S1 NUCLEASE : AFFINITY PURIFICATION, ACTIVE SITE CHARACTERIZATION AND IMMOBILIZATION

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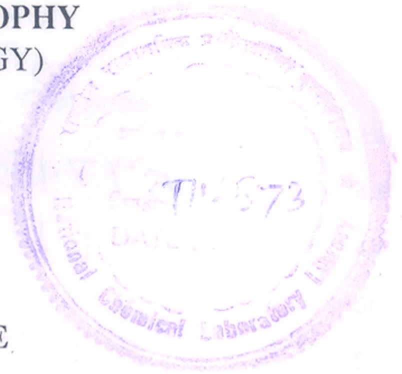

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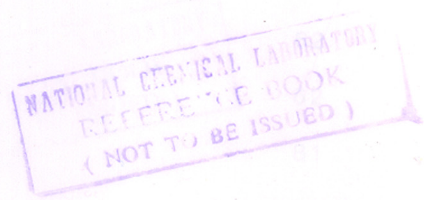

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DECLARATION

Certified that the work incorporated in the thesis entitled *"S1 nuclease : Affinity purification, active site characterization and immobilization"* submitted by Mr. Sadanand Gite was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. V. Shankar)

Research Guide

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(Sadanand Gite)

SUMMARY

Single strand specific nucleases, which act on single stranded nucleic acids and single stranded regions in double stranded nucleic acids, are multifunctional enzymes and have been isolated from microorganisms, plants and animals. Due to their high specificity and selectivity, they have found extensive application as analytical tools in molecular biology research. Though more than thirty single strand specific nucleases have been identified from various sources, only a few, namely S1 nuclease from *Aspergillus oryzae*, P1 nuclease from *Penicillium citrinum*, *Neurospora crassa* nuclease, mung bean nuclease and BAL 31 nuclease from *Alteromonas espejiana* have been sufficiently characterized. Amongst them S1 nuclease is preferred, since it can be easily prepared in large quantities from commercially available Takadiastase powder, is stable to low concentrations of denaturants often used in annealing experiments and is highly specific for single stranded nucleic acids under the right conditions.

S1 nuclease (EC 3.1.30.1) from *A. oryzae* is a sugar non-specific multifunctional enzyme, which acts on single stranded DNA (ssDNA), RNA and 3' AMP. Although, this enzyme has been purified and extensively used as an analytical tool for the characterization of nucleic acid structure, very little information is available regarding its molecular enzymology. Through competitive inhibition studies, it has

been demonstrated that different activities associated with S1 nuclease are catalyzed by the same active site but no information exists till now regarding the active site nature of the enzyme. Secondly, it has been observed that, after the nuclease treatment of nucleic acid samples, removal of residual enzyme activity from the reaction mixture is often essential and involves tedious emulsion forming phenol extraction steps, resulting in the loss of nucleic acid samples. In such cases, use of immobilized enzyme offers a distinct advantage over the soluble enzyme as it can easily be removed from the reaction mixture by physical methods. Hence, the present investigation was carried out to (a) determine the nature of the active site of S1 nuclease and (b) develop a simple immobilization procedure to obtain a highly active and stable immobilized preparation suitable for analytical purposes.

Chapter 1 : General introduction

This part comprises of literature survey with reference to single strand specific nucleases, their properties, applications and immobilization.

Chapter 2 : Affinity purification and active site characterization of S1 nuclease

A simple procedure, involving heat treatment, DEAE-Sephadex A-50, AMP-Sepharose and Bio-Gel P-60 chromatography, was developed for the purification of S1 nuclease to homogeneity, from commercially available Takadiastase pow-

der, with an overall yield of 27%. Chemical modification studies on purified S1 nuclease revealed 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, could protect the enzyme against 2, 4, 6 - trinitrobenzenesulphonic acid (TNBS) mediated inactivation whereas, this was not observed in case of either methylene blue or diethylpyrocarbonate (DEP) mediated inactivation of the enzyme. Moreover, the lysine (TNBS)-modified enzyme, having very little catalytic activity, showed a significant decrease (70%) in its ability to bind 5' AMP, a competitive inhibitor of S1 nuclease, while the histidine (DEP)-modified enzyme (having very little catalytic activity), 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 ssDNAase, RNAase and phosphomonoesterase activities associated with S1 nuclease, indicating the existence of a common catalytic site responsible for the hydrolysis of both monomeric and polymeric substrates. Modification of carboxylate groups, on the other hand, either by 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide or Woodward's Reagent K and EDTA treatment of S1 nuclease revealed that the loss of catalytic activity of the enzyme is due to the removal of zinc atoms and carboxylate groups are involved in metal binding. The data obtained with carboxylate groups modification, EDTA treatment, reconstitution with metal ions, zinc estimation

and circular dichroism analysis of the enzyme suggested that, out of the three zinc atoms present in S1 nuclease, zinc I is easily replaceable and is probably involved in the catalytic activity while zinc II and zinc III are involved in maintaining the enzyme structure.

Chapter 3 : Immobilization of S1 nuclease.

S1 nuclease is an analytically important enzyme and is extensively used for the determination of nucleic acid structure. It is a glycoprotein and contains approximately 18% carbohydrate. Moreover, it has been shown that the carbohydrate moiety of the enzyme is not essential for its catalytic activity and stability. Hence, attempts were made to bind purified S1 nuclease, covalently, through its carbohydrate moiety to aminated Bio-Gel P-2, to assess its potential as a reusable analytical tool. Purified S1 nuclease, when coupled to amino-butyl Bio-Gel P-2 (AB-Bio-Gel P-2), retained 40-50% activity of the soluble enzyme. Optimization of coupling conditions showed that the most active immobilized preparations are obtained when 50-60 U of 1 mM periodate oxidized enzyme are reacted with 1 ml (packed volume) of AB-Bio-Gel P-2 at 4°C, in presence of 20% (v/v) ethylene glycol, for 15 h, under mild agitation. Immobilization did not change the pH and temperature optima of the enzyme, but it increased the temperature stability. Immobilization brought about approximately 2-fold increase in the K_m and a slight decrease in the V_{max}. On repeated use, the bound enzyme retained 60-65% of its initial activity after

six cycles. Immobilized S1 nuclease could be stored, in wet state, for more than 45 days without any apparent loss in its initial activity. Like the soluble enzyme, both AB-Bio-Gel- and Con A-Sepharose-bound S1 nuclease could effectively remove Bam HI generated single stranded tails in pUC 8 DNA, demonstrating that both the immobilized preparations can be used as reusable analytical tools.

Publications :

1. Gite, S., Reddy, G. and Shankar, V. (1992) Active-site characterization of S1 nuclease I. Affinity purification and influence of amino-group modification. *Biochem. J.*, **285**, 489-494.

2. Gite, S., Reddy, G. and Shankar, V. (1992) Active-site characterization of S1 nuclease II. Involvement of histidine in catalysis. *Biochem. J.*, **288**, 571-575.

3. Gite, S. and Shankar, V. (1992) Characterization of S1 nuclease : Involvement of carboxylate groups in metal binding. *Eur. J. Biochem.*, **210**, 437-441.

4. Gite, S. and Shankar, V. (1993) Preparation, properties and applications of *Aspergillus oryzae* S1 nuclease covalently bound to aminobutyl-Bio-Gel P-2 through its carbohydrate moiety. *Biotechnol. Appl. Biochem.* (In press).

Chapter I

General Introduction

SINGLE STRAND SPECIFIC NUCLEASES

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 are extensively used as analytical tools for the determination of nucleic acid structure (Shishido and Ando, 1985). Though more than thirty single strand specific nucleases from various sources have been isolated till now (Table 1.1), only a few enzymes, like S1 nuclease from *Aspergillus oryzae*, P1 nuclease from *Penicillium citrinum*, BAL 31 nuclease from *Alteromonas espejiana*, *Neurospora crassa* and mung bean nucleases, have been sufficiently characterized. These enzymes are mainly used as analytical tools and very little information is available regarding their chemical nature, structure-function relationship and biological role. However, past few years have witnessed a significant progress in the molecular enzymology of some of these enzymes. The present review gives a comprehensive account of single strand specific nucleases with respect to their purification, physico-chemical properties, biological role and applications.

Purification

Since single strand specific nucleases are primarily used as analytical tools, most of the purification procedures were aimed at obtaining enzyme preparations free from contaminating nuclease(s). These enzymes occur in a

Table 1.1 : Single strand specific nucleases

Enzyme	Source	Reference
P1 Nuclease	*Penicillium citrinum*	Kuninaka *et al.* (1961)
Mung bean nuclease	Mung bean sprouts	Sung and Laskowski (1962)
Potato nuclease	Potato tubers	Björk (1965)
N. crassa nuclease	*Neurospora crassa* mycelia	Linn and Lehman (1965a)
N. crassa nuclease	*Neurospora crassa* mitochondria	Linn and Lehman (1966)
S1 nuclease	*Aspergillus oryzae*	Ando (1966)
SK nuclease	Sheep kidney	Kasai and Grunberg-Manago (1967)
Carrot nuclease	Carrot	Harvey *et al.* (1967)
Yeast nuclease	*Saccharomyces* hybrid strain	Lee *et al.* (1968)
Wheat nuclease	Wheat seedlings	Hanson and Fairley (1969)
Actinomyces nuclease	*Actinomyces* sp.	Tatarskaya *et al.* (1970)
Avena leaf nuclease	Avena leaf tissues	Wyen *et al.* (1971)

Enzyme	Source	Reference
N. crassa nuclease	*Neurospora crassa* conidia	Rabin *et al.* (1972)
Chlamydomonas nuclease	*Chlamydomonas reinhardi*	Small and Sparks (1972)
U. maydis nuclease	*Ustilago maydis*	Holloman and Holliday (1973)
Tobacco nuclease	Tobacco leaves	Oleson *et al.* (1974)
Bacillus nuclease	*Bacillus subtilis*	Kanamori *et al.* (1974a)
BAL 31 nuclease	*Alteromonas espejiana*	Gray *et al.* (1975)
Physarum nuclease	*Physarum polycephalum*	Waterborg and Kuyper (1979)
Pea seed nuclease	Pea seedlings	Wani and Hadi (1979)
Rye germ nuclease	Rye germ nuclei	Przykorska and Szarkowski (1980)
Yeast nuclease	*Saccharomyces cerevisiae* mitochondria	von Tigerstrom (1982)
N. crassa nuclease	*Neurospora crassa* mitochondria and vacuoles	Chow and Fraser (1983)
Hen liver nuclease	Hen liver nuclei	Tanigawa and Shimoyama (1983)

Enzyme	Source	Reference
Barley nuclease	Germinating barley seeds	Yupsanis and Georgatsos (1983)
Mouse mitochondrial nuclease	Mouse mitochondria	Tomkinson and Linn (1986)
SC nuclease	*Schizophyllum commune*	Martin *et al.* (1986)
Barley nuclease	Barley seeds	Brown and Ho (1986)
Streptomyces nuclease	*Streptomyces tendae*	Engel and Ullah (1988)
Wheat nuclease	Wheat chloroplasts	Kuligowska *et al.* (1988)
SP nuclease	Spinach leaves	Doetsch *et al.* (1988)
Rye nuclease	Rye germ ribosomes	Siwecka *et al.* (1989)
Flammulina nuclease	*Flammulina velutipes*	Kurosawa *et al.* (1990)
Aspergillus nuclease	*Aspergillus nidulans*	Kao *et al.* (1990)
Penicillium nuclease	*Penicillium* sp.	Kazama *et al.* (1990)
Drosophila nuclease	*Drosophila melanogaster*	Shuai *et al.* (1992)

wide variety of sources, each containing a unique set of contaminating proteins and hence it would be naive to expect a general purification scheme for all the enzymes. Nevertheless, most of the purification procedures, irrespective of the source, involve steps like concentration of the crude extract by salt or solvent precipitation and heat treatment followed by conventional purification methods like ion-exchange chromatography and gel filtration. In most of the cases though ammonium sulfate, alcohol and/or acetone are used for the concentration of the crude extract, NaCl (Lee *et al.*, 1968) and polyethylene glycol (Watanabe and Kasai, 1978) have also been used.

Single strand specific nucleases are relatively thermostable enzymes and a brief exposure of the crude enzyme preparation to high temperature (60 - 70°C) has proved to be extremely beneficial, as it not only helps in inactivating the protease(s), if any, but also in the removal of heat-labile proteins. Though, ion exchangers like DEAE- and CM-cellulose are widely used for the purification of these enzymes, phosphocellulose has also been found useful in certain cases. For example, potato tuber nuclease, in spite of its net negative charge at pH 7.5, binds to phosphocellulose due to its affinity towards phosphate groups in phosphocellulose. In this manner, this support not only acts as a cation exchanger but also as an affinity matrix (Nomura *et al.*, 1971). 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 (Sutton, 1971). Moreover, rechromatography of the partially purified enzyme preparation on DEAE-cellulose, gave a S1 nuclease preparation free of double stranded (ds) DNAase activity (Hahn and Van Ness, 1976).

Hydroxyapatite has extensively been used for the purification of several enzymes including nucleases (Bernardi, 1973). This adsorbent has been successfully used for the purification of single strand specific nucleases from carrot (Harvey *et al.*, 1970), *Chlamydomonas* (Small and Sparks, 1972), *P. 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 in 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 Shimoyama, 1983) on ssDNA bound to 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, 1986) 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.

Modern purification techniques like HPLC and FPLC have also 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).

Catalytic properties

Assay procedure : Single strand specific nucleases are usually assayed by measuring the release of acid soluble nucleotides, at 260 nm, following the hydrolysis of either heat denatured DNA or RNA. Unit of the enzyme is defined either as µmoles of nucleotides liberated (Fujimoto *et al.*, 1974a) or as µg of DNA or RNA digested (Vogt, 1973).

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

Optimum pH : In general, the pH optima of single strand specific nucleases are in the range of 4.0-9.0 and most of the enzymes show the same optimum pH for the hydrolysis of both polymeric and monomeric substrates (Table 1.2). BAL 31 nuclease however shows an optimum pH of 8.0 for double stranded (ds) DNA hydrolysis and 8.8 for single stranded (ss) DNA hydrolysis (Gray *et al.*, 1981). In case of P1 nuclease, the optimum pH for phosphomonoesterase activity varies depending on the substrate used (Fujimoto *et al.*, 1974c). Potato tuber nuclease shows an optimum pH of 8.0 for phosphomonoesterase activity while nuclease activity is maximum between pH 6.5 - 7.5 (Nomura *et al.*, 1971). Similarly, in the case of tobacco nuclease, the pH optima for the hydrolysis of ssDNA or RNA is 5.2-6.0 but phosphomonoesterase activity is optimal at pH 7.0 (Oleson *et al.*,

Table 1.2 : Properties of single strand specific nucleases

Enzyme	Molecular weight (kDa)	Optimum pH	Optimum temperature (°C)	Metal ion requirement	References
S1 nuclease	32	4.0-4.3	-	Zn^{2+}	Vogt (1973), Shishido and Habuka (1986)
P1 nuclease	42	5.3	70	Zn^{2+}	Fujimoto *et al.* (1974b, 1975a)
Mung bean nuclease	39	5.0	-	Zn^{2+}	Kowalski *et al.* (1976)
N. crassa nucleases					
a. Mycelia	55	7.5-8.5	55	Mg^{2+}, Ca^{2+}, Fe^{2+}	Linn and Lehman (1965a), Fraser (1980)
b. Conidia	72	8.5	-	Mg^{2+}	Rabin *et al.* (1972)
c. Mitochondria	-	6.0-7.5	37-45	Mg^{2+}, Mn^{2+}, Co^{2+}	Linn and Lehman (1966)

Enzyme	Molecular weight (kDa)	Optimum pH	Optimum temperature (°C)	Metal ion requirement	References
Wheat nucleases					
a. Seedling	43	4.8-5.5	-	Zn^{2+}	Hanson and Fairley (1969)
b. Chloroplast	29	7.0-8.0	-	-	Kuligowska *et al.* (1988)
U. maydis nuclease	42	7.0-8.5	52	Mg^{2+}, Ca^{2+}	Holloman and Holliday (1973)
BAL 31 nuclease	85*	8.8	60	Ca^{2+}	Gray *et al.* (1981), Wei *et al.* (1983)
Physarum nuclease	32	8.0	-	Zn^{2+}	Waterborg and Kuyper (1979)
Pea seed nuclease	42	6.5-8.0	45	Ca^{2+}, Mg^{2+}	Wani and Hadi (1979), Naseem and Hadi (1987)

* Slow [S] form of BAL 31 nuclease

Enzyme	Molecular weight (kDa)	Optimum pH	Optimum temperature (°C)	Metal ion requirement	References
SP nuclease	43	7.0	50-65	Zn^{2+}	Strickland *et al.* (1991)
SK nuclease	52	7.0-7.5	-	Mg^{2+}	Kasai and Grunberg-Manago (1967), Watanabe and Kasai (1978)
Hen liver nuclease	43	9.0	-	Mg^{2+}	Tanigawa and Shimoyama (1983)
Schizophyllum nuclease	34	7.0	-	Zn^{2+}	Martin *et al.* (1986)

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1974). *S. tendae* nuclease, on the other hand, is active over a broad range of pH i. e. 4.5 - 10.5, when assayed with ssDNA (Engel and Ullah, 1988).

Optimum temperature and temperature stability : The temperature optima of majority of single strand specific nucleases have not been reported but in the case of some of the well characterized enzymes, it is in the range of 37°C - 70°C (Table 1.2, page 14). Increase in the temperature, from 47°C - 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°C and 60°C respectively, than at 35°C 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).

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 high thermal stability has been attributed to the presence of high amount of hydrophobic amino acids.

Stability to denaturants : S1 nuclease (Vogt, 1973), BAL 31 nuclease (Gray *et al.*, 1981) and barley nuclease (Yupsa-

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nis and Georgatsos, 1983) are stable to low concentration 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).

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) and barley (Yupsanis and Georgatsos, 1983) nucleases, are either metalloenzymes or metal requiring enzymes (Table 1.2, page 14). S1 (Shishido and Habuka, 1986), P1 (Fujimoto *et al.*, 1975a), mung bean (Kowalski *et al.*, 1976), *P. polycephalum* (Waterborg and Kuyper, 1979) nucleases and a nuclease from *Penicillium* sp. (Kazama *et al.*, 1990) are zinc metalloproteins, while the enzyme from *N. crassa* is a cobalt metalloprotein (Fraser, 1980). However, *S. commune* enzyme is either a zinc or cobalt metalloprotein (Martin *et al.*, 1986). In addition, 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 enzyme from *Chlamydomonas* needs Ca^{2+} for its optimal activity (Small and Sparks, 1972). *B. subtilis* nuclease is active on dsDNA, ssDNA and rRNA, in presence of Ca^{2+} but Ca^{2+} is required only for dsDNAase activity (Kanamori *et al.*, 1974a).

Some of these enzymes require more than one divalent cation for their optimal activity (Table 1.2, page 14). Like *N. crassa* (Linn, 1967) and *U. maydis* (Holloman and Holliday, 1973) nucleases, *A. nidulans* nuclease requires three divalent cations *viz* Mg^{2+}, Mn^{2+} and Zn^{2+} for its maximum activity (Kao *et al.*, 1990). *D. 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). Rye germ ribosome nuclease though does not show an absolute requirement for divalent cations, exogenous addition of Zn^{2+} brings about a sizable stimulation of its activity (Siwecka *et al.*, 1989). Pea seed nuclease is stimulated by Mg^{2+} or Ca^{2+} to some extent but it does not show an absolute requirement for the added divalent cations (Wani and Hadi, 1979). The activity of *N. crassa* nuclease on dsDNA is dependent on Mg^{2+} concentration but its activity on ssDNA is independent of Mg^{2+}, though it is stimulated to some extent (Fraser, 1980). Moreover, the pH optimum of *N. crassa* nuclease for the hydrolysis of DNA and RNA is dependent on Mg^{2+} concentration (Rabin *et al.*, 1972). Addition of 10 mM of either Mg^{2+}, Ca^{2+} or Fe^{2+} resulted in 2.5-fold stimulation of the ssDNAase activity of *N. crassa* enzyme but it also brought about approximately 40% inhibition of the RNAase activity. The selective inhibition of RNAase activity, in presence of the aforementioned 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). *U. maydis* nuclease does not require metal ions for its activity but addition of Ca^{2+} or Mg^{2+} significantly enhances the activity (Holloman and Holliday, 1973).

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 of 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 dsDNAase activity of *N. crassa* nuclease, it had only a marginal effect on the ssDNAase activity (Fraser, 1980). Similarly, in case of *D. melanogaster* nuclease, 30 mM NaCl inhibited 50% of the dsDNAase activity whereas it required 100 mM NaCl to bring about the same level of inhibition of the ssDNAase activity (Shuai *et al.*, 1992). The inhibition of dsDNAase activity, in presence of high salt concentration, was attributed to the stabilization of the AT rich regions in dsDNA (von Hippel and Felsenfeld, 1964; Wingert and von Hipple, 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, 1980). On the contrary, NaCl inhibited *P. poly-*

cephalum 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 but spinach nuclease requires 50 - 75 mM NaCl for its maximal activity (Strickland *et al.*, 1991). Action of rye germ nuclei enzyme, on PM2 DNA, showed that it is strongly dependent on salt concentration but 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, 1978), while the enzyme from pea seed showed 35% and 85% inhibition of ssDNAase and dsDNAase activities respectively, in presence of 200 mM NaCl (Wani and Hadi, 1979). It is interesting to note that *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).

Inhibitors : Since most of the single strand specific nucleases are either metalloenzymes or metal requiring enzymes, they are strongly inhibited by metal chelators like EDTA and citrate. While 8-hydroxyquinoline inhibited pea seed nuclease, EDTA had no effect (Wani and Hadi,

1979). Inorganic phosphate and pyrophosphate inhibited S1 nuclease (Oleson and Hoganson, 1981), while inorganic phosphate inhibited the enzymes from mouse mitochondria (Tomkinson and Linn, 1986), *N. crassa* (Linn and Lehman, 1965a), *P. polycephalum* (Waterborg and Kuyper, 1979) and *U. maydis* (Holloman and Holliday, 1973). Moreover, β-mercaptoethanol and ATP inhibited *U. maydis* nuclease (Holloman and Holliday, 1973). S1 nuclease (Oleson and Hoganson, 1981) is also inhibited by 5' ribo- and deoxyribonucleotides. Compared to monoribonucleotides, monodeoxyribonucleotides are more potent inhibitors. 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). Potato tuber (Nomura *et al.*, 1971) and *B. subtilis* (Kanamori *et al.*, 1974a) nucleases were inhibited by Mn^{2+}, Co^{2+} and Zn^{2+} and wheat chloroplast nuclease by Cu^{2+} (Kuligowska *et al.*, 1988).

In case of *N. crassa* nuclease, excess amounts of a heat stable, trypsin sensitive, 24 kDa protein (purified from mycelia) non-competitively inhibited the ssDNAase activity whereas the inhibition of dsDNAase activity was competitive. In addition, this protein completely inhibited the RNAase activity (Hatahet and Fraser, 1989).

Physical properties

Molecular weight and subunit structure : Molecular weights of single strand specific nucleases are in the range of

5.5 - 140 kDa but majority of them fall in the range of 29 - 85 kDa (Table 1.2, page 14). The enzymes from *F. velutipes* (Kurosawa *et al.*, 1990), carrot (Harvey *et al.*, 1970) and yeast (von Tigerstrom, 1982) are high molecular weight proteins with a molecular weight of 91, 100 and 140 kDa, respectively, whereas rye germ ribosome nuclease (Siwecka *et al.*, 1989) is a comparatively low molecular weight protein of 20 kDa. The interesting feature of germinating barley nuclease is its low molecular weight (5.5 kDa) nature (Yupsanis and Georgatsos, 1983). It is perhaps one of the smallest enzyme reported so far.

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 15kDa and 30 and 24 kDa, respectively. 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, 57 kDa and 37.4 kDa, respectively. Mung bean nuclease showed only one band (corresponding to a molecular weight of 39 kDa), in SDS-polyacrylamide gels, in the absence of β-mercaptoethanol but in its presence the enzyme resolved into three components, corresponding to molecular weights 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).

Isoelectric point : Isoelectric focussing studies on single strand specific nucleases revealed that the pI of these enzymes show a large variation and 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 ± 0.3 (Strickland *et al.*, 1991). However, the enzyme from hen liver nuclei is a highly basic protein with a pI of 10.2 ± 0.2 (Tanigawa and Shimoyama, 1983). Isoelectric focussing studies on crude S1 nuclease showed a single band corresponding to a pI of 4.3 (Rushizky *et al.*, 1975). However, a highly purified enzyme preparation resolved into one major and two minor forms of the enzyme corresponding to a pI of 3.67, 3.35 and 3.53, respectively (Oleson and Sasakuma, 1980). The formation of multiple forms of the enzyme was attributed to the partial degradation of the enzyme during its purification from commercial Takadiastase powder or due to heating at 70°C, during the purification step (Rushizky, 1981).

Glycoprotein nature : Some of the well studied single strand specific nucleases like P1 nuclease (Fujimoto *et al.*, 1975a), S1 nuclease (Oleson and Sasakuma, 1980), mung

bean nuclease (Laskowski, 1980), pea seed nuclease (Naseem and Hadi, 1987), barley seed nuclease (Brown and Ho, 1987), rye germ ribosome nuclease (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%. 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).

Structure and function

Structure : Primary structure of two single strand specific nucleases have recently been determined. S1 nuclease consists of a single polypeptide chain of 267 amino acids crosslinked by two disulfide bonds. The disulfide (S-S) linkages probably occur between Cys 72 - Cys 216 and Cys 80 - Cys 85. The N-terminal residue is tryptophan while C-terminal is serine. It is a high mannose-containing glycoprotein and the sugar moieties are attached to asparagine 92 and 228 (Iwamatsu *et al.*, 1991). P1 nuclease is also a single polypeptide chain made up of 270 amino acids with two disulfide bonds *viz* Cys 72 - Cys 217 and Cys 80 - Cys 85. The N-terminal amino acid is tryptophan and the

C-terminal is lysine. However, P1 nuclease contains four carbohydrate moieties and they are linked to asparagine 92, 138, 184 and 197. S1 and P1 nucleases show approximately 50 - 59% sequence homology (Maekawa *et al.*, 1991). Moreover, comparison of the N-terminal sequence of barley nuclease with the N-terminal sequences of S1 and P1 nucleases revealed a considerable degree of homology (Iwamatsu *et al.*, 1991). 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).

Based on circular dichroism (CD) and optical rotatory dispersion (ORD) studies, Fujimoto *et al.* (1975b) showed that P1 nuclease consists of 29-31% 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).

P1 nuclease was crystallized recently in three different space groups using ammonium sulfate or PEG 4000 as precipitants and its structure was studied at 4.5 A° resolution (Lahm *et al.*, 1990). Subsequently, Volbeda *et al.* (1991) solved its structure at 3.3 A° and refined the data at 2.8 A° resolution. The 3-dimensional structure of P1 nuclease showed the presence of 269 amino acid residues, 3 zinc atoms and 2 N-acetyl glucosamine residues. Though 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 (Fujimoto *et al.*, 1975b). It has been shown that dsDNA and dsRNA are resistant to P1 nuclease (Fujimoto *et al.*, 1974a). Based on the 3-dimensional structure, this property of the enzyme was correlated to the inaccessibility of the zinc atoms to the phosphate groups either in dsDNA or dsRNA since they are located at the bottom of the cleft. In order to locate the substrate binding site, the native enzyme crystals were soaked in a solution of thiophosphorylated dinucleotide (dA.P(S).dA, an uncleavable substrate analog). A difference Fourier calculated between the data collected from the native crystals and soaked crystals clearly showed the density corresponding to the dinucleotide in electron density map. The distance between the two mononucleotide binding sites was approximately 20 A°. One site showed the binding of the phosphate group to one of the zinc ions. At both the sites, there was a hydrophobic binding pocket for the nucleotide base. In addition, zinc ions were co-ordinated to the nitrogen of 2 histidine residues and to the oxygen of 2 aspartic acid residues. A remarkable feature of the structural model of P1 nuclease was the presence of two buried pairs of carboxylate groups at the edge of the substrate binding cleft.

A cleavage mechanism, based on the crystal structure, suggested the involvement of a nucleophilic attack by a zinc activated water molecule.

Active site : Very little information is available regarding the active site nature of this class of enzymes. Through competitive inhibition studies, it has been demonstrated that ssDNAase, RNAase 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. A similar observation was made by Reddy and Shankar (1989) while studying the immobilization of S1 nuclease on Con A-Sepharose. Pea seed nuclease, which has two subunits, it has been suggested that the phosphomonoesterase activity resides in one of the subunits but the nuclease activity requires both the subunits (Naseem and Hadi, 1987).

Role of metal ions : Though, some of the well studied single strand specific nucleases are metalloproteins, no detailed information is available regarding the role of metal ions, except in case of P1 nuclease. P1 nuclease is a zinc metalloprotein and contains 3 zinc atoms/molecule of the enzyme (Fujimoto *et al.*, 1975a). Rokugawa *et al.* (1980a) carried out a systematic investigation on the role of metal ions in P1 nuclease by selective removal of zinc from the enzyme by treating the enzyme with EDTA 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 50 % loss of its activity towards RNA but it retained 93% activity towards 3' AMP. While the removal of the second zinc atom brought about a significant decrease in the RNAase (45%) and phosphomonoesterase (60%) activities, the removal of all the 3 zinc atoms resulted in the complete inactivation of the enzyme and a complete disruption of the enzyme structure. Based on this data, the authors concluded that while Zn I is involved in maintaining the tertiary structure required for RNA binding, Zn II is essential for maintaining the active conformation and Zn III is involved in holding the structural integrity of the enzyme. Like P1 nuclease, S1 nuclease is also a zinc metalloprotein and contains 3 atoms of zinc/molecule of the enzyme. Preliminary studies on the role of metal ions in S1 nuclease also showed that the selective removal of zinc (by EDTA treatment) results in the loss of enzyme activity. Furthermore, CD analysis of the EDTA-treated enzyme sample revealed that the loss of activity is due to conformation changes of the enzyme as a result of the removal of zinc atoms (Shishido and Habuka, 1986).

Substrate specificity and mode of action

Single strand specific nucleases are sugar non-specific, multifunctional enzymes and exhibit both exo- and endo-nuclease activities (Table 1.3). They degrade single stranded nucleic acids to all four mononucleotides and a

Table 1.3 : Substrate specificity and mode of action of single strand specific nucleases

Enzyme	Substrates	Products	Mode of action	References
S1 nuclease	ssDNA, RNA	5'Mono nucleotides	Endo-exo	Ando (1966), Oleson and Sasakuma (1980)
P1 nuclease	ssDNA, RNA	5'Mono-nucleotides	Endo-exo	Fujimoto *et al.* (1974c, 1974d)
N. crassa nuclease (mycelia)	ssDNA, RNA	5'Mono-nucleotides	Endo-exo	Fraser (1980)
U. maydis nuclease	ssDNA, RNA	5'Mono-nucleotides	Endo-exo	Holloman and Holliday (1973)
Mung bean nuclease	ssDNA, RNA	5'Mono-nucleotides	Endo-exo	Laskowski (1980)
BAL 31 nuclease	ssDNA, RNA	5'Mono-nucleotides	Endo-exo	Gray *et al.* (1981)

Enzyme	Substrates	Products	Mode of action	References
Wheat seedling nuclease	ssDNA, RNA	5'Mono-nucleotides	Endo-exo	Kroeker and Fairley (1975)
Wheat chloroplast nuclease	ssDNA, RNA	5'Oligodeoxyribo- or 3'oligoribo-nucleotides	Endo	Kuligowska *et al.* (1988)
Spinach nuclease	ssDNA, RNA	5'Mono-nucleotides	Endo	Strickland *et al.* (1991)
SK nuclease	ssDNA, RNA	5'Oligo-nucleotides	Endo	Watanabe and Kasai (1978)
Yeast nuclease	ssDNA, RNA	5'Di and tri-nucleotides	Endo	Lee *et al.* (1968)
Rye germ ribosome nuclease	ssDNA, RNA	5'Monoribo- or 3'Monodeoxyribo-nucleotides	Endo	Siwecka *et al.* (1989)

small amount of oligonucleotides by the co-operative action of exo- and endo-nuclease activities. Moreover, S1 nuclease (Oleson and Sasakuma, 1980), P1 nuclease (Fujimoto *et al.*, 1974a), and nucleases from mung bean (Mikulski and Laskowski, 1970), pea seed (Naseem and Hadi, 1987), wheat seedlings (Kroeker *et al.*, 1976), rye germ (Przykorska and Szarkowski, 1980), potato tuber (Nomura *et al.*, 1971), tobacco (Oleson *et al.*, 1974), avena leaf (Wyen *et al.*, 1971) and barley seeds (Brown and Ho, 1987) exhibit 3'phosphomonoesterase activity.

Single strand specific nucleases act on ssDNA, RNA and 3' mononucleotides but the rate of hydrolysis of these substrates vary 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.*, 1974a) 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 following order: 3'AMP > RNA > ssDNA > dsDNA (Fujimoto *et al.*, 1974a), while that of tobacco nuclease is, ssDNA > 3'AMP > RNA > dsDNA (Oleson *et al.*, 1974). Wheat seedling nuclease (Hanson and Fairley, 1969), which acts on ssDNA, RNA and 3' AMP and yeast nuclease (Lee *et al.*, 1968) which acts only on ssDNA and RNA, show same rate of hydrolysis for all the substrates.

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. Similarly, 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 very slow rate (5%). Moreover, under similar conditions poly (rA) and poly (rG) were resistant to hydrolysis. On the other hand, 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 (Vogt, 1973). 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 was attributed to the lack of ordered secondary structure. 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). A similar behaviour was also observed in the case of rye germ nuclease (Przykorska and Szarkowski, 1980). Hen liver nuclease (which is poly ADP-ribose sensitive) showed maximum activity on ssDNA and the other substrates were degraded in the following order : ssDNA = rRNA > poly (dA) > poly (dT) > poly (dC) = poly (dG) > dsDNA (Tanigawa and

Shimoyama, 1983). In general, the susceptibility of various synthetic polymeric substrates to nuclease action is correlated to pH induced changes in the secondary structure of these substrates.

The rate of hydrolysis of mononucleotides by single strand specific nucleases also varies from source to source. Mung bean nuclease cleaves 3' ribonucleotides 50 - 100 fold faster than the corresponding deoxyribonucleotides (Kole *et al.*, 1974). Additionally, the enzyme shows a preference to bases in the order of A > T (U) > C > G (Mikulski and Laskowski, 1970). P1 nuclease also hydrolyzes 3'ribonucleotides 20-50 times faster than 3' deoxyribonucleotides and the base specificity falls in the order of G > A > C ≥ U and C ≥ T > A ≥ G for ribonucleotides and deoxyribonucleotides, respectively. In addition, P1 nuclease acts on nucleoside 3' 5' diphosphates but 2'AMP is highly resistant. It was also noted that, the rate of hydrolysis of various mononucleotides is pH dependent (Fujimoto *et al.*, 1974c). Like mung bean and P1 nucleases, S1 nuclease too prefers ribonucleotides to deoxyribonucleotides and it also acts on nucleoside 3'5' diphosphates. The substrate specificity of S1 nuclease is in the order of ribonucleoside 3'5' disphosphate > ribonucleoside 3' phosphate > deoxyribonucleoside 3'5' disphosphate > deoxyribonucleoside 3' phosphate ≈ ribonucleoside 2' phosphate (Oleson and Hoganson, 1981).

Though majority of single strand specific nucleases reported so far appear to be 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. *N. crassa* (Linn and Lehman, 1965b) and *U. maydis* (Holloman, 1973) nucleases prefer guanosine or deoxyguanosine residues in a polynucleotide chain whereas P1 (Fujimoto *et al.*, 1974d), spinach (Doetsch *et al.*, 1989) and avena leaf (Wyen *et al.*, 1971) nucleases preferentially attack the linkages between 3' hydroxyl group of adenosine or deoxyadenosine and 5' phosphate group of the adjacent nucleotide. Cytidine or deoxycytidine is resistant to avena leaf nuclease (Wyen *et al.*, 1971). Mung bean nuclease, on the other hand, shows preference for AT rich regions in DNA (Johnson and Laskowski, 1970).

In general, single strand specific nucleases show exo- and endo-nucleolytic activities on both ssDNA and RNA (Table 1.3). Wheat seedling nuclease exhibits endonuclease activity toward ssDNA and exonuclease activity towards RNA (Kroeker *et al.*, 1975) whereas BAL 31 nuclease, apart from showing an endonuclease activity on single stranded nucleic acids, shortens the duplex DNA molecules from both the ends (Gray *et al.*, 1981). Yeast nuclease hydrolyzes ssDNA and RNA endonucleolytically and gives predominantly di- and tri-nucleotides as the reaction products. However, very small amount of mononucleotides are also formed. The

shorter chains of nucleic acids are hydrolyzed slowly than the longer chains while double- and triple-stranded structures are less susceptible (Lee *et al.*, 1968). *B. subtilis* nuclease is probably the only enzyme which shows exonucleolytic activity and degrades DNA from the 5' end liberating 3' mononucleotides (Kanamori *et al.*, 1974a).

Interestingly, *N. crassa* produces four major nucleases, exhibiting markedly different properties and modes of action. These enzymes are reported to be formed from a single precursor polypeptide of 90 kDa *via* differential routes of proteolysis. First is a 75 kDa protein having single strand specific, Mg^{2+} dependent exonuclease activity, identical to the one formed in conidia but not in mycelia. This enzyme shows 5'$\longrightarrow$ 3' exonuclease activity in presence of Mg^{2+} but endonuclease action in the absence of Mg^{2+}. Second is a 65 kDa endo-exonuclease, exhibiting endonuclease activity towards ssDNA and exonuclease activity towards dsDNA. Third, a 55 kDa proteolytic product, a single strand specific endonuclease, identical to the enzyme isolated from mycelia. Fourth, a 65 kDa Ca^{2+} dependent endonuclease, which cleaves both ssDNA and dsDNA but has no dsRNAase activity (Fraser, 1980). Furthermore, the enzyme isolated from *N. crassa* mitochondria showed distributive endonuclease activity towards ssDNA but processive exonuclease activity towards dsDNA (Chow and Fraser, 1983).

Single strand specific nucleases are conformation specific enzymes and act on wide variety of DNAs. S1 nuclease could convert supercoiled DNAs (form I) from polyoma virus (Germond *et al.*, 1974), simian virus (SV) 40 (Waldeck *et al.*, 1976) and mitochondria of African Green Monkey cells [VERO ATCC; CCL 81] (Koch and Bruhn, 1976) into full length linear duplex DNAs (form III). Similarly, BAL 31 (Gray *et al.*, 1975) and hen liver (Tanigawa and Shimoyama, 1983) nucleases could convert PM2 DNA (form I) to linear duplex DNA (form III), while the enzymes from *N. crassa* (Kato *et al.*, 1973), *S. tendae* (Engel and Ullah, 1988) and spinach (Doetsch *et al.*, 1989) converted supercoiled DNAs from øX 174 and pUC 19 plasmid to linear duplex DNAs. The conversion of superhelical DNA (form I) to linear duplex (form III) was shown to proceed *via* the formation of a nicked circular DNA (form II) (Méchali *et al.*, 1973; Chowdhury *et al.*, 1975; Chowdhury and Sauer, 1976). In case of BAL 31 nuclease, the conversion of PM2 DNA form I to form III depended on its superhelical density (Lau and Gray, 1979). The conversion of form I DNA to form III DNA, at least in case of S1 nuclease, was found to be independent of salt concentration (Waldeck *et al.*, 1976). Moreover, the presence of Mn^{2+} in the reaction mixture led to an increase in the specificity of S1 nuclease towards SV 40 DNA (form I) than the reaction in presence of Zn^{2+}, though both the metal ions were equally effective with ssDNA (Shishido, 1979).

S1 nuclease not only digests ssDNA but also introduces single stranded nicks in dsDNA (Godson, 1973). Covalently closed circular DNA molecules, which are not superhelical and linear duplex DNAs, are relatively resistant (Beard *et al.*, 1973). Double stranded DNA molecules are attacked only if they are either nicked or supercoiled (Germond *et al.*, 1974). S1 nuclease exhibits low activity on ds øX174 RF DNA and native calf thymus DNA. The action of this enzyme on dsDNA was attributed either to an accumulation of single stranded nicks in dsDNA or to the local melting of dsDNA (John *et al.*, 1974). Subsequently, Mykoniatis (1982) demonstrated that some local alterations in the dsDNA makes it susceptible to S1 nuclease action. S1 nuclease does not create nicks in native T5 DNA under the conditions where ssT5 DNA is completely hydrolyzed. However, incubation of native T5 DNA with high concentrations of the enzyme resulted in a few nicks (Shishido and Ando, 1975a). S1 nuclease can cleave small stretches (12 bases) in the single stranded region of λ DNA (Cos ends), in addition to heteroduplex loops (Wiegand *et al.*, 1975). In addition, S1 nuclease can cleave ssDNA even in presence of 2.5-10 % formamide, 1-5% formaldehyde, 10-25 mM glyoxal, 30% dimethylsulphoxide and 30% dimethylformamide. The susceptibility of the substrates, in presence of these reagents, was correlated to the destabilization of DNA structure (Case and Baker, 1975; Hutton and Wetmur, 1975).

Mung bean nuclease is highly specific for ssDNA and the ratio of its ssDNAase : dsDNAase activity is about 30,000 : 1, at 37°C in the absence of Mg^{2+}, for T4 DNA but it varies with the nature of the DNA used. For example, the ratio of ssDNAase : dsDNAase activity is only 2 : 1 for (dA.dT)n, suggesting that AT rich regions are more susceptible to hydrolysis. Moreover, under the above conditions, the enzyme did not hydrolyze poly (dG). poly (dC), even with 100 - fold excess enzyme (Johnson and Laskowski, 1970). However, it could create a limited number of cleavages in the interior of native T7 DNA (Kroeker *et al.*, 1976). Wheat seedling nuclease, on the other hand, hydrolyzed intact high molecular weight native DNA from *Escherichia coli* to limit polymers which were resistant to further hydrolysis. Like mung bean nuclease, this enzyme too showed a preference for AT rich regions in DNA (Kroeker and Fairley, 1975).

Limited digestion of øX 174 ssDNA with sheep kidney nuclease gave five fragments, suggesting that the øX 174 ssDNA might have some intermolecular or intramolecular double stranded regions, which are not attacked by the enzyme (Watanabe, 1978a). However, the hydrolysis of tRNA showed that this enzyme attacks all the chains and produces mainly large fragments (≈ 40 bp) containing pGp terminal, TpψpC sequences and a small amount of oligonucleotides (≈ 4-5 bp) containing large amounts of CpCpA (Kasai and Grunberg-Manago, 1967).

BAL 31 nuclease (both slow [S] and fast [F] forms) degrades ssDNA exonucleolytically while dsDNA degradation occurs in a terminally directed manner where, the removal of nucleotides takes place from both the ends of dsDNA. The ratio of the turnover number for the exonuclease activity of the [F] species, to shorten the linear duplex DNAs, is approximately 27 ± 5 times higher as compared to [S] species. Apart from terminally directed exonuclease activity, some endonuclease activity was also found to be associated with this enzyme against 5'-terminated single stranded tails generated by exonuclease action (Wei *et al.*, 1983; Zhou and Gray, 1990).

The thermostable single strand specific nuclease from *N. crassa*, which does not bind strongly to phosphocellulose, was found to be associated with an equally thermostable exonuclease capable of acting on linear dsDNA but not on circular or superhelical DNA (Fraser *et al.*, 1976). *F. velutipes* nuclease hydrolyzes polynucleotides exonucleolytically, from 5' end, yielding 5' mononucleotides. In addition, it cleaves ss- and ds-circular M13mp18 DNAs, endonucleolytically, to give ss- and ds-linear DNAs respectively (Sen *et al.*, 1991).

Action of *N. crassa* endonuclease, on UV-irradiated DNA, showed that it cleaves in the region containing pyrimidine dimers (Kato and Fraser, 1973). However, action of S1 nuclease on UV-irradiated DNA revealed that, it is

directly proportional to the dose of UV-light, apart from enzyme concentration and ionic strength of the reaction mixture (Shishido and Ando, 1974). Spinach nuclease, too, shows relatively high activity on UV-irradiated DNA compared to that on native DNA (Doetsch *et al.*, 1989). Action of S1 nuclease on substrates like platinum- and N-acetoxy-N-2-acetylaminoflurone (AAAF)-modified DNAs revealed that the susceptibility depends not only on cis- or trans-modification but also on the G + C content (Fuchs, 1975; Scovell and Capponi, 1984). Platinum-modified DNA was also susceptible to spinach nuclease (Doetsch *et al.*, 1988). Treatment of superhelical DNA with drugs like netropsin, which interacts with AT rich regions in DNAs, showed increased susceptibility to S1 nuclease action whereas intercalating drugs inhibited the enzyme action (Shishido *et al.*, 1984).

Heteroduplex DNAs with a single base mismatch were highly resistant to S1 nuclease action while heteroduplex DNAs with two base mismatches were susceptible (Silber and Loeb, 1981; Burdon and Lees, 1985). On the other hand, purine-pyrimidine mismatch, which produces a very minor distortion in the DNA structure, was susceptible to *U. maydis* nuclease (Ahmad *et al.*,1975).

P1 nuclease (Fujimoto *et al.*, 1974d) and potato tuber nuclease (Suno *et al.*, 1973), which hydrolyze single stranded nucleic acids to 5' mononucleotides endonucleolytically, recognize a nucleoside 3' phosphate moiety in

the polynucleotide chain as the substrate and cleave the bond between 3' hydroxyl and 5' phosphate. Kinetic analysis of the hydrolysis of natural substrates and a series of mono- and di-nucleoside phosphates indicated that, S1 nuclease has no specificity towards the nature of the heterocyclic base, the sugar moiety or the base sequence during the enzyme-substrate [ES] complexation. However, significant differences in the conversion of [ES] complexes to reaction products, with different substrates, suggested that the substrate's non-reactive parts may affect the productive [ES] complex structure and conformation. Two or more than two nucleotides are required for the productive [ES] complex formation. The minimum requirement of the substrate for S1 nuclease is a nucleotide, where all the groups *viz* the base, sugar and phosphate take part in binding of substrate to the enzyme (Karpeiskii *et al.*, 1982). Stereochemical analysis of the reaction mechanism of S1 nuclease showed that it proceeds *via* the inversion of configuration of the oxygen atom at phosphorus position (Potter *et al.*, 1983). Using cloned $(dG.dA)_n$ and $(dC.dT)_n$ duplexes, as model substrates, it was demonstrated that the cleavage of alternate (non B - non Z) DNA structure by S1 nuclease is length dependent, in both supercoiled and linear forms (Evans and Efstratiadis, 1986). In case of S1 nuclease reaction, it has also been shown that the substrate binds to the enzyme through 3' base, electrostatically, with a charged amino group of lysine and the

enzyme bound zinc hydrolyzes the anhydride specifically at the carboxylate groups (Witzel *et al.*, 1988).

Biological role

It is believed that, the repair of base mismatches in the heteroduplex region of DNA may have a role in gene conversion in fungi (Radding, 1978). In case of *N. crassa*, compared to wild strain, the repair deficient and UV-sensitive mutants *viz* uvs-2, uvs-3, uvs-6 and nuh-4, could not secrete endo-exonucleases (Chow and Fraser, 1979; Fraser, 1979). In addition, these mutants had a higher level of endo-exonuclease precursor, than the wild type, suggesting that these mutants may have some defect either in the protease(s) that controls 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 single strand specific nucleases in DNA recombination and repair. Similarly, UV-sensitive and recombination/repair deficient mutants of *U. maydis* also showed reduced levels of single strand specific nucleases, suggesting that these enzymes have some important role in recombination in this organism (Holloman and Holliday, 1973). Furthermore, the ability of S1, P1 and mung bean nucleases to recognize minor distortions in duplex DNA, as a result of UV-irradiation, apurinization and mutagenesis, implies that they may have a role in DNA repair (Shishido and Ando, 1985).

Applications

Single strand specific nucleases, by their ability to selectively hydrolyze single stranded nucleic acids and single stranded regions in double stranded nucleic acids, are extensively used as analytical tools in nucleic acid hybridization, nucleic acid structure, isolation of specific genes and gene manipulation. Some of the important applications of single strand specific nucleases are summarized in Table 1.4. Moreover, the combination of various single strand specific nucleases have also been used in many cases. Schaller *et al.* (1969) isolated GC core of phage fd DNA by successive digestion with *N. crassa* nuclease and *E. coli* exonuclease. While S1 nuclease and BAL 31 nuclease were used to obtain linear duplex DNA from negatively supercoiled DNAs (Beard *et al.*, 1973; Godson, 1973; Méchali *et al.*, 1973; Germond *et al.*, 1974; Shishido and Ando, 1975b; Lau and Gray, 1979), *N. crassa* and mung bean nucleases were employed to obtain nicked circular DNA molecules from supercoiled DNAs (Kato *et al.*, 1973; Wang, 1974). P1 nuclease along with mung bean nuclease, was used for studying the susceptibility of DNA in chromatin (Fujimoto *et al.*, 1979). Rye germ and wheat chloroplast nucleases were utilized to investigate the secondary structure of small RNA, namely Cop A RNA (Przykorska *et al.*, 1989).

Table 1.4 : Application of single strand specific nucleases

Enzyme	Applications	References
S1 nuclease	Isolation and estimation of double helical content of single stranded nucleic acids	Shishido and Ikeda (1971a), Shishido and Ando (1972)
	Isolation and characterization of hairpin structures and invert loops	Shishido and Ikeda (1971b), Ohtsubo and Ohtsubo (1979)
	Specific fragmentation of phage T5 and T7 DNAs	Shishido and Ando (1975a), Pavlov *et al.* (1977)
	Structural analysis of tRNAs and rRNAs	Harada and Dahlberg (1975), Khan and Maden (1976), Wrede *et al.* (1979), Baumann *et al.* (1985)
	Determination of structure of spliced RNAs and mapping spliced points in RNAs	Berk and Sharp (1978), Wrust *et al.* (1978), Favaloro *et al.* (1980), Berk (1989), Rahman and Schaup (1990)
	Removal of single stranded tails prior to T4 DNA ligase reaction	Shishido and Ando (1981)

Enzyme	Applications	References
S1 nuclease	Isolation of inserts from plasmid DNA	Hofstetter *et al.* (1976)
	Physical mapping of mutations	Shenk *et al.* (1975)
	Construction and mapping of targeted deletion mutants	Green and Tibbetts (1980), Pangyotatos and Truong (1981)
	Removal of protein attached to the DNA	Ariga *et al.* (1979)
	Screening of DNA-binding proteins and substances	Meyer *et al.* (1980), Shishido *et al.* (1980)
	Study of chemical properties of DNA-membrane attachment	Abe *et al.* (1977)
	Specific amplification of deleted mitochondrial DNA of myopathic patients	Tanaka-Yamamoto *et al.* (1989)
	Characterization of genes for antibiotic resistance	Laufs and Fock (1979)
	Degradation of nucleic acids in cell lysate	Zechel and Weber (1977)
	Study of homology of various DNA molecules	Crosa *et al.* (1973), Barth and Grinter (1975)

Enzyme	Applications	References
S1 nuclease	Isolation and purification of sea urchin rRNA genes	Joseph and Stafford (1976)
	Enzymatic synthesis of globin genes *in vitro*	Efstratiadis *et al.* (1976)
	Measurement of DNA crosslinks	Ben-Hur *et al.* (1979), Sriram and Ali-Osman (1990)
	Removal of pyridine dimers from UV-irradiated DNA	Heflich *et al.* (1979)
	Cleavage of COS end in λ DNA	Ghangas and Wu (1975)
	Study of palindromic sequences in DNA	Wilson and Thomas (1974)
	Study of DNA intercalating and non-intercalating agents	Alvi *et al.* (1985)
P1 nuclease	Industrial production of 5'nucleotides	Kuninaka *et al.* (1961)
	Isolation of eukaryotic mRNA cap structure	Furuichi *et al.* (1975)
	Base composition analysis of nucleic acids	Katayama-Fujimura *et al.* (1984)

Enzyme	Applications	References
P1 nuclease	Preparation of nucleoside triphosphate mixtures	Haynie and Whitesides (1990)
	Removal of nucleic acids during protein purification	Zabriskie and DiPaola (1988)
	Estimation of nucleotide content of vegetables	Nguyen *et al.* (1988)
	Sequence analysis of end-group labeled RNA	Silberklang *et al.* (1977)
	Post-labeling of AAAF-modified DNA	Sharma and Jain (1991)
	Analysis of tRNA structure	Aultman and Chang (1982)
SK nuclease	Preparation of dideoxynucleotides	Watanabe (1978b)
N. crassa nuclease	Isolation of pure Lac operon	Shapiro *et al.* (1969)
	Isolation of tRNA and rRNA gene hybrids	Marks and Spencer (1970), Joseph and Stafford (1976)
	Detection of sequence heterology	Bartok *et al.* (1974)

Enzyme	Applications	References
Mung bean nuclease	Conformation analysis of hairpin loops	Baumann *et al.* (1986, 1987)
	Probing of low melting regions in DNA	Kedzierski *et al.* (1973)
BAL 31 nuclease	Ordering restriction endonuclease generated DNA fragments	Legersky *et al.* (1978)
	Study of legions in DNA helix produced by carcinogens	Legersky *et al.* (1977)
B. Subtilis nuclease	5' End group reagents	Kanamori *et al.* (1974b)
Wheat Seedling nuclease	Production of large DNA fragments from intact DNA	Kroeker and Fairley (1975)

Immobilization of single strand specific nucleases

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 systems as immobilization offers important advantages like, reusability, enhanced stability, greater control of the catalytic process and the development of continuous process. Single strand specific nucleases are important analytical enzymes and are widely used for studying nucleic acid structure (Shishido and Ando, 1985). Additionally, S1 and P1 nucleases are used for the industrial production of 5' mononucleotides (Shishido and Ando, 1985). Due to the extensive applications of single strand specific nucleases, several attempts have been made to obtain highly active and stable immobilized preparations suitable for various biotechnological applications.

Analytical : One of the most important applications of immobilized enzyme technology is its use as probes in analytical systems. High sensitivity and specificity of enzymes make them excellent analytical tools and because of this property, several enzymes have been immobilized for their potential application in routine biochemical and

clinical analysis (Guilbault, 1982; Sundaram, 1982).

Reddy and Shankar (1987) bound crude S1 nuclease to gelatin-alginate composite matrix, using the free aldehyde groups on the surface of 1 % (v/v) glutaraldehyde cross-linked matrix and the immobilized preparation retained approximately 10% activity of the soluble enzyme. On the other hand, partially purified S1 nuclease (free from contaminants like RNAase T1, RNAase T2 and double strand specific nuclease), when coupled to gelatin-alginate matrix under identical conditions, did not show any detectable activity. However, the activity could be restored by coupling the enzyme in presence of a co-protein (BSA) or the substrate (ssDNA). With increase in glutaraldehyde concentration used for crosslinking the matrix, the efficiency of the bound enzyme decreased suggesting that the loss of activity of the enzyme, in the absence of co-protein or substrate, was caused by the conformational changes as a result of multiple attachment of the enzyme to the matrix. The restoration of the activity in presence of BSA was correlated to its ability to compete with S1 nuclease for free aldehyde groups on the matrix, thus preventing the multiple attachment of the enzyme to the matrix. This conclusion was supported by the results on the influence of BSA concentration on the efficiency of the immobilized preparation where, it was noted that initially the efficiency increased with increase in the BSA concentration and after attaining a maximum, it decreased. The retention of

activity in the presence of substrate however was attributed to the protection of catalytically active lysine residues (Reddy, 1989). Immobilization resulted in a decrease in the optimum pH, K_m and V_{max}. The bound enzyme showed poor stability to repeated use and lost significant amount of its initial activity (>75%) after 6 cycles. However, the bound enzyme showed high temperature optimum and stability suggesting that it can be used for studying DNA structure at elevated temperatures (Reddy and Shankar, 1987).

S1 nuclease is a glycoprotein and contains approximately 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, to overcome the low activity associated with the aforementioned system, attempts were made to bind S1 nuclease through its carbohydrate moiety. When the partially purified enzyme was bound to Con A-Sepharose, the retention of activity was high, varying essentially from 75% on low lectin matrix (1 mg Con A/ml Sepharose) to no detectable activity on high lectin matrix (8 mg Con A/ml Sepharose). However, approximately 50% activity could be restored on high lectin matrix, when the coupling was carried out in presence of glucose, suggesting that the loss of activity is probably due to the multiple attachment of the enzyme to the matrix. Though the carbohydrate moiety is not required for the activity of S1 nuclease, the

sensitivity of the partially purified enzyme to the concentration of matrix bound lectin coupled with the inhibitory effect of Con A on the soluble enzyme suggested that the loss of activity, observed in case of the enzyme bound to high lectin matrix, is due to the masking of its active site and the carbohydrate moiety is situated near the active site of the enzyme. Moreover, the parallel loss of all the three activities of the enzyme bound to matrices containing increasing concentrations of lectin suggested the involvement of a common catalytic site for the hydrolysis of both monomeric and polymeric substrates. Immobilization did not affect pH optimum but it increased the temperature optimum. A marginal increase in the K_m of the bound enzyme alongwith its high efficiency indicated the absence of significant diffusional limitations. The Con A-Sepharose-S1 nuclease conjugate showed enhanced stability to pH, temperature and 8 M urea. The bound enzyme also showed high stability to repeated use and retained more than 80% of its initial activity after 6 cycles (Reddy and Shankar, 1989).

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'-mono-

phosphate, 2-ethylinosine-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). In view of the high demand for 5' nucleotides in food and beverage industries, several attempts have been made to immobilize 5' nucleotide producing enzymes, to assess their potential, for the commercial production of mononucleotides. Though 5' nucleotide producing nucleases are widely distributed (Lehman, 1981), the enzyme of commercial interest originates from *P. citrinum*. As mentioned earlier, P1 nuclease from *P. citrinum*, is a multifunctional enzyme which acts on both RNA and DNA, liberating 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 mononucleotides. It was observed that the immobilized system could simultaneously degrade and fractionate nucleic acids. Rokugawa *et al.* (1979) bound crude P1 nuclease (P1 nuclease - malonogalactan complex) to ion-exchange celluloses by cyanogen bromide, carbodiimide and titanium chloride activation methods and the immobilized conjugates exhibited 3%, 28% and 23.5% activity respectively, of the soluble enzyme. However, when the pure enzyme (devoid of malonogalactan) was bound to cellulose by these methods, titanium

chloride-cellulose-P1 nuclease conjugate showed the maximum (83%) efficiency. The low efficiency, observed in the case of crude enzyme, was correlated to the probable interference of the malonogalactan during immobilization. However, the comparatively high efficiency (26-28%) of the crude enzyme bound through the carboxylate groups in malonogalactan to aminoethyl- and guanidinoethyl-cellulose indicated that this method can be used to obtain highly active and stable immobilized preparations suitable for large scale operations. Crude P1 nuclease bound to titanium chloride activated Whatman CF - 1 could hydrolyze 1% RNA, at pH 4.8 and 60°C, for a week. Since celluloses could not be used industrially (due to overpacking and susceptibility to microbial attack) attempts were made to bind P1 nuclease to a number of titanium activated synthetic ion-exchange resins (Rokugawa *et al.*, 1980b). Among the supports tried, the enzyme bound to porous matrices like Diaion HPK-25 (a strongly acidic cation exchange resin), Diaion HPA-10 (a strongly basic anion exchange resin) and Duolite A-4 (a weakly basic anion exchange resin) gave the best results, with efficiency values ranging from 64 - 69%, with RNA. P1 nuclease is a zinc metalloprotein and requires zinc for its activity and stability (Fujimoto *et al.*, 1975a). The enzyme bound to the aforementioned matrices showed an obligatory requirement for zinc ions whereas this was not observed in case of the enzyme bound to cyanogen bromide activated Sepharose. This property of the titanium bound enzyme was correlated to its increased flexibility. Addi-

tionally, the bound enzyme showed inferior temperature stability and this was dependent on the pH and zinc ion concentration. On continuous operation, at pH 4.8 and 60°C, the bound enzyme could hydrolyze 1% RNA for 24 days. The subsequent decrease in the activity was due to the non-specific adsorption of pigments and sediments (formed during hydrolysis) to the matrix and this could be alleviated by washing the matrix with 1 M NaCl (Rokugawa *et al.*, 1980b). In order to overcome the inferior temperature stability of P1 nuclease bound to titanium activated ion-exchange resins, Rokugawa *et al.* (1980c) 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 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).

PRESENT INVESTIGATION

As mentioned earlier, single strand specific nucleases are extensively used as analytical tools for the determination of nucleic acid structure. Among them, single strand specific nuclease from *A. oryzae* (S1 nuclease, EC 3.1.30.1) is preferred, primarily due to the fact that it can easily be prepared from commercially available Takadiastase powder, it is stable to low concentration of denaturants often used in annealing and its specificity for single stranded nucleic acids is extremely high under the right conditions (Vogt, 1980).

S1 nuclease is a sugar non-specific and multifunctional enzyme which acts on ssDNA, RNA and 3'AMP (Oleson and Sasakuma, 1980). Though this enzyme has been purified and extensively used as an analytical tool for the characterization of nucleic acid structure (Rushizky, 1981), very little information is available regarding its active site nature. Moreover, it has been reported that after the nuclease treatment of nucleic acid samples, removal of residual enzyme activity from the reaction mixture is often essential and requires repeated extractions with phenol, which in turn result in the loss of nucleic acid samples. In such circumstances, use of immobilized enzyme offers a distinct advantage over the soluble enzyme as it can easily be removed from the reaction mixture by physical methods. Hence, the present investigation with S1 nuclease was

undertaken to (a) study the nature of the active site by chemical modification and (b) develop a suitable immobilization procedure to obtain a highly active and stable immobilized preparation suitable for routine analytical purposes.

Chapter II

Affinity Purification and Active Site Characterization of S1 Nuclease

SUMMARY

A simple procedure, involving heat treatment, DEAE-Sephadex A-50, AMP-Sepharose and Bio-Gel P-60 chromatography, was developed for the purification of S1 nuclease to homogeneity, from commercially available Takadiastase powder, with an overall yield of 27%. Chemical modification studies on purified S1 nuclease revealed 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, could protect the enzyme against 2, 4, 6 - trinitrobenzenesulphonic acid (TNBS) mediated inactivation whereas, this was not observed in case of either methylene blue or diethylpyrocarbonate (DEP) mediated inactivation of the enzyme. Moreover, the lysine (TNBS)-modified enzyme, having very little catalytic activity, showed a significant decrease (70%) in its ability to bind 5' AMP, a competitive inhibitor of S1 nuclease, while the histidine (DEP)-modified enzyme (having very little catalytic activity), 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 ssDNAase, RNAase and phosphomonoesterase activities associated with S1 nuclease, indicating the existence of a common catalytic site responsible for the hydrolysis of both monomeric and polymeric substrates. Modification of carboxylate groups, on the other hand, either by

1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) or Woodward's Reagent K (WRK) and EDTA treatment of S1 nuclease, revealed that the loss of catalytic activity of the enzyme is due to the removal of zinc atoms and carboxylate groups are involved in metal binding. The data obtained with carboxylate groups modification, EDTA treatment, reconstitution with metal ions, zinc estimation and circular dichroism analysis of the enzyme suggested that, out of three zinc atoms present in S1 nuclease, zinc I is easily replaceable and is probably involved in the catalytic activity while zinc II and zinc III are involved in maintaining the enzyme structure.

INTRODUCTION

Single strand specific nuclease from *Aspergillus oryzae* (S1 nuclease, EC 3.1.30.1), is a sugar non-specific multifunctional enzyme which acts on single stranded DNA (ssDNA), RNA and 3' AMP (Oleson and Sasakuma, 1980). Although, this enzyme has been purified (Vogt, 1973; Oleson and Sasakuma, 1980; Shishido and Habuka, 1986) and extensively used as an analytical tool for the characterization of nucleic acid structure (Rushizky, 1981), very little information is available regarding its molecular enzymology. Through competitive inhibition studies, Oleson and Hoganson (1981) demonstrated that different activities associated with S1 nuclease are catalyzed by the same active site. A similar observation was also made by Reddy and Shankar (1989), while studying the immobilization of S1

nuclease on concanavalin A-Sepharose. However, no conclusive proof exists till now regarding the active site nature of the enzyme. In the case of enzyme(s) 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; Bandyopadhyay and Cheng-Wen, 1978). Moreover, the involvement of histidine in the catalytic activity of RNAases such as RNAase T1 (Irie, 1970; Takahashi, 1971), RNAase T2 (Kawata *et al.*, 1990) and RNAase A (Gundlach *et al.*, 1959; Crestfield *et al.*, 1963a, b) as well as pancreatic DNAase (Price *et al.*, 1969) has been demonstrated. S1 nuclease is a zinc metalloprotein and contains a large number of carboxylic acid residues. The role of zinc in catalysis, structure and regulation, has been demonstrated in many enzymes (Vallee and Galdes, 1984). Recently, in case of a 19 kDa protein from rat liver, which inactivates rat liver 6-phosphofructokinase, it has been shown that carboxylate groups serve as an excellent ligand for zinc co-ordination (Brand *et al.*, 1988). Carboxylic acid residues have also been implicated in the catalytic activity of RNAase T1 (Takahashi and Moore, 1982) and Staphylococcal nuclease (Weber *et al.*, 1991). Since S1 nuclease is an acidic metalloprotein acting on both ssDNA and RNA, chemical modification studies were carried out to evaluate the role of the aforementioned amino acid residues in the catalytic activity of the enzyme.

MATERIALS

Takadiastase (Uni-Sankyo, India); DEAE-Sephadex A-50 and Sepharose 4B (Pharmacia Fine Chemicals, Sweden); Bio-Gel P-10 and P-60 (Bio-Rad, USA); yeast RNA (Sisco Research Laboratories, India); sodium borohydride, formaldehyde, methylene blue and hydroxylamine hydrochloride (BDH, India); 3'AMP, 5'AMP, 2, 4, 6-trinitrobenzenesulphonic acid (TNBS), citraconic anhydride, diethylpyrocarbonate (DEP), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), imidazole, N-acetylimidazole, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and bovine serum albumin (BSA) (Sigma Chemical Co., USA); Woodward's reagent K (WRK) (Aldrich Chemical Co., USA), cyanogen bromide (Fluka AG, Germany); EDTA and uranyl acetate (Loba Chemie Indoaustranal Co., India) and perchloric acid (Qualigens, India) were used. [^{14}C]-Glycine was obtained from Bhabha Atomic Research Center (BARC), India. All other chemicals used were of analytical grade.

METHODS

DNA isolation

High molecular weight 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 local slaughter house was stored 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 9226 g for 20 min. The crude nuclear pellet obtained after centrifugation was washed 2-3 times with the homogenization buffer (till the supernatant was colourless) and then with saline EDTA (150 mM NaCl, 100 mM EDTA, pH 8.0). The washed pellet was suspended in minimum volume of saline EDTA, mixed with sodium dodecylsulphate to a final concentration of 2% (w/v) and incubated at 62°C for 30 min to facilitate the lysis of nuclei. The lysate was then brought to room temperature and 5 M sodium perchlorate was added to a final concentration of 1 M. The mixture was incubated under agitation for 30 min and then deproteinized using a mixture of chloroform and isoamyl alcohol (24:1). Subsequently, the mixture was centrifuged at 9226 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 pancreatic RNAase (50 µg/ml, made DNAase free by heating the enzyme at 80°C for 10 min) at 37°C for 1 h. After RNAase treatment, the mixture was again deproteinized and the DNA was precipitated as described above. The precipi-

tate was collected with a glass rod, dried free of ethanol, dissolved in sterile distilled water and stored at -20°C.

The UV absorption of DNA was determined in the range of 220-320 nm and only those preparations with A_{300} less than 0.1 and ratios of A_{230} : A_{260} and A_{280} : A_{260} corresponding to 0.45 and 0.55, respectively, were used. High molecular weight nature of the DNA preparation was also checked electrophoretically using 1% agarose gels.

Preparation of matrices

Aminohexyl-Sepharose (AH-Sepharose) : This was prepared according to the method of Kohn and Wilchek (1982). Sepharose 4B (10 g, wet weight) was washed and suspended in 10 ml of 60% (v/v) chilled acetone. The suspension was then cooled to -10°C and mixed with 200 mg of cyanogen bromide. Subsequently, 1 ml of 1.5 M triethylamine, in 60% (v/v) acetone, was added slowly to the above mixture (maintained at -10°C) under vigorous stirring. The entire reaction mixture was then poured into 100 ml of ice cold washing medium consisting of acetone and 100 mM HCl (1:1). The activated gel was then filtered on a Buchner funnel, washed successively with 60% (v/v) acetone, 30% (v/v) acetone and distilled water and used immediately for coupling.

Coupling of hexamethylenediamine was carried out by mixing 1 g hexamethylenediamine, in 10 ml of distilled water, with activated Sepharose 4B followed by overnight incubation at room temperature, under mild agitation. At

the end of the incubation period, the aminated gel was collected by filtration, washed extensively with 100 mM NaCl followed by distilled water, till the supernatant was free of hexamethylenediamine, as indicated by a negative colour test with TNBS (positive test is indicated by the formation of an orange colour on addition of a few drops of a 3% w/v TNBS solution prepared in saturated sodium tetraborate). The washed gel was stored in distilled water, at 4°C, till further use.

The amino group content of the matrix was determined according to the method of Antoni *et al.* (1983). The matrix (0.1 ml, packed volume) was suspended in 10 ml of 100 mM potassium tetraborate, mixed with 1 ml of 10 mM TNBS and incubated at 37°C, in a shaker water bath (75-100 rpm), for 2 h. After the incubation period, the gel was removed by centrifugation (1000 g, 10 min) and 1 ml of the supernatant was diluted with 5 ml 100 mM potassium tetraborate, followed by the addition of 0.5 ml of 30 mM glycine. Blank consisted of 1 ml supernatant, 5 ml of 100 mM potassium tetraborate and 0.5 ml distilled water instead of glycine. After incubation at 25°C for 25 min, 10 ml of cold methanol was added to the reaction mixture and the absorbance of the sample was read against the blank, at 340 nm. The amount of amino groups present on the matrix was calculated by assuming a molar absorption coefficient of 12,400 M^{-1} cm^{-1}. The amino group content of AH-Sepharose was found to be 10 μmoles/ml of matrix.

5'AMP-Sepharose : Coupling of 5' AMP to AH-Sepharose was carried out essentially according to Uchida and Shibata (1981). The ribose moiety of 5' AMP was subjected to periodate oxidation by mixing 10 ml of 9 mM 5'AMP with 5 ml of 200 mM sodium periodate. The reaction mixture was then adjusted to pH 9.0 with saturated solution of sodium tetraborate and incubated at 4°C for 1 h, in dark. AH-Sepharose (10 ml, packed volume) after washing with distilled water, 500 mM NaCl and 100 mM sodium tetraborate, was suspended in 10 ml of 100 mM of sodium tetraborate. The solution of 5'AMP-dialdehyde was then mixed with the gel suspension and left overnight at 4°C, under mild agitation. Subsequently, 50 mg of solid sodium borohydride was added to the above mixture and incubated for an additional 2 h at 4°C, with gentle agitation. The gel was then filtered on a Buchner funnel, washed successively with 1 M NaCl, distilled water and 50 mM sodium acetate buffer, pH 5.0, till the filtrate showed no detectable absorbance at 260 nm. The matrix thus prepared was stored in 50 mM acetate buffer, pH 5.0, at 4°C till further use.

The amount of 5' AMP bound to the matrix was determined by hydrolyzing 1 ml (packed volume) of the matrix with 1 M HCl, in a sealed tube, at 100°C for 1 h, followed by measuring the adenine liberated, at 263 nm. The amount of adenine was calculated by assuming a molar absorption coefficient of 12,700 M^{-1} cm^{-1} (Cohn, 1957). The matrix contained 3-4 μmoles of 5' AMP/ml of Sepharose.

Preparation of reagents

p-Nitrophenylglyoxal : This was synthesized according to the procedure of Steinbach and Becker (1954). A solution of selenius acid (3.9 g) in 2.4 ml of distilled water and 15 ml of glacial acetic acid was refluxed, at 120°C, for 1 h with 5 g of *p*-nitroacetophenone. Subsequently, the reaction mixture was cooled and the precipitated selenium was removed by filtration. The filtrate was then distilled to remove water and acetic acid. The *p*-nitrophenylglyoxal was then crystallized and recrystallized by cooling the solution of glyoxal. The purity of the product was checked by melting point and IR spectroscopy.

Glycine ethyl ester : This was prepared by mixing 5 g of glycine with 50 ml of ethanolic HCl, followed by incubation of the mixture at room temperature for 24 h. The undissolved glycine was then removed by filtration and the glycine ethyl ester formed was crystallized by cooling the filtrate to 4°C. The purity of the final product was ascertained by melting point and IR spectroscopy. [^{14}C] glycine ethyl ester was prepared in a similar manner. The specific activity of the labeled compound was 6.3 µCi/mmol.

Enzyme assays

ssDNAase activity of S1 nuclease : The standard reaction mixture of 1 ml contained 50 µg of sonicated and heat denatured buffalo liver DNA, 1 mM $ZnSO_4$, 50 mM NaCl and 5% (v/v) glycerol in 30 mM sodium acetate buffer, pH 4.6 and

appropriately diluted enzyme. The reaction was initiated by the addition of the enzyme 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 (800 g, 10 min) to sediment the precipitate. The acid soluble nucleotides in the supernatant were measured at 260 nm. The acid soluble deoxyribonucleotides were estimated by assuming a molar absorption coefficient of 10,000 M^{-1} cm^{-1} (Curtis *et al.*, 1966).

RNAase activity of S1 nuclease : RNA degrading activity of the enzyme was determined according to Uchida and Egami (1967b) with slight modifications. The standard reaction mixture of 0.75 ml contained 3 mg of yeast RNA, 1 mM $ZnSO_4$, 50 mM NaCl and 5% (v/v) glycerol in 30 mM sodium acetate buffer, pH 4.6 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 chilled uranyl reagent (0.75% w/v uranyl acetate in 25% v/v perchloric acid) and the resulting precipitate was immediately removed by centrifugation (800 g, 10 min). Subsequently, 0.1 ml of the supernatant was diluted with 2.5 ml of distilled water and the acid soluble nucleotides were measured at 260 nm. The amount of acid soluble ribonucleotides was calculated by assuming a molar absorption coefficient of 10,600 M^{-1} cm^{-1} (Curtis *et al.*, 1966).

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

Phosphomonoesterase (AMPase) activity of S1 nuclease : This was carried out using 3'AMP as the substrate. The total reaction mixture of 2 ml contained 1 mM 3'AMP, 1 mM $ZnSO_4$, 50 mM NaCl and 5% (v/v) glycerol in 30 mM sodium acetate buffer, pH 4.6 and appropriately diluted enzyme. After incubation at 37°C for 15 min, the reaction was terminated by the addition of 2 ml of freshly prepared Chen's reagent, made up of 1 volume of 12 M H_2SO_4, 2 volumes of distilled water, 1 volume of 2.5 % (w/v) of ammonium molybdate and 1 volume of 10 % (w/v) ascorbic acid (Chen *et al.*, 1956). The blue colour 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 standard assay conditions.

Assay of RNAase T1 : RNAase T1 activity determination was carried out according to Uchida and Egami (1967a) with slight modifications. The standard reaction mixture of 0.75 ml contained 0.25 ml yeast RNA (12 mg/ml), 0.3 ml of 200 mM Tris-HCl buffer (pH 7.5), 0.1 ml of EDTA (20 mM) and 0.1 ml of appropriately diluted enzyme. The reaction was

initiated by the addition of RNA, followed by incubation at 37°C for 15 min. After the incubation period, the reaction was terminated by the addition of 0.25 ml of chilled uranyl reagent (0.75% w/v uranyl acetate in 25% v/v perchloric acid) and the resulting precipitate was immediately removed by centrifugation (800 g, 10 min). Subsequently, 0.1 ml of the supernatant was diluted with 2.5 ml of distilled water and the acid soluble nucleotides were measured at 260 nm. The amount of acid soluble nucleotides was calculated by assuming a molar absorption coefficient of 10,600 for the ribonucleotide mixture (Curtis *et al.*, 1966).

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

Assay of RNAase T2 : The assay procedure was similar to that of RNAase T1 except, the pH of the assay mixture was 4.5 (Uchida and Egami, 1967b).

Protein determination

During the enzyme purification steps, protein concentrations were monitored by assuming a relationship *viz* 1 $A^{1\%}_{1\,cm,\ 280nm}$ = 10. However, for chemical modification studies, protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as standard. The blue colour developed after the addition of Folin phenol reagent was read at 660 nm.

PURIFICATION OF S1 NUCLEASE

Unless otherwise stated, all the operations were carried out at 4°C. During the purification steps, S1 nuclease activity was monitored using sonicated and heat denatured DNA.

Heat treatment : Two g of Takadiastase powder was suspended in 50 ml of buffer A (300 mM sodium acetate buffer, pH 5.5, containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% v/v glycerol) and stirred for 1 h. The insoluble material was removed by centrifugation (6800 g, 10 min). The supernatant was then heated at 70°C for 90 sec with vigorous swirling, chilled immediately on ice and used for DEAE-Sephadex chromatography.

DEAE-Sephadex A-50 chromatography : The heat treated enzyme sample was adsorbed onto a DEAE-Sephadex column (2 cm X 20 cm) equilibrated at pH 5.5 with buffer B (50 mM sodium acetate buffer containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% v/v glycerol) at a flow rate of 16 ml/h. The column was then washed with buffer B, till the flow through fractions showed no nuclease activity. Subsequently, the elution of the bound enzyme was affected with a linear gradient, 400 ml total volume, of NaCl (0-400 mM) in buffer B. Four ml fractions were collected at a rate of 16 ml/h and fractions having specific activity greater than 100 were pooled and dialyzed extensively against buffer B and used for the affinity step.

Affinity chromatography on 5'AMP-Sepharose : The dialyzed enzyme was then adsorbed onto an AMP-Sepharose column (2 cm X 8 cm) pre-equilibrated at pH 5.5 with buffer B, at a rate of 12 ml/h. The column was washed with the same buffer, till the effluent showed no nuclease activity. Subsequently, the bound enzyme was eluted with a linear gradient, 300 ml total volume, of NaCl (0-500 mM) in buffer B. Fractions of 3 ml were collected at a rate of 12 ml/h and those having specific activity greater than 500 were pooled and concentrated to 4 ml using an Amicon ultrafiltration unit fitted with PM-10 membrane.

Gel filtration on Bio-Gel P-60 : Two ml of the concentrated enzyme sample was chromatographed on a Bio-Gel P-60 column (2 cm X 125 cm) equilibrated at pH 5.0 with buffer C (30 mM sodium acetate buffer containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% v/v glycerol) at a rate of 8 ml/h. Two ml fractions were collected and those having specific activity greater than 1200 were pooled, concentrated and stored at -20°C until further use. No loss of activity was observed when the purified enzyme was stored under these conditions.

Electrophoresis

Native and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were carried out at pH 8.3 according to Laemmli (1970). After electrophoresis, the native gels were stained with Coomassie Brilliant Blue G-250 (Blakesley and Boezi, 1977) whereas the SDS-gels were stained with Coomassie Brilliant Blue R-250 and destained using methanol :

acetic acid : water (25:10:65). Isoelectric focussing (IEF) in polyacrylamide gels was performed according to Vesterberg (1972) over the pH range 3.0 - 10.0.

Molecular weight determination

Molecular weight determination was done on Bio-Gel P-60 column (1 cm X 100 cm) using ovalbumin (45,000), soybean trypsin inhibitor (20,100), myoglobin (17,000) and lysozyme (14,300) as reference proteins. Subunit molecular weight was determined using 10% (w/v) SDS-polyacrylamide gels (pH 8.3) according to Laemmli (1970) with bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000) and lysozyme (14,300) as reference proteins.

CHEMICAL MODIFICATION STUDIES

In chemical modification studies, the residual activity of the modified enzyme was determined using all the three substrates *viz* ssDNA, RNA and 3' AMP. For the modification of carboxylate groups, the purified enzyme was extensively dialyzed against deionized water and stored at -20°C. No loss of activity was observed when the enzyme was stored under these conditions. Metal ion free distilled water (Milli-Q) was used throughout the carboxylate group modification studies.

Modification of lysine residues

Reaction with TNBS : The total reaction mixture of 2 ml, containing 0.5 ml of S1 nuclease (250 μg) and 1 ml of 4% (w/v) sodium bicarbonate, was incubated with varying con-

centrations of TNBS (0.1 - 0.5 mM) at 37°C, in dark. Aliquots were withdrawn at suitable intervals and the reaction was terminated by adjusting the pH to 4.6. Subsequently, the residual activities were determined under the standard assay conditions. Enzyme samples incubated in the absence of TNBS served as control. The number of amino groups modified was determined spectrophotometrically by assuming a molar absorption coefficient of 9950 M^{-1} cm^{-1} for the trinitrophenylated lysine (Habeeb, 1966).

Citraconylation : The amino groups of S1 nuclease were reversibly blocked with citraconic anhydride according to Dixon and Perham (1968). Citraconic anhydride was diluted with dioxane and the concentration of the diluted reagent was 228 mM. Dioxane at this concentration did not have any adverse effect on the activity of S1 nuclease. Purified S1 nuclease (1 mg) in 7 ml of 100 mM sodium bicarbonate buffer (pH 8.0) was treated at room temperature with a total of 310 µl of citraconic anhydride. The reagent was added in 6 instalments and the pH of the reaction mixture was maintained at 8.0 by the addition of 1 M NaOH. After every addition, an aliquot was removed and assayed for enzyme activities and number of free amino groups. Control consisted of enzyme samples incubated under identical conditions without citraconic anhydride. The total number of amino groups in native and modified enzyme samples were estimated by TNBS method of Habeeb (1966). Decitraconylation was achieved by incubating the modified enzyme samples

at pH 4.0 and 4°C for 15 h, followed by assaying the enzyme activities.

Reductive methylation : This was carried out essentially according to Means and Feeney (1968). To 1 ml of purified S1 nuclease (200 µg) in 200 mM sodium borate buffer (pH 9.0) at 0°C, 0.1 ml of sodium borohydride (0.5 mg/ml) was added followed by the addition of 6 aliquots (20 µl each) of 0.35% (v/v) formaldehyde solution, at an interval of 10 min. At the end of the reaction, the residual activities and the number of amino groups modified were determined as described before. Enzyme incubated in the absence of formaldehyde served as control.

Modification of arginine residues

Reaction with p-nitrophenylglyoxal : S1 nuclease (500 µg) in 50 mM HEPES buffer, pH 8.0, containing 150 mM sodium ascorbate, was incubated with 25 µl of 10% (w/v) *p*-nitrophenylglyoxal in methanol at 30°C for 30 min. Subsequently, the residual activities were measured under standard assay conditions. Enzyme samples incubated in absence of *p*-nitrophenylglyoxal served as control. The number of arginine residues modified were determined according to Yamasaki *et al.* (1981).

Modification of histidine residues

Photo-oxidation : This was carried out by exposing 200 µg of the purified enzyme, in 1 ml of 50 mM sodium maleate buffer pH 7.5, in a glass test tube (1 cm X 10 cm), con-

taining different concentrations of methylene blue, to 200 W flood light bulb held at a distance of 12 cm for 30 min, at 26 ± 1 °C, followed by estimation of the residual activities. Enzyme samples treated under identical conditions, in dark, served as control.

Reaction with DEP : S1 nuclease (200 µg), in 1 ml of 50 mM sodium maleate buffer pH 6.8, was incubated at 26 ± 1 °C for 20 min, with various concentrations of DEP, freshly diluted with absolute ethanol. Aliquots were withdrawn at suitable intervals and the reaction was arrested by the addition of 10 µl of 10 mM imidazole, pH 7.5. Subsequently, the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of DEP served as control. The DEP concentration in the diluted samples was determined by mixing an aliquot of the diluted sample with 3 ml of 10 mM imidazole (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} (Melchior and Fahrney, 1970). The concentration of the diluted stock DEP solution was 10 mM. The ethanol concentration in the reaction mixture did not exceed 2 % (v/v) and had no effect on the activity and stability of the enzyme during the incubation period. S1 nuclease modification by DEP was also monitored spectrophotometrically by measuring the change 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 hydrochloride, at pH 7.0 and 4°C for 15 h and the enzyme activities were determined under standard assay conditions.

Modification of tyrosine residues

Reaction with N-acetylimidazole : S1 nuclease (100 μg) in 1 ml of 50 mM sodium borate buffer, pH 7.5, was incubated with 1 mM N-acetylimidazole for 20 min, at 26 ± 1 °C, followed by estimation of the residual activities under standard assay conditions. The enzyme incubated in the absence of N-acetylimidazole was taken as control. The number of tyrosine residues modified were calculated by using a molar absorption coefficient of 1160 M^{-1} cm^{-1} at 278 nm (Means and Feeney, 1971).

Modification of cysteine residues

Reaction with DTNB : The enzyme (100 μg) in 1 ml of 50 mM Tris-HCl buffer, pH 7.9, was incubated with 2 mM DTNB, at 26 ± 1 °C, for 20 min and the residual activities were determined under standard assay conditions. Enzyme incubated in the absence of DTNB served as control. The number of cysteine residues modified were determined at 412 nm, using a molar absorption coefficient of 13,600 M^{-1} cm^{-1} (Means and Feeney, 1971).

Inhibitor binding studies

The inhibitor binding studies on native and modified enzyme samples were carried out according to Hummel and Dreyer (1962). The TNBS- and DEP-modified enzyme samples (200 μg each) in 1 ml of 30 mM sodium acetate buffer, pH 4.6 (containing 1 mM $ZnSO_4$, 50 mM NaCl, 5% v/v glycerol and 20 μM 5' AMP), were passed through a Bio-Gel P-10 column (1 cm X 25 cm) equilibrated with the above buffer, at a rate of 0.4 ml/min. Fractions of 2 ml were collected and the absorbance at 260 nm was measured. Unmodified enzyme subjected to similar treatment was taken as control.

Modification of carboxylate groups

Reaction with EDC : The carboxylic acid groups of the enzyme were modified by incubating 1.5 mg of purified S1 nuclease, in 3 ml of distilled water (adjusted to pH 4.6 with dilute HCl), with a total of 15 mM EDC and 50 mM [^{14}C] glycine ethyl ester. EDC was added in three instalments, at an interval of 15 min, to obtain an effective concentration of 5 mM, 10 mM and 15 mM, respectively. During the course of the reaction, the pH of the reaction mixture was maintained at 4.6 by the addition of dilute HCl. After each addition, an aliquot was removed, subjected to gel filtration on Sephadex G-10 column (1 cm X 5 cm), to remove excess reagents and the residual activities were determined under standard assay conditions. The number of carboxylate groups modified were determined by measuring the radioactivity incorporated, on a LKB-RackBeta II liquid

scintillation counter. Enzyme samples incubated in the absence of EDC served as control. Zinc content in the unmodified and modified enzyme samples was determined by atomic absorption.

Reaction with WRK : This was carried out by incubating 400 µg of purified S1 nuclease in 50 mM Tris-HCl buffer, pH 7.8, with a total of 15 mM of WRK. The reagent was added in four instalments, at an interval of 15 min, to obtain an effective concentration of 2 mM, 5 mM, 10 mM and 15 mM, respectively. After every addition, an aliquot was removed and the reaction was arrested by the addition of 1 M sodium acetate buffer, pH 4.6. The reaction mixture was then dialyzed extensively against 30 mM sodium acetate buffer, pH 4.6, containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% (v/v) glycerol and the residual activities were determined under standard assay conditions. Enzyme samples incubated in the absence of WRK were taken as control. The number of carboxylate groups modified were determined by assuming a molar absorption coefficient of 7,000 M^{-1} cm^{-1} (Sinha and Brewer, 1985). The zinc content in the unmodified and modified enzyme samples was determined by atomic absorption.

Reaction with EDTA : S1 nuclease (200 µg) in 1 ml of 30 mM sodium acetate buffer, pH 4.6, was incubated with various concentrations of EDTA (1 - 10 mM) at 37°C for 30 min. Subsequently, the reaction mixture was dialyzed against the above buffer and the residual activities were determined

under standard assay conditions. Enzyme samples incubated in the absence of EDTA served as control. The zinc content of the enzyme samples was determined by atomic absorption.

Reactivation of the modified enzyme : For these studies, the enzyme samples were extensively dialyzed against 30 mM sodium acetate buffer, pH 4.6. Reactivation of the WRK modified and EDTA treated enzyme samples were then achieved by incubating the enzyme samples with 1 mM Zn^{2+} in 30 mM sodium acetate buffer, pH 4.6 at 4°C for 15 h, followed by assaying their activities and zinc content. Influence of Co^{2+} on the activity of WRK modified and EDTA treated enzyme samples was carried out in a similar manner.

Substrate protection studies

In all the chemical modification reactions, the effect of substrate protection was studied by incubating the enzyme with excess amounts of ssDNA, RNA and 3'AMP, followed by treatment with various modifying reagents.

Metal ion protection

In the case of carboxylate group modification, the effect of metal ion (Zn^{2+}) protection was studied by incubating the enzyme with 1 mM Zn^{2+} prior to WRK treatment.

Atomic absorption spectrophotometry

Zinc content of the enzyme samples was determined by atomic absorption on a Hitachi-Z-8000 Polarized Zeeman atomic absorption spectrophotometer at 213.8 nm, whereas

the cobalt content was estimated at 240.7 nm. Prior to analysis, the enzyme samples were extensively dialyzed against distilled water to remove the buffer salts, EDTA and excess reagents. The insoluble material, if any, was then removed by centrifugation (8000g, 5 min).

Circular dichroism (CD) studies

CD measurements were carried out on a Jasco J-500 A spectopolarimeter, at 20°C, in the range of 200-240 nm.

RESULTS

PURIFICATION OF S1 NUCLEASE

The results of a typical procedure for the purification of S1 nuclease to homogeneity are summarized in Table 2.1. The enzyme was purified approximately 84-fold with an overall yield of 27%. S1 nuclease obtained after DEAE-Sephadex chromatography, though showed high specific activity, contained a significant amount of RNAase activity. Subsequently, the RNAase activity was identified as that of RNAase T1, on the basis of its optimum pH, heat stability at pH 2.0 and non-glycoprotein nature. Our efforts to separate S1 nuclease from RNAase T1, on 5' AMP-Sepharose, failed as the latter not only bound to the matrix but also co-eluted with S1 nuclease. However, this step helped in removing the coloured impurities and non-nucleotide binding proteins. Finally, chromatography on Bio-Gel P-60 yielded a homogeneous S1 nuclease preparation. The purified enzyme moved as a single band in native, SDS and IEF gels, indicating its homogeneity (Fig. 2.1). The molecular weight of

Table 2.1 : Purification of S1 nuclease

Step	Activity (U)	Protein (A_{280})	Specific activity (U/A_{280})	Fold purification	Recovery (%)
Crude extract	25,000	1540	16.23	1.0	100
Heat treatment	24,000	1450	16.55	1.02	96.0
DEAE-Sephadex chromatography	12,000	109	110.1	6.78	48.0
AMP-Sepharose chromatography	10,000	14	714.3	44.0	40.0
Bio-Gel P-60 chromatography	6,800	5	1360	83.79	27.2

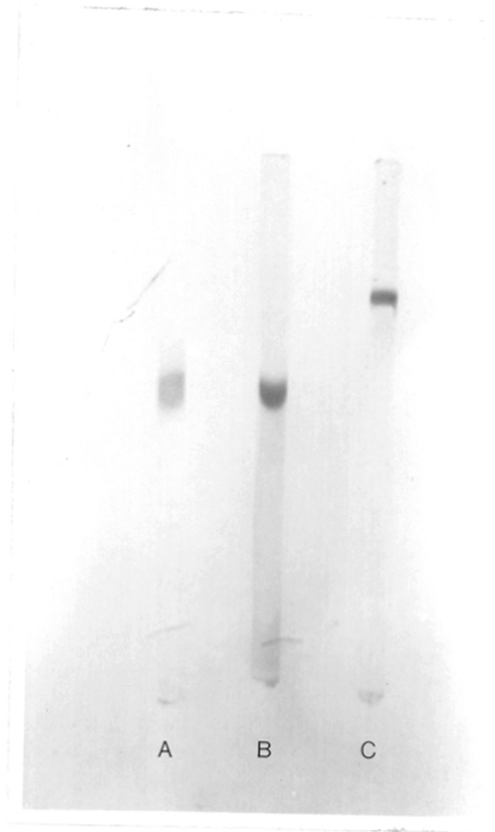

Fig. 2.1: Electrophoresis of purified S1 nuclease.

(A) 10% (w/v) polyacrylamide gel, Tris-glycine buffer, pH 8.3, current 4 mA/tube, protein loaded 75 μg

(B) SDS-polyacrylamide gel (10%, w/v), Tris-glycine buffer, pH 8.3, SDS 0.1%, current 6 mA/tube, protein loaded 75 μg

(C) IEF in 10% (w/v) polyacrylamide gel, pH range 3.0-10.0, protein loaded 50 μg.

the pure enzyme determined by gel filtration and SDS-PAGE was 32,000 and 32,360 respectively (Fig. 2.2 A and B). The pI of the purified enzyme was 4.0.

CHEMICAL MODIFICATION STUDIES

Modification of amino groups

Purified S1 nuclease when incubated with 0.5 mM TNBS at pH 8.0 for 30 min, lost 65-70% of its activity towardsssDNA, RNA and 3' AMP. The inactivation was dependent on the concentration of the reagent and the number of amino groups modified (Fig. 2.3). However, no loss of activity was observed in the control samples. The logarithm of residual activity plotted as a function of time at various TNBS concentrations was linear upto 33%, 28% and 34% of the initial activity towards ssDNA, RNA and 3' AMP respectively (Fig. 2.4). 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 plots of log (percent residual activity) *versus* reaction time and the order was determined from the slope of the plots of log (K_{app}) against log [TNBS]. These plots (insets Fig. 2.4) indicated that the loss of enzyme activity towards all the three substrates occurred as a result of modification of a single lysine residue per molecule of the enzyme.

Citraconylation of S1 nuclease resulted in 70-75% loss in its initial activity and the inactivation was concentration dependent (Fig. 2.5 A). The plot of percent

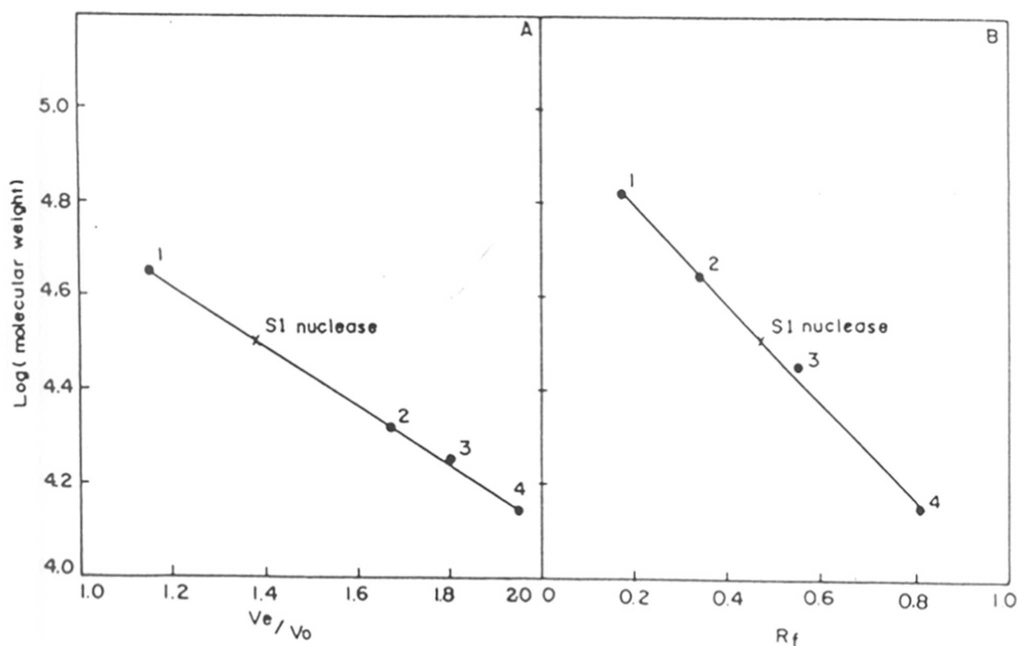

Fig. 2.2: **Molecular weight determination of S1 nuclease.**

(A) Gel filtration: Bio-Gel P-60 column (1 cm x 100 cm) was equilibrated with 30 mM sodium acetate buffer (pH 5.0). The column was calibrated with (1) ovalbumin (45,000), (2) soybean trypsin inhibitor (20,100), (3) myoglobin (17,000) and (4) lysozyme (14,300). Vo is the void volume and Ve, the elution volume.

(B) SDS-polyacrylamide gel electrophoresis: Relative mobilities of the reference proteins were plotted against the log molecular weight. The reference proteins used were (1) bovine serum albumin (66,000), (2) ovalbumin (45,000), (3) carbonic anhydrase (29,000) and (4) lysozyme (14,300).

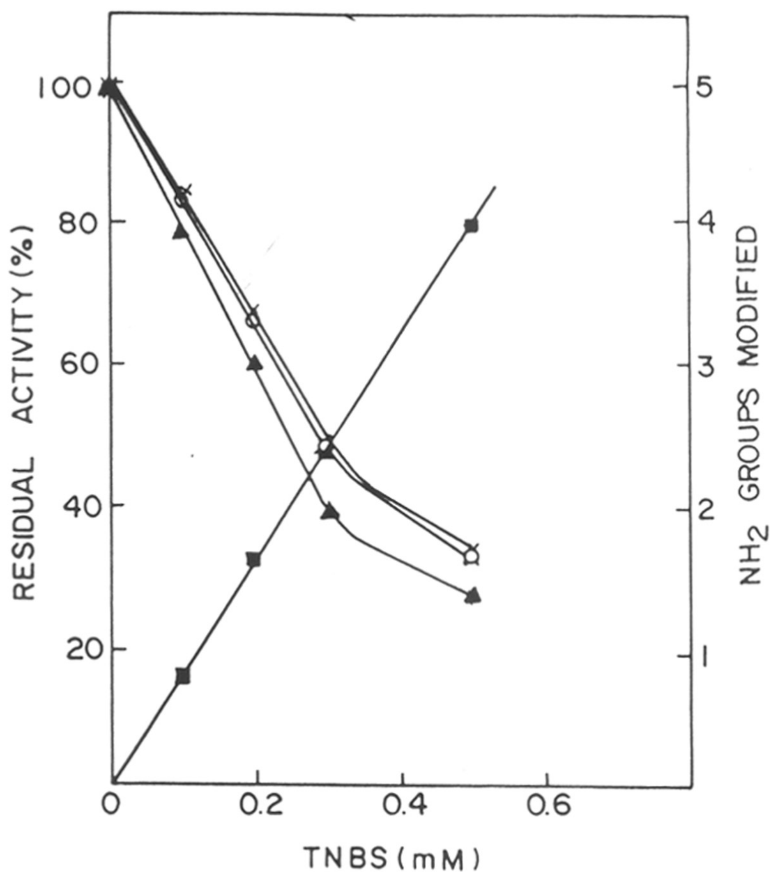

Fig. 2.3: Effect of TNBS concentration on the activities of purified Sl nuclease.

ssDNAase (o), RNAase (▲), Phosphomonoesterase (×) and number of NH_2 groups modified (■).

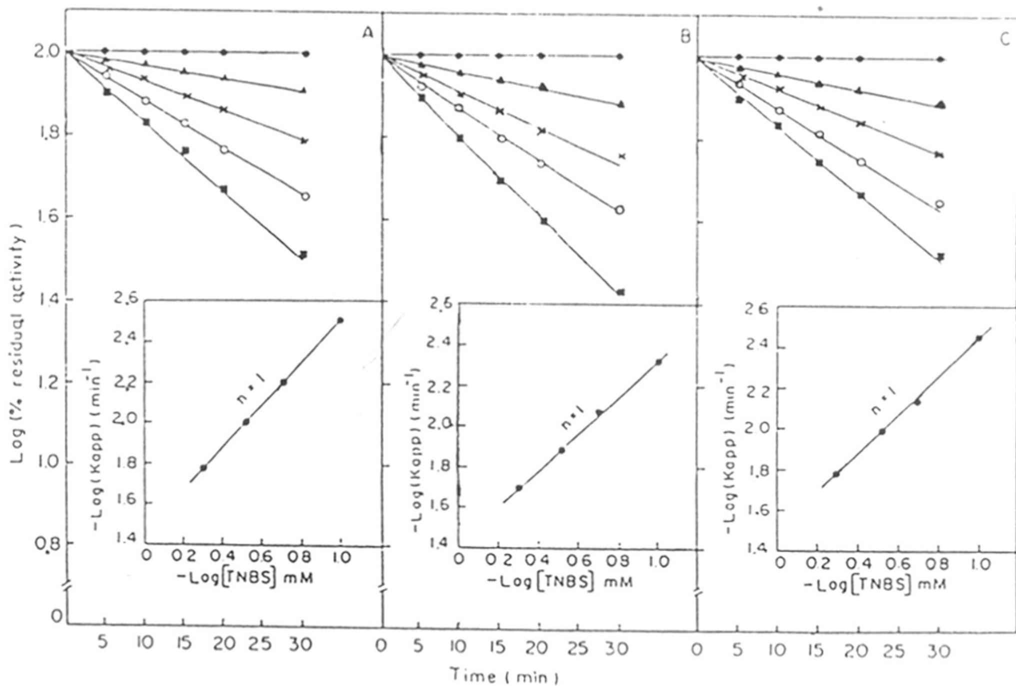

Fig. 2.4: Pseudo first-order plots for the inactivation of Sl nuclease by TNBS.

(A) ssDNAase (B) RNAase (C) Phosphomonoesterase. Concentrations of TNBS were 0 mM (●), 0.1 mM (▲), 0.2 mM (×), 0.3 mM (o) and 0.5 mM (■). Insets: second order plots of pseudo first-order rate constants (K_{app}) (min^{-1}) of inactivation at different concentrations of TNBS.

residual activity against the number of amino groups modified revealed that the loss of enzyme activity towards ssDNA, RNA and 3'AMP resulted from the modification of one lysine residue (Fig. 2.5 B). Decitraconylation of the enzyme restored 70-75% of its initial activity, suggesting the involvement of lysine in the catalytic activity of S1 nuclease. Further, reductive methylation of the enzyme resulted in 60% loss in its initial activity towards all the substrates. Estimation of free amino groups after modification showed that the loss of activity was due to the modification of one lysine residue (Table 2.2).

Table 2.2 : Effect of citraconylation and reductive methylation on the activity of S1 nuclease

Modification reaction	Lysine residues modified	Residual activity (%)		
		ssDNAase	RNAase	AMPase
Control	0	100	100	100
Citraconylation	1.0	55	60	60
Decitraconylation	0.3	75	70	70
Reductive methylation	1.2	40	40	40

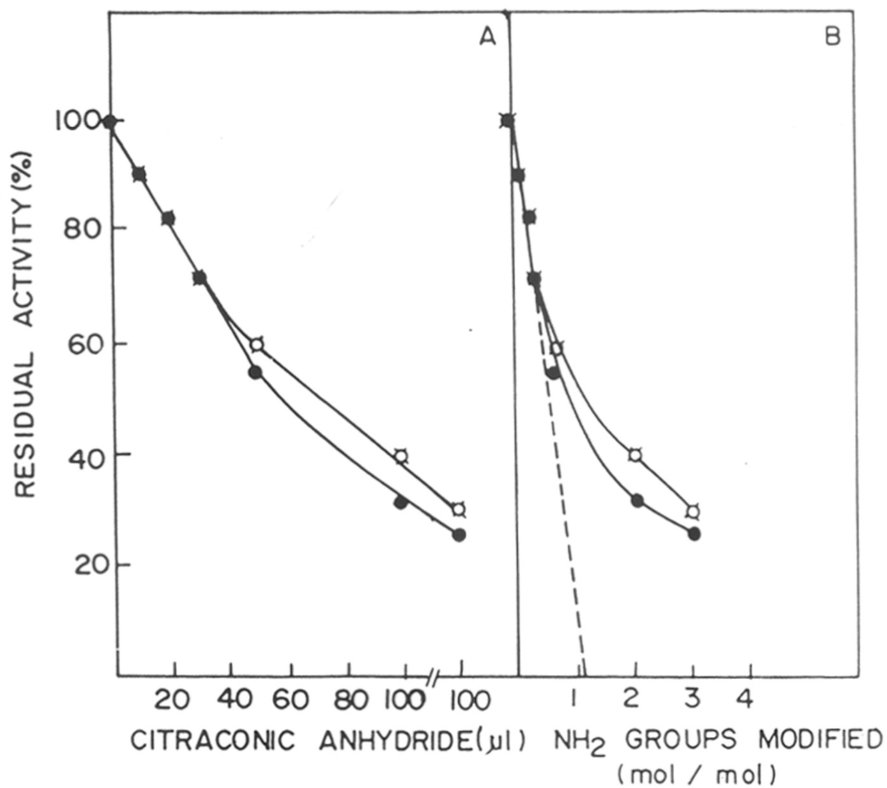

Fig. 2.5: Citraconylation of S1 nuclease

(A) Influence of citraconic anhydride concentration on the activities of purified S1 nuclease.

(B) Changes in the activities of S1 nuclease as a function of modified NH_2 groups.

ssDNAase (●), RNAase (o) and Phosphomonoesterase (✖).

TNBS mediated inactivation of S1 nuclease could be prevented to a great extent, by incubating the enzyme with excess amounts of ssDNA, RNA and 3' AMP prior to the modification reaction (Table 2.3). The CD spectra of both unmodified and lysine modified S1 nuclease were almost identical (Fig. 2.6), indicating that modification does not result in a gross change in the conformation of the enzyme. In addition, the TNBS modified enzyme showed a significant decrease in its ability to bind 5' AMP, a competitive inhibitor of S1 nuclease (Table 2.3).

Table 2.3 : Influence of lysine modification on the activity of S1 nuclease : Substrate protection and inhibitor binding studies

Incubation mixture	Residual activity (%)	5'AMP binding (%)
Enzyme	100	100
Enzyme + TNBS (0.5 mM)	32	33
Enzyme + ssDNA (1 mg) + TNBS	65	-
Enzyme + RNA (1 mg) + TNBS	62	-
Enzyme + 3'AMP (1 mM) + TNBS	65	-

Modification of arginine residues by treating the enzyme with *p*-nitrophenylglyoxal though resulted in the

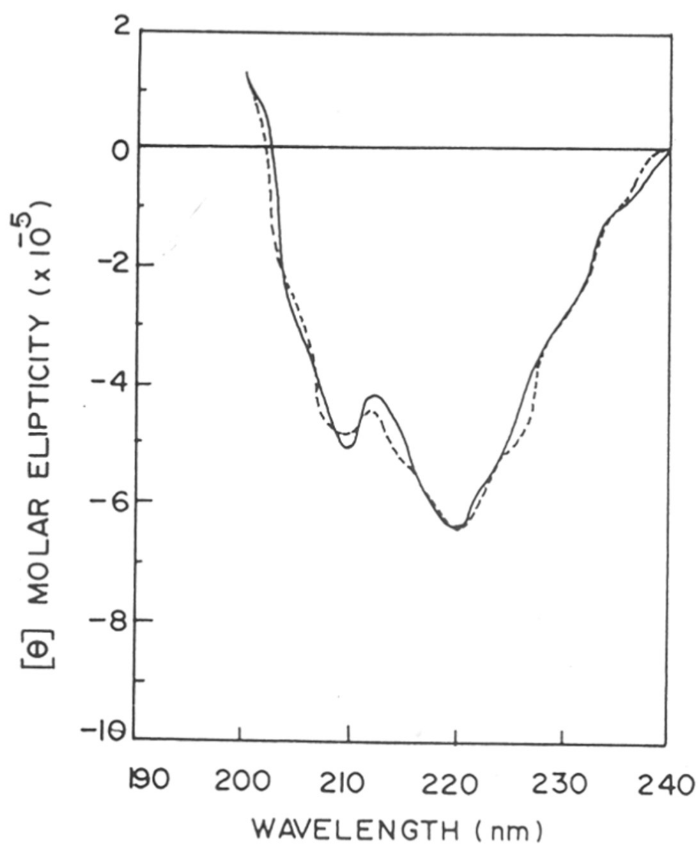

Fig. 2.6 : The CD spectra of native and TNBS treated S1 nuclease

The CD measurements were performed in a 1 mm cell at an enzyme concentration 200 µg/ml. Native enzyme (——) and TNBS treated enzyme (----).

modification of two arginine residues out of a total of three (Shishido and Habuka, 1986), it did not have any effect on the activity of purified S1 nuclease, indicating that arginine residues may not have any role in the catalytic activity of the enzyme.

Modification of histidine residues

Photo-oxidation of purified S1 nuclease, in presence of 0.2% (w/v) methylene blue, showed a pH-dependent inactivation and the maximum loss of activity was observed at pH 7.5 (Fig. 2.7). When the enzyme was irradiated with 0.2% (w/v) methylene blue at pH 7.5 for 30 min, lost 70% of its initial activity towards ssDNA, RNA and 3'AMP and the inactivation was dependent on the concentration of the reagent (Fig. 2.8). However, no loss of activity was observed in the controls.

Carbethoxylation of S1 nuclease, at pH 6.8 for 20 min, resulted in 65-75% loss of its initial activities and the inactivation was concentration dependent. No loss of activity was observed in the control samples. The logarithm of the percent residual activity plotted as a function of time at various DEP concentrations was linear upto 27%, 24% and 34% of the initial activity towards ssDNA, RNA and 3' AMP, respectively (Fig. 2.9). The DEP 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 plots of log (percent residual activity) *versus* reaction

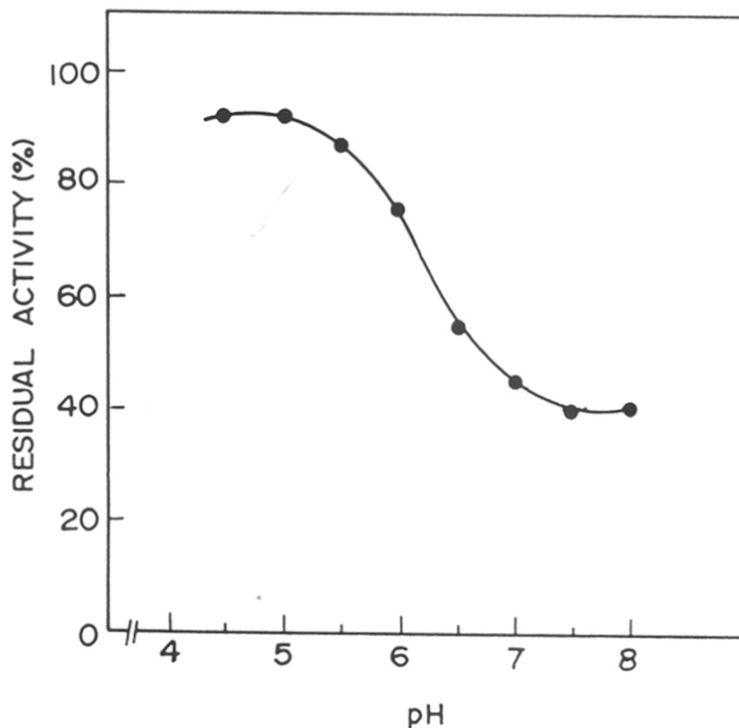

Fig. 2.7: Effect of pH on photo-oxidation of S1 nuclease.

The enzyme (100 μg/ml) was incubated at different pH (4.5-8.0) with 0.2% methylene blue at room temperature for 30 min as described under Methods. An identical sample at each pH value was kept in dark to serve as a control. Enzyme activity was measured using ssDNA as the substrate.

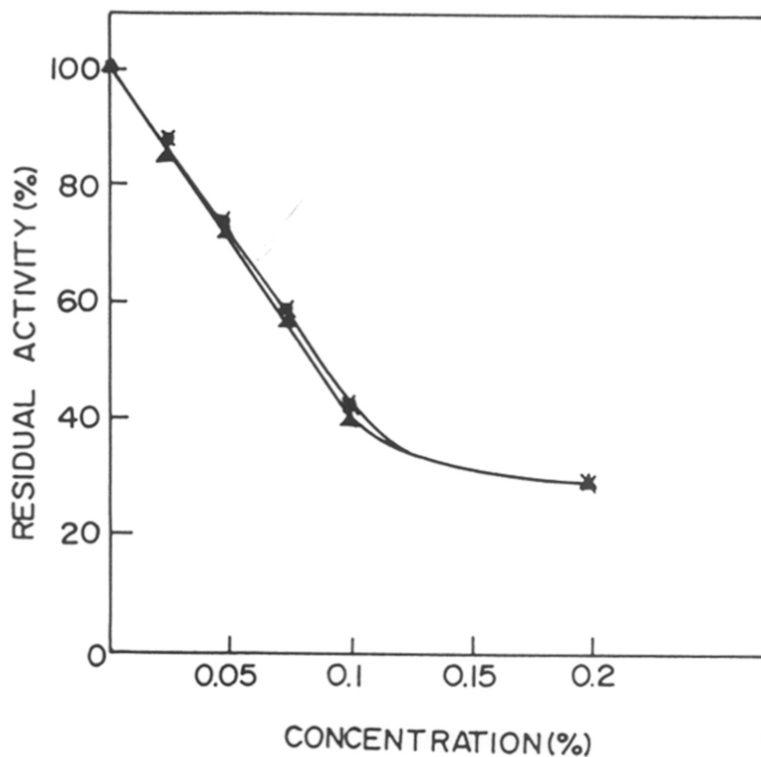

Fig. 2.8: Effect of methylene blue concentration on the activities of purified S1 nuclease.

The purified enzyme (200 µg) was incubated at pH 7.5 at room temperature with various concentrations of methylene blue for 30 min as described under Methods.

ssDNAase (●), RNAase (▲), Phosphomonoesterase (×).

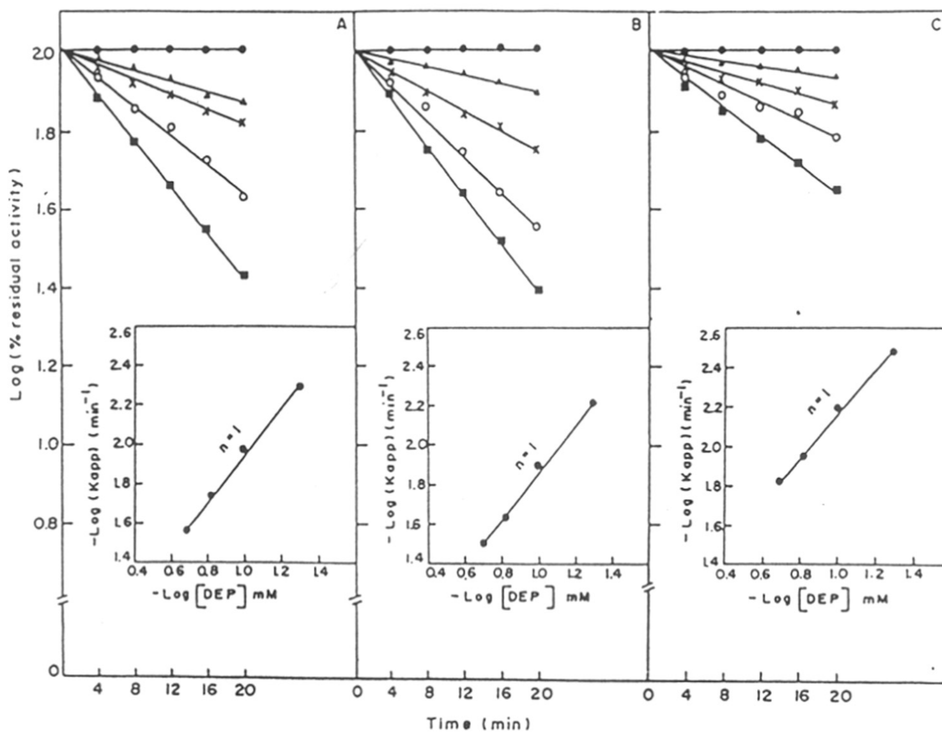

Fig. 2.9: Pseudo first-order plots for the inactivation of Sl nuclease by DEP.

(A) ssDNAase (B) RNAase (C) Phosphomonoesterase. Concentrations of DEP were 0 mM (●), 0.05 mM (▲), 0.1 mM (×), 0.15 mM (o) and 0.2 mM (■). Insets: second order plots of the pseudo first-order rate constants (K_{app}) (min^{-1}) of inactivation at different concentrations of DEP.

time and the order was determined from the slope of the plots of log (K_{app}) against log [DEP]. These plots (insets Fig. 2.9) indicated that the loss of enzyme activity towards all the three substrates occurred as a result of modification of a single histidine residue per molecule of the enzyme. Furthermore, carbethoxylation of the enzyme, as a result of DEP treatment, was accompanied by an increase in the absorbance of the modified protein at 240 nm. Based on a molar absorption coefficient of carbethoxyhistidine at 240 nm to be 3200 M^{-1} cm^{-1} (Ovadi *et al.*, 1967) and molecular weight of S1 nuclease to be 32,000 Da (Iwamatsu *et al.*, 1991), the total number of histidine residues modified was found to be 1.6. However, the plot of percent residual activity *versus* the number of histidine residues modified revealed that the loss of activity towards ssDNA, RNA and 3' AMP, resulted from the modification of a single histidine residue (Fig. 2.10). Incubation of the DEP modified enzyme with 500 mM hydroxylamine, at pH 7.0 and 4°C for 15 h, restored 80-90% of its original activity (Table 2.4).

Modification of tyrosine by treating the enzyme with N-acetylimidazole, though resulting in the modification of 6 residues out of a total of 16 (Iwamatsu *et al.*, 1991), did not have any effect on the activity of the enzyme, suggesting that tyrosine may not have a role in the catalytic activity of S1 nuclease (Table 2.4). Similarly, modification of cysteine residues of the enzyme, by treating the enzyme with DTNB, though resulting in the modifica-

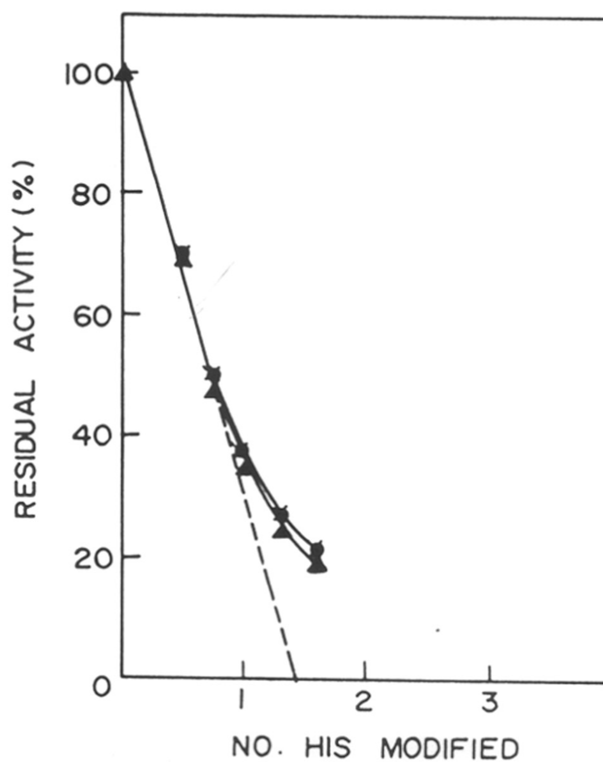

Fig. 2.10: **Plot of percent residual activity verses number of histidine residues modified.**

The number of histidine residues modified were estimated as described in the text. ssDNAase (●), RNAase (▲) and Phosphomonoesterase (✕).

tion of the only available cysteine residue (Iwamatsu *et al.*, 1991), did not affect the activity of the enzyme, showing that cysteine too may not have a role in the catalytic activity of S1 nuclease (Table 2.4).

Table 2.4 : **Effect of different modifying reagents on the activity of S1 nuclease**

Modification reaction	Number of residues modified	Residual activity (%)		
		ssDNAase	RNAase	AMPase
Control	0	100	100	100
Histidine (DEP)	1	38	35	38
Decarbethoxylation (Hydroxylamine)	-	86	82	90
Tyrosine (N-acetylimidazole)	6	100	100	100
Cysteine (DTNB)	1	100	100	100

Methylene blue and DEP mediated inactivation of S1 nuclease could not be prevented by incubating the enzyme with excess amounts of ssDNA, RNA and 3'AMP prior to the modification reaction (Table 2.5). The CD spectra of both unmodified and histidine modified S1 nuclease were almost identical (Fig. 2.11), indicating that modification does not result in a gross change in the conformation of the enzyme. In addition, the histidine modified enzyme which

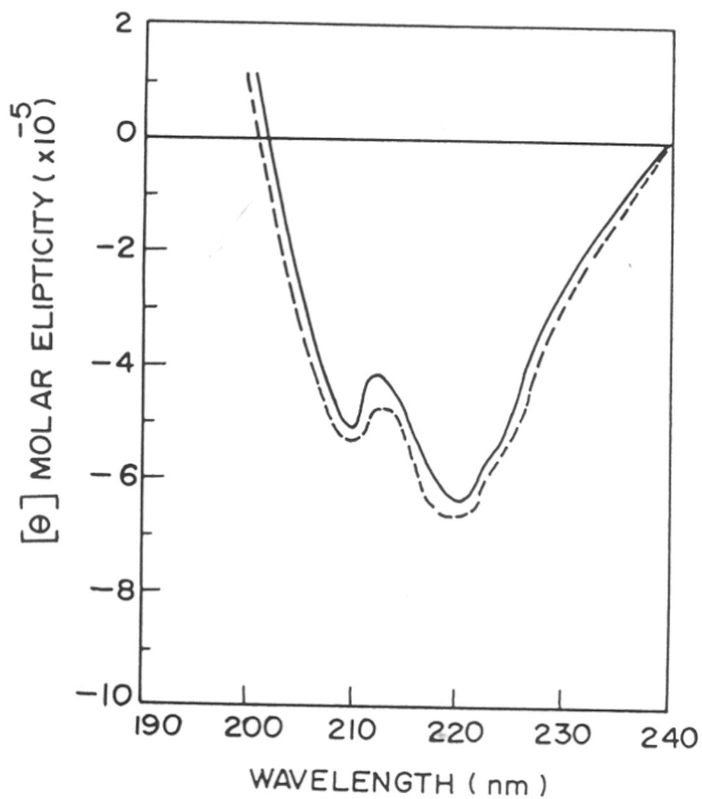

Fig. 2.11: The CD spectra of native and DEP treated S1 nuclease.

The CD measurements were performed in a 1 mm cell at an enzyme concentration 200 μg/ml. Native enzyme (——) and DEP treated enzyme (----).

had very little catalytic activity, could bind 5' AMP in a ratio of 1:1, similar to that of the unmodified enzyme. The lysine modified enzyme, on the other hand, showed approximately 70% decrease in its ability to bind 5' AMP (Table 2.5).

Table 2.5 : Influence of histidine modification on the activity of S1 nuclease: Substrate protection and inhibitor binding studies

Incubation mixture	Residual activity(%)	5'AMP binding (%)
Enzyme	100	100
Enzyme + TNBS (0.5 mM)	32	33
Enzyme + DEP (0.2 mM)	30	100
Enzyme + ssDNA (1 mg) + DEP	32	-
Enzyme + RNA (1 mg) + DEP	30	-
Enzyme + 3' AMP (1mM) + DEP	32	-
Enzyme + MB* (0.2%)	43	-
Enzyme + ssDNA (1 mg) + MB	46	-
Enzyme + RNA (1 mg) + MB	50	-
Enzyme + 3' AMP (1mM) + MB	50	-

*methylene blue

Modification of carboxylate groups

Purified S1 nuclease when incubated with 5 mM EDC at pH 4.6, lost 50-60% of its initial activity towards ssDNA, RNA and 3'AMP. However, no loss of activity was observed in the control samples. Modification of 7 carboxylate groups was accompanied by 50-60% loss in its initial activities, with a concomitant loss of 1 zinc atom per molecule of the enzyme whereas, extensive modification (i. e. 12-15 carboxylate groups), resulted not only in a significant loss in its initial activities (>80%) but also in the loss of 2.0-2.5 zinc atoms per molecule of the enzyme (Table 2.6).

Modification of the carboxylate groups of the enzyme with WRK, at pH 7.8, resulted in 95% loss in its initial activities towards all the substrates and the inactivation was concentration dependent (Fig. 2.12). No loss of activity was observed in the control samples. Determination of the number of carboxylate groups modified, showed that the enzyme retained more than 95% of its activity when 3 carboxylate groups were modified whereas, modification of 7 carboxylate groups resulted in 50-55% loss in its activity, as well as in the loss of 1 zinc atom per molecule of the enzyme. Furthermore, extensive modification of the enzyme (>10 carboxylate groups modified) resulted in more than 85% loss of its activity with a concomitant loss of 2-3 atoms of zinc per molecule of the enzyme (Fig. 2.12, Table 2.6). CD spectra of the enzyme samples showed no qualitative

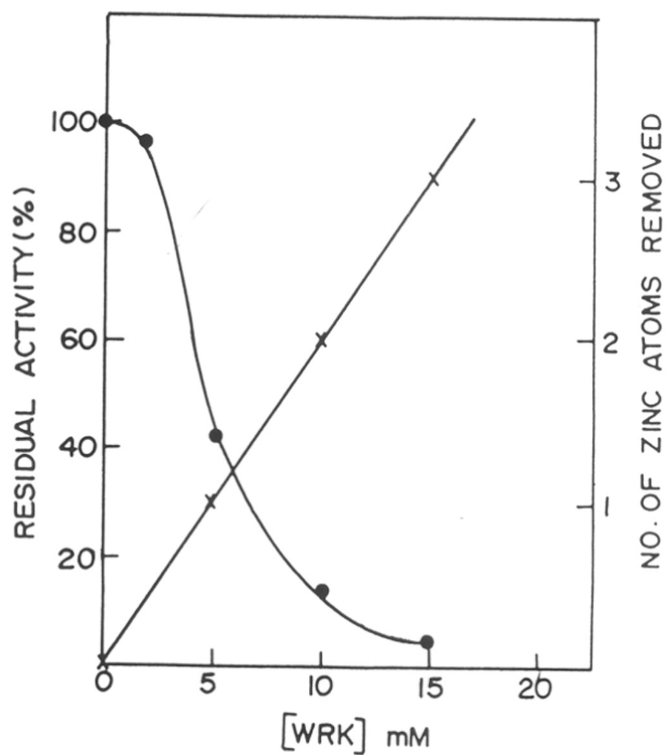

Fig. 2.12: **Effect of WRK concentration on the activity of purified S1 nuclease.**

ssDNAase (●) and number of zinc atoms removed (×).

difference when one zinc atom was removed whereas, a significant destruction of the secondary structure took place with the removal of other zinc atoms from the enzyme molecule (Fig. 2.13).

Table 2.6: Effect of different modifying reagents on the activity of S1 nuclease

Modification reaction	Number of COOH groups modified	Number of zinc atoms removed	Residual activity (%)		
			ssDNAase	RNAase	AMPase
Control	0	0	100	100	100
EDC (5 mM)	7	1.0	42	50	48
EDC (10 mM)	12	2.0	21	20	21
EDC (15 mM)	15	2.5	15	15	15
WRK (2 mM)	3	0	96	98	100
WRK (5 mM)	7	1.0	42	45	45
WRK (10 mM)	11	2.0	14	12	12
WRK (15 mM)	14	2.7	4	5	4
EDTA (1 mM)	0	1.0	45	50	50
EDTA (5 mM)	0	2.0	14	12	15
EDTA (10 mM)	0	2.8	0	0	0

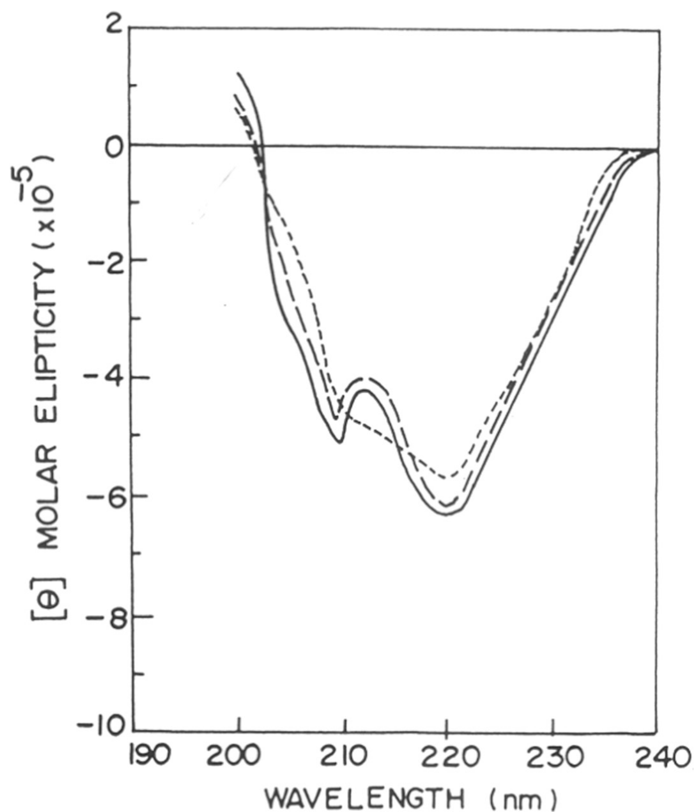

Fig. 2.13: The CD spectra of native and WRK modified Sl nuclease.

The CD measurements were performed in a 1 mm cell at an enzyme concentration 200 µg/ml.
Native enzyme (——), WRK 5 mM (— —) and WRK 15 mM (----).

Incubation of the purified S1 nuclease with different concentrations of EDTA also resulted in the loss of enzyme activity and the activity loss was dependent on the removal of zinc atoms (Table 2.6). While approximately 55% loss of activity accompanied the loss of 1 zinc atom, almost total loss of activity (>90%) occurred as a result of the removal of 2-3 zinc atoms per molecule of the enzyme. Comparison of the CD spectra of the enzyme samples showed that the removal of 1 zinc atom did not result in any significant change in the secondary structure of the enzyme whereas, the removal of the other two zinc atoms resulted in the complete collapse of its secondary structure (Fig. 2.14). In fact, a significant insolubilization (approximately 70%) of the enzyme took place as a result of removal of more than 2 zinc atoms per molecule of the enzyme.

Incubation of the EDTA treated enzyme samples with 1 mM Zn^{2+} showed that the enzyme sample devoid of one zinc atom could regain a significant amount of its activity (76%) while no reactivation was observed in case of the enzyme devoid of 2 or 3 zinc atoms. Incubation of partially inactivated enzyme (i.e. after the removal of one zinc atom) with Co^{2+} could also restore a significant amount (72%) of its original activity. Analysis of the zinc and cobalt content of the reactivated enzyme samples revealed the presence of the third zinc atom or one cobalt atom. On the contrary, in case of carboxylate groups

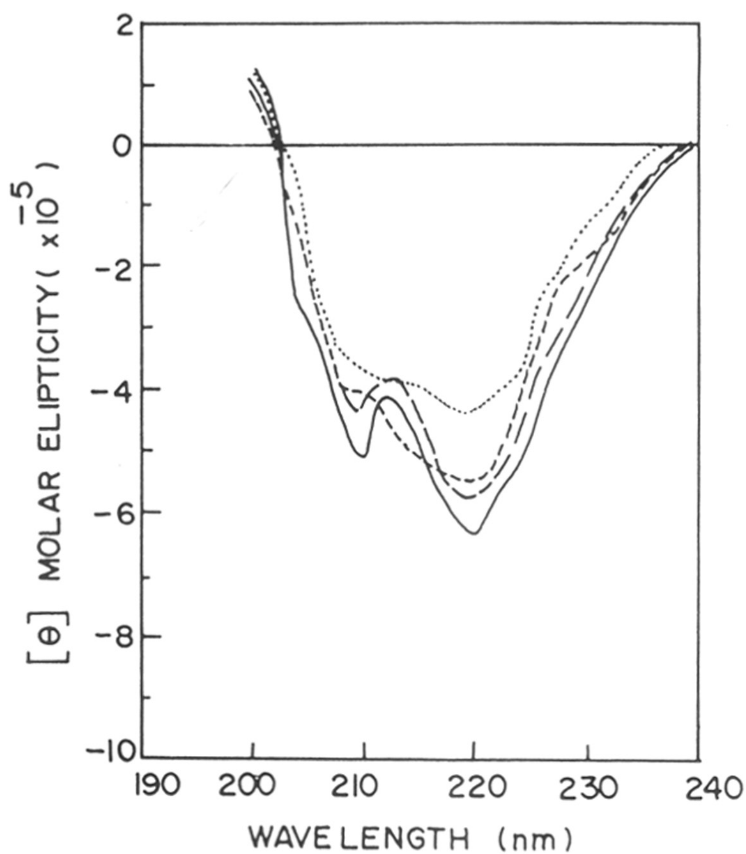

Fig. 2.14: The CD spectra of native and EDTA treated Sl nuclease.

The CD measurements were performed in a 1 mm cell at an enzyme concentration 200 µg/ml.
Native enzyme (——), EDTA 1 mM (— —), EDTA 5 mM (----) and EDTA 10 mM (····).

modified enzyme samples, zinc and cobalt did not have any effect on the activity (Table 2.7).

Table 2.7 : Reactivation of the modified S1 nuclease with zinc and cobalt

Reaction	Residual ssDNAase activity (%)	Metal ions (1 mM)	Activity after incubation (%)
Control	100	-	100
EDTA (1 mM)	42	Zn^{2+}	75
EDTA (5 mM)	14	Zn^{2+}	14
EDTA (10 mM)	0	Zn^{2+}	0
WRK (5 mM)	42	Zn^{2+}	42
EDTA (1 mM)	42	Co^{2+}	72
EDTA (5 mM)	14	Co^{2+}	14
EDTA (10 mM)	0	Co^{2+}	0
WRK (5 mM)	42	Co^{2+}	42

The loss of activity, either due to carboxylate group modification or EDTA treatment, could be prevented to a great extent by incubating the enzyme with excess amounts of ssDNA, RNA and 3'AMP, prior to the modification reaction. In case of WRK mediated inactivation of the enzyme,

pre-incubation of the enzyme with 1 mM Zn^{2+} could also protect the enzyme from inactivation to a considerable extent (Table 2.8).

Table 2.8 : **Influence of carboxylate groups modification on the activity of S1 nuclease: Substrate protection studies.**

Incubation mixture	Residual ssDNAase activity (%)
Enzyme (Control)	100
Enzyme + WRK (10 mM)	25
Enzyme + ssDNA (1 mg) + WRK	72
Enzyme + RNA (1 mg) + WRK	78
Enzyme + 3' AMP (1mM) + WRK	81
Enzyme + Zn^{2+} (1 mM) + WRK	62
Enzyme + EDTA (1 mM)	50
Enzyme + ssDNA (1 mg) + EDTA	86
Enzyme + RNA (1 mg) + EDTA	88
Enzyme + 3' AMP (1mM) + EDTA	82

DISCUSSION

Since a large amount of the enzyme is required for the structural studies, initially attempts were made to develop a simple procedure to obtain a homogeneous enzyme in good yield. In the present studies, DEAE-Sephadex chromatography was carried out at pH 5.5, as under this condition it is possible to separate RNAase T2 completely from S1 nuclease. Complete removal of RNAase T2 was essential at this step, as our previous experience showed that removal of trace amounts of RNAase T2 presented considerable difficulties due to their similar physico-chemical properties like molecular weight, glycoprotein nature and nucleotide binding (Uchida and Egami, 1971; Rushizky, 1981). However, the pooled S1 nuclease fractions obtained after DEAE-Sephadex chromatography contained a significant amount of RNAase T1 activity. Similar observations were also made by Ando (1966) and Vogt (1973) while purifying S1 nuclease on DEAE-cellulose at pH 7.0-7.5. For further purification, affinity chromatography on 5' AMP-Sepharose was preferred, as 5' AMP is a competitive inhibitor of S1 nuclease having $K_i = 2.43 \times 10^{-4}$ M (Oleson and Hoganson, 1981) and thus has desirable properties as an affinity ligand. The inability of 5' AMP-Sepharose to separate S1 nuclease and RNAase T1 can be correlated to the comparable affinities of these enzymes to the bound nucleotide. In fact, RNAase T1 has been purified on several nucleotide affinity matrices (Takahashi and Moore, 1982). Neverthe-

less, a considerable degree of purification of S1 nuclease could be achieved by this step, in addition to the removal of coloured impurities. Though, Rushizky *et al.* (1975) separated RNAase T1 and S1 nuclease on DEAE-cellulose at pH 5.0, we chose to chromatograph the enzyme on Bio-Gel P-60, as it is possible to remove RNAase T1 (molecular weight 11,000 Da) from S1 nuclease (molecular weight 32,000 Da) due to the vast difference in their molecular weights. This procedure yielded an enzyme preparation which was electrophoretically homogeneous (Fig. 2.1). The purified enzyme is a single polypeptide chain with a molecular weight of 32,000 Da (Fig. 2.2 A and B) having a pI of 4.0. Our values are consistent with that of Shishido and Habuka (1986). Oleson and Sasakuma (1980) purified S1 nuclease to near homogeneity (97-99% pure) in seven steps with 32% recovery. Subsequently, Shishido and Habuka (1986), starting from 30 mg of a partially purified S1 nuclease preparation, obtained 8 mg of pure enzyme in three steps. In comparison, our method offers a simple and rapid procedure for the purification of S1 nuclease to homogeneity with comparable yields (Table 2.1).

It has been reported that lysine and arginine residues have an essential role in the active site of enzymes binding to anionic substrates (Riordan, 1979; Richardson *et al.*, 1990). The anionic nature of the substrates of S1 nuclease prompted us to look for the possible involvement of amino groups in the catalytic activity of the enzyme.

When lysine residues of the enzyme were modified by TNBS, it lost 65-70% of its activity towards ssDNA, RNA and 3'AMP, indicating that lysine may have a role in the catalytic activity of S1 nuclease (Fig. 2.3). Kinetic analysis of TNBS inactivation revealed that the loss of activity towards all the substrates is due to the modification of a single lysine residue (Fig. 2.4). The involvement of lysine in the catalytic activity of S1 nuclease was further ascertained by acylation and reductive methylation. Citraconylation of the purified enzyme led to its rapid inactivation and 70-75% of its initial activity was lost towards ssDNA, RNA and 3'AMP (Fig. 2.5). Decitraconylation could recover a significant amount of its activity towards all the substrates. Similar results were obtained when lysine residues of S1 nuclease were converted to N, N'-dimethyllysine by reductive methylation. The loss of activity on citraconylation and reductive methylation resulted from the modification of one lysine residue per molecule of the enzyme (Table 2.2). The above results point towards the presence of lysine at or near the active site of S1 nuclease. Moreover, the comparable loss of activities of S1 nuclease as a result of lysine modification by different reagents suggests that modification does not result in a drastic change in the active site conformation of the enzyme.

Substrate protection studies on TNBS modified enzyme revealed that the inactivation could be prevented to a

considerable extent by pre-incubating the enzyme with excess amounts of ssDNA, RNA and 3'AMP (Table 2.3). Additionally, lysine modification did not bring about any gross change in the enzyme structure indicating, that the loss of enzyme activity is due to lysine modification rather than structural changes (Fig. 2.6). However, the modified enzyme showed decreased 5' AMP binding (Table 2.3). These results indicate the involvement of lysine in substrate binding. In case of RNAase A, it has been shown that lysine residues are involved in the substrate binding (Richardson *et al.*, 1990).

Since histidine has been implicated in the catalytic activity of several nucleases and DNA polymerase I (Pandey *et al.*, 1987), modification of histidine was carried out to evaluate its role in the catalytic activity of S1 nuclease. When purified S1 nuclease was incubated with methylene blue, at pH 7.5 and 26°C for 30 min, it resulted in 60-70% loss of its initial activity towards ssDNA, RNA and 3' AMP. The inhibition of the enzyme activity could be prevented by shielding the enzyme-methylene blue mixture from irradiation, indicating the presence of histidine at or near the active site. The pH-dependent inactivation of the enzyme was similar to that observed in the case of photo-oxidation of free histidine (Weil, 1965) and also in the photo-inactivation of several enzymes with histidine at their active site (Westhead, 1965; Martinez-Carrion *et al.*, 1967; Chatterjee and Noltmann, 1967), suggesting the

presence of histidine at or near the active site of S1 nuclease (Fig. 2.7).

The involvement of histidine in the catalytic activity of S1 nuclease was further ascertained by modifying the enzyme with a histidine specific reagent *viz* DEP. Modification of the enzyme with DEP also resulted in a significant loss in its activity towards ssDNA, RNA and 3' AMP, indicating that histidine may have a role in the catalytic activity of S1 nuclease. Kinetic analysis of DEP inactivation revealed that the loss of activity towards all the substrates is due to the modification of a single histidine residue (Fig. 2.9). The DEP mediated inactivation of the enzyme was accompanied by a significant increase in the absorbance of the modified enzyme at 240 nm, which is characteristic of ethoxycarboxylation of histidine residues. Determination of the number of essential histidine residues following DEP modification indicated that the modification of a single histidine is responsible for the loss of enzyme activity towards all the substrates (Fig. 2.10). Hydroxylamine treatment of the DEP modified enzyme restored a significant amount of its activity towards all the substrates, substantiating the role of histidine in the catalytic activity of S1 nuclease (Table 2.4).

Though, DEP is specific for histidine at or around neutral pH, it also reacts, to a lesser extent, with tyrosine, cysteine and lysine residues (Miles, 1977). However, N-acetylimidazole treatment of purified S1 nuclease did not

bring about any loss in the enzyme activity, suggesting that tyrosine may not have a role in activity of S1 nuclease (Table 2.4). The modification of tyrosine, as a result of DEP treatment, was further ruled out by the observation that there was no significant decrease in the absorbance of the modified protein at 278 nm. Though the above observations support the presence of histidine at or near the active site, they still do not rule out the possible involvement of cysteine. Hence, modification of cysteine was carried out with a cysteine specific reagent namely, DTNB. DTNB treated enzyme retained its full activity, ruling out the involvement of cysteine in the catalytic activity of S1 nuclease. Lysine has already been implicated in the catalytic activity of S1 nuclease (page 112). However, the loss of activity of S1 nuclease as a result of DEP treatment cannot be correlated to lysine modification, since the DEP modified enzyme could recover a significant amount of its activity in the presence of hydroxylamine. Had the inactivation of S1 nuclease been due to lysine modification, then hydroxylamine treatment would not have restored its activity.

Studies on substrate protection revealed that methylene blue and DEP mediated inactivation could not be prevented by incubation of the enzyme in presence of excess amounts of ssDNA, RNA and 3' AMP, prior to the modification reaction (Table 2.5). Additionally, DEP modification did not bring about any gross change in the enzyme structure,

indicating that the loss of enzyme activity is due to histidine modification rather than structural changes (Fig. 2.11). However, the DEP-modified S1 nuclease fully retained its ability to bind 5' AMP whereas, the TNBS modified enzyme showed a significant decrease in its ability to bind 5' AMP, under identical conditions (Table 2.5). These results point towards the involvement of histidine in catalysis rather than in substrate binding.

As mentioned earlier, S1 nuclease catalyses the hydrolysis of ssDNA, RNA and 3' AMP (Oleson and Sasakuma, 1980). Oleson and Hoganson (1981) interpreted the ability of 3'AMP to inhibit the hydrolysis of ssDNA competitively and *vice versa*, to the existence of a common catalytic site for the hydrolysis of both types of substrates. In the present studies, the parallel loss of all the three activities of S1 nuclease on lysine and histidine modification, coupled with substrate protection, decitraconylation and hydroxylamine treatment data, confirms the presence of a common catalytic site responsible for the hydrolysis of both monomeric and polymeric substrates. Our results are consistent with those of Oleson and Hoganson (1981) and Reddy and Shankar (1989).

S1 nuclease is an acidic metalloprotein and contains 3 g atoms of zinc/mole of the enzyme. It has been shown that the removal of zinc atoms by dialysis of the enzyme against EDTA results in its inactivation and a significant

amount of its activity can be restored by incubation in presence of excess Zn^{2+} or Co^{2+} (Shishido and Ando, 1985). Subsequently, Shishido and Habuka (1986) demonstrated that the removal of 2 zinc atoms resulted in the irreversible inactivation of the enzyme and the inactivation was due to the disruption of its secondary structure. The involvement of carboxylate groups in metal binding (Brand *et al.*, 1988) and in the catalytic activity of RNAase T1 (Takahashi and Moore, 1982) and Staphylococcal nuclease (Weber *et al.*, 1991) have been demonstrated. Since S1 nuclease is a zinc containing enzyme having a large number of acidic amino acids, attempts were made to evaluate the possible role of carboxylate groups in the structure-function relationship of the enzyme.

Modification of the carboxylate groups by EDC resulted in a concentration dependent inactivation of the enzyme, indicating that carboxylate groups may have a role in the catalytic activity of the enzyme. However, no loss of activity was observed in the control samples. The analysis of the zinc content of the carboxylate group modified enzyme revealed the loss of 1 - 2.5 atoms of zinc/molecule of the enzyme (Table 2.6), suggesting that the loss of activity could be due to the loss of metal ions and that carboxylate groups may have a role in the metal binding. Modification of the enzyme with WRK also resulted in a significant loss in the enzyme activity towards ssDNA, RNA and 3'AMP and the extent of inactivation was dependent on

the number of carboxylate groups modified and in turn, on the number of zinc atoms removed (Fig. 2.12; Table 2.6). To establish the correlation between enzyme activity and loss of zinc atoms, selective removal of zinc atoms was carried out by treating the enzyme with different concentrations of EDTA. The results revealed that the loss of enzyme activity was dependent on the number of zinc atoms removed (Table 2.6). However, the ability of zinc and cobalt to restore a significant amount of activity of the EDTA treated partially inactivated enzyme (depleted of 1 zinc atom) and the inability of carboxylate groups modified (WRK treated), partially inactivated enzyme (depleted of 1 zinc atom), to regain the activity in presence of the aforementioned metal ions, points toward the involvement of carboxylate groups in metal binding (Table 2.7).

Substrate and metal ion (Zn^{2+}) protection studies showed that the WRK mediated inactivation could be prevented to a considerable extent by pre-incubating the enzyme in presence of excess amounts of ssDNA, RNA, 3'AMP and Zn^{2+}. Fujimoto *et al.* (1974b) studied the inactivation of P1 nuclease by EDTA treatment and observed that the substrate could prevent the inactivation of the enzyme. Similarly, pre-incubation of S1 nuclease with excess amounts of substrates protected the enzyme from inactivation. The inability of EDTA to inactivate the enzyme in presence of substrates can be correlated to the non-availability of the metal ions in presence of substrates (Table 2.7).

Comparison of the CD spectra, of EDTA treated and WRK modified enzyme samples, revealed that while the removal of one zinc atom from the enzyme molecule did not result in a significant change in its gross conformation, a considerable disruption of the secondary structure took place with the removal of the two remaining zinc atoms (Fig. 2.13; 2.14). The latter could account for the irreversible inactivation of the enzyme. Our results are in agreement with that of Shishido and Habuka (1986). Moreover, the extent of changes in the secondary structure as a result of removal of metal ions could explain the ability of partially inactivated EDTA treated S1 nuclease to recover significant amount of its activity in presence of Zn^{2+} and Co^{2+}. Though WRK treated, partially inactivated, enzyme did not show any significant change in its secondary structure, the inability of Zn^{2+} and Co^{2+} to restore the activity can be correlated to the non-availability of the carboxylate groups for metal binding. This observation further supports our claim that carboxylate groups are involved in the metal binding.

Rokugawa *et al.* (1980a) carried out a systematic investigation on the role of zinc in P1 nuclease (a single strand specific nuclease from *P. citrinum*) by selective removal of zinc by treating the enzyme with EDTA 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 50% loss in its

activity towards RNA but it retained 93% of its activity towards 3'AMP. While the removal of the second zinc atom brought about a significant decrease in the RNAase (45%) and phosphomonoesterase (60%) activities of P1 nuclease, the removal of all the three zinc atoms resulted in the complete inactivation of the enzyme and a complete disruption of the enzyme structure. Based on this data, the authors concluded that while Zn I is involved in maintaining the tertiary structure required for RNA binding, Zn II is essential for maintaining the active conformation and Zn III is involved in holding the structural integrity of the enzyme. However, unlike in P1 nuclease, the depletion of zinc atoms was accompanied by a parallel loss of all the three activities of S1 nuclease. The removal of one zinc atom resulted in the partial loss of activity with no significant change in its secondary structure whereas, the removal of the second zinc atom resulted in the gradual unfolding of the polypeptide chain and a complete unfolding with a significant insolublization of the enzyme took place with the removal of all three zinc atoms from the enzyme molecule.

The present studies show that the inactivation of S1 nuclease as a result of either EDTA treatment or WRK modification is due to the loss of zinc atoms and carboxylate groups are involved in metal binding. Furthermore, the zinc present in S1 nuclease can be divided into two types *viz*, a loosely bound i.e. an easily replaceable (Zn I) and

strongly bound (Zn II and Zn III). The easily replaceable zinc may have a role in the catalytic activity while the strongly bound zinc atoms provide the structural integrity to the enzyme.

In conclusion, the chemical modification studies on S1 nuclease 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, histidine is involved in catalysis. Carboxylate groups, on the other hand, are involved in metal binding. Moreover, all the activities associated with S1 nuclease are catalyzed by the same active site.

Chapter III

Immobilization of S1 Nuclease

SUMMARY

Purified S1 nuclease, when covalently coupled to AB-Bio-Gel P-2, *via* its carbohydrate moiety, retained 40-50% activity of the soluble enzyme. Optimization of coupling conditions showed that the most active immobilized preparations are obtained when 50 - 60 U of 1 mM periodate oxidized enzyme are reacted with 1 ml (packed volume) of AB-Bio-Gel P-2 at 4°C, in presence of 20% (v/v) ethylene glycol, for 15 h. Immobilization did not change the pH and temperature optima of the enzyme, but it increased the temperature stability. Immobilization brought about approximately 2-fold increase in the K_m and a slight decrease in the V_{max}. On repeated use, the bound enzyme retained 60 - 65% of its initial activity after six cycles. Immobilized S1 nuclease could be stored, in wet state, for more than 45 days without any significant loss in its initial activity. Application of immobilized S1 nuclease in removing restriction endonuclease generated single stranded tails in plasmid DNA is demonstrated.

INTRODUCTION

S1 nuclease (EC 3.1.30.1) from *Aspergillus oryzae*, is an analytically important enzyme and is used extensively for the determination of nucleic acid structure (Rushizky, 1981). It has been observed that after the nuclease treatment of nucleic acid samples, removal of residual enzyme activity is essential and involves tedious emulsion forming

phenol extraction steps, which in turn result in the loss of nucleic acid samples. Under such circumstances, use of immobilized enzyme offers a distinct advantage over the soluble enzyme, as the bound enzyme can be easily removed from the reaction mixture by physical methods. S1 nuclease is a glycoprotein and contains 18% carbohydrate (Rushizky, 1981). It has been reported that coupling of glycoproteins to solid supports poses considerable difficulties due to the shielding of the reactive groups of amino acid side chains by carbohydrates. To overcome this problem, several glycoprotein conjugates have been prepared, where the carbohydrate side chains provide the point of attachment between the enzyme and the matrix (Zaborsky and Ogletree, 1974; Hsiao and Royer, 1979; Marek *et al.*, 1984; O'Shannessy and Wilchek, 1990). Such methods of binding, either by adsorption or covalent coupling, to solid supports afford high retention of enzyme activity, probably due to the fact that the carbohydrate moiety of the enzyme is not essential for its catalytic activity and hence the protein moiety is free of the restrictions imposed upon it as a result of binding. In case of S1 nuclease, it has been shown that the carbohydrate moiety of the enzyme is not essential for its catalytic activity and stability (Shishido and Habuka, 1986). In view of this, attempts were made to bind the enzyme covalently through its carbohydrate moiety and assess its potential as a reusable analytical tool.

MATERIALS

Bio-Gel P-2 (40-80 µm; Bio-Rad, USA); ethylenediamine and sodium periodate (Sisco Research Laboratories, India); butyl-, hexyl- and octyl-diamines and sodium cyanoborohydride (Aldrich Chemical Co., USA); ethylene glycol (SD Fine Chemicals Pvt. Ltd., India); toluene (BDH, India); BSA, 2, 4, 6-trinitrobenzenesulphonic acid (TNBS) and concanavalin (Con) A-Sepharose (Sigma Chemical Co., USA) and Bam HI, DNA polymerase (Klenow) and dNTPs (New England Biolabs, USA) were used. [α-^{32}P] dCTP was obtained from BARC, India. All other chemicals used were of analytical grade. High molecular weight DNA from buffalo liver was isolated as described in Chapter II (page 62).

METHODS

Enzyme assays

Single strand DNAase activity of S1 nuclease was assayed, at pH 4.6 and 37°C, by measuring the amount of acid soluble nucleotides liberated, at 260 nm, following the hydrolysis of sonicated and heat denatured DNA, as described before (Chapter II, page 67).

The immobilized enzyme was assayed by incubating appropriate amounts of the bound enzyme with 3 ml of the standard reaction mixture at 37°C, in a thermostated shaker water bath (100-125 rpm) for 15 min, followed by estimating the acid soluble nucleotides liberated at 260 nm.

One unit of the enzyme is defined as the amount of enzyme required to liberate 1.0 µmol of acid soluble nucleotides/min under standard assay conditions.

Protein determination

Protein was estimated according to Lowry *et al.* (1951) using BSA as standard. The blue colour developed after the addition of Folin phenol reagent was measured at 660 nm.

Purification of the enzyme

S1 nuclease, from Takadiastase powder, was purified to homogeneity as described in Chapter II (page 71). The specific activity of the purified enzyme, which ranged from 1200 - 1250, was used for immobilization studies.

Preparation of aminated matrices

Amination of Bio-Gel P-2 was carried out essentially according to the procedure of Inman and Dintzis (1969). In a typical experiment, 50 ml of ethylenediamine was pre-heated under mild stirring in an oil bath maintained at 90°C, kept in a fume hood. Subsequently, 5 g of dry Bio-Gel P-2 beads were gradually added and the reaction was allowed to proceed at 90°C for 5 h. However, the amination of Bio-Gel P-2 with butyl-, hexyl- and octyl-diamines were carried out by refluxing 5 g (dry weight) of Bio-Gel P-2 with 25 ml of 20% (w/v) of the respective diamine in toluene, at 120°C for 5 h. At the end of the reaction, the reaction mixtures were cooled in ice and mixed with equal

volume of crushed ice. The aminated gels were then washed extensively with 100 mM NaCl to remove the excess free amines (as indicated by a negative colour test with TNBS), followed by 50 mM sodium acetate buffer, pH 5.0 and stored in the same buffer, at 4°C, till further use.

The amino group content of aminoethyl (AE)-, aminobutyl (AB)-, aminohexyl (AH)- and aminooctyl (AO)-Bio-Gel P-2, determined according to the method of Antoni *et al.* (1983), were 189, 172, 170 and 165 µmoles/ml of the matrix, respectively.

Periodate oxidation of S1 nuclease

One ml of the purified enzyme (50 U) in 50 mM sodium acetate buffer, pH 5.0, was incubated with 1 mM sodium periodate, at 4°C for 30 min, in dark. Subsequently, the reaction was arrested by the addition of 50 µl of ethylene glycol and incubated for an additional 30 min. The reaction mixture was then dialyzed extensively against 50 mM sodium acetate buffer, pH 5.0 and used for immobilization studies.

Immobilization technique

In a typical experiment, 50 U (40 µg protein) of oxidized S1 nuclease, in 2 ml of 50 mM sodium acetate buffer, pH 5.0, was incubated with 1 ml (packed volume) of the aminated Bio-Gel P-2, at 4°C for 15 h, under mild agitation. The supernatant was decanted and the matrix was washed successively with the coupling buffer, 1 M NaCl in

the coupling buffer and finally with the assay buffer (30 mM sodium acetate buffer, pH 4.6, containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% v/v glycerol) till the washings showed no nuclease activity. The washings were pooled and concentrated to 100 µl and used for protein determination. The amount of enzyme and protein bound to the matrix was determined by estimating the difference in the enzyme activity and protein before loading and after coupling. When the immobilization was carried out in presence of ethylene glycol, it was added in optimum concentration (20% v/v) during coupling.

To determine the non-specific adsorption of the enzyme on AB-Bio-Gel P-2, a similar experiment was carried out with the native enzyme (without periodate oxidization) under identical conditions.

Determination of efficiency

Efficiency of the immobilized S1 nuclease preparation was determined by assaying appropriate amounts of the bound enzyme, under standard assay conditions, followed by calculation of the ratio of measured activity to bound activity. The specific activity of the immobilized enzyme is defined as measured activity/mg protein/ml matrix.

Immobilization on Con A-Sepharose

This was carried out according to the method of Reddy and Shankar (1989). Purified S1 nuclease (100 U, 80 µg protein), in 2 ml of 50 mM sodium acetate buffer, pH 5.0,

was incubated with 0.5 ml (packed volume) of Con A-Sepharose, at 4°C for 6 h, under mild stirring. The supernatant was decanted and the matrix was washed successively with the coupling buffer, 1 M NaCl in the coupling buffer and finally with the assay buffer (30 mM sodium acetate buffer, pH 4.6, containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% v/v glycerol) till the washings showed no nuclease activity. The washings were pooled and concentrated to 100 µl and used for protein determination. The amount of enzyme and protein bound to the matrix was determined by estimating the difference in the enzyme activity and protein before loading and after coupling. The efficiency of the bound enzyme was determined as described before. Con A-Sepharose-S1 nuclease conjugate retained 60% activity of the soluble enzyme.

Plasmid DNA isolation and end filling reaction

Plasmid (pUC 8) 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 *E. coli* harbouring pUC 8 plasmid followed by incubation at 37°C, under vigorous agitation, for 15 h. 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 1 mM EDTA, pH 8.0) and after resuspending in 10 ml of 25 mM Tris-HCl, 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 in 200 mM NaOH and incubated at 0°C for 10 min. After the incubation period, 15 ml of an ice-cold solution of 5 M 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) at room temperature, washed with absolute ethanol, dried free of ethanol, dissolved in distilled water and stored at -20°C. The purity of the isolated DNA was checked by absorbance ratio of A_{260} : A_{280} and agarose gel electrophoresis.

The pUC 8 DNA was then linearized using Bam HI restriction endonuclease. The Bam HI digest of pUC 8 (1 µg) was incubated with 0.1 U of soluble, Bio-Gel- and Con A-Sepharose-bound S1 nuclease, in 30 mM sodium acetate buffer, pH 4.6, containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% (v/v) glycerol, at 37°C for 10 min. The reaction was then terminated by the addition of 5 mM EDTA (effective concentration) and the digested DNA samples were extracted with phenol/chloroform. DNA samples incubated under identical

conditions, in the absence of S1 nuclease, served as control.

Control and S1 nuclease digested DNA samples were labeled by end filling reaction using DNA polymerase (Klenow) and [α-^{32}P] dCTP. The total reaction mixture of 20 μl contained 100 ng DNA, 10 mM dNTPs (dATP, dTTP and dGTP), 5 U of DNA polymerase (Klenow), 5 μCi [α-^{32}P] dCTP and an appropriate amount of the reaction buffer. The reaction was allowed to proceed for 2 h at 20°C and then arrested by the addition of 1 μl of 0.5 M EDTA. Subsequently, 1 μg of tRNA was added to the reaction mixture as a carrier. The salt concentration was then adjusted to 150 mM and the DNA was precipitated with absolute ethanol (-20°C, overnight). The precipitate was collected by centrifugation, washed with 70% ethanol and lyophilized. The radioactivity incorporated in the DNA samples was measured on a BETAmatics scintillation counter. The labeling of DNA was also confirmed by agarose gel electrophoresis followed by autoradiography. For comparison, 100 ng of cold DNA samples were electrophoresed in 0.8% agarose gel and visualized under UV light, after staining with ethidium bromide.

RESULTS

When 50 U of periodate oxidized S1 nuclease was reacted with 1 ml (packed volume) of AE-Bio-Gel P-2, 25 U were bound. The effectiveness factor (η) of the immobilized preparation was approximately 0.22, indicating the

efficiency of the immobilized enzyme system to be 22%. Influence of oxidation conditions revealed that optimal results are obtained when the enzyme is oxidized with 1 mM of sodium periodate, at pH 5.0 and 4°C. Sodium periodate at this concentration did not have any significant effect on the activity of the soluble enzyme. Increase in the sodium periodate concentration, though did not have any adverse effect on the activity of the soluble enzyme to a great extent, the bound enzyme showed less activity. The decrease in the efficiency, in case of enzyme samples oxidized with higher concentrations of sodium periodate (>1 mM), was due to overcrowding of the enzyme on the matrix, as there was a decrease in the specific activity of the bound enzyme (Fig. 3.1). Effect of spacer length on the activity of immobilized S1 nuclease showed that, after an initial increase the efficiency decreased with increase in the spacer length and maximum retention of the activity was obtained when the enzyme was bound to AB-Bio-Gel P-2, although there was no significant difference in the ligand density of the matrices. The decrease in the efficiency observed in case of the enzyme bound to AH-Bio-Gel P-2 and AO-Bio-Gel P-2, was due to non-specific binding of the enzyme to the matrix, as there was a decrease in the specific activity of the bound enzyme (Fig. 3.2). Since optimal results were obtained with the enzyme bound to AB-Bio-Gel P-2, further experiments were carried out with this matrix. Studies on matrix to enzyme ratio, on the activity

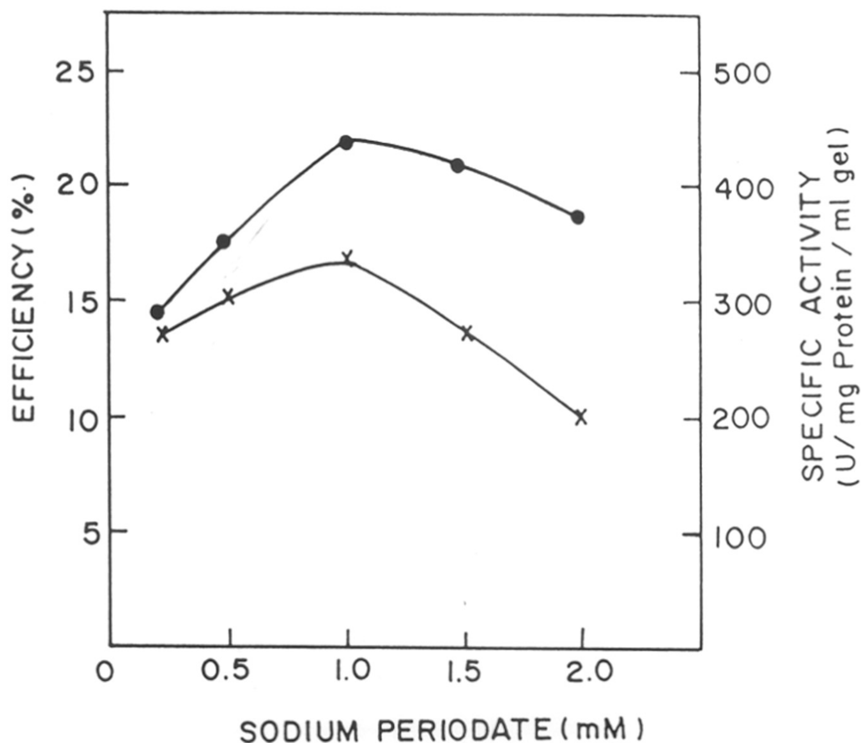

Fig. 3.1: **Effect of sodium periodate concentration on the efficiency of AE-Bio-Gel bound Sl nuclease. Efficiency (●) and specific activity (x).**

50 U of Sl nuclease were oxidized with various concentrations of sodium periodate and the immobilization was carried out as described under Methods.

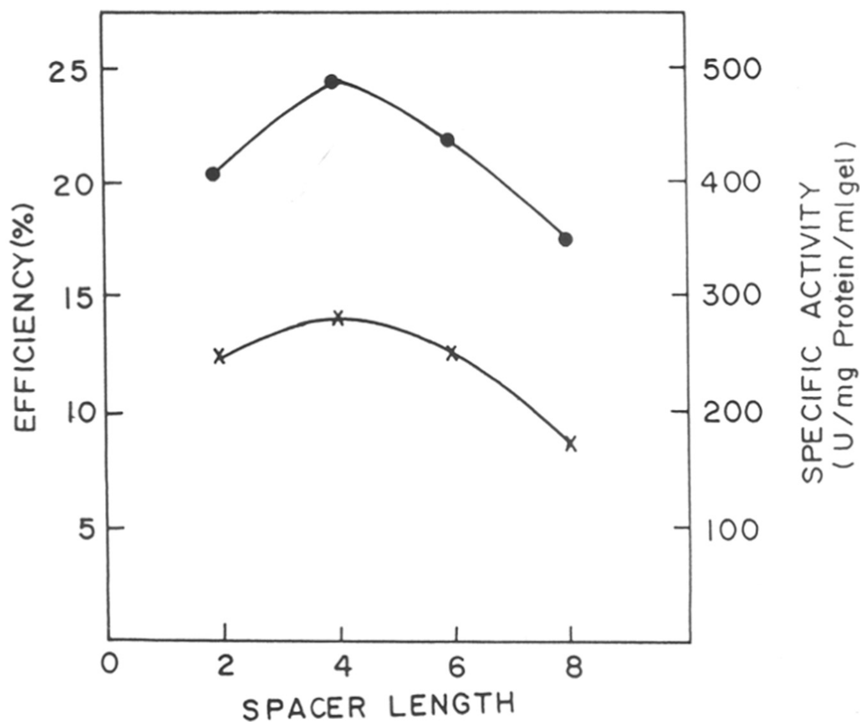

Fig. 3.2: **Effect of spacer length on the efficiency of immobilized S1 nuclease. Efficiency (●) and specific activity (✖).**

50 U of oxidized S1 nuclease were reacted with 1 ml (packed volume) of Bio-Gel P-2 having increasing spacer length, at pH 5.0 and 4°C for 15 h and efficiency of the immobilized preparations were determined as described under Methods.

of AB-Bio-Gel P-2 bound S1 nuclease, showed that maximum retention of activity is obtained when approximately 60 U of the oxidized enzyme are reacted with 1 ml (packed volume) of AB-Bio-Gel P-2. The decrease in the efficiency at higher enzyme load (>60 U) can be attributed to overcrowding of the enzyme on the matrix, as there was a decrease in the specific activity of the bound enzyme (Fig. 3.3). Periodate oxidized S1 nuclease bound to AB-Bio-Gel P-2 retained 27% activity of the soluble enzyme but the efficiency could be increased to a great extent by coupling the enzyme in presence of 20% (v/v) ethylene glycol. The results of a typical procedure for the immobilization of periodate oxidized S1 nuclease on AB-Bio-Gel P-2, under optimized conditions, are given in Table 3.1.

Table 3.1 : Immobilization of periodate oxidized S1 nuclease on AB-Bio-Gel P-2

Enzyme loaded		Enzyme bound		Activity of the complex (U expressed)	Efficiency* (%)
U	protein (μg)	U	protein (μg)		
50	41	19	16	8.4	44.2

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

When 50 U of periodate oxidized S1 nuclease were reacted with 1 ml (packed volume) of AB-Bio-Gel P-2, in

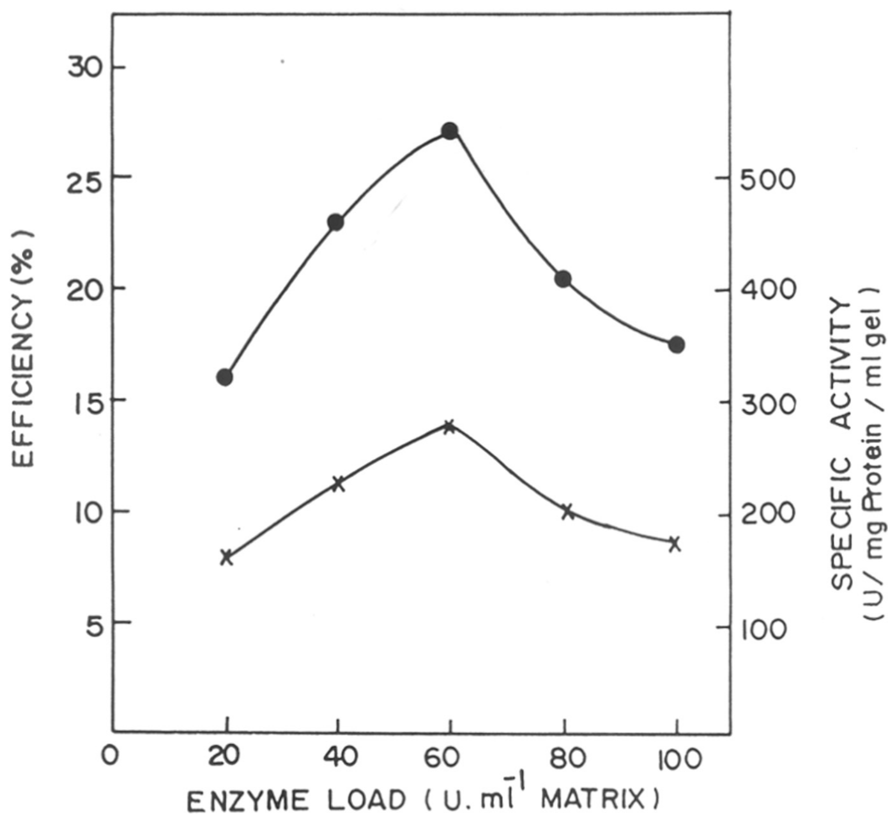

Fig. 3.3: **Effect of matrix to enzyme ratio on the efficiency of immobilized S1 nuclease. Efficiency (●) and specific activity (✖).**

One ml (packed volume) of AB-Bio-Gel P-2 was incubated with varying amounts (20-100 U), of periodate oxidized enzyme, at pH 5.0 and 4°C for 15 h and the efficiency of the immobilized preparations were determined as described under Methods.

presence of 20% (v/v) ethylene glycol, at pH 5.0 for 15 h, 19 U were bound. The effectiveness factor (η) of the immobilized preparation was 0.44, indicating the efficiency of the immobilized system to be 44% (Table 3.1).

Comparison of the pH activity profiles of soluble and immobilized S1 nuclease revealed no change in the optimum pH of the enzyme, as a result of immobilization (Fig. 3.4). Similarly, no change was observed in the optimum temperature of the enzyme due to immobilization (Fig. 3.5). Determination of kinetic parameters of soluble and immobilized S1 nuclease showed approximately 2-fold increase in the K_m and a slight decrease in the V_{max} (Table 3.2).

Table 3.2: **Kinetic data of soluble and immobilized S1 nuclease**

State of the enzyme	K_m (mg/ml)	V_{max} (µmoles/mg protein/ ml matrix)
Soluble	0.250	2500
Immobilized	0.400	2000

Temperature stability studies on soluble and immobilized S1 nuclease showed the bound enzyme to be more stable, as indicated by its inactivation pattern. While the soluble enzyme lost more than 80% of its initial activity, in 30 min at 70°C, the immobilized enzyme retained 75% of

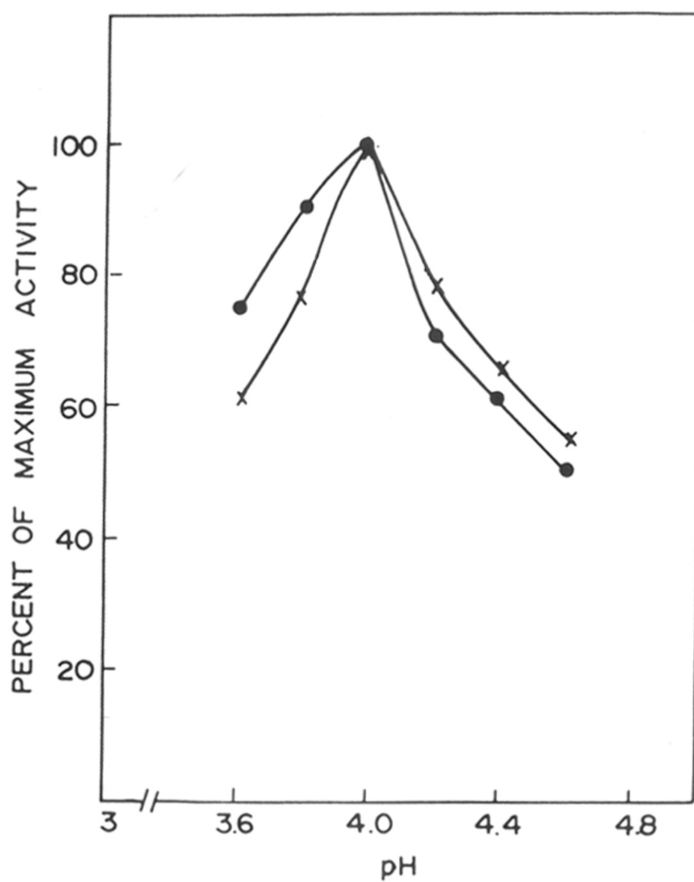

Fig. 3.4: pH activity profiles of soluble (●) and immobilized (X) Sl nuclease.

Both soluble and immobilized enzymes (1 U) were assayed in a series of pH (3.6 - 4.6) at 37°C as described under Methods.

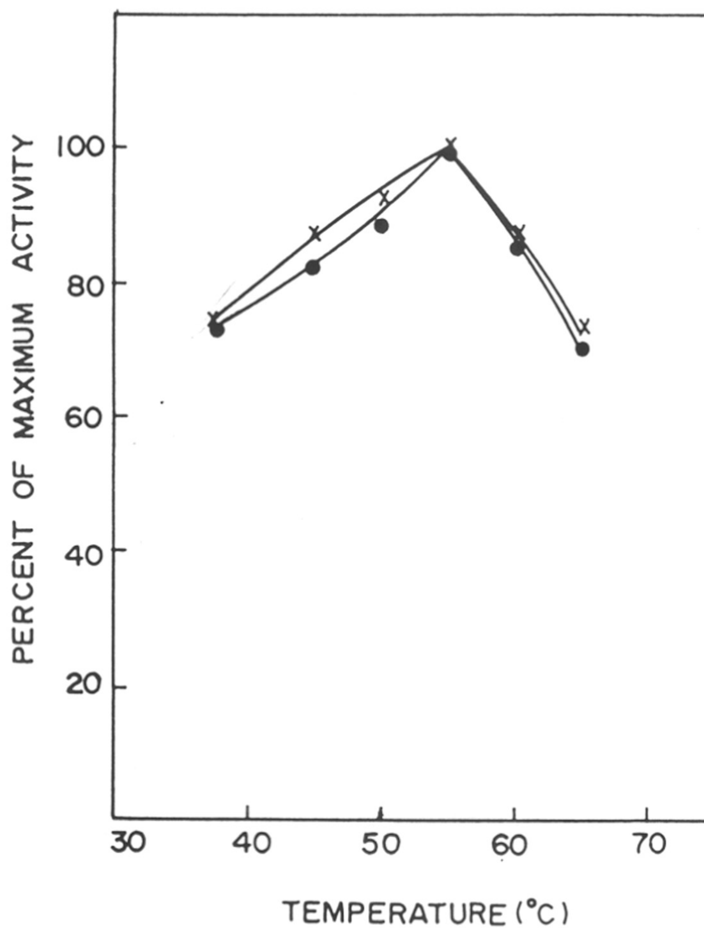

Fig. 3.5: Temperature activity profiles of soluble (●) and immobilized (×) S1 nuclease.

Appropriate amounts of soluble and immobilized enzymes (1 U) were assayed in a series of temperatures (37 - 65°C) at pH 4.6 as described under Methods.

its original activity (Fig. 3.6). Studies on repeated usability showed that the bound enzyme retained approximately 60% of its initial activity after 6 cycles. Comparable results were obtained when AB-Bio-Gel P-2 bound enzyme, stabilized by cyanoborohydride reduction (10 mM sodium cyanoborohydride at pH 5.0 and 4°C for 30 min), was subjected to repeated use (Fig. 3.7). The bound enzyme showed good storage stability and could be stored in 30 mM sodium acetate buffer, pH 4.6 (containing 1 mM $ZnSO_4$, 50 mM NaCl and 5% v/v glycerol) at 4°C, for more than 45 days, without any apparent loss in its initial activity.

The soluble and immobilized (i. e. Bio-Gel- and Con A-Sepharose-bound) S1 nuclease could effectively remove the single stranded tails from Bam HI digested pUC 8 DNA, as indicated by the inability of Klenow to label the S1 nuclease treated DNA samples (Table 3.3, Fig. 3.8).

Table 3. 3 : **Comparison of DNA labeling efficiencies of soluble and immobilized S1 nuclease.**

DNA digested with	Radioactivity incorporated CPM (%)
None	100
Soluble S1 nuclease	0.98
AB-Bio-Gel P-2 bound S1 nuclease	5.6
Con A-Sepharose bound S1 nuclease	6.3

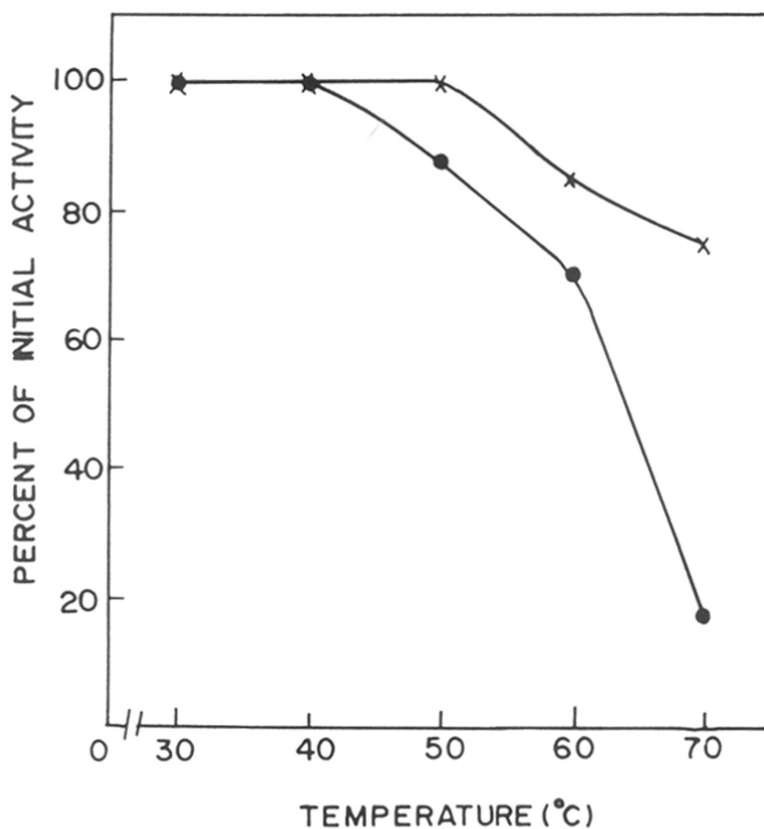

Fig. 3.6: Temperature stability of soluble (●) and immobilized (✕) Sl nuclease.

Both soluble (1 U) and immobilized (2 U) enzymes were preincubated at different temperatures, ranging from 30 - 70°C, at pH 4.6 and their residual activities were determined under standard assay conditions.

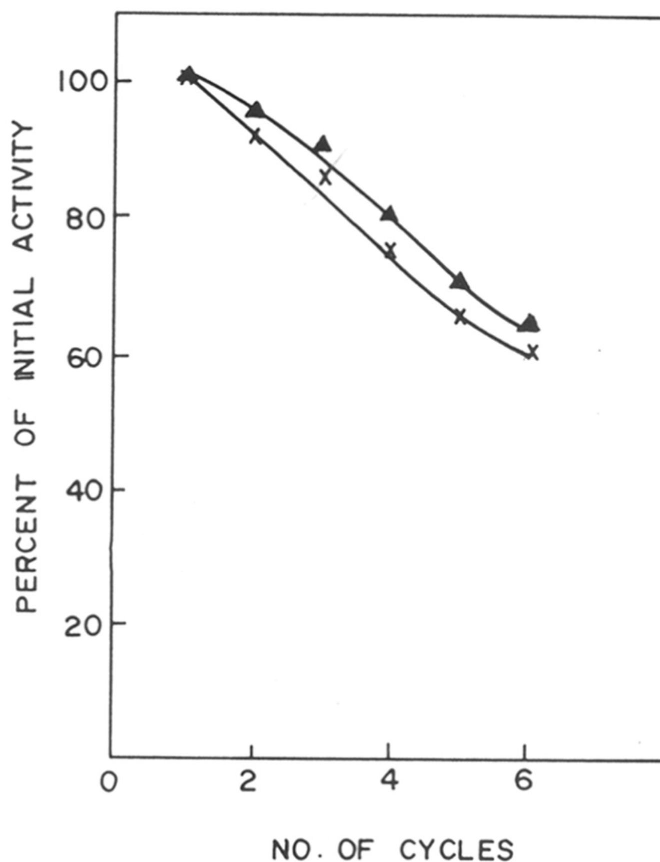

Fig. 3.7: **Effect of number of assay cycles on the activity of immobilized S1 nuclease. With (▲) and without (×) cyanoborohydride reduction.**

2 U of AB-Bio-Gel P-2 bound enzyme was assyed under standard assay conditions. After every use, the immobilized enzyme was washed free of substrate and products and used for the next assay.

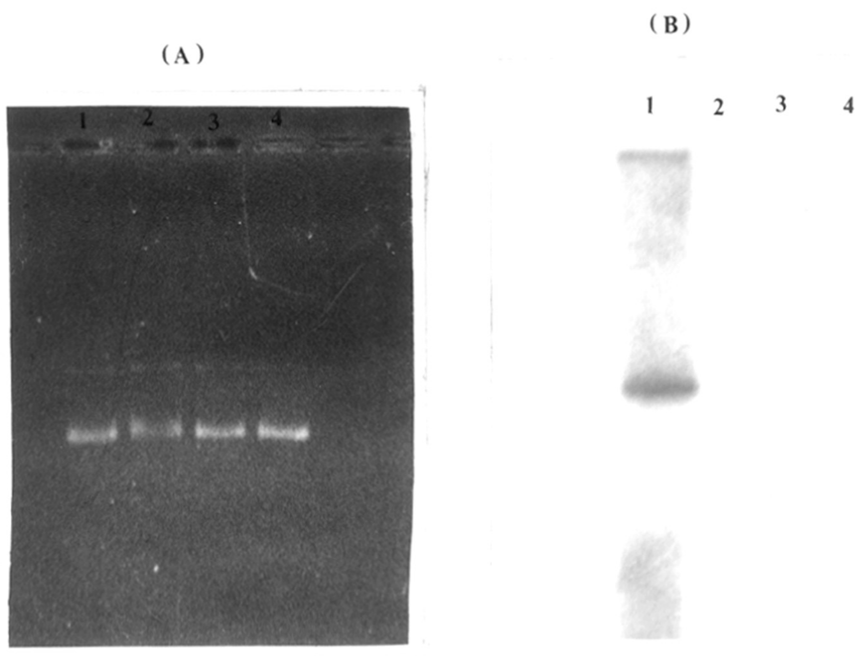

Fig. 3.8: **Agarose gel electrophoresis of S1 nuclease digested DNA and autoradiography.**

(A) DNA before labeling reaction: Lane 1 - control DNA, lane 2 - DNA digested with soluble S1 nuclease, lane 3 - DNA digested with AB-Bio-Gel P-2 bound S1 nuclease and lane 4 - DNA digested with Con A - Sepharose bound S1 nuclease.

(B) Autoradiograph of labeled DNA: Lane 1 - control DNA, lane 2 - DNA digested with soluble S1 nuclease, lane 3 - DNA digested with AB-Bio-Gel P-2 bound S1 nuclease and lane 4 - DNA digested with Con A-Sepharose bound S1 nuclease.

For experimental details please refer to Methods.

DISCUSSION

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

Optimization of coupling conditions showed that the most active preparations are obtained when approximately 60 U of 1 mM sodium periodate oxidized enzyme are reacted with 1 ml (packed volume) of AB-Bio-Gel P-2 at pH 5.0 and 4°C, for 15 h. AB-Bio-Gel P-2 bound enzyme showed only 27% activity of the soluble enzyme. Our efforts to increase

the efficiency of the immobilized preparation by coupling the enzyme in presence of competing molecules like, ethanolamine and glycine, failed to improve the retention of activity, indicating that the low efficiency is not due to the multiple attachment of the enzyme to the matrix. Moreover, increase in the spacer length, stirring speed and incubation period also failed to improve the retention of activity, showing that the low activity observed in the present case can not be correlated to diffusional limitations or to the lack of exposure of the bound enzyme to substrate.

Oleson and Sasakuma (1980) used phenyl-Sepharose for the purification of S1 nuclease, demonstrating that the enzyme can bind to hydrophobic matrices. In order to ascertain whether the observed low retention of activity, in the case of AB-Bio-Gel P-2 bound enzyme, is due to non-specific interactions of the enzyme with the spacer arm, the native enzyme (i. e. without periodate oxidation) was incubated with AB-Bio-Gel P-2. The results showed that while approximately 30% of the enzyme bound to the matrix, no binding took place on underivatized Bio-Gel P-2. In addition, the bound enzyme showed very low efficiency (3-4%). These results reveal that the low retention of the activity, observed in case of AB-Bio-Gel P-2-S1 nuclease conjugate, can be attributed to a decrease in the specific activity of the bound enzyme as a result of non-specific (hydrophobic) binding of the enzyme to the matrix. The

ability of 20% (v/v) ethylene glycol to increase the efficiency of the bound enzyme supports the above speculation.

Comparison of pH activity profiles of soluble and immobilized S1 nuclease showed no change in the optimum pH of the enzyme as a result of immobilization, indicating the absence of partitioning effects in the microenvironment of the bound enzyme. Similarly, there was no change in the optimum temperature of the enzyme after immobilization. Evaluation of kinetic parameters of soluble and immobilized S1 nuclease showed that the bound enzyme followed Michaelis-Menten kinetics and there was a slight increase in the K_m, suggesting the absence of significant diffusional barriers. However, the marginal decrease in the V_{max} can be correlated to the masking of some of the active sites during coupling. AB-Bio-Gel P-2 bound S1 nuclease showed higher temperature stability as compared to its soluble counterpart. Increase in the temperature stability of the bound enzyme can be attributed to its rigid conformation in bound form. The bound enzyme showed comparatively high stability to repeated use. In the absence of any detectable leaching (as evidenced by the absence of enzyme activity in the supernatant), in case of both the immobilized preparations (i. e. with and without cyanoborohydride reduction), the decrease in the activity after every use can be due to slight inactivation of the enzyme. The stability of the bound enzyme to storage also suggests its high stability.

One of the important applications of S1 nuclease is in the removal of restriction endonuclease derived sticky ends in DNAs before ligation (Shishido and Ando, 1985). In the present studies, like the soluble enzyme both i.e. AB-Bio-Gel- and Con A-Sepharose-bound S1 nuclease, could effectively remove the single stranded tails in Bam HI digested pUC 8 DNA, showing that the immobilized enzyme can be used as a reusable analytical tool.

A simple procedure for obtaining a highly active and stable immobilized S1 nuclease is described. The comparatively high activity and improved stability, coupled with its ability to remove the single stranded tails from DNA, makes both AB-Bio-Gel- and Con A-Sepharose-bound S1 nuclease, a useful tool for analytical purposes.

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List of Publications

1. Leon, D., **Gite, S.** and Shankar, V. (1992) Partial purification and immobilization of Ribonuclease T2. *Biotechnol. Appl. Biochem.* **16**, 11-18.

2. **Gite, S.** and Shankar, V. (1992) Affinity purification of Ribonuclease T1 on 5' AMP-Sepharose. *Biotechnol. Tech.* **6**, 245-248.

3. **Gite, S.**, Reddy, G. and Shankar, V. (1992) Active-site characterization of S1 nuclease I. Affinity purification and influence of amino-group modification. *Biochem. J.*, 285, 489-494.

4. **Gite, S.**, Reddy, G. and Shankar, V. (1992) Active-site characterization of S1 nuclease II. Involvement of histidine in catalysis. *Biochem. J.*, **288**, 571-575.

5. **Gite, S.** and Shankar, V. (1992) Characterization of S1 nuclease : Involvement of carboxylate groups in metal binding. *Eur. J. Biochem.*, **210**, 437-441.

6. **Gite, S.** and Shankar, V. (1993) Preparation and properties of RNase T1 immobilized on aminoethyl Bio-Gel P-2. *J. Biotechnol.* (In press).

7. **Gite, S.** and Shankar, V. (1993) Preparation, properties and applications of *Aspergillus oryzae* S1 nuclease covalently bound to aminobutyl-Bio-Gel P-2 through its carbohydrate moiety. *Biotechnol. Appl. Biochem.* (In press).