 µl of sample loading buffer (0.4 M sodium borate, pH 10.2) diluted 1:2 and 1 µl of the sample was subjected to analysis on a Hewlett-Packard Amino Quant with a diode array detector. Primary amino acids were detected as orthophthalaldehyde derivatives.

Mechanism of action of nuclease Rsn

This was determined viscometrically, using an Ubbelohde viscometer, according to Melgar and Goldthwait (1968). The total reaction mixture of 15 ml,

in 30 mM Tris-HCl buffer pH 7.0, contained 15 U of nuclease Rsn and 5 mg of dsDNA. The measurements were made at 37 °C, in presence of either 2mM Mg^{2+}, Mn^{2+} or Co^{2+}. The specific viscosity values (η_{sp}) obtained at different time intervals, during the enzymatic degradation, were plotted according to Bernardi and Sadron (1964) as log[((1-R)/R) * 100] against log *t* where R = η_{spt} / η_{spt_o} and *t* = time. The slope of the plot was used to determine the 'n' values.

Action of nuclease Rsn on plasmid DNA

The total reaction mixture of 50 µl contained 5 µg of pUC-18 supercoiled plasmid DNA, in 50 mM Tris-HCl buffer pH 7.0 containing 2 mM of either Mg^{2+}, Mn^{2+} or Co^{2+}, was incubated with 0.0125 U of nuclease Rsn at 37 °C. Aliquots of 10 µl were withdrawn at various time intervals and the reaction was terminated by the addition of 2 µl of the loading dye (30 % v/v glycerol, 0.25 % w/v Bromophenol Blue and 12 mM EDTA). Subsequently, the samples were subjected to electrophoresis on 1.2 % (w/v) agarose gels, at pH 8.0 (40 mM Tris-acetate buffer containing 1 mM EDTA) and the products were visualized by staining with ethidium bromide.

HPLC analysis of the reaction products of nuclease Rsn

***Action on DNA*:** The total reaction mixture of 5 ml containing 2.5 mg of either sonicated and heat denatured DNA or native DNA, in 30 mM Tris-HCl buffer pH 7.0 (containing 2 mM of either Mg^{2+}, Mn^{2+} or Co^{2+}), was incubated with 5 U of purified enzyme at 37 °C. Subsequently, 1 U of the enzyme was added at an interval of 1 h upto 3 h and incubated for 24 h. Aliquots (1 ml) were removed after 24 h and the reaction was terminated by the addition of 2 ml of chilled absolute ethanol. The mixture was left overnight at -20 °C and the undigested DNA was removed by centrifugation (6000g, 15 min). The supernatant was lyophilized, reconstituted in 100 µl of Milli Q water and subjected to HPLC.

***Determination of phosphoryl termini of the reaction products*:** The hydrolytic products of DNA, obtained following the action of nuclease Rsn, were lyophilized,

reconstituted in 100 µl of 50 mM Tris-HCl buffer pH 7.5 and incubated with either 0.05 U of snake venom phosphodiesterase or 0.1 U of spleen phosphodiesterase for 6 h at 37 °C. After the incubation period, the reaction was terminated by the addition of two volumes of chilled absolute ethanol. The samples were concentrated by lyophilization, reconstituted in Milli Q water and subjected to HPLC.

***Separation of the reaction products*:** High Performance Liquid Chromatography (Perkin Elmer Model fitted with series 410 LC pump) was carried out on a Nucleosil C18 column (120 x 4 mm, 3 µm, Macherey-Nagel, Germany). The mobile phase comprising of a linear gradient of acetonitrile, in 100 mM triethylammonium acetate pH 6.2 (0 - 15 % v/v for 20 min followed by 15 - 100 % v/v for 5 min), was used at 25 ± 1 °C and at a flow rate of 1.0 ml/min. Twenty µl of the standard or the sample solution was injected onto the column and the nucleotides were detected, at 255 nm, using a Perkin Elmer Model LC235 Diode Array Detector. The amount occupied by each peak was computed on the basis of total area occupied by each peak of the standard and sample. The nucleotides eluted in the order of 5'dCMP, 5'dTMP, 5'dGMP and 5'dAMP with retention times of ca. 4.40, 10.37, 12.53 and 15.22, respectively (data not shown).

Terminal base analysis

The total reaction mixture of 4 ml, containing 4 mg of ssDNA or dsDNA in 30 mM Tris-HCl buffer pH 7.0 (containing 2 mM Mg^{2+}), was incubated with 40 U of nuclease Rsn at 37 °C. Aliquots of 500 µl were withdrawn at different time intervals and the reaction was arrested by heat treatment at 75 °C for 15 min. DNA samples incubated in the absence of nuclease Rsn served as control. The control and nuclease Rsn treated samples were then incubated overnight with 0.2 U of calf intestinal alkaline phosphatase, in 1 ml (total volume) of calf intestinal alkaline phosphatase buffer (10 mM Tris-HCl buffer, pH 7.9, containing 50 mM NaCl, 1 mM DTT and 10 mM Mg^{2+}), at 37 °C. After the incubation period, phosphatase was removed by extraction with an equal volume of chloroform : isoamyl alcohol

(24 : 1 v/v). The aqueous layer was collected and subjected to 5' and 3' terminal nucleoside analysis. 5' deoxyribonucleotides (5'dCMP, 5'dTMP, 5'dGMP and 5'dAMP) treated with calf intestinal phosphatase served as nucleoside standards.

***3' termini*:** An aliquot (500 µl) of the sample, obtained after treatment with calf intestinal alkaline phosphatase, was incubated overnight with 0.06 U of spleen phosphodiesterase at 37 °C. The 3' terminal nucleosides obtained, on treatment with spleen phosphodiesterase, were then separated by HPLC (Waters model fitted with 515 HPLC pump) on a Symmetry C18 column (250 x 4.6 mm, 5 µm, Waters, USA). The mobile phase comprising of a discontinuous gradient of acetonitrile in 100 mM triethylammonium acetate pH 6.2 (0 % v/v for 3 min, 0 to 5 % for 5 min and continued at 5 % v/v for 5 min, 5 to 10 % v/v for 5 min and continued at 10 % v/v for 5 min, 10 to 20 % v/v for 7 min followed by 20 to 100 % v/v for 5 min), was used at 25 ± 1°C and at a flow rate of 0.8 ml/min. Twenty µl of the standard or the sample solution was injected onto the column and the nucleosides were detected, at 255 nm, using Waters 2487 Dual λ Absorbance Detector. The amount occupied by each peak was computed on the basis of total area occupied by each peak of the standard and sample. The nucleosides eluted in the order of dC, dG, dT and dA with retention times of ca. 11.14, 14.86, 16.05 and 19.03 min, respectively (data not shown).

***5' termini*:** An aliquot (500 µl) of the digested sample, obtained after treatment with calf intestinal alkaline phosphate, was incubated overnight with snake venom phosphodiesterase (0.06 U) at 37 °C. The nucleosides liberated from the 5' terminal were then analyzed by HPLC as described above.

Determination of the size of the hydrolytic products of DNA

***Action on DNA*:** The total reaction mixture of 1 ml containing 500 µg of either sonicated and heat denatured DNA or native DNA, in 30 mM Tris-HCl buffer pH 7.0 (containing 2 mM of either Mg^{2+}, Mn^{2+} or Co^{2+}), was incubated with 5 U of purified nuclease Rsn at 37 °C for 1h. Subsequently, 5 U of the enzyme was added

at an interval of 1 h upto 3 h and incubated for 24 h. Samples were then subjected to DEAE-cellulose chromatography.

***Separation of the oligonucleotide fragments*:** The oligonucleotides obtained from the above step were resolved according to Tomlinson and Tener (1963). An aliquot (1 ml) of the nuclease Rsn digest of ssDNA or dsDNA was loaded onto a DEAE-cellulose (chloride) column (1.5 x 10 cm) and washed in with 2 ml of water. The oligonucleotides were then eluted with a linear gradient of NaCl (0 - 1 M) in 2.5 mM Tris-HCl buffer, pH 7.8, containing 7 M urea. Fractions of 4 ml were collected and the absorbance read at 260 nm. Fractions were pooled, diluted with 3 to 5 volumes of water, adjusted to pH 8.0 with 1 M Tris-HCl buffer pH 8.0 and loaded onto a DEAE-cellulose (carbonate) column (1.5 x 5 cm). The column was then washed extensively with 0.02 M ammonium carbonate (pH 8.5) and the oligonucleotides were eluted with 2 M ammonium carbonate (pH 8.5). The fractions were pooled and concentrated to dryness in vacuo. To remove the ammonium carbonate, the residue was reconstituted in Milli Q water and lyophilized to dryness. This process was repeated 2-3 times to ensure complete removal of ammonium carbonate.

***Determination of the fragment length*:** The lyophilized samples, obtained from the above step, were then reconstituted in 500 µl of calf intestinal alkaline phosphatase buffer (10 mM Tris-HCl buffer, pH 7.8, containing 50 mM NaCl, 1 mM DTT and 10 mM Mg^{2+}). The amount of terminal phosphate and total phosphate were then determined by incubating the samples (100 µl each) with 0.05 U of calf intestinal alkaline phosphatase alone and together with 0.05 U of snake venom phosphodiesterase respectively, at 37 °C for 24 h, followed by estimating the inorganic phosphate (Chen *et al.* 1956). The ratio of total phosphate to terminal phosphate was determined to assign the fragment length of the oligonucleotides.

RESULTS AND DISCUSSION

Purification of nuclease Rsn

Rhizopus stolonifer produces an extracellular nuclease and an extracellular RNase in a ratio of 1:60. In the present studies, DEAE-cellulose chromatography carried out at pH 7.0 completely separated the nuclease from RNase. The subsequent affinity chromatography on Blue Sepharose yielded a homogeneous enzyme preparation. Interestingly, metal ions (Mg^{2+}, Mn^{2+} or Co^{2+}) were essential during the affinity step because the enzyme failed to bind to the matrix in their absence. The results of a typical procedure for the purification of nuclease Rsn to homogeneity are summarized in Table 2.1.

The enzyme was purified approximately 1625 fold, with an overall yield of 47 %. The purified enzyme moved as a single band in native PAGE indicating its homogeneity (Fig. 2.1). However, on SDS-PAGE, it gave two bands suggesting that it is a multimeric protein made up of unidentical subunits. The purified enzyme could be stored in 30 mM potassium phosphate buffer pH 7.0, at -20 °C, for more than two months without any apparent loss of its activity. However, repeated freezing and thawing lead to considerable loss of activity.

Table 2.1: Purification of nuclease Rsn

Step	Total Activity* (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	Recovery (%)
Crude	17330	514.12	33.71	1.00	100.00
DEAE-cellulose (pH 7.0)	9770	30.65	318.76	9.50	56.37
Blue-Sepharose (pH 7.0, in presence of 2 mM Mg^{2+})	8214	0.15	54760.00	1624.44	47.39

* Based on ssDNase activity.

Fig. 2.1: Electrophoresis of purified nuclease Rsn. 10 % (w/v) polyacrylamide gel, Tris-glycine buffer pH 8.3, current 2 mA/well, protein loaded 8 µg.

The Mr of the enzyme determined by native PAGE, on varying concentrations of acrylamide, was 67,000 (Fig. 2.2A). However, on SDS-PAGE it gave two bands of Mr 21,000 and 13,000 respectively (Fig. 2.2B), suggesting that nuclease Rsn is a tetramer made up of dimer of heterodimers. The Mr based on amino acid composition (Table 2.2) was 68,400 which is in agreement with the data obtained by native PAGE. Amino acid composition showed the absence of cysteine, a property similar to nucleases from *Staphylococcus aureus* (Taniuchi et al., 1967) and *D. melanogaster* (Shuai et al., 1992). The Mr of nuclease Rsn is comparable to nucleases from *N. crassa* mitochondria (Chow and Fraser, 1983) and *U. maydis* nuclease β (Rusche *et al.,* 1980). In contrast to enzymes from *N. crassa* mitochondria (Chow and Fraser, 1983) and mouse mitochondria (Tomkinson and Linn, 1986) which are dimers consisting of two identical subunits, nuclease Rsn is made up of two protomers with each protomer consisting of two non-identical monomers.

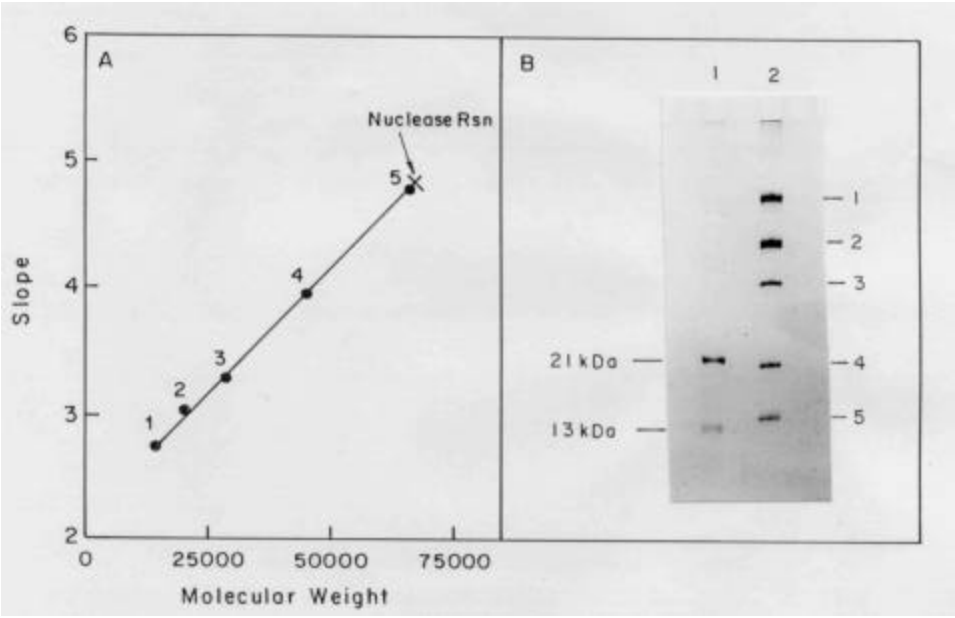

Fig. 2.2: Mr determination of nuclease Rsn.

A. **Native PAGE:** (1) α-lactalbumin (Mr 14,200) (2) soybean trypsin inhibitor (Mr 20,100) (3) carbonic anhydrase (Mr 29,000) (4) ovalbumin (Mr 45,000) and (5) bovine serum albumin (Mr 66,000).

B. **SDS-PAGE:** Lane 1, purified nuclease Rsn (10 μg). Lane 2, molecular weight markers (1) bovine serum albumin (2) ovalbumin (3) carbonic anhydrase (4) soybean trypsin inhibitor and (5) α-lactalbumin.

For details refer to Methods.

Table 2.2: Amino acid composition of nuclease Rsn

Amino acid	No. of residues / mol
Aspartic acid and Asparagine	63
Glutamic acid and Glutamine	97
Serine	130
Histidine	16
Glycine	33
Threonine	35
Alanine	68
Arginine	15
Tyrosine	10
Valine	31
Methionine	6
Phenyl alanine	16
Isoleucine	13
Leucine	26
Lysine	21
Proline	14
Tryptophan	8[a]
Cysteine	0[b]
Total	602

Determined spectrophotometrically according to:

a Spande and Witkop (1967)

b Cavillani et al. (1966)

Nuclease Rsn is an acidic protein with a pI of 4.2 ± 0.2 (Fig. 2.3). All the activities viz. ssDNase, dsDNase and RNase, focussed at same pH indicating that they are associated with the same protein.

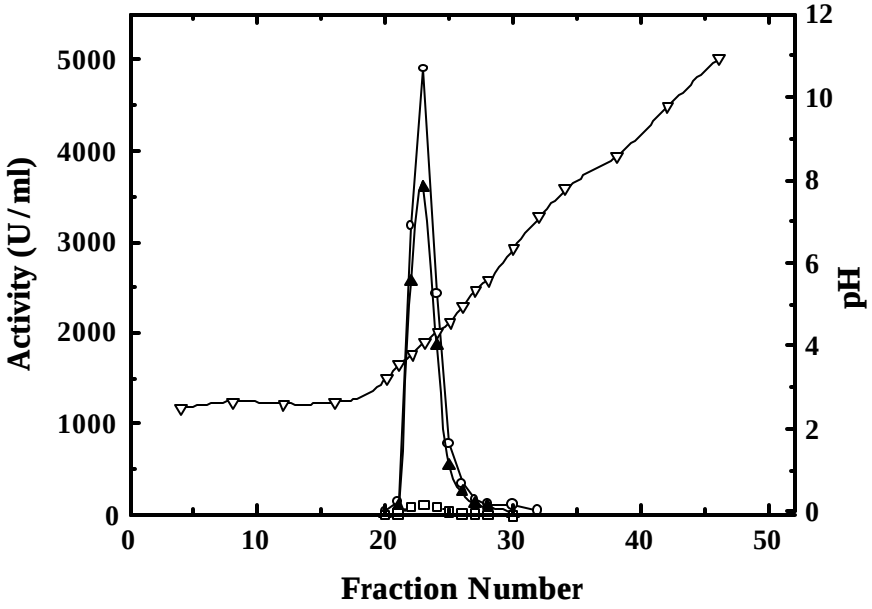

Fig. 2.3: Isoelectric focussing of nuclease Rsn. Nuclease Rsn (1000 U) was subjected to isoelectric focussing and the fractions were assayed for ssDNase, dsDNase and RNase activities. ssDNase (o), dsDNase ( ), RNase ( ) and pH (∇).

Unlike some of the well studied single-strand-specific extracellular fungal nucleases like S1 (Oleson and Sasakuma, 1980), P1 (Fujimoto *et al*., 1975) and *Syncephalastrum racemosum* (Chen *et al.*, 1993), nuclease Rsn is not a glycoprotein.

Metal ion requirement

Influence of metal ions on the activity of the enzyme revealed that, like majority of nucleases (Gite and Shankar, 1995), nuclease Rsn showed an obligate requirement of divalent cations like Mg^{2+}, Mn^{2+} and Co^{2+} and optimal activity was observed in presence of 2 mM of the aforementioned metal ions (Fig. 2.4). Other divalent cations like Zn^{2+}, Ca^{2+} and Hg^{2+} were not effective (Table 2.3).

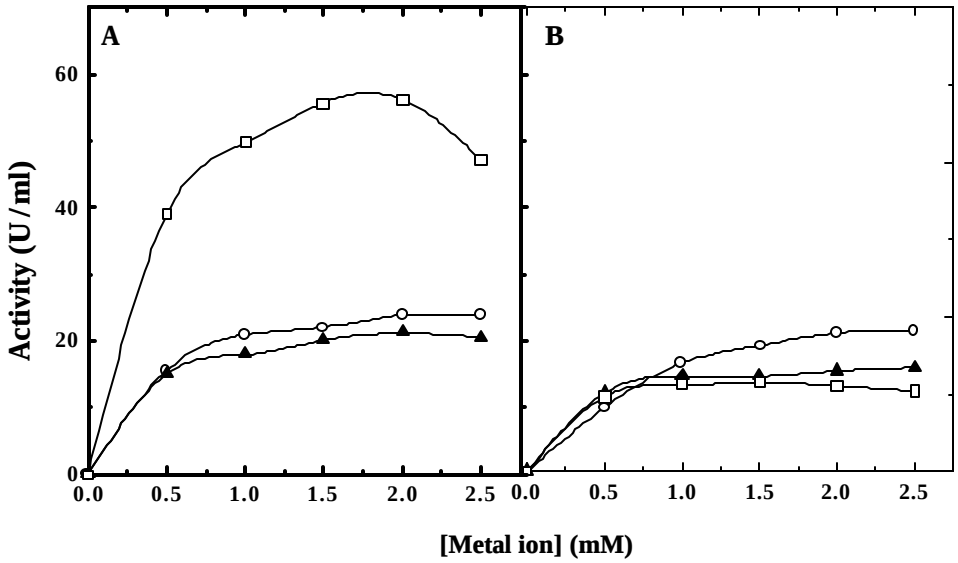

Fig. 2.4: Effect of metal ion concentration on the DNase activity of nuclease Rsn. Purified nuclease Rsn (1 U of corresponding activity) was assayed for ssDNase (A) and dsDNase (B) activities in presence of different concentrations of Mg^{2+} (O), Mn^{2+} (σ) and Co^{2+} (ÿ) as described under Methods.

The metal ion requirement of nuclease Rsn is similar to that of nucleases from *N. crassa* mitochondria (Chow and Fraser, 1983), *Sacharomyces cerevisiae* mitochondria (Dake *et al*., 1988), SM1 and SM2 from *Serratia marcescens kums* 3958 (Yonemura *et al*., 1983), *S. marcescens* (Nestle and Roberts, 1969a) and pancreatic DNase (Moore, 1981). Unlike *N. crassa* mitochondria nuclease and pancreatic DNase, no synergism was observed in presence of Mg^{2+}, Mn^{2+} and Co^{2+}. While Mg^{2+} and Mn^{2+} did not affect the ssDNase:dsDNase ratio, the enzyme showed higher preference for ssDNA (4:1) in presence of Co^{2+}. Though there was no synergism in presence of either of the above metal ions, higher ssDNase:dsDNase ratio was observed when Co^{2+} was used in combination with either Mg^{2+} or Mn^{2+} (Table 2.3). This observation suggests that Co^{2+} increases the preference of the enzyme towards ssDNA. Extensive dialysis of the enzyme against 30 mM potassium phosphate buffer pH 7.0, containing 10 mM (effective concentration) of EDTA, followed by assay in presence of 2 mM of the

aforementioned metal ions restored over 80 % of its initial activity suggesting that nuclease Rsn is not a metalloprotein. In this respect, it differs from well characterized fungal enzymes like S1, P1, *Physarum polycephalum* and *N. crassa* (mycelia) nucleases (Gite and Shankar, 1995).

Table 2.3: Effect of metal ions on the DNase activity of nuclease Rsn[#]

Metal ions (2 mM)	Activtiy on ssDNA (U/ml)	Activity on dsDNA (U/ml)	ssDNA:dsDNA
Mg^{2+}	20	12	1.66
Mn^{2+}	21	12	1.75
Co^{2+}	45	10	4.50
Ca^{2+}	0	0	
Zn^{2+}	0	0	
Cu^{2+}	0	0	
Hg^{2+}	0	0	
$Mg^{2+} + Mn^{2+*}$	20	14	1.43
$Mg^{2+} + Co^{2+*}$	37	14	2.64
$Mn^{2+} + Co^{2+*}$	28	10	2.80
$Mg^{2+} + Ca^{2+}$	23	17	1.35
$Mg^{2+} + Zn^{2+}$	0	0	
$Mg^{2+} + Cu^{2+}$	0	0	
$Mg^{2+} + Hg^{2+}$	0	0	

[#] Assays were carried out at pH 7.0 and 37 °C with sonicated and heat denatured DNA and native DNA as substrates, in presence of metal ions indicated.

* When metal ions were used in combination, the effective concentration of individual metal ions was 1 mM, so as to have a total effective concentration of 2 mM.

Optimum pH, temperature and stability

The optimum pH of purified nuclease Rsn was 7.0 for the hydrolysis of both ssDNA and dsDNA and it was not influenced by the type of metal ion used (Fig. 2.5) as observed with *U. maydis* nuclease γ (Yarnal *et al*., 1984). However, nuclease Rsn differs from *Alteromonas* BAL 31 nuclease (Gray *et al.*, 1981) and *N. crassa* mitochondria nuclease (Chow and Fraser, 1983) which exhibited different pH optima for the hydrolysis of ssDNA (pH 8.8 and pH 6.5-7.5) and dsDNA (pH 8.0 and pH 5.5 - 6.5), respectively. The pH optima of nuclease Rsn is comparable to nucleases from *Schizophyllum commune* (Martin *et al.*, 1986), sheep kidney (Watanabe and Kasai, 1978), spinach leaves (Strickland *et al*., 1991), *S. cerevisiae* mitochondria (Dake *et al.*, 1988) and pancreatic DNase (Moore, 1981).

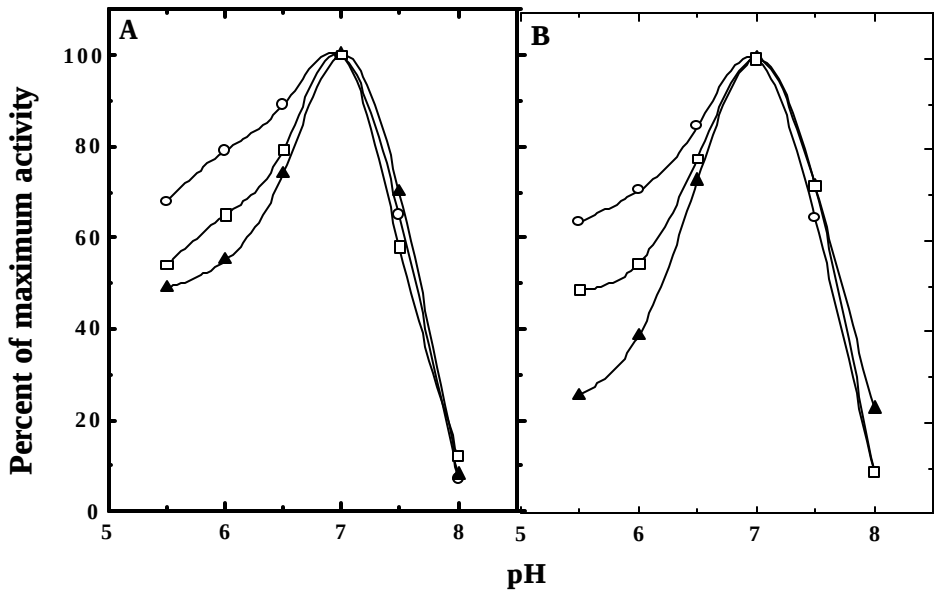

Fig. 2.5: Optimum pH of the DNase activity of nuclease Rsn. Nuclease Rsn (1 U of corresponding activity) was assayed for ssDNase (A) and dsDNase (B) activities in a series of pH (5.0 - 8.0), at 37 °C, in presence of 2mM each of Mg^{2+} (○), Mn^{2+} (σ) and Co^{2+} (ÿ) as described under Methods.

The optimum temperature of the enzyme was 40 °C for ssDNA hydrolysis and it was not influenced by the type of metal ion used (Fig. 2.6A). However, metal ions influenced the optimum temperature for the hydrolysis of dsDNA.

While in presence of Mg^{2+} the optimum temperature was 40 °C, the enzyme exhibited an optimum temperature of 45 °C in presence of Mn^{2+} and Co^{2+} (Fig. 2.6B). In this respect, nuclease Rsn differs from most of the well studied single-strand-specific nucleases. The optimum temperature of nuclease Rsn is higher compared to *S. cerevisiae* mitochondrial nuclease which exhibits an optimum temperature of 35 °C for its ss and dsDNase activities (Dake *et al.*, 1988).

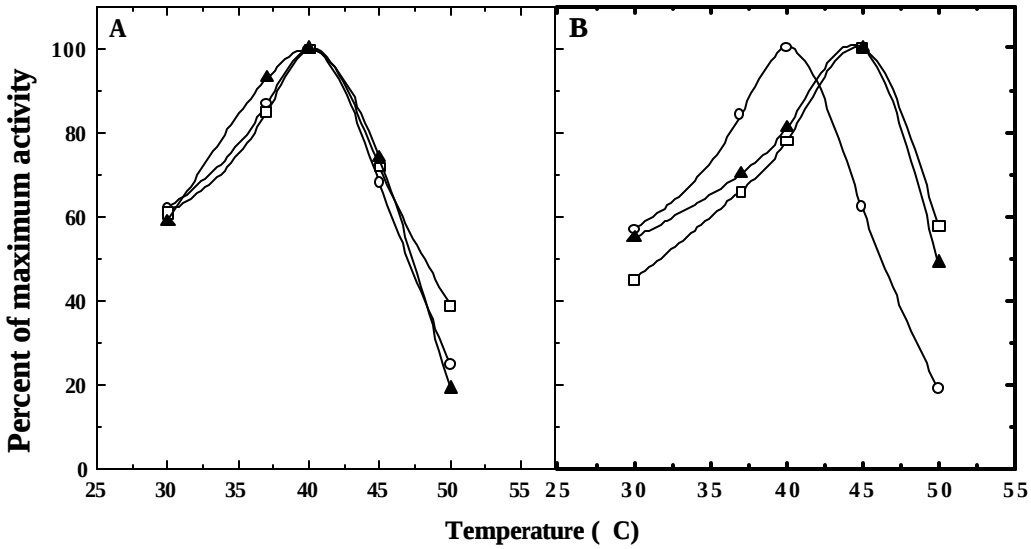

Fig. 2.6: Optimum temperature of the DNase activity of nuclease Rsn. Nuclease Rsn (1 U of corresponding activity) was assayed for ssDNase (A) and dsDNase (B) activities in a series of temperatures (30 - 50 °C), at pH 7.0, in presence of 2 mM each of Mg^{2+} (○), Mn^{2+} (σ) and Co^{2+} (ÿ) as described under Methods.

Unlike well characterized extracellular fungal enzymes like S1 (Shishido and Habuka, 1986) and P1 nuclease (Fujimoto *et al*., 1975), SM1 and SM2 from *S. marcescens kums* 3958 (Yonemura *et al.*, 1983) and *S. marcescens* (Nestle and Roberts, 1969a), nuclease Rsn showed inferior stability and under the assay conditions (pH 7.0 and 37 °C) lost more than 50 % of its initial activity in 60 min (Fig. 2.7).

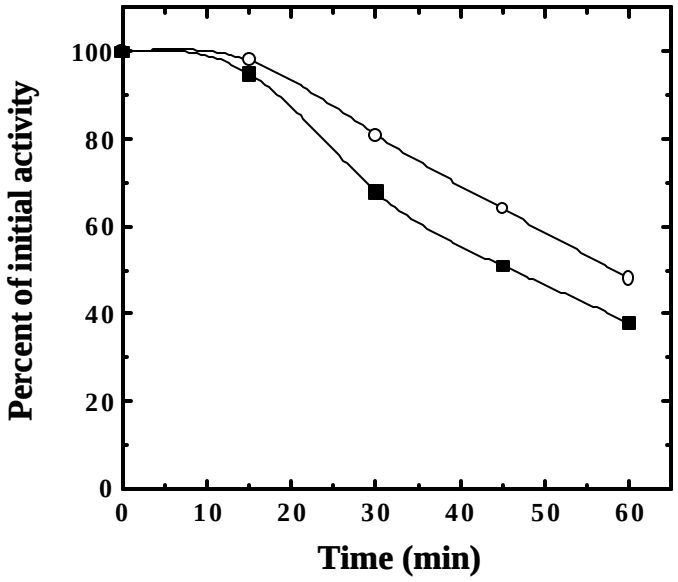

Fig. 2.7: Stability of the DNase activity of nuclease Rsn. Purified nuclease Rsn (100 U of corresponding activity) was incubated, at 37 °C and pH 7.0, for 1 h. Aliquots were removed at different time intervals and assayed for ssDNase (○) and dsDNase (▽) activities as described under Methods.

Effect of salt concentration

Salt concentration in the reaction mixture affects the activity of single-strand-specific nucleases (Gite and Shankar, 1995). Influence of salt concentration on the activity of nuclease Rsn showed that, increase in salt concentration brought about a progressive decrease in the enzyme activity and at 200 mM of either NaCl or KCl, the enzyme exhibited approximately 15 - 20 % of its maximum activity (Fig. 2.8 A, B). Similar observations were also made in case of mouse mitochondrial nuclease (Tomkinson and Linn, 1986) and nuclease from *Streptomyces glaucescens* (Aparicio *et al.*, 1992). In case of *N. crassa* nuclease, 100 to 200 mM NaCl completely inhibited the dsDNase activity whereas it had only marginal effect on the ssDNase activity (Fraser. 1980). While 150 mM NaCl inhibited the dsDNase activity of *S. cerevisiae* mitochondrial nuclease, it had no effect on the ssDNase activity (Dake *et al.*, 1988). Low concentration of NaCl

(30 mM) brought about 50 % inhibition of the dsDNase activity of *Drosophila melanogaster* nuclease whereas, it required 100 mM NaCl to bring about the same level of inhibition of the ssDNase activity (Shuai *et al*., 1992). This differential level of inhibition of ssDNase and dsDNase activity, in presence of salt, was not observed in case of nuclease Rsn.

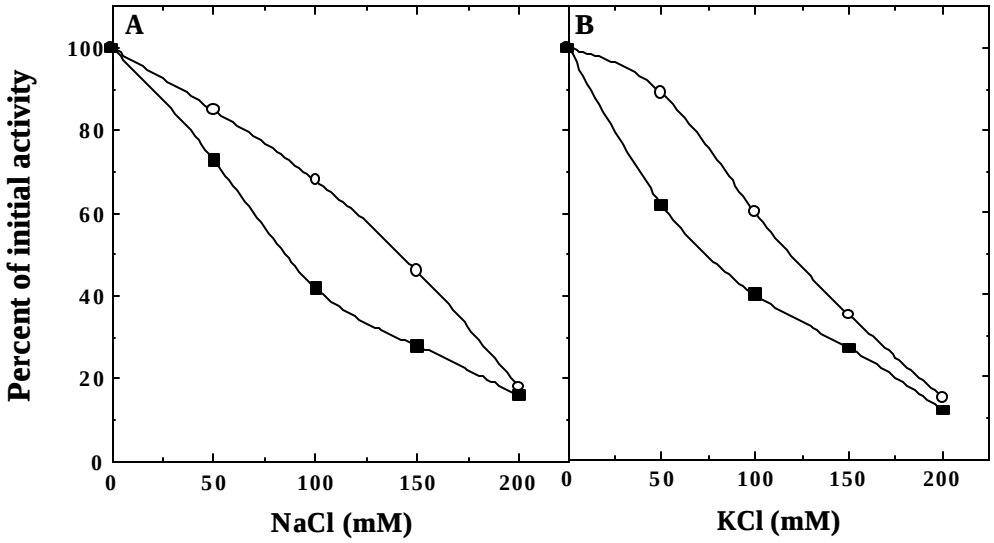

Fig. 2.8: Effect of salt concentration on the DNase activity of nuclease Rsn. Purified nuclease Rsn (1 U of corresponding activity) was assayed for ssDNase (○) and dsDNase (▽) activities in presence Mg^{2+} (2 mM) and varying concentrations of NaCl (A) and KCl (B), at 37 °C and pH 7.0, as described under Methods.

Stability to denaturants and organic solvents

The purified enzyme showed decreased stability against protein denaturants and lost significant amount of its initial activity (> 80 %) when assayed in presence of low concentrations of SDS (0.02 % w/v) and guanidine hydrochloride (200 mM). However, it showed comparatively higher stability in presence of 1M urea and retained more than 60 % of its initial activity (Table 2.4). The stability of nuclease Rsn to denaturants is inferior to enzymes like S1 nuclease (Vogt, 1973), BAL 31 nuclease (Gray *et al*., 1981) and barley nuclease (Yupsanis and Georgatsos, 1983).

Table 2.4: Effect of protein denaturants on the DNase activity of nuclease Rsn*

Denaturants	Residual activity (%)	
	ssDNase	dsDNase
Control[#]	100	100
SDS		
0.01 %	26	11
0.02 %	16	6
Urea		
1 M	96	65
2 M	61	23
3 M	20	9
Guanidine hydrochloride		
0.05 M	100	68
0.10 M	82	43
0.20 M	18	9

* Activity was determined under standard assay conditions i.e. at pH 7.0 and 37 °C, using both sonicated and heat denatured DNA and native DNA as substrates in presence of 2 mM Mg^{2+}.

[#] Enzyme assayed in the absence of denaturants served as control.

In case of endonucleases from *S. antibioticus* (Cal *et al*., 1995) and *S. glaucescens* (Aparicio *et al.*, 1992), organic solvents like dimethyl sulphoxide stimulated the enzyme activity. However, all the organic solvents tested inhibited nuclease Rsn to a varying extent with maximum inhibition occurring with formamide (Table 2.5).

Table 2.5: Effect of organic solvents on the DNase activity of nuclease Rsn*

Organic solvents	Concentration (%)	Residual activity (%)	
		ssDNase	dsDNase
Control[#]	-	100	100
Formamide	10	97	67
	20	36	12
	30	9	4
Ethylene glycol	10	91	76
	20	72	72
	30	65	49
	40	50	14
Dimethyl formamide	10	100	100
	20	85	70
	30	84	34
	40	60	24
Dimethyl sulphoxide	10	100	100
	20	68	57
	30	62	18
	40	59	0

* Activity was determined under standard assay conditions ie. at pH 7.0 and 37 °C, using both sonicated and heat denatured DNA and native DNA as substrates in presence of 2 mM Mg^{2+}.

[#] Enzyme assayed in the absence of organic solvents served as control.

The stability of nuclease Rsn towards organic solvents is inferior to that of S1 nuclease (Hutton and Wetmur, 1975). It was also noted that, while the inhibitory effect of ethylene glycol, dimethyl formamide and dimethyl sulphoxide was pronounced on the dsDNase activity, this differential inhibition was not

observed with formamide. Interestingly, in presence of high concentration of dimethyl sulphoxide (40 % v/v) the dsDNase activity of the enzyme was completely abolished but the enzyme retained approximately 60 % of its ssDNase activity (Table 2.5). This observation is of importance as the enzyme can be used as a single-strand-specific nuclease to study DNA structure in presence of dimethyl sulphoxide.

Inhibitors

In the present studies, divalent cations like Zn^{2+}, Cu^{2+} and Hg^{2+}, inhibited nuclease Rsn when assayed in presence of optimum concentration (2 mM) of Mg^{2+} (Table 2.3, Page 89). Mononucleotides, especially 5'-mononucleotides are potent inhibitors of some of the well studied enzymes like S1 nuclease (Oleson and Hoganson, 1981), *S. glaucescens* nuclease (Aparicio *et al*., 1992) and the nuclease from *S. aureus* (Cuatrecasas *et al.*, 1967b). Additionally, guanosine 5' nucleotides are strong inhibitors of nuclease Bh1 from *Basidiobolus haptosporus* (Desai and Shankar, 2000). In contrast, nuclease β from *U. maydis* was inhibited by 3' mononucleotides (Rusche *et al.*, 1980). However, nuclease Rsn did not show any inhibition when assayed in presence of 200 μM of 3' and 5' ribonucleotides or deoxyribonucleotides (Table 2.6).

Nuclease Rsn retained approximately 80 % and 30 % of its ssDNase and dsDNase activity respectively, in presence of 5 mM inorganic phosphate whereas, pyrophosphate at this concentration (5 mM) significantly inhibited both the activities (Table 2.7). Similar effects were observed with nucleases SM1 and SM2 from *S. marcescens kums* 3958 (Yonemura *et al*., 1983). The sensitivity of nuclease Rsn towards inorganic phosphate is lower compared to S1 nuclease, which showed approximately 50 % of its ssDNase activity in presence of either 2 mM phosphate or 20 μM of pyrophosphate (Shishido and Ando, 1985). In contrast, the dsDNase activity of nuclease Rsn showed higher sensitivity towards inorganic phosphate than *S. glaucescens* nuclease, which required 10 mM phosphate to bring about 62 % inhibition of its dsDNase activity (Aparicio *et al.*, 1992).

Table 2.6: Effect of mononucleotides on the DNase activity of nuclease Rsn*

Nucleotides (200 µM)	Residual activity (%)	
	ssDNase	dsDNase
Control[#]	100	100
5'mononucleotides		
dAMP	96	100
dGMP	90	90
dCMP	87	99
TMP	85	100
AMP	100	100
GMP	100	88
CMP	100	100
UMP	96	90
3'mononucleotides		
dAMP	78	73
dGMP	73	77
dCMP	90	96
TMP	77	75
AMP	100	100
GMP	99	99
CMP	89	96
UMP	87	98

* Activity was determined under standard assay conditions i.e. at pH 7.0 and 37 °C, using both sonicated and heat denatured DNA and native DNA as substrates in presence of 2 mM Mg^{2+}.

[#] Enzyme assay carried out in the absence of mononucleotides served as control.

Table 2.7: Effect of phosphate and pyrophosphate on the DNase activity of nuclease Rsn*

Phosphates	Concentration (mM)	Residual activity (%) ssDNase	Residual activity (%) dsDNase
Control#	-	100	100
Inorganic phosphate	1	94	90
	5	81	28
	10	45	5
Pyrophosphate	1	70	30
	5	20	-

* Activity was determined under standard assay conditions i.e. at pH 7.0 and 37 °C, using both sonicated and heat denatured DNA and native DNA as substrates in presence of 2 mM Mg^{2+}.

Enzyme assayed the absence of phosphates served as control.

Substrate specificity

Nuclease Rsn showed high activity on ssDNA and dsDNA but very low activity on RNA. Bis(p-nitrophenyl)phosphate was resistant to hydrolysis suggesting that nuclease Rsn is a not a typical phosphodiesterase. The inability of the enzyme to dephosphorylate 3' and 5' mononucleotides point towards the absence of phosphomonoesterase activity. Additionally, *p*-nitrophenyl phosphate was not hydrolyzed by nuclease Rsn indicating that the enzyme is devoid of any phosphatase activity (Table 2.8). The higher ssDNase:dsDNase ratio, observed in presence of all the three metal ions (Table 2.3 Page 89), shows that nuclease Rsn is a single strand preferential enzyme.

Table 2.8: Substrate specificity of nuclease Rsn

Substrate	Value* (%)
Polymeric substrates	
ssDNA	100
dsDNA	50
RNA	5
Monomeric substrates	
3'AMP	0
5'AMP	0
bis(*p*-nitrophenyl)phosphate	0
p-nitrophenyl phosphate	0

* The ratio of activities were determined based on the initial rates of hydrolysis of different substrates carried out at 37 °C, pH 7.0, in presence of 2 mM Mg^{2+}. For details refer to Methods.

Mechanism and mode of action

Viscometric studies have been employed to determine the mechanism of action of DNases (Bernardi and Cordonnier, 1965). The 'n' value, obtained from the plot of log of function of change in viscosity against log time, gives an indication of the mechanism of action. An 'n' value of approximately 1 indicates a 'single hit' mechanism i.e. cleavage of strands opposite to each other resulting in complete scission of the molecule (Bernardi and Sadron, 1964) whereas, 'n' value between 1 and 2 suggests a 'double hit' mechanism i.e. scission of the molecule will not occur until the nicks are opposite to each other (Shumaker *et al.*, 1956). Viscometric studies on the cleavage of dsDNA by nuclease Rsn, in presence of Mg^{2+}, Mn^{2+} and Co^{2+}, gave an 'n' value of 1.22, 1.03 and 1.04 respectively, showing that the cleavage of dsDNA occurs through a single hit mechanism (Fig.

2.9). Similar observations have been made in case of pancreatic DNase I, acid DNase II and *Escherichia coli* endonuclease (Bernardi and Cordonnier, 1965). However unlike DNase I, the type of metal ion used did not alter the mechanism of action of the enzyme (Melgar and Goldthwait, 1968).

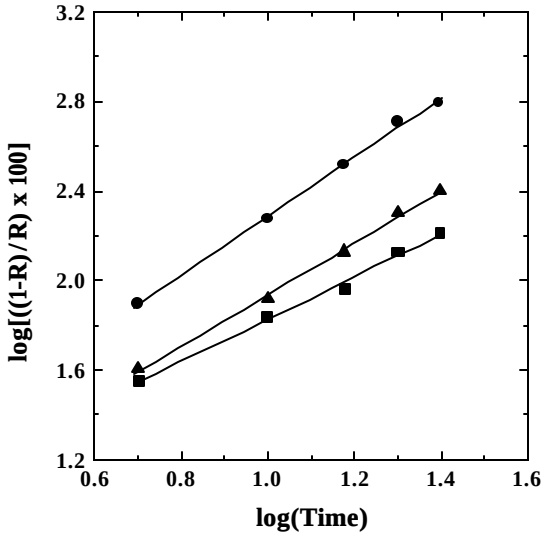

Fig. 2.9: Mechanism of action of the DNase activity of nuclease Rsn. Viscometric measurements were carried out at 37 °C with 15 ml of 30 mM Tris-HCl buffer pH 7.0, containing 15 U of nuclease Rsn and 5 mg of dsDNA, in presence of 2 mM Mg^{2+} (λ), Mn^{2+} (σ) and Co^{2+} (ν). For experimental details refer to Methods.

Like most of the single-strand-specific nucleases, the purified enzyme could convert supercoiled pUC 18 DNA (Form I) to linear duplex DNA (Form III) via nicked circular DNA (Form II). However with increase in the incubation time, degradation of Form III DNA was observed (Fig. 2.10). These observations suggest that nuclease Rsn is an endonuclease. Unlike *U. maydis* nuclease γ (Yarnall *et al.*, 1984), the type of metal ion used did not influence the cleavage pattern. However, the conversion of Form I to Form III and its further degradation in presence of Co^{2+} was slow, in comparison to Mg^{2+} and Mn^{2+}, indicating the relatively low preference of the enzyme for dsDNA in presence of Co^{2+}. Moreover, the inability of excess enzyme (4 fold) to digest Form I DNA, in the absence of metal ions, supports our earlier observation that nuclease Rsn is a metal requiring enzyme.

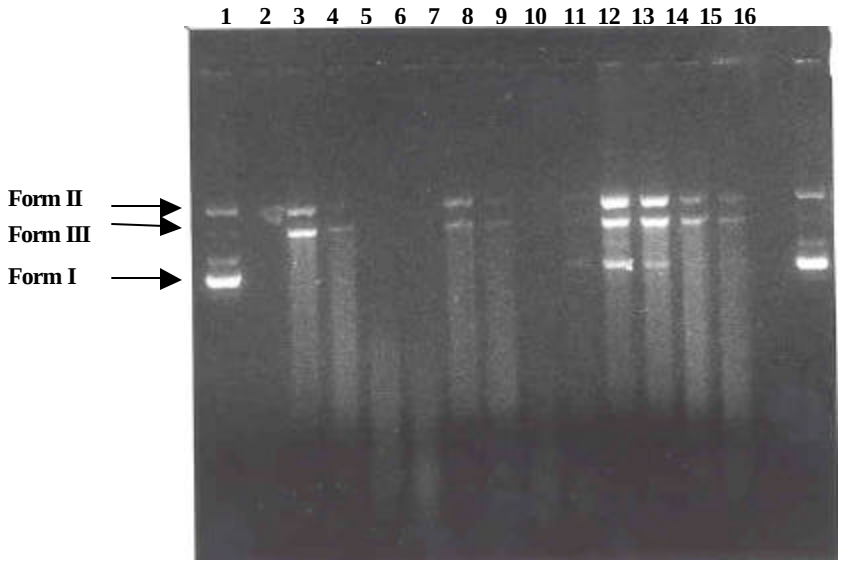

Fig. 2.10: Time course of supercoiled pUC 18 DNA digestion by nuclease Rsn. Reactions were carried out on pUC 18 DNA (1 μg) at 37 °C with 0.0025 U of nuclease Rsn. Lane 1 - pUC 18 alone, lanes 3-6 - digestion in presence of 2 mM Mg^{2+} at 1, 2, 5 and 10 min, respectively, lanes 7-10 - digestion in presence of 2 mM Mn^{2+} at 1, 2, 5 and 10 min, respectively, lanes 11-14 - digestion in presence of 2 mM Co^{2+} at 1, 2, 5 and 10 min, respectively and lane 16 - digestion with 0.01 U of enzyme in the absence of metal ion. Samples were electrophoresed on 1.2 % (w/v) agarose gels at pH 8.0.

Exhaustive digestion of either ssDNA or dsDNA by nuclease Rsn, in presence of Mg^{2+}, yielded oligonucleotides as the major end products of hydrolysis, suggesting an endo mode of action (Fig. 2.11). Similar observations have also been made in case of extracellular nuclease from *S. marcescens* (Nestle and Roberts, 1969b). BAL 31 nuclease (Gray *et al.*, 1981) as well as *U. maydis* nuclease α (Holloman *et al.*, 1981) cleaved ssDNA endonucleolytically while they exhibited exonucleolytic action on dsDNA. However, nuclease Rsn differs from these enzymes in that, it cleaves both ss and dsDNA endonucleolytically. HPLC analysis of the hydrolytic products of DNA by nuclease Rsn, in presence of Mn^{2+} and Co^{2+}, gave similar results (Fig. 2.12, 2.13). Subsequent analysis of the reaction

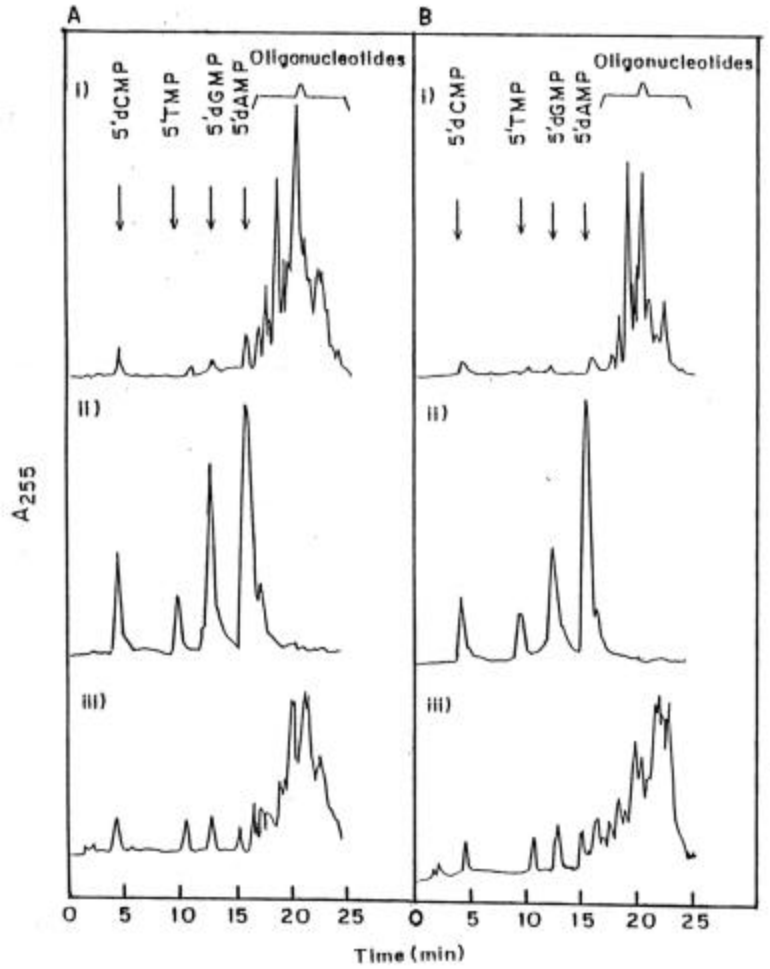

Fig. 2.11: Action of nuclease Rsn on DNA in presence of Mg^{2+}.

HPLC analysis was carried out on a Nucleosil C18 column (120 x 4 mm, 3 μm) with a linear gradient of acetonitrile in 100 mM triethylammonium acetate buffer pH 6.2 (0-15 % v/v for 20 min followed by 15 - 100 % for 5 min) as the mobile phase.

A) i) Hydrolysis of ssDNA, ii) Action of snake venom phosphodiesterase on (i) and iii) Action of spleen phosphodiesterase on (i).

B) i) Hydrolysis of dsDNA, ii) Action of snake venom phosphodiesterase on (i) and iii) Action of spleen phosphodiesterase on (i).

For experimental details refer to Methods.

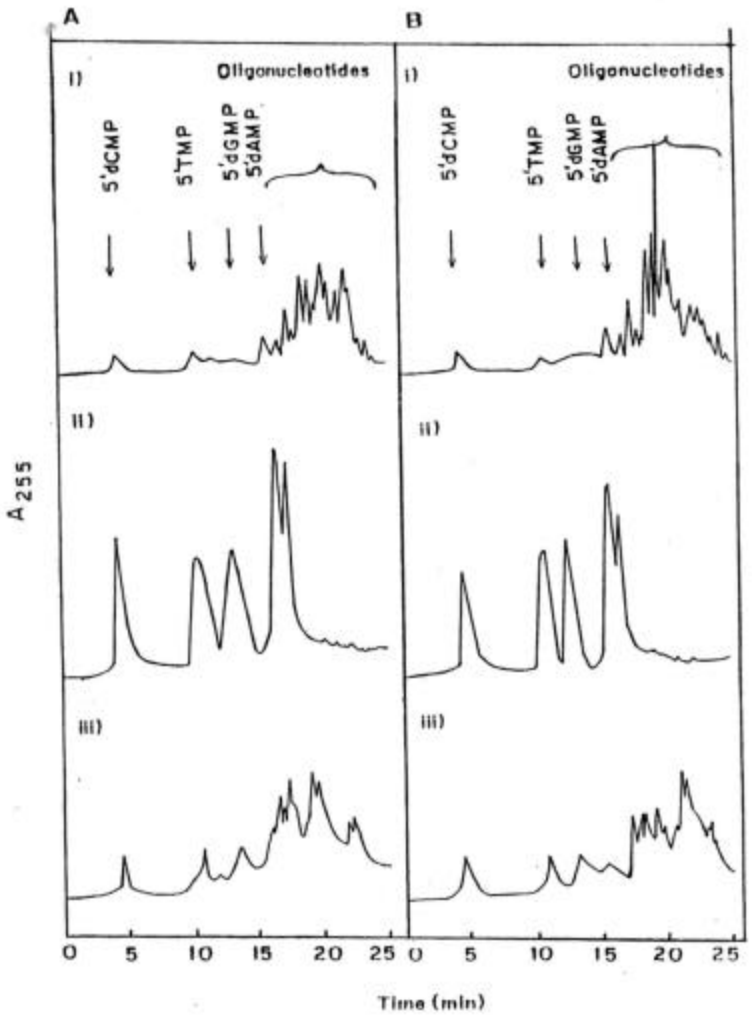

Fig. 2.12: Action of nuclease Rsn on DNA in presence of Mn^{2+}.

HPLC analysis was carried out on a Nucleosil C18 column (120 x 4 mm, 3 μm) with a linear gradient of acetonitrile in 100 mM triethylammonium acetate buffer pH 6.2 (0-15 % v/v for 20 min followed by 15 - 100 % for 5 min) as the mobile phase.

A) i) Hydrolysis of ssDNA, ii) Action of snake venom phosphodiesterase on (i) and iii) Action of spleen phosphodiesterase on (i).

B) i) Hydrolysis of dsDNA, ii) Action of snake venom phosphodiesterase on (i) and iii) Action of spleen phosphodiesterase on (i).

For experimental details refer to Methods.

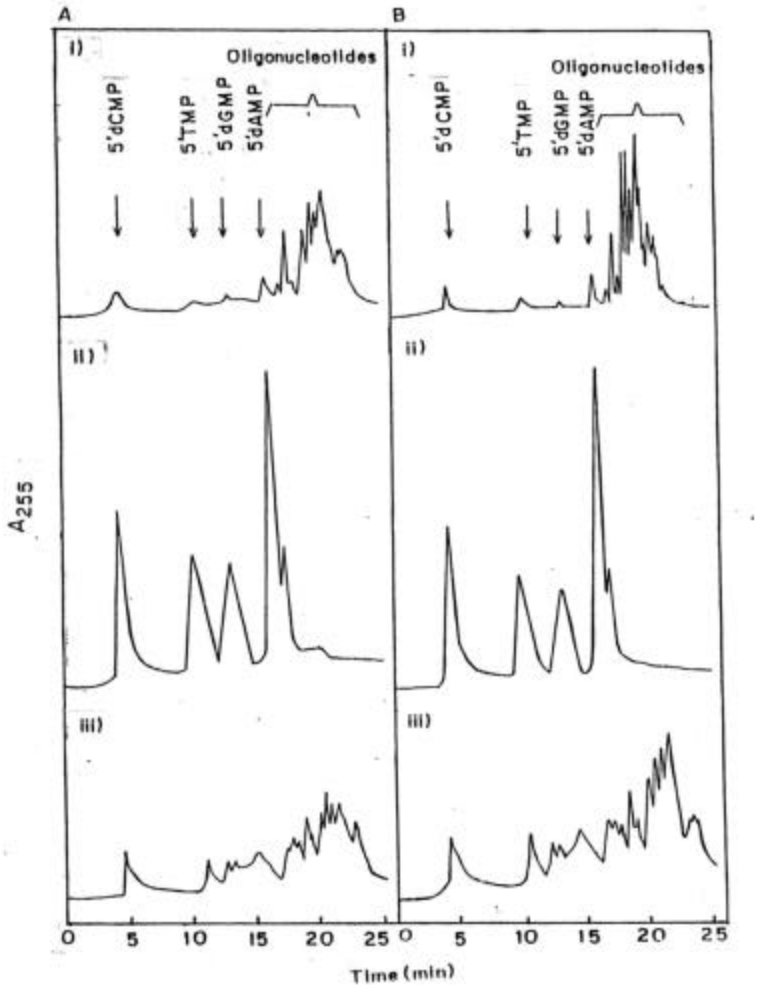

Fig. 2.13: Action of of nuclease Rsn on DNA in presence of Co^{2+}.

HPLC analysis was carried out on a Nucleosil C18 column (120 x 4 mm, 3 μm) with a linear gradient of acetonitrile in 100 mM triethylammonium acetate buffer pH 6.2 (0-15 % v/v for 20 min followed by 15 - 100 % for 5 min) as the mobile phase.

A) i) Hydrolysis of ssDNA, ii) Action of snake venom phosphodiesterase on (i) and iii) Action of spleen phosphodiesterase on (i).

B) i) Hydrolysis of dsDNA, ii) Action of snake venom phosphodiesterase on (i) and iii) Action of spleen phosphodiesterase on (i).

For experimental details refer to Methods.

products, using snake venom phosphodiesterase (which acts on oligonucleotides having free 3'-OH termini) yielded 5' mononucleotides whereas, spleen phosphodiesterase (which acts on oligonucleotides having free 5'-OH termini) failed to digest the oligonucleotides. These results suggest that, nuclease Rsn produces oligonucleotides having 3'-hydroxyl and 5'-phosphoryl termini (Table 2.9). The production of oligonucleotides, having 3'-hydroxyl and 5'-phosphate termini, as the major end products of DNA hydrolysis, suggests that the mode of action of nuclease Rsn is similar to endonucleases from *S. antibioticus* (Cal *et al*., 1995) and rat liver endoplasmic reticulum (Kouidou *et al*., 1987) and pancreatic DNase (Laskowski, 1967).

Table 2.9: Composition of the hydrolytic products of DNA by nuclease Rsn

Metal ions (2 mM)	Phosphodiesterase action*	ssDNA		dsDNA	
		5' Mono nucleotides (%)	Oligo nucleotides (%)	5' Mono nucleotides (%)	Oligo nucleotides (%)
Mg^{2+}	-	3	97	2	98
	Snake venom	90	10	93	7
	Spleen	3	97	4	96
Mn^{2+}	-	5	95	4	96
	Snake venom	88	12	90	10
	Spleen	8	92	12	88
Co^{2+}	-	4	96	9	91
	Snake venom	92	8	89	11
	Spleen	6	94	8	92

* The hydrolytic products of DNA obtained following the action of nuclease Rsn were further subjected to phosphodiesterase action (50 mM Tris-HCl, pH 7.5, for 6 h at 37 °C) and the products were analyzed by HPLC as described under Methods.

Separation of the oligonucleotides, obtained on exhaustive digestion of either ssDNA or dsDNA by nuclease Rsn in presence of either Mg^{2+}, Mn^{2+} or Co^{2+}, on DEAE-cellulose urea column gave four fractions (Fig. 2.14). Subsequent analysis of the individual fractions, for terminal phosphate content as well as total phosphate, revealed that the fractions I, II, III and IV corresponds to mono, di, tri and tetra nucleotides, respectively (Table 2.10).

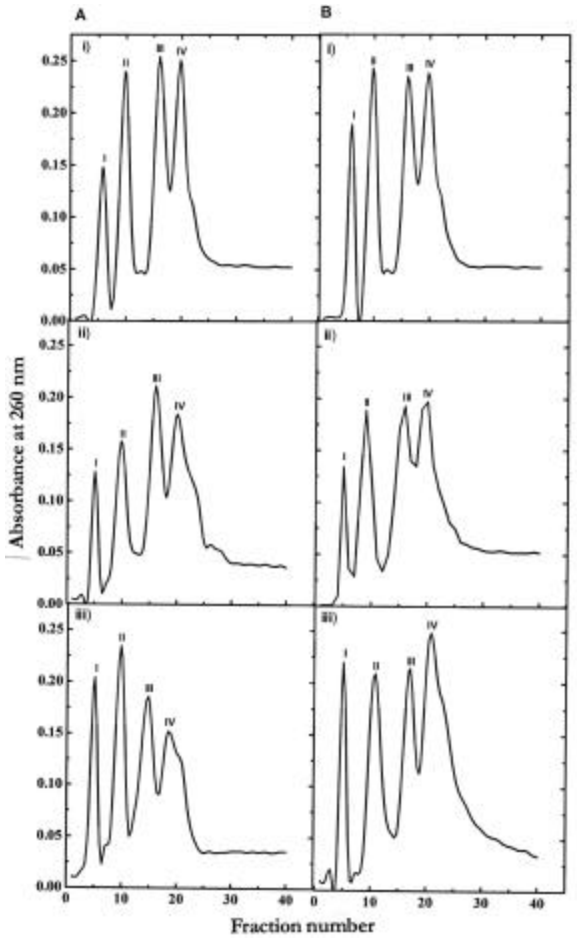

Fig. 2.14: Chromatographic profiles of the hydrolytic products of DNA. Nuclease Rsn digested DNA samples, in presence of 2 mM of Mg^{2+} (i), Mn^{2+} (ii) and Co^{2+} (iii), were resolved on DEAE-cellulose column. The elution of the bound oligonucleotides were carried out with a linear gradient of 0-1 M NaCl in 2.5 mM Tris-HCl buffer pH 7.8 containing 7 M Urea. ssDNA (A) and dsDNA (B). For details refer to Methods.

The relative percentages of the individual peaks indicated the predominance of trinucleotides (28-33 %) and tetranucleotides (35-45 %) followed by dinucleotides (20-23 %). The formation of low amount of mononucleotides (7-12 %) is consistent with our earlier observation on the exhaustive digestion of ssDNA and dsDNA (Table 2.9). In this respect, nuclease Rsn is similar to nucleases from *S. marcescens* (Nestle and Roberts, 1969b), yeast mitochondria (Morosoli and Lusena, 1980) and *A. nidulans* (Koa *et al.*, 1990) which produced di, tri and tetra nucleotides as the major end products of DNA hydrolysis with little (< 3 %) or no mononucleotides.

Table 2.10: Analysis of the fragment size of DNA[#]

Sample	Peak I		Peak II		Peak III		Peak IV	
	(%)	Ratio*	(%)	Ratio*	(%)	Ratio*	(%)	Ratio*
Mg^{2+}								
ssDNA	11	1.25	23	2.44	28	2.82	38	3.80
dsDNA	11	1.20	22	1.80	31	3.17	36	3.89
Mn^{2+}								
ssDNA	7	1.30	20	1.80	28	2.86	45	3.78
dsDNA	7	1.25	22	1.80	31	3.17	36	4.39
Co^{2+}								
ssDNA	12	0.67	22	2.17	31	2.88	35	4.12
dsDNA	11	0.85	21	2.00	33	3.36	35	3.68

Fragments obtained after DEAE-cellulose chromatography were analyzed for their terminal as well as total phosphate content as described under Methods.

* Ratio of total phosphate to terminal phosphate concentration.

Time course of the 3' terminal nucleoside analysis, of the hydrolytic products of ssDNA, showed the presence of nucleosides in the order of dT > dG > dC with very little, if any, of dA. From the initial stages of hydrolysis, the amount

of deoxythymidine was higher than other bases (Fig. 2.15 A) indicating that the enzyme prefers thymidylic acid linkages. Moreover, time course of the 5' terminal analysis revealed the presence of nucleosides in the order of dT dA > dC > dG (Fig. 2.15 B).

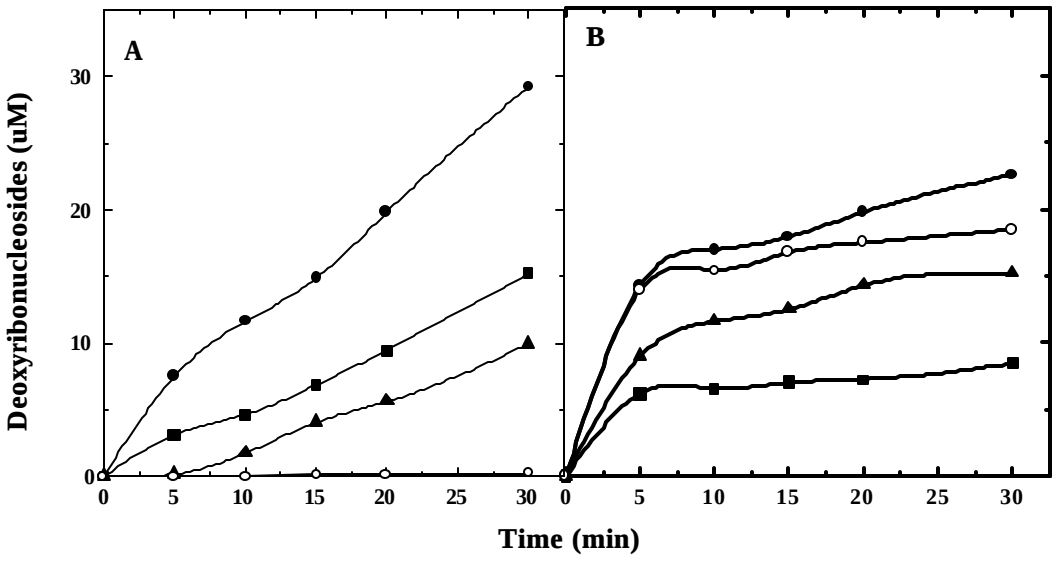

Fig. 2.15: Time course analysis of the 3' and 5' termini of the hydrolytic products of ssDNA

A) **3' termini:** The control and nuclease Rsn treated ssDNA samples were dephosphorylated with calf intestinal phosphatase, treated with spleen phosphodiesterase and the nucleosides released were then analyzed by HPLC. dT (λ), dG (ν), dC (σ) and dA (ο).

B) **5' termini:** The control and nuclease Rsn treated ssDNA samples were dephosphorylated with calf intestinal alkaline phosphatase, treated with snake venom phosphodiesterase and the nucleosides released were then analyzed by HPLC. dT (λ), dA (ο), dC (σ) and dG (ν).

For details refer to methods.

The presence of deoxythymidine and deoxyadenosine at 5' termini and deoxythymidine as the major product at the 3' termini indicate the high preference of the enzyme for dTpdT and dTpdA type of linkages. The 3' terminal base

analysis, for dsDNA, too showed a similar trend i.e. dT > dG > dC with negligible amount of dA (Fig. 2.16 A). However, the 5'terminal analysis revealed the

presence of nucleosides in the order of dT > dC > dA dG (Fig. 2.16 B). Inspite of the difference in the order of appearance of the nucleosides at the 5' termini, the preference for dTpdT linkages remained unaltered. The 5' and 3' terminal analysis, of the hydrolytic products of ssDNA and dsDNA, indicates that nucleases Rsn does not show any strict base preference and cleaves DNA in a nonspecifc manner. Additionally, very low amount of dA at the 3' terminal of the hydrolytic products of both ssDNA and dsDNA, indicates that dApdX bonds are resistant to cleavage. Like nuclease Rsn, nonspecific cleavage of DNA has also been observed with endonucleases from *S. marcescens* (Nestle and Roberts, 1969b) and yeast mitochondria (Morosoli and Lusena, 1980).

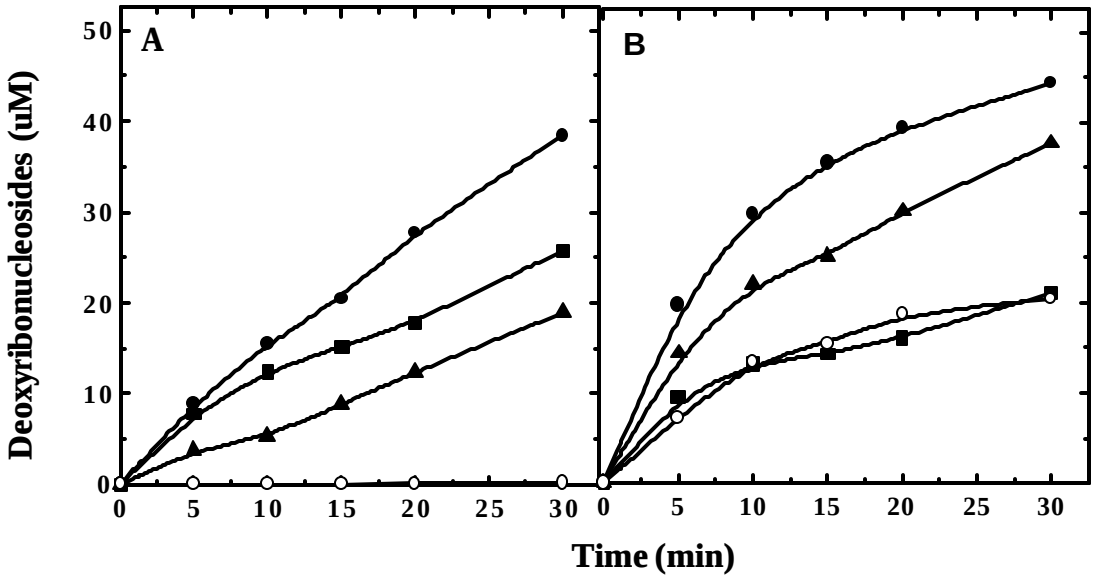

Fig. 2.16: Time course analysis of the 3' and 5' termini of the hydrolytic products of dsDNA

A. **3' termini:** The control and nuclease Rsn treated dsDNA samples were dephosphorylated with calf intestinal phosphatase, treated with spleen phosphodiesterase and the nucleosides released were then analyzed by HPLC. dT (λ), dG (ν), dC (σ) and dA (ο).

B. **5' termini:** The control and nuclease Rsn treated dsDNA samples were dephosphorylated with calf intestinal alkaline phosphatase, treated with snake venom phosphodiesterase and the nucleosides released were then analyzed by HPLC. dT (λ), dA (ο), dC (σ) and dG (ν).

For details refer to methods.

In conclusion, the present study shows that nuclease Rsn is a single strand preferential endonuclease and the preference for ssDNA can be enhanced using Co^{2+}. Furthermore, significant retention of the ssDNase activity in presence of high concentrations of organic solvents, especially dimethyl sulphoxide, suggests that under appropriate conditions it can also be used as a strict single-strand-specific enzyme. Since, nuclease Rsn is similar to pancreatic DNase with respect to metal ion requirement and mode of action, it can be used in conjunction with Mg^{2+} requiring enzymes like DNA polymerase I in nick translation reaction or techniques where limited digestion of DNA is required. The added advantage of nuclease Rsn is that, being an extracellular microbial enzyme, it is easy to purify.

CHAPTER - 3

CHARACTERIZATION OF THE ASSOCIATED RIBONUCLEASE ACTIVITY

SUMMARY

Nuclease Rsn from *Rhizopus stolonifer* is a multifunctional enzyme and hydrolyses both DNA and RNA. The RNase activity of the enzyme also showed an obligate requirement of divalent cations like Mg^{2+}, Mn^{2+} or Co^{2+} for its activity. The optimum pH and temperature, for RNA hydrolysis, were 7.0 and 35 °C respectively and it was not influenced by the type of metal ion used. RNase activity of nuclease Rsn was inhibited by Zn^{2+}, inorganic phosphate and pyrophosphate but not by 2', 3' or 5' mononucleotides. Nuclease Rsn hydrolyzed RNA endonucleolytically, liberating oligonucleotides ending in 3'-hydroxyl and 5'-phosphoryl termini as the end product of hydrolysis indicating that it cleaves at sites 5' to the phosphoribose. Additionally, the type of metal ions used did not influence the mode of action of the enzyme. The 3' and 5' terminal base analysis revealed that the RNase activity of nuclease Rsn is adenine specific and cleaves internucleotide bonds in the order of ApA >> UpA > CpA. However, GpX and XpG were resistant to hydrolysis.

INTRODUCTION

Nucleases are important analytical enzymes and have found wide application as analytical tools for the determination of nucleic acid structure. They are multifunctional enzymes and catalyze the hydrolysis of DNA and RNA. Some nucleases also exhibit either 3' or 5' phosphomonoesterase activity (Gite and Shankar, 1995). Majority of these enzymes have been extensively characterized with respect to their DNase activity and in comparison very little attention has been paid to the characterization of the associated RNase activity. Nuclease Rsn from *Rhizopus stolonifer* is a multifunctional enzyme and catalyzes the hydrolysis of both DNA and RNA. In the preceding Chapter we described the purification and extensive characterization of the DNase activity of nuclease Rsn. This Chapter describes the detailed characterization of the associated RNase activity of nuclease Rsn.

MATERIALS

2', 3' and 5' mononucleotides, dithiothreitol (DTT), β-mercaptoethanol, snake venom phosphodiesterase and spleen phosphodiesterase (Sigma, St. Louis, MO, USA); calf intestinal alkaline phosphatase (Bangalore-Genei Pvt. Ltd., Bangalore, India); uranyl acetate (Loba-Chemie Pvt. Ltd., Mumbai, India); Tris-(hydroxymethyl)methylamine (Tris) and perchloric acid (Qualigens Fine Chemicals, Mumbai, India) and HPLC grade acetonitrile (E. Merck (India) Limited, Mumbai, India) were used. All other chemicals used were of analytical grade. Commercial yeast RNA (Sisco Research Laboratories, Mumbai, India) was purified by ethanol precipitation.

METHODS

Isolation of rRNA

Isolation of rRNA, from purified RNA, was carried out according to Poulson (1973). Five g of commercial RNA (BDH Chemicals Ltd., Poole, England) was extracted 3 times with 25 ml of cold 3.0 M sodium acetate buffer pH 6.0. The residual rRNA was recovered each time by centrifugation at 8,000 g for 10 min. The rRNA pellet was finally washed with 75 % (v/v) ethanol containing 0.2 M sodium acetate. On electrophoresis, in 1.0 % (w/v) agarose gels at pH 8.0 (40 mM Tris-borate buffer containing 1 mM EDTA), the purified rRNA migrated to a position corresponding to that of 5 - 5.8 S RNA.

Determination of RNase activity

This was carried out as described in Chapter 2 (Page 74). The total reaction mixture of 0.75 ml contained 1.25 mg RNA in 200 mM Tris-HCl buffer pH 7.0 (containing 2 mM Mg^{2+}) 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 (2500 g, 15 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 nucleotides liberated was determined by assuming a molar absorption coefficient of 10,600 M^{-1} cm^{-1} for the ribonucleotides mixture (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.

Protein determination

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

Purification of nuclease Rsn

Cultivation of *R. stolonifer* and purification of nuclease Rsn was carried out as described in Chapter 2 (Page 76).

Action of nuclease Rsn on homopolyribonucleotides and RNA

The total reaction mixture of 750 µl containing 1.25 mg of either RNA or homopolyribonucleotide (poly A, poly G, poly C or poly U), in 200 mM Tris-HCl buffer pH 7.0 (containing 2 mM Mg^{2+}), was incubated with 1 U of nuclease Rsn at 37 °C. Aliquots of 75 µl were withdrawn at different time intervals and the reaction was terminated by the addition of 25 µl of MacFadyen's reagent. The precipitate formed was removed by centrifugation (2500 g, 15 min) and the acid soluble nucleotides produced were measured at 260 nm. For HPLC analysis of the cleavage products of poly A, the reaction was terminated by the addition of 10 µl of 100 mM EDTA.

HPLC analysis of the reaction products of nuclease Rsn

***Action on RNA*:** The total reaction mixture of 2 ml containing 2.5 mg of RNA, in 200 mM Tris-HCl buffer pH 7.0 (containing 2 mM of either Mg^{2+}, Mn^{2+} or Co^{2+}), was incubated with 2 U of purified enzyme at 37 °C. Subsequently, 1 U of the enzyme was added at an interval of 1 h upto 3 h and incubated for 24 h. After the incubation period, an aliquot (1 ml) was removed and the reaction was terminated

by the addition of 3 ml of chilled absolute ethanol. The mixture was left overnight at -20 °C and the undigested RNA was removed by centrifugation (6000g, 15 min). The supernatant was lyophilized, reconstituted in 200 µl of Milli Q water and subjected to HPLC.

***Determination of phosphoryl termini of the reaction products*:** The hydrolytic products of RNA, obtained following the action of nuclease Rsn, were lyophilized and reconstituted in 50 mM Tris-HCl buffer pH 7.5 and incubated with either 0.05 U of snake venom phosphodiesterase or 0.1 U of spleen phosphodiesterase overnight at 37 °C. After the incubation period, the reaction was terminated by the addition of two volumes of chilled absolute ethanol. The samples were concentrated by lyophilization, reconstituted in Milli Q water and subjected to HPLC.

***Separation of the reaction products*:** High Performance Liquid Chromatography (Waters model fitted with 515 HPLC pump) was carried out on a Symmetry C18 column (250 x 4.6 mm, 5 µm, Waters, USA). The mobile phase comprising of a linear gradient of acetonitrile in 100 mM triethylammonium acetate pH 6.2 (0 - 2.5 % v/v for 20 min, 2.5 - 50 % v/v for 10 min followed by 50 - 100 % v/v for 5 min), was used at 25 ± 1°C at a flow rate of 0.8 ml/min. Ten µl of the standard or the sample solution was injected onto the column and the nucleotides were detected, at 254 nm, using Waters 2487 Dual λ Absorbance Detector. The amount occupied by each peak was computed on the basis of total area occupied by each peak of the standard and sample. The nucleotides eluted in the order of 5'CMP, 5'UMP, 5'GMP and 5'AMP with retention times of ca. 7.52, 9.82, 16.71 and 30.77 min, respectively.

Terminal base analysis

***3' termini*:** The total reaction mixture of 250 µl, containing 5 mg of rRNA in 200 mM Tris-HCl buffer pH 7.0 (containing 2 mM Mg^{2+}), was incubated with 6 U of

nuclease Rsn at 37 °C. Aliquots of 35 µl were withdrawn at different time intervals and the reaction was terminated by the addition of 2 µl of 100 mM EDTA. The samples were treated with 20 µl of 1M sodium periodate at 26 ± 1 °C for 30 min. Subsequently, 200 µl of 1 M cyclohexylamine (pH 10.0) was added and incubated at 45 °C for 2 h. The reaction mixture was then cooled and the recovery of the free base was effected by the addition of 500 µl of chilled absolute ethanol (Neu and Heppel, 1964). The alcohol fraction was then lyophilized, reconstituted in 100 µl of Milli Q water and subjected to HPLC as described above. All four 5' ribonucleotides (5'AMP, 5'GMP, 5'CMP and 5'UMP) treated in a similar manner served as standards. The bases eluted in the order of C, U, G and A with retention times of ca. 4.83, 6.08, 9.76 and 17.39 min, respectively (data not shown).

***5' termini*:** The total reaction mixture of 250 µl, containing 5 mg of rRNA in 200 mM Tris-HCl buffer pH 7.0 (containing 2 mM Mg^{2+}), was incubated with 6 U of nuclease Rsn at 37 °C. Aliquots of 35 µl were withdrawn at different time intervals and the reaction was terminated by heat treatment (75 °C, 10 min). RNA samples incubated in the absence of nuclease Rsn served as control. The control and nuclease Rsn treated samples were then incubated with 0.4 U of calf intestinal alkaline phosphatase, in 100 µl (total volume) of calf intestinal alkaline phosphatase buffer (10 mM Tris-HCl buffer, pH 7.9, containing 50 mM NaCl, 1 mM DTT and 10 mM Mg^{2+}), at 37 °C for 1 h. After the incubation period, phosphatase was removed by extraction with an equal volume of chloroform : isoamyl alcohol (24 : 1 v/v). The aqueous layer was collected, incubated overnight with 0.01 U of snake venom phosphodiesterase at 37°C and subjected to HPLC as described earlier. The nucleosides eluted in the order of cytidine, uridine, guanosine and adenosine with retention times of ca. 9.20, 13.40, 30.71 and 31.30 min, respectively.

RESULTS AND DISCUSSION

Nuclease Rsn from *R. stolonifer* is a multifunctional enzyme and catalyzes the hydrolysis of ssDNA, dsDNA and RNA at a relative rate of approximately 100, 50 and 5, when assayed in presence of Mg^{2+}. Like the DNase activity, the RNase activity of the enzyme showed an obligate requirement of divalent cations because no activity could be detected when the assay was carried out in presence of 2 mM EDTA. However, addition of either Mg^{2+}, Mn^{2+} or Co^{2+} restored the activity and optimal activity was observed in presence of 2 mM of the aforementioned metal ions (Fig. 3.1). Other divalent cations like Ca^{2+}, Zn^{2+}, Hg^{2+} and Cu^{2+} did not have any effect (Table 3.1).

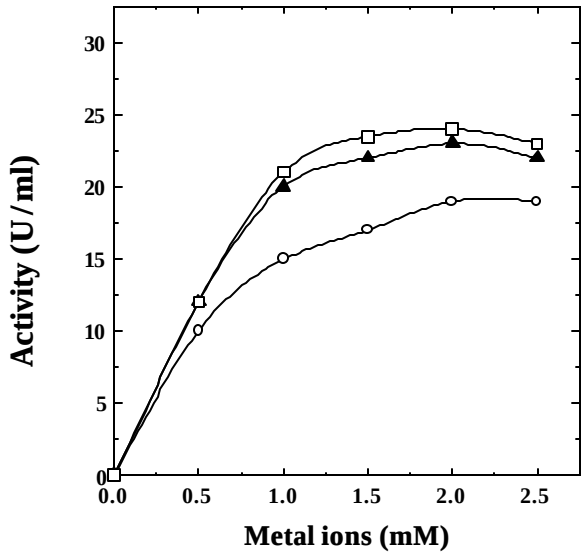

Fig. 3.1: Effect of metal ion concentration on the RNase activity of nuclease Rsn. Purified nuclease Rsn (1 U of RNase activity) was assayed in presence of different concentrations of Mg^{2+} (O), Mn^{2+} (σ) and Co^{2+} (ÿ) as described under Methods.

The metal ion requirement of the RNase activity of nuclease Rsn is similar to the associated RNase activity of *Saccharomyces cerevisiae* mitochondria nuclease (Dake *et al.,* 1988) and nucleases (SM1 and SM2) from *Serratia marcescens kums* 3958 (Yonemura *et al.*, 1983). In contrast to nuclease Rsn, the RNase activity of *S. cerevisiae* mitochondria nuclease was stimulated to some extent by Zn^{2+} and

Ca^{2+}. RNase activity of *S. marcescens kums* 3958 nucleases was optimally active in presence of 10 mM Mg^{2+} (Yonemura *et al.*, 1983) whereas, *S. cerevisiae* enzyme required 30 mM Mg^{2+} for its maximum activity (Dake *et al.*, 1988). However, RNase activity of nuclease Rsn required only 2 mM of either Mg^{2+}, Mn^{2+}

Table 3.1: Effect of metal ions on the RNase activity of nuclease Rsn[#]

Metal ions (2 mM)	Activity (U/ml)
Mg^{2+}	20
Mn^{2+}	23
Co^{2+}	24
Ca^{2+}	0
Zn^{2+}	0
Cu^{2+}	0
Hg^{2+}	0
Mg^{2+} + Mn^{2+}*	23
Mg^{2+} + Co^{2+}*	24
Mn^{2+} + Co^{2+}*	25
Mg^{2+} + Ca^{2+}	19
Mg^{2+} + Zn^{2+}	0
Mg^{2+} + Cu^{2+}	22
Mg^{2+} + Hg^{2+}	18

[#] Assays were carried out at pH 7.0 and 37 °C with RNA as substrate, in presence of metal ions indicated.

* When metal ions were used in combination, the effective concentration of individual metal ions was 1 mM, so as to have a total effective concentration of 2 mM.

or Co^{2+}, for its optimal activity. Like the dsDNase activity of nuclease Rsn, the extent of activation of the RNase activity in presence of Mg^{2+}, Mn^{2+} or Co^{2+} were comparable. Moreover, no synergism was observed in presence of Mg^{2+}, Mn^{2+} and Co^{2+} (Table 3.1). In case of *Bacillus subtilis* nuclease (a Ca^{2+} requiring enzyme), Ca^{2+} was essential only for the dsDNase activity (Kanamori *et al.*, 1973a) but nuclease Rsn required Mg^{2+}, Mn^{2+} or Co^{2+} for its ssDNase, dsDNase and RNase activities. Interestingly, addition of 10 mM of either Mg^{2+}, Co^{2+} or Fe^{2+} resulted in 2.5 fold increase in the ssDNase activity of *Neurospora crassa* nuclease but it also brought about approximately 40 % inhibition of the RNase activity (Linn, 1967). This selective inhibition of the RNase activity in presence of Mg^{2+}, Mn^{2+} or Co^{2+} was not observed with nuclease Rsn.

Optimum pH, temperature and stability

The optimum pH of purified nuclease Rsn for RNA hydrolysis was 7.0, which is similar to that of ssDNase and dsDNase activities of the enzyme and it was not influenced by the type of metal ion used (Fig. 3.2). The optimum temperature for the hydrolysis of RNA was 35 °C (Fig. 3.3), which is lower than that of ssDNase and dsDNase activities of the enzyme. However, unlike the dsDNase activity of nuclease Rsn, which showed higher optimum temperature in presence of Mn^{2+} and Co^{2+}, the type of metal ions used did not influence the optimum temperature for RNA hydrolysis. The optimum pH and temperature of the RNase activity of nuclease Rsn is comparable to the RNase activity of *S. cerevisiae* mitochondrial nuclease (Dake *et al.*, 1988).

The RNase activity of nuclease Rsn showed low stability and under the assay conditions (i.e. pH 7.0, 37 °C) lost approximately 50 % of its initial activity in 15 min (Fig. 3.4). The stability of the RNase activity of nuclease Rsn is inferior compared to the associated RNase activities of *S. cerevisiae* mitochondrial nuclease (Dake *et al.*, 1988), P1 nuclease (Fujimoto *et al.*, 1974), sheep kidney nuclease (Kasai and Grunberg-Monago, 1967) and a nuclease from chicken pancreas (Eley and Roth, 1966).

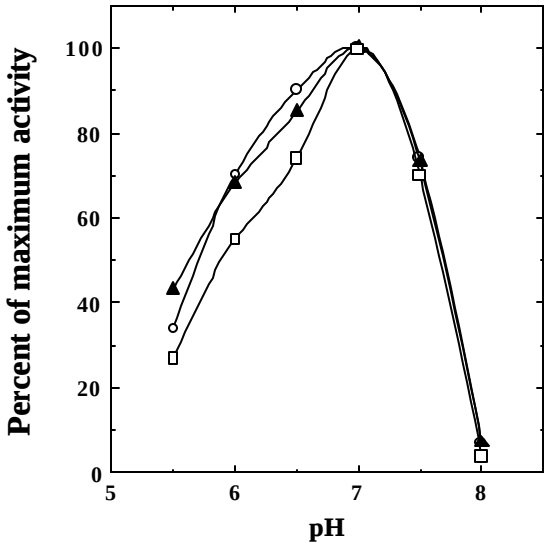

Fig. 3.2: Optimum pH of the RNase activity of nuclease Rsn. Nuclease Rsn (1 U of RNase activity) was assayed in a series of pH (5.0 - 8.0), at 37 °C, in presence of 2mM each of Mg^{2+} (O), Mn^{2+} (σ) and Co^{2+} (ÿ) as described under Methods.

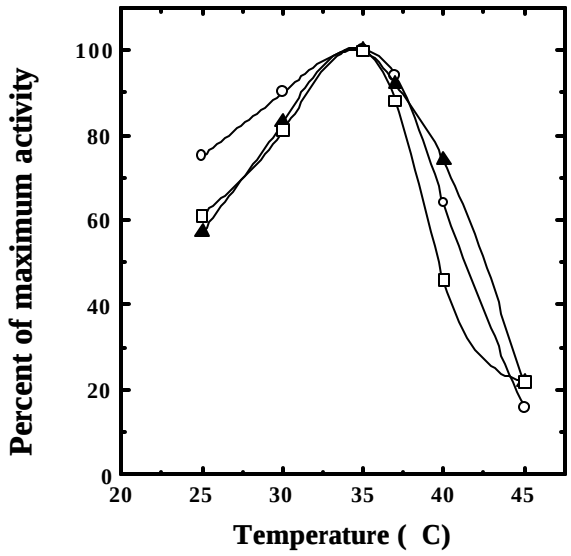

Fig. 3.3: Optimum temperature of the RNase activity of nuclease Rsn. Nuclease Rsn (1 U of RNase activity) was assayed in a series of temperatures (30 - 50 °C), at pH 7.0, in presence of 2 mM each of Mg^{2+} (O), Mn^{2+} (σ) and Co^{2+} (ÿ) as described under Methods.

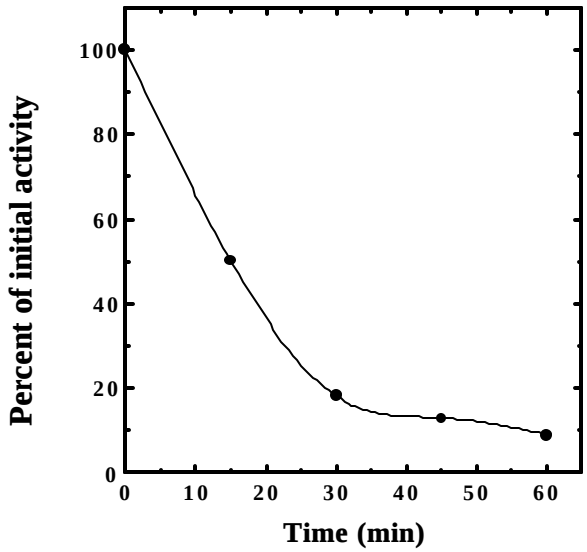

Fig. 3.4: Stability of the RNase activity of nuclease Rsn. Purified nuclease Rsn (100 U of RNase activity) was incubated, at 37 °C and pH 7.0, for 1 h. Aliquots were removed at different time intervals and assayed under standard assay conditions as described under Methods.

Effect of salt concentration

Influence of salt concentration on the RNase activity of nuclease Rsn showed that, increase in salt concentration brought about a progressive decrease in the enzyme activity and at 200 mM of NaCl or KCl, the enzyme exhibited 30 to 40 % of its maximal activity (Fig. 3.5). Susceptibility of the RNase activity of nuclease Rsn, to salt concentration, is inferior compared to sheep kidney nuclease where, a similar level of inhibition (30 - 40 %) was observed in presence of 50 mM of either NaCl or KCl (Kasai and Grunberg-Monago, 1967). In contrast, RNase activity of *S. cerevisiae* mitochondrial nuclease was insensitive to salt concentration and exhibited optimal activity in presence of 300 mM KCl (Dake *et al.*, 1988).

Inhibitors

In the present studies, the RNase activity of nuclease Rsn was inhibited only by Zn^{2+}, when tested in presence of optimal concentration of Mg^{2+}.

Interestingly Cu^{2+} and Hg^{2+}, which inhibited the DNase activity of nuclease Rsn, had no effect on the RNase activity (Table 3. 1, Page 117).

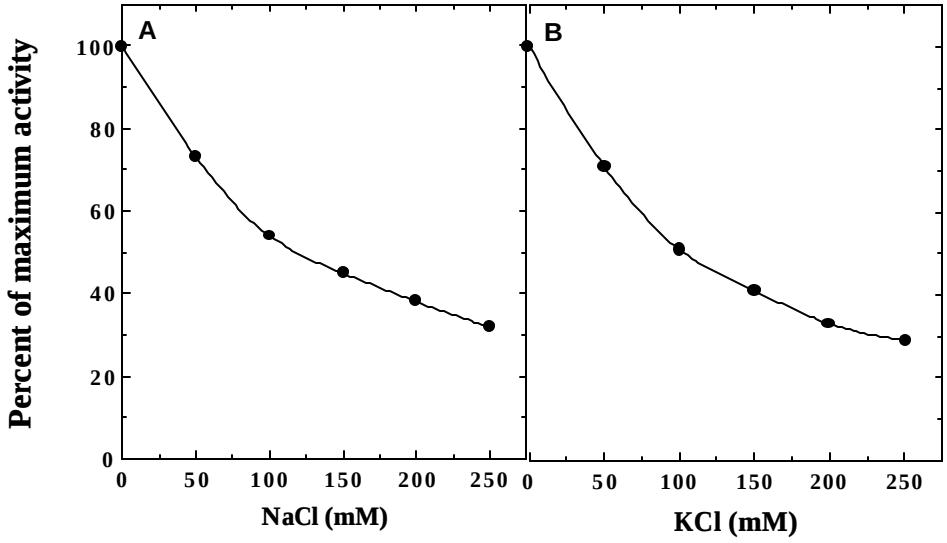

Fig. 3.5: Effect of salt concentration on the RNase activity of nuclease Rsn. Purified nuclease Rsn (1 U of RNase activity) was assayed in presence of varying concentrations of NaCl (A) and KCl (B) at pH 7.0 and 37 °C as described under Methods.

Mononucleotides, especially 5' mononucleotides are potent inhibitors of some of the well studied enzymes like S1 nuclease (Oleson and Hoganson, 1981), *S. glaucescens* nuclease (Aparicio *et al.*, 1992) and the nuclease from *Staphylococcus aureus* (Cuatrecasas *et al.*, 1967b). Additionally, guanosine 5' nucleotides are strong inhibitors of nuclease Bh1 from *Basidiobolus haptosporus* (Desai and Shankar, 2000). In contrast, nuclease β from *U. maydis* was inhibited by 3' mononucleotides (Rusche *et al*., 1980). Moreover, 2' mononucleotides are potent inhibitors of cyclizing type of RNases (Irie, 1967). However, like the DNase activity, the RNase activity of nuclease Rsn did not show any inhibition when assayed in presence of 200 µM of 2', 3' or 5' mononucleotides (Table 3.2).

Table 3.2: Effect of mononucleotides on the RNase activity of nuclease Rsn*

Nucleotides (200 µM)	Residual activity (%)
Control#	100
5' mononucleotides	
AMP	100
GMP	95
CMP	83
UMP	100
3' mononucleotides	
AMP	92
GMP	88
CMP	90
UMP	95
2' mononucleotides	
AMP	100
GMP	100
CMP	100
UMP	100

* Activity was determined under standard assay conditions i.e. at pH 7.0 and 37 °C, using RNA as substrate in presence of 2 mM Mg^{2+}.

\# Enzyme assay carried out in the absence of mononucleotides served as control.

RNase activity of nuclease Rsn retained approximately 85 % of its activity in presence of 5 mM inorganic phosphate while pyrophosphate at this concentration brought about 50 % inhibition. Moreover, the enzyme exhibited 69 % and 11 % of its initial activity in presence of 20 mM inorganic phosphate and pyrophosphate

respectively (Table 3.3), suggesting that pyrophosphate is a more potent inhibitor of the enzyme. In contrast, RNase activity of nucleases SM1 and SM2 from *S. marcescens kums* 3958 did not show any inhibition in presence of 5 mM inorganic phosphate while pyrophosphate at this concentration brought about approximately 80 % inhibition of the activity (Yonemura *et al*., 1983). Interestingly, nuclease Rsn retained significant amount of its RNase activity (50 %) in presence of 5 mM pyrophosphate whereas pyrophosphate at this concentration completely abolished the dsDNase activity (Chapter 2, Page 98), indicating the less sensitivity of the RNase activity towards pyrophosphate.

Table 3.3: Effect of phosphate and pyrophosphate on the RNase activity of nuclease Rsn*

Phosphates	Concentration (mM)	Residual activity (%)
Control #	-	100
Inorganic phosphate	5	85
	10	75
	20	69
Pyrophosphate	5	48
	10	24
	20	11

* Activity was determined under standard assay conditions i.e. at pH 7.0 and 37 °C, using RNA as substrate in presence of 2 mM Mg^{2+}.

\# Enzyme assay carried out in the absence phosphates served as control.

Action on homopolyribonucleotides and RNA

Action on homopolyribonucleotides revealed that nuclease Rsn hydrolyzed poly A rapidly whereas, RNA was cleaved at approximately 20 % the rate of

poly(A). Poly(U), poly(C) and poly(G) were resistant to hydrolysis (Fig. 3.6). However, detectable cleavage of poly(U) and poly(C) could be observed only on prolonged incubation with excess enzyme (15 fold), showing the high resistance of poly(U) and poly(C) to hydrolysis. Interestingly, poly(G) was resistant to cleavage under the above conditions (Table 3.4). The extent of hydrolysis of poly(A) with 15 fold excess enzyme in 30 min was approximately equal to that obtained with 1 U of the enzyme in 10 min (Fig. 3.6), suggesting the high susceptibility of poly(A) to cleavage. Additionally, the amount of acid soluble nucleotides liberated following the hydrolysis of poly(U) and poly(C) was approximately 8 % and 5 % that of poly(A) (120 min, Table 3.4), indicating the high preference of nuclease Rsn for adenylic acid linkages.

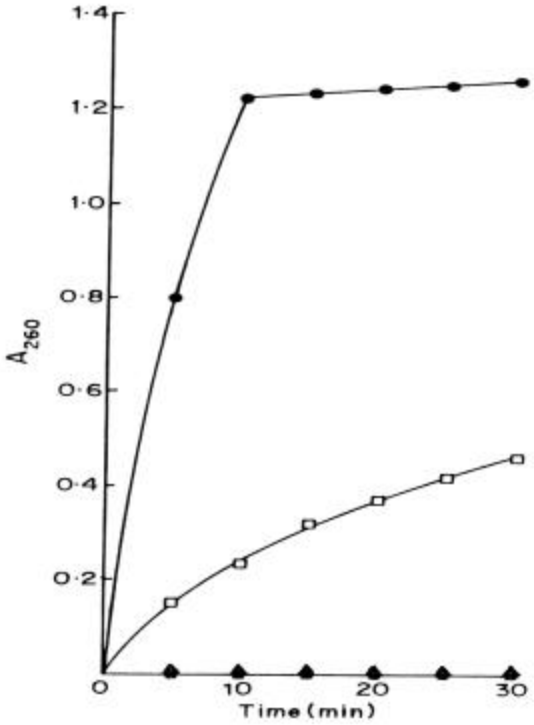

Fig. 3.6: Hydrolysis of synthetic polynucleotides and RNA by nuclease Rsn.

The rate of hydrolysis of polyribonucleotides and RNA, at pH 7.0 and 37 °C, was followed by monitoring the increase in the acid soluble nucleotides at 260 nm. Poly A (•), poly U (o), poly C (×), poly G (Δ) and RNA ( ). For experimental details refer to Methods.

Table 3.4: Hydrolysis of polynucleotides by nuclease Rsn

Substrate	Absorbance (A_{260})		
	30 min	60 min	120 min
Poly(A)	1.28	1.31	1.32
Poly(U)	0.05	0.08	0.10
Poly(C)	0.03	0.04	0.06
Poly(G)	0.00	0.00	0.01

The polyribonucleotides (1.25 mg each) were incubated with 15 U of purified nuclease Rsn at pH 7.0 and 37 °C and the hydrolysis was followed by measuring the acid soluble nucleotides at 260 nm.

Mode of action

Exhaustive digestion of RNA by nuclease Rsn, in presence of either Mg^{2+}, Mn^{2+} or Co^{2+}, yielded oligonucleotides as the end product of hydrolysis. Additionally, HPLC analysis of the hydrolytic products of poly(A) (30 min, Fig. 3.6) showed the absence of 5'AMP (data not shown). The above observations suggest an endo mode of action. BAL 31 nuclease 'F' (fast) species (Bencen *et al.*, 1984) as well as wheat seedling nuclease (Kroeker *et al.*, 1975) showed endonuclease activity towards ssDNA but exhibited exonuclease activity towards RNA. In this respect, nuclease Rsn differs from these enzymes in that, it cleaves both RNA and DNA endonucleolytically. Subsequent analysis of the reaction products, using snake venom phosphodiesterase (which acts on oligonucleotides having free 3'-OH termini) yielded 5' mononucleotides whereas, spleen phosphodiesterase (which acts on oligonucleotides having free 5'-OH termini) failed to digest the oligonucleotides (Table 3.5). These results show that, nuclease Rsn produces oligonucleotides having 3' hydroxyl and 5' phosphoryl termini.

Table 3.5: Composition of the hydrolytic products of RNA by nuclease Rsn

Metal ions (2 mM)	Phosphodiesterase action*	RNA	
		5' Mono nucleotides (%)	Oligo nucleotides (%)
Mg^{2+}	-	ND	100
	Snake venom	72	28
	Spleen	3	97
Mn^{2+}	-	ND	100
	Snake venom	66	34
	Spleen	ND	100
Co^{2+}	-	ND	100
	Snake venom	78	22
	Spleen	ND	100

* The hydrolytic products of RNA, obtained following the action of nuclease Rsn, were further subjected to phosphodiesterase action (50 mM Tris-HCl, pH 7.5, for overnight at 37 °C) and the products were analyzed by HPLC as described under Methods.

ND - Not detected.

The production of oligonucleotides, having 3' hydroxyl and 5' phosphoryl termini as the end products of RNA hydrolysis indicate that, the mode of action of nuclease Rsn is similar to endonucleases from rye germ ribosomes (Siwecka *et al.*, 1989), chicken pancreas (Eley and Roth, 1966), wheat chloroplast stromal protein (Monko *et al.*, 1994), *S. marcescens kums* 3958 (Yonemura *et al.*, 1983), sheep kidney (Kasai and Grunberg-Monago, 1967) and rat liver endoplasmic reticulum (Kouidou *et al.*, 1987*)*. In contrast, wheat choloroplast nuclease cleaved ssDNA endonucleolytically liberating oligonucleotides having 3' hydroxyl and 5' phosphoryl termini while oligonucleotides from RNA had 3' phosphoryl and 5'

hydroxyl termini (Kuligowska, *et al.*, 1988). Rye germ ribosome nuclease, on the other hand, liberated oligonucleotides ending in 3' hydroxyl and 5' phosphate from RNA and 3' phosphate and 5' hydroxyl from ssDNA, respectively (Siwecka *et al.*, 1989). However, nuclease Rsn differs from these enzymes in that, the oligonucleotides liberated following the hydrolysis of both DNA and RNA have 3' hydroxyl and 5' phosphoryl termini.

Nuclease Rsn shows approximately 5 % of the ssDNase activity on RNA. The low endonucleolytic activity of the enzyme, on RNA, suggests specific nature of cleavage. This observation coupled with the ability of the enzyme to hydrolyze only poly(A) indicated that, nuclease Rsn prefers adenylic acid linkages. Moreover, the formation of oligonucleotides with 5' phosphoryl and 3' hydroxyl termini revealed that the cleavage of RNA occurs on sites 5' to the phosphoribose. Time course of the 3' terminal base analysis of the hydrolytic products of yeast rRNA showed the presence of bases in the order of A >> U > C with the absence of G. From the initial stages of hydrolysis, the amount of adenine was considerably higher than other bases (Fig. 3.7A) indicating that the enzyme prefers adenylic acid linkages. Moreover, time course of the 5' terminal analysis revealed the presence of nucleosides in the order of adenosine >> uridine cytidine with the absence of guanosine (Fig. 3.7B). The high levels of adenosine (> 90 %) at 5' termini indicate the high preference of the enzyme for adenine residues and it cleaves 5' to the phosphoribose generating 5'pA termini (Fig. 3.8). Significant amount of uracil and cytosine at the 3' termini coupled with very low amounts (5 % each) of uridine and cytidine at the 5' termini, shows that the cleavage of uridylic acid and cytidylic acid linkages are probably influenced by the proximity of adenine residues. Moreover, the 3' and 5' terminal analysis of the oligonucleotides following the hydrolysis of rRNA, by nuclease Rsn, showed that the susceptibility of the linkages are in the order of ApA >> UpA > CpA. The absence of G either at the 3' or the 5' termini point towards the resistance of GpX and XpG to cleavage, where X is any base.

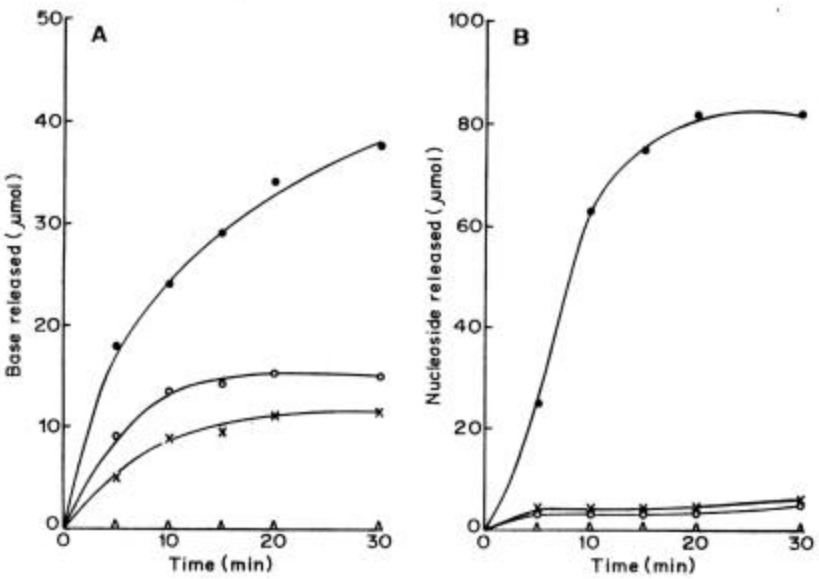

Fig. 3.7: Time course analysis of the 3' and 5' termini of the hydrolytic products of nuclease Rsn.

A) **3' termini:** The control and nuclease Rsn treated rRNA samples were subjected to periodate oxidation followed by cyclohexylamine reduction and the bases released were then analyzed by HPLC. Adenine (•), Uracil (o), Cytosine (×) and Guanine (Δ).

B) **5' termini:** The control and nuclease Rsn treated rRNA samples were dephosphorylated with calf intestinal alkaline phosphatase, treated with snake venom phosphodiesterase and the nucleosides released were then analyzed by HPLC. Adenosine (•), Uridine (o), Cytidine (×) and Guanosine (Δ).

For experimental details refer to Methods.

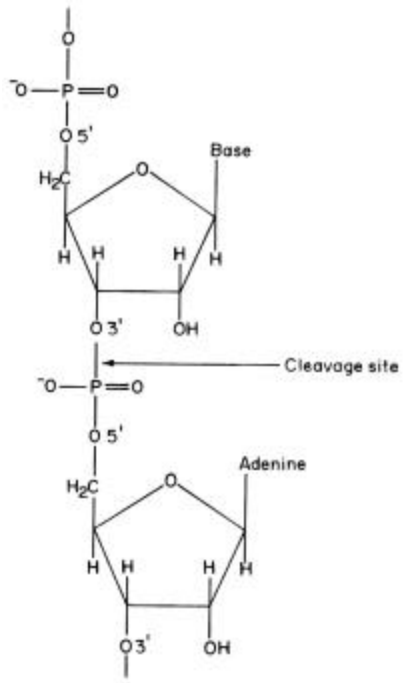

Fig. 3.8: Mode of action of nuclease Rsn

Based on the results of the 3' and 5' terminal analysis of the hydrolytic products of rRNA, by nuclease Rsn, the initial increase in the rate of hydrolysis of RNA (Fig. 3.6), can be correlated to the rapid hydrolysis of ApA bonds. However, the subsequent decrease in the reaction rate can be due to the slow rate of hydrolysis of UpA and CpA linkages.

In conclusion, the RNase activity of nuclease Rsn is adenine specific and hence it can be used in conjunction with other base specific RNases for the structural determination of RNAs.

CHAPTER - 4

ACTIVE SITE CHARACTERIZATION

SUMMARY

Chemical modification studies on purified nuclease Rsn revealed the involvement of two histidine, single tryptophan and two carboxylate residues in the catalytic activity of the enzyme. Substrates of nuclease Rsn, viz. DNA and RNA could not protect the enzyme against DEP and EDAC mediated inactivation whereas, substrate protection was observed in case of NBS-mediated inactivation of the enzyme. K_m and k_{cat} values of the partially inactivated enzyme samples suggested that while histidine and carboxylate are involved in catalysis, tryptophan is involved in substrate binding. Furthermore, fluorescence quenching studies on native and modified nuclease Rsn, using metal ions, indicated the involvement of carboxylate in metal binding. The involvement of carboxylate in catalysis coupled with its role in metal binding suggests that, cleavage of DNA and RNA by nuclease Rsn, probably follows a mechanism of metal ion mediated catalysis.

INTRODUCTION

Nuclease Rsn from *Rhizopus stolonifer* is a sugar-non-specific endonuclease, which acts on DNA and RNA. Histidine has been implicated in the catalytic activity of S1 nuclease (Gite *et al.*, 1992a), nucleases from *Serratia marcescens* family (Vincent *et al.*, 1988; Friedhoff *et al.*, 1996a; Ho *et al.*, 1998; Ho and Liao, 1999; Meiss *et al.*, 2000) as well as RNases belonging to T2 family (Sanda *et al.*, 1985; Kawata *et al.*, 1990; Rangarajan *et al.*, 1999). The involvement of carboxylate has been shown in the catalytic activity of nucleases from *Staphylococcus aureus* (Cotton *et al.*, 1979) and *S. marcescens* (Friedhoff *et al.*, 1996a) and a DNA/RNA non-specific endonuclease NucA from *Anabaena* sp. (Meiss *et al.*, 2000). In the case of enzymes acting on anionic substrates lysine and / or arginine has been implicated in substrate binding (Riordan, 1979; Richardson *et al.*, 1990). Lysine has been shown to have a role in the catalytic activity of S1 nuclease (Gite *et al.*, 1992b). Moreover, the involvement of tryptophan in the catalytic activity of RNase Rh (Sanda and Irie, 1980) and *S. marcescens* nuclease (Meiss *et al.*, 1999) has been demonstrated. Since nuclease Rsn acts both on DNA and RNA, chemical modification of histidine, carboxylate, lysine and tryptophan residues were carried out to evaluate their role in the catalytic activity of the enzyme and the results are presented in this Section.

MATERIALS

Methylene Blue and perchloric acid (Qualigens, Bombay, India); hydroxylamine hydrochloride (BDH, Bombay, India); uranyl acetate (Loba-Chemie Pvt. Ltd., Bombay, India); diethyl pyrocarbonate (DEP), N-acetylimidazole (NAI), 2,4,6-trinitrobenzenesulphonic acid (TNBS), N-bromosuccinimide (NBS), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), 3-nitro-L-tyrosine ethyl ester (NTEE) and 2-(N-morpholino)ethanesulphonic acid (MES) (Sigma, St. Louis, MO, USA) were used. All other chemicals used were of analytical grade. High Mr DNA from buffalo liver was isolated as described in Chapter 2 (Page 73). Commercial yeast RNA (Sisco Research Laboratories, Bombay, India) was purified by ethanol precipitation.

METHODS

Enzyme assays

The DNase and RNase activities of nuclease Rsn were determined as described in Chapters 2 and 3 (Pages 74, 112). The amount of acid soluble nucleotides liberated following the hydrolysis of DNA or RNA, at pH 7.0 (in presence of 2 mM Mg^{2+}) and 37 °C, were calculated by assuming a molar absorption coefficient of 10,000 $M^{-1}cm^{-1}$ and 10,600 $M^{-1}cm^{-1}$ for deoxyribonucleotide and ribonucleotide mixture, respectively (Curtis *et al.*, 1966). One unit of DNase and RNase activity is defined as the amount of enzyme required to liberate 1 µmol of acid soluble nucleotides/min under the assay conditions.

Protein determination

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

Purification of nuclease Rsn

Nuclease Rsn was purified to homogeneity as described in Chapter 2.

Chemical modification studies

In chemical modification studies, the residual activities of the modified enzyme were determined using both DNA and RNA.

***Photo-oxidation*:** This was carried out by exposing 5 µg of the purified enzyme, in 1 ml of 30 mM MES buffer pH 7.0, in a glass test tube (1 cm x 10 cm) containing different concentrations of Methylene Blue, to 200 W flood-light bulb held at a distance of 12 cm for 15 min, at 25 ± 1 °C, followed by estimation of the residual activities. Enzyme samples treated under identical conditions, in the dark, served as control.

***Reaction with DEP*:** Nuclease Rsn (50 µg), in 500 µl of 30 mM MES buffer pH 7.0, was incubated with a total of 1.5 mM DEP, at 25 ± 1 °C. The reagent was added in three installments (5 µl each) to obtain an effective concentration of 0.5, 1.0 and 1.5 mM respectively. After each addition, an aliquot (5 µl) was removed and the reaction was arrested by the addition of 10 µl 10 mM imidazole buffer, pH 7.5. Subsequently, the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of DEP served as control. The DEP concentration in the diluted sample was determined by mixing an aliquot of the sample with 3 ml of 10 mM imidazole buffer, pH 7.5, followed by monitoring the increase in the absorbance at 230 nm. The amount of N-carbethoxyimidazole formed was calculated by using a molar absorption coefficient of 3000 $M^{-1}cm^{-1}$ (Melchoir and Fahrney, 1970). The concentration of the diluted DEP solution was 50 mM. The ethanol concentration in the reaction mixture did not exceed 3 % (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. The DEP mediated modification was monitored, spectrophotometrically, by measuring the changes in the absorbance at 240 nm as described by Ovadi *et al*. (1967). The spectral scans were recorded on a Shimadzu UV-VIS Spectrophotometer in the range of 220-290 nm.

***Reaction with hydroxylamine*:** Decarbethoxylation was carried out according to Miles (1977). The DEP modified enzyme samples were incubated with 50 mM hydroxylamine, pH 7.0, at 27 ± 1 °C for 2h and the enzyme activities were determined under standard assay conditions.

***Reaction with NAI*:** The purified nuclease Rsn (50 µg), in 500 µl of 30 mM MES buffer pH 7.0, was incubated with 2 mM NAI for 20 min at 25 ± 1 °C, followed by estimation of the residual activities under standard assay conditions. The enzyme incubated in the absence of NAI served as control. The number of tyrosine residues modified were calculated by using a molar absorption coefficient of 1160 $M^{-1}cm^{-1}$, at 278 nm (Means and Feeney, 1971).

***Reaction with TNBS*:** Nuclease Rsn (50 µg), in 500 µl of 30 mM MES buffer pH 7.0, was incubated with 2 mM TNBS for 30 min at 30 ± 1 °C in dark, followed by estimation of the residual activities under standard assay conditions. The enzyme incubated in the absence of TNBS served as control. The number of lysine residues modified were calculated by using a molar absorption coefficient of 14,000 $M^{-1}cm^{-1}$, at 335 nm (Means and Feeney, 1971).

***Reaction with NBS*:** Nuclease Rsn (50 µg), in 500 µl of 50 mM sodium acetate buffer pH 5.0, was incubated with a total of 24 µM NBS, at 25 ± 1 °C. The reagent was added in six installments (5 µl each), to obtain an effective concentration of 4, 8, 12, 16, 20 and 24 µM respectively. After each addition, an aliquot (5 µl) was removed and the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of NBS served as control. The NBS mediated modification was monitored, spectrophotometrically, by measuring the changes in the absorbance at 280 nm as described by Spande and Witkop (1967). The spectral scans were recorded on a Shimadzu UV-VIS Spectrophotometer in the range of 240 – 320 nm.

***Reaction with EDAC*:** Nuclease Rsn (50 µg), in 250 µl of 30 mM MES buffer pH 6.0, was incubated with varying concentrations of EDAC (5 - 25 mM) and 5 mM NTEE at 30 ± 1 °C for 15 min. After the incubation period the samples (20 µl) were removed, made upto 1 ml with 50 mM sodium acetate buffer pH 5.5 and the residual activities were determined under standard assay conditions. To determine the incorporation of nitrotyrosyl groups, the EDAC treated enzyme samples were precipitated by the rapid addition of 5 volumes of absolute ethanol and the mixture was left at 4 °C for 3 h. The precipitated protein was collected by centrifugation (6000 g,

1 min), washed extensively with absolute ethanol, air dried, dissolved in 500 µl of 100 mM NaOH and dialyzed extensively against 100 mM NaOH. The nitrotyrosyl groups incorporated were determined, spectrophotometrically at 430 nm, by assuming a molar absorption coefficient of 4600 $M^{-1}cm^{-1}$ (Pho *et al.*, 1977). Enzyme samples incubated in the absence of EDAC served as control.

Substrate and metal ion protection

The effect of substrate and metal ion protection was studied by pre-incubating the enzyme with excess amounts of DNA, RNA or the metal ions (Mg^{2+}, Mn^{2+} and Co^{2+}) followed by treatment with the modifying reagents.

CD measurements

CD spectra of the native and chemically modified enzyme samples were recorded on a JASCO-715 Spectropolarimeter, at 25 ± 1 °C, in the range of 200-250 nm.

Fluorescence studies

Fluorescence measurements were performed on a Perkin Elmer Luminiscence Spectrometer LS 50 B, at 26 ± 1 °C, using a slit width of 5 nm for both excitation and emission. An excitation wavelength of 295 nm was used and the emission was monitored at 335 nm (λ_{max} of the enzyme). Fluorescence measurements of native and chemically modified nuclease Rsn (10 µg in 2 ml of 10 mM MES buffer pH 7.0) were carried out by titrating the enzyme samples with Mg^{2+} or Co^{2+} (from 100 mM stock) followed by monitoring the change in fluorescence at 335 nm. Fluorescence of the buffer and metal solutions were used to correct the observed fluorescence. The binding data were then analyzed to yield the best fit by hyperbolic binding curve and the K_d values were determined using the equation

$$I_F/I^\circ_F = L/(L+K_d)$$

where I°_F is the fluorescence intensity of the protein alone, I_F is the fluorescence intensity at the concentration, L, of the metal ions added.

Treatment of data by Tsou's method

The data obtained by chemical modification studies on nuclease Rsn were analyzed according to Tsou (1962), to determine the number of essential residues, using the following equation.

$$a^{1/i} = x$$

where 'a' is the fraction of the activity remaining, 'x' is the overall fraction of the essential groups remaining fully active and 'i' is the number of essential groups involved in the activity. The above equation represents the case wherein only one type of essential group is modified and all the groups of the same type are equally reactive. Successively, a, a, a, etc. against 'x' were plotted until it yielded a value of ' i ' which gave the best fit for a straight line.

RESULTS AND DISCUSSION

Modification of histidine residues

Photo-oxidation of purified nuclease Rsn, in presence of 0.001% (w/v) Methylene Blue, showed a pH-dependent inactivation of both DNase and RNase activities and the maximum loss of activity was observed at pH 7.0 (Fig. 4.1). When the enzyme was irradiated with 0.001 % (w/v) Methylene Blue, at pH 7.0 for 15 min, it lost approximately 80-90 % of its initial activity towards DNA and RNA and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. The inactivation of the enzyme could be prevented by shielding the enzyme-Methylene Blue mixture from irradiation, indicating the presence of histidine at or near the active site. The pH-dependent inactivation of the enzyme was similar to that observed in case of photo-oxidation of free histidine (Weil, 1965) and also in the photo-inactivation of several enzymes with histidine at their active site (Westhead, 1965; Gite *et al.*, 1992a; Rangarajan *et al.*, 1999), suggesting the presence of histidine at or near the active site of nuclease Rsn.

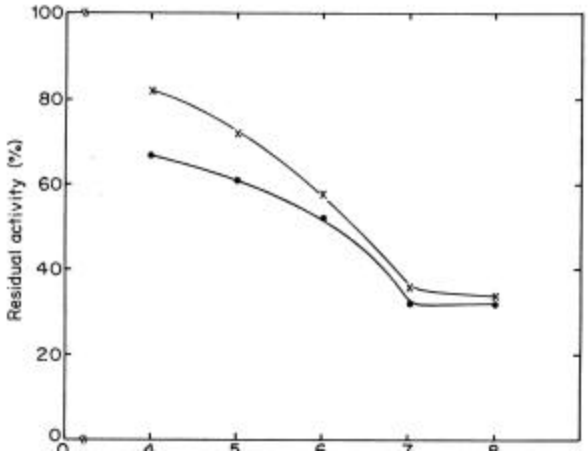

Fig. 4.1: Effect of pH on photo-oxidation of nuclease Rsn. The enzyme (5 µg/ml) was incubated in series of pH (4.0-8.0) with 0.001 % (w/v) Methylene Blue at 25 ± 1 °C for 15 min as described under Methods. An identical sample at each pH value was kept in the dark to serve as control. Enzyme activities were determined under standard assay conditions. DNase (•) and RNase (x).

Carbethoxylation of the enzyme at pH 7.0, resulted in approximately 70-80 % loss of its initial activity towards DNA and RNA and the inactivation was dependent on the concentration of the reagent. No loss of activity was observed in the control samples. Carbethoxylation of nuclease Rsn, as a result of DEP treatment, was accompanied by an increase in the absorption of the modified protein at 240 nm. Based on a molar absorption coefficient of 3200 $M^{-1}cm^{-1}$, for carbethoxyhistidine at 240 nm (Ovadi *et al*., 1967) and the Mr of 67000 for nuclease Rsn (Chapter 2, Page 84), the total number of histidine residues modified was found to be 1.8. However, the plot of percent residual activity versus the number of histidine residues modified revealed that the loss of activity towards DNA and RNA resulted from the modification of two residues per molecule of the enzyme (Fig. 4.2). These observations suggest that histidine may have a role in the catalytic activity of nuclease Rsn. To evaluate the number of essential histidine residues involved in the catalytic activity of nuclease Rsn, the modification data were analyzed according to Tsou (1962). The standard deviation from linearity, derived from the data on histidine modified samples, indicated that the best fit is obtained when ' i ' = 2, for both the

activities (Table 4.1). The above result indicated that both the residues are essential for the catalytic activity of the enzyme. Incubation of the DEP modified enzyme with 50 mM hydroxylamine pH 7.0, at 27 ± 1 °C for 2 h, failed to restore the original activity.

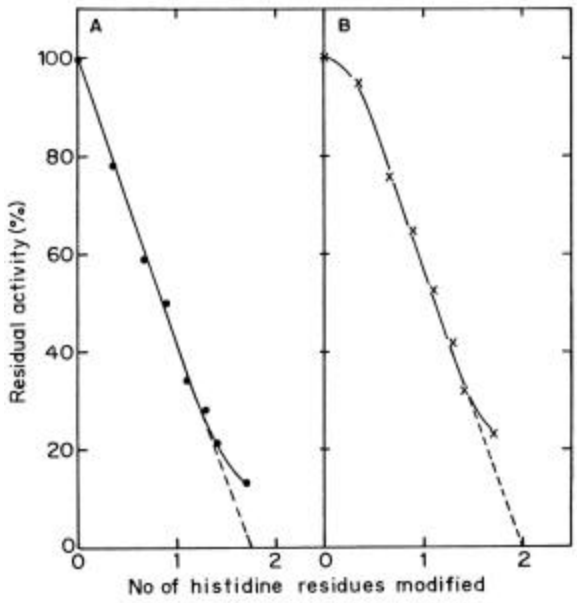

Fig. 4.2: Plot of percent residual activity versus number of histidine residues modified. DNase (A) and RNase (B). The number of histidine residues modified were estimated as described under Methods.

Table 4.1: Analysis of the DEP modification data according to Tsou*

Sample	'i' Value	Standard deviation (%)	
		DNase	RNase
Histidine modified	1	6.5 ± 0.4	4.4 ± 0.5
	2	2.5 ± 0.1	2.2 ± 0.1
	3	1.5 ± 0.2	1.5 ± 0.1

* The values given are the mean of two independent sets of experiments. For details refer to Methods.

Though DEP is specific for histidine at or around neutral pH, it also reacts to a lesser extent with tyrosine, lysine and cysteine residues (Miles, 1977). The DEP mediated inactivation of tyrosine was excluded by the observation that there was no significant decrease in the absorbance of the modified protein at 278 nm. Moreover, modification of tyrosine residues of the purified enzyme, with a tyrosine specific reagent viz. NAI, though resulting in the modification of 6 residues out of 10, did not have any significant effect on the enzyme activity suggesting that tyrosine may not have a role in the catalytic activity of nuclease Rsn. Though the above observations support the presence of histidine at or near the active site, they still do not rule out the possible involvement of cysteine. However, in the present case, the DEP mediated inactivation of the enzyme cannot be attributed to the modification of cysteine, as amino acid composition revealed the absence of cysteine (Chapter 2, Page 86). As mentioned earlier, DEP mediated inactivation of nuclease Rsn could not be reversed by incubation with hydroxylamine and this suggested that the loss of activity as a result of DEP treatment could be due to lysine modification. However, modification of the pure enzyme with a lysine specific reagent, namely TNBS, though resulting in the modification of 5 residues out of 21 did not have any significant effect on the enzyme activity (Table 4.2), indicating that lysine has no role in the catalytic activity of nuclease Rsn. It has been reported that DEP mediated modification of histidine residues can result in disubstitution of the imidazole ring which is not reversed by hydroxylamine (Lundblad, 1991). Moreover, the disubstituted histidine shows an

absorption maxima at 242 nm (Miles, 1977). In the present studies, the difference spectra of DEP modified nuclease Rsn showed a maxima at 245 nm pointing towards the probable disubstitution of the imidazole ring (Fig. 4.3). This observation also suggests that the inability of hydroxylamine to reverse the DEP mediated inactivation of the enzyme is due to disubstitution in the imidazole ring. Hence the observed loss of activity of nuclease Rsn can be correlated to the modification of histidine residues.

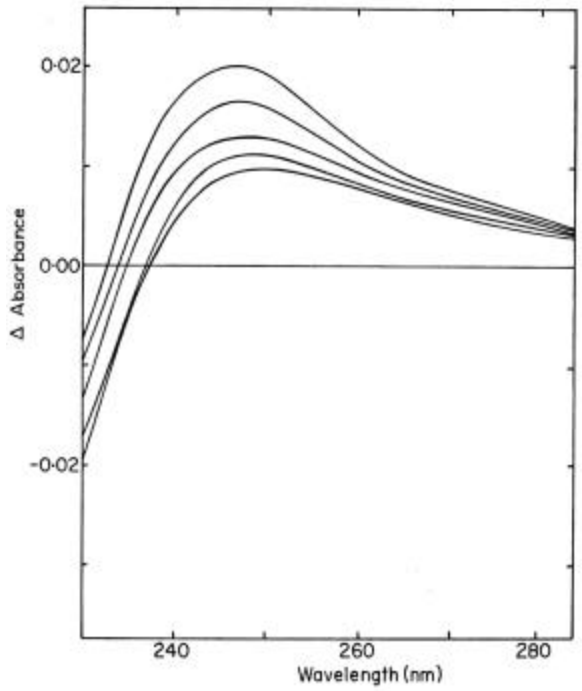

Fig. 4.3: Difference spectra of histidine modified nuclease Rsn.

For experimental details refer to Methods.

DEP mediated inactivation of nuclease Rsn could not be prevented by incubating the enzyme with excess amounts of DNA, RNA and metal ions prior to the modification reaction (Table 4.3). Additionally, DEP modification did not bring about any gross change in the enzyme structure indicating that the loss of activity of the enzyme is due to histidine modification rather than structural changes (Fig. 4.4).

Table 4.2: Effect of different modifying reagents on the activity of nuclease Rsn

Incubation mixture	Number of residues modified	Residual activity (%)	
		DNase	RNase
Control	0	100	100
Histidine (DEP)	2	15	32
Decarbethoxylation (Hydroxylamine 50 mM, pH 7.0)	-	15	30
Tyrosine (NAI)	6	70	72
Lysine (TNBS)	5	100	100

For experimental details refer to Methods.

Table 4.3: Effect of histidine modification on the activity of nuclease Rsn: substrate and metal ion protection studies *

Incubation mixture	Residual activity (%)	
	DNase	RNase
Enzyme	100	100
Enzyme + DEP (500 μM)	15	32
Enzyme + DNA (1 mg) + DEP (500 μM)	26	38
Enzyme + RNA (1 mg) + DEP (500 μM)	34	50
Enzyme + Mg^{2+} (5 mM) + DEP (500 μM)	15	35
Enzyme + Mn^{2+} (5 mM) + DEP (500 μM)	21	35
Enzyme + Co^{2+} (5 mM) + DEP (500 μM)	23	39

* The values given are the mean of three independent sets of experiments with S.D. less than 10%. For experimental details refer to Methods.

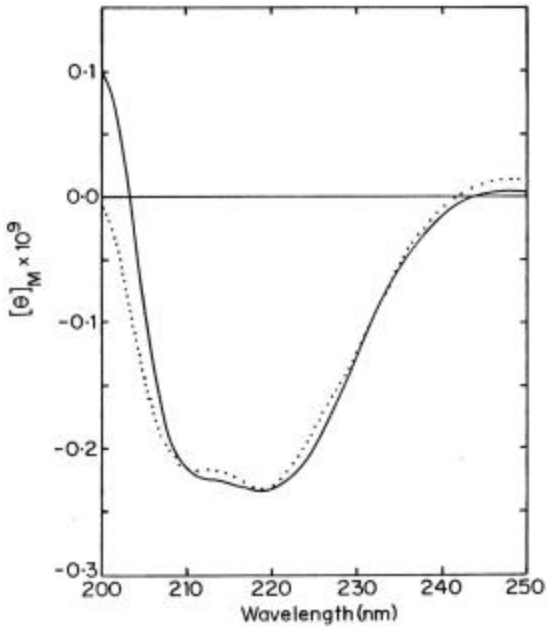

Fig. 4.4: The CD spectra of native and DEP modified nuclease Rsn. The CD measurements were performed in a 1 mm cell at an enzyme concentration of 75 µg/ml. Native enzyme (——) and DEP treated enzyme (....).

The above observations coupled with no change in the K_m values of DEP modified partly inactivated enzyme samples with a concomitant decrease in the k_{cat} (Table 4.4) suggest the involvement of histidine in catalysis. In case of P1 nuclease, histidine has been implicated in metal binding (Suck, 1992). However, fluorescence quenching studies on native and histidine modified enzyme samples with metal ions showed no difference in the extent of quenching. Additionally, no change in the emission maxima was observed for the native and histidine modified enzyme samples on titration with the metal ions. Furthermore, the dissociation constants of metal ions for native and histidine modified enzyme samples were comparable (Table 4.5), indicating that histidine residues are not involved in metal binding. The role of histidine in catalysis has also been shown in case of *S. marcescens* family nucleases (Vincent *et al.*, 1988; Friedhoff *et al.*, 1996a; Ho *et al.*, 1998; Ho and Liao., 1999; Meiss *et al.*, 2000) and S1 nuclease (Gite *et al.*, 1992a).

Table 4.4: K_m and k_{cat} values for DEP treated partially inactivated nuclease Rsn*

Activity	Residue modified	Residual activity (%)	K_m (mg/ml)	k_{cat} (min^{-1})
DNase	None	100	0.050 ± 0.003	4.53 ± 0.27 x 10^9
	Histidine	72		3.70 ± 0.13 x 10^9
		60		1.84 ± 0.21 x 10^9
		40		1.44 ± 0.09 x 10^9
RNase	None	100	1.54 ± 0.2	2.37 ± 0.18 x 10^8
	Histidine	67		1.34 ± 0.06 x 10^8
		53		1.03 ± 0.07 x 10^8
		37		0.83 ± 0.08 x 10^8

K_m and k_{cat} values were determined from Lineweaver-Burk plots.

* The values given are the mean of two independent sets of experiments with S.D. less than 10%.

Table 4.5: Fluorescence studies of native and modified nuclease Rsn*

Sample	λ_{max} (nm)	Quenching (%)	K_d values (mM)	
			Mg^{2+}	Co^{2+}
Native	335	47	6.37 + 0.77	9.72 + 2.52
Histidine modified	335	47	5.35 + 0.65	9.06 + 0.86
Tryptophan modified	335	47	8.72 + 1.40	8.35 + 1.15
Carboxylate modified	335	25	1.51 + 0.43	3.14 + 0.34

* The values given are the mean of two independent sets of experiments with S.D. less than 10%. For experimental details refer to Methods.

Modification of tryptophan residues

NBS modification of nuclease Rsn, at pH 5.0, resulted in approximately 80 – 90 % loss of its initial activity towards DNA and RNA and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. NBS mediated inactivation was accompanied by a decrease in the absorbance of the modified protein at 280 nm. Moreover, isobestic points around 260 nm and 295 nm coupled with an increase in absorption around 250 nm and 315 nm (Fig. 4.5) (Patchornik *et al*., 1958), suggested that the loss of activity

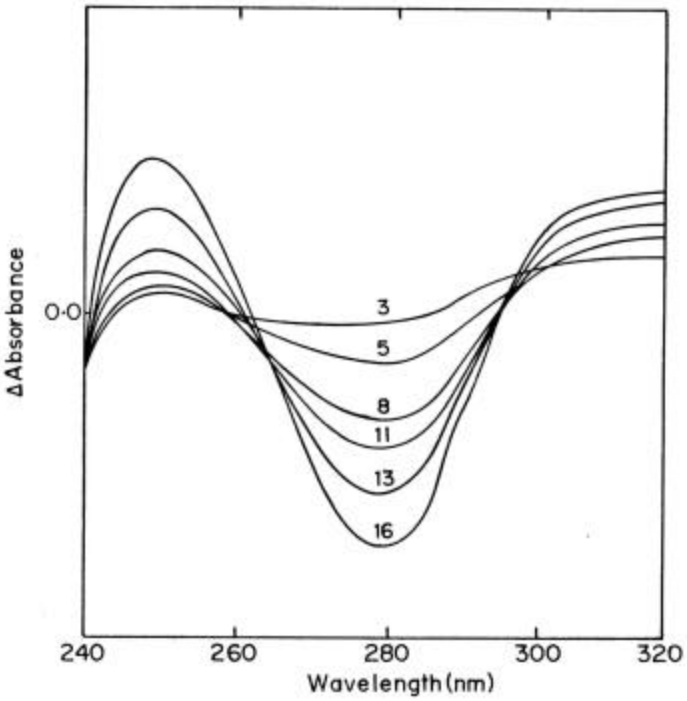

Fig. 4.5: Difference spectra of NBS modified nuclease Rsn. The number, on the spectra, indicates the fold excess of NBS added. For experimental details refer to Methods.

is due to tryptophan modification rather than cleavage. Based on a molar absorption coefficient of 5500 M^{-1} cm^{-1}, for tryptophan at 280 nm (Spande and Witkop, 1967) and the Mr of 67000 for nuclease Rsn, the total number of tryptophan residues modified were found to be 2.0. However, the plot of percent residual activity versus the number of tryptophan residues modified revealed

that the loss of activity towards DNA and RNA occurred due to the modification of a single tryptophan residue per molecule of the enzyme (Fig. 4.6). This observation suggests that tryptophan may have a role in the catalytic activity of nuclease Rsn.

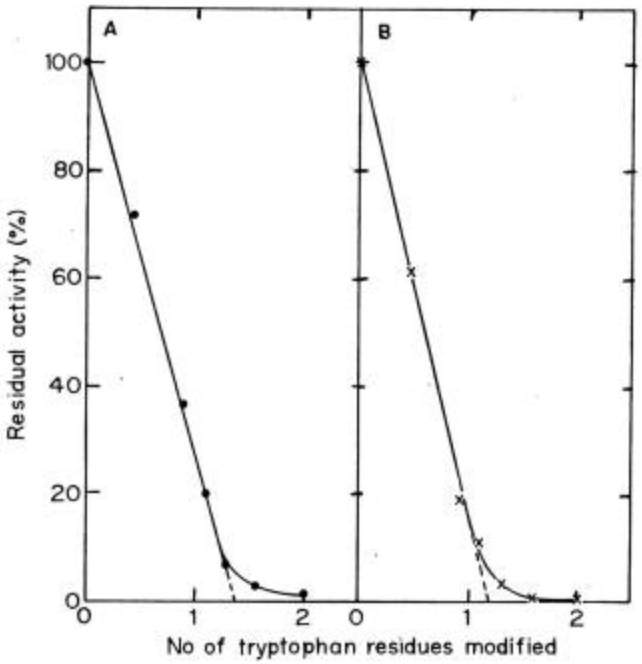

Fig. 4.6: Plot of percent residual activity versus number of tryptophan residues modified. DNase (A) and RNase (B). The number of tryptophan residues modified were determined as described under Methods.

Substrate protection studies showed that NBS mediated inactivation could be prevented by incubation of the enzyme with excess amounts of DNA and RNA, prior to the modification reaction. On the contrary, metal ion protection was not observed (Table 4.6). Moreover, NBS modification did not bring about any gross change in the enzyme structure (Fig. 4.7) indicating that the loss of enzyme activity is due to tryptophan modification rather than structural changes. Furthermore, NBS modified, partially inactivated enzyme samples showed an increase in the K_m but no change in the k_{cat} (Table 4.7), pointing towards the involvement of tryptophan in substrate

Table 4.6: Effect of tryptophan modification on the activity of nuclease Rsn: substrate and metal ion protection studies *

Incubation mixture	Residual activity (%)	
	DNase	RNase
Enzyme	100	100
Enzyme + NBS (1.25 μM)	12	17
Enzyme + DNA (1 mg) + NBS (1.25 μM)	73	96
Enzyme + RNA (1 mg) + NBS (1.25 μM)	75	88
Enzyme + Mg^{2+} (5 mM) + NBS (1.25 μM)	12	16
Enzyme + Mn^{2+} (5 mM) + NBS (1.25 μM)	11	17
Enzyme + Co^{2+} (5 mM) + NBS (1.25 μM)	12	15

* The values given are the mean of three independent sets of experiments with S.D. less than 10%. For experimental details refer to Methods.

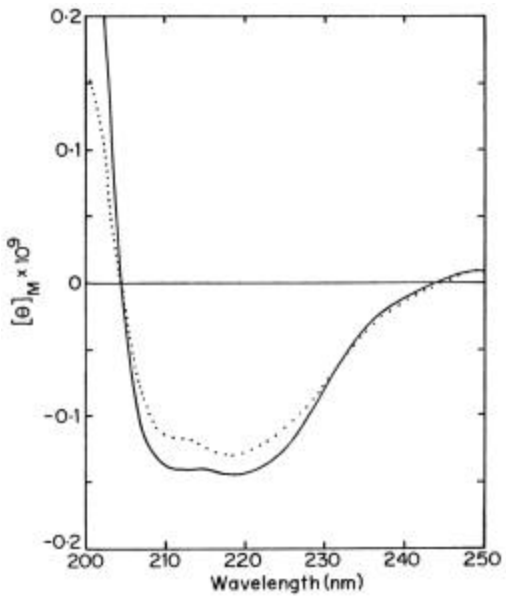

Fig. 4.7: The CD spectra of native and NBS modified nuclease Rsn. The CD measurements were performed in a 1 mm cell at an enzyme concentration of 75 μg/ml. Native enzyme (——), NBS treated enzyme (....).

binding. In case of P1 nuclease, tryptophan has been implicated in metal binding (Suck, 1992). However, in the present case, fluorescence quenching studies on native and tryptophan modified enzyme samples, with metal ions, showed a similar level of quenching with no apparent change in the emission maxima as well as the dissociation constants (Table 4.5) suggesting that tryptophan has no role in metal binding. Involvement of tryptophan in substrate binding / base stacking has also been demonstrated with *S. marcescens* nuclease (Meiss *et al.*, 1999) and RNase Rh (Ohgi *et al.*, 1996).

Table 4.7: K_m and k_{cat} values for NBS treated partially inactivated nuclease Rsn*

Activity	Residue modified	Residual activity (%)	K_m (mg/ml)	k_{cat} (min^{-1})
DNase	None	100	0.033 ± 0.001	2.14 ± 0.09 x 10^9
	Tryptophan	83	0.042 ± 0.001	
		60	0.071 ± 0.005	
		48	0.127 ± 0.015	
RNase	None	100	0.97 ± 0.06	0.97 ± 0.01 x 10^8
	Tryptophan	79	1.59 ± 0.08	
		60	2.29 ± 0.07	
		47	3.43 ± 0.57	

K_m and k_{cat} values were determined from Lineweaver-Burk plots.

* The values given are the mean of two independent sets of experiments with S.D. less than 10%.

Modification of carboxylate residues

Purified nuclease Rsn, when incubated with 25 mM EDAC at pH 6.0 for 15 min, lost about 50 - 60 % of its initial activity towards DNA and RNA and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in the control samples. Based on a molar absorption coefficient of 4600 $M^{-1}cm^{-1}$, for nitrotyrosyl groups at 430 nm (Pho *et al.*, 1977) and the Mr of 67000 for nuclease Rsn, the total number of carboxylate residues modified were found to be 2.8. However, the plot of percent residual activity versus the number of carboxylate residues modified revealed that the loss of activity towards DNA and RNA resulted from the modification of 4 and 2 residues, respectively per molecule of the enzyme (Fig. 4.8) indicating the probable involvement of carboxylate residues in the catalytic activity of nuclease Rsn. However Tsou's analysis, of the carboxylate modification data, gave a best fit when ' i ' = 2 for both the activities, suggesting that only two carboxylate residues are essential for the catalytic activity of the enzyme (Table 4.8).

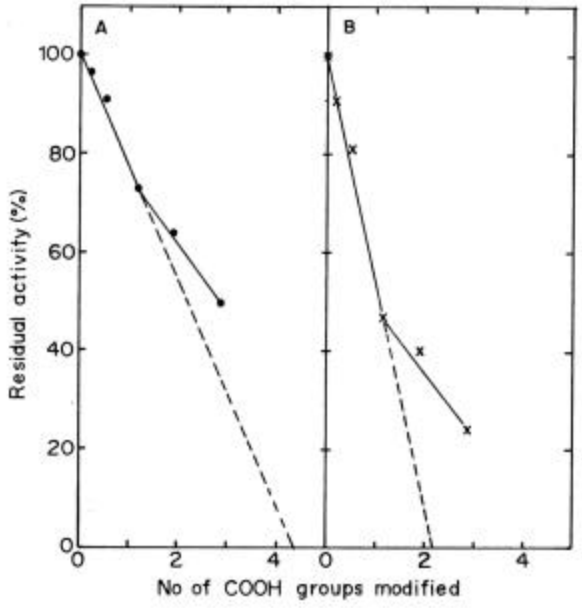

Fig. 4.8: Plot of percent residual activity versus number of carboxylate residues modified. DNase (A) and RNase (B). The number of carboxylate residues modified were determined as described under Methods.

Table 4.8: Analysis of the EDAC modification data according to Tsou*

Sample 'i' Value		Standard deviation (%)	
		DNase	RNase
Carboxylate modified	1	2.5 ± 0.2	6.6 ± 0.5
	2	1.1 ± 0.1	3.8 ± 0.2
	3	0.7 ± 0.1	2.9 ± 0.1

* The values given are the mean of two independent sets of experiments.

EDAC mediated inactivation could not be prevented by incubating the enzyme with excess amounts of DNA and RNA prior to the modification reaction. On the contrary, metal ions could prevent, to some extent, the EDAC mediated inactivation of the enzyme (Table 4.9).

Table 4.9: Effect of carboxylate modification on the activity of nuclease Rsn: substrate and metal ion protection studies *

Incubation mixture	Residual activity (%)	
	DNase	RNase
Enzyme	100	100
Enzyme + EDAC (25 mM)	18	8
Enzyme + DNA (1 mg) + EDAC (25 mM)	15	4
Enzyme + RNA (1 mg) + EDAC (25 mM)	14	10
Enzyme + Mg^{2+} (5 mM) + EDAC (25 mM)	47	32
Enzyme + Mn^{2+} (5 mM) + EDAC (25 mM)	55	39
Enzyme + Co^{2+} (5 mM) + EDAC (25 mM)	64	28

* The values given are the mean of three independent sets of experiments with S.D. less than 10%. For experimental details refer to Methods.

The CD spectra of unmodified and EDAC modified enzyme samples were almost identical suggesting that, the loss of activity is due to carboxylate modification rather than structural changes (Fig. 4.9).

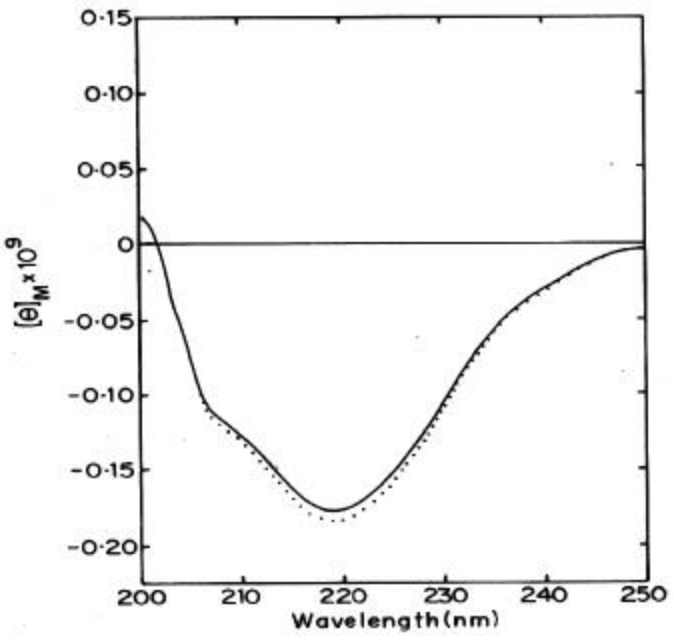

Fig. 4.9: The CD spectra of native and EDAC modified nuclease Rsn. The CD measurements were performed in a 1 mm cell at an enzyme concentration of 75 μg/ml. Native enzyme (——), NBS treated enzyme (....).

Additionally, determination of the K_m and k_{cat} values of partially inactivated enzyme samples showed an increase in the k_{cat} with no change in the K_m compared to the unmodified enzyme (Table 4.10). The above results point towards the involvement of carboxylate in catalysis. Fluorescence quenching studies on native and EDAC modified nuclease Rsn, with metal ions, revealed a significant difference in the level of quenching. EDAC modified enzyme samples were quenched approximately 25 % whereas the native enzyme was quenched approximately 47 %. Additionally, EDAC modified enzyme samples showed a significant decrease in the dissociation constant (approximately 3 - 4 fold) with respect to the native enzyme

Table 4.10: K_m and k_{cat} values for EDAC treated partially inactivated nuclease Rsn*

Activity	Residue modified	Residual activity (%)	K_m (μg/ml)	k_{cat} (min^{-1})
DNase	None	100	0.050 ± 0.002	$1.97 \pm 0.03 \times 10^9$
	Carboxylate	86		$1.62 \pm 0.01 \times 10^9$
		68		$1.25 \pm 0.05 \times 10^9$
		36		$0.72 \pm 0.01 \times 10^9$
RNase	None	100	1.25 ± 0.2	$1.57 \pm 0.02 \times 10^8$
	Carboxylate	82		$0.96 \pm 0.04 \times 10^8$
		67		$0.73 \pm 0.01 \times 10^8$
		34		$0.67 \pm 0.02 \times 10^8$

K_m and k_{cat} values were determined from Lineweaver-Burk plots.

* The values given are the mean of two independent sets of experiments with S.D. less than 10%.

(Table 4.5, Page 142). These observations suggest the involvement of carboxylate groups in metal binding. Similar observations have been made in case of nucleases from *S. aureus* (Cotton *et al.*, 1979), S1 nuclease (Gite and Shankar, 1992) and *S. marcescens* (Miller *et al.*, 1999), as well as nucleases from *Anabaena* sp. (Meiss *et al*., 2000) and shrimp hepatopancreas (Wang *et al.*, 2000). The involvement of carboxylate residues in catalysis as well as in metal binding suggests that the hydrolysis of DNA and RNA, by nuclease Rsn, probably occurs via metal ion mediated catalysis.

In conclusion, chemical modification studies, on nuclease Rsn, revealed that the active site of the enzyme consists of a substrate binding site, a hydrolytic site and a metal binding site. While

tryptophan is involved in substrate binding, histidine and carboxylate residues are involved in catalysis and metal binding respectively. Nuclease Rsn is a non-specific multifunctional endonuclease which shows an obligate requirement of metal ions for its activity. Nucleases, showing obligate requirement of metal ions for their activity, exhibit metal ion mediated mechanism for the hydrolysis of nucleic acids. In such enzymes, carboxylate groups have been shown to be involved in metal coordination (Horton *et al.*, 1998; Miller *et al.*, 1999). X-ray crystallographic studies, on type II restriction endonuclease EcoRV (a dimer), showed that the binding of metal ion occurs through two Asp and one Glu residues, which forms a common motif for this class of enzymes (Horton *et al.*, 1998). Moreover, in case of *S. marcescens* nuclease (a dimer), the interaction of Asn119 and Glu127 with Mg-water cluster was shown to be responsible for the catalytic activity of the enzyme. Furthermore, the residues involved in contact with water cluster coordination (required for the integrity of magnesium binding site), are conserved throughout in the *Serratia* endonuclease family (Miller *et al.*, 1999). The properties of nuclease Rsn viz. obligate requirement of metal ions (Mg^{2+}, Mn^{2+} or Co^{2+}), dimeric nature of the protein, substrate specificity and mode of action, closely resembles that of *S. marcescens* nuclease. Moreover chemical modification studies, on nuclease Rsn, showed the involvement of similar residues as that of *S. marcescens* nuclease in the catalytic activity of the enzyme. Hence it is reasonable to assume that, like *S. marcescens* nuclease, nuclease Rsn may also follow the metal-water cluster mediated mechanism for the hydrolysis of DNA and RNA.

GENERAL DISCUSSION

Nucleic acids, the vital biomolecules present in cell, act as carriers of genetic information. For the effective transfer of genetic information from one generation to another, they have to undergo processes such as replication, recombination and repair. Nucleases, the enzymes which hydrolyze the phosphodiester linkages in nucleic acids, participate in the aforementioned cellular events. Due to their ability to recognize a wide variety of nucleic acid structures, considerable efforts have been made to evaluate their role in different cellular processes as well as their application as analytical tools to study nucleic acid structure. Majority of these enzymes share common properties like multiple activity and metal ion requirement. Though these enzymes have been extensively studied with respect to their catalytic properties, very little attention was paid to their structure-function relationship. However, amino acid sequences of typical endonucleases from *S. marcescens*, *S. cerevisiae*, *B. taurus*, *C. echinulata var echinulata*, *S. racemosum*, *S. pombe*, *Anabaena* sp. and shrimp hepatopancreas showed significant homology in their primary structure. The comparison of primary sequences, conserved sequences and residues involved in the active site of non-specific endonucleases will advance our understanding on the evolutionary aspect of these enzymes. Moreover, detection, purification and extensive characterization of non-specific nucleases will not only provide a convenient tool to probe nucleic acid structures but also advance our knowledge on the phyicochemical characteristics and biological role of this class of enzymes.

Extracellular nuclease (nuclease Rsn) from *Rhizopus stolonifer* (NCIM 880) is a non-specific multifunctional enzyme and catalyzes the hydrolysis of ssDNA, dsDNA and RNA in a ratio of approximately 2:1:0.1. It is a tetramer of Mr 67 kDa and each protomer consists of two unidentical subunits of Mr 13 and 21 kDa. On isoelectric focussing, all the activities of the enzyme viz. ssDNase, dsDNase and RNase showed the same pI suggesting that, these are associated with the same protein.

The ssDNase, dsDNase and RNase activities of nuclease Rsn showed an obligate requirement of divalent cations like Mg^{2+}, Mn^{2+} and Co^{2+}. Moreover, the enzyme exhibited higher preference for ssDNA in presence of Co^{2+} whereas, the

dsDNase and RNase activities of the enzyme were not influenced by the type of metal ions used. Nuclease Rsn showed the same pH optima for the hydrolysis of ssDNA, dsDNA and RNA and it was not influenced by the type of metal ions used. Although, the optimum temperature of the enzyme for ssDNA and dsDNA hydrolysis was 40 °C, in presence of Mg^{2+}, it showed higher optimum temperature (45 °C) for dsDNA hydrolysis in presence of Mn^{2+} and Co^{2+}. However, the RNase activity of the enzyme showed an optimum temperature of 35 °C and it was not influenced by the type of metal ions used. Nuclease Rsn exhibited a similar sensitivity to metal chelators, inorganic phosphate and pyrophosphate.

Mode and mechanism of action showed that nuclease Rsn is an endonuclease and cleaves dsDNA through a single hit mechanism. The end products of both ssDNA and dsDNA hydrolysis were predominantly tri and tetranucleotides ending in 3' hydroxyl and 5' phosphoryl termini suggesting it to be a non-specific endonuclease. Moreover, the type of metal ions used did not influence the mode and mechanism of action of the enzyme. On the contrary, the RNase activity of nuclease Rsn cleaved RNA endonucleolytically with a strong preference for ApA linkages. However, guanylic acid linkages were resistant to cleavage.

The active site characterization of nuclease Rsn revealed that, it consists of a hydrolytic site, a substrate binding site and a metal ion binding site. While tryptophan is involved in substrate binding, histidine and carboxylate residues are involved in catalysis and metal binding, respectively.

In general, the properties of nuclease Rsn namely, dimeric nature, obligate requirement of metal ions, substrate specificity, mode of action and active site nature are comparable to *S. marcescens* family of non-specific endonucleases. Hence nuclease Rsn can be assigned to the class EC. 3.1.30.2.

REFERENCES

Anfinsen, C. B., Cuatrecasas, P. and Taniuchi, H. (1971) In: *The Enzymes* (Boyer, P. D. ed.) 3rd edn., **4**, pp. 177-204, Academic Press, New York.

Aparicio, J. F., Hardisson, C. and Sanchez, J. (1992) *Biochem. J.* **281**, 231-237.

Apte, P. S., Deshpande, P. S. and Shankar, V. (1993) *World J. Microbiol. Biotechnol.* **9**, 205-209.

Araki, T. (1903) *Z. Physiol. Chem.* **38**, 84-92.

Arnone, A., Bier, C. J., Cotton, F. A., Day, V. W., Hazen, E. E. Jr., Richardson, D. C., Richardson, J. S. and Yonath A. (in part) (1971) *J. Biol. Chem.* **246**, 2302-2316.

Arnott, S. and Selsing, E. (1974) *J. Mol. Biol.* **88**, 551-552.

Ball, T. K., Saurugger, P. N. and Benedik, M. J. (1987) *Gene* **57**, 183-192.

Ball, T. K., Sih, Y. and Benedik, M. J. (1992) *Nucleic Acids Res.* **20**, 4971-4974.

Bannikova, G. E., Blagova, E. V., Dementiev, A. A., Morgunova, E. Y., Mikchailov, A. M., Shlyapnikov, S. V., Varlamov, V. P. and Vainshtein, B. K. (1991) *Biochem. Mol. Biol. Int.* **24**, 813-822.

Bartok, K., Fraser, M. J. and Fareed, G. C. (1974) *Biochem. Biophys. Res. Commun.* **60**, 507-514.

Bauer, W. and Vinograd, J. (1974). In: *Basic Principles of Nucleic Acid Chemistry* (Cantoni, G.L. and Davis, D.R. eds) **2**, pp 265-303, Academic Press, New York.

Baumann, U., Frank, R., and Blöcker, H. (1986) *Eur. J. Biochem.* **161**, 409-413.

Bencen, G. H., Wei, C-F., Robberson, D. L. and Gray, H. B. Jr. (1984) J. *Biol. Chem*. **259**, 13584-13589.

Benedik, M. J. and Strych, U. (1998) *FEMS Microbiol. Lett.* **165**, 1-13.

Bernard, E. A. (1969) *Ann. Rev. Biochem.* **38**, 677-682.

Bernardi, A. and Bernardi, G. (1968) *Biochim. Biophys. Acta* **155**, 371-377.

Bernardi, G. (1973) *Methods Enzymol*. **27**, 471-479.

Bernardi, G. and Cordonnier, C. (1965) *J. Mol. Biol.* **11**, 141-143.

Bernardi, G. and Sadron, C. (1964) *Biochemistry* **3**, 1411-1418.

Biedermann, K., Jepsen, P. K., Riise, E. and Svendsen, I. (1989) *Carlsberg Res. Commun.* **54**, 17-27. (*Chem. Abstr*. 112:174481)

Blum, H., Beier, H. and Gross, H.J. (1987) *Electrophoresis* **8**, 93-99.

Boyd, J. B. and Mitchell, H.K. (1965) *Anal. Biochem.* **13**, 28-42.

Brown, D. R., Hurwitz, J., Reinberg, D. and Zipursky, S. L. (1985) In: *Nucleases* (Linn, S.M. and Roberts, R.J. eds.) 2nd edn., pp. 187-209, Cold Spring Harbor Laboratory Press, New York.

Brown, P. H. and Ho, T-H. D. (1986) *Plant. Physiol.* **82**, 801-806.

Brown, P. H. and Ho, T-H. D. (1987) *Eur. J. Biochem.* **168**, 357-364.

Bruice, T. W., Wise, J. G., Rosser, D. S. E. and Sigman, D. S. (1991) *J. Am. Chem. Soc.* **113**, 5446-5447.

Cal, S., Aparicio, J. F., De Los Reyes-Gavilan, C. G., Nicieza, R. G. and Sanchez, J. (1995) *Biochem. J.* **306**, 93-100.

Chacko, R., Deshpande, M. and Shankar, V. (1996) *Curr. Microbiol.* **32**, 246-251.

Chaiken, I. M. and Sanchez, G. R. (1972) *J. Biol. Chem.* **247**, 6743-6747.

Chen, C. B. and Sigman, D.S. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7147-7151.

Chen, C. B. and Sigman, D.S. (1987) *Science* **237**, 1197-1201.

Chen, C. B. and Sigman, D.S. (1988) *J. Am. Chem. Soc.* **110**, 6570-6572.

Chen, L-Y., Ho, H-C., Tsai, Y-C. and Liao, T-H. (1993) *Arch. Biochem. Biophys.* **303**, 51-56.

Chen, P. S. Jr., Toribara, T. Y. and Warner, H. (1956) *Anal. Chem.* **28**, 1756-1758.

Chen, Y-C., Suh, Y., Riise, E., Kartman, B., Jin, S. and Benedik, M. J. (1996) *Gene* **172**, 9-16.

Chesbro, W. R., Stuart, D. and Burke, J. J. II (1966) *Biochem. Biophys. Res. Commun.* **23**, 783-792.

Chinnathambi, S., Lachke, A. and Radhakrishnan, S. (1995) *J. Chromatogr. A.* **705**, 400-402.

Chou, M-Y. and Liao, T-H. (1990) *Biochim. Biophys. Acta* **1036**, 95-100.

Chow, T. Y-K. and Fraser, M. J. (1983) *J. Biol. Chem.* **258**, 12010-12018.

Chow, T. Y-K. and Resnick, M. A. (1987) *J. Biol. Chem.* **262**, 17659-17667.

Compton, M. M. and Cidlowski, J. A. (1987) *J. Biol. Chem.* **262**, 8288-8292.

Cordis, G. A., Goldblatt, P-J. and Deutscher, M. (1975) *Biochemistry* **14**, 2596-2693.

Côtè, J. and Ruiz-Carillo, A. (1993) *Science* **261**, 765-769.

Côtè, J., Renaud, J. and Ruiz-Carillo, A. (1989) *J. Biol. Chem.* **264**, 3301-3310.

Cotton, F. A. and Hazen, E. E. Jr. (1971) In: *The Enzymes* (Boyers, P. D. eds.) **3**, pp. 153-170, Academic Press, New York.

Cotton, F. A., Hazen, E. A. and Legg, M. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2551-2555.

Cuatrecasas, P., Edelhoch, H. and Anfinsen, C. B. (1967a) *Proc. Natl. Acad. Sci. USA* **58**, 2043-2050.

Cuatrecasas, P., Fuchs, S. and Anfinsen, C. B. (1967b) *J. Biol. Chem.* **242**,1541-1547.

Cuatrecasas, P., Fuchs, S. and Anfinsen, C. B. (1967c) *J. Biol. Chem.* **242**, 3063-3067.

Cuatrecasas, P., Fuchs, S. and Anfinsen, C. B. (1969a) *J. Biol. Chem.* **244**, 406-412.

Cuatrecasas, P., Wilchek, M. and Anfinsen, C. B. (1968) *Science* **162**, 1491-1493.

Cuatrecasas, P., Wilchek, M. and Anfinsen, C. B. (1969b) *Biochemistry* **8**, 2277-2284.

Cunningham, L., Catlin, B. W. and Privat de Gar'lhe, M. (1956) *J. Am. Chem. Soc.* **78**, 4642-4645.

Curtis, P. J., Burdon, M. G. and Smellie, R. M. S. (1966) *Biochem. J.* **98**, 813-817.

Dake, E., Hofmann, T. J., McIntire, S., Hudson, A. and Zassenhaus, H. P. (1988) *J. Biol. Chem*. **263**, 7691-7702.

Desai, N. A. and Shankar, V. (2000) *Eur. J. Biochem.* **267**, 5123-5135.

Dirksen, M. L. and Dekker, C. A. (1960) *Biochem. Biophys. Res. Commun.* **2**, 147-152.

Doerner, K.D. and White, B.A. (1990) *Anal. Biochem.* **187**, 147-150.

Doetsch, P.W., McGray, W.H. Jr. and Valenzuela, M.R.L. (1989) *Biochim. Biophys. Acta* **1007**, 309-317.

Drew, H. R. (1984) *J. Mol. Biol.* **176**, 535-557.

Eaves, G. N. and Jeffries, C. D. (1963) *J. Bacteriol.* **85**, 273-278.

Eley, J. and Roth, J. S. (1966) *J. Biol. Chem.* **241**, 3063-3069.

Felsenfeld, G. and Sandeen, G. (1962) *J. Mol. Biol.* **5**, 587-610.

Filiminova, M. N., Balaban, N. P., Sharipova, F. R. and Leshchinskaya, I. B. (1980) *Biokhimiya* **45**, 2097-2103.

Filiminova, M. N., Baratova, L. A., Vospel'nikova, M. D., Zheltova, A. O. and Leshchinskaya, I. B. (1981) *Biokhimiya* **46**, 1660-1666.

Filiminova, M. N., Dementyev, A. A., Leshchinskaya, I. B., Bakulina, G. Yu, and Shlyapnikov, S. V. (1991) *Biokhimiya* **50**, 508-520.

Filiminova, M. N., Garusov, A. V., Smetanina, T. A., Andreeva, M. A., Bogomolnaya, L. and Leshchinskaya, I. B. (1996) *Biochemistry (Moscow)* **61**, 1274-1278.

Fox, K. R. and Waring, M. J. (1987) *Biochim. Biophys. Acta* **909**, 145-155.

Frank, J. J., Hawk, J. A. and Levy, C. C. (1975) *Biochim. Biophys. Acta* **390**, 117-124.

Franke, I., Meiss, G. and Pingoud, A. (1999) *J. Biol. Chem.* **274**, 825-832.

Franke, I., Meiss, G., Blecher, D., Gimadutdinow, O., Urbanke, C. and Pingoud, A. (1998) *FEBS Lett.* **425**, 517-522.

Fraser, M. J. (1979) *Nucleic Acids Res.* **6**, 231-246.

Fraser, M. J. (1980) *Methods. Enzymol.* **65**, 255-263.

Fraser, M. J. and Low, R.L. (1993) In: *Nucleases* (Linn, S. M., Lloyd, R. S. and Roberts, R. J. eds.), 2nd edn., pp. 171-207, Cold Spring Harbor Laboratory Press, New York.

Fraser, M. J., Chow, T. Y-K., Cohen, H. and Koa, H. (1986) *Biochem. Cell Biol.* **64**, 106-116.

Fraser, M. J., Tjeerde, R. and Matsumoto, K. (1976) *Can. J. Biochem.* **54**, 971-980.

Friedhoff, P., Franke, I., Krause, K. L. and Pingoud, A. (1999a) *FEBS Lett.* **443**, 209-214.

Friedhoff, P., Franke, I., Meiss, G., Wende, W., Krause, K. L. and Pingoud, A. (1999b) *Nature Struct. Biol.* **6**, 132-133.

Friedhoff, P., Gimadutdinow, O. and Pingoud, A. (1994a) *Nucleic Acids Res.* **22**, 3280-3287.

Friedhoff, P., Gimadutdinow, O., Rüter, T., Wende, W., Urbanke, C., Thole, H. and Pingoud, A. (1994b) *Protein Expr. Purif.* **5**, 37-43. (*Chem. Abstr.* 120:185847)

Friedhoff, P., Kolmes, B., Gimadutdinow, O., Wende, W., Krause, K.L. and Pingoud, A. (1996a) *Nucleic Acids Res.* **24**, 2632-2639.

Friedhoff, P., Meiss, G., Kolmes, B., Pieper, U., Gimadutdinow, O., Urbanke, C. and Pingoud, A. (1996b) *Eur. J. Biochem.* **241**, 572-580.

Fujimoto, M., Fujiyama, K., Kuninaka, A. and Yoshino, H. (1974) *Agric. Biol. Chem*., **38,** 777-783.

Fujimoto, M., Kuninaka, A. and Yoshino, H. (1975) *Agric. Biol. Chem*. **39**, 1991-1997.

Gaido, M. L. and Cidlowski, J. A. (1991) *J. Biol. Chem.* **266**, 18580-18585.

Galcheva-Gargova, Z., Davidov, V. and Dessev, G. (1985) *Arch. Biochem. Biophys.* **240**, 464-469.

Geiduschek, E. P. and Daniels, A. (1965) *Anal. Biochem.* **11**, 133-136.

Gite, S. and Shankar, V. (1992) *Eur. J. Biochem.* **210**, 437-441.

Gite, S. U. and Shankar, V. (1995) *Crit. Rev. Microbiol.* **21**, 101-122.

Gite, S., Reddy, G. and Shankar, V. (1992a) *Biochem. J.* **285**, 489-494.

Gite, S., Reddy, G. and Shankar, V. (1992b) *Biochem. J.* **288**, 571-575.

Gray, H. B. Jr. and Lu, T. (1993) In: *Methods in Molecular Biology* (Burrel, M. M. ed.) **16**, pp. 231-251, Humana Press, Totowa.

Gray, H. B. Jr., Ostrander, D. A., Hodnett, J. L., Legerski, R. J. and Robberson, D. L. (1975) *Nucleic Acids Res.* **2**, 1459-1492.

Gray, H. B. Jr., Winston, T. P., Hodnett, J. L., Legerski, R. J., Nees, D. W., Wei, G F. and Robberson, D. L. (1981) In: *Gene Amplification and Analysis* (Chirikjian, J. G. and Papas, T. S. eds.) **2**, pp. 169-203, Elservier/North Holland, New York.

Grissom, C. B. and Markley, J. L. (1989) *Biochemistry* **28**, 2116-2124.

Gudding, R. (1980a) *Acta Vet. Scand*. **21**, 229-241 (*Chem. Abstr*.93:163241).

Gudding, R. (1980b) *Acta Vet. Scand*. **21**, 267-277 (*Chem.Abstr*. 93:110305).

Hale, S. P., Poole, L. B. and Gerlt, J. A. (1993) *Biochemistry* **32**, 7479-7487.

Hall, J., Hüsken, D. and Häner, R. (1996) *Nucleic Acids Res.* **24**, 3522-3526.

Harosh, I., Mezzina, M., Harris, P. V. and Boyd, J. B. (1992) *Eur. J. Biochem.* **210**, 455-460.

Hatahet, Z. and Fraser, M. J. (1989) *Biochem. Cell. Biol.* **67**, 632-641.

Hedrick, J. L. and Smith, A. J. (1968) *Arch. Biochem. Biophys.* **126**, 155-164.

Hegg, E. L. and Burstyn, J. N. (1998) *Coord. Chem. Rev.* **173**, 133-165.

Heins, J. N., Suriano, J. R., Taniuchi, H. and Anfinsen, C. B. (1967) *J. Biol. Chem.* **242**, 1016-1020.

Hejazi, A. and Falkiner, F.R. (1997) *J. Med. Microbiol*. **46**, 903-912 (*Chem. Abstr.* 128: 20317).

Hess, D. (1975) In: *Genetic Manipulations with Plant Material* (Ledoux, L. ed.) pp. 519-537, Plenum Press, New York and London.

Hess, D. (1987) In: *Pollen Development and Cytology* (Giles, K. L. and Prakash, J. eds.), pp. 367-395, Academic Press, New York.

Hibler, D. W., Stolowich, J. N., Reynolds, M. A., Gerlt, J. A., Wilde, J. A. and Bolton, P. H. (1987) *Biochemistry* **26**, 6278-6286.

Ho, H-C. and Liao, T-H. (1999) *Biochem. J.* **339**, 261-267.

Ho, H-C., Shiau, P-F., Liu, F-C., Chung, J-G. and Chen, L-Y. (1998) *Eur. J. Biochem.* **256**, 112-118.

Holloman, W. K., Rowe, T. C. and Rusche, J. R. (1981) *J. Biol. Chem*. **256**, 5087-5094.

Holstein, S. E. H., Kobert, B, Hillmer, S, Brown, P. H., Ho, T-H. D. and Robinson, D. G. (1991) *Physiol. Plant.* **83**, 255-265.

Horney, D.L. and Webster, D.A. (1971) *Biochim. Biophys. Acta* **247**, 54-61.

Horton, N. C., Newberry, K. J. and Perona, J. J. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 13489-13494.

Hutton, J. R. and Wetmur, J. G. (1975) *Biochem. Biophys. Res. Commun*. **66**, 942-948.

Igawa, T., Sumaoka, J. and Komiyama, M. (2000) *Nucleosides Nucleotides* **19**, 891-902.

Ikeda, S., Maeda, N., Ohshima, T. and Takata, N. (1996) *Biochem. Mol. Biol. Int.* **40**, 1017-1024.

Imagawa, H., Toryu, H., Ozawa, T. and Takino, Y. (1982) *Agric. Biol. Chem.* **46**, 1261-1269.

Irie, M. (1967) *J. Biochem. (Tokyo)* **62**, 509-518.

Irisawa, M. and Komiyama, M. (1995) *J. Biochem. (Tokyo)* **117**, 465-466.

Ito, K., Matsuura, Y. and Miniamura, N. (1994) *Arch. Biochem. Biophys.* **309**, 160-167.

Iwanoff, L. (1903) *Z. Physiol. Chem.* **39**, 31.

Iwasaki, M., Igarashi, H. and Yutsudo, T. (1997) *Microbiol.* **143**, 2449-2455.

Janski, A. M. and Oleson, A. E. (1976) *Anal. Biochem.* **71**, 471-480.

Jeferies, C.D., Holloman, D.F. and Guse, D.G. (1957) *J. Bacteriol.* **73**, 5690- 5698.

Joseph, D.R. and Stafford, D.W. (1976) *Biochim. Biophys. Acta.* **335**, 173-184.

Judice, J. K., Gamble, T. R., Murphy, E. C., de Vos, A. M. and Schultz, P. G. (1993) *Science* **261**, 1578-1581.

Kacian, D. L. and Spiegelman, S. (1974) *Anal. Biochem.* **58**, 534-540.

Kanamori, N., Cozzarelli, N. R. and Okazaki, R. (1973a) *Biochim. Biophys. Acta* **335**, 173-184.

Kanamori, N., Sakabe, K. and Okazaki, R. (1973b) *Biochim. Biophys. Acta* **335**, 155-172.

Kasai, K. and Grunberg-Manago, M. (1967) *Eur. J. Biochem*. **1**, 152-163.

Kawata, Y., Sakiyama, F., Hayashi, F. and Kyogoku, Y. (1990) *Eur. J. Biochem.* **187**, 255-262.

Khorana, H. G. (1961) In: *The Enzymes* (Boyer, P. D., Lardy H. and Myrback, K. eds.) **5**, pp. 79-94, Academic Press, New York.

Kilpatrick, M. W., Wei, C. F., Gray, H. B. Jr. and Wells, R. D. (1983) *Nucleic Acids Res.* **11**, 3811-3822.

Koa, H., Fraser, M. J. and Kafer, E. (1990) *Biochem. Cell Biol.* **68**, 387-392.

Kobayashi, H., Inokuchi, N., Koyama, T., Tomita, M. and Irie, M. (1995) *Biosci. Biotechnol. Biochem*. **59**, 1169-1171.

Kohen, R., Szyf, M. and Chevion, M. (1986) *Anal. Biochem*. **154**, 485-491.

Kolmes, B., Franke, I., Friedhoff, P. and Pingoud, A. (1996) *FEBS Lett*. **397**, 343-346.

Komiyama, M. (1995) *J. Biochem. (Tokyo)* **118**, 663-670.

Komiyama, M., Matsumoto, Y., Hayashi, N., Matsumura, K., Takeda, N. and Watanabe, K. (1993) *Polym. J.* **25**, 1211-1214. (*Chem. Abstr*. 120:185530)

Komiyama, M., Shiiba, T., Takahashi, Y., Takeda, N., Matsumura, K. and Kodama, T. (1994a) *Supramol. Chem.* **4**, 31-34. (*Chem. Abstr*. 123:106465)

Komiyama, M., Takeda, N., Shiiba, T., Takahashi, Y., Matsumoto, Y. and Yahsiro, M. (1994b) *Nucleosides Nucleotides* **13**, 1297-1309.

Kouidou, S. A., Triantos, A. A., Papageorgiou, G. R., Kavoukopoulos, E. M., Vavatsi, N. A. and Trakatellis, A. C. (1987) *Int. J. Biochem*. **19**, 859-864.

Krämer, R. (1999) *Coord. Chem. Rev.* **182**, 243-261. (*Chem. Abstr*. 130:308201)

Kroeker, W. D., Hanson, D. M. and Fairley, J. L. (1975) *J. Biol. Chem*. **250**, 3767-3772.

Krupp, G. and Gross, H. J. (1979) *Nucleic Acids Res.* **6**, 3481-3490.

Kuligowska, E., Klarkowska, D. and Szarkowski, J. W. (1988) *Phytochemistry* **27**, 1275-1279.

Kunitz, M. (1940) *J. Gen. Physiol.* **24**, 15-30.

Kunitz, M. (1950) *J. Gen. Physiol.* **33**, 349-355.

Kwong, S. and Fraser, M. J. (1978) *Can. J. Biochem.* **56**, 370-377.

L'vova, T. H., Avrosimova-amel-ianchik, N. M. and Tatarskaia, R. I. (1976) *Biokimiya* **41**, 2077-2081.

Laemmli, U.K. (1970) *Nature (London)* **227**, 680-685.

Laskowski, M. (1959) *Ann. N.Y. Acad. Sci.* **81**, 776-783.

Laskowski, M. and Seidel, M.D. (1945) *Arch. Biochem.* **7**, 465-472.

Laskowski, M. Sr. (1967) *Adv. Enzymol.* **29**, 165-220.

Laskowski, M. Sr. (1982) In: *Nucleases* (Linn, S. and Roberts, R. J. eds.), pp. 1-21, Cold Spring Harbor Press, New York.

Lau, P. P. and Gray, H. B. Jr. (1979) *Nucleic Acids Res.***6**, 331-357.

Legerski, R. J., Hodnett, J. L. and Gray, H. B. Jr. (1978) *Nucleic Acids Res.* **5**, 1445-1464.

Levy, C. C., Hieter, P. A. and LeGendre, S. M. (1974) *J. Biol. Chem*. **249**, 6762-6769.

Liao, T-H. (1975) *J. Biol. Chem.* **250**, 3721-3724.

Libson, A. M., Gittis, A. G. and Lattman, A. E. (1994) *Biochemistry* **33**, 8007-8016.

Lin, J., Wang, W. and Liao, T. (1994) *Biochim. Biophys. Acta* **1209**, 209-214.

Linn, S. (1967) *Methods Enzym ol.* **12A**, 247-255.

Linn, S. (1982) In: *Nucleases* (Linn, S. and Roberts, R. J. eds.) pp. 355-370, Cold Spring Harbor Press, New York.

Linn, S. and Lehman, I. R. (1965a) *J. Biol. Chem.* **240**, 1287-1293.

Linn, S. and Lehman, I. R. (1965b) *J. Biol. Chem.* **240**, 1294-1304.

Linn, S. and Lehman, I. R. (1966) *J. Biol. Chem.* **241**, 2694-2699.

Loll, P. J. and Lattman, E. E. (1989) *Proteins: Struct. Funct. Genet.* **5**, 183-201.

Loll, P. J. and Lattman, E. E. (1990) *Biochemistry* **29**, 6866-6873.

Loll, P. J., Quirk, S., Lattman, E. E. and Garavito, R. M. (1995) *Biochemistry* **34**, 4316-4324.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275.

Lu, T. and Gray, Jr., H. B. (1995) *Biochim. Biophys. Acta* **1251**, 125-138.

Lundblad, R. L. (1991) In: *Chemical reagents for protein modification*.(Lundblad, R. L. ed.) 2nd edn., pp. 105-128, CRC Press Inc., Boca Raton.

Lunin, V.Y., Levdikov, V.M., Shlyapnikov, S.V., Blagova, E.V., Lunin, V.V., Wilson, K.S. and Mikhailov, A.M. (1997) *FEBS Lett.* **412**, 217-222.

Maeda, M. and Taga, N. (1976) *Can. J. Biochem.* **22**, 1443-1452.

Marks, A. and Spencer, J.H. (1970) *J. Mol. Biol.* **51**, 115-130.

Martin, C. E. and Wagner, R. P. (1975) *Can. J. Biochem.* **53**, 823-825.

Martin, S. A., Ullrich, R. C. and Meyer, W. L. (1986) *Biochim*. *Biopys. Acta* **867**, 67-75.

Matousek, J. and Tupy, J. (1984) *Biol. Plant.* **26**, 62-73.

Matousek, J., Trnena, L., Oberhauser, R., Lichtenstein, C.P. and Nellen, Wolfgang (1996) *Biol. Chem. Hoppe-Seyler* **375**, 261-269.

Matsumoto, Y. and Komiyama, M. (1992) *Chem. Express*. **7**, 785-788. (*Chem. Abstr*. 118:119104)

McCall, M., Brown, T. and Kennard, O. (1985) *J. Mol. Biol.* **78**, 453-471.

Means, G. E. and Feeney, R. E. (1971) In: *Chemical modification of proteins* (Means, G. E. and Feeney, R. E.) pp 214-230. Holden-Day, Inc., California.

Mehdi, S. and Gerlt, J. A. (1982) *J. Am. Chem. Soc*. **104**, 3223-3225.

Mehra, U. and Ranjekar, P.K. (1979) *Ind. J. Biochem. Biophys.* **16**, 56-60.

Meiss, G., Franke, I., Gimadutdinow, O., Urbanke, C. and Pingoud, A. (1998) *Eur. J. Biochem.* **251**, 924-934,

Meiss, G., Friedhoff, P., Hahn, M., Gimadutdinow, O. and Pingoud, A. (1995) *Biochemistry* **34**, 11979-11988.

Meiss, G., Gast, F-U. and Pingoud, A. M. (1999) *J. Mol. Biol.* **288**, 377-390.

Meiss, G., Gimadutdinow, O., Haberland, B. and Pingoud, A. (2000) *J. Mol. Biol.* **297**, 521-534.

Melchoir, W. B. Jr. and Fahrney, D. (1970) *Biochemistry* **9**, 251-258.

Melgar, E. and Goldthwait, D. A. (1968) *J. Biol. Chem.* **243**, 4409-4416.

Mikulski, A. J., Sulkowski, E., Stasiuk, L. and Laskowski, Sr., M. (1969) *J. Biol. Chem.* **244**, 6559-6565.

Miles, E. W. (1977) *Methods Enzymol.* **47**, 431-442.

Miller, M. and Krause, K. (1996) *Protein Sci.* **5**, 24-33.

Miller, M. D., Cai, J. and Krause, K. L. (1999) *J. Mol. Biol.* **288**, 975-987.

Miller, M. D., Tanner, J., Alpaugh, M., Benedik, M. J. and Krause, K. L. (1994) *Nat. Struct. Biol.* **1**, 461-468.

Mishra, N.C. (1995) in *Molecular Biology of Nucleases,* (Mishra, N.C. ed) pp 1-21.CRC Boca Raton, Florida.

Mittra, B., Sadhukhan, P. K. and Majumder, H. K. (1998) *J. Biochem (Tokyo)* **124**, 1198-1205.

Monko, M., Kuligowska, E. and Szarkowski, J.W. (1994) *Phytochemistry* **37**, 301-305.

Montague, J. W., Gaido, M. L., Frye, C. W. and Cidlowski, J. A. (1994) *J. Biol. Chem*. **269**, 18877-18880.

Montague, J. W., Hughes, F. M. Jr. and Cidlowski, J. A. (1997) *J. Biol. Chem*. **272**, 6677-6684.

Moore, S. (1981) In: *The Enzymes* (Boyer, P.D. ed.), **14**, pp. 281-296, Academic Press, New York.

Morosoli, R. and Lusena, C. V. (1980) *Eur. J. Biochem.* **110**, 431-437.

Muro-Pastor, A. M., Flores, E., Herrero, A. and Wolk, C. P. (1992) *Mol. Microbiol.* **6**, 3021-3030.

Nestle, M. and Roberts, W. K. (1969a) *J. Biol. Chem.* **244**, 5213-5218.

Nestle, M. and Roberts, W. K. (1969b) *J. Biol. Chem.* **244**, 5219-5225.

Neu, H.C. and Heppel, L.A. (1964) *J. Biol. Chem* . **239**, 2927-2934.

Nicieza, R. G., Huergo, J., Connolly, B. A. and Sanchez, J. (1999) *J. Biol. Chem.* **274**, 20366-20375.

Nomura, A., Suno, M. and Mizuno, Y. (1971) *J. Biochem. (Tokyo)* **70**, 993-1001.

Nunez, M., Bautista, L., Medina, M., and Gaya, P. (1988) *J. Appl. Bacteriol.* **65**, 29-34(*Chem. Abstr*. 110: 6595).

Ohgi, K., Takeuchi, M., Iwama, M. and Irie, M. (1996) *J. Biochem (Tokyo)* **119**, 9-15.

Oleson, A. E. and Hoganson, E. D. (1981) *Arch. Biochem. Biophys.* **211**, 478-484.

Oleson, A. E. and Sasakuma, M. (1980) *Arch. Biochem. Biophys.* **204**, 361-370.

Ovadi, J., Libor, S. and Elodi, P. (1967) *Acta Biochim. Biophys. (Budapest)* **2**, 455-458.

Patchornik, A., Lawson, W. B. and Witkop, B. (1958) *J. Am. Chem. Soc.* **80**, 4747-4748.

Pedersen, J., Andersen, J., Roepstorff, P., Filimonova, M. and Biedermann, K. (1993a) *Biotechnol. Appl. Biochem* . **18**, 389-399.

Pedersen, J., Filiminova, M. N., Roepstorff, P. and Biedermann, K. (1995a) *Biochemistry (Moscow)* **60**, 450-461.

Pedersen, J., Filiminova, M. N., Roepstorff, P. and Biedermann, K. (1995b) *Bioorg. Khim*. **21**, 336-344.

Pedersen, J., Filimonova, M., Roepstroff, P. and Biedermann, K. (1993b) *Biochim. Biophys. Acta* **1202**, 13-21.

Pho, D. B., Roustan, C., Tot, A. N. T. and Pradel, L-A. (1977) *Biochemistry* **16**, 4533-4537.

Pollack, Y., Kasir, J., Shemer, R., Metzger, S., and Szyf, M. (1984) *Nucleic Acids Res.* **12**, 4811-4824.

Potter, B. V. L., Romaniuk, P. J. and Eckstein, F. (1983) *J. Biol. Chem.* **258**, 1758-1760.

Poulson, R. (1973) In: *The Ribonucleic Acids* (Stewart P. R., and Letham, D. S. eds.) pp. 253-256, Springer-Verlag, Berlin.

Pritchard, A.E., Kowalski, D. and Laskowski, M. Sr. (1977) *J. Biol. Chem.* **252**, 8652-8659.

Privat *de* Garhille, M. and Laskowski, N. (1956) *J. Biol. Chem.* **223**, 661-680.

Przykorska, A. K., Hauser, C. R. and Gray, Jr., H. B. (1988) *Biochim. Biophys. Acta* **949**, 16-26.

Przykorska, A., Kuligowska, E., Gerhart, E., Wagner, H., Szarkowksi, J. W. and Nordstrom, K. (1989) *Biochemistry* **28**, 1585-1588.

Rangarajan, S., Chacko, R. and Shankar, V. (1999) *Biochim. Biophys. Acta* **1428**, 372-380.

Razzell, W. E. (1963) *Methods Enzymol.* **6**, 236-258.

Razzell, W. E. and Khorana, W. E. (1959) *J. Biol. Chem.* **234**, 2105-2113.

Razzell, W. E. and Khorana, W. E. (1961) *J. Biol. Chem.* **236**, 1144-1149.

Richardson, R.M., Pares, X. and Cuchillo, C.M. (1990) *Biochem. J.* **267**, 593-599.

Riordan, J.F. (1979) *Mol. Cell. Biochem* . **26**, 71-92.

Romier, C., Domingues, R, Lahm, A., Dahl, O. and Suck, D. (1998) *Proteins: Struct. Funct. Genet.* **32**, 414-424.

Rosenthal, A. L. and Lacks, S. A. (1977) *Anal. Biochem.* **80**, 76-90.

Roth, J. S. and Milstein, S. W. (1952) *J. Biol. Chem.* **196**, 489-500.

Ruiz-Carrillo, A. and Renaud, J. (1987) *EMBO J.* **6**, 401-407.

Rusche, J. R., Rowe, T. C. and Holloman, W. K. (1980) *J. Biol. Chem.* **255**, 9117-9123.

Sachs, D. H., Berzofsky, J. A., Pisetsky, D. S. and Schwartz, R. H. (1978) *Springer Semin. Immunopathol.* **1**, 51-83 (*Chem. Abstr*. 89:105550).

Sakaguchi, R., Joho, K. and Shishido, K. (1985) *FEBS Lett.* **191**, 59-62.

Sanda, A. and Irie, M. (1980) *J. Biochem. (Tokyo)* **87**, 1079-1087.

Sanda, A., Takizawa, Y. and Irie, M. (1985) *Chem. Pharm. Bull.* **33**, 4515-4521.

Schmidt, G. (1955) In: *The Nucleic Acids* (Chargaff, E. and Davidson, J. N. eds.) **1**, pp. 555-626, Academic Press, New York.

Schumaker, V. N., Richards, E. G. and Schachman, H. K. (1956) *J. Am. Chem. Soc.* **78**, 4230-4236

Serpersu, E. H., Hibler, E. W., Gerlt, J. A. and Mildvan, A. S. (1989) *Biochemistry* **28**, 1539-1548.

Serpersu, E. H., McCracken, J., Peisach, J. and Mildvan, A. S. (1988) *Biochemistry* **27**, 8034-8044.

Serpersu, E. H., Shortle, D. and Mildvan, A. S. (1986) *Biochemistry* **25**, 68-77.

Serpersu, E. H., Shortle, D. and Mildvan, A. S. (1987) *Biochemistry* **26**, 1289-1300.

Shapiro, J., Machattie, L., Eron, L., Ihler, G., Ippen, K. and Beckwith, J. (!969) *Nature (London)* **224**, 768-774.

Shiebel, E., Shwarz, H. and Braun, V. (1989) *J. Biol. Chem.* **264**, 16311-16320.

Shiiba, T., Yonezawa, K., Takeda, N., Matsumoto, Y., Yashiro, M. and Komiyama, M. (1993) *J. Mol. Catal.* **84**, L21-L25. (*Chem. Abstr.* 120:26249)

Shimada, H., Inokuchi, N., Koyama, T., and Irie, M. (1991) *Chem. Pharm. Bull.* **39**, 2633-2637.

Shishido, K. (1985) *Biochim. Biophys. Acta* **826**, 147-150.

Shishido, K. and Ando, T. (1985) In: *Nucleases* (Linn, S.M. and Roberts, R.J. eds.), pp. 155-185, Cold Spring Harbor Laboratory Press, New York.

Shishido, K. and Habuka, N. (1986) *Biochim. Biophys. Acta* **884**, 215-218.

Shlyapnikov, S. V., Lunin, V. V., Perbandt, M., Polyakov, K. M., Lunin, V. Y., Levdikov, V. M., Betzel, C. and Mikhailov, A. M. (2000) *Acta Crystallogr. D Biol. Crystallogr.* **56**, 567-72.

Shuai, K., Das Gupta, C. K., Hawley, R. S., Chase, J. W., Stone, K. L. and Williams, K. R. (1992) *Nucleic Acids Res.* **20**, 1379-1385.

Sigman, D. S., Bruice, T. W., Mazumder, A. and Sutton, C. L. (1993) *Acc. Chem. Res.* **26**, 98-104 (*Chem. Abstr.* 118: 120100).

Sigman, D. S., Graham, D. R., D'Aurora,V., and Stern, A. M. (1979) *J. Biol. Chem.* **254**, 12269-12272.

Sissi, C., Mancin, F., Palumbo, M., Scrimin, P., Tecilla, P. and Tonellato, U. (2000) *Nucleosides Nucleotides* **19**, 1265-1271.

Siwecka, M. A. (1997) *Acta. Biochim. Polon.* **44**, 61-68.

Siwecka, M. A., Rytel, M. and Szarkowski, J.W. (1989) *Acta Biochim. Polon.* **36**, 45-62.

Sluka, J. P., Horvath, S. J., Bruist, M. F., Simon, M. I. and Dervan, P. B. (1987) *Science* **238**, 1129-1132.

Spande, T. F. and Witkop, B. (1967) *Methods Enzymol.* **11**, 498-506.

Srivastava, T. K., Friedhoff, P., Pingoud, A. and Katti,, S. B. (1999) *Nucleosides Nucleotides* **18**, 1945-1960.

Stanczyk, S. M. and Bolton, P. M. (1992) *Biochemistry* **31**, 6396-6401.

Steffens, J. J., Siewers, I. J., Benkovic, S. J. (1975) *Biochemistry* **14**, 2431-2340.

Stersky, A. K., Szabo, R., Todd, E. C. D., Thacker, C., Dickie, N. and Akhtar, M. (1986) *J. Food Prot.* **49**, 428-435 (*Chem.Abstr*. 105:77691).

Stevens, A. and Hilmoe, R. J. (1960a) *J. Biol. Chem.* **235**, 3016-3022.

Stevens, A. and Hilmoe, R. J. (1960b) *J. Biol. Chem.* **235**, 3023-3027.

Stoscheck, C.M. (1990) *Methods Enzymol.* **182**, 50-68.

Strickland, J. A., Marzilli, L. G., Puckett, J. M., Jr. and Doetsch, P. W. (1991) *Biochemistry* **30**, 9749-9756.

Suck, D. (1992) In: *Molecular Structure and Life* (Kyogoku, Y. and Nishimura, Y. eds.), pp. 141-156, Japan Sci. Soc. Press, Tokyo.

Sulkowski, E. and Laskowski, M. Sr. (1968) *J. Biol. Chem.* **243**, 4917-4921.

Sundaram, S. P., Kumari, S. L. S. and Murthy, K. V. (1982) *Indian J. Med. Res.* **75**, 19-22 (*Chem. Abstr*. 96: 177612).

Suno, M., Nomura, A. and Mizuno, Y. (1973) *J. Biochem. (Tokyo)* **73**, 1291-1297.

Taniuchi, H. and Anfinsen, C. B. (1968) *J. Biol. Chem.* **243**, 4778-4786.

Taniuchi, H. and Bohnert, J. L. (1975) *J. Biol. Chem.* **250**, 2388-2394.

Taniuchi, H., Anfinsen, C.B. and Sodja, A. (1967) *J. Biol. Chem.* **242**, 4752-4758.

Thomas, C. A. Jr. (1956) *J. Am. Chem. Soc*. **78**, 1861-1868.

Tomkinson, A. E. and Linn, S. (1986) *Nucleic Acids Res.* **14**, 9579-9593.

Tomlinson, R. V. and Tener, G. M. (1963) *Biochemistry* **2**, 967-702.

Tomoyeda, M., Eto, Y. and Yoshino, T. (1969) *Arch. Biochem. Biophys.* **131**, 191-202.

Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Tsou, C-L. (1962) *Sci. Sin.* **11**, 1535-1558.

Tucker, P. W., Hazen, E. E. Jr. and Cotton, F. A. (1978) *Mol. Cell. Biochem.* **22**, 67-77 .

Tucker, P. W., Hazen, E. E. Jr. and Cotton, F. A. (1979a) *Mol. Cell. Biochem.* **23**, 3-16.

Tucker, P. W., Hazen, E. E. Jr. and Cotton, F. A. (1979b) *Mol. Cell. Biochem.* **23**, 67-86.

Uhlmann, E. and Smith, J.A. (1987) *Nucleosides Nucleotides* **6**, 331-334.

van der Westhuizen, A. J., Gliemeroth, A. K., Wenzel, W. and Hess, D. (1987) *J. Plant. Physiol.* **131**, 373-384.

Vincent, R. D., Hofmann, T. J. and Zassenhaus, J. P. (1988) *Nucleic Acids Res.* **16**, 3297-3312.

Vischi, M. and Marchetti, S. (1997) *Theor. Appl. Genet.* **95**, 185-190.

Vogt, V. M. (1973) *Eur. J. Biochem* . **33**, 192-200.

von Hippel, P. H. and Felsenfeld, G. (1964) *Biochemistry* **3**, 27-35.

von Tigerstrom, R. G. (1980) *Can. J. Biochem.* **26**, 1029-1032.

von Tigerstrom, R. G. (1982) *Biochemistry* **21**, 6397-6403.

Wang, W-Y., Liaw, S-H. and Liao, T-H. (2000) *Biochem. J.* **346**, 799-804.

Watanabe, T. and Kasai., K. (1978) *Biochim. Biophys. Acta* **520**, 52-60.

Weber, D. J., Mullen, G. P. and Mildvan, A. S. (1991) *Biochemistry* **30**, 7425-7437.

Weber, D. J., Serpersu, E. H., Shortle, D. and Mildvan, A. S. (1990) *Biochemistry* **29**, 8632-8642.

Wei, C.-F., Legerski, R. J., Alianell, G. A., Robberson, D. L. and Gray, H.B. Jr. (1984) *Biochim. Biophys. Acta* **782**, 408-414.

Wei, C-F., Alianell, G. A., Bencen, G. H. and Gray, H. B. Jr. (1983) *J. Biol. Chem.* **258**, 13506-13512.

Weil, L. (1965) *Arch. Biochem. Biophys.* **110**, 57-68.

Weir, A. F. (1993) In: *Methods in Molecular Biology* (Burell, M. M. eds.), **16**, pg. 7-16, Humana Press, New Jersey.

Westhead, E. W. (1965) *Biochemistry* **4**, 2139-2144.

Wilcox, D.E. (1996) *Chem.Rev.* **96**, 2435-2458.

Williams, E. J. (1961) *J. Biol. Chem.* **236**, 1130-1134.

Wingert, L. and von Hippel, P. H. (1968) *Biochim. Biophys. Acta* **157**, 114-126.

Yarnall, M., Rowe, T. C. and Holloman, W. K. (1984) *J. Biol. Chem.* **259**, 3026-3032.

Yonemura, K., Matsumoto, K. and Maeda, H. (1983) *J. Biochem. (Tokyo)* **93**, 1287-1295.

Yupsanis, T. and Georgatsos, J. B. (1983) *Int. J. Biochem.* **15**, 959-963.

Zhou, X. and Gray, Jr., H. B. (1990) *Biochim. Biophys. Acta* **1049**, 83-91.

Zimmermann, S.B. (1982) *Ann. Rev. Biochem.* **51**, 395-427.