IMMOBILIZATION OF ENZYMES: PURIFICATION AND IMMOBILIZATION OF S1 NUCLEASE

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DEDICATED TO MY MOTHER

CONTENTS

DECLARATION

Certified that the work incorporated in the thesis entitled: "Immobilization of enzymes: Purification and immobilization of S1 nuclease" submitted by Mr. L. Gurucharan Reddy was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

V. Shankar
Research Guide

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L. Gurucharan Reddy

SUMMARY

Single strand specific nucleases are wide spread in origin and have been isolated from various sources like bacteria, fungi, yeast, plants and animals. These enzymes exhibit high selectivity for single stranded nucleic acids and single stranded regions in double stranded nucleic acids. Due to this property, single strand specific nucleases have found extensive application as an analytical tool in nucleic acid hybridization, analysis of nucleic acid structure, isolation of specific genes and gene manipulation. Though single strand specific nucleases with comparable properties have been isolated from Neurospora crassa, Penicillium citrinum, mung bean sprouts and yeast, S1 nuclease from Aspergillus oryzae is preferred since it can be easily prepared from commercially available α-amylase powder (Takadiastase), it is stable to low concentrations of denaturants often used in annealing and its specificity for single stranded nucleic acids is extremely high under right conditions.

S1 nuclease from Aspergillus oryzae is a sugar non-specific and multifunctional enzyme which acts on single stranded DNA, RNA and mononucleotides. Though competitive inhibition studies have shown that different activities associated with S1 nuclease are catalysed by the same active site, no conclusive proof exists till now. Secondly, it has been observed that after S1 nuclease treatment

of nucleic acid samples, removal of the residual enzyme activity from the reaction mixture is essential and requires repeated extractions with phenol which in turn results in the loss of nucleic acid samples. In such cases, use of immobilized enzymes offer a distinct advantage over the soluble enzyme since it can be removed easily from the reaction mixture by physical methods. Hence the present investigation with S1 nuclease was carried out to (a) study the nature of the catalytic site by chemical modification and (b) develop immobilization procedures to obtain highly active and stable immobilized preparations suitable for routine analytical purposes.

Chapter 1 : General Introduction

This part comprises of literature survey pertaining to single strand specific nucleases, their applications and immobilization of different nucleases.

Chapter 2 : Purification and Active Site Characterization of S1 Nuclease

Since the existing procedures for the purification of S1 nuclease are tedious, attempts were made to develop a simple and rapid procedure to obtain a homogeneous enzyme preparation suitable for chemical modification studies. In this method, Takadiastase powder, after extraction and heat treatment, was subjected to ammonium sulfate precipitation. The fraction containing S1 nuclease was chromatographed on DEAE-cellulose at pH 5.0,

a procedure in which RNase T1, RNase T2 and S1 nuclease get separated. High specific activity fractions obtained from this step were pooled and rechromatographed on DEAE-cellulose under the above conditions to obtain the purified enzyme. The enzyme preparation showed a single band both in the native and SDS-PAGE indicating its homogeneity.

When the pure enzyme was treated with different amino acid modifying reagents, only diethylpyrocarbonate (DEPC), N-bromosuccinimide (NBS), p-hydroxymercuribenzoate (pHMB) and trinitrobenzenesulfonic acid (TNBS) inhibited all the three activities, namely, ssDNase, RNase and phosphomonoesterase, associated with S1 nuclease, indicating the involvement of a single catalytic site. However, substrate protection was observed only against TNBS inactivation, further indicating that the hydrolytic site and the phosphate binding sites are different. Results on chemical modification studies showed that while histidine, tryptophan and cysteine may be involved in the hydrolytic site, lysine is involved in the phosphate binding site.

Chapter 3 : Immobilization of S1 Nuclease

(A) Binding Through Protein Moiety: Crude S1 nuclease was coupled to gelatin-alginate composite matrix using residual free aldehyde groups on the surface of glutaraldehyde crosslinked matrix. The immobilized enzyme retained

approximately 10% activity of the soluble enzyme. When the partially purified enzyme was bound to the matrix, the bound enzyme lost all its activity. However, the activity could be restored when the coupling was carried out in presence of a coprotein or substrate. Immobilization brought about a shift in the optimum pH of S1 nuclease towards acid side (3.8) from 4.3 for the soluble enzyme. Also, the optimum temperature increased from 55°C to 65°C after immobilization. Bound S1 nuclease exhibited superior pH and temperature stabilities. Immobilization brought about a two-fold decrease in the Michaelis-Menten constant (K_m). The bound enzyme showed poor stability to repeated use.

(B) Binding Through Carbohydrate Moiety: S1 nuclease is a high mannose containing glycoprotein and contains approximately 18% carbohydrate. It has been shown that carbohydrate moiety is not essential for the catalytic activity of the enzyme. Hence, attempts were made to immobilize S1 nuclease through its carbohydrate moiety. When partially purified S1 nuclease was bound through its carbohydrate moiety to Concanavalin A-Sepharose containing increasing amounts of lectin, the retention of activity was high, varying essentially from 75% on "low lectin" matrix (1 mg of Con A/ml of Sepharose) to no detectable activity on "high lectin" matrix (8 mg Con A/ml of Sepharose). However, approximately 50% activity could

be restored on "high lectin" matrix when the binding was carried out in presence of glucose suggesting that the loss of activity on "high lectin" matrix is due to the conformational changes brought about by the multiple attachment of the enzyme to the matrix. Interaction of Con A with S1 nuclease was used to predict the nature of the carbohydrate moiety and its location with respect to the active site of the enzyme. Immobilization resulted in an increase in the optimum temperature, pH and temperature stabilities, but it did not affect the pH optimum. A marginal increase in the apparent K_m was observed. The bound enzyme also exhibited enhanced stability towards 8 M urea. On repeated use, Con A-Sepharose-S1 nuclease conjugate retained more than 80% of its initial activity after 6 cycles.

PUBLICATIONS

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CHAPTER I

GENERAL INTRODUCTION

1. SINGLE STRAND SPECIFIC NUCLEASES

Single strand specific nucleases which act on single stranded nucleic acids and single stranded regions in double stranded nucleic acids are widely distributed in plants, animals and microorganisms. They exhibit high selectivity for single stranded nucleic acids and produce mono- and oligonucleotides terminating in 5'-phosphoryl and 3'-hydroxyl groups. Though more than twenty single strand specific nucleases from various sources have been isolated till now (Table 1.1), only few enzymes like S1 nuclease from Aspergillus oryzae, P1 nuclease from Penicillium citrinum, Neurospora crassa, Alteromonas espejiana BAL 31 and mung bean nucleases, have been well studied (Shishido and Ando, 1985).

1.1 **Substrate specificity and mode of action:** Most of the single strand specific nucleases are sugar non-specific, multifunctional enzymes and exhibit both exo- and endonuclease activities. They degrade single stranded nucleic acids to all four mononucleotides and small amount of oligonucleotides by the cooperative action of both exo- and endonucleolytic activities. In addition, some of the single strand specific nucleases viz. S1 nuclease (Oleson and Hoganson, 1981), P1 nuclease (Fujimoto et al., 1974a), mung bean nuclease (Mikulski and Laskowski, 1970), pea seed nuclease (Naseem and Hadi, 1987), wheat

Table 1.1 : Single strand specific Nucleases

Name of the enzyme	Source	Reference
P1 nuclease	*Penicillium citrinum*	Kuninaka *et al.*, 1961
Mung bean nuclease	Mung bean sprouts	Sung and Laskowski, 1962
Exonuclease I	*E. coli*	Lehman and Nussbaum 1964
Shark liver nuclease	*Mustelus canis*	Ashe *et al.*, 1965
N. crassa nuclease	*Neurospora crassa*	Linn and Lehman, 1965
S1 nuclease	*Aspergillus oryzae*	Ando, 1966
Sheep kidney nuclease	Sheep	Kasai and Grunberg-Manago, 1967
Staphylococcal nuclease	*Staphylococcus aureus*	Cuatrecasas *et al.*, 1967
Phage SP3 Exonuclease	*Bacillus subtilis* infected with phage SP3	Trilling and Aposhian, 1968
Saccharomyces nuclease	*Saccharomyces* (hybrid strain)	Lee *et al.*, 1968
Wheat seedling nuclease	Wheat seedlings	Hanson and Fairley, 1969
Phage T4 Endonuclease IV	*E. coli* infected with phage T4	Sadowski and Hurwitz, 1969
Endonuclease I	*E. coli* infected with phage T7	Center and Richardson, 1970
U. maydis nuclease	*Ustilago maydis*	Holloman and Holliday, 1973

Name of the enzyme	Source	Reference
Exonuclease VII	*E. coli*	Chase and Richardson, 1974
BAL 31 nuclease	*Alteromonas espejiana* BAL 31	Gray *et al.*, 1975
Single strand specific nuclease	Mouse ascites cells	Otto and Knippers, 1976
Single strand specific nuclease	Human KB cells	Wang *et al.*, 1978
B. subtilis DNase	*Bacillus subtilis*	Cobianchi *et al.*, 1978
P. polycephalum nuclease	*Physarum polycephalum*	Waterborg and Kuyper, 1979
Pea seed nuclease	Pea seedlings	Naseem and Hadi, 1987
Single strand specific nuclease	*Streptomyces tendae*	Engel and Ullah, 1988
Single strand specific nuclease	*Coprinus cinereus*	Lu *et al.*, 1988

seedling nuclease (Hanson and Fairley, 1969), *Physarum polycephalum* nuclease (Waterborg and Kuyper, 1979) and sheep kidney nuclease (Kasai and Grunberg - Manago, 1967; Watanabe and Kasai, 1978) exhibit phosphomonoesterase activity. Single strand specific nucleases act on single stranded DNA (ssDNA), RNA and mononucleotides but the rate of hydrolysis of these substrates vary depending

on the source of the enzyme. While S1 and mung bean nucleases prefer ssDNA to RNA and 3'AMP (Oleson and Sasakuma, 1980; Mikulski and Laskowski, 1970), P1 nuclease shows higher activity towards 3'AMP and RNA (Fujimoto et al., 1974a). However, wheat seedling nuclease acts on all the three substrates at an equal rate (Hanson and Fairley, 1969). Though majority of the single strand specific nucleases are reported to be base non-specific, enzymes from N. crassa (Linn, 1967), P. citrinum (Fujimoto et al., 1974b), Ustilago maydis (Holloman and Holliday, 1973) and mung bean (Johnson and Laskowski, 1970) show some preference to bases in the initial stage of hydrolysis. While N. crassa and U. maydis nucleases prefer guanosine or deoxyguanosine residues in a polynucleotide chain, P1 nuclease preferentially attacks the linkage between 3'-hydroxyl group of adenosine or deoxyadenosine and 5'-phosphoryl group of adjacent nucleotide. However, mung bean nuclease shows preference for Ap↓Nd and T(U)↓pN in single stranded nucleic acids. Action of S1, P1 and mung bean nucleases on synthetic oligonucleotides showed that the rate of hydrolysis is pH dependent and this was attributed to pH induced changes in the secondary structure of the substrates (Shishido and Ando, 1985). Most of the single strand specific nucleases show exo- and endonuclease activities on both ssDNA and RNA but wheat seedling nuclease exhibits endonuclease activity

towards DNA and exonuclease activity towards RNA (Hanson and Fairley, 1969). However, the enzyme from Alteromonas BAL 31 apart from showing an endonuclease activity on single stranded nucleic acids, shortens duplex DNA molecules from both ends (Gray et al., 1975; Legerski et al., 1978). P1 nuclease (Fujimoto, 1974a), pea seed nuclease (Naseem and Hadi, 1987) and mung bean nuclease (Laskowski, 1980) act on native DNA also, but the latter shows some preference for AT-rich regions in double stranded DNA. N. crassa on the other hand, produces four major nucleases exhibiting markedly different properties and modes of action. These nucleases are reported to be formed from a single precursor polypeptide of 90 KD via different routes of proteolysis. First is a 75 KD, single strand specific, Mg^{2+} dependent exonuclease, identical to the one formed in conidia but not in mycelia. Second is a 65 KD endoexonuclease exhibiting endonuclease activity towards ssDNA and exonuclease activity on double stranded DNA. Third, a 55 KD proteolytic product, is a single strand specific endonuclease, identical to the enzyme isolated from mycelium. Fourth is a 65 KD, Ca^{2+} dependent endonuclease that cleaves both single and double stranded DNA but has no RNase activity (Fraser, 1980).

It has also been observed that NaCl concentration in the assay medium affects the activity of certain nucleases. For example, the activity of Alteromonas BAL 31

nuclease is maximal between 0 - 2 M NaCl and the enzyme retains 40% of the activity in the presence of 4.4 M NaCl (Gray et al., 1981). However, in case of N. crassa nuclease, NaCl concentration in the range of 0.1 - 0.2 M showed only slight inhibition with denatured DNA, but strongly inhibited its action on native DNA (Fraser, 1980). S1 nuclease on the other hand is optimally active at 0.1 M NaCl. The enzyme is relatively insensitive to ionic strength between 0.01 - 0.2 M NaCl and in 0.4 M NaCl, it degrades ssDNA at 55% of the maximal rate. The stringency of S1 nuclease for ssDNA is maximal at high salt concentrations (Vogt, 1980).

The rate of hydrolysis of mononucleotides by single strand specific nucleases varies from source to source. Mung bean nuclease cleaves the esters of ribonucleotides 50 - 100 fold faster than the corresponding deoxyribonucleotides. In addition, it shows preference to bases in the order A > T(U) > C > G (Mikulski and Laskowski, 1970). S1 nuclease too hydrolyzes 3'ribonucleotides at a higher rate than 3'deoxyribonucleotides. The enzyme also shows some activity on 2'AMP. The substrate specificity falls in the following order: ribonucleoside 3'5'bisphosphate > ribonucleoside 3'phosphate > deoxyribonucleoside 3'5'bisphosphate > deoxyribonucleoside 3'phosphate ≃ ribonucleoside 2'phosphate (Oleson and Hoganson, 1981). P1 nuclease shows high activity towards 3'mononucleotides but 2'nuc-

leotides are resistant to hydrolysis. The enzyme hydrolyzes 3'phosphomonoester bonds of various kinds of 3'mononucleotides, nucleoside 3'5'diphosphates, coenzyme A and oligonucleotides bearing 3'terminal phosphate. However, the rate of hydrolysis of various mononucleotides is pH dependent (Fujimoto et al., 1974b).

Preliminary studies with S1, P1 and mung bean nucleases have shown that the hydrolysis of both polymeric and monomeric substrates is catalyzed by a single active site (Oleson and Hoganson, 1981; Fujimoto et al., 1974a; Kowalski et al., 1976). However, in case of pea seed nuclease, which has two subunits, it has been speculated that the monoesterase activity resides in one of the subunits and the diesterase activity requires both the subunits (Wani and Hadi, 1987).

1.2 Properties

1.2.1 pH optimum: The pH optima of single strand specific nucleases generally are in the range of 4.3 - 8.8. While S1 nuclease (Vogt, 1973), P1 nuclease (Kuninaka et al., 1961), mung bean nuclease (Sung and Laskowski, 1962) and wheat seedling nuclease (Hanson and Fairley, 1969) have an optimum pH in the range of 4.3 - 5.5, that of N. crassa (Fraser, 1980), P. polycephalum (Waterborg and Kuyper, 1979), U. maydis (Holloman and Holliday, 1973), Coprinus cinereus (Lu et al., 1988), pea seed nuclease (Wani and

Hadi, 1979) and sheep kidney nuclease (Watanabe and Kasai, 1978) fall in the range of 7.0 - 8.8. Alteromonas BAL 31 nuclease on the other hand shows an optimum pH of 8.0 for duplex DNA and 8.8 for ssDNA (Gray et al., 1981). The optimum pH for phosphomonoesterase activity of S1 nuclease (Shishido and Habuka, 1986), mung bean nuclease (Mikulski and Laskowski, 1970) and pea seed nuclease (Naseem and Hadi, 1987) is similar to that of the one observed with ssDNA and RNA, while that of P1 nuclease varies depending upon the nucleotides used (Fujimoto et al., 1974b).

1.2.2 Temperature optimum: Temperature optima of most of the well studied nucleases fall in the range of 45-75°C and they exhibit high thermal stability. The high thermal stability of S1, P1 and mung bean nucleases has been attributed to the high content of hydrophobic amino acids and glycoprotein nature (Shishido and Ando, 1985).

1.2.3 Stability: S1 and Alteromonas BAL 31 nucleases are stable to low concentrations of denaturants like SDS and urea. Though P1 and mung bean nucleases are susceptible to guanidine hydrochloride and SDS, the inhibition of P1 nuclease by urea and guanidine hydrochloride is reversible (Shishido and Ando, 1985).

1.2.4 Metal ion requirement: S1 nuclease (Vogt, 1973), P1 nuclease (Kuninaka et al., 1961), U. maydis nuclease

(Holloman and Holliday, 1973) and nucleases from P. polycephalum (Waterborg and Kuyper, 1979) and wheat seedlings (Hanson and Fairley, 1969) are zinc metalloproteins, whereas N. crassa nuclease has Co^{2+} as the prosthetic group (Fraser, 1980). While the activity of N. crassa nuclease on native DNA is dependent on Mg^{2+}, its activity on ssDNA is independent of Mg^{2+}, though it is stimulated to some extent. In addition, the pH optima of N. crassa nucleases for the hydrolysis of DNA and RNA is dependent on Mg^{2+} concentration (Rabin et al., 1972). In the case of Alteromonas nuclease (Gray et al., 1981) Ca^{2+} appears to be a prosthetic group and Mg^{2+} is required for the activity. Coprinus cinereus nuclease requires Mg^{2+} and/or Ca^{2+} for its activity. Though Mg^{2+} is more effective than Ca^{2+}, the enzyme shows maximum activity in the presence of both the metal ions (Lu et al., 1988). Pea seed nuclease activity is stimulated by Mg^{2+} but it has no absolute requirement for metal ions (Wani and Hadi, 1979). Similarly, U. maydis enzyme does not require metal ions for its activity but it is inhibited by EDTA. However, addition of excess metal ions like Mg^{2+} or Ca^{2+} in presence of EDTA prevents inhibition (Holloman and Holliday, 1973).

1.2.5 Inhibitors: Being metal requiring enzymes, single strand specific nucleases are strongly inhibited by metal chelating agents like EDTA and citrate. While 8-hydroxy-

quinoline inhibits pea seed nuclease, EDTA has no effect on the enzyme activity (Wani and Hadi, 1979). Inorganic phosphate inhibits S1, N. crassa and P. polycephalum nucleases and in addition N. crassa and U. maydis nucleases are inhibited by thiols and ATP. S1 nuclease is also inhibited by 5'ribo and deoxyribonucleotides (Shishido and Ando, 1985).

1.2.6 Molecular weight and subunit structure: Molecular weights of single strand specific nucleases are in the range of 32,000 - 85,000 (Shishido and Ando, 1985). Most of the enzymes consist of a single polypeptide chain, but pea seed and Coprinus cinereus enzymes are made up of two unidentical subunits of 30 KD, 24 KD and 12 KD, 14 KD, respectively (Naseem and Hadi, 1987; Lu et al., 1988). Mung bean nuclease shows only one band (corresponding to 39 KD) in SDS gels but gel electrophoresis in presence of SDS and mercaptoethanol gives 3 bands corresponding to molecular weights of 39 KD, 25 KD and 15 KD. Since the intact species and the cleaved species migrate as a single band prior to reduction, it has been suggested that the intact species are held together by a disulphide bond(s). However, both cleaved and intact forms of the enzyme are equally active on ssDNA, RNA and 3'mononucleotides (Laskowski, 1980).

1.2.7 Glycoprotein nature: Some of the well studied single strand specific nucleases like S1 nuclease (Vogt,

1973), P1 nuclease (Fujimoto et al., 1975), pea seed nuclease (Naseem and Hadi, 1987) and mung bean nuclease (Kowalski and Laskowski, 1976) are glycoproteins and their carbohydrate content varies from 17 - 29%.

1.3 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 molecular biology research. Among the single strand specific nucleases, S1 nuclease from A. oryzae is the most widely used enzyme. S1 nuclease has been used for (i) the estimation of double helical content of various single stranded nucleic acids (Shishido and Ando, 1972); (ii) the isolation of the double stranded regions of single stranded nucleic acids (Shishido and Ikeda, 1970); (iii) the isolation of two types of hairpin structures of phage f1 DNA rich in AT and GC base pairs (Shishido and Ikeda, 1971a, b); (iv) the isolation of invert repeats from a number of alkali denatured eukaryotic DNAs (Wilson and Thomas, 1974); (v) the isolation and characterization of double stranded stem sequences from the inversion loop formed after the denaturation of transposable elements (Ohtsubo and Ohtsubo, 1976); (vi) the removal of single stranded tails prior to T_4DNA ligase treatment in the construction of recombinant DNA molecules between different restriction

endonuclease derived fragments or an *in vitro* deletion mutant by exonuclease digestion (Shishido and Ando, 1981); (vii) increasing the ligation efficiency of the sticky ends produced by the action of EcoRI and HpaII along with the Klenow fragments (Shishido and Ando, 1981); (viii) recovering the inserts from hybrid plasmids constructed by poly dA:poly dT tailing method (Hofstetter *et al.*, 1976); (ix) specifically cleaving the hairpin structure present at the 3'terminus of the complementary strand formed during cDNA synthesis (Efstratiadis *et al.*, 1976); (x) construction of the targetted deletion mutants *via* D loops (Green and Tibetts, 1980); (xi) the determination of spliced RNA structure and mapping of spliced points (Berk and Sharp, 1978) and (xii) the selective fragmentation of phage T7 at AT-rich regions (Povlov *et al.*, 1977).

Shaller *et al.* (1986) isolated GC core of phage fd DNA by successive digestion with *N. crassa* and *E. coli* exonucleases. While S1 and BAL 31 nucleases were used to obtain linear duplex DNA from negative superhelical DNA (Beard *et al.*, 1973; Godson, 1973; Mechali *et al.*, 1973; Germond *et al.*, 1974; Shishido and Ando, 1975a and Lau and Gray, 1979), *N. crassa* (Kato *et al.*, 1972) and mung bean nucleases (Wang, 1974) were used to obtain nicked circular DNA molecules from supercoiled DNA. The ability of S1 nuclease to recognize nicks in

double stranded DNA has been used for the generation of specific fragments in phage T5 DNA (Shishido and Ando, 1975b; Wiegand et al., 1975). Legerski et al. (1978) exploited the remarkable property of BAL 31 nuclease to digest duplex DNA from both ends, for the development of a convenient method for ordering restriction fragments. N. crassa nuclease was used for the isolation of pure lac operon DNA (Shapiro et al., 1969), in addition to the isolation of rRNA and tRNA gene hybrids (Kohne, 1968; Sgaramella et al., 1968; Marks and Spencer, 1970).

Other applications of single strand specific nucleases include the physical mapping of mutants (Shenk et al., 1975; Bartok et al., 1974), isolation of mRNA cap structures (Furuchi et al., 1975), determination of the susceptibility of DNA in chromatin to single strand specific nucleases (Fujimoto et al., 1979), recognition and cleaving of physically and chemically induced alterations in DNA (Shishido and Ando, 1974; Wiegand et al., 1975; Kato and Fraser, 1973; Legerski et al., 1977; Yamasaki et al., 1977), tRNA sequence analysis (Harada and Dahlberg, 1975; Tal, 1975) and production of mononucleotides (Kuninaka et al., 1961; Ando, 1966).

1.4 IMMOBILIZED NUCLEASES

The advent of immobilized enzyme technology has led to increased efforts to replace the conventional enzymic processes with immobilized enzymes as immobili-

zation (a) enables the enzymes to process a large amount of substrate, since it can be easily removed from the mixture of substrate and product, thus enabling the enzyme to be reused (b) in general imparts greater stability to the enzyme, so that it can be used for the development of continuous process and (c) permits the economical utilization of an otherwise cost prohibitive enzyme.

Nucleases are important analytical enzymes and are extensively used for studying nucleic acid structure. Their application depends on the specificity and mode of action of the particular enzyme. Thus, restriction endonucleases which cleave DNAs at defined base sequences, conformation specific enzymes like S1 nuclease, which hydrolyse single stranded nucleic acids, non-specific nuclease like micrococcal nuclease and RNases like T1, T2 and RNase A are used as routine analytical tools. In addition, S1 and P1 nucleases are used for the industrial production of mononucleotides. Due to the extensive applications of nucleases, several attempts have been made to develop methods to obtain highly active and stable immobilized preparations suitable for various biotechnological applications.

Hoffman et al. (1970) bound polynucleotide phosphorylase to cyanogen bromide (CNBr) activated cellulose and the bound enzyme retained 26% activity of the soluble

enzyme. The immobilized system could be used more than 40 times for the polymerization of nucleoside diphosphates. tRNA nucleotidyltransferase covalently coupled to CNBr-activated Sepharose could repair pCpCpA sequence of tRNA. Though the properties of the bound enzyme were similar to that of the soluble enzyme, the former showed increased stability against inactivation either by temperature or proteolytic digestion (Litvak et al., 1971). Glitz et al. (1974) bound RNase T1 and U1 to agarose and observed that the band patterns obtained after RNA digestion with the immobilized enzymes were identical to those obtained with the soluble enzyme suggesting the utility of these immobilized enzymes in RNA sequence analysis. Moorman et al. (1976) covalently bound DNase to CNBr-activated Sepharose and used the immobilized preparation to remove the endogeneous DNA in the extracts of E. coli, so that the extracts could be used for cell free transcription-translation system. Lee et al. (1978) compared the action patterns of Sepharose bound BamHI and EcoRI on several viral DNAs with that of the soluble enzyme and observed no change. The thermal and storage stabilities of the bound enzymes were superior to that of the soluble enzymes. DNase I covalently bound to Sepharose 4B was used to digest chromatin from hypotonically lysed nuclei from rooster liver. RNA Pol II released by this method was atleast 2 times more active in in vitro transcription

than that of the enzyme prepared by conventional method (Kastern et al., 1979). T_4 DNA ligase bound to 2,2,2-trifluoroethanesulfonyl chloride and CNBr-activated Sepharose 4B could ligate the restriction endonuclease generated DNA fragments with sticky as well as blunt ends, though immobilized preparation took longer time to complete ligation (16-18 h) compared to the free enzyme. The longer time required by the immobilized enzyme to complete ligation was correlated to diffusional restrictions. While the enzyme bound to Sepharose by both the methods showed the same efficiency (17%), the storage stability of the enzyme bound by CNBr-activation method was inferior. It was also observed that if the lysine groups of the enzyme (which are involved in the ATP binding) are not protected through adenylation, it results in immobilized preparations with low activity (Bulow and Mosbach, 1982). Singh (1984) entrapped PstI in low melting agarose containing 1,6-hexamethylenediamine and the immobilized preparation could effectively linearize pBR322 DNA. Crosslinking of the immobilized preparation with 1,5 bis (N-acetylamino-N-succinimideoxy carbonyl) pentene did not affect the enzyme activity. While polymerization in presence of acrylamide resulted in a considerable reduction in the activity of PstI, EcoRI under identical conditions exhibited high activity. Acetylation of amino groups of PstI with hydroxysuccinimide

acetate led to total inactivation of the enzyme. It was also observed that covalent coupling of PstI to Sepharose 4B results in the inactivation of the enzyme. The loss of activity of PstI in both cases was attributed to the possible modification of amino groups required for the catalytic activity. Wingard and Egler (1984) coupled lysine to several restriction enzymes, namely EcoRI, BamHI and BglI, using water soluble carbodiimide to assess the feasibility of immobilizing these enzymes through their carboxylic groups to solid supports. Since the coupling of lysine to enzyme did not bring about any loss of activity, the authors concluded that carbodiimide coupling through the enzyme carboxylic groups may be useful for the immobilization of restriction enzymes. SalI and PvuII bound to CNBr-activated Sepharose were active on DNA from phage λ and in addition they showed good storage stability (Bakh and Semina, 1985). Soboleva et al. (1985) immobilized RNase T1 and T2 on CM-cellulose for studying the influence of immobilization on their synthetic activities. It was observed that immobilization did not significantly alter their synthetic activities. It was interesting to note that inspite of the high lysine content (23 residues) RNase T2 reacted poorly with CM-cellulose azide matrix whereas RNase T1 which has only one lysine residue, bound most efficiently. To design glutaraldehyde based methods to immobilize restriction

enzymes, Olszewski and Wasserman (1986) investigated the effect of glutaraldehyde on the activity of restriction endonucleases like BamHI, HindIII, EcoRI and Tth 111 I and observed that while Tth 111 I was most sensitive (with activity losses occurring at very low glutaraldehyde concentrations), HindIII was most stable. However, addition of BSA to the incubation mixture generally had a stabilizing effect. Nasri and Thomas (1987) studied the effect of several chemical reagents on the activity of restriction endonucleases PvuII and HindIII. Treatment of PvuII and HindIII with carbodiimide and glutaraldehyde inactivated the enzyme, which could not be prevented by pBR322 DNA. However, Mg^{2+} protected the enzyme against carbodiimide inactivation. While N-ethylmaleimide caused slight inhibition of PvuII activity, it had no effect on HindIII enzyme. When PvuII and HindIII were covalently coupled to various supports through amine, carboxyl and phenolic groups, only the enzyme bound through the phenolic groups showed some activity.

Kirwan et al. (1974) bound RNase A to silica ceramic monoliths and observed a significant reduction (20 times) in the time of hydrolysis of RNA, when the substrate was in the form of aerosol rather than in solution. Exonuclease A5 bound to inorganic supports like Chromosorb P, porous glass and Silochrome retained 75% activity of the soluble enzyme. The bound enzyme could be used

for the production of 5'mononucleotides for over one month (Varlamov et al., 1975). To design a continuous process for the production of IMP and GMP, phosphodiesterase from Penicillium sp. and AMP deaminase from Aspergillus sp. were separately immobilized on porous ceramic. The immobilized system could convert 4% RNA solution to mononucleotides with 85% efficiency for 30 days. However, the conversion of AMP to IMP was 100% (Noguchi et al., 1977). Minh-Nguy and Annie (1977) immobilized pancreatic RNase and alkaline phosphatase on CNBr-activated cellulose and used the immobilized system for the manufacture of oligonucleotides and polynucleotides with specific terminations.

Fujishima et al. (1977) bound P1 nuclease to an anion exchange resin (Unilex A-885) and used the immobilized nuclease for the hydrolysis of RNA to mononucleotides. It was observed that the immobilized system could simultaneously degrade and fractionate nucleic acids. Isao (1976) immobilized RNase on collagen-alumina beads and used the immobilized system to examine RNA hydrolysis in aerosol. It was observed that RNA hydrolysis increased with decrease in the space velocity. Rokugawa et al.(1979) immobilized crude P1 nuclease (P1 nuclease-malonogalactan complex) to CNBr, N,N-dicyclohexylcarbodiimide and $TiCl_4$ activated celluloses and the immobilized preparations showed 3%, 28% and 23.5% activity respectively of the

soluble enzyme. However, when the pure enzyme (devoid of malonogalactan) was bound to celluloses by above methods, titanium cellulose-P1 nuclease conjugate showed maximum efficiency (83%). The low efficiency observed in case of crude enzyme was attributed to the probable interference of malonogalactan during the immobilization procedure. Since celluloses could not be used industrially, attempts were made to bind P1 nuclease to a number of Ti activated cation and anion exchange resins. Among them, Diaion HPK-25 (strongly acidic cation exchanger) Diaion HPA-10 (strongly basic anion exchanger) and Duolite A-4 (weakly basic anion exchanger) gave the best results with efficiency values ranging from 64-70%. The efficiency of the immobilized preparation depended on the activation conditions of the matrix. On continuous operation at pH 4.8 and 60°C, the immobilized nuclease could hydrolyze 1% RNA for more than 24 days. The matrix could be reused after regeneration (Rokugawa et al., 1980a). In order to overcome the inferior temperature stability of the Ti activated ion exchange resin-P1 nuclease complexes, Rokugawa et al. (1980b) coupled P1 nuclease to silica gel, porous glass beads and Pumice stone activated with various transition metal salts. Among the immobilized preparations the enzyme bound to Ti activated Pumice showed high efficiency and superior tempera-

ture stability. P1 nuclease immobilized on p-aminobenzenesulfonylethyl(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 preparation to be 30 times more active than the soluble enzyme (Yuan et al., 1980).

Manhua (1984) bound bovine pancreatic RNase A on agarose beads and stabilized the immobilized preparation with α-napthol. The immobilized preparation was used for synthesizing oligonucleotides with improved yields. The bound enzyme could be stored for more than one year. RNase entrapped in an ultrafiltration membrane could hydrolyze RNA with simultaneous separation of substrate and the reaction products (Magdalena, 1987). Onishi et al. (1988) designed a bioreactor, with a column of flocculant cells of the moderate halophile Micrococcus varians subsp. halophilus, with adsorbed halophilic nuclease H, for the production of 5'mononucleotides from RNA. An interesting feature of the flocculated cells was that they preferentially adsorbed much of the exogeneous nuclease, excluding adsorbed 5'nucleotidase. Desalting treatment of the flocculated cells in presence of $MgSo_4.7H_2O$ resulted in the selective inactivation of 5'nucleotidase without any affect on nuclease H activity.

Barnett and Bull (1959) bound RNase to glass, Dowex 50 (cation exchanger) and Dowex 2 (anion exchanger) and did not observe any change in the pH optima. Patel et al. (1969) bound DNase onto various supports and observed that the enzyme bound to polygalacturonic acid yielded the best results with an efficiency of 45%. Messing (1970) immobilized RNase on the surface of porous glass and observed that after an initial leaching period, the bound enzyme exhibited a constant level of activity over a period of 111 days. However, the activity level was considerably below the predicted value and this was attributed to the probable inactivation of the enzyme as a result of reaction of the amino groups at the active site with the silanol groups of the matrix during adsorption. Pancreatic RNase coupled to CNBr-activated Sepharose showed upto 90% and 45% activity of the soluble enzyme when tested aganst cytidine 2'3'-cyclic phosphate and yeast RNA respectively (Axén et al., 1971). Lee et al. (1971) linked RNase T1 to diazotized p-aminobenzyl cellulose, CNBr-activated Sepharose 2B and Sephadex G-200 and studied the properties of Sepharose bound enzyme. No change in the Km and specificity was observed as a result of immobilization. Smith et al. (1973) observed that when polynucleotide phosphorylase was bound to either mercerized cellulose or Sepharose 4B by CNBr-activation, the amount of enzyme bound in presence of collidine-HCl

buffer was more than that of the one obtained with Tris-HCl buffer. The increased binding obtained in presence of collidine buffer was attributed to steric hindrance by 2 and 6-methyl groups of collidine which must be preventing the base from reacting with the activated matrix. However, in case of Tris-HCl buffer, the amino groups might be competing with enzyme for binding sites on the matrix. Ohgi et al. (1974) immobilized RNase M from Aspergillus saitoi and RNase VS1 from seminal vesicles onto CNBr-activated Sepharose 4B and the bound enzymes retained 7.2% and 10% activity respectively of the soluble enzymes. Bound RNase M and VS1 showed high pH stability and stability to urea denaturation. In case of both the enzymes, immobilization resulted in an increase in the K_m. RNase, snake venom DNase and E. coli phosphomonoesterase bound to CM-cellulose azide showed high storage stability both individually and in mixtures. The immobilized preparations could hydrolyze synthetic and natural substrates (Shalina et al., 1976). Purified pancreatic RNase covalently coupled to CM-cellulose showed high activity towards RNA, in addition to its ability to hydrolyse cyclic 3'5' phosphates (Babkina et al., 1976). Hofstee (1977) bound DNase I and a number of other enzymes (with pI in the acidic range) to butyl and octyl Sepharoses at pH 8.0 and found octyl-Sepharose bound enzymes to be more stable. It was also observed that RNase

(pI 9.45) could not be bound to the above matrices since the enzyme was positively charged at pH 8.0. Christin et al. (1977) covalently coupled RNase A through a varying number of bonds to Sepharose CL 4B activated with different amounts of CNBr and observed that increase in the number of points of attachments between the enzyme and the matrix brought about a decrease in the specific activity, though the bound protein increased. Differential scanning calorimetric studies showed that the weakly bound enzyme exhibited a similar behaviour as that of the soluble enzyme. However, the strongly bound enzyme showed a higher transition temperature and broader endotherm. RNase T1 bound to Enzacryl AH by acid azide method showed 45% and 77% activity towards RNA and 2'3'cyclic GMP respectively. Compared to the soluble enzyme, Enzacryl AH bound RNase T1 showed superior pH and temperature stabilities (Ito et al., 1977a). RNase T1 bound to copolymers of acrylamide and divinylbenzene by acid azide method retained most of the activity of the soluble enzyme. The bound enzyme showed superior temperature and pH stabilities (Ito et al., 1977b). Chirikjian (1979) immobilized restriction endonucleases, to various water insoluble matrices, through the introduction of multifunctional crosslinking groups. Aldehyde groups generated following the periodate oxidation of cotton were utilized to bind

BamHI. No change in the enzymic properties was observed as a result of immobilization. However, Bam HI and EcoRI bound to CNBr-activated agarose showed enhanced temperature stability. EcoRI bound to CNBr-activated Sepharose 4B showed high thermal and storage stabilities. Guisan and Ballesteros (1979) bound micrococcal nuclease to CNBr-activated Sepharose 4B by coupling through the amino, carboxyl and tyrosyl or histidyl groups of the enzyme and observed that the enzyme bound through amino groups showed maximum efficiency (1.28%) compared to the preparations bound through carboxyl (0.2%) and tyrosyl/histidyl groups (0.015%), when assayed using DNA as substrate. However, when Sepharose-NH_2-nuclease was assayed using a low molecular weight substrate, nitrophenyl-pdTp, the efficiency increased to 70% indicating the presence of diffusional limitations. When bovine pancreatic RNase was bound to silanized glass beads through glutaraldehyde there was no change in the specificity and optimum pH. However, increasing the bead volume resulted in a decrease in the specific activity of the bound enzyme (Jerzy et al., 1980). Zhongyi et al. (1981) immobilized Pl nuclease on diazonium salts of p-aminobenzenesulfonylethyl-ECD-agar and the immobilized preparation retained 18-35% activity of the soluble enzyme. Coupling of the enzyme in presence of NH_4^+ ions increased the activity of the bound enzyme. Pl nuclease bound covalently to cellulose

showed an optimum temperature of 70°C, in addition to a slight shift in the optimum pH towards acid side. Immobilization resulted in an increase in the apparent K_m for RNA. Kitao et al. (1981) bound bovine pancreatic DNase on human erythrocytes by chromium chloride method and the immobilized preparation retained 100% activity of the soluble enzyme. The bound enzyme showed good stability to repeated use. However, when the binding was carried out with 4'4'-diisothiocyano 2,2'-stilbene disulfonate or ethyl-3- (dimethylaminopropyl) carbodiimide-HCl, the bound enzyme lost its activity. Guisan and Ballesteros (1981) observed that the initial rate of hydrolysis of nucleic acids by Sepharose bound micrococcal nuclease is strongly influenced by diffusional limitations, which could be overcome by changing the particle and pore size and the enzyme load on the matrix. It was also observed that steric and diffusional limitations brought about a change in the course of reaction and selectivity to hydrolytic products, compared to the soluble enzyme. Manecke and Polakowski (1981) employed RNase to study the effect of protein denaturing agents on binding and its pH dependence, for coupling to terephthaldehyde crosslinked poly(vinyl alcohol) derivatized with 1-fluoro-4-nitrobenzene and reduced with sodium dithionite. It was observed that though coupling either at alkaline pH or in presence of 8 M urea increased the

binding, the bound enzyme showed less activity. EcoRI bound to CNBr-activated Sepharose 4B exhibited good thermal and storage stabilities and could be used repeatedly (Chirikjian, 1982). Cashion et al. (1982a) attached restriction endonucleases, DNA polymerase, RNA ligase, S1 nuclease, AMV reverse transcriptase etc. by hydrophobic bonds to tritylated agarose or Sepharose, with high retention of activity. The authors further showed that due to weak binding forces involved between the enzyme and the support, both can be reused (Cashion et al. 1982b). Ballesteros et al. (1986) used acetone instead of dioxane for the activation of agarose with tosyl chloride, since use of acetone reduces activation time and the handling of dioxane. Staphylococcal nuclease bound to tosylated agarose showed high retention of activity. The activity of RNase covalently coupled to or included in collagen membrane was dependent on the method of immobilization. Bound RNase showed superior pH, temperature and operational stabilities (Jeana et al., 1987). When RNase T2 was bound to Concanavalin A-Sepharose through its carbohydrate moiety, the retention of enzyme activity was high, ranging from 70% at low enzyme load to approximately 9% at high enzyme load. Compared to the soluble enzyme, the immobilized RNase T2 showed enhanced stability towards temperature and metal ions (Reddy and Shankar, 1988).

PRESENT INVESTIGATION

Single strand specific nucleases by their ability to selectively hydrolyse single stranded nucleic acids and single stranded regions in double stranded nucleic acids are widely used as an analytical tool for nucleic acid hybridization, analysis of nucleic acid structure, isolation of specific genes and gene manipulation (Shishido and Ando, 1985). Though single strand specific nucleases with comparable properties have been isolated from N. crassa, P. citrinum, P. polycephalum, mung bean sprouts, wheat seedlings and pea seedlings; S1 nuclease from Aspergillus oryzae is preferred, since it can be easily prepared in large amounts from commercially available crude α-amylase powder, it is stable to low concentrations 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 mononucleotides. Though the enzyme has been purified and extensively used as an analytical tool for the characterization of nucleic acid structure, very little information is available regarding the nature of its catalytic site. It has also been observed that after S1 nuclease treatment of nucleic acid samples, removal of the residual enzyme activity from the reaction mixture is essential and requires

repeated extractions with phenol, which in turn results in the loss of nucleic acid samples. In such circumstances, use of immobilized enzymes offer a distinct advantage over the soluble enzyme as it can be easily removed from the reaction mixture by physical methods. Hence the present investigation with S1 nuclease was undertaken to (a) study the nature of the catalytic site by chemical modification and (b) develop suitable immobilization procedures to obtain highly active and stable immobilized preparation suitable for routine analytical purposes.

CHAPTER 2

PURIFICATION AND ACTIVE SITE CHARACTERIZATION OF S1 NUCLEASE

SUMMARY

A simple and rapid procedure was developed to purify S1 nuclease to homogeneity from commercially available Takadiastase powder with an overall yield of 20%. When the purified enzyme was treated with various amino acid modifying reagents, only rose bengal, diethylpyrocarbonate, N-bromosuccinimide, 2-hydroxy-5-nitrobenzylbromide, N-ethylmaleimide, 2-hydroxymercuribenzoate, 2,4,6 trinitrobenzene sulfonic acid and pyridoxal 5'-phosphate inhibited all the three activities, namely single strand DNase, RNase and phosphomonoesterase, associated with S1 nuclease, showing the involvement of a single catalytic site responsible for the hydrolysis of all the three substrates. However, substrate protection was observed only against trinitrobenzene sulfonic acid and pyridoxal 5'-phosphate inactivation indicating the presence of separate hydrolytic and phosphate binding sites. Chemical modification studies suggest that while histidine, tyrptophan and cysteine are involved in the hydrolytic site, lysine is involved in the phosphate binding site. Based on our chemical modification studies and the inhibition studies of Oleson and Sasakuma (1980), the existence of three phosphate binding subsites and a closely situated hydrolytic site is predicted.

INTRODUCTION

Single strand specific nuclease from Aspergillus oryzae (S1 nuclease, EC 3.1.30.1), first described by Ando (1966), is a sugar non-specific and multifunctional enzyme which acts on single stranded DNA, RNA and 3'AMP at relative rates of 100:52:13, respectively (Oleson and Sasakuma, 1980). Though the enzyme has been purified (Vogt, 1973; Rushizky et al., 1975; Oleson and Sasakuma, 1980) and extensively used as an analytical tool for the characterisation of nucleic acid structure (Rushizky, 1981), very little information is available regarding its molecular enzymology. Through competitive inhibition studies Oleson and Hoganson (1981) showed that the hydrolysis of polynucleotides and mononucleotides is carried out by a single catalytic site. However, no reports are available till now regarding the nature of the catalytic site of S1 nuclease. The importance of histidine and cysteine residues of transcriptional regulatory proteins in DNA binding and that of cysteine and tryptophan in case of single strand DNA binding proteins has been receiving considerable attention recently (Klug and Rhodes, 1987; Casas-Finet et al., 1988). Since S1 nuclease is also a single strand nucleic acid binding protein, chemical modification studies were carried out to determine (a) the type of amino acid residues responsible for catalysis, and (b) whether all the three activities

associated with S1 nuclease reside at a single catalytic site.

MATERIALS AND METHODS

Materials:

DEAE-Cellulose (Whatman, U.K.); Takadiastase (Sankyo, Japan); RNA (Sisco Research Laboratories, India); adenosine 3' monophosphate (3'AMP), diethylpyrocarbonate (DEPC), pyridoxal 5'-phosphate (PLP), 2-hydroxy-5-nitrobenzyl bromide (HNBB), N-bromosuccinimide (NBS) and p-hydroxymercuribenzoate (pHMB) (Sigma Chemical Co., USA); N-ethylmaleimide (NEM) (Fluka, Switzerland); 2,4,6 trinitrobenzene sulfonic acid (TNBS) and sodium borohydride (BDH, India), were used. All other chemicals used were of analytical grade.

Methods:

Isolation of DNA: Unless otherwise stated, all the operations were carried out at 0 - 4°C. Buffalo liver DNA was isolated by a combination of the methods of Mehra and Ranjekar (1979) and Marmur (1961). Approximately 100 g of liver tissue was suspended in minimum volume of 0.05 M Tris-maleate buffer, pH 6.0 (containing 0.5 M sucrose, 0.003 M $CaCl_2$ and 0.1% Triton X -100) and homogenized. 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 and finally with saline EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0). The washed pellet was subsequently suspended in minimum volume of saline EDTA, mixed with sodium dodecylsulfate 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 gently agitated for 30 min and then deproteinized using a mixture of chloroform:isoamyl alcohol (24:1 v/v). 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 (0.15 M sodium chloride, 0.015 M trisodium citrate, pH 7.0). From the DNA preparation, RNA contamination was removed by incubation with pancreatic RNase (50 µg/ml, made DNase free by heating the enzyme at 80°C for 10 min) at 37°C for 1 h. After RNase treatment, the mixture was again deproteinized and the DNA was precipitated as described above. The precipitate was collected with a glass rod, dried free of ethanol, dissolved in SSC and stored at 4°C.

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

High molecular weight nature of the DNA preparations was also checked electrophoretically using 1% agarose gel.

Preparation of the substrate: High molecular weight buffalo liver DNA dissolved in the assay buffer (0.03 M sodium acetate pH 4.6, 0.05 M NaCl, 1 mM $ZnSO_4$ and 5% glycerol) was sonicated in cold (4°C) for approximately 10 min using Virsonic cell disruptor (model 16-850). Sonicated DNA was then denatured by heating at 100°C for 10 min, rapidly cooled in ice and used for the assay.

DNase assay of S1 nuclease: This was carried out by a slightly modified procedure of Vogt (1973). The standard reaction mixture of 1 ml contained 40 µg of sonicated and heat denatured buffalo liver DNA, 1 mM $ZnSO_4$, 0.05 M NaCl and 5% glycerol in 0.03 M sodium acetate buffer, pH 4.6 and appropriately diluted enzyme. The reaction was initiated by addition of the enzyme followed by incubation at 37°C for 30 min. The reaction was terminated by the addition of 1 ml of chilled 10% perchloric acid and 1 ml of 0.1% BSA. The mixture was left in ice

for 10 min and then centrifuged to sediment the precipitate. The acid soluble nucleotides in the supernatant were measured at 260 nm.

RNase activity of S1 nuclease: RNA degrading activity of the enzyme was assayed in the same manner as that of single strand DNase activity. However, in place of perchloric acid, 10% TCA was added to precipitate the unreacted RNA to avoid the hydrolysis of the substrate. The acid soluble nucleotides were measured at 280 nm since TCA absorbs strongly at 260 nm.

One unit of the enzyme is defined as the amount of enzyme required to digest 10 µg of DNA or RNA in 30 min under the assay conditions.

Phosphomonoesterase activity of S1 nuclease: Phosphomonoesterase activity of the enzyme was assayed using 3'AMP as the substrate (Oleson and Sasakuma, 1980). The total reaction mixture of 1 ml contained 1 mM 3'AMP, 1 mM $ZnSO_4$, 0.05 M NaCl and 5% glycerol in 0.03 M sodium acetate buffer, pH 4.6 and appropriately diluted enzyme. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 0.2 ml of a freshly prepared cold solution of 5% SDS and 0.5 N H_2SO_4. The inorganic phosphate liberated was determined according to Bartlett (1959).

One unit of the enzyme is defined as the amount of enzyme required to liberate 1 μmole of phosphate in 30 min under the assay conditions.

Assay of RNase T2: RNase T2 activity determination was carried out according to the procedure of Uchida and Egami (1967a). The standard assay mixture of 0.70 ml contained, 0.25 ml of 0.2 M sodium acetate, pH 4.5, 0.1 ml of 20 mM EDTA, 0.25 ml of RNA (12 mg/ml) and 0.1 ml appropriately diluted enzyme. The reaction was initiated by the addition of RNA and the mixture was incubated for 15 min at 37°C. The reaction was terminated by the addition of 0.25 ml uranyl reagent (0.75% uranyl acetate in 25% perchloric acid) and the resulting precipitate was immediately removed by centrifugation. 0.2 ml of the supernatant was diluted to 5.0 ml with distilled water and the acid soluble nucleotides were measured at 260 nm. An increase in absorption values of 1.0 is defined as 1 unit of enzyme.

Protein determination:

(a) Protein estimation according to Lowry et al. (1951) was carried out using crystalline bovine serum albumin as standard. The blue color developed after the addition of Folin reagent, was read at 660 nm.

(b) Protein estimation by optical method was done according to Kalckar (1947) using the following equation :

Protein concentration(mg/ml) = 1.45 D_{280} - 0.74 D_{260}

where D_{280} and D_{260} are the optical densities at 280 and 260 nm respectively.

PURIFICATION OF S1 NUCLEASE

Purification of S1 nuclease was carried out by a slightly modified procedure of Vogt (1973) and Rushizky (1975). Unless otherwise stated all the operations were carried out at 4°C. During the purification steps, S1 nuclease activity was monitored by using single stranded DNA as substrate.

Heat treatment: 10 g Takadiastase powder was suspended in 50 ml of buffer A (0.3 M sodium acetate buffer, pH 4.6 containing 0.1 mM $ZnSO_4$, 0.05 M NaCl and 5% glycerol) and stirred for 1 h. The insoluble material was removed by centrifugation at 9226 g for 10 min and the pH of the dark brown supernatant was adjusted to 5.0. The solution was then heated at 70°C, by vigorous swirling, for approximately 90 sec and chilled immediately in ice. The precipitated proteins were removed by centrifugation at 9226 g for 10 min and the supernatant was used for the next step.

Ammonium sulfate fractionation: The supernatant obtained after heat treatment was brought to 0.65 saturation by the addition of 21.5 g of solid ammonium sulfate and the mixture was stirred for 1 h. The precipitated protein

was removed by centrifugation and discarded. The supernatant containing the enzyme activity was brought to 0.95 saturation by adding 13.5 g of solid ammonium sulfate. The mixture was stirred for 3 h and the undissolved ammonium sulfate crystals were removed by decantation. The precipitate obtained after the centrifugation of the decanted solution was dissolved in minimum amount of buffer B (0.03 M sodium acetate pH 5.0, containing 1 mM $ZnSO_4$, 0.05 M NaCl and 5% glycerol) and dialyzed extensively against the same buffer to remove the ammonium sulfate.

DEAE-Cellulose chromatography: The dialyzed enzyme solution was then adsorbed at a rate of 15 ml/h onto a DEAE-cellulose column (1.2 x 13 cm) at pH 5.0 (equilibrated with buffer B). 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 - 0.35 M) in buffer B. 5 ml fractions were collected at a flow rate of 15 ml/h and those fractions having activity greater than 500 U/ml were pooled and concentrated to 5 ml, using an Amicon ultrafiltration unit fitted with PM 10 membrane. The concentrated enzyme solution was then rechromatographed on a fresh DEAE-cellulose column (1.2 x 13 cm) under similar conditions as described above. High activity fractions (> 500 U/ml)

were pooled, concentrated and stored at -20°C. No loss of activity was observed over a period of 2-3 months when the purified enzyme was stored under these conditions.

Gel electrophoresis: Analytical disc gel electrophoresis at pH 4.3 and 8.9 was carried out using 7.5% polyacrylamide gels according to Davis (1964). After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250 as described by Blakesley and Boezi (1977). SDS-PAGE was carried out using 15% polyacrylamide gels and 0.1% SDS according to Weber and Osborn (1969). The gels were stained with Coomassie Brilliant Blue R-250 and destained with a solution containing methanol, acetic acid and water (50:10:40).

CHEMICAL MODIFICATION STUDIES

In chemical modification studies, the residual activity of the modified enzyme was determined against all the three substrates viz. ssDNA, RNA and 3'AMP, since one of the main objectives of this work was to determine whether the above substrates are hydrolysed by the same catalytic site.

1. Modification of histidine residues:

(a) Photo-oxidation: This was carried out at room temperature by exposing 400 µg of the enzyme in 20 mM maleate buffer, pH 6.8, containing different concentrations of rose bengal to 200 W flood light bulb held at 10 cm,

for various time intervals. Enzyme samples treated under similar conditions in dark served as control.

(b) Carbethoxylation: S1 nuclease (400 µg) in 20 mM maleate buffer, pH 6.8, was incubated at 37°C for 10 min with varying concentrations of DEPC diluted in absolute alcohol. Ethanol at this concentration did not have any adverse effect either on the activity or on the stability of the enzyme during the incubation period. After incubation, excess DEPC and ethanol were removed by extensive dialysis and the residual activity was estimated. Enzyme samples treated under similar conditions without DEPC, served as control.

Decarbethoxylation: S1 nuclease (400 µg) was treated with 1 mM DEPC as described above. The reactivation of the modified protein was achieved by incubating the enzyme with 400 mM hydroxylamine hydrochloride at pH 6.8 and 4°C for 16 h. Subsequently, the enzyme samples were extensively dialyzed and assayed for the residual activity.

2. Modification of tryptophan residues:

(a) Reaction with N-bromosuccinimide (NBS): The enzyme (400 µg) in 20 mM sodium acetate buffer, pH 4.0 was incubated with different concentrations of NBS at 37°C for 30 min. The enzyme samples were then extensively dialyzed before determining the residual activity. A

control sample containing no NBS was subjected to identical treatment.

(b) Reaction with 2-hydroxy-5-nitrobenzylbromide (HNBB): HNBB was prepared in acetone just prior to use and protected from light. 400 µg enzyme in 20 mM sodium acetate buffer at pH 4.0 was incubated with 3 mM HNBB for 30 min at 37°C. The modified enzyme was then dialyzed to remove the low molecular weight products and the residual activity was determined under standard assay conditions. An equal amount of untreated enzyme incubated under identical conditions was used as control.

3. Modification of cysteine residues:

(a) Reaction with N-ethylmaleimide (NEM): The enzyme (400 µg) in 20 mM Tris-HCl buffer, pH 7.0, was incubated with various concentrations of NEM at 30°C for 30 min. After the reaction, the samples were dialyzed and the residual activity was determined. The enzyme samples incubated in absence of NEM were taken as control.

(b) Reaction with p-hydroxymercuribenzoate(pHMB): S1 nuclease (400 µg) in 20 mM maleate buffer, pH 6.8 was incubated with varying concentrations of pHMB for 30 min at 37°C. Control contained an equal amount of enzyme incubated under identical conditions, without pHMB. Subsequently, the samples were dialyzed and the residual activity was measured.

4. Modification of lysine residues:

(a) Reaction with 2,4,6 trinitrobenzene sulfonic acid (TNBS): The total reaction mixture of 3 ml contained 1 ml of enzyme (600 µg), 1 ml of 4% sodium bicarbonate and 1 ml of 0.1% of TNBS. This mixture was incubated at room temperature for 2 h in dark. After the incubation period, the reaction mixture was extensively dialyzed and the residual activity was determined. An equal amount of untreated enzyme incubated under similar conditions served as control.

(b) Reaction with pyridoxal 5'-phosphate (PLP): S1 nuclease (600 µg) was incubated with 0.5 mM of PLP at 30°C and pH 6.8 for 15 min. The reaction mixture was then cooled to 0°C and two drops of decyl alcohol was added to avoid foaming. Subsequently, excess amount of solid sodium borohydride (approximately 5 - 6 mg) was added and the mixture was incubated for another 5 min at 0°C. The samples were extensively dialyzed before estimating the residual activity. Control consisted of enzyme incubated under identical conditions without PLP.

Substrate protection studies:

In all the above experiments, effect of substrate protection was studied by preincubating the enzyme with excess amount of ssDNA, RNA and 3'AMP followed by treatment with the various modifying reagents.

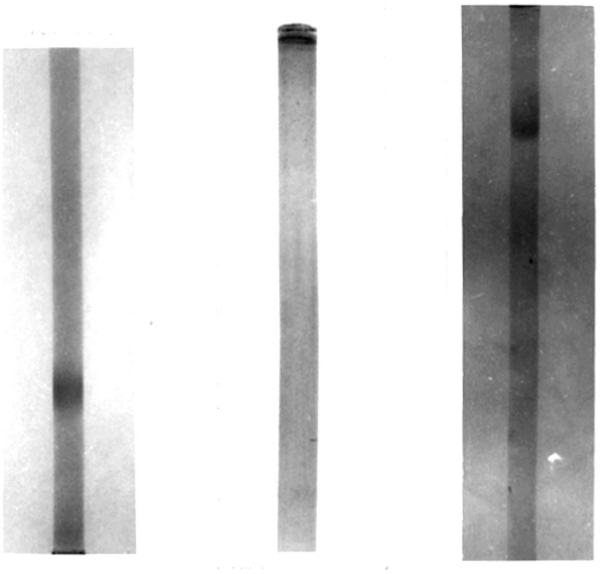
RESULTS

The results of a typical purification procedure are given in Table 2.1.

Table 2.1 : Purification of S1 nuclease

Step	Volume (ml)	Total protein as A_{280}	Total activity (U)	Specific activity (U/A_{280})	Fold purification	Recovery (%)
Crude	50	3200	100,000	31.25	1.0	100
Heat treatment	49	3000	100,000	33.33	1.06	100
Ammonium sulfate	10	320	60,000	187.5	6.0	60
DEAE-cellulose pH 5.0	5	50	30,000	600	19.2	30
DEAE-cellulose pH 5.0	5	15	20,000	1333.3	42.6	20

The enzyme was purified approximately 43 fold with a yield of 20%. Though the enzyme obtained after the first DEAE-cellulose step showed high specific activity, it did contain trace amounts of RNase T2 and some other proteins. However, rechromatography on DEAE-cellulose at pH 5.0 yielded a homogeneous preparation. The purified enzyme moved as a single band both in native and SDS-PAGE indicating its homogeneity (Fig. 2.1).



A B C

Fig. 2.1: Polyacrylamide gel electrophoresis (PAGE) of purified S1 nuclease:

A. 7.5% polyacrylamide gel, Tris-glycine buffer, pH 8.9, 4 mA/tube; protein loaded 60 μg.

B. 7.5% polyacrylamide gel; β-alanine acetic acid buffer, pH 4.3; 4 mA/tube; protein loaded 60 μg.

C. SDS-PAGE; 0.01 M sodium phosphate buffer, pH 7.3; SDS, 0.1%, 2-mercaptoethanol, 5%; 8 mA/tube; protein loaded 60 μg.

When the purified enzyme was subjected to photo-oxidation in presence of rose bengal, it lost about 90% activity and the inactivation was dependent on the concentration of rose bengal (Table 2.2).

Table 2.2 : Effect of rose bengal on the activity of S1 nuclease

Concentration of the reagent (%)	Time (min)	Residual activity (%)
Control	20	100
0.05	10	50
0.05	20	20
0.10	10	42
0.10	20	11

The results indicate the presence of histidine at or near the active site of S1 nuclease, as rose bengal is known to be specific for histidine residues at neutral pH. This observation was further confirmed by treating the enzyme with DEPC. Reaction of S1 nuclease with DEPC inactivated the enzyme completely and the inactivation was dependent on the concentration of the reagent. However, 10-15% activity could be restored when DEPC inactivated enzyme was treated with hydroxylamine hydrochloride (Table 2.3), indicating the presence of histidine at or near the active site.

Table 2.3 : Effect of DEPC on S1 nuclease activity

Concentration of the reagent (mM)	Residual activity (%)
Control	100
0.10	73.8
0.25	53.8
0.50	30.0
1.0	0.0
1.0 + ssDNA (250 μg)	0.0
1.0 + RNA (250 μg)	0.0
1.0 + 3'AMP (5 mM)	0.0
Hydroxylamine hydrochloride treatment	15.0

S1 nuclease lost its activity completely when incubated with NBS and the inactivation was dependent on the concentration of the reagent, indicating the involvement of tryptophan in the catalytic activity. The inactivation could also be seen in presence of HNBB (which is very specific to tryptophan residues at pH 4.0) substantiating the above observation (Table 2.4).

Table 2.4 : Effect of tryptophan modifying reagents on S1 nuclease activity

Reagent used	Concentration (mM)	Residual activity (%)
Control		100
NBS	0.1	100
"	0.5	88
"	1.0	50
"	3.0	0
"	3.0 + ssDNA (250 µg)	0
"	3.0 + RNA (250 µg)	0
"	3.0 + 3'AMP (5 mM)	0
HNBB	0.1	77
"	1.0	55
"	3.0	0
"	3.0 + ssDNA (250 µg)	0
"	3.0 + RNA (250 µg)	0
"	3.0 + 3'AMP (5 mM)	0

Treatment of the enzyme with NEM and pHMB also led to the inactivation of the enzyme which again was dependent on the concentration of the reagent used, showing the importance of cysteine residues for the catalytic activity (Table 2.5).

Table 2.5 : Effect of cysteine modifying reagents on S1 nuclease activity

Reagent used	Concentration (mM)	Residual activity (%)
Control	-	100
NEM	2.5	100
"	5.0	95
"	7.5	75
"	10.0	50
"	10.0 + ssDNA (250 µg)	50
"	10.0 + RNA (250 µg)	50
"	10.0 + 3'AMP (5 mM)	50
pHMB	2.5	100
"	5.0	50
"	7.5	0
"	7.5 + ssDNA (250 µg)	0
"	7.5 + RNA (250 µg)	0
"	7.5 + 3'AMP (5 mM)	0

In all the above modification studies, the substrates of S1 nuclease, viz. ssDNA, RNA and 3'AMP could not protect the enzyme from inactivation (Table 2.2 - 2.5).

When the amino groups of the enzyme were modified using TNBS, it lost about 65% of its activity. However,

TNBS inactivation could be prevented by preincubating the enzyme with either ssDNA, RNA or 3'AMP, suggesting the involvement of amino groups at the substrate binding site of S1 nuclease. The involvement of amino groups was further ascertained by using a more specific reagent, namely PLP to modify the amino groups present at the substrate binding site. Like TNBS, PLP too inhibited the enzyme and the inhibition could be prevented by pre-incubating the enzyme with the substrates (Table 2.6).

Table 2.6 : Effect of lysine modifying reagents on S1 nuclease activity

Reagent used	Concentration	Reagent (%)
Control	-	100
TNBS	0.1%	33.3
"	0.1% + ssDNA (250 µg)	100
"	0.1% + RNA (250 µg)	100
"	0.1% + 3'AMP (5 mM)	75
PLP	0.1 mM	51
"	0.5 mM	21
"	0.5 mM + ssDNA (250 µg)	100
"	0.5 mM + RNA (250 µg)	100
"	0.5 mM + 3'AMP (5 mM)	100

DISCUSSION

Since a large amount of enzyme is required for the structural studies, initially, attempts were made to develop a simple procedure to obtain a homogeneous enzyme with good recovery. In the present studies, DEAE-cellulose chromatography at pH 5.0 was preferred to pH 7.0, because under these conditions RNase T1, RNase T2 and S1 nuclease get separated from each other (Rushizky *et al.*, 1975). However, it was observed that the pooled fractions obtained after this step did contain trace amounts of RNase T2 activity and some other proteins. The inability of this procedure to effectively separate RNase T2 and other proteins from S1 nuclease can be correlated to the relative impurity of the starting material. Though Rushizky *et al.* (1975) removed RNase T2 by adsorption on CM-cellulose at pH 4.4, we chose to rechromatograph the enzyme on DEAE-cellulose under similar conditions (pH 5.0), as it is possible to remove RNase T2 in addition to other proteins. This procedure in fact gave a homogeneous preparation as evidenced by the gel patterns (Fig. 2.1). The less number of steps required in this procedure, compared to other reported procedures, to obtain the homogeneous enzyme can be attributed to the high specific activity of the starting material (Table 2.1).

For the photo-oxidation studies, rose bengal was preferred to methylene blue as it is reported to be more

specific to histidine residues at neutral pH (Westhead, 1965; Bellin and Yankus, 1968). When the enzyme was treated with rose bengal, it lost approximately 90% of its activity, which could be prevented by shielding the enzyme - rose bengal mixture from irradiation, indicating the presence of histidine at or near the catalytic site. Modification of histidine residues with DEPC also resulted in total loss of activity. Inactivation of the enzyme was accompanied by a significant increase in the absorption of the modified protein at 240 nm, which is characteristic of ethoxycarboxylation of histidine residues (Means and Feeney, 1971). Though DEPC is specific to histidine, it also reacts (to a lesser extent) with arginine, tyrosine and cysteine residues. However, N-acetylimidazole and phenylglyoxal did not bring about any inhibition of the enzyme indicating that arginine and tyrosine may not be involved in the catalysis. The modification of tyrosine was further ruled out by the observation that there was no significant decrease in the absorbance of the DEPC treated enzyme at 278 nm (Means and Feeney, 1971). 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 the enzyme with cysteine specific reagents like NEM and pHMB was carried out. NEM treated enzyme showed approximately 50% loss of activity indicating

the involvement of cysteine in the catalysis. Though NEM is specific to thiol groups, it is also known to react with amino groups at alkaline pH and to a lesser extent with histidine residues (Smyth et al., 1960). In the present case, reaction with amino groups may not have taken place as the reaction with NEM was carried out at neutral pH. To further confirm the presence of cysteine, inhibition with a more specific reagent namely pHMB was studied. pHMB treated enzyme not only lost its activity completely but also showed a characteristic increase in absorption at 255 nm, which is typical of mercaptide formation. These observations also point towards the presence of cysteine at or near the active site. Reaction of the enzyme with N-bromosuccinimide led to the inactivation of enzyme which was accompanied by a sharp decrease in absorption of the modified protein at 280 nm. Moreover, the absorbance at 260 nm of the modified protein was higher than at 280 nm, which is typical of tryptophan modification. NBS is also known to react with tyrosine, histidine and cysteine in addition to tryptophan (Ramachandran and Witkop, 1967). With the ruling out of the involvement of tyrosine for the catalytic activity, the NBS mediated inhibition could either be due to the exclusive modification of tryptophan residues or as a result of a cumulative modification of histidine, cysteine and tryptophan residues.

Modification of S1 nuclease with HNBB also resulted in loss of activity, further confirming the presence of tryptophan at or near the active site.

In all the above studies involving the modification of histidine, cysteine and tryptophan, the substrates viz. ssDNA, RNA, 3'AMP could not protect the enzyme from inactivation showing that the catalytic site may be different from the substrate binding site. In addition, modification of any of these residues individually resulted in the total loss of activity towards ssDNA, RNA, and 3'AMP, indicating the presence of a single catalytic site responsible for the hydrolysis of these substrates. These observations are in agreement with those of Oleson and Hoganson (1981).

It has been reported that enzymes catalysing the hydrolysis of phosphate containing substrates have phosphate binding domains in their structure, consisting mainly of amino groups (Rippa et al., 1967). The presence of phosphate groups in all the three substrates (ssDNA, RNA, 3'AMP), of S1 nuclease prompted us to look for the importance of amino groups in the phosphate binding domain. When the lysine groups of the enzyme were modified with TNBS, it lost approximately 65% of its activity towards all the three substrates. The involvement of lysine residues at the substrate binding site were also ascer-

tained by treating the enzyme with PLP. Since PLP has a phosphate group, it specifically attacks the phosphate binding site of the enzyme and forms Schiff base with the amino groups of lysine. The loss of activity after the modification of lysine residues could primarily be due to the blocking of the substrate binding site rather than inactivation of the enzyme. The inactivation of S1 nuclease with TNBS and PLP could be prevented by pre-incubating the enzyme with ssDNA, RNA and 3'AMP. This observation, apart from confirming the presence of separate catalytic and substrate binding sites, also shows the involvement of lysine residues in the substrate binding site.

The substrates catalysed by S1 nuclease contain three components namely, a base, a sugar and a phosphate in their structure and hence the enzyme should have a nucleotide binding site consisting of three recognition sites. S1 nuclease is a base and sugar non-specific enzyme. However, the enzyme hydrolyses ssDNA approximately twice the rate of RNA, indicating that the enzyme prefers deoxyribosugars to ribosugars. This in turn suggests that only sugar and phosphate binding domains may be of consequence in substrate binding and subsequent catalysis. Our results on substrate protection studies have shown that only TNBS and PLP mediated inactivation of the enzyme is prevented by the substrates.

Though this gives an impression of the existence of separate substrate binding and catalytic sites, it is reasonable to assume that the active site (i.e. the nucleotide binding site) might be made up of a phosphate binding site responsible for substrate binding and a hydrolytic site responsible for the catalysis. Pyrophosphate is a more potent inhibitor of S1 nuclease though inorganic phosphate inhibits the enzyme (Shishido and Ando, 1985). Oleson and Sasakuma (1980) studied the effect of various nucleotides on the activity of S1 nuclease and observed that compared to 5'NMP and 5'NDPs, 5'NTPs especially 5'dNTPs, are the most potent inhibitors of the enzyme. This observation suggests that the phosphate binding site should consist of three sub-sites. The end products of S1 nuclease digestion of ssDNA and RNA are 5'mononucleotides and 3'mononucleotides act as substrates. This indicates that the phosphate binding site and the hydrolytic site are situated near to each other. Cuatrecasas et al. (1968, 1969) also postulated a similar kind of active site for Staphylococcal nuclease, consisting of three non-equivalent phosphate binding subsites and a closely related phosphodiester hydrolytic site. It should also be noted that while 3'mononucleotides act as substrates, 5'nucleotides inhibit the enzyme (Oleson and Sasakuma, 1980). This can be correlated to the different configuration and the increased distance between

sugar and phosphate moieties in 5'nucleotides compared to 3'nucleotides. However, the inhibition caused by 5'nucleotides could be due to the blocking of phosphate binding site of the enzyme. All these observations point towards the importance of phosphate binding domain in the active site of S1 nuclease.

Recently, increasing attention is being paid to the role of 'zinc fingers' in DNA binding proteins. Though the zinc finger proteins are known to interact predominantly with DNA, the ability of TFIIIA, a zinc finger containing protein from Xenopus, to bind both DNA and RNA suggests the versatility not demonstrated for the helix turn helix (Evans and Hollenberg, 1988). S1 nuclease is also a nucleic acid binding protein requiring zinc for its activity. It contains three gram atoms of zinc per mole of the enzyme and removal of zinc results in the loss of its secondary structure. Circular dichroism and amino acid analysis data have shown that S1 nuclease has less of α-helical structure (25%) and consists of a large number of hydrophobic amino acids (Shishido and Habuka, 1986). Through chemical modification studies, we have shown that histidine and cysteine residues are required for the enzyme activity. These observations point towards the possible presence of 'zinc fingers' in S1 nuclease. Involvement of histidine in DNA binding

has been shown in the case of transcriptional regulatory proteins (Parraga et al., 1988). However, present studies with S1 nuclease indicates the involvement of histidine in catalysis rather than in substrate binding. If histidine had some role in DNA binding, then inorganic phosphate would not have brought about complete inhibition of the enzyme. All the above observations lead to the question as to whether zinc fingers, apart from binding to nucleic acids, have a role in catalysis. Further studies, especially amino acid sequence determination are required to prove this speculation.

CHAPTER 3

IMMOBILIZATION OF S1 NUCLEASE

A : Binding Through Protein Moiety

SUMMARY

S1 nuclease was coupled to gelatin-alginate composite matrix using the residual free aldehyde groups on the surface of glutaraldehyde crosslinked matrix. The immobilized enzyme retained approximately 10% activity of the soluble enzyme. When partially purified enzyme was bound to the matrix, the immobilized preparation did not show any detectable enzyme activity. However, the activity could be restored when the coupling was carried out in the presence of a coprotein or substrate. The optimum pH of the immobilized S1 nuclease shifted to 3.8 from 4.3 for the soluble enzyme. Also, the optimum temperature increased to 65°C after immobilization. Bound S1 nuclease showed increased pH and temperature stabilities. Immobilization brought about a two-fold decrease in Michaelis-Menten constant (K_m). The bound enzyme showed poor stability to repeated use.

INTRODUCTION

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 due to this property, several enzymes have been immobilized for their potential application in routine biochemical and clinical analysis (Guilbault, 1982; Sundaram, 1982). Among the several single strand specific nucleases S1 nuclease has extensively been used, as it can be prepared easily from commercially available crude α-amylase powder, it is stable to low concentrations of denaturants often used in annealing and its specificity for single stranded nucleic acids is extremely high under the right conditions (Vogt, 1980). Though P1 nuclease has been immobilized on various supports (Rokugawa et al., 1979, 1980), no information is available regarding the immobilization of S1 nuclease. Hence, attempts were made to develop a suitable method to obtain a highly active and stable immobilized S1 nuclease, useful for routine analytical purposes. In the present studies, binding the enzyme covalently to a suitable matrix was preferred as it is possible to obtain an active and stable immobilized preparation. Gelatin was chosen as the matrix since it has been shown to be a useful support for immobilization of biologically active macromolecules (Wang

et al., 1982; Parascandola and Scardi, 1981; Kennedy et al., 1984) and is readily available at a low price. Being a protein, gelatin has an added advantage of stabilizing the immobilized enzyme (Kennedy et al., 1984).

MATERIALS AND METHODS

Materials:

Yeast RNA (Sisco Research Laboratories, India), DEAE-cellulose and CM-cellulose (Whatman, U.K.), glutaraldehyde (Fluka AG, Switzerland), sodium alginate and bovine serum albumin (Loba Chemie Indoaustranal Co., India) and Takadiastase (Unisankyo, India) were used. Gelatin was obtained from the local market. All other chemicals used were of analytical grade. High molecular weight DNA from buffalo liver was isolated as described in Chapter 2.

Methods:

Assay of S1 nuclease: S1 nuclease was assayed at pH 4.6 and 37°C by measuring the increase in the absorbance at 260 nm following the hydrolysis of single stranded DNA, as described before (Chapter 2).

Assay of RNase T1: RNase T1 activity determination was carried out according to the procedure of Uchida and Egami (1967b). The standard assay mixture of 0.70 ml contained , 0.25 ml of RNA (12 mg/ml), 0.25 ml of 0.2 M Tris-HCl pH 7.5, 0.1 ml of 20 mM EDTA and 0.1 ml of

appropriately diluted enzyme. The reaction initiated by the addition of RNA, followed by incubation for 15 min at 37°C. The reaction was arrested by the addition of 0.25 ml uranyl reagent (0.75% uranyl acetate in 25% perchloric acid) and the resulting precipitate was immediately removed by centrifugation. 0.2 ml of the supernatant was then diluted to 5.0 ml with distilled water and the absorbance of the acid soluble nucleotides was measured at 260 nm. An increase in the absorption values of 1.0 is defined as 1 unit of enzyme.

Assay of RNase T2: This was performed as described in Chapter 2.

Purification of S1 nuclease: This was carried out essentially as described in Chapter 2. From the enzyme obtained after the first DEAE-cellulose chromatography, RNase T2 contamination was removed by adsorption onto CM-cellulose at pH 4.4, a procedure in which S1 nuclease is not bound (Rushizky et al., 1975). The partially purified enzyme thus obtained was free from contaminants like RNase T1 and RNase T2 and was used for immobilization studies.

Immobilization technique: Immobilization of S1 nuclease on glutaraldehyde crosslinked gelatin-alginate composite matrix using free aldehyde groups on the surface of the crosslinked matrix was carried out by a slightly modified method of Kennedy et al. (1985). The gelatin-

alginate composite beads were prepared according to Brodelius and Nilsson (1980).

A 6% (w/v) solution of gelatin in 2% (w/v) alginate, maintained at 50 - 55°C, was passed under pressure through an extruder having a finely drawn needle (Klein and Vorlop, 1984) into a 3% solution of $CaCl_2$. The microbeads formed were left in $CaCl_2$ solution for about 2 h, under mild stirring. The beads were then washed free of excess $CaCl_2$ and incubated with 1% (v/v) glutaraldehyde in sodium bicarbonate buffer (pH 9.5, 0.01 M) at room temperature for 1 h for crosslinking. The beads were then washed free of excess glutaraldehyde with distilled water and immediately used for coupling.

In a typical experiment, 1 g (wet weight) of crosslinked beads were incubated with 150 U of partially purified S1 nuclease (350 µg protein) in 3 ml sodium acetate buffer (pH 5.5, 0.03 M) containing 120 mg of BSA, for approximately 4 h at 4°C, with occassional agitation. The supernatant was decanted and the beads were washed successively with coupling buffer, 0.5 M NaCl in the coupling buffer and finally with assay buffer (sodium acetate buffer, pH 4.6, 0.03 M). The amount of enzyme bound was determined by estimating the difference in the enzyme activity before loading on the matrix and after coupling.

Assay of the immobilized S1 nuclease: The immobilized enzyme was assayed by incubating 1 g (wet wt.) of the matrix with 3 ml of the standard reaction mixture at 37°C, in a thermostated shaker water bath (75 - 100 rpm) for an appropriate time (10 - 15 min) followed by measuring the acid soluble nucleotides at 260 nm after precipitation of the unreacted DNA.

One unit of the enzyme is defined as the amount of enzyme required to digest 10 µg of single stranded DNA in 30 min under the assay conditions.

RESULTS

When 150 U of crude S1 nuclease (enzyme obtained after ammonium sulfate fractionation) was reacted with 1 g (wet wt.) of glutaraldehyde crosslinked gelatin-alginate composite matrix, 50 U were bound. The effectiveness factor (η) of the immobilized preparation was approximately 0.1 indicating the efficiency of the system to be 10%. With increase in glutaraldehyde concentration used for crosslinking, the efficiency decreased significantly (Fig. 3A.1). Since 1% glutaraldehyde crosslinked matrix gave optimum results, with respect to stability of the beads and efficiency of the immobilized preparation, further experiments were carried out using 1% glutaraldehyde crosslinked matrix. It was also observed that the efficiency of the immobilized S1 nuclease decreased with increase in enzyme

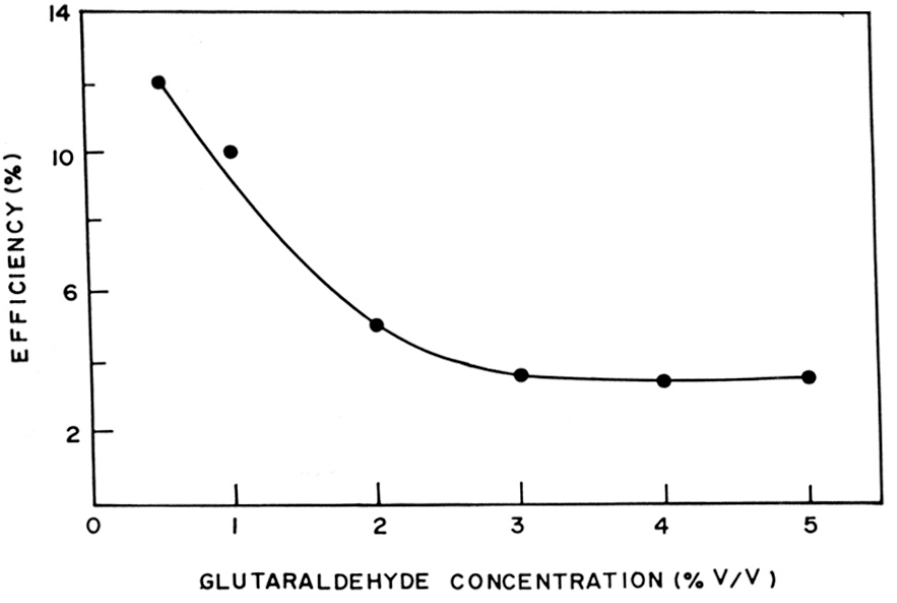


Fig. 3A.1 : **Effect of glutaraldehyde concentration on the efficiency of immobilized S1 nuclease:**

1 g (wet wt.) of the matrix was crosslinked with varying concentrations of glutaraldehyde (0.5 - 5% v/v) in 0.01 M sodium bicarbonate buffer pH 9.5 at room temperature for 1 h. Coupling of S1 nuclease to the crosslinked matrix was carried out by incubating 150 U of the crude enzyme with 1g (wet wt.) of the crosslinked matrix at pH 5.5, followed by determining the efficiency of the immobilized system.

load.

However, when partially purified enzyme was bound to 1% glutaraldehyde crosslinked matrix under similar conditions (as that of crude enzyme) it did not show any detectable activity. The activity could be restored when the coupling was carried out in presence of a co-protein or substrate. It was also observed that the concentration of the coprotein plays a role in the optimal retention of activity (Fig. 3A.2). The results of a typical immobilization procedure of partially purified S1 nuclease on glutaraldehyde crosslinked gelatin-alginate composite matrix are given in Table 3A.1.

Table 3A.1 : Immobilization of S1 nuclease on glutaraldehyde crosslinked gelatin-alginate composite matrix

Enzyme loaded (U)	Enzyme bound (U)	Activity of the complex	Efficiency* (%)
150	85	8	9.4

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

When 150 U of partially purified S1 nuclease were reacted with 1 g (wet wt.) of 1% (v/v) glutaraldehyde

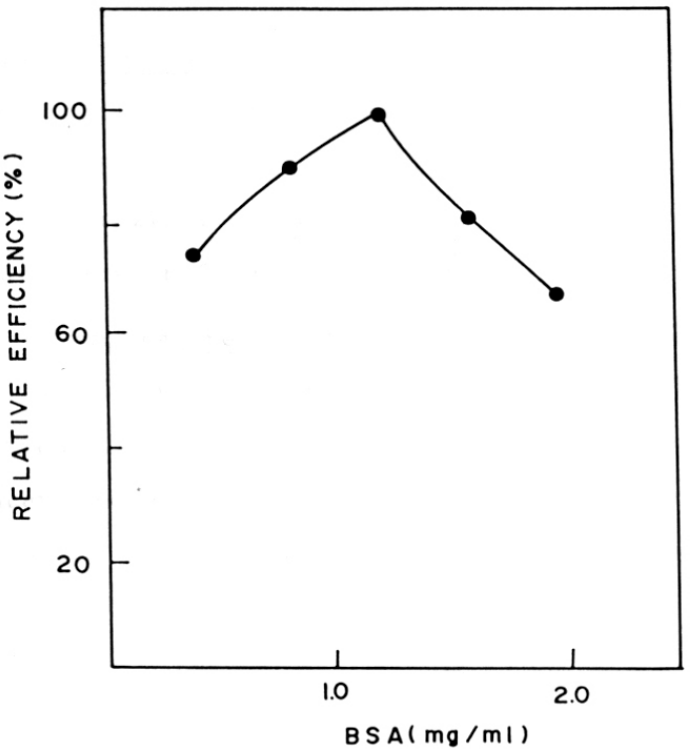


Fig. 3A.2 : **Effect of BSA concentration on the efficiency of bound S1 nuclease:**

1 g (wet wt.) of 1% (v/v) glutaraldehyde crosslinked matrix was incubated with 150 U of partially purified enzyme, in presence of varying amounts of BSA, at pH 5.5 and the efficiency of the immobilized system was determined.

crosslinked matrix at pH 5.0, 85 U were bound. The effectiveness factor (η) of the immobilized preparation was 0.094 indicating the efficiency of the system to be 9.4 (Table 3A.1).

Effect of pH on the reaction rate: Comparison of pH activity profiles of soluble and immobilized S1 nuclease showed a shift in the optimum pH to 3.8 from 4.3 for the soluble enzyme. However, there was no change in the pH activity profiles (Fig. 3A.3).

Effect of temperature on the reaction rate: Determination of temperature activity profiles of soluble and immobilized S1 nuclease showed an increase in the optimum temperature to 65°C from 55°C for the soluble enzyme. No change in the temperature activity profiles was noted (Fig. 3A.4).

Effect of substrate concentration: Determination of kinetic parameters of the soluble and immobilized S1 nuclease showed a two-fold decrease in the K_m and approximately four-fold decrease in the V_{max} as a result of immobilization (Fig. 3A.5, Table 3A.2).

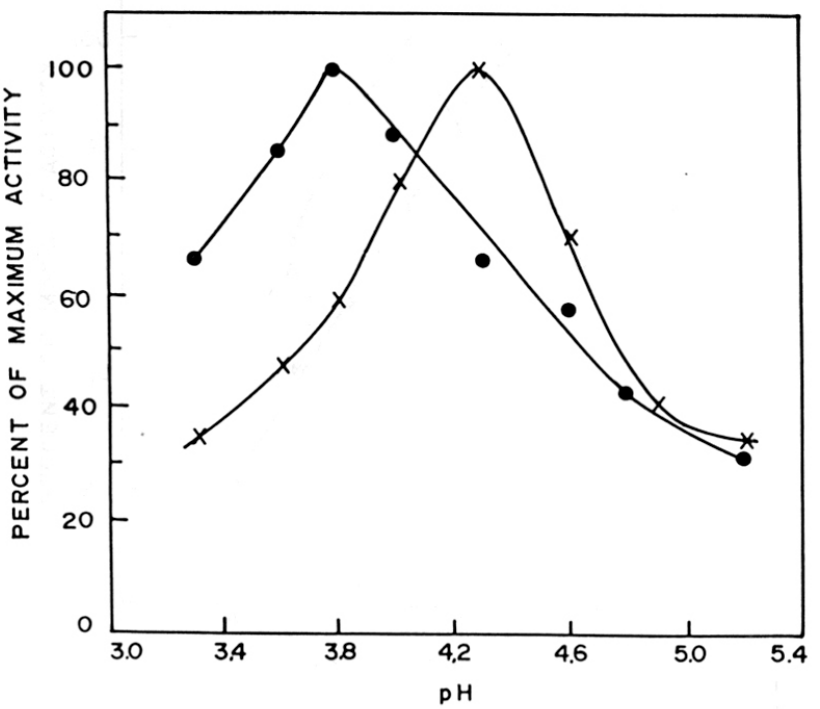


Fig. 3A.3: **pH activity profiles of soluble (x) and immobilized (●) S1 nuclease:**

Soluble ($\backsim$ 2 U) and immobilized (8-10 U) enzymes were assayed in a series of pH (3.3 - 5.3) at 37°C as described under Methods.

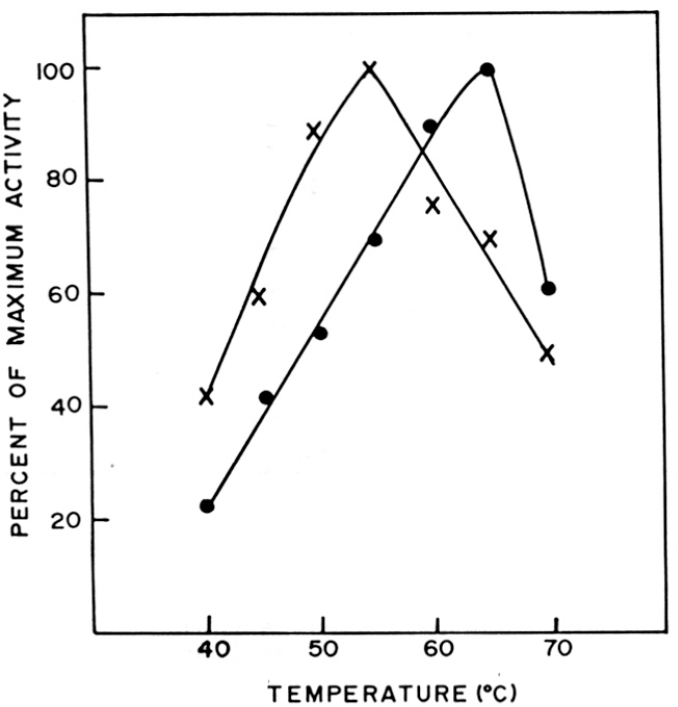


Fig. 3A.4 : **Temperature activity profiles of soluble (x) and immobilized (●) S1 nuclease:**

Soluble (~ 2 U) and immobilized (8-10 U) enzymes were incubated in a series of temperatures (40-70°C) at pH 4.6 and their activities were determined.

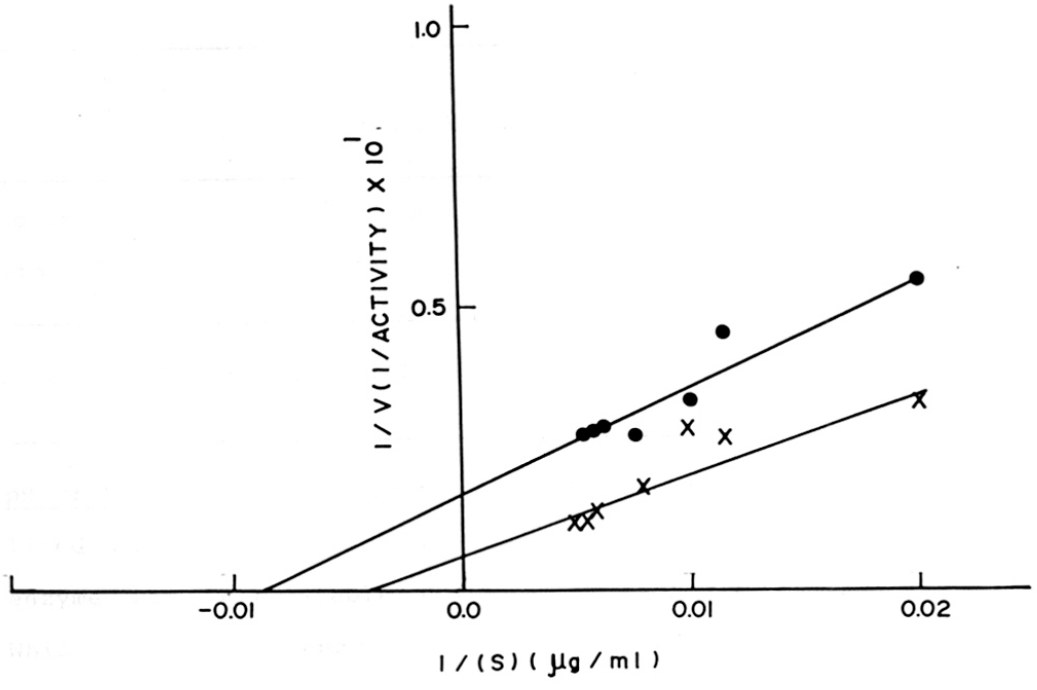


Fig. 3A.5 : Lineweaver-Burk plots for soluble (x) and immobilized (●) S1 nuclease:

Soluble (∽ 2 U) and immobilized S1 nuclease (8 - 10 U) were assayed in a series of substrate concentrations ranging from 50-200 ug of ssDNA at pH 4.6 and 37°C.

Table 3A.2 : Kinetic data of soluble and immobilized Sl nuclease

State of the enzyme	K_m (ug/ml)	V_{max}* (U)
Soluble	266	200
Immobilized	144	58

*Soluble - U/ml; Immobilized - U/g (wet wt.) of the matrix

pH stability: pH stability of both soluble and immobilized Sl nuclease is shown in Fig. 3A.6. The bound enzyme was more stable compared to soluble enzyme. While the soluble enzyme lost more than 50% of its activity at pH 5.3, the immobilized preparation retained its complete activity.

Temperature stability: Comparison of the temperature stability of both soluble and immobilized enzymes showed the immobilized enzyme to be more stable. While the soluble enzyme lost its activity completely at 70°C, the immobilized enzyme retained more than 40% of its initial activity (Fig. 3A.7).

Stability to repeated use: Gelatin-alginate bound Sl nuclease showed poor stability to repeated use and lost more than 50% of its initial activity after five cycles

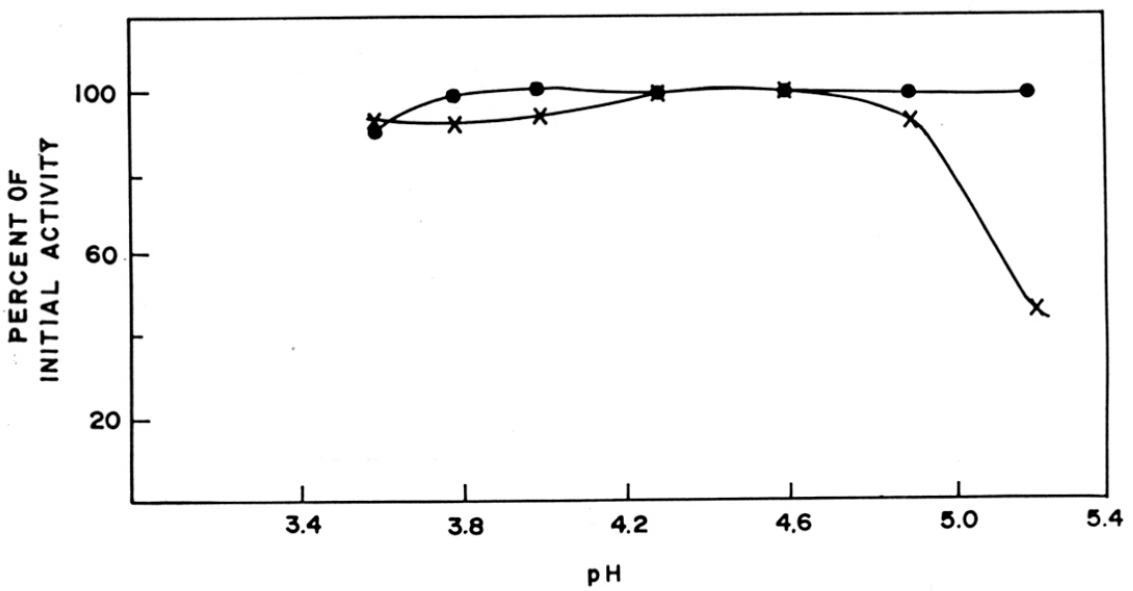


Fig. 3A.6 : pH stability of soluble (x) and immobilized (●) S1 nuclease:

Approximately 2 U of soluble and 8 - 10 U of immobilized enzymes were preincubated at different pH (3.6 - 5.2) for 3 h at room temperature and their activities were determined under standard assay conditions.

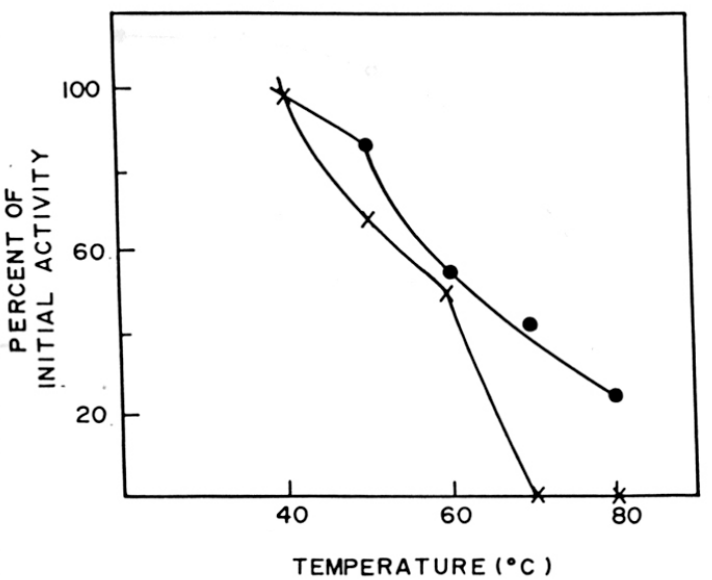


Fig. 3A.7 : **Temperature stability of soluble (x) and immobilized (●) S1 nuclease:**

Both soluble (∽ 2 U) and immobilized (8 - 10 U) enzymes were preincubated at different temperatures ranging from 35-70°C at pH 4.6 and their activities were determined under standard assay conditions.

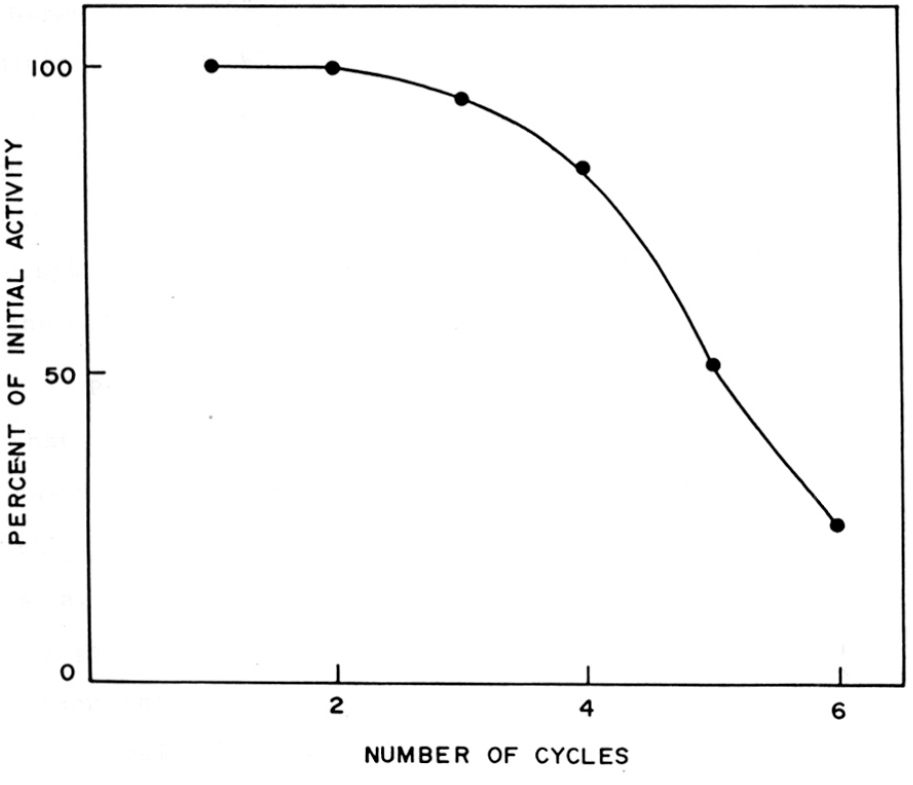


Fig. 3A.8 : **Effect of number of assay cycles on the activity of immobilized S1 nuclease:**

8 - 10 U (1 g wet wt.) of the immobilized preparation was assayed under standard assay conditions. After every use, the immobilized enzyme was washed free of substrate and products and used for the next assay.

(Fig. 3A.8). Slight disintegration of the matrix was also observed after every use.

DISCUSSION

Among the several methods available for binding enzymes covalently to insoluble supports, binding via glutaraldehyde was preferred as this method is simple, non-toxic and can be carried out over a wide range of pH (Marty, 1985). Though alkaline pH is favoured for glutaraldehyde activation of the matrix, the subsequent coupling of the enzyme can be achieved depending on the stability of the enzyme in question.

Preliminary experiments using crude enzyme showed that optimum results are obtained when 150 U of the enzyme is reacted with 1 g (wet wt.) of 1% (v/v) glutaraldehyde crosslinked matrix, at pH 5.0 and 4°C for 4 h. When partially purified S1 nuclease was bound to 1% glutaraldehyde crosslinked gelatin-alginate matrix under the above conditions, it did not show any detectable activity. However, the activity could be restored when the coupling was carried out in presence of a coprotein (BSA) or substrate (ssDNA). The loss of activity in the absence of coprotein could be due to conformational changes as a result of multiple attachment of of the enzyme to the matrix rather than diffusional limitations, since crude S1 nuclease bound to Con A-

Sepharose under similar conditions retained 50-70% activity of soluble enzyme. The decrease in efficiency observed in case of crude enzyme bound to matrices crosslinked with increasing concentrations of glutaraldehyde (Fig. 3A.1) supports this speculation. The effect of BSA in the restoration of activity can be explained on the basis that it must be competing with S1 nuclease for free aldehyde groups on the surface of the matrix, thus preventing the multiple attachment of the enzyme to the support. The effect of increasing concentration of BSA on the efficiency of immobilized S1 nuclease (Fig. 3A.2) supports this explanation. Fig. 3A.2 shows that initially efficiency increases with increase in BSA concentration and after attaining a maximum it decreases. The decrease in efficiency could presumably be caused by overcrowding on the matrix and/or by protein-protein interactions. When an enzyme or a protein is bound to an insoluble matrix via glutaraldehyde, primarily ε-amino groups of lysine are involved in the binding. We have shown the involvement of lysine residues at the substrate binding domain of S1 nuclease (Chapter 2). Hence the ability of ssDNA to restore the activity can be attributed to the protection of the lysine groups from reacting with the aldehyde groups on the matrix.

Immobilization of S1 nuclease to glutaraldehyde crosslinked gelatin-alginate composite matrix resulted

in a slight shift in the pH optimum towards the acid side (Fig. 3A.3). This implies that the carrier surface is somewhat positively charged either due to charged residual amino groups or metal cations (Ca^{2+} present in calcium alginate). Similarly, immobilization resulted in an increase in the optimum temperature to 65°C from 55°C for the soluble enzyme. Evaluation of kinetic parameters showed a two-fold decrease in the K_m and a four-fold decrease in the V_{max} as a result of immobilization (Fig. 3A.5, Table 3A.2). The decrease in the K_m of immobilized S1 nuclease can be attributed to the increase in the substrate concentration in the microenvironment of the immobilized enzyme as a result of attraction of negatively charged substrate towards positively charged matrix. Decrease in the V_{max} can be due to blocking or masking of some of the catalytic sites during coupling.

The bound enzyme showed high pH and temperature stabilities, which can be correlated to its rigid conformation in the bound form. However, the bound enzyme showed poor stability to repeated use and lost significant amount of its activity after 5 cycles. The decrease in the activity can be attributed to loss of enzyme due to the slight disintegration of the matrix after every use.

A relatively simple me od for the preparation

of active and stable immobilized S1 nuclease is described. The high optimum temperature and temperature stability will be useful in DNA reassociation kinetic studies in which the incubation temperature varies from 60 - 75°C.

B : Binding Through Carbohydrate Moiety

SUMMARY

Partially purified S1 nuclease was bound through its carbohydrate moiety to Con A-Sepharose containing increasing amounts of lectin. The retention of activity was high varying essentially from 75% on 'low lectin' matrix (1 mg Con A/ml of Sepharose), to no detectable activity on 'high lectin' matrix (8 mg Con A/ml of 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 on 'high lectin' matrix is due to conformational changes brought about by the multiple attachment of the enzyme to the matrix. Interaction of Con A with S1 nuclease was used to predict the nature of the carbohydrate moiety and its location with respect to the active site of the enzyme. Immobilization resulted in an increase in the optimum temperature, pH and temperature stabilities, but it did not effect the pH optimum. A marginal increase in the apparent K_m was observed. The bound enzyme also showed enhanced stability towards 8 M urea. On repeated use, the bound enzyme retained more than 80% of its initial activity after six cycles. These results are discussed taking into consideration the factors affecting immobilized enzymes. In addition, the potential use of immobilized S1 nuclease is discussed.

INTRODUCTION

S1 nuclease is an analytically important enzyme and is used extensively as an analytical tool in molecular biology research. It has been observed that after S1 nuclease treatment of the nucleic acid samples, removal of residual enzyme activity from the reaction mixture is essential and involves repeated extractions with phenol, which in turn results in loss of nucleic acid samples. In such cases, use of immobilized enzymes offer a distinct advantage over soluble enzymes since the immobilized enzyme can be easily removed from the reaction mixture by physical methods. With this objective in mind, though we could successfully immobilize partially purified S1 nuclease on glutaraldehyde cross-linked gelatin-alginate composite matrix, the bound enzyme showed only 10% activity of the soluble enzyme (Chapter 3A). S1 nuclease is a glycoprotein and it contains 18% carbohydrate (Rushizky, 1981). It has been reported that coupling of glycoproteins to insoluble matrices is manifested with a number of problems due to the shielding of the reactive groups of amino acid side chains by carbohydrates. To overcome this difficulty, several glycoprotein enzyme adducts have been prepared, in which the carbohydrate side chains provide the point of attachment between the enzyme and the matrix (Hsiao and Royer, 1979). Such methods of binding either

by adsorption or covalent coupling to solid supports afford high retention of enzyme activity, presumably due to the fact that the carbohydrate moiety of the enzyme is not essential for the catalytic activity and its protein moiety is therefore free of the restrictions imposed upon it as a result of immobilization. It has been shown recently by Shishido and Habuka (1986) that carbohydrate moiety of S1 nuclease is not essential for its catalytic activity. This observation coupled with the ability of S1 nuclease to bind strongly to Con A-Sepharose (Shishido and Habuka, 1986) prompted us to immobilize S1 nuclease through its carbohydrate moiety, the details of which are presented in this Section.

MATERIALS AND METHODS

Materials:

DEAE-Cellulose, CM-cellulose (Whatman, U.K.), Sepharose 4B (Pharmacia Fine Chemicals, Sweden), Concanavalin A (CSIR Biochemicals, India), 3'AMP and α-methylmannoside (Sigma Chemical Co., U.S.A.), Takadiastase (Sankyo, Japan), glutaraldehyde 50% v/v (Fluka AG, Switzerland) and vinylsulfone (Aldrich Chemical Co., U.S.A.) were used. All other reagents used were of analytical grade. High molecular weight buffalo liver DNA was isolated as described in Chapter 2.

Methods:

Partial purification of S1 nuclease was carried out as described in Chapter 3A.

Preparation of Con A-Sepharose: Sepharose 4B containing varying amounts of lectin (1-5 mg of Con A/ml of Sepharose) was prepared by vinylsulfone activation method of Sairam and Porath (1976) modified by Mawal et al. (1985). Sepharose 4B was first vacuum dried and then washed extensively with distilled water to remove the preservatives. 10 ml of Sepharose 4B was mixed with 10 ml of 1 M sodium bicarbonate, pH 11.0 and 2 ml of divinylsulfone (DVS) and the mixture was incubated for approximately 90 min under gentle agitation. Subsequently, excess DVS was removed by extensive washing with 0.3 M sodium bicarbonate (pH 10.0). Coupling of Con A to the activated Sepharose was carried out by reacting the desired amount of protein with the matrix at pH 10.0 (sodium bicarbonate buffer, 0.3 M) for 6-8 h at room temperature. After the incubation, the matrix was washed successively with 0.3 M sodium carbonate, 0.3 M NaCl, 0.3 M glycine and 0.03 M sodium acetate buffer (pH 5.0). By this procedure, quantitative binding of Con A to Sepharose could be achieved. Con A-Sepharose containing 8 mg Con A/ml of Sepharose was obtained commercially from Sigma Chemical Co., USA.

Immobilization technique: Binding of partially purified S1 nuclease to Con A-Sepharose was carried out as follows: One ml of Con A-Sepharose in acetate buffer, pH 5.0 (0.03 M) containing 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ was incubated with 200 U (464 µg protein) of partially purified enzyme at 4°C for 6-8 h, under mild agitation. The supernatant was collected by centrifugation (4000 rpm) and the matrix was extensively washed, initially with the coupling buffer and then with the assay buffer (acetate buffer, pH 4.6, 0.03 M) till the washings showed no detectable nuclease activity. The amount of enzyme bound to the matrix was determined by estimating the difference in the enzyme activity before loading on the matrix and after coupling. However, when the immobilization was carried out in presence of glucose, it was added in optimum concentrations during coupling.

Covalent coupling of S1 nuclease: Covalent coupling of partially purified S1 nuclease to 'low lectin' matrix (1 mg of Con A/ml of Sepharose) was carried out by incubating 5 ml suspension of Con A-Sepharose-S1 nuclease conjugate (200 U) with 0.1% (v/v) glutaraldehyde (effective concentration) in acetate buffer, pH 5.0 (0.03M) at 30°C for 1 h, with occassional agitation. Subsequently, the crosslinking process was arrested by rapidly mixing the reaction mixture with 0.01% ethanolamine

(effective concentration) followed by incubation at 30°C for 1 h. The ethanolamine treated preparation was then centrifuged, washed thoroughly with acetate buffer, pH 5.0 (0.03 M) and suspended in the assay buffer. S1 nuclease covalently bound to the matrix was determined by incubating the crosslinked enzyme preparation with 0.5 M ∝-methylmannoside for 1 h followed by assaying the solubilized nuclease activity under standard assay conditions, after the removal of the matrix. The amount of enzyme bound to the matrix was determined by estimating the difference in the activity before loading on the matrix and after coupling. For comparison, soluble enzyme was subjected to crosslinking under identical conditions.

Assay of soluble and immobilized S1 nuclease: Single stranded DNA, RNA and 3'AMP hydrolysing activities associated with S1 nuclease were assayed as described in Chapter 2.

Con A-Sepharose bound S1 nuclease was assayed similarly by incubating appropriate amounts of the bound enzyme with 3 ml of the standard reaction mixture containing the respective substrates at pH 4.6 and 37°C in a thermostated shaker water bath (75 - 100 rpm) for 30 min followed by estimating the products formed.

Determination of efficiency: Efficiency of the immobi-

lized enzyme was determined by assaying appropriate amounts of the immobilized preparation (4-5 U) using ssDNA, under standard assay conditions followed by calculating the ratio of measured activity to bound activity (Chapter 3A).

RESULTS

Effect of lectin concentration: In a typical experiment, when 200 U of partially purified S1 nuclease were reacted with 1 ml of 'low lectin' matrix (1 mg Con A/ml of Sepharose), 40 U were bound. The effectiveness factor (η) of the preparation was approximately 0.75 indicating that the efficiency of the preparation to be 75%. Though increase in the lectin concentration on the matrix (maintaining the enzyme to matrix ratio constant) was accompanied by an increase in the bound activity (Table 3B.1), it also resulted in a progressive decline in the efficiency of the immobilized preparation to such an extent that the enzyme bound to 'high lectin' matrix (8 mg Con A/ml of Sepharose) showed no detectable activity (Fig. 3B.1).

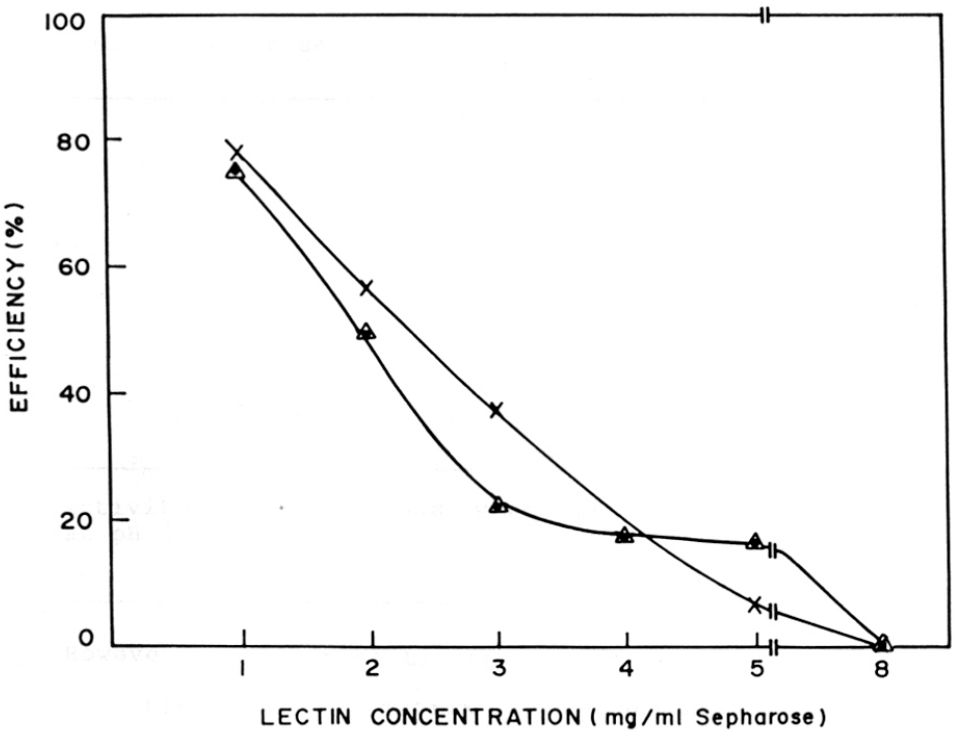


Fig. 3B.1 : Effect of lectin concentration on the efficiency of immobilized S1 nuclease:

Assays were performed by incubating 4-5 U of the immobilized preparation with ssDNA (●) RNA (▲) and 3'AMP (x) at pH 4.6 and 37°C followed by determining the efficiency of the immobilized system.

Table 3B.1 : Effect of lectin concentration on the bound activity*

Con A concentration mg/ml Sepharose	Enzyme loaded (U)	Enzyme bound (U)
1	200	40
2	200	50
3	200	80
4	200	165
5	200	175
8	200	200

*Activity determinations were carried out using ssDNA at pH 4.6 and 37°C.

However, it was possible to recover about 50% activity on 'high lectin' matrix when the immobilization was carried out in presence of glucose (Fig. 3B.2).

Soluble conjugates of S1 nuclease-Concanavalin A obtained by the addition of Con A to the soluble enzyme (keeping the enzyme concentration constant) also showed a progressive decrease in the activity with increase in lectin concentration (Fig. 3B.3). No precipitation of the S1 nuclease-Con A complex was observed in the concentration range tried.

Effect of glutaraldehyde crosslinking: Crosslinking

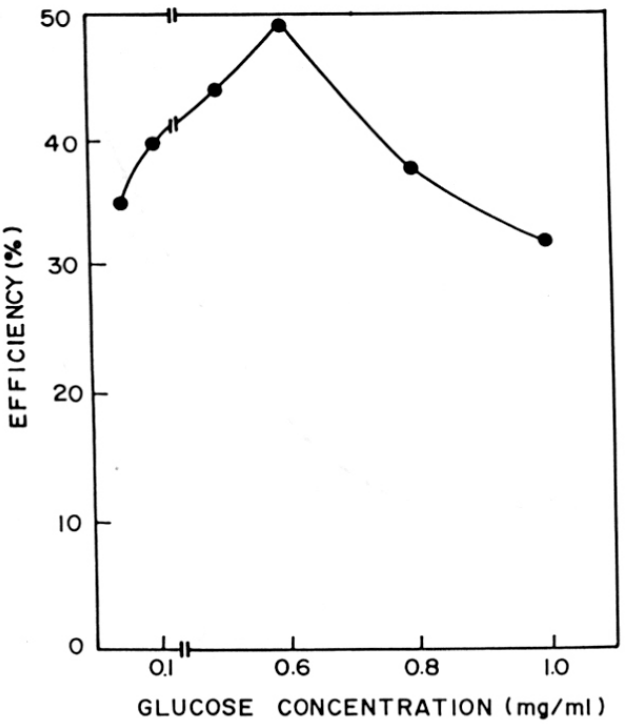


Fig. 3B.2 : Effect of glucose concentration on the efficiency of S1 nuclease bound to 'high lectin' matrix:

Experiments were carried out by incubating 200 U of the enzyme with 1 ml of 'high lectin' matrix and varying amounts of glucose (0.05 - 1 mg) at pH 5.0 and 4°C for 6-8 h as described under Methods. Immobilized S1 nuclease was assayed at pH 4.6 and 37°C using ssDNA as substrate.

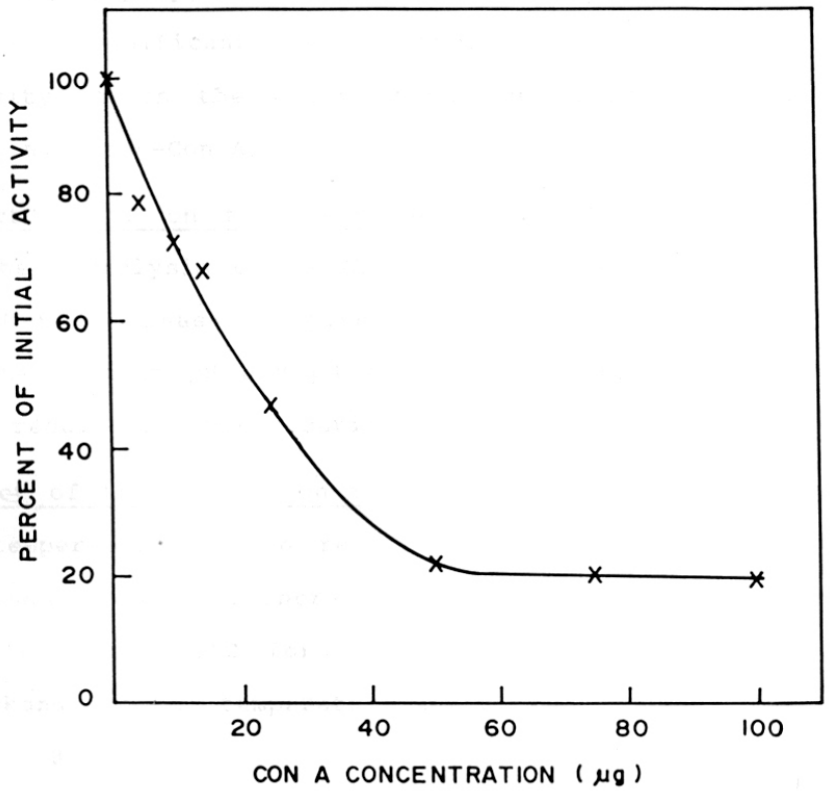


Fig. 3B.3 : Effect of Con A concentration on the activity of soluble S1 nuclease:

Experiments were performed by adding varying concentrations of Con A (0 - 100 µg) to 20 U of the enzyme at pH 5.0 and 30°C followed by assaying the activity of the complex at pH 4.6 and 37°C using ssDNA as substrate.

of 'low lectin' bound enzyme with 0.1% (v/v) glutaraldehyde brought about a decrease in the efficiency of the immobilized preparation from 75% to 20% but it did not have any significant effect either on the soluble enzyme activity or on the activity of the soluble conjugate of S1 nuclease-Con A.

Effect of pH on the reaction rate: Influence of pH on the hydrolysis of ssDNA by both soluble and immobilized S1 nuclease is given in Fig. 3B.4. No change in the optimum pH and pH activity profile was observed as a result of immobilization.

Effect of temperature on the reaction rate: The effect of temperature on the reaction rate of immobilized S1 nuclease showed an increase in the temperature optimum to 70°C from 55°C for the soluble enzyme. However, no change in the temperature activity curve was observed (Fig. 3B.5).

Effect of substrate concentration: Effect of substrate concentration on the activity of both soluble and immobilized enzyme showed a marginal increase in Michaelis-Menten constant (K_m) and a three-fold decrease in the V_{max} as a result of immobilization (Table 3B.2). K_m was calculated from Lineweaver-Burk plots.

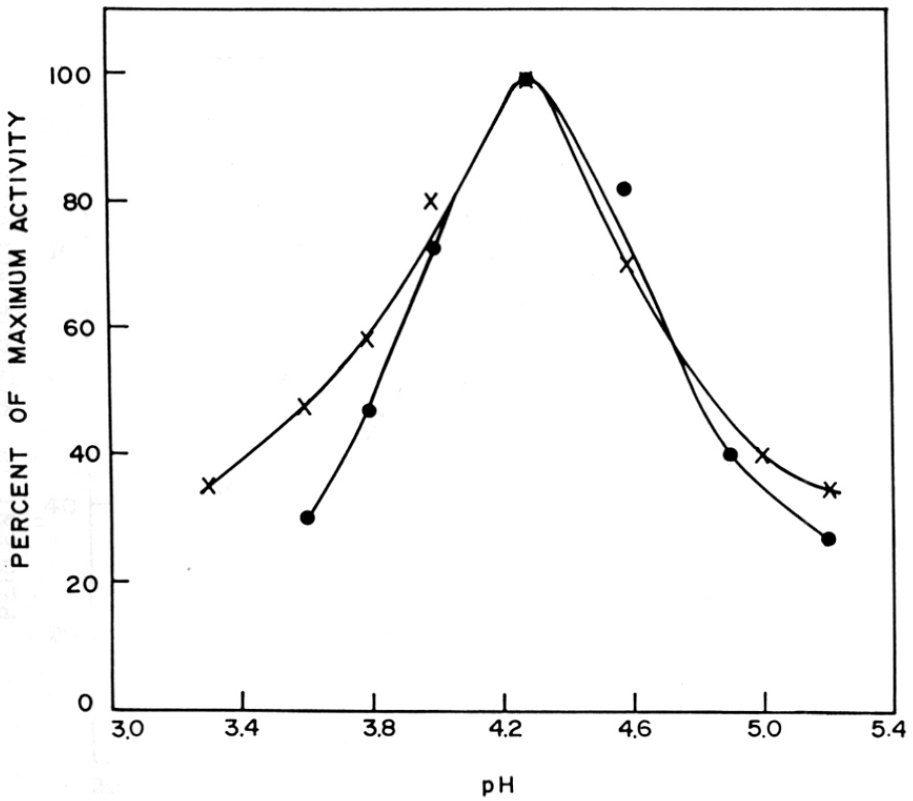


Fig. 3B.4 : pH activity profiles of soluble (x) and immobilized (●) S1 nuclease:

Soluble (∽ 2 U) and immobilized (4-5 U) enzymes were assayed in a series of pH (3.3 - 5.2) at 37°C using ssDNA as substrate.

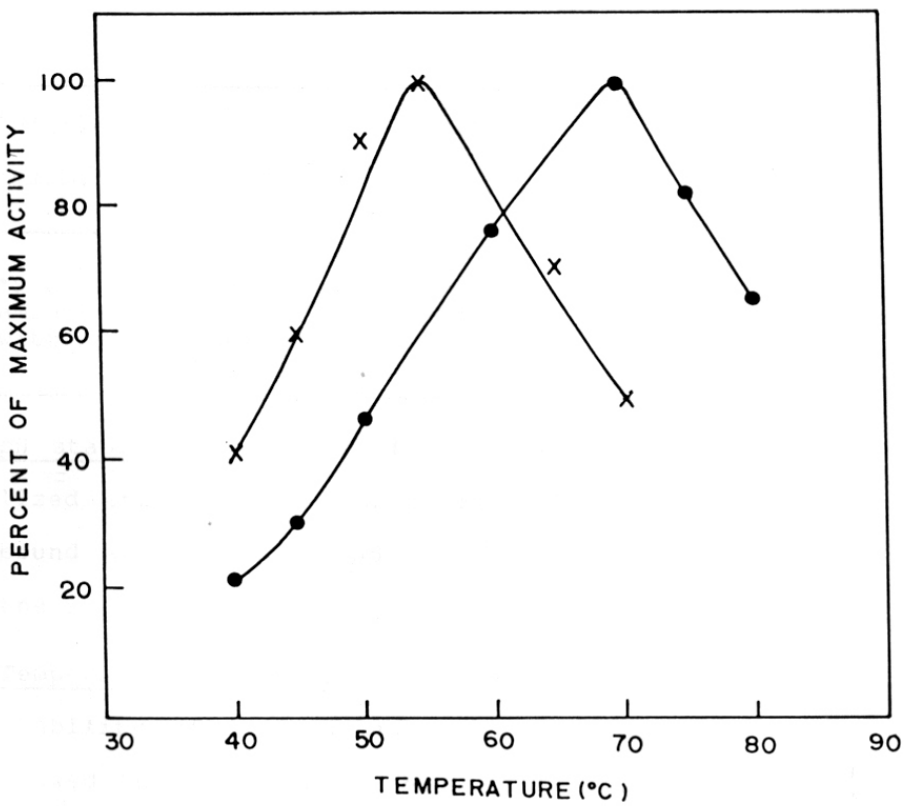


Fig. 3B.5 : Temperature activity profiles of soluble (x) and immobilized (●) S1 nuclease:

Both soluble (∽ 2 U) and immobilized (4-5 U) enzymes were assayed at different temperatures, ranging from 40 - 80°C at pH 4.6 and their activities were determined using ssDNA as substrate.

Table 3B.2 : Kinetic data of soluble and immobilized S1 nuclease*

State of the enzyme	K_m (ug/ml)	V_{max} (U/ml)
Soluble	266.6	166.6
Immobilized	312.5	60.6

*Experiments were carried out by incubating appropriate amounts of soluble (2 U) and immobilized (4-5 U) enzymes with ssDNA (50-200 µg) at pH 4.6 and 37°C.

pH stability: pH stability of both soluble and immobilized enzymes is shown in Fig. 3B.6. Con A-Sepharose bound enzyme was found to be more stable compared to the soluble enzyme.

Temperature stability: Comparison of the temperature stability of both soluble and immobilized S1 nuclease showed the immobilized enzyme to be more stable. While the soluble enzyme completely lost its activity at 70°C, the immobilized enzyme retained more than 85% of its initial activity (Fig. 3B.7).

Stability to denaturants: Effect of 8 M urea on the activity of both soluble and immobilized S1 nuclease at 4°C and 30°C showed that the stability of S1 nuclease increased as a result of immobilization at both the temperatures (Table 3B.3).

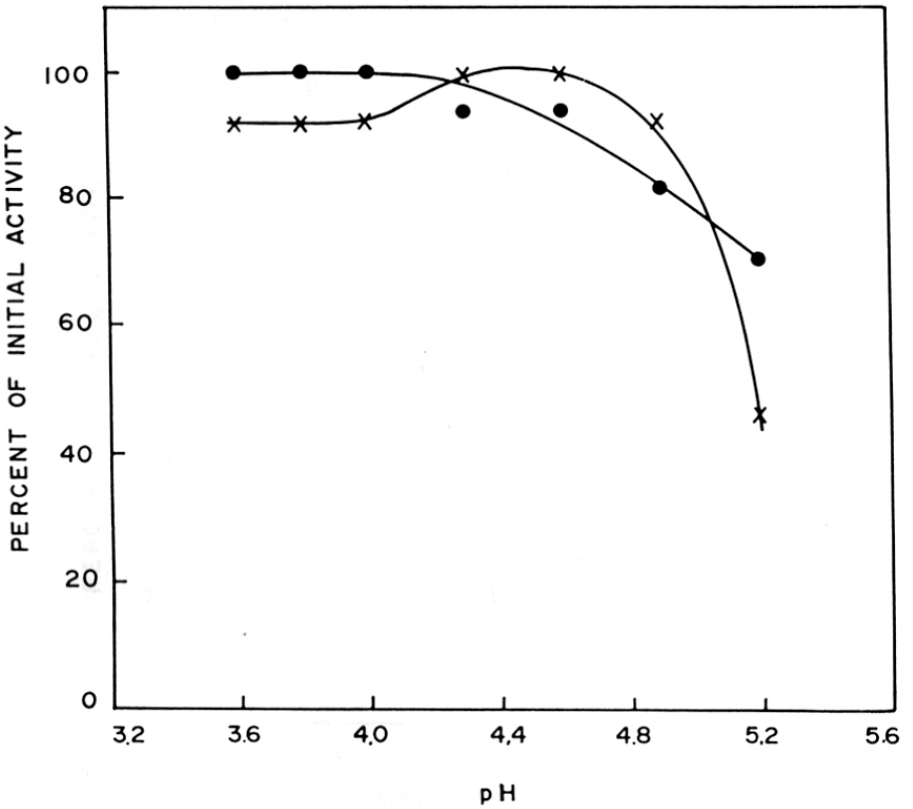


Fig. 3B.6 : pH stability of soluble (x) and immobilized (•) S1 nuclease:

Approximately 2 U of soluble and 4-5 U of immobilized enzymes were preincubated at different pH (3.6 - 5.2) for 1 h at room temperature and their activities were determined at pH 4.6 and 37°C using ssDNA as substrate.

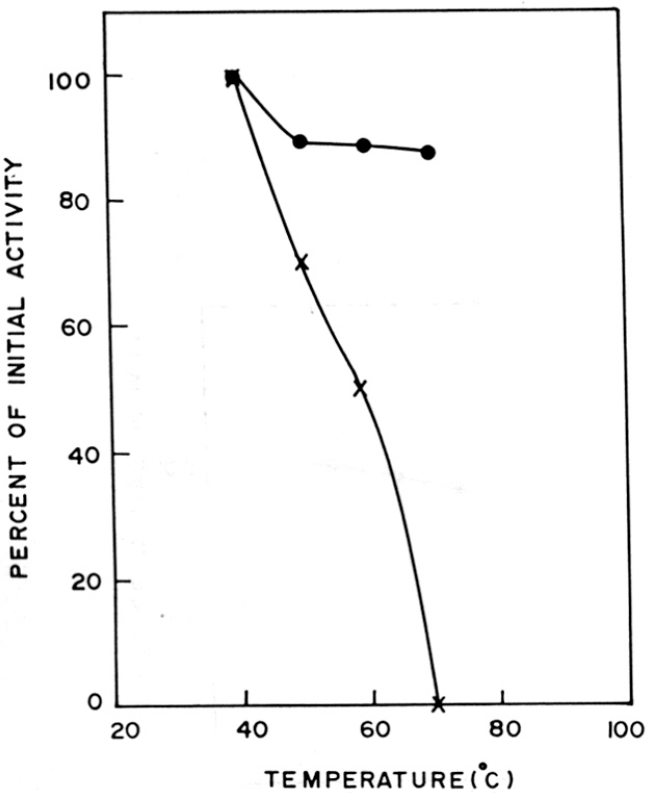


Fig. 3B.7 : Temperature stability of soluble (x) and immobilized (●) S1 nuclease:

Approximately 2 U of soluble and immobilized (4-5 U) enzymes were preincubated at temperatures ranging from 40-80°C for 1 h at pH 4.6 and their activities were determined at 37°C using ssDNA as substrate.

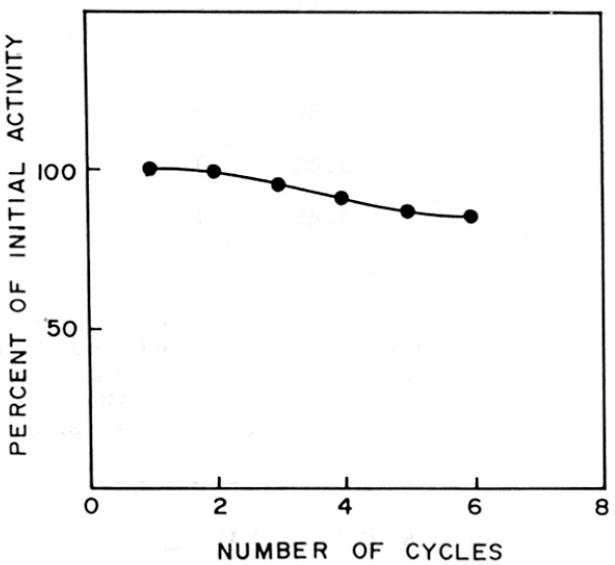


Fig. 3B.8 : Effect of number of assay cycles on the activity of immobilized S1 nuclease:

0.1 ml (4-5 U) Con A-Sepharose bound S1 nuclease was assayed at pH 4.6 and 37°C using ssDNA as substrate. After every cycle, the immobilized enzyme was washed free of substrate and products and used for the next assay.

Table 3B.3 : Effect of 8 M urea on the activity of soluble and immobilized S1 nuclease*

Incubation time (min)	Residual activity (%)			
	Soluble enzyme		Immobilized enzyme	
	4°C	30°C	4°C	30°C
0	100	100	100	100
15	77.7	72.2	100	100
30	72.2	66.5	100	100
45	77.0	55.5	100	100
60	77.0	25.0	100	100
90	72.0	25.0	100	100

*Both soluble and immobilized S1 nuclease (15-20 U) were preincubated in presence of 8 M urea and the residual activity was measured under standard assay conditions using ssDNA as substrate. Activity in absence of 8 M urea was taken as 100%

Stability to repeated use: Con A-Sepharose bound S1 nuclease could be used repeatedly upto 6 cycles without any apparent loss in its activity (Fig. 3B.8). The slight decrease in the activity was mainly because of the loss of gel particles after every use rather than enzyme inactivation.

DISCUSSION

The partially purified S1 nuclease bound to 'low lectin' matrix showed approximately 75% activity of

the soluble enzyme when assayed using both high molecular weight (ssDNA and RNA) and low molecular weight (3'AMP) substrates. However, the enzyme bound to 'high lectin' matrix did not show any detectable activity. The loss in activity of an enzyme after immobilization can be attributed to diffusional restrictions, conformational changes as a result of multiple attachment to the matrix, steric hindrance, partitioning effects etc. While these factors cannot be avoided, attempts can be made to minimize them. In immobilization studies, the matrix characteristics and the method of attachment play an important role in determining the properties of the bound enzyme. On this background, Sepharose was selected as the matrix since it does not have any charged groups responsible for partitioning effects. Secondly, divinylsulfone activation of the matrix was preferred because during activation an unavoidable simultaneous crosslinking occurs providing a spacer arm on the surface of the matrix which in turn reduces the steric and diffusional restrictions.

In the present studies, the observed loss of activity on 'high-lectin' matrix can be attributed to (a) conformational changes brought about by the multiple attachment of the enzyme to the matrix, (b) steric hindrance due to the shielding of the active site by the carbohydrate side chains as a result of binding to Con A-

Sepharose and (c) overcrowding of the enzyme on the matrix. However, the ability of glucose to restore approximately 50% activity on 'high lectin' matrix (without any detectable decrease in the bound activity) shows that the loss of activity on 'high lectin' matrix is due to the multiple attachment of the enzyme to the matrix rather than overcrowding. The effect of glucose in the restoration of activity can be explanied on the basis that it must be competing with S1 nuclease for Con A on the matrix, thus preventing the multiple attachment of the enzyme to the support. The effect of increasing glucose concentration on the efficiency (Fig. 3B.2) supports this explanation. Hence, to obtain a highly active immobilized preparation, it is essential to avoid the multiple attachment of the enzyme to the matrix. This can be achieved either by reducing the lectin concentration on the matrix or by immobilizing the enzyme in presence of a competing molecule like glucose or mannose. The observed increase in efficiency of the immobilized preparation with decrease in lectin concentration on the matrix supports the above speculation. The substantial loss of activity of the 'low lectin' bound enzyme coupled with the insensitivity of the soluble enzyme to glutaraldehyde crosslinking further substantiates our view that the loss of activity of the enzyme is due to conformational changes as a result of the

multiple attachment of the enzyme to the matrix. Since the enzyme bound to 'low lectin' matrix showed maximum efficiency, further studies were carried out using 'low lectin' bound enzyme.

The inhibitory effect of Con A on the activity of soluble enzyme (Fig. 3B.3) shows that though the carbohydrate moiety is not essential for the catalytic activity of S1 nuclease, its binding to Con A masks the active site of the enzyme and thus interferes in the activity by reducing the accessibility of the substrate to the active site. This also indicates that the carbohydrate moiety is situated near the active site of the enzyme. Insensitivity of bovine plasma amine oxidase to Con A was interpreted on the basis that carbohydrate moiety is not essential for its catalytic activity and it is situated away from the active site of the enzyme (Ishizaki and Yasunobo, 1980).

Carbohydrate moiety of glycoproteins consists of two main classes of oligosaccharides viz. complex oligosaccharides and high mannose containing oligosaccharides (Merkle and Cummings, 1987). S1 nuclease appears to be a high mannose containing glycoprotein, since an increase in the Con A concentration on the matrix brings about significant loss of activity of the bound enzyme. If the enzyme were to contain complex oligosaccharides,

it would not have been very sensitive to changes in the lectin concentration. In addition, the requirement of high concentration of ∝-methylmannoside (0.5 M) to elute the bound enzyme substantiates the above explanation Our results are in consistance with those of Shishido and Habuka (1986) who attributed the strong affinity of S1 nuclease to Con A to its high mannose content.

According to Oleson and Sasakuma (1980), S1 nuclease catalyses the hydrolysis of ssDNA, RNA and 3'AMP at relative rates of 100, 52 and 13 respectively. Oleson and Hoganson (1981) interpreted the ability of 3'AMP to competitively inhibit the hydrolysis of ssDNA and vice versa, to the existance of a common catalytic site for both types of substrates. In the present studies, the similar efficiency pattern obtained using ssDNA, RNA and 3'AMP (Fig. 3B.1) also indicates that the hydrolysis of all three substrates are catalysed by the same active site. If the active sites were different, then it would have resulted in different efficiency patterns. This result is in agreement with the observations of Oleson and Hoganson (1980) and our chemical modification studies (Chapter 2).

Action of 8 M urea on the soluble enzyme showed that while the enzyme retained more than 70% of its activity at 4°C, it lost 75% of its activity at 30°C.

The sensitivity of the soluble enzyme at 30°C could be due to the flexing of the enzyme molecules at higher temperatures resulting in the exposure of the interior bonds to urea. However, the enhanced stability of the immobilized S1 nuclease to 8 M urea can be attributed to the rigidity or tight conformation in the bound form. Though rigidity in the bound form prevents the flexing of the molecule at higher temperatures, it might not be preventing the local changes in the conformation required for the catalysis because, if the latter was not the case, then the immobilized enzyme would not have been active at all. The superior temperature and pH stabilities of immobilized S1 nuclease also point towards the rigidity of its conformation in the bound form. In addition, the repeated usability and absence of leaching in presence of high salt concentration (0.35 M NaCl) shows the high stability of the immobilized preparation.

Evaluation of the kinetic parameters showed that the immobilized enzyme follows Michaelis-Menten kinetics and there is a marginal increase in the K_m as a result of binding. This observation, coupled with the high efficiency of the immobilized S1 nuclease indicates that steric and diffusional limitations may not have a role. However, the decrease in the apparent V_{max} can be attributed to the blocking of some active sites

during immobilization and this in turn could account for 25-30% decrease in the efficiency.

A simple procedure for obtaining a highly active and stable immobilized S1 nuclease is described. High efficiency and improved stability makes S1 nuclease Con A-Sepharose conjugate a potentially useful system for routine analytical purposes. The immobilized system can also be used for the production of 5'mononucleotides.

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