EXTRACELLULAR NUCLEASE FROM

BASIDIOBOLUS HAPTOSPORUS (89-3-24) :

PURIFICATION AND CHARACTERIZATION

A THESIS SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

BY

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PUNE 411 008 (INDIA)

NOVEMBER 2000

DECLARATION

Certified that the work incorporated in the thesis entitled **"Extracellular nuclease from *Basidiobolus haptosporus* (89-3-24) : Purification and characterization"** submitted by ***Miss. Neelam A. Desai*** 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

ACKNOWLEDGMENT

I take this opportunity to gratefully acknowledge my guide Dr. V. Shankar for his guidance and keen interest during the course of this investigation.

I am equally obliged to Dr. M. I. Khan for his valuable suggestions, which proved to be turning points in the successful completion of this work.

I would also like to extend my sincere thanks to :

Dr. M. C. Srinivasan, emeritus scientist, Division of Biochemical Sciences, NCL, for his kind gift of the culture Basidiobolus haptosporus (89-3-24). It was because of his interest in the microbial world and urge to isolate new strains that I had an opportunity to learn many interesting aspects of the different microbial strains isolated by him.

Dr. K. N. Ganesh, Head, Organic Chemistry (S), NCL, for providing synthetic deoxyribooligonucleotides and for permission to use HPLC and CD facilities.

Dr. S. K. Rawal, Division of Plant Tissue Culture, NCL, for HPLC and lyophilisation facilities.

Dr. (Mrs.) J.N. Nandi, Dr. D.T. Mourya and Mr. Pradeep, National Institute of Virology, Pune, for their kind co-operation and help in conducting FPLC, protein blotting and glycoprotein staining experiments.

Dr. Dinkar Salunke, National Institute of Immunology, New Delhi, for N-terminal sequencing.

Dr. C.G. Suresh, Dr. (Miss) Aditi Pant, Dr. M.V. Deshpande, Dr. (Mrs.) M. V. Rele, Dr. (Mrs.) H. Sivaraman and Dr. (Mrs.) Vidya Gupta, Division of Biochemical Sciences, NCL, and Dr. Krishna Sastry, National Center for Cell Sciences, Pune, for their timely help.

Mr. Modak, Mr. Kamthe and Mr. Karanjkar for efficient maintenance of equipments.

Head, Division of Biochemical Sciences, for his encouragement and support. Director, NCL, for permitting me to submit this work in the form of a thesis and Council of Scientific and Industrial Research, India, for financial assistance.

A number of colleagues and friends have always extended their helping hand at various stages during the course of this work. I would like to specially thank Rao, Ramchander, Vrinda, Bhushan, Mukund, Anjali and Pradeep for their help. It was indeed fortunate to have Mrs. Sreedevi U. as my room partner whose caring and helpful nature made my stay in the hostel comfortable and memorable.

Discussions with my lab mates Rangarajan, Rohini and Rajashree have always helped in critically evaluating my work. I am thankful to them for their support and co-operation. The atmosphere in the lab was always cheerful because of Dr. (Mrs.) Sumedha Deshmukh.

I would like to specially thank Mr. and Mrs. Deshpande and Nagnath who despite their busy schedule, always went out of their way to help me. I appreciate the helpful nature of Ashish, Anand and Vineet.

My mother has been a source of inspiration and the driving force during all these years. No words can suffice to acknowledge the tremendous moral support and sacrifices of my parents. It was because of the love and blessings of my family that I could successfully complete this work.

All this would have been impossible without the strong emotional support and co-operation by my husband Dr. Subray Hegde, who has incessantly helped me through all the ups and downs during the course of this investigation.

Finally, I would like to thankfully acknowledge Professor M.V. Hegde, for his interest in my work, encouragement and timely suggestions.

Neelam A. Desai.

DEDICATED AFFECTIONATELY TO

MY PARENTS

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CHAPTER 1

GENERAL INTRODUCTION

SINGLE-STRAND-SPECIFIC NUCLEASES

Historical perspectives

Nucleic acids act as carriers of genetic information from one generation to the other. In order that the genetic information is faithfully transferred to the next generation, the nucleic acids have to undergo processes such as replication and recombination. All living systems contain a set of enzymes called nucleases, capable of interacting with nucleic acids and hydrolyzing the phosphodiester linkages. The enzymatic breakdown of nucleic acids was first observed in early twentieth century (Araki, 1903) and the term "nucleases" was coined for enzymes involved in the degradation of nucleic acids. However, it was not until 1940, that Kunitz (1940, 1950) described two groups of nucleases, based on sugar specificity. Different schemes of classification were proposed in an attempt to overcome the shortcomings of the earlier ones (Kunitz, 1950 and Laskowski, 1959, 1967). However, with the discovery of newer nucleases and multifunctional enzymes like micrococcal nuclease and snake venom phosphodiesterase, the classification of Kunitz was found to be inadequate. Soon, a new class of sugar non-specific nucleases had to be added to the list as per new evidences. Hence, in order to overcome these shortcomings, Bernard (1969) and Laskowski (1959, 1982) suggested that nucleases be classified 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 oligo nucleotides terminating in a 3'- or a 5'- phosphate and
(iv) The nature of the bond hydrolyzed.

However, these schemes of classification did not make any provision for the difference in double-stranded (ds) and single-stranded (ss) cleavage. The credit for the discovery of nucleases hydrolyzing single-stranded nucleic acids goes to Lehman (1960). With the realization of the varied and complex nature of the catalytic activities of different nucleases, it became obvious that

exceptions did exist in almost every category of each of the proposed classification schemes which lead Laskowski (1982) to comment that the issue of classification expired because "the progress just overgrew all boundaries".

Single-strand-specific nucleases are ubiquitous in distribution. They exhibit high selectivity for single-stranded nucleic acids and single-stranded regions in double-stranded nucleic acids (Shishido and Ando, 1985) and hence they are widely used as probes for the structural determination of nucleic acids, mapping mutations and studying the interactions of DNA with various intercalating agents (Drew, 1984). Intracellularly, some of them have been implicated in recombination, repair (West, 1985) and replication (Brown *et. al.*, 1985). Although, their widespread use has lead to the isolation of more than 30 single-strand-specific nucleases from various sources, only a few enzymes such as S1 nuclease from *Aspergillus oryzae*, P1 nuclease from *Penicillium citrinum*, BAL 31 nuclease from *Alteromonas espejiana*, *Neurospora crassa, Ustilago maydis* and mung bean nucleases have been characterized sufficiently. Off late, a number of these enzymes have been cloned, their crystal structures solved and their interaction with different substrates has been well established. The present review gives a comprehensive account of single-strand-specific nucleases studied to date.

Occurrence and localization

It is well known that nucleases play an important role in the four R's i.e. recombination, replication, restriction and repair. Hence every living organism must produce one or the other type of nucleases. Single-strand-specific nucleases have been isolated from a wide variety of sources and these include microbes, plants and animals. Many of these enzymes are intracellular but microbial enzymes like S1 nuclease, P1 nuclease and nucleases from *A. espejiana* (Gray *et* al., 1975), *Serratia marcescens* (Benedik and Strych, 1998), *Thermus thermophilus* HB8 (Takahashi and Uchida, 1978) and *Anabaena* sp. PCC 7120 (Muro-Pastor *et al*., 1992) are secreted extracellularly. *Bacillus subtilis* 16-8S produces a single-strand-specific

DNase that is associated with the cell wall membrane fraction. This enzyme is secreted into the medium in large amounts when the cells are converted to protoplasts (Birnboim, 1966). In contrast to the extracellular nucleases of *A. oryzae*, viz. S1 nuclease, RNase T1 and RNase T2, nuclease O is found intracellularly in the mycelia (Uozumi *et al*., 1969). In case of *N. crassa* (Fraser and Cohen, 1983) and *Aspergillus nidulans* (Kao *et al*., 1990), the endoexonucleases are found in various organelles like mitochondria, vacuoles, conidia, mycelia and nuclei. Endonucleases from *Streptomyces antibioticus* (DeLos Reyes-Gavilan *et al*., 1988a) and *Streptomyces glaucescens* (Aparicio *et al*., 1988) are located in the periplasmic space between the cytoplasmic membrane and the cell wall. Although nucleases α (Holloman *et al*., 1981), ß (Rusche *et al*., 1980) and γ (Yarnall *et al*., 1984) from *U. maydis* differ in their physicochemical properties, all of them are located intracellularly. In case of the basidiomycetous fungi such as *Flamulina velutipes* (Kurosawa *et al*., 1990), *Coprinus cinereus* (Lu, *et al.,* 1988) and *Lentinus edodes* (Shimada *et al*., 1991), the enzyme is located in the fruiting body. Recently Kitamura *et al*. (1997), using immunohistochemical techniques, demonstrated that *C. cinereus* endonuclease is distributed in the surface gills of the fruiting body, which contain the meiotic tissues.

In plants, single-strand-specific nucleases have been isolated from various cellular components viz. rye germ nuclei (Przykorska and Szarkowski, 1980), wheat chloroplasts (Kuligowska *et al*., 1988), stroma, thylakoid membrane, envelope membranes of leaf chloroplasts (Monko *et al*., 1994) and germinating alfalfa seeds (Yupsanis *et al*., 1996; Christou *et al*., 1998). Their presence has also been shown in the endoplasmic reticulum, golgi apparatus, protein bodies and vacuoles of aleurone layer of barley seeds (Holstein *et al*., 1991), bound to chromatin in the embryo axis of germinating pea (Weir and Bryant, 1989), cell wall of potato tubers (Nguyen *et al*., 1988), and leaves of tobacco (Oleson *et al*., 1974), Avena (Wyen *et al*., 1971), spinach (Doetsch *et al*., 1989) and tea (Hiroshi *et al*., 1982). Moreover, they have also been isolated from mung bean sprouts (Sung and Laskowaski, 1965) and

germinating pea seeds (Wani and Hadi, 1979). Interestingly, the nuclease from pollen of *Petunia hybrida* was considered as extracellular since, it was easily excreted into the medium during the germination of pollen grains (van der Westhuizen *et al*., 1987).

In case of trypanosomes viz. *Leishmania donovani* (Dwyer and Gottlieb, 1984), *Crithidia luciliae* (Gottlieb *et al*., 1988) and *Crithidia facsiculata* (Shlomai and Linial, 1986) the nuclease activity is localized on the surface membrane. Among the animals, they are found in various organelles viz. sheep kidney (Healy *et al*., 1963), hen liver nuclei (Tanigawa and Shimoyama, 1983) and mouse mitochondria (Tomkinson and Linn, 1986).

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*., 1974a) or µg of RNA or DNA digested (Vogt, 1973).

Viscometric and hyperchromicity measurments 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*** **:**

(i) This method is based on the measurement of either increase in the acid soluble radioactivity or decrease in the acid insoluble radioactivity due to

mono / oligonucleotides liberated following the hydrolysis of radiolabelled nucleic acids (DNA / RNA). Unit of the enzyme is defined as the amount of enzyme required to render 1 nmol of labelled substrate acid soluble, under the assay conditions (Roth and Milstein, 1952).

(ii) Alternatively, the radiolabelled substrate is bound to plastic depression plates through anti-DNA antibodies and the release of radiolabel after the nuclease action is measured (Mishra, 1995).

(iii) 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. ***Fluorometric methods*** **:** The release of free ethidium bromide following the action of the enzyme on ETBr -DNA / RNA complex is measured. Similar quantitation can be made on a microtiter plate (Friedhoff *et al*., 1996).

E. ***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).

F. ***Assay of 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).

C. ***Detection of phosphohydrolase activities*** **:** Zlotnick and Gottlieb (1986) adapted the sensitive colorimetric method of Lanzetta *et al*. (1979), that determines Pi in the range of 0.5 - 10 nmol, for detection of several phosphohydrolase activities in polyacrylamide gels. This procedure (Zlotnick and Gottlieb, 1986) which results in the formation of a malachite green-phosphomolybdate complex was used for enzymes such as acid and alkaline phosphatase, nucleotidase and ATPase. This method has an advantage over that of Abrams and Baron (1967) which uses the Fiske-

Subbarow reagents (1925) and the procedure described by McLaughlin *et al*. (1976) where the inorganic phosphate liberated after enzyme action is detected after precipitation with lead nitrate.

Purification

As mentioned earlier, with few exceptions, a majority of the nucleases are located intracellularly. Depending on their source, the crude nuclease preparation contains a unique set of contaminating proteins and hence it is difficult to postulate a general purification scheme for all enzymes. During the initial purification steps one of the primary aims is to get rid of the coloured impurities contributed by the pigments of the organelles (e.g. leaf, carotenoids, pancreas etc.) in case of intracellular nucleases or the media constituents in case of extracellular nucleases. This is achieved by precipitation with alcohol and acetone or with ammonium sulfate. These procedures in addition to removal of some of the contaminants are useful in the concentration of the crude extract. Moreover, sodium chloride (Lee *et al*., 1968) and polyethylene glycol (Watanabe and Kasai, 1978) have been used for this purpose.

Single-strand-specific nucleases are relatively thermostable enzymes and a brief exposure of the crude enzyme preparation to high temperature (60 to 70 °C) have proven to be extremely beneficial, as it not only helps in inactivating the protease(s), if any, but also in removing heat labile proteins. Although ion exchangers such as DEAE- and CM-cellulose are widely used for the purification of these enzymes, phosphocellulose has been found useful in certain cases. For example, potato tuber nuclease, despite its net negative charge at pH 7.5, binds to phosphocellulose due to affinity toward 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. Single-strand-specific nucleases, in general, are relatively easy to purify to a level where they are free from contaminating nucleases and this can be achieved in one or two chromatographic steps. In case of S1 nuclease, the most widely used enzyme, it has been shown that a single chromatographic step on DEAE-

cellulose (pH 7.0) is sufficient to remove most of the contaminating nucleolytic activity. Moreover, rechromatography of the partially purified enzyme preparation on DEAE-cellulose, gave an enzyme preparation free of ds DNase activity (Sutton, 1971).

Hydroxyapatite has been used extensively for the purification of single-strand-specific nucleases from carrot (Harvey *et al*., 1970), *Chlamydomonas* (Small and Sparks, 1972), *Physarum polycephalum* (Waterborg and Kuyper, 1979), hen liver (Tanigawa and Shimoyama, 1983) and mouse mitochondria (Tomkinson and Linn, 1986). Furthermore, enzymes like S1 nuclease (Oleson and Sasakuma, 1980) and *U. maydis* nuclease (Holloman *et al*., 1981) have been purified on hydrophobic matrices like Phenyl- and Octyl- Sepharose, respectively.

Affinity chromatography has also been employed for the purification of some of the single-strand-specific nucleases. The glycoprotein nature of nucleases from *A. oryzae* (Oleson and Sasakuma, 1980), pea seed (Naseem and Hadi, 1987) and spinach (Strickland *et al*., 1991) have been exploited for their purification on concanavalin (Con) A-Sepharose. The preference of single-strand-specific nucleases for single-stranded nucleic acids has been utilized for the purification of S1 nuclease (Slor, 1975), *N. crassa* nuclease (Chow and Fraser, 1983) and hen liver nuclease (Tanigawa and Shimayoma, 1983) on ssDNA bound ɒ cellulose and Sepharose or entrapped in acrylamide. In this case, the chromatographic operation is generally carried out under conditions where the enzyme is either not active or shows very little activity. While heparin agarose was used for the purification of yeast mitochondrial (von Tigerstrom, 1982), *S. commune* (Martin *et al*., 1986) and barley seed (Brown and Ho, 1987) nucleases, Affi-gel Blue and poly (U)-Sepharose were employed for the purification of spinach nuclease (Strickland, *et al*., 1991). Kurosawa *et al*., (1990) used ApUp-agarose for the purification of *F. velutipes* nuclease whereas Hb-Sepharose was used for the purification of *S. tendae* nuclease (Engel and Ullah, 1988). Gray *et al*. (1981) purified fast (F) form of BAL 31 nuclease on 5'AMP-agarose. Similarly, S1 nuclease was purified

using 5'-AMP Sepharose (Gite *et al*., 1992 a). Immunoaffinity purification involving the use of anti-S1 nuclease antibodies bound to Sepharose has also been used for the purification of S1 nuclease (Gite and Shankar, 1995a).

Modern purification techniques like HPLC and FPLC have been successfully utilized for the purification of single-strand-specific nucleases from *F. velutipes* (Kurosawa *et al*., 1990), *Penicillium* sp. (Kazama *et al*., 1990) and spinach (Strickland *et al*., 1991).

Physicochemical properties

Optimum pH and pH stability

The optimum pH of a nuclease is an important criterion that determines its potential as an analytical tool. Experiments on nucleic acids are best done at or around neutral pH. The pH optima of single-strand-specific nucleases range from 4-9. Some of the widely used and well-studied enzymes like S1, P1 and mung bean nucleases have acid pH optima in the range of 4.0 - 5.0. Having an acid pH optimum is disadvantageous since lower pH values lead to considerable depurination of DNA. In contrast, nucleases from *Aspergillus sojae* (Suzuki and Sakaguchi, 1974), *B. subtilis* (Doly and Anagnastapolus, 1976), *T. thermophilus* (Takahasi and Uchida, 1978) and *Proteus mirabilis* (Goebel and Helsinki, 1971) have pH optima on the alkaline side in the range of 9-10. Although S1 nuclease exhibited an acidic pH optimum, the intracellular nuclease O, exhibited a broad pH optimum of 7.2 to 8.2. Most of the enzymes exhibit the same pH optimum for the hydrolysis of both monomeric and polymeric substrates (Gite and Shankar, 1995b). However, enzymes like BAL 31 nuclease (Gray *et al*., 1981), *N. crassa* (mitochondria) nuclease (Linn and Lehman, 1966) and *U. maydis* nuclease α (Holloman *et al*., 1981), showed different pH optima for the hydrolysis of ssDNA (8.8, 6.5-7.5 and 8.0) and dsDNA (8.0, 5.5-6.5 and 5.0), respectively. 3'-nucleotidase-nuclease from potato tubers (Nomura *et al*., 1971) showed different pH optima for nucleotidase (pH 8.0) and nuclease (pH 6.5 - 7.5) activities whereas, wheat chloroplast nuclease showed an optimum pH of 7.8 and 6.8 for the hydrolysis

of denatured DNA and RNA, respectively (Kuligowska *et al*., 1988). Similarly, tobacco nuclease hydrolyzed ssDNA and RNA optimally at pH 5.2 to 6 but the phosphomonoesterase activity was optimal at pH 7.0 (Oleson *et al*., 1974). Nucleases Le1 and Le3 from *L. edodes* (Shimada *et al*., 1991, Kobayashi *et al*., 1995) and P1 nuclease from *P. citrinum* (Fujimoto *et al*., 1974a) showed different pH optima for the hydrolysis of different mononucleotides. In contrast, S1 nuclease exhibited the same pH optimum for hydrolysis of the monomeric as well as polymeric substrates (Oleson and Hoganson, 1981). *Streptomyces tendae* was active over a broad range of pH (4.5 - 10.5) when assayed with ssDNA (Engel and Ullah, 1988). Yupsanis *et al*. (1996) isolated two nucleases from alfalfa seeds with optimum pH of 5.5 and 7.0. P1 nuclease from *P. citrinum* showed high stability between pH 5 - 8 (Fujimoto *et al*., 1974a) whereas, nuclease from *A. sydowii* was stable in the pH range of 5 - 9 (Ito *et al*., 1994).

The optimum pH of some of the nucleases is also dependent on factors such as the ionic strength and the presence of metal ions. For example, the optimum pH of the endonuclease from *P. polycephalum* (Waterborg and Kuyper, 1979) increased from 7.0 to 8.5 with increase in ionic strength of the buffer whereas, nuclease γ from *U. maydis* (Yarnall *et al*., 1984) showed an optimum pH of 8.0 and 9.0 in presence of Mg^{2+} and Mn^{2+}, respectively.

Optimum temperature and temperature stability

The temperature optima of most of the well-characterized single-strand-specific nucleases are in the range of 37 - 70 °C (Gite and Shankar, 1995b). Increase in the temperature, from 47 - 62 °C, did not significantly affect the rate of reaction of *N. crassa* (Linn and Lehman, 1965a) and spinach (Strickland *et al*., 1991) nucleases. S1 nuclease, however, showed 2- and 3-fold increase in the activity, on ssDNA at 45 and 60 °C respectively, than at 35 and 37 °C (Vogt, 1973; Liou *et al*., 1986). Pea seed nuclease exhibited an optimum temperature of 45 °C for nuclease activity and 60 °C for phosphomonoesterase activity (Naseem and Hadi, 1987). Nuclease from *T.*

thermophilus is perhaps the only enzyme having a very high temperature optimum of 85 °C (Takahashi and Uchida, 1978).

Single-strand-specific nucleases, in general, are thermostable enzymes and in case of some of the well-characterized enzymes like S1 nuclease (Shishido and Habuka, 1986), P1 nuclease (Fujimoto *et al*., 1975a) and mung bean nuclease (Laskowski, 1980), the increased thermal stability has been attributed to the presence of high amount of hydrophobic amino acids.

Metal ion requirement

Most of the single-strand-specific nucleases, with the exception of *F. velutipes* (Kurosawa *et al*., 1990), tobacco (Oleson *et al*., 1974), barley (Yupsanis and Georgatsos, 1983) nucleases and nuclease ß from *U. maydis* (Holloman *et al*., 1981), are either metalloenzymes or metal requiring enzymes (Gite and Shankar, 1995b). S1 (Shishido and Habuka, 1986), P1 (Fujimoto *et al*., 1975a), mung bean (Kowalski *et al*., 1976) and *P. polycephalum* (Waterborg and Kuyper, 1979) nucleases, nuclease PA3 from *Penicillium* sp. (Kazama *et al*., 1990) and a 3'-nucleotidase / nuclease from *C. luciliae* (Neubert and Gottlieb, 1990) are zinc metalloproteins while, the enzyme from *N. crassa* is a cobalt metalloprotein (Fraser, 1980). *S. commune* nuclease is either a zinc or cobalt metalloprotein (Martin *et al*., 1986) whereas, the enzyme from *A. sydowii* is a calcium and magnesium metalloprotein (Ito *et al*., 1995). S1 nuclease, the extracellular nuclease from *A. oryzae* showed a requirement of Zn^{2+} for the activity whereas the intracellular nuclease O required Mg^{2+} for its activity (Uozumi *et al*., 1969). Although nuclease α from *U. maydis* (Holloman *et al*., 1981) and wheat chloroplast nuclease (Kuligowska, 1988) did not require metal ions for their activity, nuclease α was stimulated 4-fold by Co^{2+} whereas, the ssDNase activity of wheat chloroplast nuclease showed only slight stimulation (20 %) in presence of Mg^{2+}. Similarly, the 3'-nucleotidase activity of pea seed nuclease did not show an obligate requirement of metal ions for its activity but was stimulated approximately 2-fold in presence of $MgCl_2$ and $CaCl_2$ (Naseem and Hadi, 1987). The acid and

neutral nucleases from alfalfa seedlings also did not require metal ions for their activity but exhibited differential sensitivity towards metal ions. Thus, the acid nuclease was highly stimulated by Zn^{2+} whereas, the neutral nuclease was strongly inhibited in its presence. Similarly, Mn^{2+} and Ni^{2+} stimulated the acid nuclease slightly but brought about approximately 50 % inhibition of the neutral nuclease (Yupsanis *et al*., 1996). Nucleases from yeast (von Tigerstrom, 1982), mouse mitochondria (Tomkinson and Linn, 1986) and *Actinomyces* sp. (Tatarskaya *et al*., 1970) require Mg^{2+} for their optimal activity whereas, the enzymes from *Chlamydomonas* (Small and Sparks, 1972) and *B. subtilis* need Ca^{2+} for its optimal activity (Kanamori *et al*., 1974 a). *A. sydowii* nuclease was optimally active in the presence of 20 mM Mg^{2+}, 0.4 mM Mn^{2+} or 2 mM Co^{2+}. The relative activities of this enzyme in presence of optimum concentrations of Mg^{2+}, Mn^{2+} or Co^{2+} were 100, 14 and 8 %, respectively (Ito *et al*., 1994).

Some of these enzymes require more than one divalent cation for their optimal activity. Like *N. crassa* (Linn, 1967) and *U. maydis* (Holloman and Holliday, 1973) nucleases, *A. nidulans* (Kao *et al*., 1990) nuclease too requires three divalent cations viz. Mg^{2+}, Mn^{2+} and Zn^{2+} for its maximum activity. *Drosophila melanogaster* nuclease requires Mg^{2+} and Mn^{2+} (Shuai *et al*., 1992) while the enzyme from carrot shows maximum activity in presence of Mg^{2+}, Mn^{2+}, Ca^{2+} and Zn^{2+} (Harvey *et al*., 1967). 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). Moreover, the pH optimum of *N. crassa* nuclease for the hydrolysis of dsDNA and RNA is dependent on Mg^{2+} concentration (Rabin *et al*., 1972). 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. On the other hand Co^{2+}, which appears to be a cofactor of the enzyme, stimulated its activity

3-fold towards all the substrates (Linn, 1967). The meiotic nuclease I from *C. cinereus* (Lu *et al*., 1988) needs Mg^{2+} and / or Ca^{2+} as co-factors. While Ca^{2+} is more efficient than Mg^{2+}, the enzyme shows maximum activity when both the cations are used in combination. Meiotic endonucleases II and III require Mg^{2+} as a cofactor but for meiotic endonuclease III, Ca^{2+} can also function as a co-factor (Lu and Sakaguchi, 1991; Kitamura *et al*., 1997).

Effect of salt concentration

It has been reported that salt concentration in the reaction mixture can affect the activity of single-strand-specific nucleases. For example, the activity of BAL 31 nuclease is maximum in the range 0-2 M NaCl and the enzyme shows only 40 % of its activity in presence of 4.4 M NaCl (Gray *et al*., 1981). While 100 - 200 mM NaCl completely inhibited the dsDNase activity of *N. crassa* nuclease, it had only marginal effect on the ssDNase activity (Fraser, 1980). Similarly, in case of *D. melanogaster* nuclease, 30 mM NaCl inhibited 50 % of the dsDNase activity whereas it required 100 mM NaCl to bring about the same level of inhibition of the ssDNase activity (Shuai *et al*., 1992). The inhibition of the dsDNase activity, in presence of high salt concentration, was correlated to the suppression of localized melting by electrostatic stabilization of the DNA, especially the stabilization of AT regions (von Hippel and Felsenfeld, 1964; Wingert and von Hippel, 1968). S1 nuclease, on the other hand, is optimally active at 100 mM NaCl. The enzyme is relatively insensitive to salt concentration between 10 - 200 mM NaCl and in 400 mM NaCl, it degrades ssDNA at 55 % of the maximal rate. The stringency of S1 nuclease is maximum at high salt concentrations (Vogt, 1973). On the contrary, NaCl inhibited *P. polycephalum* nuclease (Waterborg and Kuyper, 1979) while both KCl and NaCl inhibited mouse mitochondrial nuclease (Tomkinson and Linn, 1986). Mung bean (Johnson and Laskowski, 1970) and *Actinomyces* sp. (Tatarskaya *et al*., 1970) nucleases are optimally active in the range of 20 - 50 mM NaCl (Strickland *et al*., 1991). Action of rye germ nuclei nuclease, on PM2 DNA, showed that it is strongly dependent on salt

concentration but the presence of high salt (>100 mM) results in a significant inhibition of the activity (Przykorska and Szarkowski, 1980). Similarly action of S1 nuclease, on PM2 DNA, was found to be more specific in presence of NaCl concentrations greater than 200 mM (Gonikberg, 1978). Sodium chloride in the range of 50-150 mM completely inhibited the enzyme from sheep kidney (Watanabe and Kasai, 1979). *Chlamydomonas* nuclease showed significant inhibition in presence of 10 mM NaCl but KCl at this concentration had no effect on the enzyme activity (Small and Sparks, 1972).

Stability to denaturants

S1 nuclease (Vogt, 1973) and barley nuclease (Yupsanis and Georgatsos, 1983) are stable to low concentrations of denaturants like SDS and / or urea. Though P1 nuclease is susceptible to guanidine hydrochloride and SDS, the inhibition of the enzyme by urea and guanidine hydrochloride is reversible (Shishido and Ando, 1985). Gray *et al*. (1975) showed that the S 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 maximal activity in presence of 4M urea whereas, *P. polycephalum* (Waterborg and Kuyper, 1982) nuclease was stable in presence of 5M urea. Nuclease from *A. sydowii* was completely inactivated in presence of 4M urea due to the dissociation of the protein into subunits (Ito *et al*., 1995). However, the inactivation with urea was completely reversible in presence of 10 mM Ca^{2+}.

Effect of organic solvents

Organic solvents like formamide, dimethylformamide, dimethylsulfoxide and glyoxal interact with DNA and reduce its overall stability. Formaldehyde (Utiyama and Doty, 1971) and glyoxal (Broude and Budowsky, 1971) bring about chemical modification of the nucleotides in unpaired strands of DNA. Formaldehyde has been widely used to prevent

interstrand renaturation (Inman and Schnös, 1970). Isolation of single-strand-specific nucleases, exhibiting high stability in presence of organic solvents, have added a new dimension to these studies as they can be used as probes for the determination of the secondary structure of DNA in presence of various organic solvents. For example, the use of formamide has enabled the visualization, via electron microscopy, of nonbushed single-stranded regions in DNA (Davis and Hyman 1971; Davis *et al*., 1971). S1 nuclease showed high stability in presence of 60 % (v/v) formamide, 30 % (v/v) dimethylformamide, 50 % (v/v) dimethylsulfoxide and 2 % (v/v) formaldehyde (Hutton and Wetmur, 1975). Moreover, Case and Baker (1974) showed that S1 nuclease exhibits high stability in presence of 100 - 250 mM glyoxal and hence could also be used to obtain thermal-melting profiles in presence of formamide. Similarly, Muhich and Simpson (1986) demonstrated that mung bean nuclease can linearize kDNA minicircles, from trypanosomes, in presence of 40 - 50 % (v/v) formamide. Nucleases from *S. glaucescens* (Aparicio *et al*., 1992) and *S. antibioticus* (Santiago *et al*., 1995) exhibited more than 2-fold stimulation of their activity in presence of dimethylsulfoxide.

Inducers, activators and inhibitors

As mentioned earlier, the key role of nucleases is in replication and recombination processes and hence many of these enzymes are produced constitutively. Optimization of the growth conditions can enhance their levels. However, in case of barley nuclease it was observed that gibberellic acid brought about an 8-fold increase in the *de novo* synthesis of the enzyme in aleurone layers (Brown and Ho, 1987). The trypanosome *C. luciliae* (Neubert and Gottlieb, 1990) is incapable of *de novo* purine synthesis and produces a 3'-nucleotidase / nuclease which provides purine nucleosides to these parasites. The enzyme activity increases upto 1000-fold when the organism is maintained in a medium depleted of purines and / or inorganic phosphate. Moreover, cycloheximide (a protein synthesis inhibitor) and actinomycin D (a RNA synthesis inhibitor) inhibited the enzyme synthesis.

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 of both BAL 31 and S1 nuclease was considerably reduced in its presence (Shishido, 1985). Spermidine stimulated the RNase activity of yeast mitochondrial nuclease (Dake *et al*.,1988) whereas, it had no effect on the endonuclease from *S. glaucescens* (Aparicio *et al*., 1992). Both the acid and neutral nucleases from alfalfa seedlings showed similar sensitivity to polyamines and the inhibition was in the order of spermine > spermidine > putrescine. In case of nuclease I from rye germ ribosomes, it was noted that low concentrations (0.1 mM) of polyamines such as putrescine and spermidine inhibited the ribonuclease activity whereas, higher concentrations (2.5 mM) had a stimulatory effect (Siwecka *et al*., 1989). Similar observations were made with Staphylococcal nuclease (Frank *et al*., 1975). Thus, at low concentrations, polyamines act by changing the electrostatic potential of the enzyme-substrate complex and participate in the regulation of nucleic acid levels, in cells, by controlling the nuclease activity (Douzou and Maurel, 1977).

Most of the single-strand-specific nucleases are either metalloenzymes or metal requiring enzymes and hence they are strongly inhibited by metal chelators like EDTA, EGTA, citrate and 8-hydroxyquinoline. While 8-hydroxyquinoline inhibited pea seed nuclease, EDTA had no effect (Wani and Hadi, 1979). However, its 3'-nucleotidase activity was strongly inhibited by EDTA (Naseem and Hadi, 1987). Similarly, the ssDNase activity of wheat chloroplast nuclease was strongly inhibited by EDTA but it had no significant effect on the RNase activity (Kuligowska *et al*., 1988). In contrast, nuclease α from *U. maydis* (Holloman *et al*., 1981) was inhibited by EDTA and ß-mercaptoethanol while, nuclease ß from *U. maydis* (Rusche *et al*., 1980) was insensitive to EDTA and 1, 10-phenanthroline and reducing agents like DTT and ß-mercaptoethanol. Metal ions like Mn^{2+}, Co^{2+} and Zn^{2+} inhibited the nucleases from potato tubers (Nomura *et al*., 1971) and *B. subtilis* (Kanamori

et al., 1974a). Sheep kidney nuclease was inhibited by *p*-chloromercuribenzoate (Watanabe and Kasai, 1978) whereas $HgCl_2$ and $CoCl_2$ inhibited pea seed nuclease (Naseem and Hadi, 1987). Anions such as chloride, phosphate, succinate, bromide, carbonate, oxalate, propionate and sulfate activated the endonuclease from *Schizophyllum commune* while, fluoride, pyrophosphate, citrate, poly(vinyl sulfate) and inorganic phosphate strongly inhibited the enzyme activity (Martin *et al*., 1986). Neither divalent cations nor metal chelators affected the 5'-nucleotidase activity of *L. donavani* nuclease whereas, EDTA inhibited its 3'-nucleotidase activity. Moreover, compared to the 3'-nucleotidase activity, the 5'-nucleotidase activity of the enzyme was strongly inhibited by fluoride, tartarate and molybdate (Gottlieb and Dwyer, 1983). Netropsin, a bactericidal and antiviral compound, was found to enhance the single-strand-specific endonuclease activity of BAL 31 nuclease but inhibited its exonuclease activity (Sakaguchi *et al*., 1985). Netropsin selectively interacts with the AT rich sequences in double-stranded (ds) DNA and can induce the reversal from Z-form and other non-B form to B-form of DNA. It also enhances the susceptibility of negatively superhelical DNA to S1 nuclease but increasing concentration of intercalating agents like ethidium bromide, adriamycin and actinomycin-D and DNA-binding substances such as proflavine, charterusin and chromycin inhibited its activity (Shishido *et al*., 1984).

Endoexonuclease from *N. crassa* (mycelia) was inhibited by a heat-stable, trypsin-sensitive, cytosolic 24 kDa polypeptide (Hatahet and Fraser, 1989). 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*. Similarly, a heat-stable protein found in the fresh mycelia of *A. oryzae* inhibited the intracellular nuclease O but failed to inhibit the extracellular enzymes like S1 nuclease and RNases T1 and T2. This polypeptide inhibitor

was reported to regulate the intracellular levels of nuclease during the active growth phase of *A. oryzae* (Uozumi *et al*., 1976). The endonuclease NucA from *Anabaena* sp. was inhibited by its polypeptide inhibitor NuiA, while the related *Serratia* nuclease was inhibited only at a 10-fold molar excess of the inhibitor. Cleavage of the monomeric substrate 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*., 1998).

The hydrolysis of polymeric substrates by single-strand-specific nucleases shows autoretardation due to end-product inhibition. S1 nuclease was inhibited competitively by 5' ribo- and deoxyribonucleotides, with deoxyribonucleotides being the more potent inhibitors (Oleson and Hoganson, 1981). Similarly, nuclease ß from *U. maydis* was inhibited by 3'-nucleotides (Rusche *et al*., 1980). Interestingly, 1 to 5 mM ATP inhibited the nicking activity of meiotic nuclease III from *C. cinereus* only in presence of Ca^{2+} but was ineffective in presence of Mg^{2+} (Kitamura *et al*., 1997). In addition to mononucleotides, the 3'-nucleotidase activity of S1 nuclease (Oleson and Hoganson, 1981) and the 3'-nucleotidase-nuclease from potato tubers (Suno *et al*., 1973) was inhibited by polymeric substrates like ssDNA and ssDNA, RNA and poly A, respectively. In contrast, the 3'-nucleotidase activity of pea seed nuclease was stimulated 4.5-fold in presence native DNA whereas with denatured DNA, the stimulation was only 2.6-fold suggesting that DNA acts as a positive modulator of the nucleotidase activity (Naseem and Hadi, 1987).

Molecular mass and subunit structure

Mr of single-strand-specific nucleases are in the range of 5.5 - 140 kDa but majority of them fall between 29 - 85 kDa (Gite and Shankar, 1995b). The enzymes from *F. velutipes* (Kurosawa *et al*., 1990), carrot (Harvey *et al*., 1970) and yeast (von Tigerstrom, 1982) are high molecular mass proteins with a Mr of 91, 100 and 140 kDa respectively. Rye germ ribosome nuclease is

comparatively a low Mr protein of 20 kDa (Siwecka *et al*., 1989) whereas, germinating barley nuclease with a Mr of 5.5 kDa is perhaps one of the smallest enzyme reported so far (Yupsanis and Georgatsos, 1983).

Most of the single-strand-specific nucleases consist of a single polypeptide chain but mung bean (Laskowski, 1980) and pea seed (Naseem and Hadi, 1987) nucleases are made up of two unidentical subunits of 25 and 15 kDa and 30 and 24 kDa, respectively. Similarly, the nuclease from *A. sydowii* is made up of three unidentical subunits of 80, 50 and 25 kDa (Ito *et al*., 1994). On the contrary, the enzymes from *N. crassa* mitochondria (Chow and Fraser, 1983), yeast mitochondria (von Tigerstrom, 1982) and mouse mitochondria (Tomkinson and Linn, 1986) are made up of two identical subunits of 33 kDa, 57kDa and 37.4 kDa, respectively. Mung bean nuclease showed only one band corresponding to a Mr of 39 kDa, on SDS-PAGE, in the absence of ß-mercaptoethanol but in its presence the enzyme resolved into three components, corresponding to a Mr of 39 kDa, 25 kDa and 15 kDa. Since the intact and cleaved species migrated as a single band prior to reduction it was suggested that the cleaved species are held together by disulfide bond(s). However, both the cleaved and intact forms of the enzyme are equally active on ssDNA, RNA and 3'AMP (Laskowski, 1980). Shrimp DNase (subsequently designated as a nuclease) is a monomer of Mr 45 kDa and is highly crosslinked by 18 disulfide bridges (Lin *et al*., 1994). It exhibits different Mr, on SDS-PAGE, depending on the treatment of the sample with SDS and ß-mercaptoethanol. The four forms of shrimp DNase resulting from treatment under various conditions of thermal denaturation, exposure to SDS and reduction of disulfides, are depicted in Fig. 1.1. Since shrimp DNase is an acidic protein with large number of disulfide bridges, the native Form I (22 kDa) is represented by a molecule with the acidic groups exposed to the surrounding environment and the disulfides buried inside. When the protein was subjected to heat treatment with ß-mercaptoethanol, in the presence and absence of SDS, the molecule changed into an inactive Form III (45 kDa).

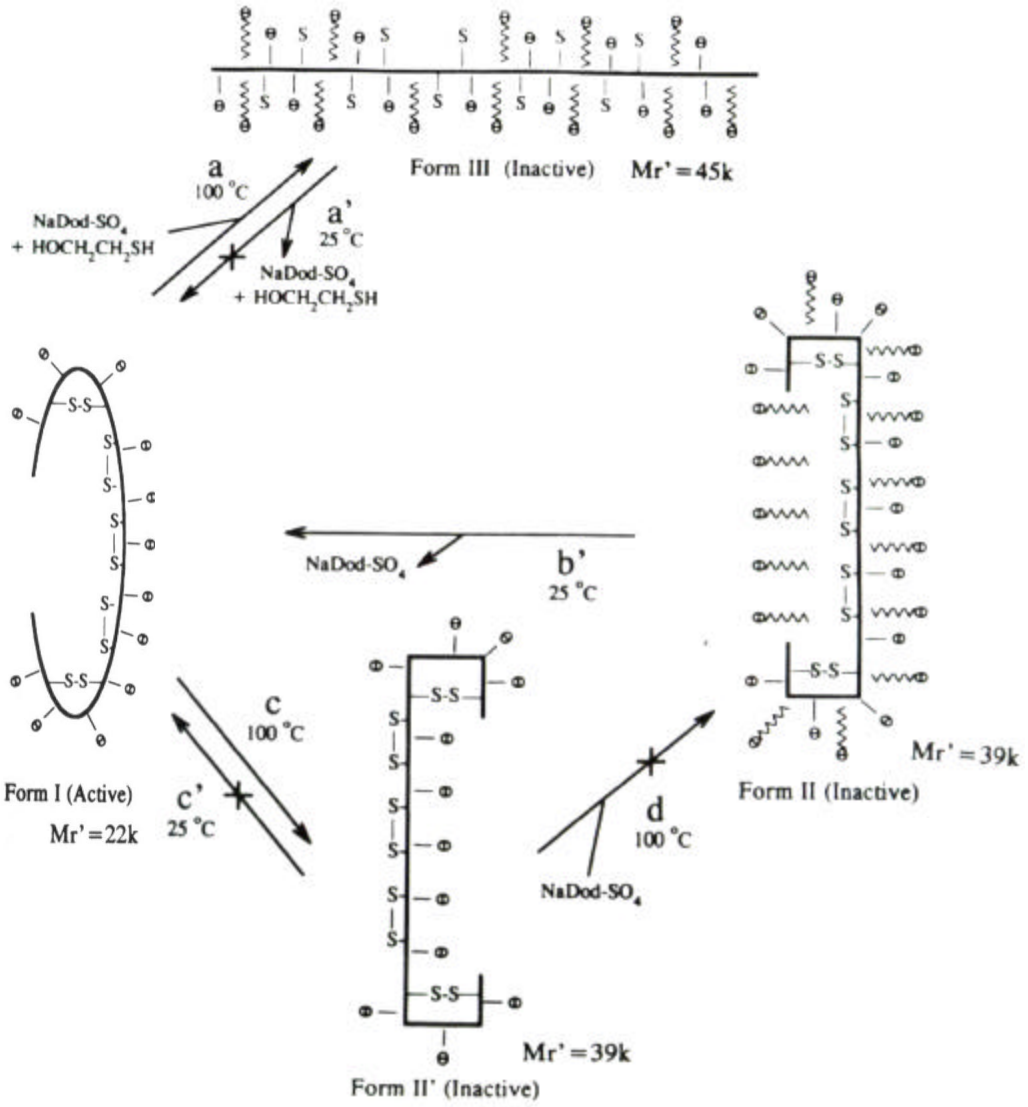

Fig. 1.1. A scheme for folding and unfolding pathways of shrimp DNase in presence and absence of SDS and ß-mercaptoethanol (Adapted from Lin *et al*., 1994).

Form III could not refold and re-oxidize to form the active Form I, even after the removal of SDS and ß-mercaptoethanol. When Form III was subjected to heat treatment, in the absence of ß-mercaptoethanol but in the presence of SDS, 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).

Interestingly, *N. crassa* produces four different nucleases in sorbose-containing liquid culture medium which are derived via different routes of proteolysis from a single inactive precursor polypeptide of Mr 90 kDa (Fraser *et al*., 1975; Kwong and Fraser, 1978; Chow and Fraser, 1979; Fraser, 1979; Käfer and Fraser, 1979; Fraser *et al*., 1980). The first is a 75 kDa single-strand-specific nuclease requiring Mg^{2+}, identical to that from conidia but not found in mycelia. The second is a 65 kDa protein and the third one is a 55 kDa enzyme identical to that originally isolated from the mycelia by Linn and Lehman (1965 a,b). The fourth enzyme, secreted by mycelia, is a 65 kDa Ca^{2+}-dependent endonuclease that has no RNase activity. In addition, a single-strand binding endoexonuclease of Mr 31 - 33 kDa has been purified from mitochondria, vacuoles and a mixture of these organelles (Chow and Fraser, 1983). The extracellular nuclease from *A. espejiana* sp. BAL 31 has been isolated as two distinct proteins, the "fast" (F) and "slow" (S) species, with Mr of 109 and 85 kDa, respectively (Wei *et al*., 1983).

Isoelectric point

The pI of single-strand-specific nucleases are in the range of 4.0-10.2. P1 (Fujimoto *et al*., 1974a), S1 (Rushizky *et al*.,1975), BAL 31 (Wei *et al*., 1983) and rye germ ribosome (Siwecka *et al*., 1989) nucleases are acidic proteins having a pI of 4.5, 4.3, 4.2 and 4.8, respectively. Spinach nuclease is a basic protein with a pI of 7.7 $\pm$ 0.3 (Strickland *et al.*, 1991). However, the enzyme from hen liver nuclei is a highly basic protein with a pI of 10.2 $\pm$ 0.2 (Tanigawa and Shimayoma, 1983). Highly purified preparation of S1 nuclease showed one major band and two minor forms corresponding to a pI of 3.67, 3.35 and 3.53, respectively (Oleson and Sasakuma, 1980) while crude S1 nuclease showed a single band corresponding to a pI of 4.3 (Rushizky *et al*.,

1975). The formation of multiple forms of the purified enzyme was attributed to the partial degradation of the enzyme during its purification from commercial Takadiastase powder or due to heat treatment at 70 °C, during the purification step (Rushizky, 1981). Although nuclease I from *Nicotiana tabacum* is a monomer of 35 kDa, two forms of the enzyme with pI of 5.2 and 5.6 could be resolved by electrofocussing. Both these forms did not exhibit any significant difference in their catalytic properties (Oleson *et al*., 1982). In contrast, acid and neutral nucleases from alfalfa seedlings although exhibited different pH optima, their pI values were in the acidic range (4.9 and 5.3, respectively) (Christou *et al*., 1998).

Glycoprotein nature

Some of the well studied single-strand-specific nucleases like P1 (Fujimoto *et al*., 1975a), S1 (Oleson and Sasakuma, 1980), mung bean (Laskowski, 1980), pea seed (Naseem and Hadi, 1987), barley (Brown and Ho, 1987) and rye germ ribosome nucleases (Siwecka *et al*., 1989), a nuclease from *Penicillium* sp. (Kazama *et al*., 1990) and spinach nuclease (Strickland *et al*., 1991) are glycoproteins and their carbohydrate content varies from 17-29 %. Compared to aforementioned nucleases, tobacco nuclease I has a very low carbohydrate content (9 %). Preliminary studies on the carbohydrate moiety of P1 nuclease revealed that it consists of mannose, galactose and glucosamine in a ratio of 6 : 2 : 1 (Fujimoto *et al*., 1975a). Rye germ ribosome nuclease contains 28 % carbohydrate and the carbohydrate moiety was shown to contain fucose, mannose and glucosamine (Siwecka *et al*., 1989). In case of S1 nuclease, out of two carbohydrate moieties, one of them is a high mannose type (Iwamatsu *et al*., 1991). Glycoproteins are known to exhibit anomalous behavior on gel-filtration and SDS PAGE, leading to incorrect estimation of their Mr (Trimble and Maley, 1977). Nucleases PA1, PA2 and PA3 from *Penicillium* sp. showed a Mr of 35000, 33000 and 32000 respectively on SDS PAGE (Kazama *et al*., 1990). Since the amino acid composition of all the species were very similar, it was concluded that the difference in Mr of these

enzymes was due to differential glycosylation. Trimble and Maley (1977) attributed the difference in the Mr of native and deglycosylated forms of P1 and mung bean nucleases to the carbohydrate moiety. Most glycoproteins are also known to be resistant to the action of proteases (Birkeland and Christensen, 1975). This is supported by the observation that pea seed nuclease, after treatment with trypsin for 1h, lost only 30 % of its initial activity whereas, DNase I which is a non-glycosylated protein was inactivated completely within 10 min of trypsin digestion (Naseem and Hadi, 1987).

Substrate specificity

Although several nucleases that act on single-stranded nucleic acids have been reported todate, it is difficult to clearly demarcate between strict single-strand-specific nucleases, single-strand-preferential nucleases and those which cleave both single- and double-stranded nucleic acids with equal efficiency. This is because any enzyme from the aforementioned category can act on a variety of substrates under different experimental conditions. Despite this, single-strand-specific nucleases owing to their high specificity for single-stranded nucleic acids have formed a distinct group of enzymes. They are sugar non-specific, multifunctional enzymes and exhibit high selectivity for single-stranded (ss) DNA and RNA. Some of them also show 3'- or 5'-phosphomonoesterase activity. However, the rate of hydrolysis of these substrates varies depending on the source of the enzyme. Thus, S1 (Oleson and Sasakuma, 1980), mung bean (Mikulski and Laskowski, 1970) and tobacco (Oleson *et al*., 1974) nucleases prefer ssDNA to RNA and 3'AMP whereas P1 (Fujimoto *et al*., 1974 a), PA3 (Kazama *et al*., 1990), Le1 (Hiroko *et al*, 1991), Le3 (Kobayashi *et al*., 1995) and potato tuber (Nomura *et al*., 1971) nucleases show higher activity on 3'AMP and RNA. The substrate specificity of P1 nuclease falls in the order of 3'AMP > RNA > ssDNA > dsDNA (Fujimoto *et al*., 1974a), while that of tobacco nuclease is ssDNA > 3'AMP > RNA > dsDNA (Oleson *et al*., 1974). Similarly, the 3'-nucleotidase / nuclease from *C. luciliae* hydrolyzed RNA faster than ssDNA with no

detectable hydrolysis of dsDNA (Neubert and Gottlieb, 1990). In contrast to majority of plant nucleases which prefer RNA to DNA, the acid and neutral nucleases from alfalfa seeds preferred ssDNA to RNA and hydrolyzed these substrates in the order of ssDNA > RNA > dsDNA and they also exhibited 3'-nucleotidase activity (Yupsanis *et al*., 1996). Many of these enzymes are also capable of hydrolyzing double-stranded nucleic acids, though at high enzyme concentrations. A comparative study of nucleases exhibiting high selectivity for single-stranded nucleic acids, based on their ssDNase **:** dsDNase activity ratios and kinetic constants, indicated that mung bean nuclease has the highest preference for ssDNA (30,000 : 1) followed by S1 (10,000 : 1) and *N. crassa* (250 - 4000 **:**1) nucleases (Martin *et al*., 1986). Nucleases α (Holloman *et al*., 1981) and ß (Rusche *et al*., 1980) from *U. maydis* showed high activity on ssDNA while nuclease γ preferred dsDNA as substrate (Yarnall *et al*., 1984).

The nuclease from *Staphylococcus aureus* (Cotton and Hazen, 1971) hydrolyzes both DNA and RNA but has greater affinity for DNA. Although, the activity on denatured DNA is greater than on native DNA, the single-strand specificity of the enzyme is not very high. Nuclease Rsn from *Rhizopus stolonifer* hydrolyzes various substrates in the order of ssDNA > dsDNA >> RNA (Rangarajan and Shankar, 1999) and hence can be classified as a single-strand-preferential enzyme because it shows higher activity on ssDNA. Moreover, the ratio of ssDNase **:** dsDNase activity varied with the type of metal ion used in the reaction mixture and the enzyme exhibited approximately 1.66, 1.75 and 4.50-fold higher activity on ssDNA in the presence of Mg^{2+}, Mn^{2+} and Co^{2+}, respectively. Similarly, endonuclease M from the kinetoplasts of the protozoan parasite *L. donovani* hydrolyzed ssDNA 2-fold faster than dsDNA suggesting it to be a single-strand-preferential enzyme. Moreover, the enzyme degraded single-stranded RNA rapidly but the RNA : DNA hybrids were 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 dsRNA-DNA hybrid. However, in presence of 10-fold-excess enzyme, the resulting RNA : DNA hybrid was also deaved (Mittra

et al., 1998). Wheat seedling nuclease (Hanson and Fairely, 1969), which acts on ssDNA, RNA and 3'AMP and yeast nuclease (Lee *et al*., 1968) which acts only on ssDNA and RNA, showed the same rate of hydrolysis for all the substrates. Additionally, other well-studied sugar non-specific nucleases from *S. marcescens* (Nestle and Roberts, 1969 a,b), *Anabaena* (Muro-Pastor *et al*., 1992), *Syncephalastrum racemosum* (Chen *et al*., 1993) and *Saccharomyces cerevisiae* (Zassenhaus and Denniger, 1994) hydrolyzed ssDNA, dsDNA and RNA at a similar rate.

Mode of action

Although single-strand-specific nucleases 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 -

Endonucleases **:** They attack the internal phosphodiester bonds of nucleic acids with or without free termini. Endonucleases can also act on covalently closed circular DNA. They show a distributive mode of action and the products of hydrolysis are oligonucleotides and / or mononucleotides.

Exonucleases **:** These enzymes require a free terminus for their action and are incapable of hydrolyzing covalently closed circular substrates. The products of hydrolysis are predominantly mononucleotides and the mode of attack is processive.

Endoexonucleases **:** This group of enzymes exhibit both exo and endo mode of action.

Although single-strand-specific nucleases hydrolyze both DNA and RNA either endonucleolytically or exonucleolytically, some enzymes exhibit different mode of action on these substrates. For example, nucleases from wheat chloroplasts (Kuligowska *et al*., 1988), wheat chloroplast stromal protein (Monko *et al*, 1994), rye germ ribosomes (Siwecka *et al*., 1989), nucleoplasm of rye germ nuclei (Przykorska and Szarkowski, 1980), barley (Brown and Ho, 1987) and yeast (Lee *et al*., 1968) hydrolyze ssDNA and RNA

endonucleolytically. In contrast, *A. sydowii* nuclease degrades both DNA and RNA exonucleolytically in 3'∏5' direction (Ito *et al*., 1994) whereas, *B. subtilis* enzyme cleaves ssDNA exonucleolytically from the 5'-end (Kanamori *et al*., 1974a). However, wheat seedling nuclease exhibits endonuclease activity towards ssDNA but exonuclease activity towards RNA (Kroeker *et al*., 1975). Similarly, nuclease from *F. velutipes* exhibits endonucleolytic activity on ss and dsDNA but RNA and linear polynucleotides are degraded exonucleolytically (Sen *et al*., 1991). Meiotic nuclease I from *C. cinereus* is strictly an endonuclease (Lu *et al*., 1988) whereas, meiotic nuclease II exhibits single-strand-specific endonuclease as well as an exonuclease activity on ssDNA (Lu and Sakaguchi, 1991). Nuclease ß from *U. maydis* exhibits both endo and exo mode of action on DNA. The high proportion of mononucleotides in the initial stages of hydrolysis of ssDNA by nuclease ß is indicative of an exo mode of action. However, it hydrolyzes ssDNA in a distributive manner, suggesting an endo mode of action. Moreover, the enzyme hydrolyzes linear DNA in an exo fashion from the 5'-end (Rusche *et al*., 1980). Nuclease α from *U. maydis* (Holloman *et al*., 1981) and BAL 31 nuclease (Gray *et al*., 1981), on the other hand, hydrolyze ssDNA endonucleolytically and shorten the linear duplex DNA from both 3' and 5' ends. In contrast to *U. maydis* nucleases α and ß, nuclease γ does not exhibit any exonucleolytic activity on DNA (Yarnall *et al*., 1984). As mentioned earlier, *N. crassa* produces four major nucleases and all of them exhibit different modes of action. The 75 kDa nuclease exhibits a 5'∏3' exonuclease activity on DNA in presence of Mg^{2+} but in the absence of Mg^{2+}, cleaves DNA endonucleolytically. The 65 kDa endoexonuclease exhibits endonuclease activity towards ssDNA but exonuclease activity towards dsDNA whereas, the 55 kDa product cleaves ssDNA endonucleolytically. However, the enzyme isolated from *N. crassa* mitochondria shows distributive endonuclease activity towards ssDNA but processive exonuclease activity towards dsDNA (Chow and Fraser, 1983).

The end products of hydrolysis of DNA and RNA, by single-strand-specific nucleases, are 5' or 3'-mononucleotides and / or oligonucleotides terminating in 5' or 3'-phosphoryl termini. However, the same enzyme does not produce both 5' and 3'-phosphorylated end products. S1 (Ando, 1966; Oleson and Sasakuma, 1980), P1 (Fujimoto *et al*., 1974c, 1974d), *N. crassa* (Fraser, 1980), mung bean (Laskowski, 1980) and wheat seedling (Kroeker and Fairely, 1975) nucleases produce 5'-mononucleotides as the end products of DNA and RNA hydrolysis. The oligonucleotides produced in the initial stages of hydrolysis, by these enzymes, have 3'-OH and 5'- PO_4 termini. In contrast, nuclease ß from *U. maydis* hydrolyzes ssDNA and RNA liberating 3'-mononucleotides (Rusche *et al*., 1980). Although BAL 31 nuclease (Gray *et al*., 1981) and nuclease α from *U. maydis* (Holloman *et al*., 1981) hydrolyze linear duplex DNA from both 3'- and 5'- PO_4 termini, the end products of hydrolysis are 5'-mononucleotides. Wheat chloroplast nuclease hydrolyzes ssDNA endonucleolytically, liberating oligonucleotides with 3'-OH and 5'-PO_4 termini while oligonucleotides liberated after RNA hydrolysis have 3'- PO_4 and 5'-OH termini (Kuligowska *et al*., 1988). Rye germ ribosome nuclease, 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 (Monko *et al*., 1994). The end products of poly(A) or synthetic deoxyoligonucleotide hydrolysis, by the acid nuclease from alfalfa seeds, are 3'-mononucleotides and oligonucleotides terminating in 3'-PO_4 whereas those of the neutral nuclease contain only oligonucleotides with 5'-PO_4 termini. Nuclease from *S. commune* acts on DNA endonucleolytically to produce dinucleotides bearing 5'- PO_4 termini (Martin *et al*., 1986). The endonucleolytic cleavage of ssDNA by meiotic nuclease II generates oligonucleotides with 3'- PO_4 termini and these oligonucleotides are resistant to the associated exonuclease activity of the enzyme. However, after removal of the 3'-PO_4 with alkaline phosphatase, the exonuclease activity of the enzyme degrades the linear ssDNA in 3'∏5' direction, generating 5'-mononucleotides. Hence, the authors proposed that the endonuclease activity is responsible for generating single-stranded nicks and / or double-stranded

breaks which will not be further degraded by the exonuclease activity, so that such single-stranded nicks and double-stranded breaks can participate as substrates in the subsequent recombination events. This was correlated to the appearance of high levels of nuclease activity in meiotic prophase (Lu and Sakaguchi, 1991).

Conformation specificity

Action on polynucleotides **:** Action of single-strand-specific nucleases on synthetic polynucleotides revealed that the rate of hydrolysis varies with the source of the enzyme and is strongly pH dependent . Fujimoto *et al*. (1974c) noted that P1 nuclease could readily hydrolyze poly (A) and poly (C) at pH 6.0 but these substrates were highly resistant to enzymatic attack at pH 4.5. On the contrary, poly (U) and poly (I) were hydrolyzed rapidly at pH 4.5 but very slowly at pH 6.0. S1 nuclease could hydrolyze poly (rU) at pH 4.6, at a rate similar to that of ssDNA but poly (rC) was degraded at a slower rate (5 %). Under similar conditions, poly (rA) and poly (rG) were resistant to hydrolysis. However, at pH 6.4, the enzyme could degrade poly (rC) and poly (rA) at a rate of 30 % and 50 %, respectively to that of ssDNA (Shishido and Ando, 1985). Mung bean nuclease showed higher activity on poly (U) than poly (A) at pH 5.0 (Mikulski and Laskowski, 1970) and the susceptibility of the former attributed to the lack of ordered secondary structure. Divalent cations influence the secondary structure of the polynucleotides. *N. crassa* (mycelia and conidia) nuclease showed specificity for polynucleotides lacking an ordered structure. Poly (dC) in the presence or absence of Mg^{2+} and poly (dI) in the absence of Mg^{2+} form random coil at pH 8.2 and were hydrolyzed by the *N. crassa* nuclease at rates comparable to those of denatured DNA. However, alternating polymer poly (dIdC), which exists in the range of helix to coil transition in the absence of Mg^{2+} at 37 °C, was hydrolyzed at a slower rate. On the other hand, poly (dAdT) and poly (dI) exist in the helical form in presence of Mg^{2+} and were degraded at a rate similar to that of native DNA. Poly (dG) and poly (dGdC) which formed highly ordered structures at pH 8.2 were totally

resistant to hydrolysis (Linn and Lehman, 1965b). Wheat chloroplast nuclease hydrolyzed various synthetic polymeric substrates in the order of poly (A) > poly (U) > poly (C) > poly (G) > poly (dA) > poly (dT) > poly (dC) > poly (dG) (Kuligowska *et al*., 1988). However, the nuclease from a fraction of wheat chloroplast stromal protein catalyzed the hydrolysis of polynucleotides in the order of poly (U) > poly (A) > poly (C) > poly (G) > poly (dA) > poly (dT) whereas, poly (dG) and poly (dC) were resistant to hydrolysis (Monko *et al*., 1994). Nuclease I from rye germ ribosomes showed high specificity for poly (C) while the remaining ribopolynucleotides were hydrolyzed in the order of poly (A) > poly (U) = poly (G) (Siwecka *et al*., 1989). Similarly, the relative rates of hydrolysis of various synthetic polyribonucleotides by the acid and neutral nucleases from alfalfa seeds were in the order of poly (U) > poly (A) > poly (C) > poly (G) and poly (A) ≥ poly (U) > poly (C) > poly (G), respectively, probably because the single-stranded character of the substrates decreased in the same order. Although rye germ ribosome nuclease hydrolyzed the double stranded deoxyribo heteropolymer 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. Moreover, this enzyme also lacked RNase H activity (Yupsanis *et al*., 1996). Sawai *et al*. (1978) noted that the nuclease from carrot tissue cultures is the only plant nuclease that exhibits RNase H activity. Like rye germ ribosome nuclease (Siwecka *et al*., 1989), barley nuclease (Brown and Ho, 1987) hydrolyzed the polynucleotides in the order of poly (C) > poly (U) > poly (A) > poly(A).poly(U) > poly (G) = poly(G).poly(C). A chromatin bound deoxyribonuclease from the embryo axis of germinating pea exhibited maximum activity on the purine analogue polymer, poly (dI), followed by poly (dA), poly (dT) and poly (dG) suggesting its preference towards purines (Weir and Bryant, 1989). The enzyme hydrolyzed the synthetic alternating copolymer poly (dA-dT) : poly (dA-dT) 10-fold faster than the duplex copolymer poly (dG-dC) : poly (dG-dC). This observation coupled with the limited extent of hydrolysis of native DNA suggested that the sites of action of

the DNase in native DNA are the regions that exhibit "structural breathing" i.e. transient single-stranded regions in DNA (von Hippel and Felsenfeld, 1964). Such regions are the AT rich regions in DNA. Similar observations were made in case of mung bean nuclease (Johnson and Laskowski, 1970). In contrast, nuclease II bound to rye germ ribosomes 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) > poly (G) (Siwecka, 1997).

Action on supercoiled and covalenlty closed DNA **:** Closed circular duplex DNA exists in a supercoiled form in plasmid and phage DNAs and is a consequence of torsional strain, which at sufficiently high negative superhelical density, promotes unwinding of helical twists (Bauer and Vinograd, 1974). In the absence of strand breakage, the unwinding of one turn of the double helix allows the untwisting of one negative supercoil. Negative supercoiling of DNA, in prokaryotes, is essential for cell growth and is required to promote strand unwinding and separation which occur during DNA replication, transcription and recombination (Wells *et al*., 1980; Gellert, 1981). As stated earlier, single-strand-specific nucleases have been shown to play an important role in DNA replication and recombination. Thus, it is likely that some sites in supercoiled DNA that exist transiently as single-stranded regions are susceptible to single-strand-specific nucleases. Almost all of the single-strand-specific nucleases reported so far have been shown to cleave supercoiled DNA from various sources (Gite and Shankar, 1995b). These enzymes nick the supercoiled DNA (Form I) to give rise to relaxed circular DNA (Form II) and then to linear duplex DNA (Form III). However, the rate at which the Form II DNA is further converted to Form III DNA varies among the different nucleases. Thus S1 (Méchali *et al*., 1973) mung bean (Kowalski and Sanford, 1982) and *N. crassa* (mitochondria and vacoules) (Chow and Fraser, 1983) nucleases showed a high degree of specificity for Form I DNA and converted it rapidly to Form II DNA. In case of mung bean nuclease,

28,000-fold excess enzyme was required to cleave the relaxed topoisomer (Form II) to Form III DNA whereas, with S1 nuclease very high concentrations were required for the conversion of Form II DNA to Form III DNA. The aforementioned enzymes cut each strand of DNA only once i.e. they first nick superhelical DNA in one strand and then cleave the strand opposite the nick to generate unit length linear Form III DNA. It is interesting to note that snake venom phosphodiesterase also exhibits single-strand-specific endonuclease activity with a similar preference (10,000-fold) for supercoiled over relaxed PM2 DNA. However, unlike mung bean nuclease, it does not accumulate the nicked circular DNA but cuts Form II DNA exactly opposite to the nick on the opposite strand. Thus, snake venom phosphodiesterase can be used as an excellent tool for the specific cleavage of the strand opposite nicks containing 3'-OH and 5'-PO_4 termini in duplex DNA (Pritchard *et al*., 1977). In case of *N. crassa* nuclease, the enzyme action can be controlled by adjusting the concentration of Mg^{2+} ions in the reaction mixture. Low concentrations of the enzyme, in presence of 0.1 mM Mg^{2+}, 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 and subsequently, Form III DNA was degraded exonucleolytically. In contrast, meiotic nuclease III from *C. cinereus*, in presence of 0.5 mM of Mg^{2+}, could not only nick supercoiled pBR322 DNA but also simultaneously produce the linear duplex DNA (Form III). Though with increase in Mg^{2+} concentration (1 to 10 mM), increasing amounts of Form II and III DNA were observed, their ratio remained unchanged. However, at higher Mg^{2+} concentrations (>10 mM), the formation of Form III was gradually inhibited and further increase in Mg^{2+} concentration (> 50 mM) showed a corresponding increase in Form II DNA. In case of nuclease α from *U. maydis*, the ratio of the rate of hydrolysis of superhelical DNA to that of the relaxed DNA was highest (approximately 140-fold) when the reactions were carried out in the presence of 40-100 mM NaCl (Holloman *et al*., 1981). In the absence of added salt, hydrolysis of Form II DNA proceeded at approximately one-tenth the rate

of hydrolysis of Form I DNA. The nicks generated by S1 (Shishido and Ando, 1981), *C. cinereus* (Lu *et al*.,1988) and mung bean (Kowalski and Sanford, 1982) nucleases and nuclease γ from *U. maydis* (Yarnall *et al*., 1984) were single base nicks, since they could be ligated by T4 DNA ligase to yield covalently closed circular DNA. In contrast, the nicks generated in supercoiled DNA by BAL 31 nuclease (Przykorska *et al*., 1988) and snake venom phosphodiesterase (Pritchard *et al*., 1977) could not be ligated back to covalently closed DNA, since they were extended into gaps by the exonuclease action of these enzymes.

Certain inverted repeats in supercoiled DNA adopt a hairpin or cruciform configuration (Gierer, 1966; Hsieh and Wang, 1975). Asakura *et al*. (1985) demonstrated that such inverted repeats are found in yeast 2μ DNA and S1 nuclease cleaves at the center of the pallindrome 3 which adopts a cruciform structure (Figure 1.2). Panayotatos and Wells (1981) showed that such cruciform structures also occur in naturally occurring DNA sequences such as those of pBR322 and pVH51 plasmid DNAs and they exhibit a similar cleavage pattern on treatment with endonucleases such as S1 and the T7 gene 3 product.

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 (Gray *et al*., 1981). Initial nicking rates of PM2 Form I DNA by BAL 31 nuclease are readily measurable at superhelical densities as low as -0.02, whereas the initial nicking with nucleases from *N. crassa* and mung bean requires more superhelicity (Shishido and Ando, 1985). Nicking of positively supercoiled DNA by BAL 31 nuclease becomes detectable at superhelical densities between 0.15 and 0.19.

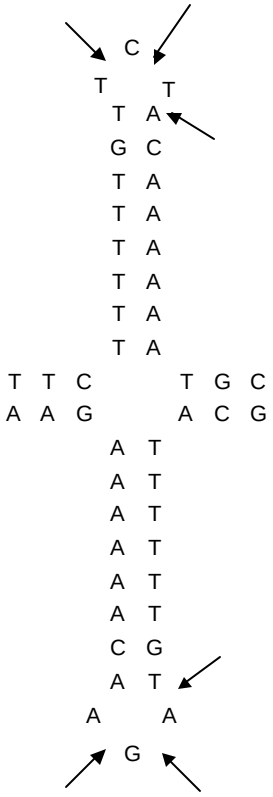

Fig. 1.2. Cleavage sites of S1 nuclease at palindrome 3 in supercoiled pYK2121 DNA. Arrows indicate possible cleavage sites (Adapted from Asakura *et al*., 1985).

An endonuclease from *Salmonella typhimurium* was shown to cleave both positively and negatively supercoiled DNA. However, when the supercoiled DNA was converted to fully relaxed form with the help of ethidium bromide, no further conversion of this relaxed DNA was observed (Zargar and Chakravorty, 1996). Similarly, meiotic nuclease I from *C. cinereus* could not hydrolyze the relaxed PM2 DNA generated by the action of topoisomerase I (Lu *et al*., 1988). *C. fasciculata* nicking enzyme cleaves a single phosphodiester bond in duplex DNA circles, from various sources, only in their supercoiled form but not following their relaxation by topoisomerases. However, the requirement of DNA supercoiling was not observed with natural

kinetoplast DNA (Shlomai and Linial, 1986). The capacity of the enzyme to activate a relaxed DNA topoisomer for nicking is an intrinsic property of the sequence-directed bend naturally present in kinetoplast DNA. The 211 base pair fragment of the bent region of *C. fasciculata* kinetoplast DNA served as the unique binding site for the nuclease. Sequence analysis of the nicking sites, in both strands of the 211 base pair bent fragment, revealed the presence of 32 nicking sites within the sequence. Twenty six of the 32 nicking sites available in this sequence were located within the dinucleotide sequence ApA (10), TpT (10), ApT (5) and TpA (1) in both strands. This observation indicated that the preferred site of cleavage lies in the A+T rich region (Linial and Shlomai, 1988). Similar observations were made with mung bean nuclease (Johnson and Laskowski, 1970). Nuclease hypersensitivity of a variety of supercoiled DNAs occurs extensively in specific A+T rich sequences and is influenced by temperature and ionic strength (Sheflin and Kowalski, 1984, 1985; Umek and Kowalski, 1987,1988) and this hypersensitivity can shift to sequences that form cruciforms or Z-DNA under different conditions (Kowalski, 1984). Kowalski *et al*. (1988) demonstrated that the stable DNA unwinding, as opposed to transient unwinding or breathing, is the reason for single-strand-specific nuclease hypersensitivity of specific A+T rich regions.

The DNA polymer (dC-dG)n . (dC-dG)n exists in a left handed conformation in presence of high salt concentrations (Zimmerman, 1982). Furthermore, 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. S1 nuclease specifically recognizes and cleaves the junction between right and left-handed regions (Singleton *et al*., 1982, 1983, 1984). However, its use as a B-Z junction probe is restricted to supercoiled DNA due to its inhibition in presence of high salt concentrations. Kilpatrick *et al*. (1983) showed that BAL 31 nuclease cleaves the B-Z junction in presence of high salt concentrations but it does not cleave DNA under conditions where (dC-dG)n

blocks exist in B conformation. Among them, S1 nuclease is a more specific probe since it lacks the exonucleolytic activity. In contrast, the DNA / RNA non-specific *Serratia* (Meiss *et al*., 1999) and *Anabaena* (Meiss *et al*., 1998) nucleases, prefer double-stranded A form of the nucleic acids.

Endonucleases are also capable of cleaving covalently closed circular DNA. Thus, S1, mung bean, *F. velutipes* and *U. maydis* α, ß and γ nucleases hydrolyzed covalently closed circular ssDNA. Meiotic nuclease II from *C. cinereus* (Lu and Sakaguchi, 1991) though brought about the endonucleolytic fragmentation of circular M13 ssDNA, it did not result in the formation of acid soluble products, since the 3'-PO_4 termini generated were refractory to further processing by the associated exonuclease activity of the enzyme. While the nature of the digestion products and the mode of degradation of linear DNA suggested an endonucleolytic action, the single-strand-specific nuclease from *U. maydis* failed to cleave the circular ϕX174 ssDNA and polyoma Form I and Form II DNAs. However, the resistant circular DNAs could be made susceptible by converting them into linear form. Hence the authors opined that the nuclease probably requires DNA with free ends for its activity (Holloman and Holliday, 1973).

Action on oligonucleotides that give rise to single-stranded loops **:** Duplex DNA molecules with covalently sealed (hairpin) ends are formed as a consequence of several biological processes including replication of some phage and viruses (van der Ende *et al*., 1981; Reddy and Bauer, 1989), site-specific recombination (Meyer *et al*., 1988; Nash and Robertson, 1989) and retroviral integration and transposition (Mazumder *et al*., 1994). Hairpins have also been implicated as intermediates in the excision of transposable elements in plants and recently in the rearrangement of T cell receptor and V(D)J recombination (Roth *et al*., 1992; Bockheim *et al*., 1996). Kabotyanksi *et al*. (1995) demonstrated the use of P1 and mung bean nucleases in opening the hairpin ends and concluded that the action of these enzymes is dependent on the sequence of the oligonucleotides that form the hairpins. Thus, P1 nuclease

preferably cleaves 3' of A residues whereas, mung bean nuclease cleaves 3' of A or T residues in DNA. Interestingly, it was observed that these enzymes do not efficiently remove the short single-stranded tails generated from the hairpins following the initial cleavage. The inability of S1 nuclease to remove short single-stranded extensions (Shishido and Ando, 1985; Esteban *et al*. 1992) is in accordance with the observations of Kabotyanski *et al*., (1995). Mung bean nuclease showed a strong preference for opening the hairpin bends, at pH 7.4, compared to the single-stranded 21-mer substrate. Even at very high enzyme concentrations, the single-stranded 21-mer polynucleotide was highly resistant to cleavage. However, at pH 5.3, both the substrates were highly susceptible to the enzyme action (Kabotyanski *et al*., 1995). Similar observations were made with P1 nuclease (Baumann *et al*., 1986). Based on these observations, the authors (Baumann *et al*., 1986) opined that hairpin opening by P1 and mung bean nucleases is remarkably efficient compared to the degradation ssDNA. Moreover, in case of mung bean nuclease the cleavage in the loop region was dependent on the stacking of the bases adjoining the loop. In presence of high concentrations of Mg^{2+}, the double helical loop is stabilized and hence there is restricted access of the enzyme to the loop bases adjacent to the helical regions. This was correlated to the reduced exposure of the bases, to the nuclease action, in presence of high concentrations of Mg^{2+} (Baumann *et al*., 1986). S1 nuclease, owing to a similar ability to cleave the loops, has been used as a probe for the anticodon loop of tRNA (Gilham, 1964; van Boom *et al*., 1982). Drew (1984) using oligonucleotide substrates that form double strands as well as hairpin loops demonstrated the ability of five nucleases to distinguish several different DNA backbone configurations (Fig. 1.3). DNase I can cut only one strand at a time and so it should turn over into a second orientation to cleave the opposite strand (Fig. 1.3a). Nevertheless, it appears that DNase I prefers a double-stranded phosphate track of suitable gauge as a binding site. Where the groove widens into a loop or where a well-defined groove ceases to exist near the 5'-end, the cleavage rate of the enzyme decreases dramatically. S1 nuclease and

micrococcal nuclease (Fig. 1.3 a, b) are very similar in their specificities. The difference between these two enzymes is that S1 nuclease does not show any base preference in the single-stranded regions, in the loop or at the ends of the hairpin whereas, micrococcal nuclease cuts preferably in the AT rich regions. Copper / phenanthroline (a chemical nuclease; Fig.1.3d) prefers a duplex to a loop, probably because it intercalates between base pairs. DNase II (Fig. 1.3e) does not require a double-stranded track as DNase I nor does it require an exposed phosphate group like S1 and micrococcal nuclease. It has a narrow binding domain and can reach down into the groove of the double helix so as to bind one strand with minimal interference from the opposing strand. However, the above studies indicated that the binding sites of the nuclease on the substrate and the site of cleavage might vary. For example, micrococcal nuclease binds two phosphate residues to the 3' side of where it cuts (Anfinsen *et al*., 1971). S1 nuclease, on the other hand, cuts symmetrically about the tip of the loops and so its cutting site is near its binding site. Similarly, Lilley (1983) showed that S1 nuclease cuts oligomer hairpins and duplexes near 5' and 3' ends in unpaired loops. It was also noted that when two hairpins oppose each other to from a cruciform structure, the unpaired loops become more sensitive to S1 nuclease than any other structural feature(s). It appears that the accessibility of S1 nuclease to the substrate is substantially restricted in the central region of the cruciform structure as compared to the exposed and protruding loops. Lilley (1983) used a small, single-strand-specific chemical (bromoacetaldehyde) to probe the unpaired bases and found that the region of single-strand near the base of the cruciform structure, 10-15 base-pairs on either side of the loop tip, became more reactive than the loop itself. This observation was consistent with the notion that S1 nuclease requires a greater degree of phosphate exposure than other single-strand-binding proteins such as micrococcal nuclease and DNase II. Based on the preference for various bonds in oligonucleotide substrates, the structural specificities of five different nucleases are summarized in Table 1.1.

Fig. 1.3. DNA configurations recognized by five different nucleases. (Adapted from Drew, 1984)

Table 1.1: Structural specificities of different nucleases. (Adapted from Drew, 1984).

Enzyme	Bond attacked	Structure attacked	Preferred conformation
DNAase I	O-3'-P	Two sugar-phosphate strands that are closely spaced	Minor groove of duplex
S1 nuclease	O-3'-P	Exposed single-strand	Ends of duplex, tip of loop
Micrococcal nuclease	O-5'-P	Exposed single-strand, unpaired A or T base.	5' end of duplex, side of loop, weakly in duplex
Copper-phenanthroline	Sugar ring	Base pair step	Duplex
DNase II	O-5'-P	Slightly exposed, stacked single-strand	Duplex with wide minor groove, stacked loop.

Using oligonucleotide substrates containing a run of five dG bases and substitution of the dG bases with deoxyguanosine analogues, Cal *et al*. (1996) showed that *S. antibioticus* nuclease interacts with both strands of DNA and also contacts the nucleic acid in both the major and minor grooves.

Base / linkage specificity **:** Though majority of single-strand-specific nucleases reported so far are base non-specific, the enzymes from *N. crassa*, *U. maydis*, *P. citrinum*, mung bean, avena leaf and spinach show some base specificity during the initial stages of hydrolysis. An endoexonuclease from *N. crassa* (conidia) (Linn and Lehman, 1965b) and *U. maydis* (Holloman, 1973) and nuclease C from *Chlamydomonas reinhardtii* (Ogawa and Kuroiwa, 1987)

showed a preference for guanylic acid linkages in ssDNA. However, *S. antibioticus* nuclease (Cal *et al*., 1996) showed a preference for runs of dG bases in dsDNA but not in ssDNA. Double stranded oligonucleotides containing sequences of four or more consecutive deoxyguanosine residues were preferentially hydrolyzed, with the strongest cutting site occurring at GGG↓GG. Moreover, 5'terminal analysis of the cleavage products of dsDNA by *S. antibioticus* nuclease (Cal *et al*., 1996) as well as that of a site specific single-strand-specific endonuclease from *Chlamydomonas* sp. (Burton *et al*., 1977) showed the predominance of dG followed by dT suggesting that after d(GpG) linkages d(GpT) linkages are preferred. Several nucleases are known which are not strictly single-strand-specific but show preference for runs of dG bases. These include the extracellular nuclease from *S. marcescens* (Meiss *et al*., 1998) and endonuclease G from mammalian nuclei and mitochondria (Ruiz-Carillo and Renaud, 1987; Low *et al*., 1988; Côtè *et al*., 1989; Côtè and Ruiz-Carillo, 1993). However, the other properties of these enzymes do not match those of other single-strand-specific nucleases. Thus, *S. marcescens* nuclease cuts runs of dG bases in ds DNA but not in d(A).d(T) tracts whereas, in ssDNA it readily cleaves the d(A).d(T) tracts. Although endonuclease G recognizes dG/dC tracts, both the purine and pyrimidine strands are cleaved with equal efficiency. However, in case of guanylic acid preferential nucleases from *N. crassa* (Linn and Lehman, 1965b) and *C. reinhardtii* (Ogawa and Kuroiwa, 1987), cytidylic acid linkages were resistant to cleavage. In contrast, *S. glaucescens* nuclease recognized the dinucleotide sequence 5'-CC-3' in dsDNA and cleaved 3' to the first C and also demonstrated a marked preference for certain 5'-CC-3'. At a higher enzyme concentration (> 10-fold), the sequences 5'-GC-3', 5'-CG-3' and 5'-GG-3' were cleaved along with other 5'-CC-3' sequences that were previously resistant. Similarly, a site-specific single-strand endonuclease activity induced by NYs-1 virus infection in a *Chlorella*-like green alga, recognized the two base sequence 5'-CC-3' and cleaved 5' to the first C. In contrast to *S. glaucescens* nuclease, the *Chlorella* enzyme exhibited an absolute specificity for the dinucleotide sequence CpC in

dsDNA and was stimulated by ATP. It cleaved 5'-C^{m}C-3' (where ^{m}C is methylcytosine) sequences but not 5'-^{m}CC-3' sequences. The enzyme generated breaks in dsDNA whenever two 5'-CC-3' sequences on opposite strands were close enough for the two strands to separate. However, when the 5'-CC-3' sequences on opposite strands were further apart only a portion of the strands separated following enzyme action. Moreover, it did not act on single-stranded nucleic acids (Xia *et al*., 1988). Single-strand-specific P1 (Fujimoto *et al*., 1974d), spinach (Doetsch *et al*., 1989) and avena leaf (Wyen *et al*., 1973) nucleases preferentially attack adenylic acid linkages in DNA. Moreover P1 nuclease prefers ribodinucleoside monophosphates compared to deoxyribonucleoside monophosphates. Using a combination of 16 dinucleoside monophosphates as substrates, Box *et al*. (1993) demonstrated that P1 nuclease shows maximum specificity for dinucleosides having adenosine at the 5'end and cytidine at the 3'end. Thus, dinucleoside monophosphates having adenosine either at the 5' or the 3'end were hydrolyzed in the order of d(ApC) > d(CpA) > d(ApG) > d(ApT) > d(ApA) > d(GpA) > d(TpA). In addition, dinucleoside monophosphates of the type d(TpN) were highly resistant to cleavage followed by d(GpN). Furthermore, dinucleoside monophosphates having either deoxycytidine or deoxyadenosine as the 5'-nucleoside were most susceptible to hydrolysis. Based on these observations, the authors (Box *et al*., 1993) concluded that P1 nuclease prefers the AC linkages in DNA. These results also indicated that in a polymeric substrate, the bases adjacent to the most preferred base also influence the specificity of the enzyme. A study of the rates of hydrolysis of dinucleoside monophosphates by nuclease I from *Nicotiana tabacum* (Oleson *et al*., 1982) revealed a strong preference for purine nucleosides as the 5' residue with slight preference for uridine as the 3' residue. Similar observations were made with barley nuclease (Brown and Ho, 1987). Action of the acid and neutral nucleases from alfalfa seeds, on dinucleoside monophosphates and deoxydecanucleotides, suggested that they differ in their preference for the phosphodiester linkages. Acid nuclease hydrolysed all dinucleoside monophosphates except UpU, CpC, CpA and CpG

to their respective 3'mononucleotides and nucleosides while, the neutral nuclease cleaved all dinucleoside monophosphates with the exception of UpU, UpC, UpG, CpA and CpG to their corresponding 5'mononucleotides and nucleosides. Moreover, the action of these enzymes on deoxydecanucleotides showed that the acid nuclease exhibited a preference for various linkages in the order of Cp↓C > Tp↓C > Gp↓T > Tp↓T > Ap↓C > Cp↓G > Gp↓G whereas the neutral nuclease hydrolyzed various bonds in the order of T↓pG > G↓pG > G↓pA > T↓pC > G↓pT > C↓pG > A↓pC (Yupsanis *et al*., 1996). In contrast the nuclease from germinated lentil exhibited a preference for T↓pT > T↓pG > G↓pT > G↓pC > A↓pG > G↓pG linkages (Kefalas and Yupsanis, 1995). However, recent studies on the action of acid and neutral nucleases from alfalfa seeds, on a synthetic 36-mer deoxynucleotide, revealed that the acid nuclease showed an initial preference for Cp↓C > Cp↓A > Gp↓T > Gp↓C bonds while the neutral nuclease for Ap↓T > Cp↓T = Tp↓C bonds. According to the authors (Christou *et al*., 1998), the probable reason for the observed differences in the bond preference of these two enzymes in the 10-mer and 36-mer substrates, is that the specificity of a nuclease depends not only on the neighbouring bases but also on the size of the substrates.

Three site-specific nucleases have been isolated from *Saccharomyces cerevisiae*. An endonuclease, designated YZ endo, cleaved at sites corresponding to the *in vivo* double strand breaks occurring at the mating type interconversion (Kostricken *et al*., 1983b). YZ endo generates a site-specific double strand break having 4 base 3' extensions terminating in 3'-hydroxyl groups. The cleavage occurs in the Z1 region near the YZ junction of the mating type locus. The second endonuclease, namely SceII, was present in all the strains of *S. cerevisiae* examined by Kostricken *et al*. (1983a). The cleavage site of SceII is unrelated to the YZ endo cleavage site. The third nuclease, Endo SceI, introduces a double-strand break in a 26 base pair consensus sequence (Shibata *et al*., 1984). The endonuclease from *Chlamydomonas* sp. showed specificity for certain sites in adenovirus-2 DNA and the initial cleavage occurred at a site containing deoxythymidine residues.

Hence the authors (Burton *et al*., 1977) proposed that, after an initial endonucleolytic cleavage at a preferred site on one strand of duplex DNA, the enzyme moves along the DNA molecule displacing the DNA strand until it reaches another recognition site where a second single-strand cleavage occurs producing a gap in the duplex DNA and an excised oligonucleotide. Holdsworth *et al*.(1989) characterized a novel site specific endonuclease from *C. fasciculata* viz. Endo A, whose preferred site of cleavage was within the TpC dinucleotide of the sequence ANATC on one strand of the double stranded DNA probe, where N is any nucleotide.

***Action on modified substrates* :** Single-strand-specific nucleases in addition to hydrolyzing natural substrates like DNA and RNA, act on alkylated, depurinated and UV-irradiated substrates and DNAs having single base lesions, mismatched bases and heteroduplexes. *N. crassa* endonuclease cleaved UV-irradiated DNA in the region containing pyrimidine dimers (Kato and Fraser, 1973). The action of S1 nuclease on UV-irradiated DNA revealed that the cleavage consisted of both single and double-stranded breaks (Shishido and Ando, 1974). Double-stranded DNA breaks were also observed following the hydrolysis of γ-irradiated DNA (Martin, 1981; Andrews *et al*., 1984). The above studies suggested that S1 nuclease recognizes alterations in the double helical structure produced by UV irradiation rather than specifically attack the UV-induced photoproducts. Moreover, its action on UV-irradiated DNA was dependent on enzyme concentration, ionic strength of the reaction mixture and this was directly proportional to the dose of UV-light. Hence S1 nuclease has been used to monitor chemically induced disruption of DNA secondary structure (Shishido and Ando, 1974). The enzyme also acts on DNA treated with alkylating agents (Rizvi *et al*., 1982; 1986), N-*acetoxy-N-2-acetylaminofluorene* (Fuchs, 1975) and cisplatin (Butour *et al*., 1990). SP nuclease from spinach nicks UV irradiated duplex DNA at adenine residues in the vicinity of 2-7 nucleotides on the same strand, in response to the formation of $TC_{(6-4)}$ photoproducts but not cyclobutane type pyrimidine dimers. DNA

damaged with *cis*-diaminedichloroplatinum and N-acetoxy-N-acetyl-2 aminofluorene was also cleaved at adenine by SP nuclease. This suggested that the enzyme does not cleave DNA in response to specific adenine modification but rather incises DNA at adenine residues in the vicinity of helical distortions produced by photoproducts, platinum and other modifications. Hence it was concluded that SP nuclease might be involved in repair of the dimers formed due to UV irradiation (Doetsch *et al*., 1988). Enzymatic hydrolysis of the intradimer phosphodiester bond may constitute the initial step in the repair of UV-induced cyclobutane pyrimidine dimers in human cells. UV-irradiation of the trinucleotide d-TpTpT results in the formation of two isomeric compounds containing a cis-syn-cyclobutane dimer. Action of various nucleases on these isomers showed that snake venom phosphodiesterase hydrolyzed only the 3'-phosphodiester group in the 5'-isomer (d-T<p>TpT) but was totally inactive toward the 3'-isomer (d-TpT<p>T). In contrast, calf spleen phosphodiesterase acted only on the 3'-isomer by cleaving the 5'-internucleotide bond. Kinetic analysis revealed that (i) the activity of snake venom phosphodiesterase was unaffected by a dimer 5' to the phosphodiester linkage (ii) the action of calf spleen phosphodiesterase was partially inhibited by a dimer 3' to the phosphodiester bond, and (iii) *E. coli* phr B-encoded DNA photolyase reacted twice as fast on d-T<p>TpT than d-TpT<p>T. Mung bean, S1 and P1 nucleases cleaved the 5'-internucleotide linkage but not the intradimer phosphodiester bond in d-TpT<p>T. Similarly, both phosphate groups in d-T<p>TpT were resistant to mung bean and S1 nuclease. Interestingly, incubation of d-T<p>TpT with P1 nuclease, however, generated the novel compound d-T<>d-pTpT containing a severed intradimer phosphodiester linkage. Thus, P1 nuclease represents the first single-strand-specific enzyme known to hydrolyze an intradimer phosphodiester linkage (Liuzzi *et al*., 1989).

Oleykowski *et al*. (1999) showed that mismatched DNA duplex is also an excellent substrate for SP nuclease. In base substitution mismatches, SP nuclease incises at all mismatches except those containing a guanine residue.

It also cleaves at insertions / deletions of one or more nucleotides. In case of extrahelical loop containing one nucleotide, the preference for cleavage of nucleotides was A>>T~ C but not G. In contrast, the CEL I family of nucleases from the leaves of broccoli, cabbage, cauliflower and celery could incise substrates with extrahelical loops containing all four nucleotides. This property of nuclease CEL I from celery was used for the detection of mutations and polymorphisms of the *BRCA1* gene in a number of women affected with either breast and / or ovarian cancer and reporting a family history of these ailments. The principle of mismatch recognition by CEL I appears to be different from T4 endonuclease VII which actually is a resolvase that nicks one strand at the site of a mismatch and then in the strand opposite the nick (Solaro *et al*., 1993). However, CEL I nuclease nicks only one strand of DNA, in a mismatch heteroduplex, at the site of the mismatch. In contrast, heteroduplex DNAs containing single base mismatches are highly resistant to S1, P1 and mung bean nucleases (Chaudhry and Weinfeld, 1995). Under conditions where little or no non-specific DNA degradation was observed, all three nucleases could generate double strand breaks when the bistranded abasic sites were 1 to 3 bases apart. Structural studies indicated that the disruption caused by the introduction of an abasic site in duplex DNA extends to the immediate adjacent base pairs (Withka *et al*., 1991; Goljer *et al*., 1992). With the abasic sites 1 to 3 base pairs apart, destabilization of the duplex would effectively extend over 4 to 6 base pairs, making the DNA susceptible to aforementioned nucleases. However, when these abasic sites were further apart, they were not cleaved by S1, P1 and mung bean nucleases since these sites were again considered as single abasic sites (Chaudhry and Weinfeld, 1995). A single apurinic site was sufficient to elicit BAL 31 catalyzed cleavage of duplex DNA wherein the F form was 2.5-fold more efficient in cleaving the depurinated DNA than the S species (Wei *et al*., 1984). Nuclease α from *U. maydis* cleaved depurinated, deaminated and UV-irradiated DNA at considerably higher rates than the untreated DNA. Heteroduplexes containing single mismatched base pairs were cleaved at the same rate as those of the normal

substrates in presence of 10 mM Mg^{2+} and 50 mM NaCl. However, when the reaction conditions were changed by replacing Mg^{2+} and NaCl with 0.2 mM Co^{2+} and lowering the temperature from 37 to 20 °C, the heteroduplex DNA was cleaved somewhat faster than the control DNA. The higher activity of the enzyme under changed experimental conditions was correlated to two kinds of structural changes in DNA (Holloman *et al*., 1981). First, a few negative superhelical turns may have been introduced by carrying out the reaction at a temperature 17 °C lower than the ligation reaction (Wang, 1969; Upholt *et al*., 1971). Secondly, at the concentration employed, Co^{2+} might destabilize the DNA helix and may cause some tilting of bases (Luck and Zimmer, 1972; Zimmer *et al*., 1974). These structure-distorting treatments coupled with the known destabilizing effect of the mismatch may render the heteroduplex DNA more susceptible to nuclease α than the homoduplex DNA (Holloman *et al*., 1981). Similarly, Naseem and Hadi (1987) showed that pea seed nuclease hydrolyzed alkylated and depurinated DNA at rates higher than that of native DNA. Moreover, the enzyme preferentially hydrolyzed depurinated DNA at apurinic sites than apyrimidinic sites. Weinfeld *et al*. (1989, 1990) studied the hydrolysis of phosphodiester bonds adjacent to apurinic site, by S1, P1 and mung bean nucleases, using dinucleotides lacking either a 5' or a 3' base as substrates. Partial depurination of d-ApA produced two A_{260} absorbing isomers, d-SpA and d-ApS (where S represents the depurinated deoxyribose sugar). S1, P1 and mung bean nucleases hydrolyzed the d-ApS isomer but not the d-SpA isomer, indicating that they interact with the base 5' to the internucleotide phosphate group. Stacking between aromatic amino acids of nucleases and nucleic acid bases plays an important role in the interaction of single-strand-specific nucleases with their substrates. Weinfeld *et al*. (1993) studied the requirement of DNA base aromaticity for five enzymes, viz. T4 polynucleotide kinase, P1 and S1 nucleases, and snake venom and calf spleen phosphodiesterases, acting on ssDNA. The modified substrates contained either cis-5R, 6S-dihydro-5, 6-dihydroxythymidine (thymidine glycol) or a mixture of 5R and 5S isomers of 5,6-dihydrothymidine. It was observed that

for all the enzymes, except snake venom phosphodiesterase, the parent molecules were better substrates than the dihydrothymidine derivatives while, the thymidine glycol compounds were very poor substrates. Snake venom phosphodiesterase acted on the unmodified and dihydrothymidine molecules almost at the same rate. P1 and S1 nucleases hydrolyzed the molecules containing 5R-dihydrothymidine approximately 50-times faster than those containing the S-isomer. The other enzymes displayed no measurable stereospecificity. Potter *et al*. (1983a) used S1 nuclease for determining the stereochemical course of hydrolysis of DNA to assess the presence or absence of a covalent enzyme intermediate in reactions catalyzed by single-strand-specific nucleases. Action of S1 nuclease on distereoisomers of d[Tp(S)A] indicated that the hydrolytic reaction proceeds with the inversion of configuration of the oxygen atom at phosphorous position. Similar results were obtained with P1 nuclease (Potter *et al*., 1983b) indicating that the hydrolytic reaction does not involve the formation of a covalent enzyme substrate intermediate.

***Structure specific DNA cleavage* :** Several important pathways of DNA metabolism involve the transient formation of branched DNA structures that contain free 5'-single-stranded ends. Such structures can arise during replication, recombination and repair. Processes such as nick translation or removal of Okazaki fragments from the lagging strand (a short stretch of ribonucleotides that act as primer for the synthesis of lagging strand) during DNA replication results in the formation of a displaced strand or a bifurcation termed as "flap". These flap structures are cleaved by the 5'-3' exonucleases. termed as structure specific 5'nucleases and many of them are physically associated with DNA polymerases (Fig. 1.3 A, B).

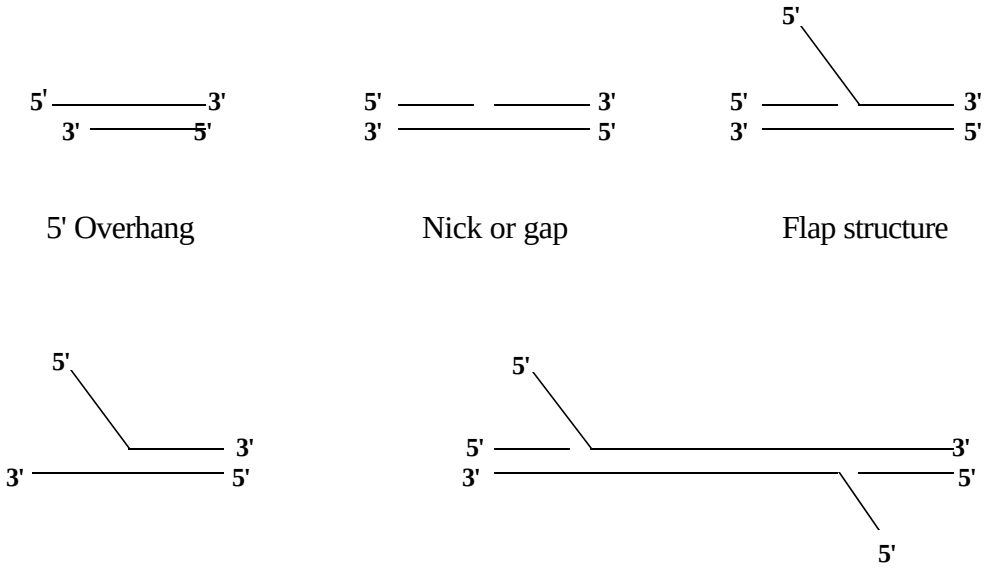

Fig. 1.3 A. Substrates of 5'-3' exonucleases.

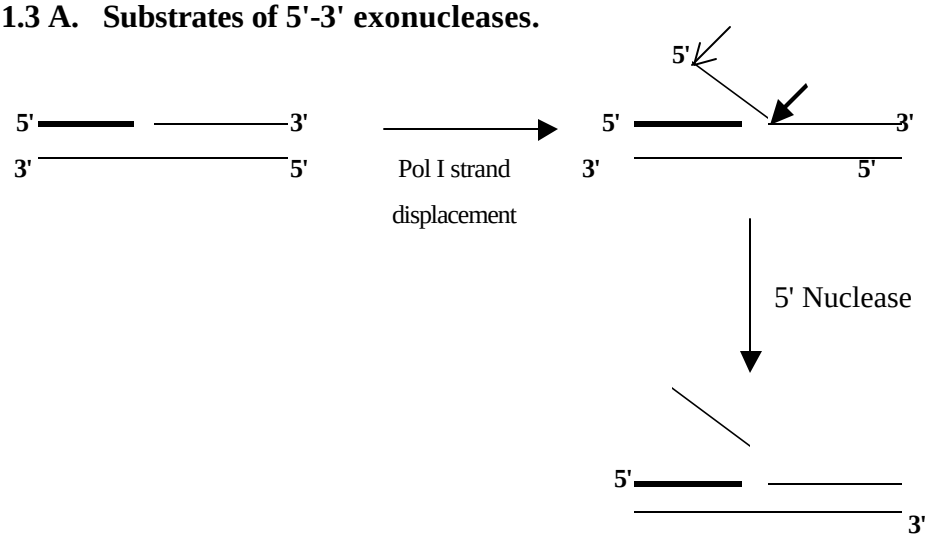

Fig. 1.3. B. Action of 5' nuclease on flap structure during nick translation.

The invading primer is shown in bold line. It is extended by the action of Pol I and the displaced strand forms a flap structure. The endonucleolytic cleavage by 5' nuclease is indicated by bold arrow whereas the faded arrow indicates exonucleolytic attack. (Adapted from Ceska and Sayers, 1998).

Two well-studied enzymes of bacteriophage origin namely, the λ exonuclease and T5 D15 5'-3' exonuclease hydrolyze DNA in 5'-3' direction and require a divalent cation for its activity (Ceska and Sayers, 1998). The λ exonuclease releases only mononucleotides from the 5'-end of duplex DNA provided that a 5'-terminal phosphate group is present but it cannot hydrolyze ssDNA or RNA. It does not act on substrates that have 5'-single-stranded overhangs and shows no evidence of endonuclease activity (Carter and Radding, 1971). In contrast, T5 exonuclease digests dsDNA and ssDNA, releasing mono -, di and tri - nucleotides as well as oligonucleotides and can also act on DNA-RNA hybrids (Sayers and Eckstein, 1990). A mammalian enzyme, designated as flap endonuclease I (FEN-1), characterized by Harrington and Lieber (1994) and previously identified as DNase IV by Lindahl *et al*. (1969) exhibit properties that are very similar to those of prokaryotic 5' nucleases. Lyamichev *et al*. (1993) proposed that this enzyme gains access to the cleavage site by moving from the free end of a 5' extension to the bifurcation of the duplex, where the endonucleolytic cleavage takes place. Single-stranded 5' arms upto 200 nucleotides long are cleaved from such a duplex.

Associated phosphomonoesterase activity

Most of the extracellular single-strand-specific nucleases exhibit an associated phosphomonoesterase activity. The main role of nucleotidases is to scavenge nucleosides and phosphates for growth which in turn is useful for the survival of the organism under environmentally stressed conditions. This is supported by the observation that high levels of 3'-phosphomonoesterase activity were induced in trypanosomes in response to depletion of purines and inorganic phosphate in the growth medium (Neubert and Gottlieb, 1990). S1 (Oleson and Hoganson, 1981), P1 (Fujimoto *et al*., 1974c), mung bean (Kole *et al*., 1974), pea-seed (Naseem and Hadi, 1987) and potato tuber (Suno *et al*., 1973) nucleases possess 3'-phopshomonesterase activity. However, nuclease ß from *U. maydis* dephosphorylates 5'-mononucleotides (Rusche *et al*., 1980). The 3'-nucleotidase activity of all the single-strand-specific nucleases reported

todate shows a strong preference for ribonucleotides over deoxyribonucleotides. In fact, 3'deoxyribomononucleotides were resistant to the 3'nucleotidase / nuclease from *C. luciliae* (Neubert and Gottlieb, 1990). Thus, mung bean (Kole *et al*., 1974), P1 (Fujimoto *et al*., 1974c) and tobacco (Oleson *et al*., 1982) nucleases cleave 3' ribonucleotides 20-50 fold faster than the 3'-deoxyribonucleotides. The preference for ribonucleotides was correlated to the presence of 2'-OH group in the ribonucleotides (Kole *et al*., 1974). In addition, some nucleases exhibit a base preference for the hydrolysis of various 3'mononucleotides. Thus, P1 nuclease hydrolyzed various 3'-ribonucleotides in the order of G > A > C ≥ U whereas, the 3'-deoxyribomononucleotides were hydrolyzed in the order of C ≥ T > A ≥ G. P1 nuclease also acts on nucleoside 3',5' diphosphates but 2'AMP is highly resistant to cleavage (Fujimoto *et al*., 1974c). The substrate specificity of S1 nuclease is in the order of ribonucleoside 3',5' diphosphate > ribonucleoside 3' phosphate > deoxyribonucleoside 3',5' diphosphate > deoxyribonucleoside 3' phosphate ≈ ribonucleoside 2' phosphate (Oleson and Hoganson, 1981). Base specificity of tobacco nuclease is in the order of A > G >> U > C and a greater affinity was observed when the substrate contained an additional phosphate at the 5'-position. Similar observations were made in case of S1 (Oleson and Hoganson, 1981), and mung bean (Kole *et al*., 1974) nucleases.

Structure and function

The N-terminal amino acid sequences of six single-strand-specific nucleases have been deduced and all of them have tryptophan as the N-terminal residue (Fig.1.4). The C terminal amino acid residues of S1 (Iwamatsu *et al*., 1991), P1 (Maekawa *et al*., 1991) and PA3 nucleases (Tabata *et al*., 1991) are serine, leucine and isoleucine, respectively. The amino acid sequence of a nuclease from *Penicillium* sp. was found to be identical to that of P1 nuclease except that Thr 190 was replaced by Ile in P1 nuclease (Tabata *et al*., 1991). The complete amino acid sequences of S1 and P1 nucleases have been determined and they showed approximately 50 % homology (Iwamatsu *et al*., 1991).

However, the N-terminal amino acid sequence of the intracellular nuclease O from *A. oryzae* showed no sequence similarity with the extracellular S1 nuclease (Motoaki, *et al*., 1996). Similarly, the N-terminal sequence of meiotic endonuclease I from *C. cinereus* did not show any homology with the aforementioned nucleases (Charlton *et al*., 1992).

	1									10									20
Lentinus edodes (Le1)	**W**	**G**	A	**I**	**G**	**H**	**E**	**T**	**V**	G	Y	**V**	**A**	M	F	L	S	P	
Lentinus edodes (Le3)	**W**	**G**	M	**L**	**G**	**H**	**E**	**L**	**V**	G	F	**I**	**A**	S	N	L	D	P	S	F
Aspergillus oryzae (S1)	**W**	**G**	N	**L**	**G**	**H**	**E**	**T**	**V**	A	Y	**I**	**A**	Q	S	F	V	A	S	S
Penicillium citrinum (P1)	**W**	**G**	A	**L**	**G**	**H**	**A**	**T**	**V**	A	Y	**V**	**A**	Q	H	Y	V	S	P	E
Penicillium sp. (PA3)	**W**	**G**	A	**L**	**G**	**H**	**A**	**T**	**V**	A	Y	**V**	**A**	Q	H	Y	V	S	P	E
Barley nuclease	**?**	**G**	K	**E**	**G**	**H**	**Y**	M	T	N	L	**I**	**A**	D	G	F	L			

	21									30	
Lentinus edodes (Le1)											
Lentinus edodes (Le3)	V	W									
Aspergillus oryzae (S1)	T	E	S	F	C	Q	N	**I**	**L**	**G**	.....
Penicillium citrinum (P1)	A	A	S	W	A	Q	G	**I**	**L**	**G**	.....
Penicillium sp. (PA3)	A	A	S	W	A	Q	G	**I**	**L**	**G**	

Fig. 1.4. Comparison of N-terminal amino acid sequence of single-strand-specific nucleases.

Although a large number of single-strand-specific nucleases have been reported todate, structural studies have been limited to only S1 and P1 nucleases. S1 nuclease consists of a single polypeptide chain of 267 amino acids cross-linked by two disulfide bonds. The disulfide (S-S) linkages probably occur between Cys 72 to Cys 216 and Cys 80 to Cys 85. It is a high mannose containing glycoprotein and the sugar moieties are attached to asparagine 92 and 228 (Iwamatsu *et al*., 1991). P1 nuclease also consists of a single polypeptide chain made up of 270 amino acids with two disulfide bonds Cys 72 to Cys 217 and Cys 80 to Cys 85. P1 nuclease contains four

carbohydrate moieties and they are linked to asparagine 92, 138, 184 and 197. Based on circular dichroism (CD) and optical rotatory dispersion (ORD) studies, Fujimoto *et al*.(1975b) showed that P1 nuclease consists of 29 to 31 % α-helix , 6% ß sheet and 63 % random coil whereas, S1 nuclease consists of 25 % α-helix, 31 % ß-sheet and 44 % random coil (Shishido and Habuka, 1986). Recently, S1 nuclease has been cloned and the full gene sequence deduced (Lee *et al*., 1995). Also the nuclease O gene (nuc O) of the intracellular nuclease from *A. oryzae* was isolated and characterized (Motoaki *et al*., 1996). Similarly, meiotic endonuclease I from *C. cinereus* was also cloned by Charlton *et al*. (1992). Comparison of the gene sequences of S1 nuclease and nuclease O from *A. oryzae* did not reveal any sequence similarity.

P1 nuclease was crystallized in three different space groups using ammonium sulphate or PEG 4000 as precipitants and its structure was studied at 4.5 Å resolution (Lahm *et al*., 1990). Subsequently, Volbeda *et al*. (1991) solved its structure at 3.3 Å and refined the data at 2.8 Å. The three dimensional structure of P1 nuclease showed the presence of 269 amino acid residues, 3 zinc atoms and 2 *N*-acetylglucosamine residues. Although the amino acid sequence showed the presence of 270 amino acids in the polypeptide chain, the C-terminal lysine could not be located in the electron density map. The main chain folding of the enzyme was very similar to that of phospholipase C from *Bacillus cereus*, with 56 % of the structure exhibiting α-helical conformation. This value, however, shows a considerable variation from the one obtained from CD data.

Coordination and function of metal ions **:** P1 nuclease is a zinc metalloprotein and contains three Zn^{2+} atoms per molecule of the enzyme. Rokugawa *et al*. (1980a) carried out a systemic investigation on the role of metal ions, in P1 nuclease, by selective removal of zinc from the enzyme by EDTA treatment and noted that the activity loss towards RNA and 3'AMP is related to the removal of the number of zinc atoms. The removal of one zinc atom from the enzyme resulted in a 50 % loss of its activity towards RNA but

it retained 93 % activity towards 3'AMP. While the removal of second zinc atom brought about further decrease in the RNase activity (45 %) and a significant decrease in the phosphomonoesterase activity (60 %), the removal of all the three zinc atoms resulted in complete inactivation of the enzyme and a complete disruption of the enzyme structure. Based on these observations the authors concluded that, while zinc I is involved in maintaining the tertiary structure required for RNA binding, zinc II is essential for maintaining the active conformation and zinc III is involved in holding the structural integrity of the enzyme. Like P1 nuclease, S1 nuclease is also a zinc metalloprotein and contains three atoms of zinc per molecule of the enzyme. Shishido and Habuka (1986) demonstrated that the removal of two zinc atoms from the enzyme results in the irreversible inactivation of the enzyme and the inactivation is due to the disruption of its secondary structure. Subsequently, Gite and Shankar (1992) showed the involvement of carboxylate groups in metal binding. Based on the data obtained with carboxylate group modification, EDTA treatment, reconstitution with metal ions, zinc estimation and CD analysis of the enzyme suggested that of the three zinc atoms present in S1 nuclease, zinc I is easily replaceable and is probably involved in catalysis whereas zinc II and III are involved in maintaining the enzyme structure.

The crystal structure of P1 nuclease revealed that the zinc cluster is located at the bottom of the substrate binding cleft. It consists of a relatively inaccessible dinuclear site with two zinc ions separated by 3.2 Å and a more exposed single site roughly 5 Å away from the other two zinc ions. Both zinc ions of the dinuclear pair are coordinated to four protein ligands and a bridging water molecule, while the more exposed zinc ion (ZnII) is linked to only three amino acid ligands (His126, His149 and Asp53) and two water molecules (Fig. 1.5). The coordination of ZnII resembles those observed in all structurally characterized catalytically active zinc sites reported so far (Valle and Auld, 1990), suggesting that it is directly involved in catalysis. The dinuclear zinc pair probably has an important function in stabilizing the fold of P1 nuclease.

It tightly links together three regions which are far apart in the amino acid sequence (Suck, 1992).

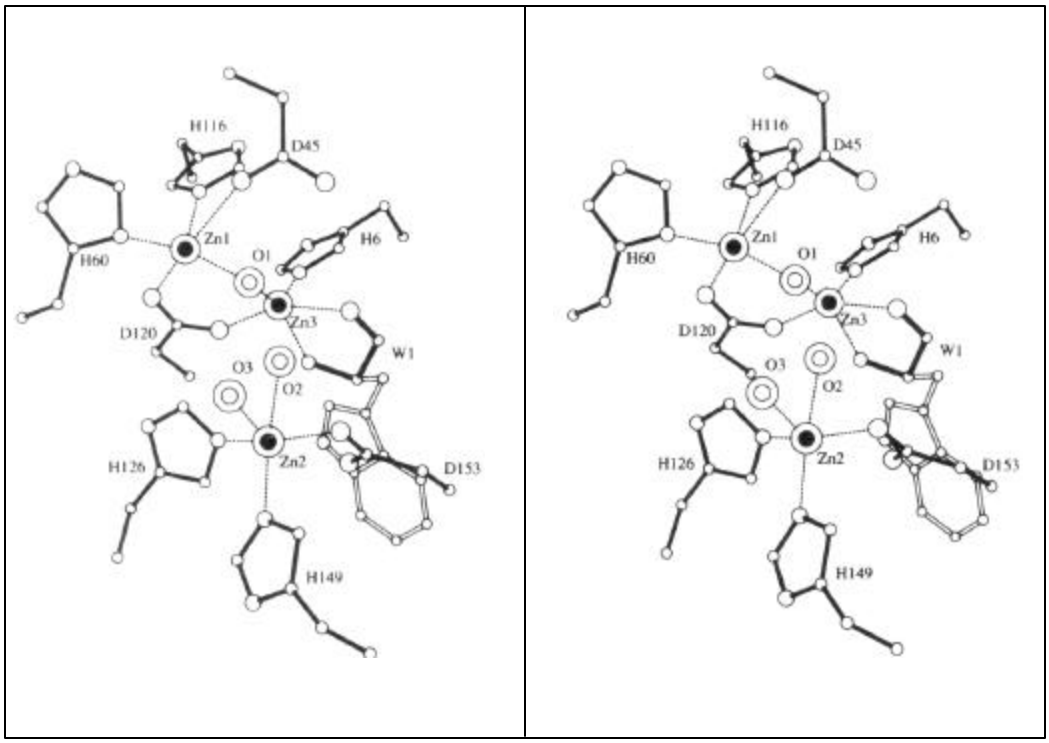

Fig. 1.5. Coordination of zinc ions in P1 nuclease. 01, 02, 03 represent the water ligands (Adapted from Suck, 1992).

Endonuclease from *S. marcescens* is a Mg^{2+} dependent DNA / RNA non-specific nuclease whereas, *Staphylococcal* nuclease is a Ca^{2+} requiring enzyme and both the enzymes do not exhibit any preference for single-stranded nucleic acids. Although the discussion of all the properties of these non-specific nucleases is outside the scope of this review, they are noteworthy, since extensive studies on these enzymes have provided insights into the substrate binding and catalytic mechanism. In case of *Serratia* nuclease, Mg^{2+} atom is coordinated with Asn119 and five solvent molecules arranged in an octahedron around the central metal atom. His89 and Glu127 are in close proximity to Mg^{2+} but do not directly interact with the metal ion (Miller *et al*.,

1999). On the other hand in *Staphylococcal* nuclease, Ca^{2+} is coordinated to Asp40 and Glu43 and a water molecule (Tucker *et al*., 1979).

***Substrate binding and catalysis* :** Although considerable work has been done on the substrate specificity and mode of action, very little information is available regarding the active site nature of single-strand-specific nucleases. Through competitive inhibition studies, it was demonstrated that the ssDNase, RNase and phosphomonoesterase activities associated with S1 nuclease (Oleson and Hoganson, 1981) and P1 nuclease (Fujimoto *et al*., 1974b) are catalyzed by the same active site. Reddy and Shankar (1989) while studying the immobilization of partially purified S1 nuclease on Con A-Sepharose made a similar observation. In case of pea seed nuclease, which has two subunits, it has been suggested that the phosphomonoesterase activity resides in one of the subunits while, the nuclease activity requires both the subunits (Naseem and Hadi, 1987). Chemical modification studies on purified S1 nuclease showed the involvement of a single lysine and histidine residue in the catalytic activity of the enzyme. The substrates of S1 nuclease viz. ssDNA, RNA and 3'AMP protected the enzyme against 2-,4-,6-trinitrobenzenesulfonic acid (TNBS)-mediated inactivation whereas, this was not observed in the case of either Methylene Blue or diethylpyrocarbonate (DEP)-mediated inactivation of the enzyme. Moreover, the lysine (TNBS)-modified enzyme showed a significant decrease (70 %) in its ability to bind 5'AMP, a competitive inhibitor of S1 nuclease, while the histidine (DEP)-modified enzyme could effectively bind 5'AMP, suggesting the involvement of lysine in substrate binding and histidine in catalysis. Furthermore, lysine and histidine modification was accompanied by a concomitant loss of ssDNase, RNase and phosphomonesterase activities of the enzyme indicating the existence of a common catalytic site for the hydrolysis of both monomeric and polymeric substrates (Gite *et al*., 1992 a,b).

Substrate binding in P1 nuclease was studied by soaking the tetragonal crystals with single-stranded dithiophosphorylated R-stereoisomers of di-, tetra- and hexanucleotides as substrate analogues (Romier *et al*., 1998). The

studies revealed the presence of two nucleotide-binding sites, one at the active site close to the catalytic zinc and the second approximately 20 Å away at the periphery of the molecule. At both sites, the base recognition involved stacking interactions with exposed aromatic residues as well as hydrogen bonding contacts with the carboxylate groups. Thus, interaction occurred with Phe61 and Val132 at the first binding site and with Tyr144 and Tyr155 at the second site. However, the hydrogen bonding occured between the adenine residues of the distereoisomer and Asp63 and Asp146 of the protein molecule (Suck, 1992). Stacking interactions have been observed in many ssDNA and RNA binding proteins as well as nucleases of the *Serratia* family and this property can be considered as a hallmark of this class of proteins (Nagai, 1996; Suck, 1997; Meiss *et al*., 1999). Similarly, differences in the contacts of the substrate with amino acid side chains in the protein were correlated to the observed differences in base specificity and pH optima for the hydrolysis of various substrates by P1 nuclease. Since no reports exist on site-directed mutagenesis of single-strand-specific nucleases, the exact residues involved in substrate binding and catalysis have not been delineated. However, for the *Serratia* endonuclease family, the active site is characterized by histidine, arginine, asparagine and glutamic acid and these residues are conserved in this family. While histidine acts as a general base, Mg^{2+} ion bound to a carboxylate acts as a general acid and asparagine is involved in the stabilization of the penta-coordinate transition state. Three arginines are implicated in substrate binding (Meiss *et al*., 1999).

In case of the structure-specific bacteriophage T5 nuclease, lysine has been implicated in catalysis. Crystal structures of three representative enzymes of this class of nucleases showed two divalent-metal-binding sites typically separated by 8–10 Å. Site-directed mutagenesis was used to investigate the role of three lysine residues (Lys83, Lys196, and Lys215) situated close to two metal-binding sites in bacteriophage T5 5'-3' exonuclease. Neither Lys196 nor Lys215 were essential for either the exo- or the endonuclease activity but mutation of these residues increased the dissociation constant for the substrate

from 5 nM to 200 nM (Lys196A) and 50 nM (Lys215A), indicating that they might be involved in substrate binding. Biochemical analysis demonstrated that Lys83 is essential for the exonucleolytic activity on ssDNA but not for the endonucleolytic cleavage of flap structures. Hence the authors suggested that Lys83 probably acts as a general base. Moreover, the enzyme exhibited different pH optima for both the endo- and exo-nuclease activities suggesting that this multifunctional enzyme uses different mechanisms for both endonuclease and exonuclease activities (Garforth *et al*., 1999). In general, it can be assumed that for both single-strand-specific as well as non-specific nucleases, histidine and carboxylate are involved in catalysis while lysine / arginine is involved in substrate binding

***Water assisted metal ion catalysis* :** As mentioned earlier, the crystal structure of majority of metallonucleases shows the presence of water molecule(s) at the active site. It may be that the metal water cluster itself is a conserved structural element in these enzymes. The water molecule acts as a nucleophile and attacks the phosphodiester bond in concert with the metal ion and the surrounding amino acid side chains. An assisting function of amino acid side chains in either orienting or activating the attacking water molecule has been proposed for a number of zinc dependent enzymes (Kim and Lipscomb, 1990). In the two metal ion mechanism proposed by Beese and Steitz (1991) for the 3',5'-exonuclease activity of *E. coli* polymerase I, catalytic RNA (Steitz and Steitz, 1993) and alkaline phosphatase (Kim and Wyckoff, 1991), a free phosphate oxygen is replaced in the solvent molecule bridging the two metal ions. One of the metal ions activated the attacking nucleophile while the other stabilized the leaving O3'-oxyanion. A similar mechanism has been proposed for the two divalent-metal-binding sites situated in the active site of the *Taq* 5' nuclease (Kim *et al*., 1995). In case of 3'-5' exonuclease activity of Pol I, it was shown that one metal ion could promote the formation of a hydroxide ion while the second stabilized the pentacoordinate transition state (Pandey *et al*., 1997). However with T4 RNase H (Bhagwat *et al*., 1997) and FEN-1 (Shen *et*

al., 1997) site directed mutagenesis studies revealed that, only one metal site is required for catalysis and the other is involved in substrate binding. Furthermore, the metal sites in T5 exonuclease were too far apart to participate in the postulated two-metal mechanism (8.1 Å in T5 exonuclease versus 3.9 Å in 3'-5' exonuclease of Pol I) (Ceska *et al*., 1996; Garforth *et al*., 1999). The conservation of metal ion-water cluster has also been shown for the *Serratia* family nucleases (Miller *et al*., 1999). Most researchers studying *Serratia* endonuclease have proposed that DNA cleavage proceeds by attack of an active site water molecule at the phosphorous atom of the bridging phosphate via the phosphorane formation followed by cleavage of the 3' O-P bond (Miller *et al*., 1994; Kolmes *et al*., 1996; Lunin *et al*., 1997). Miller *et al*. (1999) proposed two schemes for the hydrolysis of phosphodiester bond by *Serratia* endonuclease - (i) an unligated water molecule may be directly activated by His89 or (ii) magnesium bound water is activated by His89 wherein magnesium may alter the pKa of the bound water and produce a more nucleophilic hydroxide ligand and this activated water molecule may mediate the hydrolysis of the phosphodiester bond.

In contrast to the two metal ion mechanism proposed for the aforementioned nucleases, Romier *et al*. (1998) proposed a three metal ion mechanism to explain the mechanism of action of P1 nuclease (Fig. 1.6). Accordingly, the scissile phosphate of the substrate sitting between the three zinc atoms binds close to ZnII with its free oxygens replacing the two water molecules and the base 5' to the cleaved bond stacks against Phe61 and forms hydrogen bonding contacts 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. 1.6). Thus all the three zinc ions are important for catalysis (Romier *et al*., 1998).

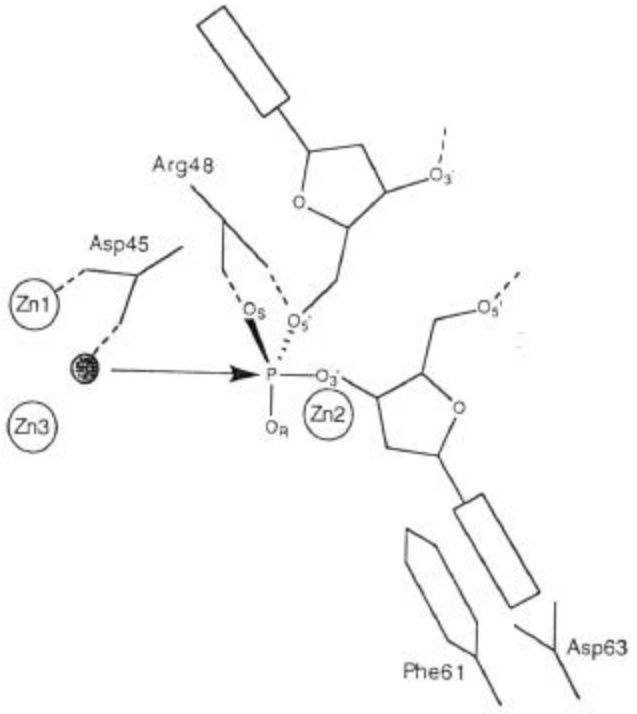

Fig. 1.6. Proposed catalytic mechanism of P1 nuclease. (Adapted from Romier *et al*., 1998).

S1 nuclease from *A. oryzae* is highly homologous to P1 nuclease (49.3 % sequence identity), contains 3g atoms of Zn^{2+} per mole of the enzyme and also requires Zn^{2+} for its activity. However, they differ in their pH optima and preference for various substrates. All the zinc ligands, as well as Phe 61 and Asp63 at the active site binding pocket are conserved in S1 nuclease. On the other hand, Arg48 in P1 nuclease is replaced by a lysine in S1 nuclease. Moreover, Gite *et al*. (1992a) have implicated lysine in substrate binding in S1 nuclease. Based on these observations, it can be assumed that arginine in P1 nuclease and lysine in S1 nuclease has a similar function. However, Tyr144, Asp146 and Tyr155, which form the Tyr-site in P1 nuclease, are replaced by

Glu, Asn and Thr, respectively in S1 nuclease. Although the structure of P1 nuclease-ssDNA complex clearly demonstrated the role of Tyr-site in nucleotide binding, they fail to provide a convincing explanation for its general function. Studies on the crystal structure of S1 nuclease, in presence of its substrate analogs, might help in evaluating the significance of this site in S1 nuclease (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 via an in-line SN-2 mechanism. In contrast, chemical nucleases are redox-active coordination complexes that nick nucleic acids under physiological conditions by oxidative attack on the ribose or deoxyribose moiety. Coordination complexes that accomplish strand scission in the presence of oxygen or reducing agents include the tetrahedral 1,10-phenanthroline-cuprous ion (Sigman *et al*., 1979), ferrous-EDTA either free or linked to DNA-binding ligands (Hertzberg and Dervan, 1984; Tullis and Domborski, 1986; Groves and Farell, 1989), various metalloporphyrin derivatives (Ward *et al*., 1986; Le Doan *et al*., 1986; Groves and Farell, 1989) and octahedral complexes of 4,7-diphenyl-diphenyl-1, 10-phenanthroline (Barton, 1986). DNA scission by uranyl acetate (Nielsen *et al*., 1988) and by the octahedral complexes of 1,10-phenanthroline and its derivatives prepared from ruthenium or cobalt is light dependent (Barton, 1986). Chemical nucleases of biological origin include bleomycin, an antibiotic from *Streptomyces verticillus* (Stubbe and Kozarich, 1987), calichaemycin from *Micromonospora echinospora* and neocarcinostatin (Zein *et al*., 1988). Sigman (1990) and Sigman and Chen (1990) have reviewed the exact mechanism of action of these artificial nucleases. Moreover, the mechanism of their potentiation and interaction with nucleic acids have been extensively described by Pratviel *et al*. (1995). The most widespread use of chemical nucleases has been as footprinting agents since they provide certain advantages

viz. better definition of the protected sequences (van Dyke and Dervan, 1983), the potential for inferring minor and major groove contacts (Kuwabara *et al*., 1986), greater sensitivity to protein-induced changes in DNA structure (Spassky and Sigman, 1985) and the capability of reacting within acrylamide gel (Kuwabara and Sigman, 1987). Papavassiliou (1995) has extensively reviewed their use as probes for studying DNA-protein interactions. 1,10-phenanthroline-cuprous ion exhibits a strong preference for the single-stranded loops of stem-loop structures in RNA (Murakawa *et al*., 1989) whereas, methidium-propyl-EDTA prefers double-stranded regions in DNA (Kean *et al*., 1985; White and Draper, 1985). Both these chemical nucleases have been used for the analysis of RNA structure and also in RNA protein interactions (Huber, 1993). 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. The importance of DNA nicking, in recombination, is strongly suggested by the formation of nicks or gaps in DNA during meiotic prophase essential for preparing the substrates for the formation of DNA heteroduplexes. Nucleases have been shown to play an important role in the formation of nicks during meiotic recombination. Holloman and Holiday (1973) observed that certain mutant strains of *U. maydis*, unable to carry out allelic recombination, showed reduced nuclease levels and hence suggested its role in recombination. High levels of endonuclease activity were observed in the basidiocarp of *C. cinereus* during the late S-phase and early karyogamy (Lu and Li, 1988). The authors also demonstrated that cofactors such as Mg^{2+} and Ca^{2+} or high temperature

enhanced the nicking activity during these meiotic phases leading to increased frequency of recombination. In contrast, DNA polymerase b peaked at late pachytene but the nuclease levels were low. Both the endonuclease and the polymerase activities were shown to be essential for meiotic recombination in *C. cinereus* (Sakaguchi and Lu, 1982). Similarly, *N. crassa* nuclease has been implicated in recombination (Chow and Fraser, 1979). The last step in recombination is repairing of nicks and gaps and at times may contain mismatched base pairs that are corrected by excision repair. The low levels of *Ustilago* nuclease in the recombination deficient mutants suggested its role in excision repair (Holloman and Holliday, 1973). Studies on nuclease α from *U. maydis* showed that it could recognize base mismatches and cleave the heteroduplexes, pointing towards its role in mismatch repair (Holloman *et al*., 1981). In the case of *N. crassa*, compared with a 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). Furthermore, the ability of S1, *Ustilago*, and *Alteromonas* nucleases to recognize minor distortions in DNA, brought about by UV-irradiation, apurinization or carcinogenic and mutagenic agents, point towards their probable role in DNA repair. The branched DNA structures formed during replication, recombination and repair are recognized and cleaved by 5'-3' exonucleases. The DNA strand displaced from the site of a nick by DNA polymerase during nick translation gives rise to flap structure which is cleaved by these structure-specific nucleases. Okazaki fragments on the lagging strand formed during replication yields similar structures in which the displaced 5'end consists of short stretches of ribonucleotides that form the primers for lagging strand synthesis. These

structures are also cleaved by the 5'-3' exonuclease activity. Nucleases have also been implicated in the restriction of invading pathogens by degrading the incoming nucleic acids. For example, nucleases from *S. antibioticus* and *S. glaucescens* circumstantially restrict the growth of actinophages (Aparicio *et al*., 1988; De los Reyes-Gavilan *et al*., 1988a,b).

An important role of the extracellular nucleases is scavenging of nucleosides and phosphate for growth. Such enzymes exhibit an associated phosphomonoesterase activity. The role of nucleases in nutrition has been demonstrated in the trypanosome *C. luciliae* (Neubert and Gottlieb, 1990). Nucleases from *S. antibioticus* and *S. glaucescens* were shown to play an important role in the recycling of nucleotides from the substrate mycelium to aerial mycelium (De los Reyes-Gavilan, 1991; Aparicio *et al*., 1992; Cal *et al*., 1995). Recently, Nicieza *et al*. (1999) isolated two extracellular nucleases from *S. antibioticus*, with Mr of 18 and 34 kDa, which were nutritionally regulated and reached 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 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 proposed to have a key role in gluco-corticoid stimulated apoptosis (Compton and Cidlowski, 1987; Gaido and Cidlowski, 1991). The *Streptomyces* 18kDa nuclease showed a requirement of both Mg^{2+} and Ca^{2+} and like NUC-18 was inhibited by Zn^{2+} and aurintricarboxylic acid. Interestingly, the N-terminal sequence of the 18kDa protein showed striking similarity to proteins of the cyclophilin family (Montague *et al*., 1994). Native cyclophilins also degrade DNA in a Ca^{2+}, Mg^{2+} dependent manner and their role in apoptosis has been reviewed by Montague *et al*. (1997).

Programmed cell death, termed as apoptosis, is a phenomenon occurring universally in all unicellular and multicellular organisms (Greenberg, 1994; Ameisen, 1996; Hochman, 1997). Its regulation is essential for the

normal development as it serves to remove surplus cells and virally infected or tumor cells (Vaux and Strasser, 1996; White, 1996; Anderson, 1997). An early biochemical change recognized as a hallmark of apoptosis is internucleosomal DNA cleavage. Recently, the generation of large DNA fragments (~ 30-50 kb) and single-strand nicking also have been reported to be associated with apoptosis. Several endonucleases such as DNase I, DNase II, NUC18, NUC58, NUC40 and 27 and 37 kDa endonucleases have been suggested as enzymes responsible for the characteristic DNA cleavage during apoptosis (Huang *et al*., 1997; Lei and Kwong-Hung, 1997; Famulski *et al*., 1999; Qing *et al*., 1999). Most of the nucleases, having a role in apoptosis, can efficiently degrade double-stranded nucleic acids, are Ca^{2+}/ Mg^{2+} dependent enzymes that produce 3'-OH DNA breaks. However, they are inhibited by Zn^{2+} and aurintricarboxylic acid.

DNA / RNA non-specific nucleases like those 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, nuclease 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. However, the role of single-strand-specific nucleases in diseases has not been postulated. Bufe *et al*. (1994,1995) discovered that the major group V allergen of grass pollen, Phelum pratense 5b (Phlp5b) from timothy grass, showed ribonuclease activity. Subsequently, 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. However, the detailed characterization of Phlp5b nuclease indicated that it is specific for ssDNA and RNA. Interestingly, the enzyme did not degrade dsDNA but showed functional characteristics of a topoisomerase (Bufe *et al*., 1999). Phlp5b is located in the cytosol (Grote *et al*., 1998) and is quickly released from the pollen grain once

the cell is humidified (Vrtala *et al*., 1993). The interesting feature of this allergen is that the C-terminal 13kDa component showed significantly higher nuclease activity than the full-length holoallergen as seen from the crystallization studies. Moreover, studies on the active site nature revealed the involvement of two tyrosine residues and a region of four amino acids strikingly similar to those from the active site region of topoisomerase I from *E. coli*, confirming that the pollen allergen has topoisomerase activity (Bufe *et al*., 1996). *Fusarium solani* f. spp. *pisi* is a pathogen of pea (*Pisum sativum*) while *Fusarium solani* f. spp *phaseoli* is a pathogen of bean (*Phaseolus vulgaris*). These species produced a heat-stable nuclease, activated by Mn^{2+} and Ca^{2+} and a marked stimulation of nuclease production occurred during macroconidium germination on pea pod endocarp surfaces (Gerhold *et al*.,1993). Hence, the authors postulated that the nuclease production by these species is stimulated by the host and might contribute to the virulence towards host plants.

Applications

***Analytical* :** Since their discovery, single-strand-specific nucleases, 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. S1 nuclease is the most widely used enzyme in molecular biology research. Its high selectivity for single-stranded nucleic acids has been employed for the estimation of double-helical content of various single-stranded nucleic acids (Shishido and Ando, 1972) and isolation and characterization of double-stranded regions of single-stranded nucleic acids (Shishido and Ikeda, 1970; 1971a, 1971b). It has also been used for the removal of ssDNA in genetic manipulation, viz. S1 mapping to determine transcriptional initiation sites (Berk and Sharp, 1977; Viville and Mantovani, 1998) and two dimensional analysis of complexible transcripts (Berk and Sharp 1977; Weaver and Weissman, 1979; Favalaro *et al*., 1980; Sharp *et al*., 1980). A variety of

methods were developed to screen genetic mutations using single-strand-specific nucleases (Orita *et al*., 1989; Weinstein *et al*., 1990; Prior *et al*., 1993). Due to the ability of S1 nuclease to cleave base pair mismatches in DNA / DNA heteroduplexes, it was used for heteroduplex analysis of PCR products (Howard *et al*.,1999). Moreover, S1, *N. crassa,* and BAl 31 nucleases can recognize structural alterations induced by various mutagenic agents and introduce single- and double-stranded breaks in DNA and hence can be used for the detection of locally altered structures in DNA (Shishido and Ando, 1985). Hatakeyama *et al*. (1998) immobilized the DNA probe on latex particles and noted that DNAs containing single point mutations failed to bind strongly to the immobilized probe, as opposed to native DNAs. The ability of S1 nuclease to cleave unhybridized and loosely hybridized DNA regions on the probe was exploited for the detection of fully complementary hybrids. Some other applications of S1 nuclease include the removal of single-stranded tails prior to DNA ligation (Shishido and Ando, 1981), isolation of inserts from plasmid DNA (Hofstetter *et al*., 1976) screening of DNA binding proteins and substances (Meyer *et al*., 1980; Shishido *et al*., 1980), study of pallindromic sequences in DNA (Wilson and Thomas, 1974), enzymatic synthesis of globin genes *in vitro* (Efstratiadis *et al*., 1976) and detection of non-B secondary structures (Marcel *et al*., 1998). S1 nuclease was used for locating the 5' and 3' termini of mRNA and DNA templates, the 5' and 3' splice junctions in relation to sites of cleavage with restriction enzymes in cloned genes or double-stranded cDNA and to quantitate the amount of specific classes of mRNA in RNAs extracted from tissues or cultured cells (Berk, 1989). It has also been found useful for the structural analysis of tRNAs and rRNAs (van Boom *et al*., 1982). The application of S1 nuclease for the analysis of RNA has been extensively reviewed by Lefebvre and Viville, (1998). Similarly, plant nucleases such as the rye germ nuclease (Przykorska *et al*., 1991, 1992; El Adlouni *et al*., 1993) and wheat chloroplast nuclease in combination with rye germ nuclease (Przykorska *et al*., 1989; Przykorska, 1995) were used for the structural determination of various tRNAs.

P1 nuclease from *P. citrinum* has been used for the isolation of eukaryotic mRNA cap structure (Furuichi *et al*., 1975), base composition analysis of nucleic acids (Katayama-Fujimura *et al*., 1984), removal of nucleic acids during protein purification (Zabriskie and DiPaola, 1988), sequence analysis of end-group labeled RNA (Silberklang *et al*., 1977), and analysis of tRNA structure (Aultman and Chang, 1982).

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 for the detection of sequence heterology (Bartok *et al*., 1974). BAL 31 nuclease shows exonuclease activity and shortens duplex DNA from both 3' and 5' ends. This property was exploited for ordering restriction endonuclease generated DNA fragments (Legerski *et al*., 1978).

Industrial **:** Monosodium glutamate has long been used as a food flavouring agent. However, with the realization that the addition of an equimolar mixture of 5'GMP and 5'IMP to monosodium glutamate can significantly increase the flavour enhancing capacity has led to a considerable interest in the production of 5'mononucleotides. Additionally, the derivatives of IMP and GMP, like 2-methylinosine-5'-monophosphate, 2-N-methyl-guanosine-5'monophosphate and 2-N,N-dimethylguanosine-5'-monophosphate, when fortified with L-glutamic acid or L-homocysteic acid not only act as strong flavour enhancers but also resist the deterioration effect of the enzymes present in food (Gutcho, 1970). P1 nuclease from *P. citrinum* which degrades RNA to 5' mononucelotides has been used for the industrial production of 5'-mononucleotides from yeast RNA (Kuninaka *et al*., 1961). Sheep kidney nuclease which converts single-stranded nucleic acid to 5'dinucleotides and trinucleotides as the final products, can also be used for the production of all the deoxydinucleotides (Watanabe, 1978).

***Immobilization* :** Biotechnological applications of enzymes are limited by the high cost of their production and stabilization on storage. As they are soluble, their recovery from the mixture of substrates and products for reuse is not economically practical and this renders the costly enzymatic process even more costly. However, with the advent of immobilized enzyme technology, increasing efforts are being made to replace the conventional enzymatic reactions with immobilized enzymes as immobilization offers important advantages like, reusability, enhanced stability, greater control of the catalytic process and the development of continuous process. Taking into consideration the importance of single-strand-specific nucleases as analytical tools and their use in industry, several attempts have been made to obtain highly active and stable immobilized preparations suitable for various biotechnological applications. Although, Reddy and Shankar (1993) have extensively reviewed the various applications of immobilized nucleases, a few representative examples of immobilized single-strand-specific nucleases are given below.

Knorre *et al*. (1973) bound *E. coli* phosphomonoesterase and snake venom phosphodiesterase (pretreated with $ZnCl_2$, at pH 8.6, to inactivate 5' nucleotidase) covalently to DEAE-cellulose to assess its utility for the quantitative determination of nucleotide composition and 5'terminal analysis of oligonucleotides. The prior inactivation of 5' nucleotidase was essential as it eliminated the use of pure snake venom phosphodiesterase for immobilization. The immobilized system could be used effectively for the analysis of deoxyribo- and ribo-oligonucleotides with 3' and 5' phosphate groups. The use of immobilized system offers additional advantages in that, the analysis of oligonucleotides by successive use of phosphodiesterase and phosphomonoesterase eliminates the need for the isolation of dephosphorylated intermediates.

S1 nuclease is a glycoprotein and contains 18 % carbohydrate (Rushizky, 1981). It has been shown that the carbohydrate moiety of the enzyme is not essential for its activity and stability (Shishido and Habuka, 1986). Hence, Gite and Shankar (1993) bound purified S1 nuclease to Con A

Sepharose and aminobutyl-Bio-Gel P-2 through its carbohydrate moiety with high retention of activity and stability. They demonstrated that like the soluble enzyme, both aminobutyl-Bio-Gel P-2 and Con A-Sepharose bound S1 nuclease could effectively remove the single-stranded tails from Bam H1 digested pUC 8 DNA, suggesting its utility as a reusable analytical tool.

In view of the high demand for 5' nucleotides in food and beverage industries, several attempts were made to immobilize 5' nucleotide producing enzymes, namely P1 and S1 nucleases, to assess their potential for the commercial production of 5'mononucleotides. Fujishima *et al*. (1977) bound P1 nuclease to an anion exchange resin (Unilex A-885) and used the immobilized preparation for the hydrolysis of RNA to 5'-mononucleotides. It was observed that the immobilized system could simultaneously degrade and fractionate nucleic acids. Rokugawa *et al*. (1980b) coupled P1 nuclease to inorganic supports like silica gel, porous glass and pumice stone activated with various transition metal salts and noted that the enzyme bound to titanium activated pumice stone showed high efficiency and superior temperature stability. The activity of the bound enzyme depended on various parameters like coupling pH, matrix to enzyme ratio and concentration of titanium chloride used for the activation of the matrix. The bound P1 nuclease could hydrolyze 1 % RNA for 25-28 days and the support could be repeatedly used after regeneration. P1 nuclease immobilized on *p*-aminobenzylsulfonylethyl (*p*-ABSE)-cellulose showed optimum activity at pH 4.8 and 75 °C, in addition to high storage stability. Large scale hydrolysis of RNA, using immobilized nuclease, revealed the immobilized enzyme to be 30 times more active than the soluble enzyme (Yuan *et al*., 1980). Similarly, ribo- and deoxyribonucleoside-5'-monophosphates were obtained by direct hydrolysis of nucleic acids, in a fluid bed bioreactor containing immobilized S1 nuclease (Khomov *et al*., 1997).

Present Investigation

As mentioned earlier, nucleic acids act as carriers of genetic information from one generation to the other. In order that the genetic information is faithfully transferred to the next generation, they have to undergo processes such as replication, recombination and repair. All living systems contain a set of enzymes called nucleases, which interact with nucleic acids and hydrolyze the phosphodiester linkages. Nucleases exhibiting high selectivity for single stranded nucleic acids and single-stranded regions in double-stranded nucleic acids are classified as single-strand-specific nucleases. Though single-strand-specific nucleases are ubiquitous in distribution, the enzymes of analytical interest originate mainly from fungal sources. However, most of the analytically important single-strand-specific 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 single-strand-specific nucleases with optimum pH at or around neutrality and which do not require metal ions for activity. Screening of several fungal cultures revealed that a strain of *Basidiobolus haptosporus* (89-3-24) produces high levels of extracellular nuclease, when grown on YPG medium. The crude enzyme showed high activity on sonicated and heat denatured DNA than on native DNA, at pH 7.0, in the absence of metal ions. 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 2

PURIFICATION AND CHARACTERIZATION OF THE DEOXYRIBONUCLEASE ACTIVITY

SUMMARY

An extracellular nuclease from *Basidiobolus haptosporus* (designated as nuclease Bh1) was purified to homogeneity by ammonium sulfate precipitation, heat treatment, negative adsorption on DEAE-cellulose, chromatography on Phenyl-Sepharose followed by FPLC on Phenyl Superose, with an overall yield of 26 %. The Mr of the purified enzyme, determined by gel filtration was 41,000 whereas by SDS-PAGE (after deglycosylation) it was 30,000. It is a glycoprotein with a pI of 6.8. The optimum pH and temperature, for DNA hydrolysis, were 8.5 and 60 °C respectively. Nuclease Bh1 neither showed an obligate requirement of metal ions for its activity nor the activity was stimulated in presence of metal ions but is a metalloprotein. The enzyme was inhibited by Zn^{2+}, Ag^{2+}, Hg^{2+}, Fe^{3+}, Al^{3+}, inorganic phosphate, pyrophosphate, DTT, ß- mercaptoethanol, NaCl and KCl. It was stable to high concentrations of organic solvents and urea but susceptible to low concentrations of SDS and guanidine hydrochloride. Nuclease Bh1 is a multifunctional enzyme and its substrate specificity is in the order of ssDNA ≈ 3'AMP >> RNA > dsDNA. The studies on the mode of action showed that it cleaved supercoiled pUC 18 DNA and phage M13 DNA, endonucleolytically, generating single base nicks. The enzyme hydrolyzed DNA with preferential liberation of 5'dGMP, suggesting it to be a guanylic acid preferential endoexonuclease. 5'dGMP, the end product of hydrolysis, was a competitive inhibitor of the enzyme. The absence of 5'dCMP in the hydrolytic products of DNA coupled with the resistance of $(dC)_{10}$ and deoxyribodinucleoside monophosphates having cytosine either at the 3' or the 5' end indicates that G-linkages are resistant to cleavage.

INTRODUCTION

Single-strand-specific nucleases are widely distributed in microorganisms, plants and animals. They exhibit high selectivity for single stranded nucleic acids and single-stranded regions in double stranded nucleic

acids and hence they are widely used as probes for the structural determination of nucleic acids, mapping mutations and studying the interactions of DNA with various intercalating agents (Drew, 1984). They have also been implicated in recombination, repair (West, 1985) and replication (Brown *et al.*, 1985). Though single-strand-specific nucleases are ubiquitous in distribution, the enzymes of analytical interest originate mainly from fungal sources. However, only few enzymes like S1 nuclease from *Aspergillus oryzae*, P1 nuclease from *Penicillium citrinum*, BAL 31 nuclease from *Alteromonas espejiana*, *Neurospora crassa, Ustilago maydis* and mung bean nucleases have been sufficiently characterized (Shishido and Ando, 1985). Most of the analytically important single-strand-specific nucleases have an acid optimum pH and show an obligate requirement of metal ions for their activity which inturn prevents their use in chelating buffers or in presence of metal chelators. Hence, there is a need to look for single-strand-specific nucleases with optimum pH at or around neutrality and which do not require metal ions for activity. Screening of several fungal cultures revealed that a strain of *Basidiobolus haptosporus* (89-3-24) produces high levels of extracellular nuclease, when grown on YPG medium. The crude enzyme showed high activity on sonicated and heat denatured DNA than on native DNA, at pH 7.0, in the absence of metal ions. The purification of this single-strand-specific nuclease and extensive characterization of its deoxyribonuclease activity are described in this chapter.

MATERIALS

DEAE – Cellulose (DE-52) (Whatman Paper Ltd., Maidstone, U.K); Phenyl-Sepharose CL 4B, Sephadex G-75, Pharmalytes and gel-filtration molecular weight markers (Pharmacia Fine Chemicals, Uppasala, Sweden); calf intestinal alkaline phosphatase and M13 phage DNA (M13mp18, covalently closed single stranded circular DNA) (Bangalore Genei, Bangalore, India); T4 DNA ligase, snake venom phosphodiesterase, spleen phosphodiesterase, endoglycosidase H, SDS molecular mass markers, sodium bis (*p*-nitrophenyl) phosphate, all 2', 3' and 5' mononucleotides, FPLC grade

ammonium sulfate, CAPS [3-(cyclohexylamino-1-propanesulfonic acid)], PVDF (polyvinylidene difluoride) membrane and Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO, USA) and *p*-nitrophenyl phosphate (Loba Chemie Pvt. Ltd, Mumbai, India) were used. All other chemicals used were of analytical grade. Yeast RNA (BDH Chemicals Ltd., Poole, England) was purified by precipitating twice with absolute ethanol.

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 the operations were carried out at 0 - 4 °C. Fresh buffalo liver, obtained from the local slaughterhouse, was frozen in liquid nitrogen. Subsequently, 100 g of liver was chopped into small pieces and suspended in minimum volume of 50 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 at 9000 g for 20 min. The crude nuclear pellet, obtained after centrifugation, was washed 2-3 times with the homogenization buffer (till the supernatant was colorless) 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 SDS to a final concentration of 2 % (w/v) and incubated at 62 °C for 30 min to facilitate the lysis of nuclei. The lysate was then brought to room temperature and 5M sodium perchlorate was added to a final concentration of 1M. 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 at 9000 g for 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 SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0). From the DNA preparation, RNA contamination was removed by incubation with purified

RNase Rs from *Rhizopus stolonifer* (100 U / ml) at 37° C for 1h. 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 purity of the DNA preparation was determined by measuring the absorption 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 molecular weight nature of the DNA was also checked electrophoretically using 1 % (w/v) agarose gels.

Plasmid pUC18 DNA was isolated according to Birnboim and Doly (1979). The inoculum was prepared in Luria broth (containing 100 µg / ml ampicillin) by inoculating a single colony of *Escherichia coli* harbouring pUC18 plasmid followed by incubation at 37° C, under vigorous agitation, for 15h. The inoculum was then transferred aseptically into a 500 ml conical flask containing 100 ml of the above medium and incubated at 37° C under vigorous shaking, till the culture reached late log phase (i.e. $OD_{660} \approx 0.6$). Subsequently, the cells were harvested by centrifugation (4000 g, 10 min, 4°C), washed with ice-cold STE buffer (100 mM NaCl, 10 mM Tris-HCl and 1mM EDTA, pH 8.0) and after resuspending in 10 ml of 25 mM Tris-HCl buffer, pH 8.0 (containing 50 mM glucose, 10 mM EDTA and 10 mg lysozyme) was left at room temperature for 5 min. This suspension was then mixed with 20 ml of a freshly prepared solution of 0.1 % (w/v) SDS and incubated at 0°C for 10 min. After the incubation period, 15 ml of an ice-cold solution of 5M potassium acetate (pH 4.8) was added and the mixture was allowed to stand at 0°C for an additional 10 min. The supernatant obtained after centrifugation (12,000 g, 20 min, 4°C) was mixed with 0.6 volume of isopropanol and left at room temperature, for 15 min, for the precipitation of plasmid DNA. The precipitated DNA was collected by centrifugation (12,000 g, 30 min), washed with absolute ethanol, dissolved in 20 mM Tris-HCl buffer pH 8.0 and stored at -20°C. The purity of the isolated DNA was checked by absorbance ratio of A_{260} : A_{280} and agarose gel [1 % (w/v)] electrophoresis.

Synthesis of deoxyribodinucleoside monophosphates and deoxyribo-oligonucleotides **:** All 16 deoxyribodinucleoside monophosphates [$d(N_pN)$]s and deoxyribo-decamers [$(dT)_{10}$, $(dG)_{10}$, $(dA)_{10}$ and $(dC)_{10}$] were synthesized, at 0.2 μmol scale, on a Pharmacia GA plus DNA synthesizer using standard phosphoramidite chemistry. They were purified on NAP-10 and NAP-25 columns (Pharmacia), respectively and their concentrations were determined by absorbance at 260 nm. Their purity was checked by reverse phase HPLC.

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 8.5, containing 2% (v/v) glycerol 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 % (w/v) 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 was 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.

Ki was determined from Lineweaver-Burk plots.

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 8.5 and appropriately diluted enzyme. The reaction was initiated by the addition of RNA followed by incubation at 37 °C for 15 min. The reaction was then arrested by the addition of 0.25 ml of MacFadyen's reagent [0.75 % (w/v) uranyl acetate in 25 % (v/v) perchloric

acid]. The mixture was left on ice for 10 min and the precipitate was removed by centrifugation (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.

Phosphodiesterase activity of nuclease Bh1 **:** 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 30 mM Tris-HCl buffer, pH 8.5, containing 2 % (v/v) glycerol 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 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.

Phosphomonoesterase activity of nuclease Bh1 **:** 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 8.5, containing 2 % (v/v) glycerol 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 water : 12 M H_2SO_4 : 2.5 % (w/v) ammonium molybdate: 10 % (w/v) ascorbic acid in the ratio of 2:1:1:1 (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.

***Phosphatase activity of nuclease Bh1* :** 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 8.5, containing 2 % (v/v) glycerol 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

During enzyme purification steps, protein concentrations were determined using the formula 1.55 A_{280} – 0.76 A_{260} = protein (mg / ml) (Stoschek, 1990). However, for N-terminal analysis and deglycosylation experiments, the protein concentration was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Microorganism and growth

Basidiobolus haptosporus (89-3-24) was kindly provided by Dr. M.C. Srinivasan, Division of Biochemical Sciences, National Chemical Laboratory. The culture was routinely maintained on MGYP slants [0.3 % (w/v) malt extract, 1 % (w/v) glucose, 0.3 % (w/v) yeast extract and 0.5 % (w/v) peptone]. The fermentation was carried out by inoculating a 1 cm diameter colony, from a 24h old MGYP slant, into 100 ml liquid YPG medium [0.1 % (w/v) yeast extract, 0.5 % (w/v) peptone and 2 % (w/v) glucose] in 500 ml Erlenmeyer flasks followed by incubation at 30 °C on a rotary shaker (200 rpm). After 96 h of fermentation, the extracellular broth was collected by filtration on

Whatman No. 1, concentrated using an Amicon ultrafiltration unit fitted with YM-3 membrane and used as the source of enzyme.

Purification of nuclease Bh1

Unless otherwise stated, all the operations were carried out at 6 ± 1°C. During the purification steps, the enzyme activity was monitored using sonicated and heat denatured DNA at pH 7.0.

Ammonium sulfate precipitation and heat treatment **:** The concentrated broth (1 L) was brought to 0.9 saturation by the addition of solid ammonium sulfate, under constant stirring and left overnight. The precipitated protein was collected by centrifugation (5000g, 20 min), dissolved in minimum volume of buffer A [30 mM Tris-HCl buffer pH 7.0, containing 5 % (v/v) glycerol] and dialyzed extensively against the same buffer. The precipitate obtained after dialysis, if any, was removed by centrifugation (8,000 g, 10 min) and the supernatant was subjected to heat treatment (60°C, 5 min). The heat treated sample was then subjected to DEAE-cellulose chromatography.

DEAE-cellulose chromatography **:** The enzyme obtained from the above step was loaded onto a DEAE-cellulose column (2.5 x 130 cm), pre-equilibrated with buffer A, at a flow rate of 15 ml/h. The unadsorbed fractions containing the nuclease activity (specific activity > 10,000 U / mg) were pooled, concentrated by Amicon ultrafiltration and dialyzed extensively against buffer A containing 22.4 % (w/v) ammonium sulfate and used for the next step.

Phenyl-Sepharose chromatography **:** The enzyme obtained after DEAE-cellulose chromatography was loaded onto a Phenyl-Sepharose column (1.5 x 15 cm), pre-equilibrated with buffer A containing 22.4 % (w/v) ammonium sulfate, at a flow rate of 15 ml / h. The column was then washed with the same buffer till the flow-through fractions showed no DNase activity. The bound enzyme was then eluted with buffer A containing decreasing concentrations of

ammonium sulfate [18, 13.5, 11.2, 9.0, 4.5, 2.0 and 0 % (w/v), respectively]. The nuclease activity separated into two peaks. The major nuclease activity (comprising of 70 - 75 % of the total nuclease activity), which eluted with buffer A containing 9.0 % (w / v) ammonium sulfate, was collected and the fractions having specific activity above 1,00,000 U/mg were pooled, concentrated by Amicon ultrafiltration and dialyzed extensively against 10 mM buffer A containing 20 % (w/v) ammonium sulfate. The precipitate obtained after dialysis, if any, was removed by centrifugation (8000g, 15 min) and the supernatant was used for FPLC.

FPLC on Phenyl Superose **:** The enzyme obtained from the above step was chromatographed on Phenyl Superose HR 5/5 (1 ml column, Pharmacia Fine Chemicals, Uppasala, Sweden), pre-equilibrated with 10 mM buffer A containing 20 % (w/v) ammonium sulfate, at a flow rate of 0.5 ml / min. The bound enzyme was then eluted by a decreasing gradient, 40 ml total volume, of ammonium sulfate [20.0 – 0.0 % (w/v)] in the above buffer. The eluted enzyme was then extensively dialyzed against 30 mM buffer A and stored at - 20 °C.

Electrophoresis

Native PAGE of the purified nuclease was carried out in 7.5 % (w/v) polyacrylamide gel, at pH 4.3, according to Reisfeld *et al*. (1962). SDS-PAGE was carried out, at pH 8.3, according to Laemmli (1970) in 10 % (w/v) polyacrylamide gel. After electrophoresis, the bands were visualized by silver staining according to Blum *et al*. (1987).

The pI of the enzyme was determined by density gradient single column isoelectric focussing according to Chinnathambi *et al*. (1995) using Pharmalytes in the pH range of 6-8.

Carbohydrate content

Purified nuclease Bh1 (100 µg in 400 µl water) was incubated with 400 µl of 5 % (w/v) phenol for 10 min at room temperature. Two ml of sulfuric acid was then added and the mixture was allowed to cool for 20 min at room temperature. The colour developed was then measured spectrophotometrically, at 490 nm, using mannose as standard (Dubois *et al.*, 1956).

Glycoprotein staining

After SDS-PAGE, the gel and the nitrocellulose membrane were sandwiched between 3 mm Whatmann paper and placed in a blotting cassette. The tank was filled with Tris-glycine buffer [25 mM Tris, 192 mM glycine and 20 % (v/v) methanol] pH 8.3 and the electrotransfer was carried out for 45 min at 100V. After the electrotransfer, the nitrocellulose membrane was immersed in 50 % (v/v) methanol for 20 min followed by extensive washing with water to remove the methanol. The blot was then subjected to oxidation with periodic acid (20 min), followed by water wash for 5 min and the bands were stained with Schiff's stain (Fuchsin-sulfite reagent, carbohydrate staining kit, Sigma, USA). The colouring reaction was quenched by subjecting the blot to reducing solution (20 min), followed by 2-5 min water wash.

Deglycosylation of nuclease Bh1

Ten µg of FPLC purified protein, after extensive dialysis against Milli Q water, was denatured by incubation at 100 °C for 5 min in presence of 0.1 % (w/v) SDS and 1mM β- mercaptoethanol. To the denatured protein, in 40 µl of 50 mM sodium citrate buffer pH 5.5, 0.03 U of Endoglycosidase H was added (in 2 instalments, at an interval of 4h) and incubated at 37 °C for 12 h. The reaction mixture was then chilled on ice and the deglycosylated and control (10µg of denatured protein incubated in the absence of Endoglycosidase H) samples were analyzed on SDS-PAGE at pH 8.3 [10 % (w/v) polyacrylamide gel].

Mr determination

Gel filtration : Mr of the purified enzyme was determined on a Sephadex G – 75 column (1.5 x 80 cm) pre-equilibrated with 30 mM Tris-HCl buffer, pH 7.0, containing 5 % (v/v) glycerol and 200 mM NaCl, using bovine serum albumin (Mr 67,000), ovalbumin (Mr 43,000), chymotrypsinogen A (Mr 25,000) and RNase A (Mr 13,700) as reference proteins.

SDS-PAGE : This was carried out in 10 % (w/v) polyacrylamide gels, at pH 8.3, according to Laemmli (1970) using bovine serum albumin (Mr 66,000), ovalbumin (Mr 45,000), glyceraldehyde–3–phosphate dehydrogenase (Mr 36,000), carbonic anhydrase (Mr 29,000) and trypsinogen (Mr 24,000) as reference proteins.

Amino acid analysis

Salt-free lyophilized enzyme sample (100 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.

N-terminal analysis

Purified enzyme (200 µg) was extensively dialyzed against Milli Q water and subjected to SDS-PAGE at pH 8.3 [10 % (w/v) polyacrylamide gel] according to Laemmli (1970). Subsequently, the protein was electroblotted onto a PVDF membrane, in 10 mM CAPS buffer pH 11.0 [containing 10 % (v/v) methanol], under a constant current of 250 mA for 40 min as described by LeGendre *et al*. (1993). After the electrotransfer, the PVDF membrane was washed several times with Milli Q water and stained with Coomassie Brilliant Blue R-250. The N-terminal amino acid sequence was determined by

subjecting the blot to Edman degradation on an automated protein sequencer (Applied BioSystems, USA).

Atomic absorption spectrophotometry

Zinc content of purified nuclease Bh1 was determined by atomic absorption on a ICP-OES (Perkin Elmer P-1000) atomic absorption spectrophotometer at 213.8 nm, whereas the cobalt content was estimated at 240 nm. Prior to analysis, the enzyme samples were extensively dialyzed against Milli Q water to remove the buffer salts. The insoluble material, if any, was removed by centrifugation (8000 g, 5 min).

Action on plasmid pUC18 and phage M13 DNAs

The reaction mixture of 20 µl each, containing 400 ng of pUC 18 supercoiled plasmid DNA or 200 ng of covalently closed single-stranded circular phage M13 DNA, in 30 mM Tris-HCl buffer pH 8.5, was incubated with appropriate amounts of nuclease Bh1 (0.1 - 2 U) at 37º C. Samples were removed at different time intervals and the reaction was terminated by the addition of 3 µl of stop solution [10 mM EDTA, 0.25 % (w/v) BPB and 25 % (v/v) glycerol]. The samples were then analyzed on 1.2 % (w/v) agarose gels, containing 3 µg/ml ethidium bromide, at pH 8.1 (40 mM Tris-acetate buffer containing 1 mM EDTA) and 60 V for 3h.

Ligation reaction

Supercoiled pUC18 DNA (400 ng) was incubated with 0.1 U of nuclease Bh1 at pH 8.5 (30 mM Tris-HCl buffer) and 37 ºC for 10 min and the reaction was stopped by the addition of 2mM 5'GMP (effective concentration). To the relaxed DNA thus generated 20 U of T4 DNA ligase were added and incubated at 20 ºC for 1h and overnight. Ligation was then analyzed on 1.2 % (w/v) agarose gels, at pH 8.1, as mentioned above.

HPLC analysis of the reaction products of nuclease Bh1

Action on DNA **:** The total reaction mixture of 10 ml, containing 5 mg of either native or sonicated and heat denatured buffalo liver DNA, was incubated with appropriate amounts of nuclease Bh1 at pH 8.5 and 37 °C. Aliquots (1 ml) were removed at fixed time intervals and the reaction was terminated by the addition of 2 volumes of chilled absolute ethanol. The mixture was left overnight at –20 °C and the undigested DNA was removed by centrifugation (8000g, 15 min). The supernatant was lyophilized, reconstituted in 100 µl of Milli Q water and subjected to HPLC.

HPLC (Perkin Elmer Model fitted with 410 LC pump) was performed on a Nucleosil C18 column (120 x 3 mm, 3µm, 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 0.8 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 LC235 Diode Array Detector and the peaks were recorded on a Perkin Elmer LCI – 100 recorder. The amount occupied by each nucleotide 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. 4.38, 10.38, 12.87 and 16.25 min, respectively (data not shown).

Determination of the phosphomonoester termini of the reaction products **:** The hydrolytic products of DNA, obtained following the action of nuclease Bh1, were lyophilized, reconstituted in 40 µl of 30 mM Tris-HCl buffer pH 7.5 and incubated with 0.1 U of either snake venom phosphodiesterase or spleen phosphodiesterase at 37 °C for 15 min. The reaction was then terminated by the addition of two volumes of chilled absolute ethanol. The samples were concentrated by lyophilization, reconstituted in 20 µl Milli Q water and subjected to HPLC analysis as mentioned above.

Action on deoxyribooligonucleotides **:** The total reaction mixture of 500 µl, in 30 mM Tris-HCl buffer pH 8.5 containing 0.5 O.D. of the decamer [$(dT)_{10}$, $(dG)_{10}$, $(dA)_{10}$ or $(dC)_{10}$], was incubated with 5 - 50 U of nuclease Bh1 at 37 °C for 1h. After the incubation period, the reaction was terminated by the addition of 500 µl of 10 % (v/v) chilled perchloric acid and 500 µl of 0.2 % (w/v) BSA. The mixture was left on ice for 10 min and then centrifuged (2500 g, 15 min) to remove the precipitate. The acid soluble deoxyribonucleotides in the supernatant were measured at 260 nm.

The time course of hydrolysis of $(dT)_{10}$ was carried out by incubating 1.0 O.D. of $(dT)_{10}$, in 100 µl of 30 mM Tris-HCl buffer pH 8.5, with 2U of nuclease Bh1 at 37 °C. Aliquots (20 µl) were removed at different time intervals and the reaction was terminated by the addition of two volumes of chilled ethanol. The mixture was left overnight at -20 °C. The samples were then lyophilized, reconstituted in 20 µl Milli Q water and subjected to HPLC as mentioned above.

Action on deoxyribodinucleoside monophosphates **:** The total reaction mixture of 100 µl, in 30 mM Tris-HCl buffer pH 8.5 containing 0.5 O.D. of each dinucleoside monophosphate, was incubated with 20 U of nuclease Bh1, at 37 °C for 12h. After the incubation period, the reaction was arrested by the addition of two volumes of chilled absolute ethanol. The samples were lyophilized, reconstituted in 20 µl 1X calf intestinal alkaline phosphatase buffer (10 mM Tris-HCl buffer, pH 8.2, containing 50 mM NaCl, 10 mM $MgCl_2$ and 1mM DTT) and incubated with 0.1 U of calf intestinal alkaline phosphatase for 1h at 37° C. The reaction was terminated by heat treatment (75°C, 10 min) and the products were analyzed by HPLC on a Symmetry C18 column (250 x 4.6 mm, 5 µm, Waters model fitted with 515 HPLC pump) at 25 ± 1 °C. Twenty µl of the standard or the sample solution were injected onto the column and the column was washed with 100mM triethylammonium acetate, pH 6.2, for 6 min at a flow rate of 0.3 ml/ min. Subsequently, the nucleosides were eluted with a linear gradient of acetonitrile in 100 mM

triethylammonium acetate, pH 6.2 [0- 10 % (v/v) for 15 min, 10-12 % (v/v) for 25 min, 12-15 % (v/v) for 35 min, 15-20 % (v/v) for 40 min followed by 20 - 100 % (v/v) for 5 min], at a flow rate of 0.8 ml/ min. The nucleosides were detected, at 255 nm, using a Waters 2487 Dual λ Detector and the peaks were visualized using a computer attached to the system. The nucleosides eluted in the order of dC, dT, dG and dA with retention times of ca. 11.32, 23.53, 24.18 and 25.62 min, respectively (data not shown).

RESULTS AND DISCUSSION

Purification of nuclease Bh1

Basidiobolus haptosporus produces extracellular ssDNase and RNase in a ratio of 1:1. Heat treatment of the ammonium sulfate precipitated and dialyzed enzyme resulted in a significant loss of RNase activity (indicated by DNase to RNase ratio) suggesting that the major extracellular RNase activity is not associated with the DNase activity. Subsequently, successive chromatography on DEAE-cellulose and Phenyl-Sepharose helped in the removal of colored impurities and excess carbohydrate, respectively. Finally, FPLC on Phenyl Superose yielded a homogenous enzyme preparation. The results of a typical procedure for the purification of nuclease Bh1 are summarized in Table 2.1.

The enzyme was purified 6000 fold with an overall yield of 26 % and specific activity of approximately 6,00,000. The constant ssDNase : RNase ratio observed in steps following heat treatment suggests that both ssDNase and RNase activities are associated with the same protein (Table 2.1). The purified enzyme moved as a single band in native PAGE indicating its homogeneity (Fig. 2.1A). The purified enzyme could be stored in 30 mM Tris-HCl buffer pH 7.0 containing 5 % (v/v) glycerol, at -20° C, for 6 months without any apparent loss of activity.

The Mr of the purified enzyme, determined by gel-filtration, was 41,000 (Fig. 2.1 B). However, on SDS-PAGE, it gave two bands of Mr 37,000

and 32,000 respectively (Fig. 2.2 A, lane 3). The purified enzyme is a glycoprotein and contains 15 % neutral sugar. Moreover, glycoprotein staining of the transblotted protein, after SDS-PAGE, indicated that both the bands are glycosylated (Fig.2.2 B).

Table 2.1 : Purification of nuclease Bh1

Step	Total activity (U)	Total protein (mg)	Sp.activity (U / mg)	Fold purification *	Recovery (%)	ssDNAse : RNase
Crude (ssDNase) (RNase)	5,00,000 (5,00,000)	5000.00	100	1.00	100.00	1.00
Ammonium sulfate precipitation and heat treatment	4,10,640 (11,090)	217.60	1887 50	19.13	83.29	37.02
DEAE-cellulose (pH 7.0)	2,78,800 (6948)	9.29	30,000 748	304.36	56.55	40.12
Phenyl-Sepharose (pH 7.0)	2,00,736 (5,000)	0.81	2,48,128 5,530	2516.51	40.71	40.14
FPLC on Phenyl Superose (pH 7.0)	1,28,400 (3,198)	0.22	5,97,209 14,536	6056.88	26.04	40.15

* Based on ssDNAse activity

Values in brackets indicate RNase activity.

Association of carbohydrate with proteins often impedes their physical and structural characterization and leads to incorrect estimation of their Mr not only by SDS-PAGE but also by gel-filtration. In case of P1 nuclease,

carboxypeptidase and yeast invertase, it was demonstrated that in the native confirmation, the protein moiety contains sterically restricted oligosaccharide cores, which become accessible to Endoglycosidase H only after denaturation of the protein (Trimble and Maley, 1977). Thus the extent of deglycosylation was shown to be more effective when it was carried out on the denatured rather than native protein.

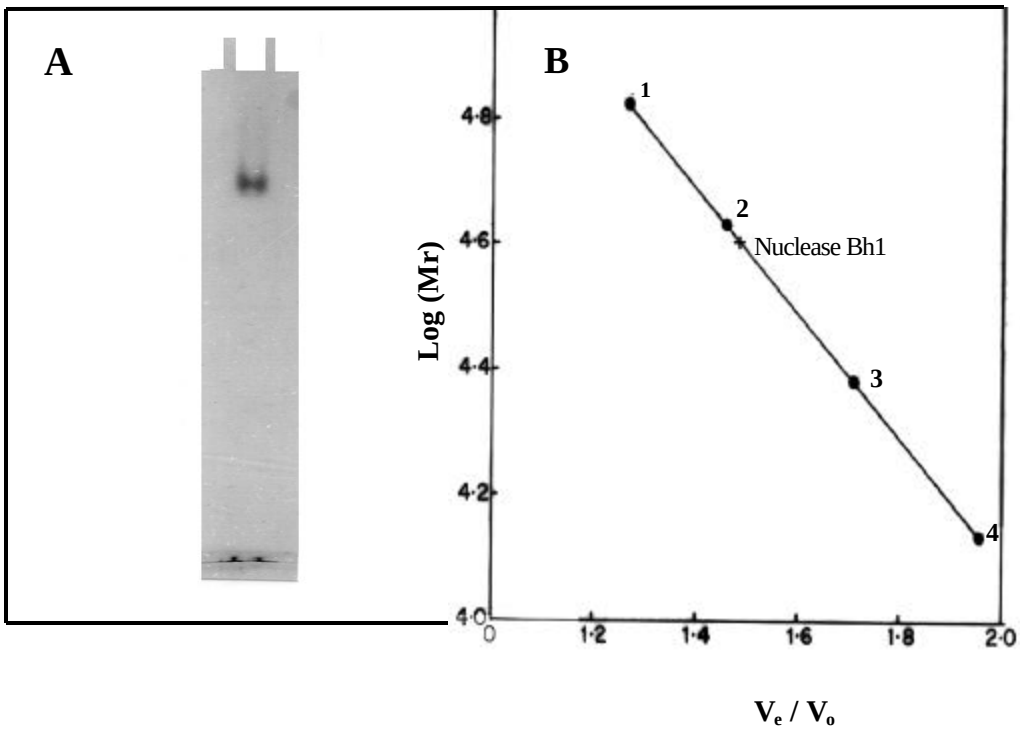

Fig. 2.1 : Electrophoresis of purified nuclease Bh1.

A. Native PAGE.

7.5 % (w/v) polyacrylamide gel, ß-alanine buffer pH 4.3, current 2 mA / well, protein loaded 20 µg.

B. Mr determination of nuclease Bh1 by gel filtration.

(1) bovine serum albumin (Mr 67,000), (2) ovalbumin (Mr 43,000), (3) chymotrypsinogen A (Mr 25,000) and (4) RNase A (Mr 13,700).

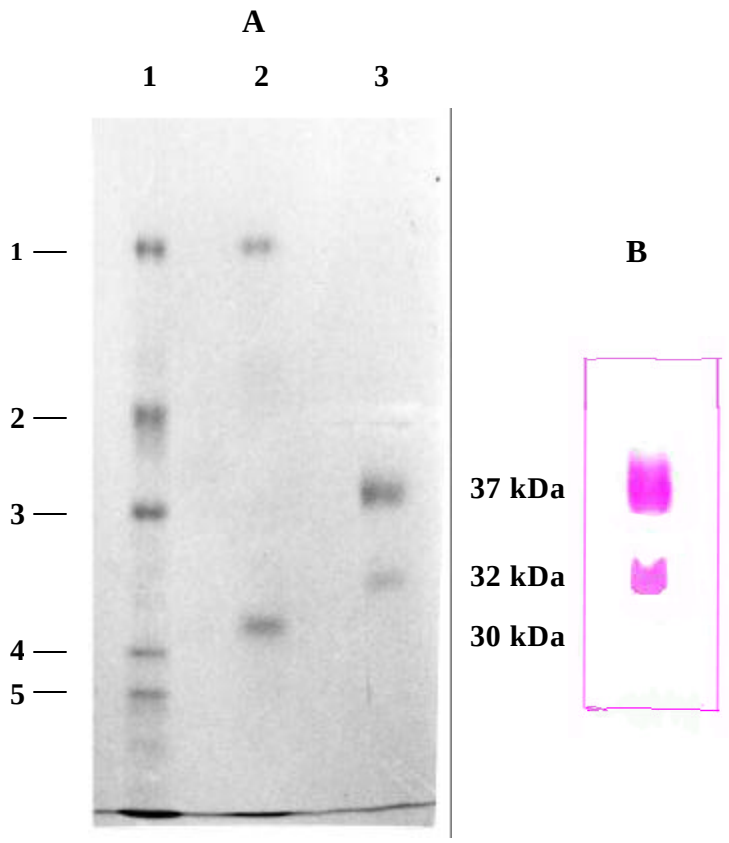

Fig. 2.2 : Mr determination of nuclease Bh1 by SDS-PAGE.

A. Lane 1: Molecular weight markers (1) bovine serum albumin (Mr 66,000), (2) ovalbumin (Mr 45,000), (3) glyceraldehyde–3–phosphate dehydrogenase (Mr 36,000), (4) carbonic anhydrase (Mr 29,000) and (5) trypsinogen (Mr 24,000).

Lane 2: Deglycosylated nuclease Bh1 (protein loaded 10 µg).

Lane 3: Nuclease Bh1 before deglycosylation (protein loaded 10 µg).

The band of Mr 66,000 in lane 2 corresponds to BSA present as stabilizer in Endoglycosidase H preparation.

B. **Glycoprotein staining :** protein loaded 10 µg.

In the present studies, when heat denatured nuclease Bh1 was subjected to deglycosylation with Endoglycosidase H, it migrated as a single band of Mr 30,000 (Fig. 2.2A, lane 2). Furthermore, partial N-terminal sequence of both the bands were identical suggesting that the 2 bands of purified nuclease Bh1, observed on SDS-PAGE, are due to differential glycosylation. Mr of nuclease Bh1 determined from amino acid composition was 32,334 which is in agreement with the one obtained by SDS-PAGE. Amino acid composition showed the presence of 6 cysteine residues. All of them seem to be involved in disulfide bond formation because modification of the native enzyme with DTNB (2mM, 1h, 25 °C) did not reveal the presence of free cysteine. Table 2.2. gives the amino acid composition of nuclease Bh1. Like nuclease Bh1, RNase Trv from *Trichoderma viridae* also showed 3 bands of Mr 34,500, 30,000 and 27,500 on SDS-PAGE which after deglycosylation migrated as a single band of Mr 27,500 (Inada *et al*., 1991). Nucleases PA1, PA2 and PA3 from *Penicillium* sp. showed a Mr of 35000, 33000 and 32000 respectively. Since the amino acid composition of all the species were very similar, it was concluded that the difference in the Mr of all the 3 proteins is due to differential glycosylation (Kazama *et al*., 1990). Also, the apparent high Mr of nuclease Bh1 on Sephadex G-75 can be correlated to its glycoprotein nature. The Mr of nuclease Bh1 is comparable to single-strand-specific nucleases viz. PA3, mung bean, S1, P1, pea seed, wheat chloroplast and *N. crassa* (mitochondria) (Gite and Shankar, 1995b).

Comparison of the partial N-terminal sequence (first 10 amino acids) with other nucleases (Fig. 2.3), showed that nuclease Bh1 exhibits 70 % homology with nuclease Le3 from *Lentinus edodes* (Kobayashi *et al.*, 1995) whereas, it shows 50 % homology with nuclease Le1 from *Lentinus edodes* (Hiroko *et al*., 1991), S1 nuclease (Iwamatsu *et al*., 1991), P1 nuclease (Maekawa *et al*., 1991), nuclease PA3 (Kazama *et al*., 1990) and barley nuclease (Brown and Ho, 1987).

Purified nuclease Bh1 is a neutral protein with a pI of 6.8 ± 0.1. Moreover, all the three activities of nuclease Bh1 viz. ssDNase, RNase and

3'AMPase focussed at the same pH (Fig. 2.4) suggesting that all the three activities are associated with the same protein.

Table 2.2 : Amino acid composition of nuclease Bh1

Amino acid	No. of residues / mol
Aspartic acid and asparagine	42
Threonine	15
Serine	15
Glutamic acid and glutamine	28
Proline	10
Glycine	33
Alanine	27
Valine	8
Methionine	3
Isoleucine	14
Leucine	23
Tyrosine	9
Phenylalanine	11
Lysine	23
Histidine	5
Arginine	13
Tryptophan	8[a]
Cysteine	6[b]
Total	**293**

Determined spectrophotometrically by : a. Spande and Witkop (1967).

b. Cavillani et al. (1966).

Name of the species											Homology (%)
Basidiobolus haptosporus		**W**	**G**	**L**	**L**	**G**	**H**	L	**L**	**T**	**G**	
Lentinus edodes	(Le3)	**W**	**G**	M	**L**	**G**	**H**	E	**L**	V	**G**	70
Lentinus edodes	(Le1)	**W**	**G**	A	I	**G**	**H**	E	T	V	**G**	50
Aspergillus oryzae	(S1)	**W**	**G**	N	**L**	**G**	**H**	E	T	V	A	50
Penicillium citrinum	(P1)	**W**	**G**	A	**L**	**G**	**H**	A	T	V	A	50
Barley nuclease		**?**	**G**	**L**	E	**G**	**H**	W	M	**T**	N	50
Penicillium sp.	(PA3)	**W**	**G**	A	**L**	**G**	**H**	A	T	V	A	50

Fig. 2.3 : Comparison of N-Terminal sequence of nuclease Bh1 with other single - strand - specific nucleases.

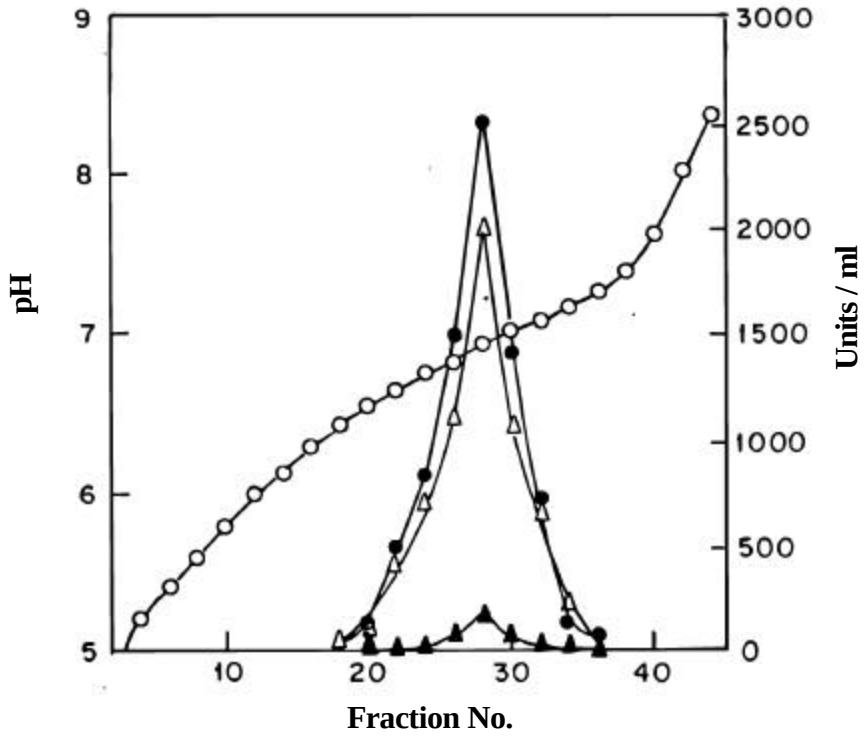

Fig. 2.4 : Isoelectric focussing of nuclease Bh1.

Nuclease Bh1 (5,000 U) was subjected to isoelectric focussing and the fractions were assayed for ssDNase, 3'AMPase and RNase activities. ssDNase (•), 3'AMPase (Δ) and RNase (σ), pH (o).

Optimum pH, temperature and stability

The optimum pH of purified nuclease Bh1 was 8.5 for the hydrolysis of both ssDNA and dsDNA (Fig. 2.5A). Its optimum pH is comparable to *N. crassa* (mycelia and conidia), *U. maydis* α and *Alteromonas* BAL 31 nucleases (Gite and Shankar, 1995b).

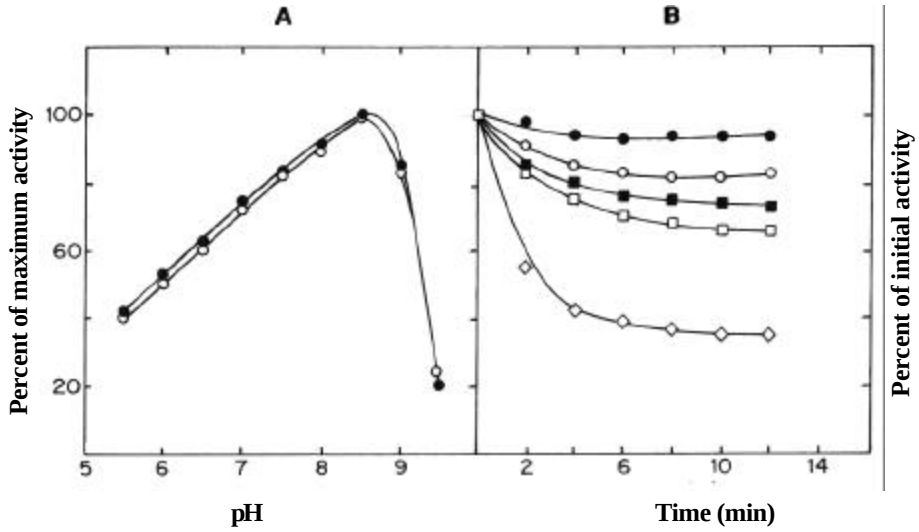

Fig.2.5 : Optimum pH and pH stability of the DNase activity of nuclease Bh1.

A. Optimum pH : Purified nuclease Bh1 (1U) was assayed for ssDNase (•) and dsDNase (o) activities in a series of pH (5.5 - 9.5) at 37° C as described under Methods.

B. pH stability : Purified nuclease Bh1 (2000 U of ssDNase activity) was incubated in a series of buffers (sodium acetate pH 5 - 5.5; immidazole pH 6.0-6.5 and Tris-HCl pH 7-9.0) at 37 °C for 24 h. Aliquots were removed at different time intervals and assayed under standard assay conditions as described under Methods.

pH 7.0 (•), pH 8.0 (o), pH 9.0 (v), pH 6.0 ( ) and pH 5.0 ( ).

However, nuclease Bh1 differs from BAL 31 nuclease and *N. crassa* (mitochondria) nuclease, 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 5.5 - 6.5), respectively (Gite and Shankar, 1995b).

The purified enzyme showed high stability and retained significant amount of its activity (> 70 %) between pH 6.0 and 9.0 for 12 h at 37°C (Fig. 2.5B). However, at pH 7.0 and 37°C, it retained its full activity for 24h. The pH stability of nuclease Bh1 is comparable to S1 and P1 nucleases.

Optimum temperature of purified nuclease Bh1 was 60 °C (Fig. 2.6A), for ssDNA hydrolysis, which is similar to that of BAL 31 nuclease S (slow) form. The optimum temperature of nuclease Bh1 is higher compared to most of the single-strand-specific nucleases (Gite and Shankar, 1995b).

Nuclease Bh1 retained its full activity for 15 min at 60 °C. However, at its optimum pH and temperature (i.e. pH 8.5 and 60 °C) the enzyme showed a half-life of 45 min (Fig. 2.6B). The temperature stability of nuclease Bh1 is superior to that of P1 nuclease but comparable to that of S1 nuclease (Gite and Shankar, 1995b).

Metal ion requirement

Influence of metal ions on the activity of nuclease Bh1 revealed that unlike majority of the single-strand-specific nucleases, it neither showed an obligate requirement of divalent metal ions for its activity nor the activity was stimulated in presence of metal ions (Table 2.3). In this respect, nuclease Bh1 is similar to nuclease ß from *U. maydis* (Rusche *et al*., 1980). Although nuclease α from *U. maydis* (Holloman *et al*., 1981) and wheat chloroplast nuclease (Kuligowska *et al*., 1988) did not show an obligate requirement of metal ions for their activity, nuclease α was stimulated 4- fold by Co^{2+} whereas, the ssDNase activity of wheat chloroplast nuclease showed only slight stimulation (20 %) in presence of Mg^{2+}. In fact, nuclease Bh1 was inhibited by low concentrations of metal ions like Zn^{2+}, Ag^{2+}, Hg^{2+}, Al^{3+} and Fe^{3+} and among them Zn^{2+}, Hg^{2+} and Ag^{2+} were more potent inhibitors.

However, metal ions like Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+} and Ba^{2+} did not have any effect. Nuclease Bh1 was also inhibited by low concentrations of metal chelators like EDTA and EGTA, 8-hydroxyquinoline and 1,10 o-phenanthroline (Table 2.4).

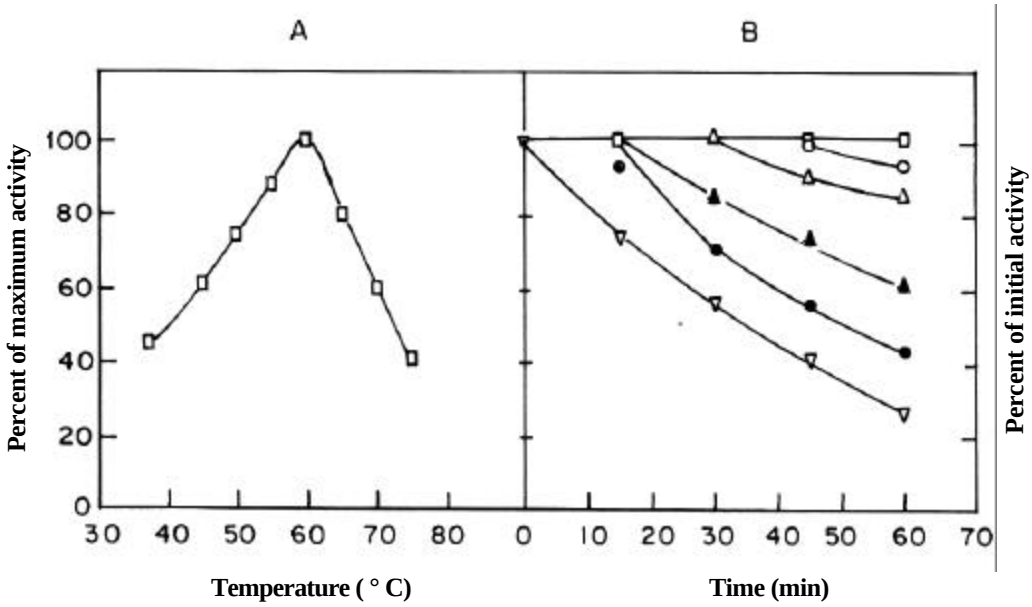

Fig.2.6 :Optimum temperature and temperature stability of the DNase activity of nuclease Bh1.

A. Optimum temperature : Purified nuclease Bh1 (1U) was assayed for ssDNase activity ( ) in a series of temperatures (37 - 75 °C) as described under Methods.

B. Temperature stability : Purified nuclease Bh1 (2000 U of ssDNase activity) was incubated in Tris-HCl buffer, pH 8.5 at different temperatures (37 - 65 °C) for 1h. Aliquots were removed at different time intervals and assayed under standard assay conditions as described under Methods. 37°C ( ), 45°C (o), 50°C (Δ), 55°C (σ), 60 °C (•) and 65°C (∇).

Table 2.3 : Effect of metal ions on the DNase activity of nuclease Bh1

Metal ion (1mM)	Residual activity (%)	
	ssDNase	dsDNase
Control (no metal ion added)	100.00	100.00
Ag^{2+}	10.60	10.00
Al^{3+}	35.80	38.00
Ba^{2+}	87.77	85.48
Ca^{2+}	97.00	98.25
Co^{2+}	92.22	95.00
Cu^{2+}	94.44	92.00
Fe^{3+}	32.22	30.00
Hg^{2+}	0.00	0.00
Mg^{2+}	99.44	98.00
Mn^{2+}	100.00	99.00
Zn^{2+}	0.00	0.00

Extensive dialysis of the enzyme against 30 mM Tris-HCl buffer, pH 7.0, containing 2 mM EDTA (effective concentration) followed by further dialysis against Tris-HCl buffer (to remove EDTA) resulted in complete loss of its activity. However, a significant amount of activity (80 - 90 %) could be restored by incubation with 10 mM Zn^{2+}, for 5-6 h, at 4°C. On the contrary, Co^{2+} at this concentration yielded only 30 % of its initial activity. Other divalent cations like Mg^{2+}, Mn^{2+} and Ca^{2+} were not effective. Despite the inhibitory nature of Zn^{2+}, the ability of Zn^{2+} to restore significant amount of activity of the EDTA treated nuclease Bh1 suggests that it is a zinc metalloprotein. In this respect, it is similar to well studied single-strand-specific nucleases like S1, P1, mung bean, *Physarum polycephalum* and PA3

(Gite and Shankar, 1995b). Moreover, the ability of Zn^{2+} to readily restore the activity of the EDTA treated nuclease Bh1 suggests that Zn^{2+} is loosely bound to the protein.

Table 2.4 : Effect of metal chelators on the DNase activity of nuclease Bh1.

Metal Chelator	Residual Activity (%)
Control *	100.00
EDTA (1 mM)	2.25
EGTA (1 mM)	5.60
8-hydroxyquinoline (200 µM)	45.00
1,10 o-phenanthroline (200 µM)	43.25
Citrate (30 mM)	35.00

* Enzyme assayed in the absence of metal ions served as control.

Effect of salt concentration

Salt concentration in the reaction mixture has been reported to affect the action of single-strand-specific nucleases. Influence of salt concentration on the ssDNase activity of nuclease Bh1 showed that the activity was maximum between 25 and 50 mM of both NaCl and KCl (Fig. 2.7). However, further increase in the salt concentration (> 75 mM) was accompanied by a progressive decrease in the activity and at 150 mM of NaCl and KCl the enzyme exhibited approximately 35 % and 20 % of its initial activity, respectively. The sensitivity of nuclease Bh1 to salt concentration is higher compared to S1 nuclease and BAL 31 nuclease, which showed maximum activity in presence of 100 mM and 2 M of NaCl, respectively (Gite and Shankar, 1995b). On the contrary, the dsDNase activity of nuclease Bh1 gradually decreased with increase in the salt concentration and in presence of 150 mM of either NaCl or KCl the enzyme exhibited 20 - 25 % of its activity,

respectively. Similar observations have been made in case of pea seed nuclease (Wani and Hadi, 1979).

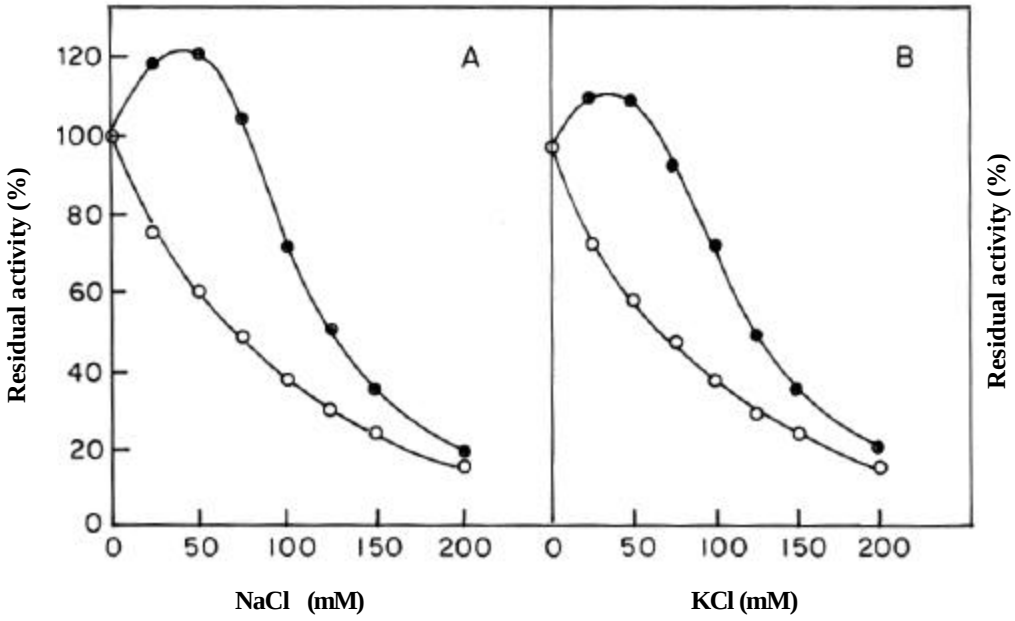

Fig. 2.7 : Effect of salt concentration on the DNase activity of nuclease Bh1.

A . Purified nuclease Bh1 (10U of dsDNase activity) was assayed for ssDNase (•) and dsDNase (o) activities in the presence of varying concentrations of NaCl under standard assay conditions.

B . Purified nuclease Bh1 (10U of dsDNase activity) was assayed for ssDNase (•) and dsDNase (o) activities in the presence of varying concentrations of KCl under standard assay conditions

In case of *N. crassa* (mitochondria) nuclease, 100-200 mM NaCl completely inhibited the dsDNase activity whereas it had only marginal effect on the ssDNase activity. The high sensitivity of the dsDNase activity towards low salt concentrations can be correlated to the suppression of localized melting by electrostatic stabilization of the DNA, especially the stabilization of AT rich regions (von Hippel and Felsenfeld, 1964).

Effect of thiol reagents

DTT and ß- mercaptoethanol inhibited the activity of nuclease Bh1, to a significant extent (>70 %), suggesting that disulfide bridges are essential for its activity. Among them, DTT was a more potent inhibitor because 1 mM of the reagent could bring about approximately 80 % loss of its initial activity whereas, it required 10 mM of ß-mercaptoethanol to bring about comparable level (70 %) of inhibition (Table 2.5). Wheat chloroplast nuclease retained 55 % and 29 % of its initial activity in presence of 5mM ß-mercaptoethanol and DTT respectively, suggesting that like nuclease Bh1 the enzyme shows higher sensitivity towards DTT (Kuligowska *et al*., 1988). On the contrary, nucleases from *Streptomyces antibioticus* (Santiago *et al*., 1995) and *Streptomyces glaucescens* (Aparicio *et al*., 1992) required DTT or ß-mercaptoethanol for their optimal activity whereas thiol reagents had no effect on the activity of nuclease ß from *U. maydis* (Rusche *et al*., 1980).

Table 2.5 : Effect of thiol reagents on the ssDNase activity of nuclease Bh1

Thiol reagent (mM)	Residual activity (%)
Control*	100.00
Dithiothreitol (DTT)	
1	20.00
ß-mercaptoethanol (ß-Me)	
2	88.00
4	68.25
6	51.77
8	44.13
10	30.53

* Enzyme assayed in the absence of the above reagents served as control.

Stability to denaturants

The purified enzyme showed high stability against protein denaturants and retained significant amount of its initial activity in presence of low concentrations of SDS (0.04 % w/v) and guanidine hydrochloride (500 mM) (Table 2.6). However, the enzyme showed higher stability in presence of urea and retained approximately 80 % of its activity in presence of 4M urea. The stability of nuclease Bh1 to SDS is inferior to that of BAL 31 nuclease because the latter was active in presence of 5 % (w/v) SDS. Moreover, BAL 31 nuclease retained 60 % of its activity in presence of 4M urea (Gray *et al*., 1981) whereas, *P. polycephalum* nuclease was stable in presence of 5M urea (Waterborg and Kuyper 1982). The stability of nuclease Bh1 to urea is lower than that of *P. polycephalum* nuclease but comparable to that of BAL 31 nuclease.

Effect of organic solvents

Isolation of single-strand-specific nucleases, exhibiting high stability in presence of organic solvents, have added a new dimension to these studies as they can be used as probes for the determination of secondary structure of DNA in presence of various organic solvents. For example, the use of formamide has enabled the visualization, via electron microscopy, of nonbushed single-stranded regions in DNA (Davis and Hyman 1971; Davis *et al*., 1971). In the present studies, nuclease Bh1 retained its full activity in presence of 50 % (v/v) formamide and 80 % and 88 % activity in presence of 40 % (v/v) dimethylformamide and 10 % (v/v) dimethylsulfoxide, respectively (Table 2.7). However, compared to S1 nuclease (Hutton and Wetmur, 1975), nuclease Bh1 showed decreased stability in presence of 40 % (v/v) dimethylsulfoxide. Nucleases from *S. glaucescens* (Aparicio *et al*., 1992) and *S. antibioticus* (Santiago *et al*., 1995) showed more than 2-fold stimulation of their activity in presence of 10 % (v/v) dimethylsulfoxide, whereas no such stimulation was seen in case of nuclease Bh1. The high stability of the ssDNase activity of nuclease Bh1 in presence of high concentrations of organic

solvents is of importance as the enzyme can be used to study DNA structure in presence of organic solvents.

Table 2.6 : Effect of denaturants on the ssDNase activity of nuclease Bh1.

Denaturant (concentration)	Residual activity (%)
SDS (%)	
Control*	100.00
0.02	92.10
0.04	82.51
0.06	68.92
0.08	54.04
0.10	24.09
Guanidine hydrochloride (M)	
Control*	100.00
0.25	98.00
0.50	95.00
0.75	75.00
1.00	50.00
1.50	25.00
2.00	5.00
Urea (M)	
Control*	100.00
1	95.00
2	95.00
3	88.00
4	80.00
5	65.00

* Enzyme assayed in the absence of the above reagents served as control.

Table 2.7 : Effect of organic solvents on the ssDNase activity of nuclease Bh1.

Organic solvent	Formamide	Dimethylformamide	Dimethylsulfoxide
(% v/v)		Residual activity (%)	
Control*	100.00	100.00	100.00
10	120.00	104.00	88.64
20	130.00	100.00	75.47
30	122.00	92.02	63.48
40	120.00	81.40	42.00
50	100.00	60.47	30.00

* Enzyme assayed in the absence of the above reagents served as control.

Inhibitors

It has been reported that mononucleotides are potent inhibitors of single-strand-specific enzymes like S1 nuclease and *U. maydis* nuclease ß. S1 nuclease was inhibited by adenosine 5'-nucleotides (Wiegand *et al*., 1975) whereas *U. maydis* nuclease ß was inhibited by adenosine 3'-nucleotides (Rusche *et al*., 1980). In the present studies, nuclease Bh1 was inhibited only by guanosine 5'-nucleotides and riboguanosine nucleotides were stronger inhibitors than deoxyriboguanosine nucleotides (5'GMP > 5'dGMP). However, in case of S1 nuclease, deoxyadenosine nucleotides were more potent inhibitors than the corresponding riboadenosine derivatives (5'dAMP > 5'AMP). The observation that deoxyguanosine was a weaker inhibitor than 5'dGMP suggested that the phosphate residue in 5'dGMP is responsible for stronger inhibition. Moreover, increased inhibition observed with increase in the number of the phosphate residues i.e. 5'GTP > 5'GDP > 5'GMP, supports the role of phosphate in the inhibition of nuclease Bh1. Guanosine 5'-nucleotides inhibited the ssDNase activity of nuclease Bh1 competitively in the

order of 5'GTP > 5'GDP > 5'GMP > 5'dGMP (Fig. 2.8) with Ki values of 2.75, 5.83, 28.28 and 40.4 µg, respectively.

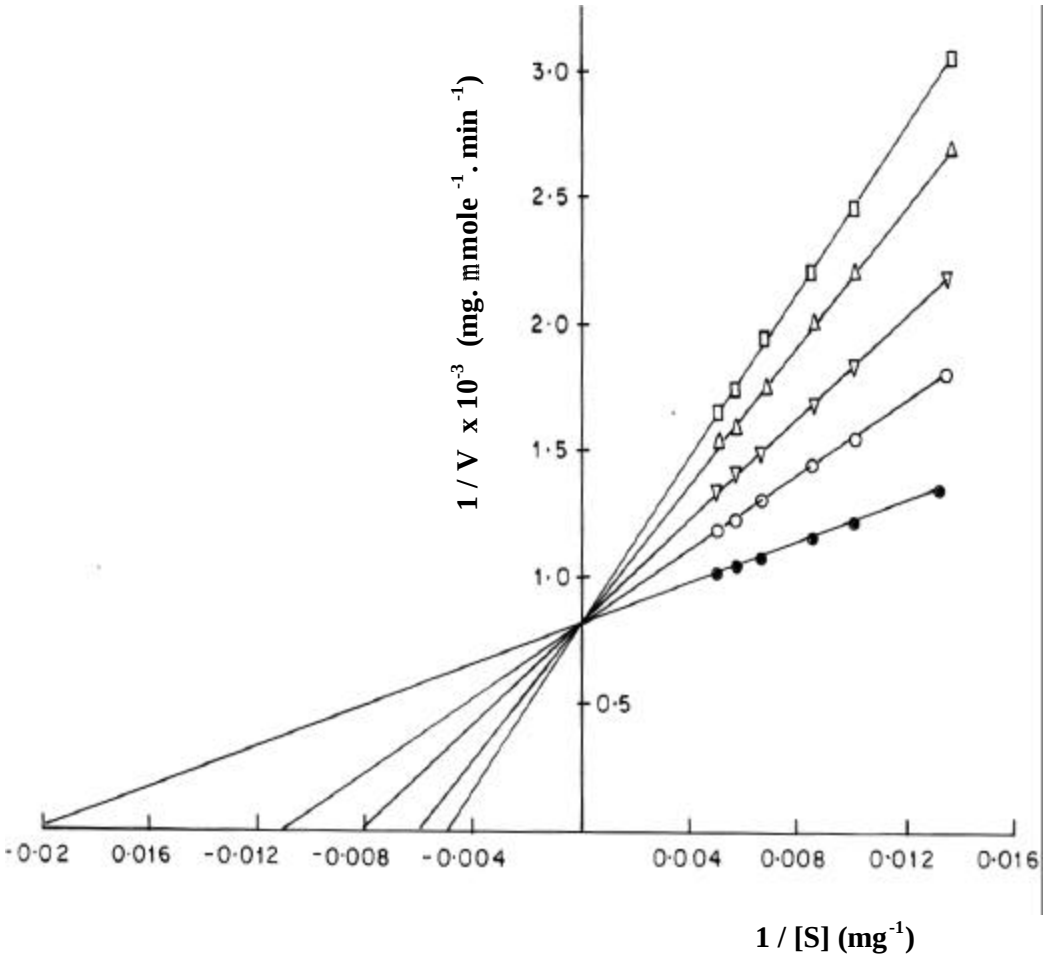

Fig. 2.8 : Competitive inhibition of ssDNase activity of nuclease Bh1 by guanosine 5' nucleotides.

Reaction mixtures containing ssDNA (25-200 µg) and the inhibitors were incubated with 1U of the ssDNase activity of nuclease Bh1 at pH 8.5 and 37 °C and the acid soluble deoxyribonucleotides formed were measured at 260 nm as described under Methods. No inhibitor (•), 100 µM 5'dGMP (∇), 100 µM 5'GMP (Δ), 10 µM 5'GDP (o), 5 µM 5'GTP ( ).

The role of phosphate in the inhibition of nuclease Bh1 is supported by the observation that pyrophosphate is a stronger inhibitor of the enzyme than inorganic phosphate. Nuclease Bh1 retained approximately 50 % and 25 % of its ssDNase activity in presence of 5 mM inorganic phosphate and pyrophosphate, respectively. The sensitivity of nuclease Bh1 towards inorganic phosphate and pyrophosphate is lower compared to S1 nuclease, which showed approximately 50 % of its activity in presence of either 2 mM inorganic phosphate or 20 µM pyrophosphate (Gite and Shankar, 1995b).

Substrate specificity

Nuclease Bh1 showed high activity on ssDNA and 3'AMP but very low activity on RNA and dsDNA. Bis (*p*-nitrophenyl) phosphate was resistant to hydrolysis, indicating that nuclease Bh1 is not a typical phosphodiesterase. Moreover, the resistance of *p*-nitrophenylphosphate points toward the absence of non-specific phosphatase activity. The high activity of the enzyme on sonicated and heat denatured DNA is suggestive of the high single-strand specificity of the enzyme (Table 2.8).

Single-strand-specific nucleases act on ssDNA, RNA and 3'mononucleotides but the rate of hydrolysis of these substrates varies depending on the source of enzyme. S1, mung bean and toboccao nucleases prefer ssDNA to RNA and 3'AMP (Gite and Shankar, 1995b) while P1 (Fujimoto *et al*., 1974a), PA3 (Kazama *et al*., 1990), Le1 (Hiroko *et al*., 1991) and Le3 (Kobayashi *et al*., 1995) nucleases show higher activity on RNA and 3'AMP. Wheat seedling nuclease, on the other hand, does not show any difference in the rates of hydrolysis of ssDNA, RNA and 3'mononucleotides (Hanson and Fairely, 1969). The high preference of nuclease Bh1 for ssDNA shows that like S1 nuclease, it is a single-strand-specific enzyme. However, unlike S1 nuclease, which hydrolyzed ssDNA faster than RNA, dsDNA and 3'AMP, nuclease Bh1 hydrolyzed these substrates in the order of ssDNA ≈ 3'AMP >>RNA > dsDNA (Table 2.8). The very low activity of nuclease Bh1, on double stranded DNA, can be correlated to the susceptibility of partly

unwound single-stranded regions in the double-stranded DNA. Based on ssDNase / dsDNAse ratio, it is reasonable to assume that the stringency of nuclease Bh1 for single-stranded DNA (250 - 300) is comparable to that of P1 nuclease (250) and *U. maydis* (200) nuclease. However, it is less than that of mung bean nuclease (30,000) and S1 nuclease (10,000) (Martin *et al*., 1986).

Table 2.8 : Substrate specificity of nuclease Bh1.

Substrate	Value (%)*
Polymeric Substrates	
1. Single stranded DNA	100
2. RNA	4
3. Double stranded DNA	<1
Monomeric Substrates	
1. 3'AMP	100
2. 5'AMP	0
3. bis-*p*-nitrophenylphosphate	0
4. *p*-nitrophenylphosphate	0

* The ratio of activities was determined based on initial rates of hydrolysis of different substrates carried out under standard assay conditions. For details, refer to Methods.

Mode of Action

Action on DNA : Low concentrations of nuclease Bh1 could convert supercoiled pUC 18 DNA (Form I) to linear duplex DNA (Form III) via nicked circular DNA (Form II) (Fig. 2.9A) and could readily linearize single-stranded covalently closed form of M13 DNA (Fig.2.9B) suggesting an endo mode of action. With increase in the incubation time, no detectable degradation of Form III DNA was observed. Degradation of Form III DNA

was observed only when Form I DNA was treated with excess (20 times) enzyme (Fig. 2.10).

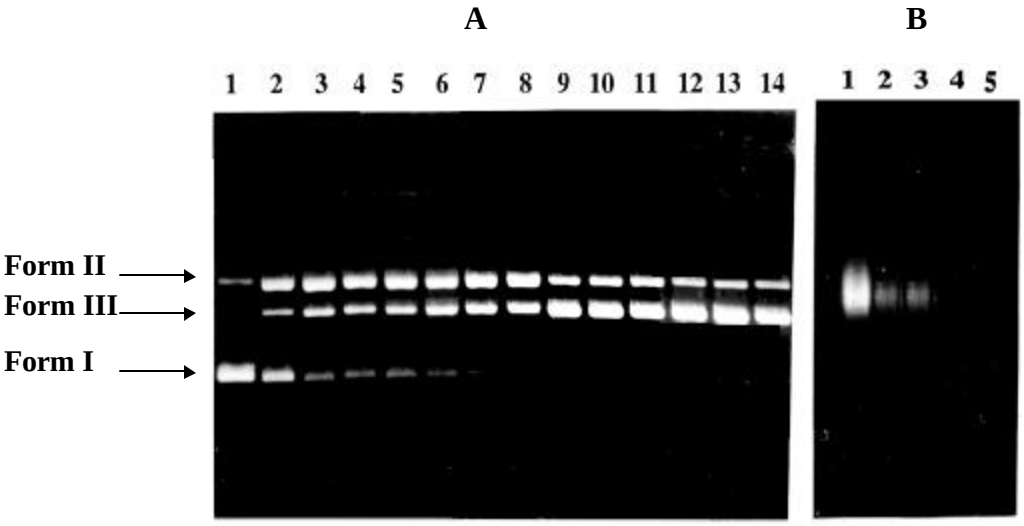

Fig. 2.9:Action of nuclease Bh1 on plasmid pUC 18 and phage M13 DNAs.

A. Time course of digestion of pUC 18 DNA by nuclease Bh1.

Lane 1: pUC 18 alone; lane 2: digestion with 0.1 U of nuclease Bh1 for 2 min; lanes 3-5: digestion with 0.15 U of nuclease Bh1 for 5, 10 and 15 min, respectively; lanes 6-8: digestion with 0.2 U of nuclease Bh1 for 5, 10 and 15 min, respectively; lanes 9-11: digestion with 0.4 U of nuclease Bh1 for 5, 10 and 15 min, respectively; lanes 12-14: digestion with 1 U of nuclease Bh1 for 5,10 and 30 min, respectively.

B. Time course of covalently closed single stranded circular phage M13 DNA digestion by nuclease Bh1.

Lane 1: phage M13 DNA alone; lanes 2-5: digestion of M13 phage DNA with 0.01 U of nuclease Bh1 for 2, 5, 10 and 15 min respectively.

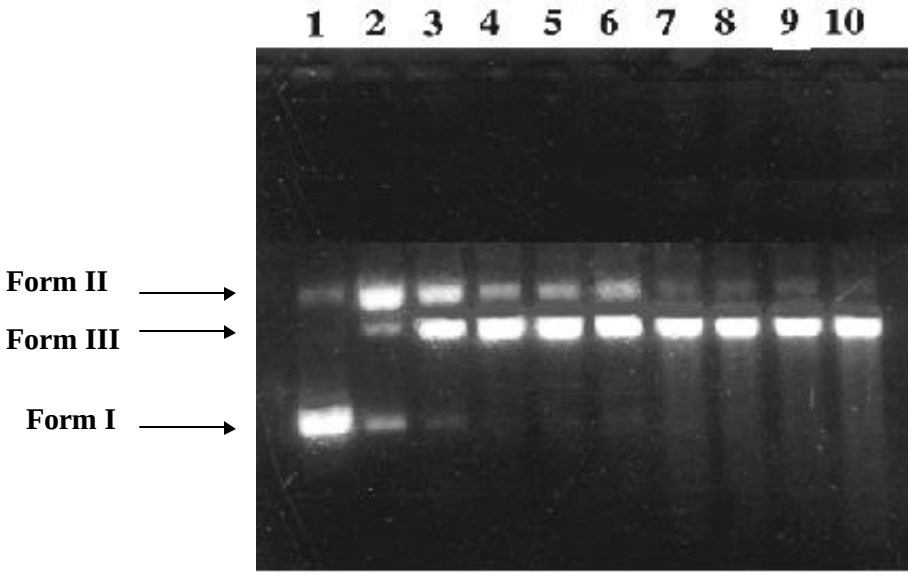

Fig. 2.10 : Resistance of linear duplex (Form III) DNA to nuclease Bh1.

Lane 1: pUC 18 alone; lane 2: digestion with 0.1 U of nuclease Bh1 for 2 min; Lane 3: digestion with 0.4 U of nuclease Bh1 for 15 min; lane 4-6: digestion with 1 U of nuclease Bh1 for 10, 15 and 20 min, respectively; lanes 7-10: digestion with 2 U of nuclease Bh1 for 10, 15, 20 and 30 min, respectively.

The inability of low concentrations of nuclease Bh1 to degrade Form III DNA, even after prolonged incubation, points toward the high single-strand specificity of the enzyme. Conversion of Form I DNA to Form II DNA was due to single base nicks because the latter could be ligated with T4 DNA ligase to yield covalently closed circular Form (Fig. 2.11). The above observations also suggest that the conversion of Form I DNA to Form III DNA, by nuclease Bh1, follows a double hit mechanism i.e. scission of the molecule will not occur unless both the strands are cleaved. Similar observation was made in case of S1 nuclease (Shishido and Ando, 1981). In contrast, the nicks

generated by BAL 31 nuclease, on pBR322 Form I DNA, could not be ligated since they were extended into gaps by the exonuclease action of the enzyme (Przykorska *et al*., 1988).

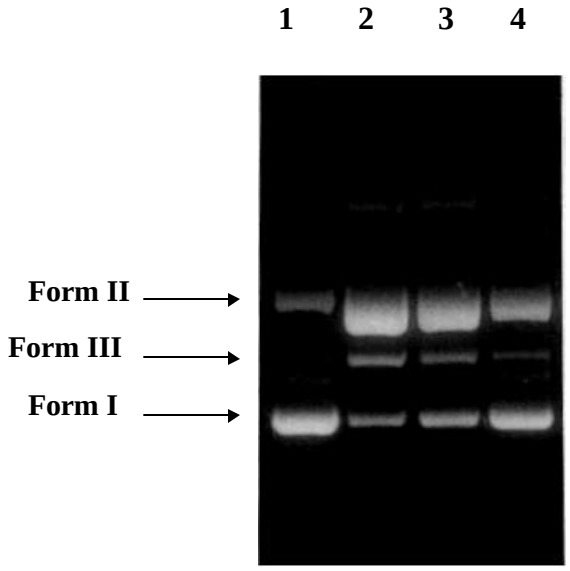

Fig. 2.11. Ligation of nicked circular DNA (Form II) by T4 DNA ligase.

The nicked circular DNA generated by the action of nuclease Bh1 on supercoiled DNA (400 ng), was incubated with 20 U of T4 DNA ligase and analyzed by agarose gel electrophoresis. For experimental details refer to Methods.

Lane 1: pUC 18 alone; lane 2: digestion with 0.1 U of nuclease Bh1 for 10 min; Lanes 3,4: Covalently closed circular form generated by the action of T4 DNA ligase at 1h and 15h.

The time course analysis of the hydrolytic products of ssDNA, with low concentrations of nuclease Bh1 (6 U/ml), showed the predominance of oligonucleotides (75-80 %) compared to mononucleotides (20-25 %). Among the mononucleotides, there was a large preponderance of 5'dGMP (15-17 %) with small amounts of 5'dTMP (2-3 %). It was also observed that after an initial rapid increase, the reaction slowed down considerably and after 4h there

was no detectable increase in the mononucleotide levels (Fig. 2.12A). This suggested end product inhibition as reported in case of S1 nuclease (Wiegand *et al*., 1975) and *U. maydis* nuclease ß (Rusche *et al*., 1980). In the present studies, the predominance of 5'dGMP in the hydrolytic products coupled with its ability to inhibit nuclease Bh1 competitively indicate end product inhibition rather than depletion of the substrate. No significant increase in the 5'dGMP and 5'dTMP levels, inspite of dosing with the enzyme (*i.e.* incremental addition of the enzyme at fixed time intervals), can be correlated to the inability of low concentrations of the enzyme to overcome the inhibition by 5'dGMP. Interestingly, neither 5'dAMP nor 5'dCMP could be detected during the entire duration of hydrolysis (Fig. 2.12A).

Analysis of the hydrolytic products of ssDNA, with 10 - fold excess enzyme (60 U / ml), showed that in addition to 5'dGMP (17.4 %), low levels of 5'dTMP (4.8 %) and 5'dAMP (3.8 %) could be detected (Fig. 2.12B). As observed with low concentrations of the enzyme (Fig. 2.12A), the reaction rate slowed down considerably after 4h. Periodic dosing of the enzyme (60 U/ml), resulted in a significant increase in 5'dGMP levels (Fig. 2.12B) accompanied by a marginal increase in 5'dTMP suggesting that, inhibition by 5'dGMP can be overcome with high concentrations of the enzyme. However, there was no increase in 5'dAMP levels and 5'dCMP was absent.

Time course analysis of the products following the hydrolysis of dsDNA with low concentrations of nuclease Bh1 (equivalent to 6 U / ml of dsDNase activity) showed that the nucleotides were liberated in the order of 5'dGMP (32 %) > 5'dTMP (20 %) > 5'dAMP (11 %) (Fig. 2.13A). Comparable results were obtained when the hydrolysis of dsDNA was followed using high enzyme concentration (60 U/ml of dsDNase activity) except that 5'dTMP levels almost equaled that of 5'dGMP (Fig.2.13B) accompanied by a marginal increase in the levels of 5'dAMP. However, 5'dCMP was absent.

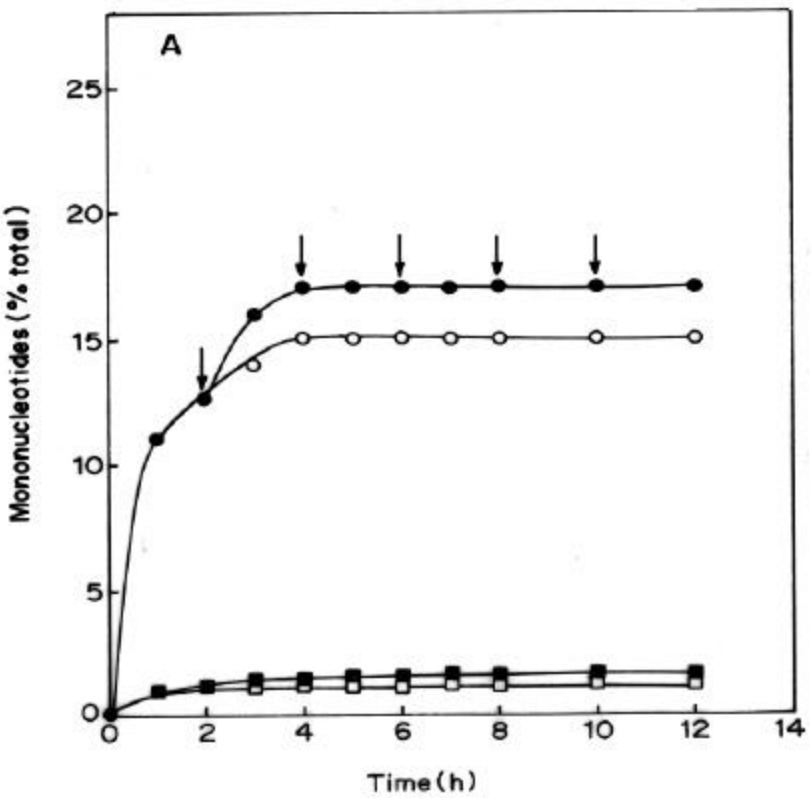

Fig. 2.12 A : Time course of hydrolysis of ssDNA with low concentrations of nuclease Bh1.

The total reaction mixture of 10 ml, containing 5 mg of sonicated and heat denatured buffalo liver DNA, was incubated with 6U nuclease Bh1 at pH 8.5 and 37 °C. Aliquots (1 ml) were removed at fixed time intervals and the products analyzed by HPLC. For experimental details refer to Methods.

Without dosage - 5'dGMP (○), 5'dTMP (Y).
With dosage - 5'dGMP (λ), 5'dTMP (ν).

The position of the arrow indicates the addition of nuclease Bh1.

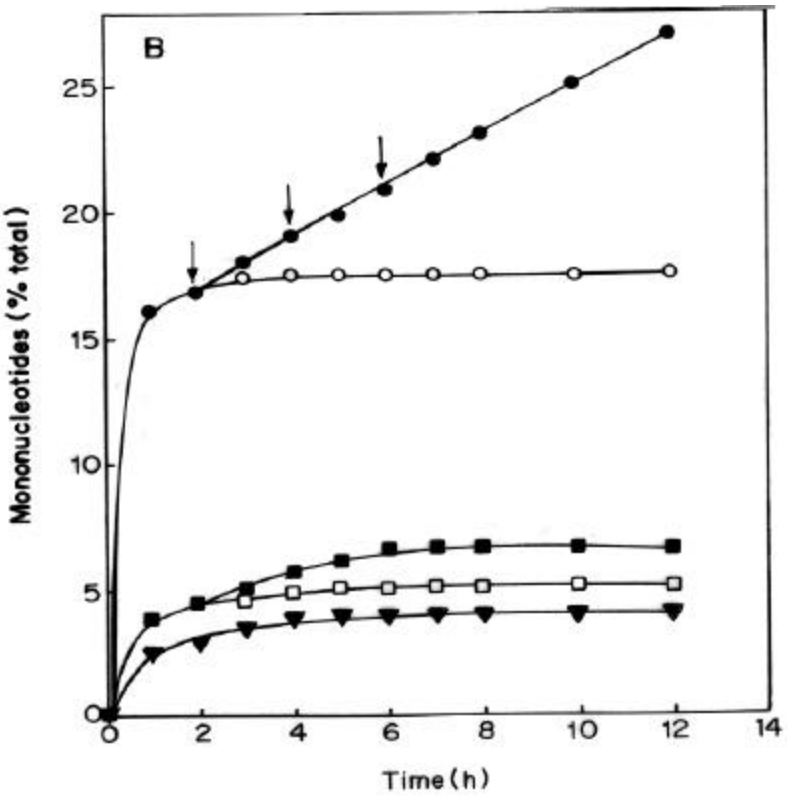

Fig. 2.12 B : Time course of hydrolysis of ssDNA with high concentrations of nuclease Bh1.

The total reaction mixture of 10 ml, containing 5 mg of sonicated and heat denatured buffalo liver DNA, was incubated with 60U of nuclease Bh1 at pH 8.5 and 37 °C. Aliquots (1 ml) were removed at fixed time intervals and the products analyzed by HPLC. For experimental details refer to Methods.

without dosage - 5'dGMP (o), 5'dTMP (Y), 5'dAMP (∇).
with dosage - 5'dGMP (λ), 5'dTMP (ν), 5'dAMP (τ)

The position of arrows indicates the addition of nuclease Bh1.

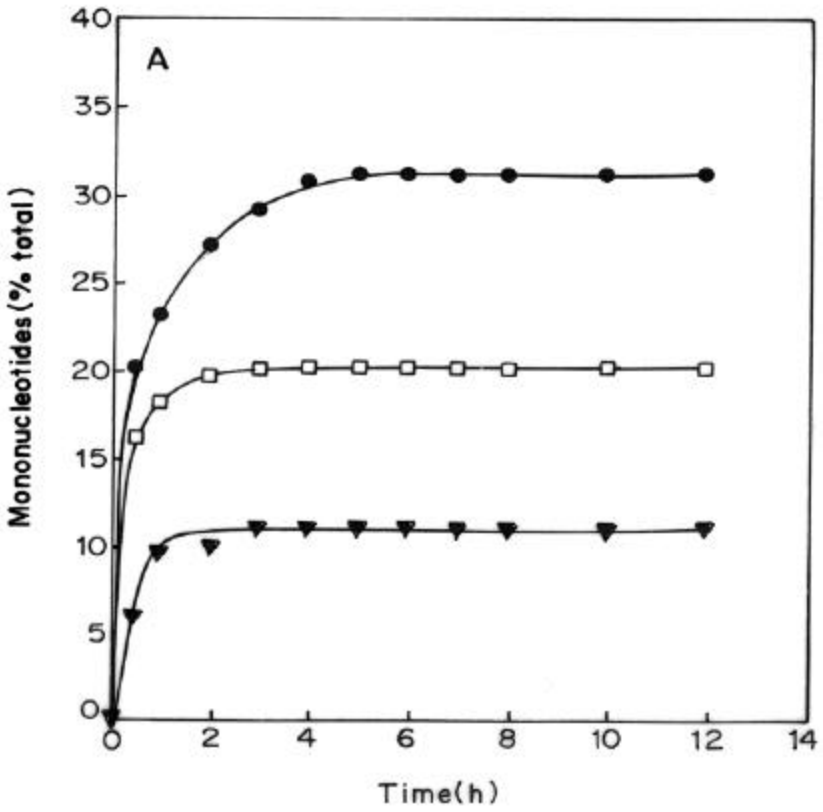

Fig. 2.13 A : Time course of hydrolysis of dsDNA with low concentrations of nuclease Bh1.

The total reaction mixture of 10 ml, containing 5 mg of native buffalo liver DNA, was incubated with 6U of nuclease Bh1 at pH 8.5 and 37 °C. Aliquots (1 ml) were removed at fixed time intervals and the products analyzed by HPLC. For experimental details refer to Methods.

5'dGMP (λ), 5d'TMP(Y), 5'dAMP (τ)

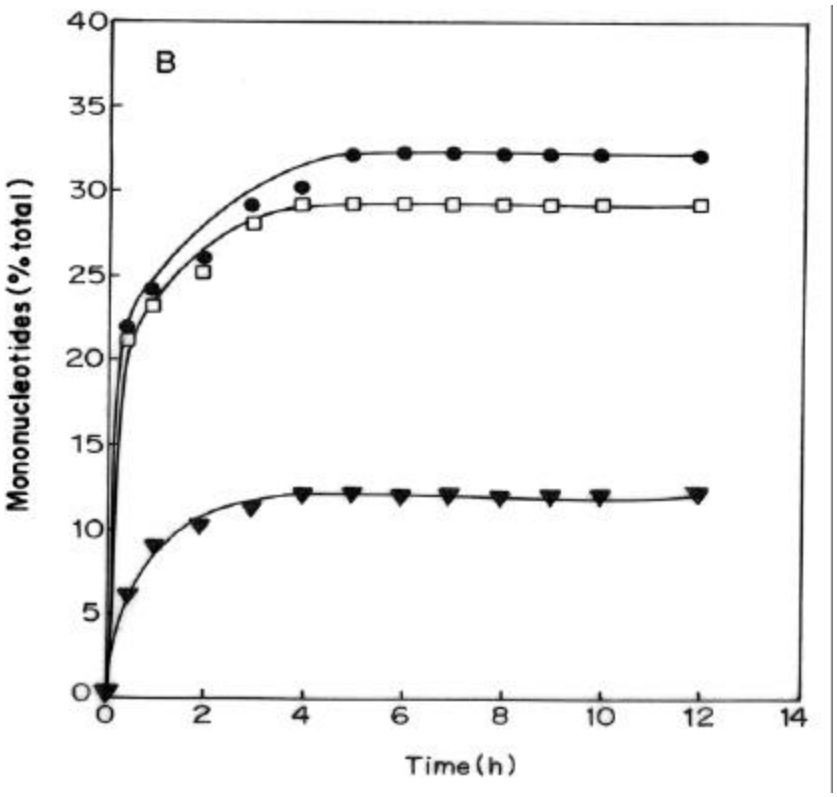

Fig. 2.13 B : Time course of hydrolysis of dsDNA with high concentrations of nuclease Bh1.

The total reaction mixture of 10 ml, containing 5 mg of native buffalo liver DNA, was incubated with 60 U of nuclease Bh1 at pH 8.5 and 37 °C. Aliquots (1 ml) were removed at fixed time intervals and the products analyzed by HPLC. For experimental details refer to Methods.

5'dGMP (λ), 5d'TMP(Y), 5'dAMP (τ)

Throughout the course of hydrolysis of ssDNA with high concentration of nuclease Bh1, with and without dosage, the proportion of oligonucleotides was higher than that of the mononucleotides. Moreover, the

time course of hydrolysis of (dT_{10}) showed that 5'dTMP could be detected only after 2h. These observations suggest predominantly an endo mode of action. The mode of action of nuclease Bh1 is similar to that of wheat chloroplast nuclease (Kuligowska *et al*., 1988). In contrast, with P1 nuclease, the proportion of mononucleotides, in the hydrolytic products of DNA, was greater than that of oligonucleotides from the initial stages of hydrolysis (Fujimoto *et al*.,1974b) whereas, with *N. crassa* (mitochondria) nuclease, complete conversion of DNA to mononucleotides was observed only with high amounts of enzyme (Linn and Lehman, 1965b). *U. maydis* nuclease α (Holloman *et al*., 1981) and BAL 31 nuclease (Gray *et al*., 1981) cleaved ssDNA endonucleolytically but they showed exonucleolytic action on dsDNA. Nuclease Bh1 differs from these enzymes in that, it cleaves both ss and ds DNA endonucleolytically.

HPLC analysis of the hydrolytic products of DNA, with low and high enzyme concentrations, revealed that from the initial stages of hydrolysis the 5'dGMP levels were significantly high suggesting that, nuclease Bh1 is a guanylic acid preferential enzyme. Low levels of 5'dTMP and 5'dAMP can be correlated to the relative resistance of thymidylic and adenylic acid linkages to hydrolysis. Interestingly, irrespective of the amount of enzyme added, 5'dCMP could not be detected in the hydrolytic products of both ss and ds DNA. HPLC analysis of the products of alcohol soluble and alcohol precipitable oligonucleotides (obtained after exhaustive digestion of DNA), after treatment with snake venom phosphodiesterase, showed high levels of 5'dCMP indicating that cytidylic acid linkages are resistant to cleavage.

An endoexonuclease from *N. crassa* (conidia) (Linn and Lehman, 1965b) and nuclease C from *Chlamydomonas reinhardtii* (Ogawa and Kuroiwa, 1987) showed a similar preference for guanylic acid linkages in ssDNA. In both cases, there was a difference in the relative amounts of the mononucleotides produced by these enzymes though all four nucleotides were present during the entire duration of hydrolysis. In contrast, in case of nuclease Bh1, with low levels of enzyme, there was a complete absence of

5'dAMP and 5'dCMP in the hydrolytic products (Fig. 2.12A), indicating an absolute preference for guanylic acid linkages followed by those between guanylic and thymidylic acid. Although, cytidylic acid linkages were resistant to hydrolysis by *N. crassa* and *C. reinhardtii* nucleases, low levels of 5'dCMP could be detected in the hydrolytic products of DNA. However, in case of nuclease Bh1, 5'dCMP could not be detected in the hydrolytic products of both ss and ds DNA, indicating that cytidylic acid linkages are resistant to cleavage.

Action of nuclease Bh1 on DNA and deoxyribodecamers showed that they were hydrolyzed in the order of ssDNA > $(dT)_{10}$ > $(dG)_{10}$ (Table 2.9). However, $(dA)_{10}$ and $(dC)_{10}$ were resistant to cleavage. Detectable hydrolysis of $(dA)_{10}$ was observed with 5-fold excess enzyme. On the contrary, $(dC)_{10}$ was resistant to cleavage even in presence of 10-fold excess enzyme. ss DNA was hydrolyzed 1.66 fold faster than the decamers [$(dA)_{10}$] while the dinucleoside monophosphates required 4-fold excess enzyme (20 U) for 25 - 100 % hydrolysis suggesting that, nuclease Bh1 prefers high molecular weight substrates.

Table 2.9 : Hydrolysis of oligodeoxynucleotides by nuclease Bh1.

Substrate	Enzyme (U)	A_{260}	Hydrolysis (%)
ssDNA	5	0.250	100
$(dT)_{10}$	5	0.150	60
$(dG)_{10}$	5	0.110	44
$(dA)_{10}$	25	0.055	22
$(dC)_{10}$	50	0.000	0

Determination of the 3' and 5'-phosphoryl terminii of oligonucleotides produced following the hydrolysis of both ss and ds DNA, using snake venom phosphodiesterase (which acts on oligonucleotides having free 3'-OH and 5'-PO_4 terminii) yielded 5'mononucleotides (> 95 %) while spleen phosphodiesterase (which acts on oligonucleotides having free 5'-OH and 3'-PO_4 terminii), failed to digest the oligonucleotides. These results suggest that nuclease Bh1 produces oligonucleotides having 3'-hydroxyl and 5'-phosphoryl terminii. The ability of T4 DNA ligase (which requires an exposed 3'-OH and 5'-PO_4 group) to ligate nicked circular DNA generated by nuclease Bh1, supports the above observation.

Action on deoxyribodinucleoside monophosphates **:** Action of nuclease Bh1 on deoxyribonucleoside monophosphates resulted in the formation of the corresponding nucleoside and a 5'-mononucleotide. For example, hydrolysis of d(TpG) resulted in the formation of dT and 5'dGMP whereas, hydrolysis of d(GpT) resulted in the formation of dG and 5'dTMP. Moreover, the formation of oligonucleotides with 5'-phosphoryl and 3'-hydroxyl termini indicated that the cleavage of DNA occurs on sites 5' to the phosphoribose as illustrated below -

$$d[N_pN'] \longrightarrow dN + 5'\text{-}N'MP$$

Time course analysis of the hydrolytic products of DNA, by nuclease Bh1, showed that the nucleotides are liberated in the order of 5'dGMP > 5'dTMP > 5'dAMP with the absence of 5'dCMP. This suggested that the enzyme showed preference to certain linkages. Hence, to assess the linkage specificity, the action of nuclease Bh1 on a series of dinucleoside monophosphates was studied. The results showed that the homodimers were hydrolyzed in the order of d(GpG) > d(TpT) > d(ApA). While d(GpG) was hydrolyzed completely, d(CpC) was resistant to cleavage (Fig. 2.14).

5' \ 3'	d**G**	d**T**	d**A**	d**C**
d**G**	100	79	52	0
d**T**	65	53	54	0
d**A**	35	40	25	0
d**C**	0	0	0	0

Fig. 2.14 : Hydrolysis of deoxyribodinucleoside monophosphates by nuclease Bh1. The numbers indicate percent hydrolysis.

It was also observed that the type of base present at the 5'end of the dinucleoside monophosphate influenced the cleavage efficiency. For example, dinucleoside monophosphates with deoxyguanosine at the 5'end were cleaved more efficiently than the ones with thymidine or deoxyadenosine as the 5'nucleoside [d(GpT) > d(TpG) > d(ApG)]. Additionally, the enzyme showed higher preference for dinucleoside monophosphates with deoxyguanosine at the 5'end than when it was present at the 3'end [d(GpT) > d(TpG), d(GpA) > d(ApG)]. Dinucleoside monophosphates having the more preferred base (dG > dT > dA) at the 5'end were hydrolyzed to a greater extent than when they were present at the 3'end [d(GpT) > d(TpG), d(GpA) > d(ApG), d(TpA) > d(ApT)]. Dinucleoside monophosphates viz. d(TpT), d(TpA) and d(GpA) were cleaved to a similar extent while d(TpG) and d(GpT) were hydrolyzed to a greater extent (Fig. 2.14) indicating that the internucleotide bonds between dT and dG are more susceptible than those between dT and dA. On the contrary,

dinucleoside monophosphates with deoxycytidine either at the 3' or the 5' end were resistant to hydrolysis.

Based on the results of the action of nuclease Bh1 on dinucleoside monophosphates and oligodeoxyribonucleotides, the appearance of different mononucleotides during the course of hydrolysis of ssDNA with nuclease Bh1 can be explained as follows. The hydrolysis of ssDNA with low and high enzyme concentrations (Fig. 2.12 A, B) showed a high preference for guanylic acid linkages. The complete hydrolysis of d(GpG) coupled with the surge in the appearance of 5'dGMP on periodic dosing (Fig. 2.12B) as well as the preference of the enzyme for dinucleoside monophosphates in the order of d(GpG) > d(GpT) > d(TpG), point towards the preference of nuclease Bh1 for G-stretches in ssDNA. Similar observations were made in case of *S. antibioticus* nuclease (Cal *et al*., 1996) which showed preference for runs of dG bases in dsDNA but not in ssDNA. Moreover, 5' terminal analysis of the cleavage products of dsDNA by *S. antibioticus* nuclease as well as that of ssDNA by a site specific single-strand-specific endonuclease from *Chlamydomonas* sp. (Burton *et al*., 1977) showed the predominance of G followed by T, suggesting that after d(GpG) linkages d(GpT) linkages are preferred. Similar observations have been made with nuclease Bh1.

Among the homodimers, after d(GpG), d(TpT) was more susceptible to cleavage. However, d(GpT) and d(TpG) were hydrolyzed to a greater extent than d(TpT). Hence the low levels of 5'dTMP, in the hydrolytic products of ssDNA with low enzyme concentrations (Fig. 2.12A), can be attributed to the preferential hydrolysis of linkages between dT and dG rather than those between d(TpT). Among the other dinucleoside monophosphates, d(TpT), d(TpA) and d(GpA) were cleaved to a similar extent while d(TpG) and d(GpT) were hydrolyzed to a greater extent (Fig.2.14) indicating that internucleotide bonds between dT and dG are more susceptible than those between dT and dA or dG and dA. This perhaps explains the absence of 5'dAMP, in the hydrolytic products of ssDNA, with low levels of nuclease Bh1 (Fig. 2.12A). Moreover, increase in the 5'dTMP but not 5'dAMP levels, during

the course of hydrolysis of dsDNA, with high enzyme concentrations (Fig. 2.13B), substantiate the above observation. These conclusions are supported by our observations on the homodeoxyoligoribonucleotides. Inspite of its preference for $d(G_pG)$, nuclease Bh1 hydrolyzed $(dT)_{10}$ to a greater extent than $(dG)_{10}$ (Table 2.6). This can be correlated to the tendency of oligomers of deoxyguanosine to aggregate and form secondary structures, which are not preferred by nuclease Bh1. The resistance of $(dA)_{10}$ to cleavage with 5-fold excess enzyme (Table 2.9) supports our observation that the hydrolysis of ssDNA with low enzyme concentration shows the presence of only 5'dGMP and 5'dTMP (Fig. 2.12A).

The absence of 5'dCMP, in the hydrolytic products of DNA (Fig. 2.12, 2.13) can be correlated to the resistance of dinucleoside monophosphates with cytosine either at the 3' or the 5'end to cleavage. Moreover, the resistance of $(dC)_{10}$ to cleavage coupled with high amounts of 5'dCMP in the oligonucleotides remaining after exhaustive digestion of DNA with nuclease Bh1, substantiates the above observation.

CONCLUSION

The present studies show that nuclease Bh1 is a high stringency single - strand -specific enzyme which does not require metal ions for its activity and stability. In addition, its high stability to pH, temperature, high concentrations of urea and organic solvents, suggests that it can be used under a wide variety of experimental conditions, for the analysis of DNA structure. Since the enzyme activity is not affected by metal ions like Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+}, it can be used in conjunction with enzymes requiring these metal ions viz. restriction endonucleases and topoisomerases.

CHAPTER 3

CHARACTERIZATION OF THE ASSOCIATED RIBONUCLEASE ACTIVITY

SUMMARY

Nuclease Bh1 from *Basidiobolus haptosporus* hydrolyzes ssDNA and RNA in a ratio of approximately 1: 0.05. The optimum pH and temperature, for RNA hydrolysis, were 8.5 and 60 °C, respectively. The RNase activity of nuclease Bh1 neither showed an obligate requirement of metal ions for its activity nor it was activated in the presence of metal ions. The enzyme was inhibited by Zn^{2+}, Ag^{2+}, Hg^{2+}, Fe^{3+} and Al^{3+}, inorganic phosphate, pyrophosphate, DTT, ß- mercaptoethanol, NaCl, KCl, metal chelators and competitively by guanosine 5'-nucleotides. It was stable to high concentrations of urea but susceptible to low concentrations of SDS and guanidine hydrochloride. Nuclease Bh1 hydrolyzed homoribopolynucleotides in the order of poly A > poly U > poly [A].poly [U]. However, poly G and poly C were resistant to cleavage. The enzyme cleaved RNA to 5' mononucleotides with preferential liberation of 5'AMP indicating it to be an adenylic acid preferential endoexonuclease. The absence of 5'CMP in the hydrolytic products of RNA coupled with the resistance of poly C to hydrolysis suggested that C-linkages are resistant to cleavage.

INTRODUCTION

Nucleases are multifunctional enzymes and catalyze the hydrolysis of DNA and RNA. 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 associated RNase activity. Nuclease Bh1 from *Basidiobolus haptosporus* is a multifunctional enzyme and catalyzes the hydrolysis of DNA, RNA and 3'AMP. In the preceding chapter we described the purification and detailed characterization of the deoxyribonuclease activity of nuclease Bh1. The detailed characterization of the associated RNase activity of this enzyme is described in this chapter.

MATERIALS

2', 3' and 5' mononucleotides, homoribopolynucleotides, poly[A].poly[U], dithiothreitol (DTT), β-mercaptoethanol, snake venom phosphodiesterase and spleen phosphodiesterase (Sigma Chemical Co., St. Louis, MO, USA); 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. Commercial yeast RNA (BDH Chemicals Ltd., Poole, England) was purified by ethanol precipitation. All other chemicals used were of analytical grade.

METHODS

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 8.5 and appropriately diluted enzyme. The reaction was initiated by the addition of RNA followed by incubation at 37 °C for 15 min. The reaction was then arrested by the addition of 0.25 ml of MacFadyen's reagent [0.75 % (w/v) uranyl acetate in 25 % (v/v) perchloric acid]. The mixture was left on ice for 10 min and the precipitate was removed by centrifugation (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.

Purification of nuclease Bh1

Cultivation of *Basidiobolus haptosporus* and purification of nuclease Bh1 was carried out as described in Chapter 2.

Action on homoribopolynucleotides and RNA

The total reaction mixture of 600 µl, containing 1.25 mg of RNA or the polynucleotide (poly A, poly U, poly G, poly C or poly[A]. poly[U]), in 200 mM Tris-HCl buffer pH 8.5, was incubated with 1U of nuclease Bh1 at 37 °C. Aliquots of 60 µl were withdrawn at different time intervals and the reaction was terminated by the addition of 20 µl of MacFayden's reagent. The precipitate obtained was removed by centrifugation (2500g, 20 min) and the acid soluble nucleotides produced were measured at 260 nm.

HPLC analysis of the reaction products

***Action on RNA* :** The total reaction mixture of 10 ml, containing 12.5 mg of RNA, in 200 mM Tris-HCl buffer pH 8.5, was incubated with 6U of nuclease Bh1, at 37 °C. Samples (750 µl) were removed at an interval of 1h 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, 20 min). The supernatant was lyophilized, reconstituted in 100 µl of Milli Q water and re-precipitated with chilled ethanol as described above. The supernatant after lyophilization was reconstituted in 100 µl Milli Q water and subjected to HPLC.

***Determination of phosphomonoester termini of the reaction products* :** The hydrolytic products of RNA, obtained following the action of nuclease Bh1, were lyophilized, reconstituted in 40 µl of 30 mM Tris-HCl buffer pH 7.5 and incubated with 0.1 U of either snake venom phosphodiesterase or spleen phosphodiesterase at 37 °C for 15 min. The reaction was then terminated by the addition of two volumes of chilled absolute ethanol. The samples were

concentrated by lyophilization, reconstituted in 20 µl Milli Q water and subjected to HPLC.

Separation of the reaction products **:** High Performance Liquid Chromatography (Hewlett-Packard Ti 1050 series model) was carried out on a LiChroCART LiChrospher 100 RP 18 column (250 x 4 mm, 5 µm, Merck, Germany). The mobile phase comprising of a linear gradient of acetonitrile in 100 mM triethyl ammonium acetate, pH 6.2 [0 - 5 % (v/v) for 40 min, 5 - 20 % (v/v) for 60 min followed by 20 - 100 % (v/v) for further 10 min], was used at 25 ± 1° C and at a flow rate of 0.8 ml / min. Standard or the sample solution (3-5 µl) was injected onto the column and the nucleotides were detected, at 255 nm, using a Jasco UV-Vis 270 detector. The amount occupied by each peak was computed on the basis of total area occupied by each peak of the standard and the sample. The nucleotides eluted in the order of 5'CMP, 5'UMP, 5'GMP and 5'AMP with retention times of ca. 6.63, 9.16, 15.77 and 28.07 min, respectively.

RESULTS AND DISCUSSION

Nuclease Bh1 from *Basidiobolus haptosporus* catalyzes the hydrolysis of ssDNA and RNA at a relative rate of approximately 100 and 5. The pI of the RNase activity of nuclease Bh1 was 6.8, which is similar to that of the ssDNase activity (Chapter 2, Fig. 2.4). The optimum pH for RNA hydrolysis was 8.5 (Fig. 3.1A), which is similar to that of the ssDNA hydrolysis. RNase activity of *Neurospora crassa* (conidia) (Linn and Lehman, 1965a) and *Alteromonas* BAL 31 (Bencen *et al*., 1984) nucleases exhibited a similar pH optima. In contrast, wheat chloroplast nuclease showed an optimum pH of 7.8 and 6.8 for the hydrolysis of denatured DNA and RNA, respectively (Kuligowska *et al*., 1988). The RNase activity of nuclease Bh1 showed high stability and retained a significant amount of its initial activity (> 70 %)

between pH 6 and 9 (Fig. 3.1B). However, at pH 7.0 and 37 °C, the enzyme retained its full activity for 24h.

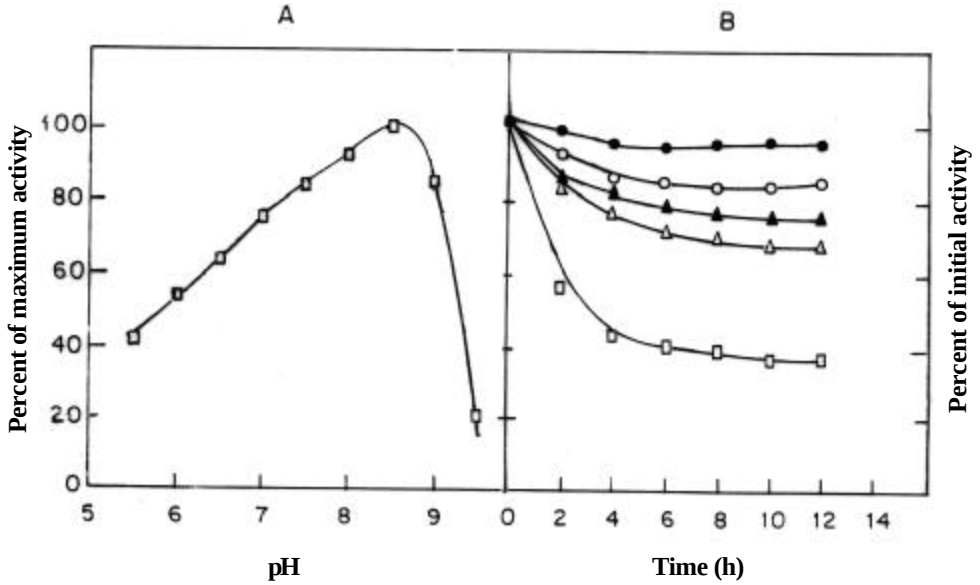

Fig. 3.1 : Optimum pH and pH stability of the RNase activity of nuclease Bh1.

A : Optimum pH : Purified nuclease Bh1 (1U) was assayed for RNase activity ( ) in a series of pH (5.5 - 9.5) at 37° C as described under Methods.

B : pH stability : Purified nuclease Bh1 (200 U of RNase activity) was incubated in a series of buffers (sodium acetate pH 5 - 5.5; immidazole pH 6.0 - 6.5 and Tris-HCl pH 7 - 9.0) at 37°C for 24 h. Aliquots were removed at different time intervals and assayed under standard assay conditions as described under Methods.

pH 7.0 (●), pH 8.0 (o), pH 9.0 (σ), pH 6.0 (Δ) and pH 5.0 ( ).

The optimum temperature for RNA hydrolysis was 60°C, which is similar to that of the ssDNase activity of the enzyme (Fig. 3.2A). Nuclease Bh1 was stable for 15 min at 60 °C but lost 50 % of its initial activity in 45 min (Fig. 3.2B).

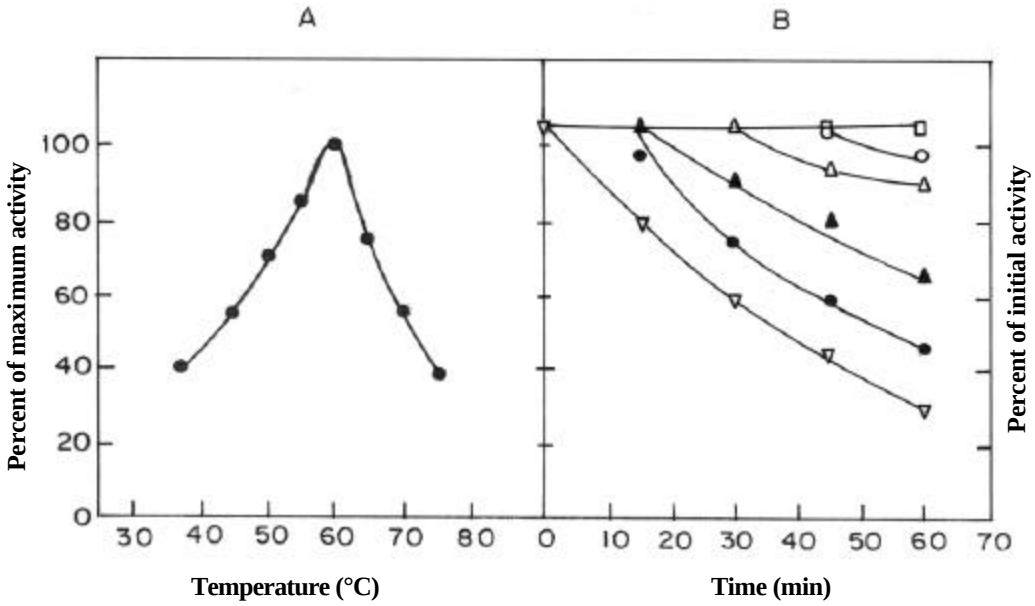

Fig. 3.2 : Optimum temperature and temperature stability of the RNase activity of nuclease Bh1.

A. Optimum temperature : Purified nuclease Bh1 (1U) was assayed for RNase (•) activity at a series of temperatures (37 - 75 °C) as described under Methods.

B. Temperature stability : Purified nuclease Bh1 (200 U of RNase activity) was incubated in Tris-HCl buffer, pH 8.5 at different temperatures (37 - 65 °C) for 1h. Aliquots were removed at different time intervals and assayed under standard assay conditions as described under Methods. 37°C ( ), 45°C (o), 50°C (∆), 55°C (σ), 60 °C (•) and 65°C (∇).

The pH and temperature stability of the RNase activity of nuclease Bh1 is similar to its ssDNase activity (Chapter 2) and comparable to that of S1 nuclease (Shishido and Ando, 1985).

Metal ion requirement

Like the ssDNase activity, the RNase activity of nuclease Bh1 neither showed an obligate requirement of metal ions for its activity nor was the activity stimulated in presence of metal ions. However, it was inhibited by low concentration of metal ions like Zn^{2+}, Ag^{2+}, Hg^{2+}, Al^{3+} and Fe^{3+} (Table 3.1).

Table 3.1 : Effect of metal ions on the RNase activity of nuclease Bh1.

Metal ion (1mM)	Residual activity (%)
Control *	100.00
Ag^{2+}	9.00
Al^{3+}	39.20
Ba^{2+}	83.47
Ca^{2+}	91.29
Co^{2+}	97.10
Cu^{2+}	90.08
Fe^{3+}	40.28
Hg^{2+}	1.75
Mg^{2+}	95.00
Mn^{2+}	95.00
Zn^{2+}	3.50

* Enzyme assayed in the absence of metal ions served as control.

In case of wheat chloroplast nuclease, 10 mM Mg^{2+} slightly stimulated the ssDNase activity (20 %) whereas the RNase activity was completely inhibited (Kuligowska *et al*., 1988). In case of nuclease Bh1, no such differential effect of metal ions was seen.

Metal chelators like EDTA, EGTA, 8-hydroxyquinoline, 1,10 o-phenanthroline and citrate strongly inhibited the RNase activity of nuclease Bh1 (Table 3.2).

Table 3.2 : Effect of metal chelators on the RNase activity of nuclease Bh1.

Metal Chelator	Residual activity (%)
Control *	100.00
EDTA (1 mM)	20.40
EGTA (1 mM)	35.00
8-hydroxyquinoline (200 µM)	20.00
1,10 o-phenanthroline (200 µM)	35.00
Citrate (30 mM)	25.00

* Enzyme assayed in the absence of the above reagents served as control.

Extensive dialysis of the enzyme against 30 mM Tris-HCl buffer, pH 7.0, containing 2 mM EDTA (effective concentration) followed by further dialysis against Tris-HCl buffer, pH 7.0 (to remove EDTA) resulted in complete loss of the RNase activity. However, incubation with 10 mM Zn^{2+} for 5-6h at 4 °C, restored a significant amount (70 -80 %) of its activity, supporting our earlier observation (Chapter 2) that nuclease Bh1 is a zinc metalloprotein. In case of wheat chloroplast nuclease, the ssDNase activity was strongly inhibited by EDTA but it had no significant effect on the RNase activity (Kuligowska *et al*., 1988). However, in case of nuclease Bh1, both ssDNase and RNase activities exhibited a similar sensitivity to EDTA.

Effect of salt concentration

The RNase activity of nuclease Bh1 retained its full activity in presence of 25 mM of both NaCl and KCl. Further increase in salt concentration (>50 mM) resulted in a decrease in the RNase activity and at 200 mM of NaCl and KCl, the enzyme exhibited approximately 50 % and 40 % of its initial activity, respectively (Fig. 3.3).

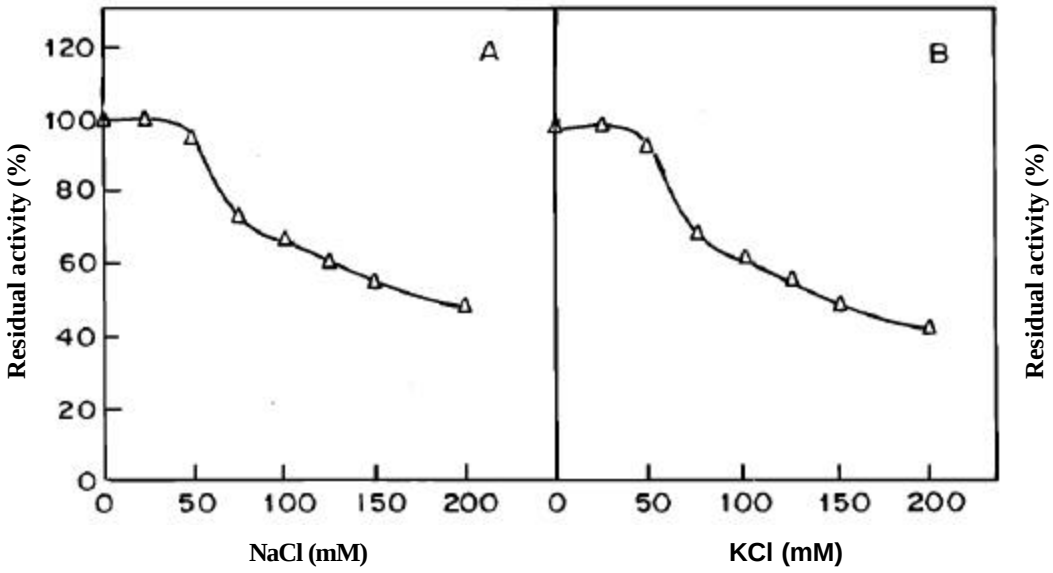

Fig. 3.3 : Effect of salt on the RNase activity of nuclease Bh1.

A . Purified nuclease Bh1 (10U of RNase activity) was assayed in presence of varying concentrations of NaCl under standard assay conditions.

B . Purified nuclease Bh1 (10U of RNase activity) was assayed in presence of varying concentrations of KCl under standard assay conditions

However, the ssDNase activity of nuclease Bh1 showed higher sensitivity to salt concentration because increased level of inhibition (35 and 20 %) was observed in presence of 150 mM of NaCl and KCl (Chapter 2). Susceptibility

of the RNase activity of nuclease Bh1, to salt concentration, is inferior compared to that of 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-Manago, 1967). In contrast, RNase activity of *Saccharomyces cerevisiae* mitochondrial nuclease was insensitive to salt concentration and exhibited optimal activity in presence of 300 mM KCl (Dake *et al*., 1988).

Effect of thiol reagents

DTT and ß-mercaptoethanol inhibited the RNase activity of nuclease Bh1 to a significant extent (>70 %) suggesting that disulfide bridges are essential for its activity. Among them, DTT was a stronger inhibitor as 1mM of the reagent could bring about approximately 80 % inhibition of the activity whereas, it required 10 mM of ß-mercaptoethanol to bring about comparable level (70 %) of inhibition (Table 3.3).

Table 3.3 : Effect of thiol reagents on the RNase activity of nuclease Bh1.

Thiol reagent (mM)	Residual activity (%)
Control*	100.00
Dithiothreitol (DTT)	
1	20.25
ß-mercaptoethanol (ß-Me)	
2	81.19
4	70.01
6	57.06
8	45.89
10	29.50

* Enzyme assayed in the absence of the above reagents served as control.

Stability to denaturants

The RNase activity of nuclease Bh1 showed high stability in presence of low concentrations of protein denaturants and retained 82 % and 75 % of its initial activity in presence of 0.04 % (w/v) SDS and 500 mM guanidine hydrochloride, respectively. However, the enzyme exhibited higher stability in presence of 4M urea and retained 80 % of its initial activity (Table 3.4). The ssDNase activity of nuclease Bh1 also showed a similar stability to the denaturants (Chapter 2). The stability of the RNase activity of nuclease Bh1 to urea is inferior to that of the RNase activity of *Physarum polycephalum* nuclease (Waterborg and Kuyper, 1982).

Inhibitors

Among all the nucleotides tested the RNase activity of nuclease Bh1 was inhibited only by guanosine 5' -nucleotides. In contrast to S1 nuclease (5'dAMP > 5'AMP), RNase activity of nuclease Bh1 showed a stronger inhibition in the presence of 5'GMP than 5'dGMP. The observation that deoxyguanosine (dG) is a weaker inhibitor than 5'dGMP suggested that the phosphate residue in 5'dGMP is responsible for stronger inhibition. Moreover, increased inhibition observed with increase in the number of phosphate residues i.e. 5'GTP > 5'GDP > 5'GMP supports the role of phosphate in the inhibition of the RNase activity of nuclease Bh1. Guanosine 5'-nucleotides inhibited the RNase activity of nuclease Bh1 competitively in the order of 5'GTP > 5'GDP > 5'GMP > 5'dGMP (Fig. 3.4) with Ki values of 173, 228, 241and 284 µg, respectively.

The role of phosphate in the inhibition of the RNase activity of nuclease Bh1 is supported by the observation that pyrophosphate is a stronger inhibitor of the enzyme than inorganic phosphate. Nuclease Bh1 retained 53 % and 25 % of its RNase activity in presence of 5 mM inorganic phosphate and pyrophosphate, respectively. Similar observations were made in case of the ssDNase activity of the enzyme (Chapter 2).

Table 3.4 : Effect of denaturants on the RNase activity of nuclease Bh1.

Denaturant (concentration)	Residual activity (%)
SDS (%)	
Control*	100.00
0.02	92.10
0.04	82.51
0.06	68.92
0.08	54.04
0.10	24.09
Guanidine hydrochloride (M)	
Control*	100.00
0.25	98.00
0.50	95.00
0.75	75.00
1.00	50.00
1.50	25.00
2.00	5.00
Urea (M)	
Control*	100.00
1	95.00
2	95.00
3	88.00
4	80.00
5	65.00

* Enzyme assayed in the absence of the above reagents served as control.

In contrast, the RNase activity of nucleases SM1 and SM2 from *Serratia marcescens kums* 3958 did not show any inhibition in presence of 5mM inorganic phosphate while pyrophosphate at this brought about approximately 80 % inhibition of the activity (Yonemura *et al*., 1983). This differential level of inhibition in presence of inorganic phosphate and pyrophosphate was not observed with nuclease Bh1.

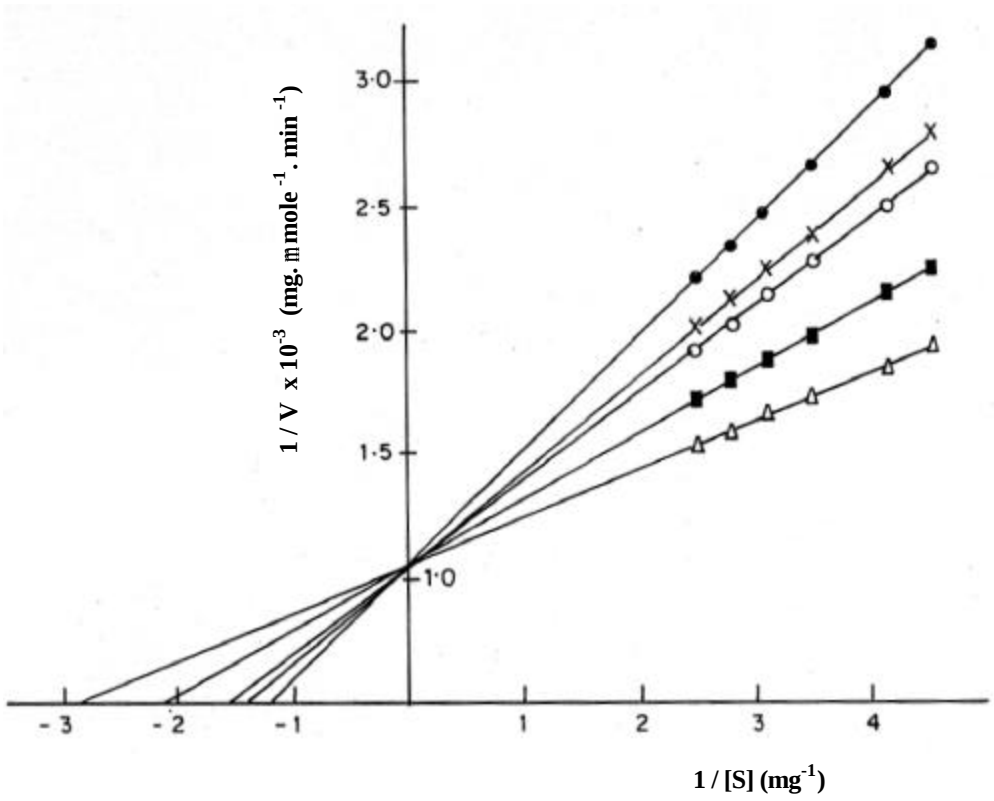

Fig. 3.4 : Inhibition of RNase activity of nuclease Bh1 by guanosine 5'-phosphates.

Reaction mixtures containing RNA (0.22 - 0.4 mg) and the inhibitors were incubated with 1U the of RNase activity of nuclease Bh1 at pH 8.5 and 37 °C and the acid soluble ribonucleotides formed were measured at 260 nm as described under Methods.

No inhibitor (Δ), 150 µM 5'dGMP (v), 100 µM 5'GMP (o), 50 µM 5'GDP (x), 50 µM 5'GTP (•).

Action on homoribopolynucleotides and RNA

Action on homoribopolynucleotides revealed that, nuclease Bh1 hydrolyzed poly A rapidly while poly U, RNA and poly[A] . poly[U] were cleaved approximately 65 %, 32 % and 16 % the rate of poly A, respectively. Poly C and poly G were resistant to hydrolysis (Fig. 3.5A).

Despite the ability of nuclease Bh1 to readily hydrolyze poly A and poly U, the low rate of hydrolysis of poly[A] . poly[U], suggests that it is a single strand preferential enzyme.

Specificity and mode of action

Time course analysis of the hydrolytic products of RNA showed that, nuclease Bh1 liberated 5'mononucleotides in the order of 5'AMP > 5'UMP > 5'GMP. From the initial stages of hydrolysis, the amount of 5'AMP liberated was higher than other nucleotides (Fig.3.5B). This observation coupled with the ability of the enzyme to rapidly hydrolyze poly A suggests that the RNase activity of nuclease Bh1 prefers adenylic acid linkages. In contrast, the DNase activity of nuclease Bh1 showed preference for guanylic acid linkages (Chapter 2). The ssDNase and RNase activities of *N. crassa* nuclease were guanylic acid preferential (Linn and Lehman, 1965b) while those of wheat chloroplast nuclease were adenylic acid preferential (Kuligowska *et al*., 1988). In contrast, nuclease Bh1 exhibited different base preference with ssDNA and RNA.

Interestingly, throughout the course of RNA hydrolysis, 5' CMP could not be detected in the hydrolytic products. Moreover, HPLC analysis of the oligonucleotides (obtained after exhaustive digestion of RNA with nuclease Bh1) after treatment with snake venom phosphodiesterase showed high levels of 5'CMP (data not shown). These observations coupled with the inability of nuclease Bh1 to hydrolyze poly C point towards the resistance of cytidylic acid linkages to cleavage. Similar observations were made with the DNase activity of nuclease Bh1 (Chapter 2).

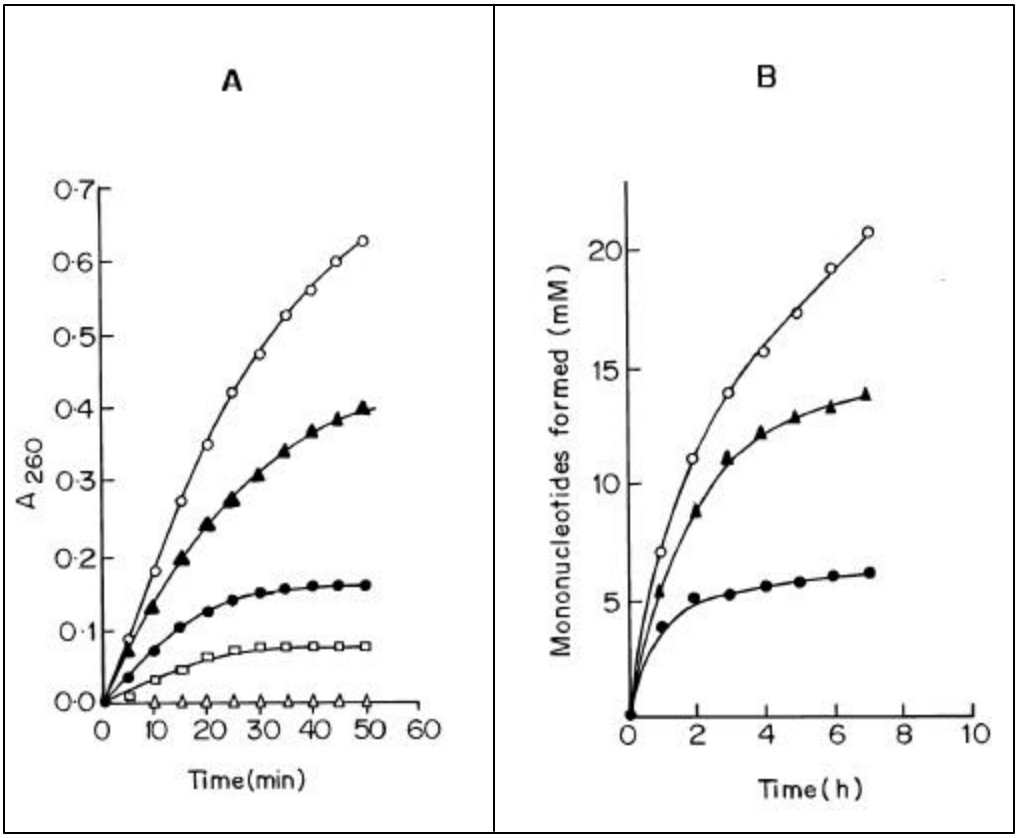

Fig. 3.5 : Time course of hydrolysis of homoribopolynucleotides and RNA by nuclease Bh1.

A : Hydrolysis of polynucleotides and RNA.

The reaction mixture containing 1.25 mg of either polynucleotides or RNA and 1 U of the RNase activity of nuclease Bh1 was incubated at pH 8.5 and 37 °C. Samples were withdrawn at different time intervals and the amount of acid soluble ribonucleotides liberated were measured at 260 nm as described under Methods.

Poly A (o), poly U (σ), RNA (•), poly[A].poly[U] ( ), poly G and ploy C (Δ).

B : Release of 5' mononucleotides from RNA.

The total reaction mixture of 10 ml containing 12.5 mg of RNA, in 200 mM Tris-HCl buffer pH 8.5, was incubated with 6U of the RNase activity of nuclease Bh1, at 37 °C. Samples (750 µl) were removed at an interval of 1h and the products formed were analyzed by HPLC. For details refer to Methods.

5'AMP (o), 5'UMP (σ), 5'GMP (•).

Analysis of the hydrolytic products of RNA showed that, during the initial stages of hydrolysis (i.e. upto 2h) the proportion of oligonucleotides (approximately 60%) was higher compared to mononucleotides (approximately 40 %). However, with increase in the incubation period, the proportion of mononucleotides increased indicating an endoexo mode of action. In contrast, the ssDNase activity of nuclease Bh1 showed predominantly an endo mode of action because throughout the course of hydrolysis, the proportion of oligonucleotides was higher than that of mononucleotides (Chapter 2). Wheat seedling nuclease cleaved ssDNA endonucleolytically but exhibited exonucleolytic action on RNA (Hanson and Fairely, 1969). Wheat chloroplast nuclease, on the other hand, showed endo mode of action on both ssDNA and RNA (Kuligowska *et al*., 1988). Nuclease Bh1 differs from these enzymes in that, it shows predominantly an endo mode of action on ssDNA but an endoexo mode of action on RNA.

Determination of 3' and 5' phosphoryl termini of the oligonucleotides produced following the exhaustive digestion of RNA, using snake venom phosphodiesterase (which acts on oligonucleotides having free 3'-OH and 5'-PO_4 termini), yielded 5'-mononucleotides (>95 %) while spleen phosphodiesterase (which acts on oligonucleotides having free 3'-PO_4 and 5'-OH termini) failed to digest the oligonucleotides. These results show that, nuclease Bh1 produces oligonucleotides having 3'-hydroxyl and 5-phosphoryl termini. 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 Bh1 is similar to endoexonuclease from *N. crassa* (Linn and Lehman, 1965b) and endonucleases from rye germ ribosomes (Siweka *et al*., 1989) and wheat chloroplast stromal protein (Monko *et al*.,1994). In contrast, wheat chloroplast 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 (Monko *et al*., 1994). However, nuclease Bh1 differs from these enzymes in that, the oligonucleotides liberated following the hydrolysis of both DNA and RNA have 3'-hydroxyl and 5'-phosphoryl termini.

CONCLUSION

The RNase activity of nuclease Bh1 is a single-strand-preferential endoexonuclease and shows preference for adenylic acid linkages in RNA. Moreover, cytidylic acid linkages are resistant to cleavage.

CHAPTER 4

CHARACTERIZATION OF THE ASSOCIATED 3'-PHOSPHOMONOESTERASE ACTIVITY

SUMMARY

Nuclease Bh1 from *Basidiobolus haptosporus* hydrolyzes ssDNA and 3'AMP in a ratio of approximately 1:1. The optimum pH and temperature for the hydrolysis of 3'AMP and other 3'-ribonucleotides were 8.5 and 60 °C, respectively. The 3'-nucleotidase activity of nuclease Bh1 neither showed an obligate requirement of metal ions for its activity nor it was activated in the presence of metal ions. The enzyme was inhibited by Zn^{2+}, Ag^{2+}, Hg^{2+}, Fe^{3+} and Al^{3+}, DTT, ß-mercaptoethanol and metal chelators. It was stable to high concentrations of urea but susceptible to low concentrations of SDS and guanidine hydrochloride. Nuclease Bh1 dephosphorylated 3'-ribonucleotides in the order of 3'UMP > 3'AMP >> 3'GMP >> 3'CMP. However, 3'-deoxyribonucleotides were resistant to cleavage. Moreover, 3'AMP and pAp were hydrolyzed at a similar rate indicating that the presence of phosphate at the 5'-end of the nucleotide does not influence the cleavage efficiency of the enzyme. Guanosine 5'-nucleotides as well as ssDNA and RNA competitively inhibited the 3'AMPase activity of nuclease Bh1 suggesting the presence of a common catalytic site for the hydrolysis of both monomeric and polymeric substrates.

INTRODUCTION

Nucleases are multifunctional enzymes and catalyze the hydrolysis of DNA and RNA. Some of them also exhibit either 3' or 5' phosphomonoesterase activity (Shishido and Ando, 1985). Secretion of nucleases into the culture media indicates that one of the main biological role of extracellular fungal enzymes is to provide nucleosides and phosphate for growth. In this respect, it is a common observation that most of the extracellular nucleases show an associated phosphomonoesterase activity. Extracellular nuclease from *Basidiobolus haptosporus* (nuclease Bh1) is a multifunctional enzyme and

catalyzes the hydrolysis of DNA, RNA and 3'AMP (Chapter 2). Characterization of the phosphomonoesterase activity is of interest because it gives a better insight into the interactions of the enzyme with low molecular weight substrates and this can be useful in the elucidation of the mechanism of enzyme action. The characteristics of the associated 3'-nucleotidase activity of nuclease Bh1 are described in this chapter.

MATERIALS

2', 3' and 5' mononucleotides, 2':3'-cyclic nucleotides, 3':5'-cyclic nucleotides, adenosine 3',5'diphosphate, dithiothreitol (DTT) and β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA); calf intestinal alkaline phosphatase (Bangalore Genei, Bangalore, India) and HPLC grade acetonitrile (E. Merck (India) Limited, Mumbai, India) were used. All other chemicals used were of analytical grade.

METHODS

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 8.5, containing 2 % (v/v) glycerol 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 water : 12 M H_2SO_4 : 2.5 % (w/v) ammonium molybdate: 10 % (w/v) ascorbic acid in the ratio of 2:1:1:1 (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.

Kinetic parameters and the inhibition constants (Ki) were determined from Lineweaver-Burk plots.

Action of nuclease Bh1 on cyclic nucleotides

The total reaction mixture of 2 ml contained 1mM of either 2':3' cAMP or 3':5' cAMP in 30 mM Tris-HCl buffer, pH 8.5, containing 2 % (v/v) glycerol and appropriately diluted enzyme. After incubation at 37 °C for 30 min, the reaction was arrested by adjusting the pH of the reaction mixture to 9.5 by the addition of 100 µl of 1M glycine-NaOH buffer. One ml of the assay mixture was then incubated with 0.05 U of calf intestinal alkaline phosphatase, for 1h at 37 °C and the inorganic phosphate liberated was estimated according to Chen *et al*. (1956).

Purification of nuclease Bh1

Cultivation of *Basidiobolus haptosporus* and purification of nuclease Bh1 was carried out as described earlier (Chapter 2).

HPLC analysis of the reaction products

Action on adenosine 3',5' diphosphate

The total reaction mixture of 100 µl in 30 mM Tris-HCl buffer, pH 8.5, containing 1mM adenosine 3', 5' diphosphate, was incubated with 1 U of nuclease Bh1 at 37 °C for 1h and the reaction was then terminated by the addition of two volumes of chilled absolute ethanol. The samples were lyophilized, reconstituted in 20 µl Milli Q water and subjected to HPLC on a Symmetry C18 column (250 x 4.6 mm, 5 µm, Waters model fitted with 515 HPLC pump) at 25 ± 1 °C. Twenty µl of the standard or the sample solution were injected onto the column and the column was washed with 100 mM triethylammonium acetate, pH 6.2, for 6 min at a flow rate of 0.3 ml / min. Subsequently, the product, 5'AMP and unhydrolyzed adenosine 3', 5' diphosphate were eluted with a linear gradient of acetonitrile in 100 mM triethylammonium acetate, pH 6.2 [0- 15 % (v/v) for 30 min followed by 20 - 100 % (v/v) for 5 min], at a flow rate of 0.8 ml / min. The nucleotides eluted in the order of 5'AMP followed by adenosine 3', 5' diphosphate with retention times of ca. 20.82 and 21.12 min, respectively.

RESULTS AND DISCUSSION

Nuclease Bh1 catalyzes the hydrolysis of ssDNA and 3'AMP at a relative rate of approximately 1:1. The pI of the 3'AMPase activity of nuclease Bh1 was 6.8, which is similar to that of the ssDNase and RNase activities of the enzyme (Chapter 2, Fig. 2.4).

The optimum pH for the 3'-phosphomonoesterase activity of nuclease Bh1 was 8.5 and it did not change with the nucleotide used (Fig. 4.1A). In contrast, nucleases Le1 and Le3 from *Lentinus edodes* (Shimada *et al*., 1991, Kobayashi *et al*., 1995) and P1 nuclease from *Penicillium citrinum* (Fujimoto *et al*., 1974a) showed different pH optima for the hydrolysis of different nucleotides. Moreover, unlike 3'-nucleotidase-nuclease from potato tubers (Nomura *et al*., 1971), which showed different pH optima for nucleotidase (pH 8.0) and nuclease (pH 6.5 - 7.5) activities, nuclease Bh1 showed the same pH optima (8.5) for the hydrolysis of both monomeric and polymeric substrates. The 3'-nucleotidase activity of nuclease Bh1 showed high stability and retained a significant amount of its initial activity (> 70 %) between pH 6 and 9 (Fig. 4.1B). However, at pH 7.0 and 37°C, the enzyme retained its full activity for 24h. The stability of the 3'-nucleotidase activity of nuclease Bh1 is comparable to that of the 3'-nucleotidase-nuclease from potato tubers and similar to its ssDNase (Chapter 2, Fig. 2.5B) and RNase (Chapter 3, Fig. 3.1B) activities.

The optimum temperature for the hydrolysis of all the four 3'-ribonucleotides was 60°C (Fig. 4.2A), which is similar to that of the ssDNase and RNase activities of the enzyme (Chapter 2, Fig. 2.6A and Chapter 3, Fig. 3.2A). On the contrary, the optimum temperature of pea seed nuclease for the hydrolysis of 3'AMP was 60°C (Naseem and Hadi, 1987) while that of ssDNA hydrolysis was 45°C (Wani and Hadi, 1979). The 3'-nucleotidase activity of nuclease Bh1 was stable for 15 min at 60°C but lost 50 % of its initial activity in 45 min. The temperature stability of 3'-nucleotidase activity of nuclease

Bh1 is inferior compared to that of the 3'-nucleotidase-nuclease from potato tubers (Nomura *et al*., 1971).

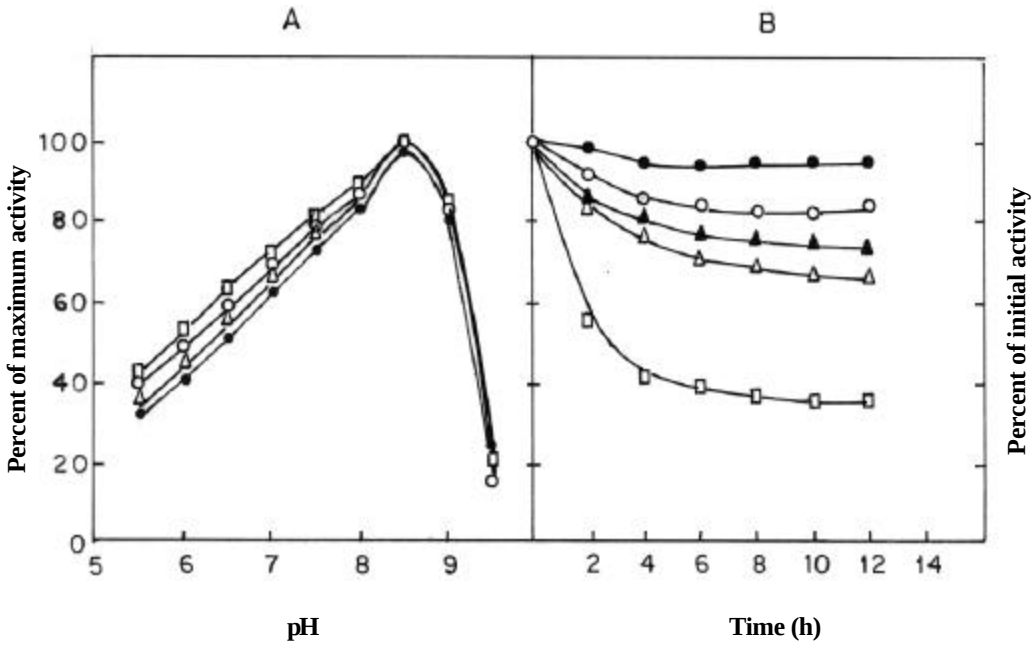

Fig.4.1 : Optimum pH and pH stability of the 3'-phosphomonoesterase activity of nuclease Bh1.

A. Optimum pH: Purified nuclease Bh1 (1U) was assayed for 3'AMPase ( ), 3'UMPase (o), 3'GMPase (Δ) and 3'CMPase (•) activities in a series of pH (5.5 - 9.5) at 37° C as described under Methods.

B. pH stability : Purified nuclease Bh1 (2000 U of 3'AMPase activity) was incubated in a series of buffers (sodium acetate pH 5 - 5.5; immidazole pH 6.0 - 6.5 and Tris-HCl pH 7 - 9.0) at 37 °C for 24 h. Aliquots were removed at different time intervals and assayed under standard assay conditions as described under Methods. pH 7.0 (•), pH 8.0 (o), pH 9.0 (σ), pH 6.0 (Δ) and pH 5.0 ( ).

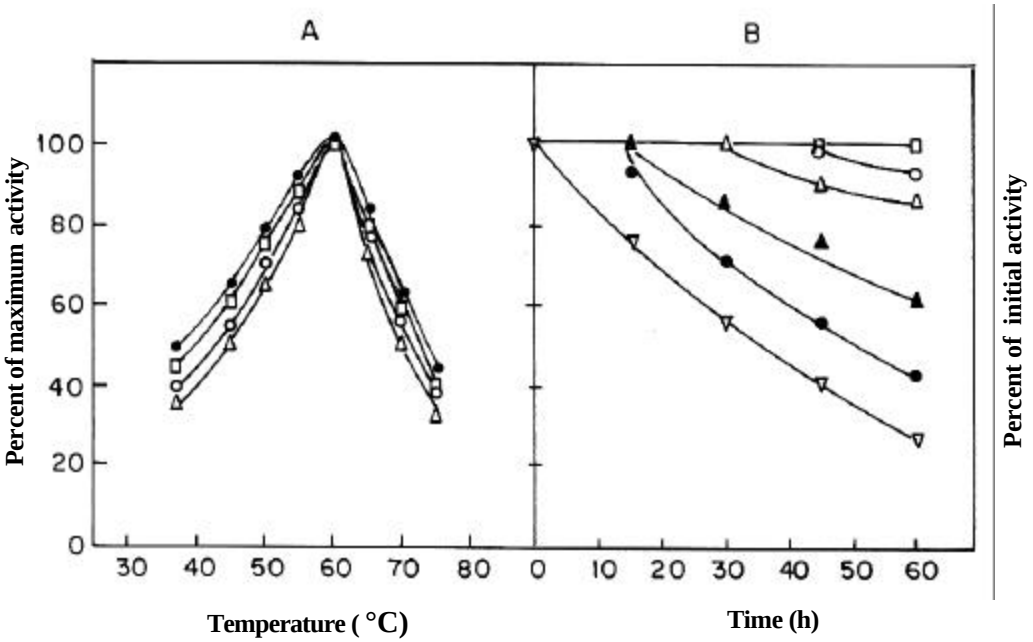

Fig. 4.2 : Optimum temperature and temperature stability of the 3'-phosphomonoesterase activity of nuclease Bh1.

A. Optimum temperature : Purified nuclease Bh1 (1U) was assayed for 3'AMPase ( ), 3'UMPase (o), 3'GMPase (Δ) and 3'CMPase (•) activities in a series of temperatures (37 - 75 °C) as described under Methods.

B. Temperature stability : Purified nuclease Bh1 (2000 U of 3'AMPase activity) in Tris-HCl buffer, pH 8.5 was incubated at different temperatures (37 - 65 °C) for 1h. Aliquots were removed at different time intervals and assayed for the 3'AMPase activity under standard assay conditions as described under Methods. 37°C ( ), 45°C (o), 50°C (Δ), 55°C (σ), 60 °C (•) and 65°C (∇).

Metal ion requirement

Like the ssDNase and RNase activities, the 3'-nucleotidase activity of nuclease Bh1 neither showed an obligate requirement of metal ions for its activity nor was the activity stimulated in presence of metal ions. However, it was inhibited by low concentration of metal ions like Zn^{2+}, Ag^{2+}, Hg^{2+}, Al^{3+} and Fe^{3+} (Table 4.1).

Table 4.1 : Effect of metal ions on the 3'-phosphomonoesterase activity of nuclease Bh1*.

Metal ion (1mM)	Residual activity (%)
Control **	100.00
Ag^{2+}	13.60
Al^{3+}	45.00
Ba^{2+}	95.47
Ca^{2+}	93.75
Co^{2+}	98.00
Cu^{2+}	91.25
Fe^{3+}	50.09
Hg^{2+}	0.00
Mg^{2+}	98.00
Mn^{2+}	93.00
Zn^{2+}	0.00

* Assays were carried out at pH 8.5 and 37 °C with 3'AMP as substrate as described under Methods.

** Enzyme assayed in the absence of metal ions served as control.

Similar observations were made in case of 3'-nucleotidase-nuclease from potato tubers (Nomura *et al*., 1971). Although the 3'-nucleotidase activity of

pea seed nuclease did not show an obligate requirement of metal ions for its activity, it was stimulated approximately two fold in the presence of $MgCl_2$ and $CaCl_2$. The 3'-nucleotidase activity of pea seed nuclease was strongly inhibited by $CoCl_2$ (Naseem and Hadi, 1987) whereas, its DNase activity showed only marginal inhibition (Wani and Hadi, 1979). In case of nuclease Bh1, the sensitivity towards different metal ions was similar for both the nuclease and the nucleotidase activities.

Metal chelators like EDTA, EGTA, 8-hydroxyquinoline, 1,10 o-phenanthroline and citrate strongly inhibited the 3'-nucleotidase activity of nuclease Bh1 (Table 4.2).

Table 4.2 : Effect of metal chelators on the 3'-phosphomonoesterase activity of nuclease Bh1*.

Metal Chelator	Residual activity (%)
Control **	100.00
EDTA (1 mM)	2.00
EGTA (1 mM)	5.00
8-hydroxyquimoline (200 µM)	29.00
1,10 o-phenanthroline (200 µM)	43.00
Citrate (30 mM)	24.00

* Assays were carried out at pH 8.5 and 37 °C with 3'AMP as substrate as described under Methods.

** Enzyme assayed in the absence of the above reagents served as control.

Extensive dialysis of the enzyme against 30 mM Tris-HCl buffer pH 7.0 containing 2 mM EDTA (effective concentration) followed by further dialysis against Tris-HCl buffer, pH 7.0 (to remove EDTA) resulted in complete loss of

the 3'AMPase activity. However, incubation with 10 mM Zn^{2+} for 5-6h at 4°C, restored a significant amount (70 -80 %) of its activity. Moreover, atomic adsorption of the purified protein showed that nuclease Bh1 contains 4 atoms of zinc per mol of the protein, confirming our earlier observation that nuclease Bh1 is a zinc metalloprotein. Sensitivity to EDTA was also observed in case of 3'-nucleotidase / nuclease from *Crithidia luciliae* (Neubert and Gottlieb, 1990). The 3'-nucleotidase activity of pea seed nuclease was strongly inhibited by EDTA (Naseem and Hadi, 1987) whereas, EDTA had no effect on the DNase activity (Wani and Hadi, 1979). However, in case of nuclease Bh1, the ssDNase, RNase and the 3'-nucleotidase activities exhibited a similar sensitivity to EDTA.

Effect of thiol reagents

Dithiothreitol and ß-mercaptoethanol inhibited the 3'AMPase activity of nuclease Bh1 to a significant extent (>70 %) suggesting that disulfide bridges are essential for its activity. Among them, DTT was a stronger inhibitor as 1mM of the reagent could bring about 80 % inhibition of the activity whereas, it required 10 mM of ß-mercaptoethanol to bring about comparable level (~70 %) of inhibition (Table 4.3). Similar results were obtained with the ssDNase and RNase activities of nuclease Bh1 (Chapter 2, 3).

Stability to denaturants

The nucleotidase activity of nuclease Bh1 showed high stability in presence of low concentrations of protein denaturants and retained approximately, 75 % of its initial activity in presence of 0.04 % (w/v) SDS and 750 mM guanidine hydrochloride, respectively. However, the enzyme exhibited higher stability in presence of 4M urea and retained 80 % of its initial activity (Table 4.4). Comparable results were obtained for the ssDNase and RNase activities of nuclease Bh1 (Chapter 2, 3).

Table 4.3 : Effect of thiol reagents on the 3'-phosphomonoesterase activity of nuclease Bh1*.

Thiol reagent (mM)	Residual activity (%)
Control**	100.00
Dithiothreitol (DTT)	
1	19.90
ß-mercaptoethanol (ß-Me)	
2	85.00
4	70.00
6	45.92
8	33.88
10	28.34

* Assays were carried out at pH 8.5 and 37 °C with 3'AMP as substrate as described under Methods.

** Enzyme assayed in the absence of the above reagents served as control.

Inhibitors

It has been reported that mononucleotides are potent inhibitors of single-strand-specific nucleases. In the present studies, among all the nucleotides tested the 3' -nucleotidase activity of nuclease Bh1 was inhibited only by guanosine 5' -nucleotides. The 3'-nucleotidase activity of nuclease Bh1 showed a stronger inhibition in presence of 5'-ribonucleotides than 5'-deoxyribonucleotides whereas in case of S1 nuclease, 5'-deoxyribonucleotides were more potent inhibitors (Oleson and Hoganson, 1981). The observation that deoxyguanosine (dG) is a weaker inhibitor than 5'dGMP suggested that the phosphate residue in 5'dGMP is responsible for stronger inhibition.

Table 4.4 : Effect of denaturants on the 3'-phosphomonoesterase activity of nuclease Bh1*.

Denaturant (concentration)	Residual activity (%)
SDS (%)	
Control**	100.00
0.02	89.24
0.04	75.39
0.06	69.24
0.08	54.55
0.10	26.52
Guanidine hydrochloride (M)	
Control**	100.00
0.25	100.00
0.50	98.00
0.75	75.00
1.00	50.00
1.50	22.00
2.00	7.00
Urea (M)	
Control**	100.00
1	98.00
2	92.00
3	84.00
4	80.00
5	60.00

* Assays were carried out at pH 8.5 and 37 °C with 3'AMP as substrate as described under Methods.

** Enzyme assayed in the absence of the above reagents served as control.

Moreover, increased inhibition observed with increase in the number of phosphate residues i.e. 5'GTP > 5'GDP > 5'GMP supports the role of phosphate in the inhibition of the 3'AMPase activity of nuclease Bh1. Similar observations were made in case of the ssDNase and RNase activities of nuclease Bh1 (Chapter 2, 3). Guanosine 5'-nucleotides inhibited the 3'AMPase activity of nuclease Bh1 competitively (Fig. 4.3) in the order of 5'GTP > 5'GDP > 5'GMP > 5'dGMP with Ki values of 0.54, 0.68, 2.94, 7.99 µM, respectively. Moreover, the 3'AMPase activity of nuclease Bh1 was inhibited competitively by ssDNA and RNA with Ki values of 2.32 and 91.28 µg, respectively (Fig. 4.4), suggesting the presence of a common catalytic site for the hydrolysis of both monomeric and polymeric substrates. Similar observations were made in case of S1 nuclease (Oleson and Hoganson, 1981) and 3'-nucleotidase-nuclease from potato tubers (Suno *et al*., 1973).

Substrate Specificity

The action of nuclease Bh1 on different nucleotides is shown in Table 4.5. The enzyme catalyzed the hydrolysis of 3'-phosphomonoester groups only from ribonucleoside monophosphates. 5'-mononucleotides (ribo as well as the deoxyribo) and 2'-mononucleotides were resistant to hydrolysis (Table 4.5). S1 nuclease (Olenson and Hoganson, 1981) and mung bean nuclease (Kole *et al*., 1974), hydrolyzed 3'-ribonucleotides faster than 3'-deoxyribonucleotides and the increased rate was attributed to the presence of 2'-OH group. However, in case of nuclease Bh1, the 3'-deoxyribomononucleotides were resistant to cleavage. Similar observations were made with 3'-nucleotidase / nuclease from the *C. luciliae* (Neubert and Gottlieb, 1990). Nuclease Bh1 hydrolyzed 3'-ribomononucleotides in the order of 3'UMP > 3'AMP >> 3'GMP >> 3'CMP. Moreover, kinetic parameters for hydrolysis of these substrates (Table 4.6) were in accordance with the observed rates of hydrolysis (Table 4.5). Although the affinity for 3'AMP was slightly more than that of 3'UMP, the V_{max} for the latter was approximately 1.5 times higher than that of 3'AMP, suggesting its higher rate of hydrolysis. Compared to 3'AMP, 3'GMP was

hydrolyzed at a slow rate while 10-fold excess enzyme was required to obtain detectable hydrolysis of 3'CMP, pointing towards the high resistance of 3'CMP to cleavage. This is substantiated by our observation that the Km and K_{cat} for 3'CMP was approximately 68 and 3.5 fold higher than that of 3'AMP. The low activity on 3'GMP can be correlated to its acting as an inhibitor analogue since 5'GMP is a competitive inhibitor of nuclease Bh1.

Table 4.5 : Substrate specificity of the 3'-phosphomonoesterase activity of nuclease Bh1*.

Nucleotide	Hydrolysis (%)
3'AMP	100
3'UMP	130
3'GMP	25
3'CMP	5
adenosine 3',5' diphosphate	100
3'-deoxyribomononucleotides	0
5'-ribomononucleotides	0
5'-deoxyribomononucleotides	0
2'-ribomononucleotides	0
3':5' and 2':3' cyclic mononucleotides	0

* Assays were carried out at pH 8.5 and 37 °C with different nucleotides as substrates as described under Methods.

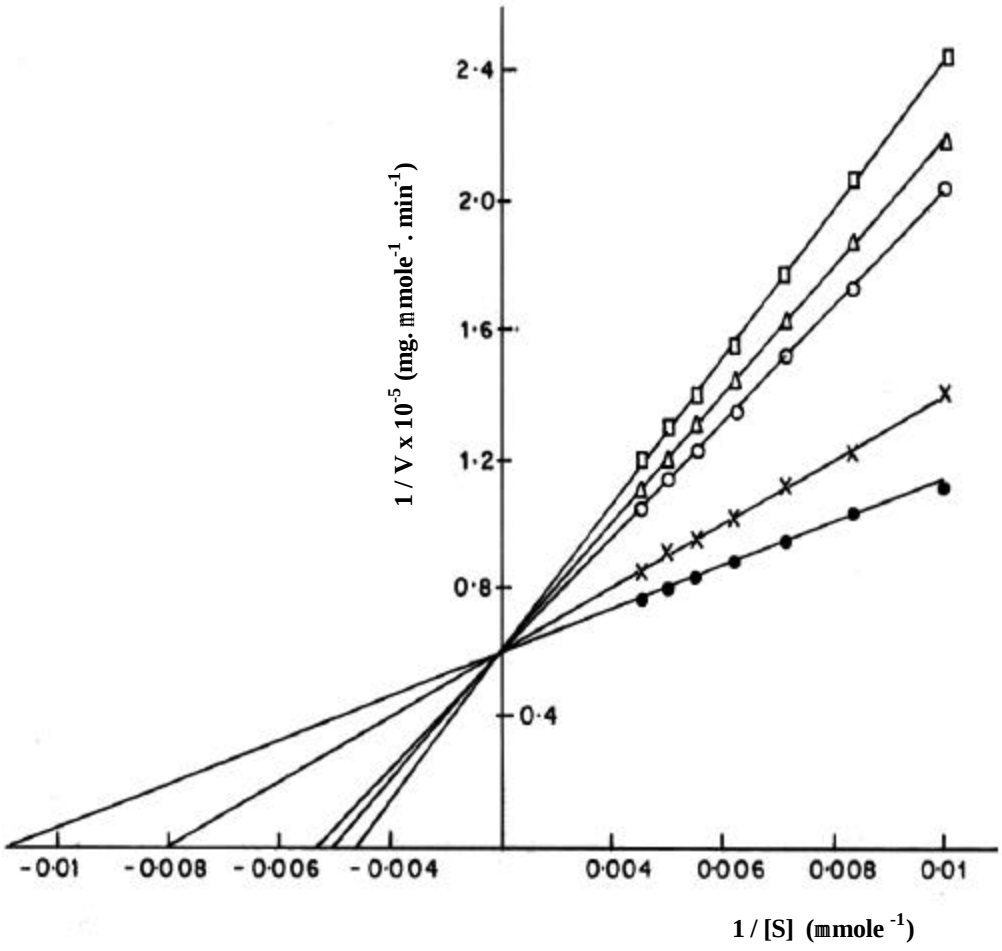

Fig.4.3: Inhibition of the 3'-AMPase activity by guanosine 5'-phosphates.

Reaction mixtures containing 3'AMP (100 - 200 µM) and the inhibitors were incubated with 1U of the 3'AMPase activity of nuclease Bh1 at pH 8.5 and 37 °C and the inorganic phosphate liberated was measured at 660 nm as described under Methods.

No inhibitor (•), 150 µM 5'dGMP (x), 100 µM 5'GMP (o), 50 µM 5'GDP (Δ), 50 µM 5'GTP ( ).

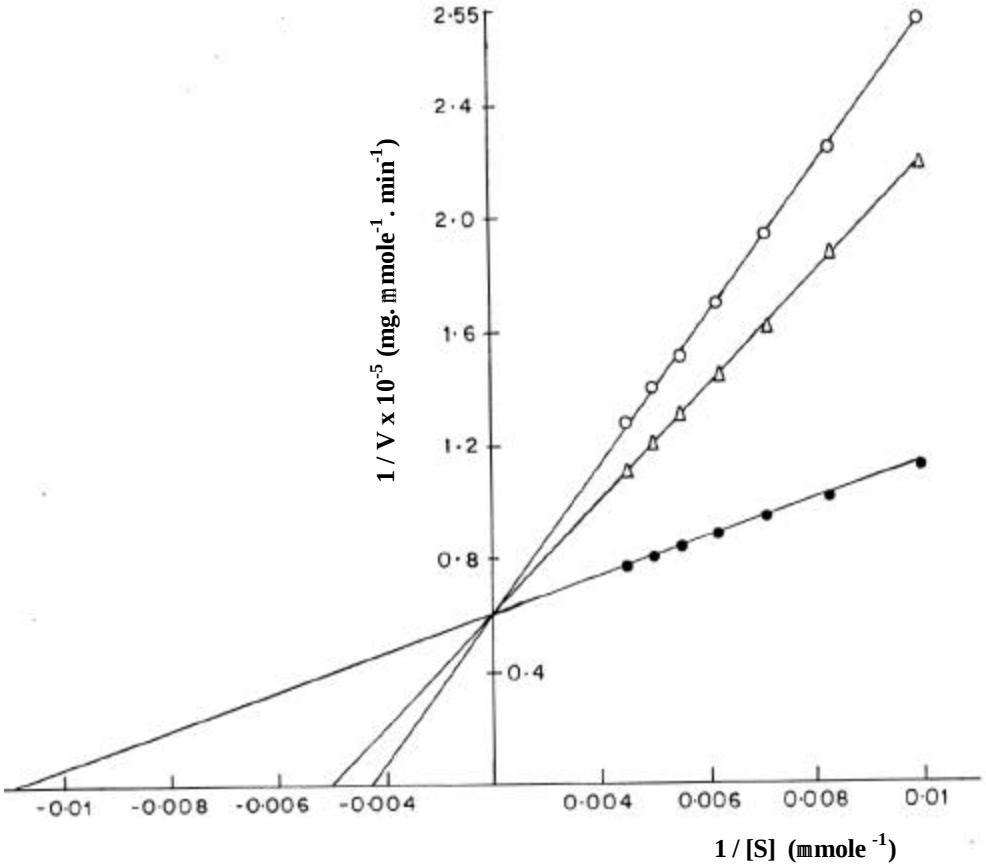

Fig. 4.4 : Inhibition of the 3'-AMPase activity by ssDNA and RNA.

Reaction mixtures containing 3'AMP (100 - 200 µM) and ssDNA (5 µg) or RNA (250 µg) were incubated with 1U of the 3'AMPase activity of nuclease Bh1 at pH 8.5 and 37 °C and the inorganic phosphate liberated was measured at 660 nm as described under Methods.

No inhibitor (•), 5 µg ssDNA (o), 250 µg RNA (Δ)

Table 4.6 : Kinetic parameters for the hydrolysis of 3'-nucleotides by nuclease Bh1*.

Substrate	Km (µmoles)	Vmax ($\mu moles.min^{-1}.mg^{-1}$) x 10^5	Vmax/ Km	K_{cat} x 10^{10}
3'AMP	92.00	5.71	0.062	6.54
3'UMP	100.00	8.40	0.082	9.62
3'GMP	125.00	3.30	0.026	3.78
3'CMP	6250.00	1.67	0.0002	1.91
pAp	95.00	5.80	0.061	6.53

* Assays were carried out at pH 8.5 and 37 °C with different nucleotides as substrates as described under Methods.

In case of S1 and mung bean nucleases (Oleson and Hoganson, 1981; Kole *et al*., 1974) presence of a 5'-phosphomonoester group or a 5'-substituent in 3'-AMP, increased the rate of hydrolysis. In contrast, 3'-nucleotidase / nuclease from *C. luciliae* hydrolyzed pAp at half the rate of 3'AMP (Neubert and Gottlieb, 1990). However, in case of nuclease Bh1, the presence of a 5'-phosphomonoester group did not affect the rate of hydrolysis of pAp. Moreover, similar Km and K_{cat} values for 3'AMP and pAp suggest that the presence of an additional phosphomonoester group does not affect the turnover of the diphosphate (Table 4.6). HPLC analysis of the hydrolytic products of pAp, by nuclease Bh1, showed the presence of only 5'AMP. Moreover, 5'AMP was not dephosphorylated even on prolonged incubation with the enzyme, substantiating our earlier observation that nuclease Bh1 does not cleave 5'-mononucleotides.

Phosphodiesterases [EC 3.1.4.1] attacking both the ribo- and deoxyribo-internucleotide bonds can be classified into two main groups, *viz*. 5'-phosphate formers and 3'-phosphate formers, depending on the nature of the mononucleotide produced. Hydrolysis of ssDNA and RNA, by nuclease Bh1, lead to the formation of 5'-mononucleotides (Chapter 2,3). Additionally, depending on the basic structural component by which the enzyme recognizes the substrate, four possible modes of action can be envisaged (Fig. 4.5).

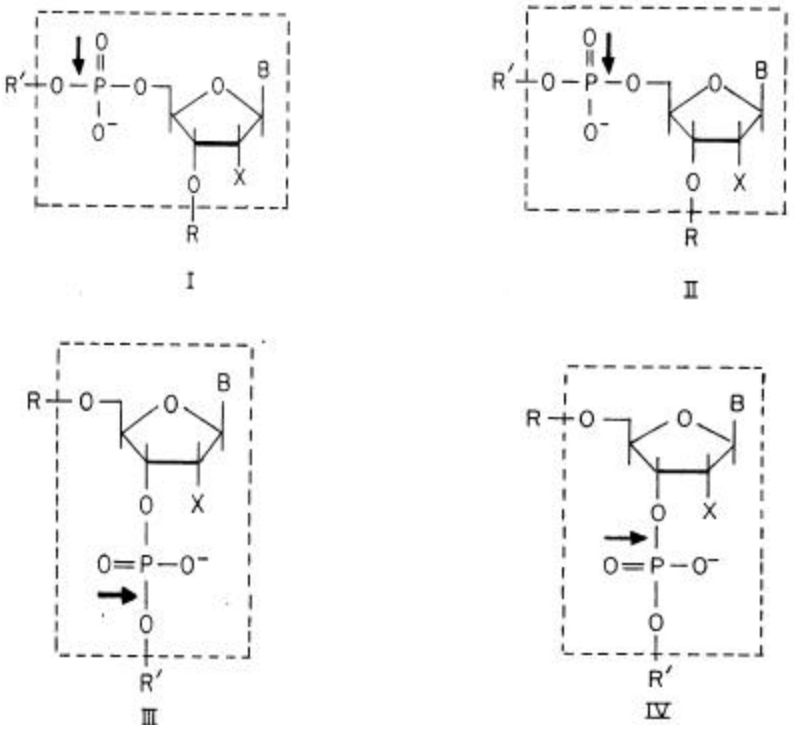

Fig. 4.5 : Mode of action of nuclease Bh1 in comparison with the other phosphodiesterases.

B = base, X = H / OH, R = H or nucleotide and R' = H or nucleoside. The basic structural unit which is recognized by the enzyme is enclosed in the box, indicated by dashed line. The position of the arrow indicates the bond that is hydrolyzed by the enzyme. (Adapted from Suno *et al.*,1973).

Class I : The enzymes belonging to this class recognize the nucleoside 5'-phosphate, require a free 3'-OH group and cleave 5' to the phosphoribose generating 5'-mononucleotides. e.g. Snake venom phosphodiesterase.

Class II : These enzymes recognize nucleoside 5'-phosphate and cleave the 5' O-P bond generating the corresponding nucleoside. e.g. Staphylococcal nuclease.

Class III : Enzymes of this class of phosphodiesterases, recognize nucleoside 3'-phosphate, require a free 5'-OH group and cleave 3' to the phosphoribose producing 3'-mononucleotides. e.g. Spleen phosphodiesterase.

Class IV : The enzymes belonging to this class, recognize the nucleoside 3'-phosphate and attack the 3' O-P bond, resulting in the formation of the corresponding nucleoside.

The action of nuclease Bh1 on 3'-mononucleotides with the formation of the corresponding nucleoside coupled with the action on adenosine diphosphate (pAp), also suggests that nuclease Bh1 recognizes the nucleoside 3'-phosphate group and attacks the 3'O-P bond in the mononucleotide substrate. Hence, nuclease Bh1 belongs to class IV phosphodiesterases where, in the mononucleotide substrate, R' will correspond to H.

CONCLUSION

The phosphomonoesterase activity of nuclease Bh1 is 3'-ribonucleotide specific because it hydrolyzes only 3'-ribonucleotides. Moreover, 3'CMP is highly resistant to cleavage. The ability of ssDNA and RNA to competitively inhibit the 3'AMPase activity of nuclease Bh1 suggests the presence of a common catalytic site for the hydrolysis of both monomeric and polymeric substrates.

CHAPTER 5

ACTIVE SITE CHARACTERIZATION

SUMMARY

Chemical modification studies on purified nuclease Bh1 revealed the involvement of a single lysine and carboxylate residue in the catalytic activity of the enzyme. Substrates of nuclease Bh1 *viz*. ssDNA, RNA and 3'AMP and the competitive inhibitor, 5'GMP, could protect the enzyme against TNBS-mediated inactivation whereas, substrate and inhibitor protection was not observed against EDC-mediated inactivation of the enzyme. Moreover the lysine modified enzyme, having very little catalytic activity, showed a significant decrease in its ability to bind 5'GMP, while the carboxylate modified enzyme (having very little catalytic activity) could effectively bind 5'GMP, suggesting the involvement of lysine in substrate binding and carboxylate in catalysis. Furthermore, lysine and carboxylate modification was accompanied by a concomitant loss of ssDNase, RNase and phosphomonoesterase activities of nuclease Bh1, indicating the existence of a common catalytic site for the hydrolysis of both monomeric and polymeric substrates. Chemical modification studies also revealed that histidine has no role in the catalytic activity of nuclease Bh1.

INTRODUCTION

Nuclease Bh1 is a sugar non-specific multifunctional enzyme, which acts on ssDNA, RNA and 3'AMP. In case of enzymes acting on anionic substrates, lysine and / or arginine has been implicated in substrate binding (Riordan, 1979; Richardson *et al*., 1990). Lysine residues have also been implicated in the binding of ssDNA to ssDNA binding proteins (Anderson *et al*., 1975; Bandopadhyay and Cheng-Wen, 1978). The involvement of histidine has been shown in the catalytic activity of S1 nuclease (Gite *et al*., 1992b), nucleases from shrimp hepatopancreas (Wang *et al*., 2000) and pancreatic DNase (Price *et al*., 1969) as well as RNases belonging to T2 family (Kawata *et al*., 1990; Sanda *et al*., 1985; Rangarajan *et al*., 1999). Moreover, carboxylate has been shown to have a role in the catalytic activity of nucleases

from *Staphylococcus aureus* (Cotton *et al*., 1979) and *Serratia marcescens* (Kolmes *et al*., 1996) and a DNA / RNA non-specific endonuclease nucA from *Anabaena* sp. (Meiss *et al*., 2000). Since nuclease Bh1 acts on ssDNA and RNA, chemical modification of lysine, carboxylate and histidine were carried out to evaluate their role in the catalytic activity of the enzyme.

MATERIALS

Methylene Blue and hydroxylamine hydrochloride (BDH, Mumbai, India); uranyl acetate (Loba Chemie Pvt. Ltd., Mumbai, India); perchloric acid (Qualigens, Mumbai, India); diethyl pyrocarbonate (DEP), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC), 3'AMP and 5'GMP (Sigma Chemical Co., St. Louis, MO, USA); N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 2[N-Morpholino]ethanesulfonic acid (MES) (Sisco Research Laboratories Pvt. Ltd., Mumbai, India) and Bio-Gel-P-10 (Bio-Rad, Richmond, CA, USA.) were used. High Mr DNA from buffalo liver was isolated as described in Chapter 2. Commercial yeast RNA (BDH Chemicals Ltd., Poole, England) was purified by ethanol precipitation. All other chemicals used were of analytical grade.

METHODS

Enzyme assays

The ssDNase and RNase activities of nuclease Bh1 were determined as described earlier (Chapter 2,3). The amount of acid soluble nucleotides liberated following the hydrolysis of ssDNA or RNA, at pH 8.5 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 deoxyribonucleotides and ribonucleotides mixture, respectively (Curtis *et al*., 1966). One unit of ssDNase and RNase activity is defined as the amount of enzyme required to liberate 1 µmol of acid soluble nucleotides / min under the assay conditions.

The phosphomonoesterase activity of nuclease Bh1 was assayed by measuring the amount of inorganic phosphate liberated following the hydrolysis of 3'AMP, at pH 8.5 and 37°C (Chapter 4). 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.

Protein determination

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

Purification of nuclease Bh1

Nuclease Bh1 was purified to homogeneity as described earlier (Chapter 2).

Chemical Modification studies

In chemical modification studies, the residual activity of the modified enzyme was determined using all three substrates *i.e.* ssDNA, RNA and 3'AMP.

Reaction with TNBS

The total reaction mixture of 1 ml, containing 10 µg of nuclease Bh1 in 30mM HEPES buffer pH 8.0, was incubated with varying concentrations of TNBS (25-100 µM) at 37 °C in the dark. Aliquots (10 µl) were removed at suitable time intervals and the reaction was terminated by the addition of 990 µl of 50 mM MES / HEPES buffer pH 6.0. Subsequently, the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of TNBS served as control.

Reaction with EDC

Nuclease Bh1 (10 µg), in 500 µl of 30 mM HEPES buffer pH 7.5, was incubated with varying concentrations of EDC (5 - 20 mM) and 5 fold excess

(25-100 mM) of glycine methyl ester at 28 ± 2 °C for 40 min. Aliquots (10 µl) were removed at suitable time intervals and the reaction was arrested by the addition of 990 µl of MES / HEPES buffer pH 6.0. The residual activities were then determined under standard assay conditions. Enzyme samples incubated in the absence of EDC served as control.

Photo-oxidation

This was done by exposing 10 µg of the purified enzyme, in 1ml of 30 mM MES / HEPES buffer pH 6.0 in a glass test tube (1 cm x 10 cm), containing different concentrations of Methylene Blue (0.01 - 0.2 %) to 200 W flood-light bulb held at a distance of 12 cm for 15 min, at 25 ± 1 °C, followed by estimation of residual activities. Enzyme samples treated under identical conditions, in the dark, served as control.

Reaction with DEP

Purified nuclease Bh1 (5µg) in 1 ml of 30 mM MES / HEPES buffer, pH 6.0, was incubated at 28 ± 2 °C for 20 min with varying concentrations of DEP (0.1 - 2.0 mM), freshly diluted with absolute ethanol. Aliquots (5 µl) were withdrawn at suitable time intervals and the reaction was arrested by the addition of 50 µl of 10 mM imidazole buffer, pH 7.5. The residual activities were then 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 125 mM. The ethanol concentration in the reaction mixture did not exceed 1 % (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. The DEP-mediated reaction was also monitored,

spectrophotometrically, by measuring the changes in the absorbance at 240 nm as described by Ovadi *et al*. (1967).

Reaction with hydroxylamine

Decarbethoxylation was carried out according to Miles (1977). The DEP modified enzyme samples were incubated with 500 mM hydroxylamine, pH 7.0, at room temperature for 6 h and the enzyme activities were determined under standard assay conditions.

Substrate protection studies

In all the chemical modification reactions, the effect of substrate protection was studied by pre-incubating the enzyme with excess amounts of ssDNA, RNA and 3'AMP as well as with the competitive inhibitor, 5'GMP, followed by treatment with various modifying reagents.

CD measurements

The CD spectra of native and chemically modified enzyme samples were recorded on a JASCO-715 Spectropolarimeter, at 25 °C, in the range of 190 - 250 nm.

Inhibitor binding studies

The inhibitor binding studies on native and modified enzyme samples were carried out according Hummel and Dreyer (1962). The TNBS and EDC modified enzyme samples (300 µg each) in 1 ml of 30 mM Tris-HCl buffer pH 7.0 [containing 5 % (v/v) glycerol] and 20 µM 5'GMP were passed through a Bio-Gel P-10 column (1 x 25 cm) equilibrated with the above buffer, at a flow rate of 0.5 ml / min. Fractions of 1.5 ml were collected and the absorbance at 260 nm was measured. Unmodified enzyme subjected to similar treatment was taken as control.

RESULTS AND DISCUSSION

Modification of lysine residues

Purified nuclease Bh1 when incubated with 75 µM TNBS, at pH 8.0 for 25 min, lost 75-80 % of its activity towards ssDNA, RNA and 3'AMP and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in control samples. The logarithm of residual activity plotted as a function of time at various TNBS concentrations was linear upto 20 %, 17 % and 22 % of the initial activity towards ssDNA, RNA and 3'AMP, respectively (Fig. 5.1). TNBS-mediated inactivation followed pseudo-first-order kinetics at any fixed concentration of the reagent. The pseudo-first-order rate constants were calculated from the slope of plot of log (percent residual activity) versus reaction time and the order of the reaction was determined from the slope of the plots of log(k_{app}) against log[TNBS]. These plots (insets Fig. 5.1) indicated that the loss of enzyme activity towards all the three substrates occurred as a result of modification of a single lysine residue. The above results suggest the involvement of lysine in the catalytic activity of nuclease Bh1.

Substrate protection studies showed that, the TNBS-mediated inactivation could be prevented by incubation of the enzyme with excess amounts of ssDNA, RNA, 3'AMP and the competitive inhibitor 5'GMP, prior to the modification reaction. The CD spectra of both unmodified and lysine modified nuclease Bh1 were almost identical (Fig.5.2) indicating that the loss of enzyme activity is due to lysine modification rather than structural changes. Additionally, the TNBS modified enzyme, having 20 % residual activity, showed a significant decrease in its ability to bind 5'GMP, a competitive inhibitor of nuclease Bh1 (Table 5.1). These results suggest the involvement of lysine in substrate binding. Involvement of lysine in substrate binding has also been demonstrated in case of the S1 nuclease (Gite *et al*., 1992a) and RNases from bovine pancreas (Richardson *et al*., 1990; Trautwein *et al*., 1991) and *Bacillus intermedius* (Yakovlev *et al*., 1994).

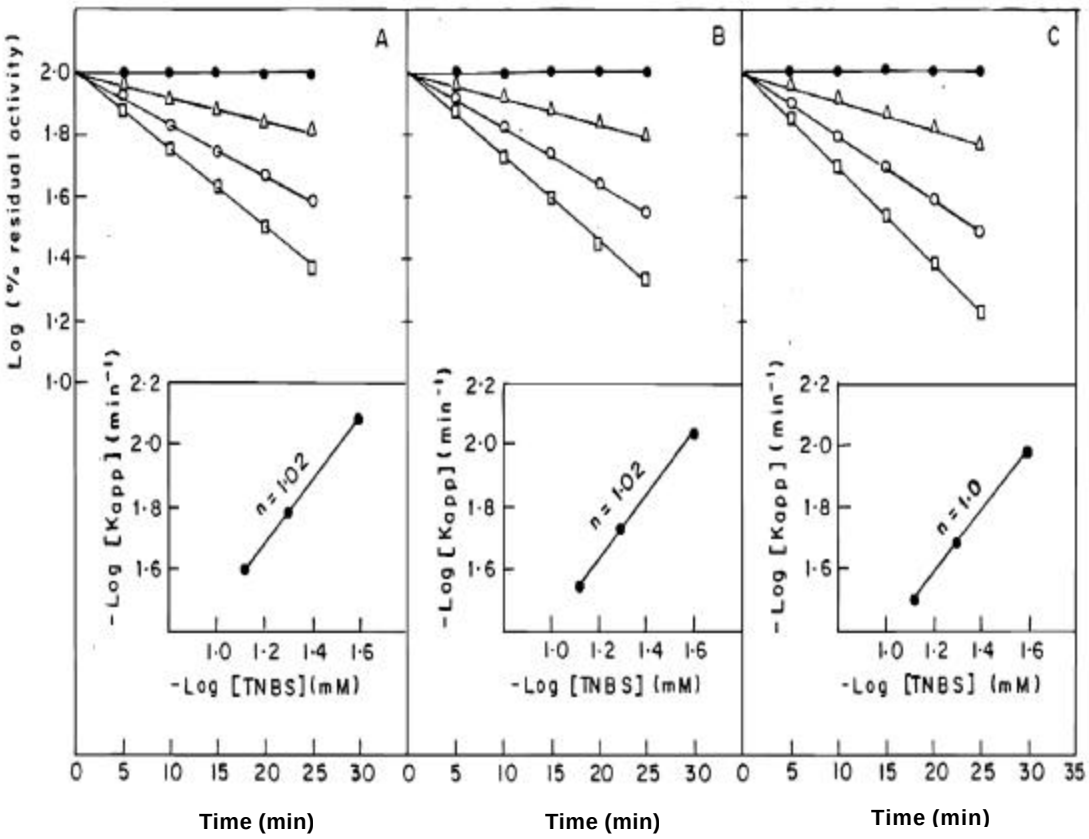

Fig. 5.1 : Pseudo-first-order plots for the inactivation of nuclease Bh1 by TNBS.

A. ssDNase **B.** RNase **C.** phosphomonoesterase.

Concentrations of TNBS were : 0 µM (•), 25 µM (Δ), 50 µM (o) and 75 µM ( ). Inset : Second-order plots for pseudo-first-order rate constants of inactivation. $(k_{app})(min)^{-1}$ at different concentrations of TNBS.

Table 5.1 : Influence of lysine modification on the activity of nuclease Bh1 : Substrate protection and inhibitor binding studies.

Incubation mixture	Residual activity (%)	5'GMP binding (%)
Enzyme	100	100
Enzyme + TNBS (75 µM)	20	20
Enzyme +ssDNA (1mg) + TNBS	95	-
Enzyme + RNA (1mg) + TNBS	96	-
Enzyme + 3'AMP (5 mM) + TNBS	90	-
Enzyme + 5'GMP (1 mM) + TNBS	95	-

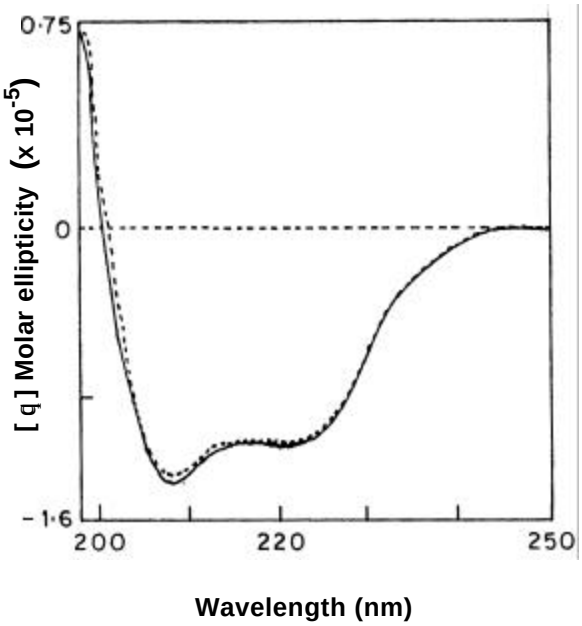

Fig. 5.2 : The CD spectra of native and TNBS modified nuclease Bh1.

The CD measurements were performed in a 1 mm cell at an enzyme concentration of 150 µg / ml. Native enzyme (—), TNBS treated enzyme (-----).

Modification of carboxylate groups

Purified nuclease Bh1, when incubated with 15 mM EDC, at pH 7.5 for 40 min, lost 70-80 % of its initial activity towards ssDNA, RNA and 3'AMP and the inactivation was dependent on the concentration of the reagent. However, no loss of activity was observed in control samples. The logarithm of residual activity plotted as a function of time at various EDC concentrations was linear upto 28 %, 20 % and 30 % of the initial activity towards ssDNA, RNA and 3'AMP, respectively (Fig. 5.3). EDC-mediated inactivation followed pseudo-first-order kinetics at any fixed concentration of the reagent and the order of the reaction (insets Fig. 5.3) indicated that the loss of enzyme activity towards all the three substrates occurred as a result of modification of a single carboxylate residue. These results indicate the involvement of carboxylate in the catalytic activity of the enzyme.

EDC mediated inactivation of nuclease Bh1 could not be prevented by incubation of the enzyme with excess amounts of ssDNA, RNA, 3'AMP and 5'GMP prior to the modification reaction. Moreover, the CD spectra of both unmodified and carboxylate modified nuclease Bh1 were almost identical (Fig. 5.4) showing that the loss of activity is due to carboxylate modification rather than structural changes. Furthermore, the carboxylate modified enzyme, having only 20-25 % residual activity, could effectively bind 5'GMP (Table 5.2) pointing towards the involvement of carboxylate in catalysis. Carboxylate groups have also been implicated in the catalytic activity of nucleases from *S. marcescens* (Kolmes *et al.*,1996) and *Anabaena* sp. (Meiss *et al*., 2000), Staphylococcal nuclease (Weber *et al*., 1991), RNase Rh (Sanda *et al*., 1985) and RNase M (Watanabe *et al*., 1983).

Modification of histidine residues

Photo-oxidation of nuclease Bh1 with 0.2 % (w/v) Methylene Blue at pH 6.0 and for 30 min, did not result in the loss of activity. Moreover, modification of histidine residues with DEP (at pH 6.0 for 30 min) though resulted in the modification of two residues out of a total of five did not have

any effect on the activity of the enzyme, suggesting that histidine has no role in the catalytic activity of nuclease Bh1.

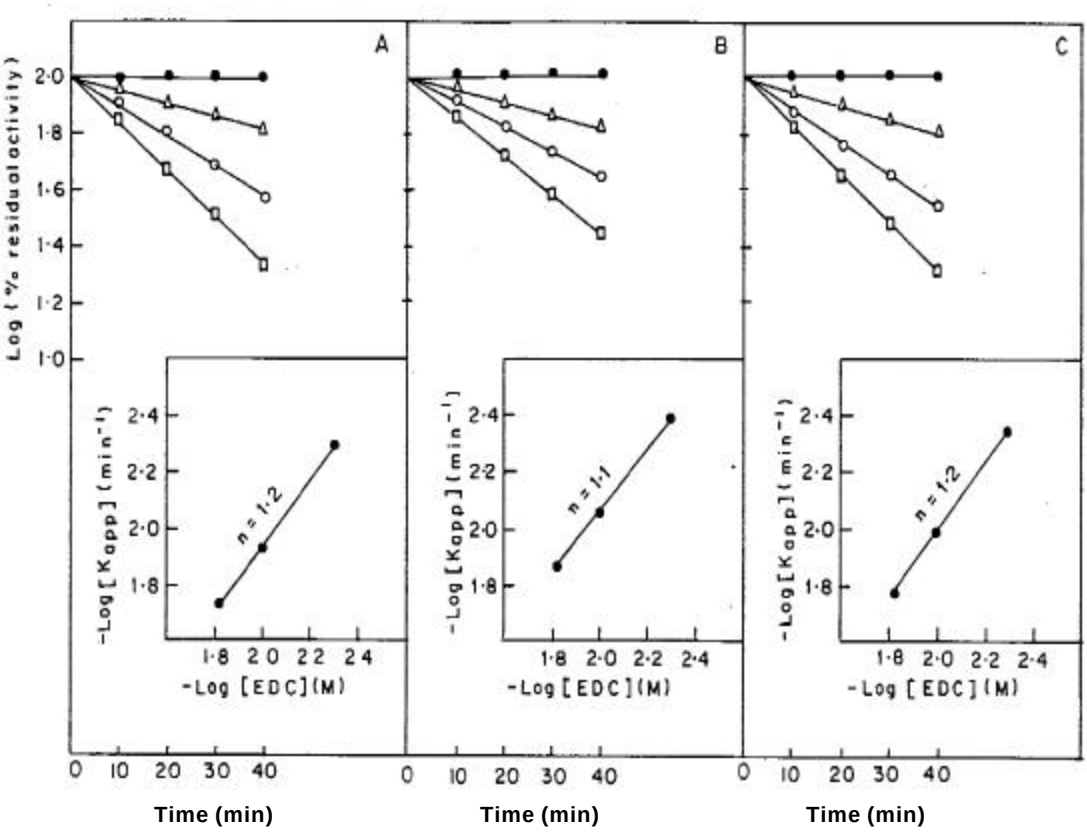

Fig. 5.3 : Pseudo-first-order plots for the inactivation of nuclease Bh1 by EDC.

A. ssDNase **B.** RNase **C.** phosphomonoesterase.

Concentrations of EDC were : 0 mM (•), 5 mM (Δ), 10 mM (o) and 15 mM ( ). Inset : Second-order plots for pseudo-first-order rate constants of inactivation. $(k_{app})(min)^{-1}$ at different concentrations of EDC.

Table 5.2 : Influence of carboxylate modification on the activity of nuclease Bh1 : Substrate protection and inhibitor binding studies.

Incubation mixture	Residual activity (%)	5'GMP binding (%)
Enzyme	100	100
Enzyme + EDC (15 mM)	30	100
Enzyme + ssDNA (1mg) + EDC	35	-
Enzyme + RNA (1 mg) + EDC	35	-
Enzyme + 3'AMP (5 mM) + EDC	30	-
Enzyme + 5'GMP (5 mM) + EDC	30	-

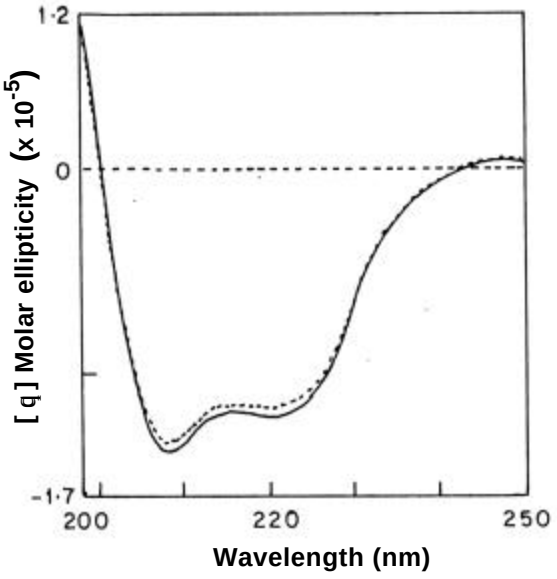

Fig. 5.4: The CD spectra of native and EDC modified nuclease Bh1.

The CD measurements were performed in a 1 mm cell at an enzyme concentration of 150 µg / ml. Native enzyme (—), EDC treated enzyme (------).

Histidine has been implicated in the catalytic activity of S1 nuclease (Gite *et al*., 1992b), nucleases from *S. marcescens* (Kolmes *et al*., 1996), *Anabaena* sp. (Meiss *et al*., 2000) and shrimp hepatopancreas (Wang *et al*., 2000), with the exception of a ribonuclease from the extremophile archaebacterium *Sulfolobus acidocaldarius* (Kulms *et al*., 1995). However, in case of *Sulfolobus acidocaldarius*, the amino acid composition revealed the absence of histidine. In the present case, though amino acid analysis revealed the presence of five histidines (Chapter 2), chemical modification studies showed that histidine does not have any role in the catalytic activity of nuclease Bh1. This is probably the first report of a single-strand-specific fungal nuclease where histidine is not involved in the catalytic activity of the enzyme.

As mentioned earlier, nuclease Bh1 catalyzes the hydrolysis of ssDNA, RNA and 3'AMP (Chapter 2). The ability of ssDNA and RNA to competitively inhibit the hydrolysis of 3'AMP (Chapter 4) suggests the existence of a common catalytic site for the hydrolysis of both types of substrates. In the present studies, parallel loss of all three activities of nuclease Bh1 on lysine and carboxylate modification (Fig.5.1, 5.3) coupled with substrate and inhibitor protection data (Table 5.1, 5.2), confirm the presence of a common catalytic site responsible for the hydrolysis of both monomeric and polymeric substrates.

Conclusion

Chemical modification studies, on nuclease Bh1, revealed that the catalytic site of the enzyme consists of a substrate binding site and a hydrolytic site. While lysine is involved in substrate binding, carboxylate is involved in catalysis. Moreover, the active site nature of nuclease Bh1 is atypical because it does not show the involvement of histidine in the catalytic activity of the enzyme. Furthermore, all the activities associated with nuclease Bh1 are catalyzed by the same active site.