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Influence of Lectin Concentration on the Catalytic Properties of S1 Nuclease Bound to Concanavalin A-Sepharose

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ABSTRACT

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 the "low lectin" matrix (1 mg Con A/mL of Sepharose), to no detectable activity on the "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 the presence of glucose, suggesting that the loss of activity on the "high lectin" matrix is caused by 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 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 showed enhanced stability toward 8 *M* urea. On repeated use, the bound enzyme retained more than 80% of its initial activity after 6 cycles. These results are discussed taking into consideration the factors affecting immobilized enzymes. In addition, the potential use of immobilized S1 nuclease as an analytical tool is discussed.

Index Entries: Single strand specific nuclease from *Aspergillus oryzae;* S1 nuclease immobilization; binding through carbohydrate

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moiety; S1 nuclease immobilization on Con A-Sepharose; influence of lectin concentration; immobilization in presence of glucose; immobilization of nuclease.

INTRODUCTION

Single strand specific nucleases, by their ability to selectively hydrolyze single stranded nucleic acids and single stranded regions in double stranded nucleic acids, are widely used as analytical tools for nucleic acid hybridization, analysis of nucleic acid structure, isolation of specific genes, and gene manipulation (*1*). 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 prepared easily in large amounts from commercially available crude α-amylase powder. Also valuable are its stable-to-low concentrations of denaturants (often used in annealing), and its extremely high specificity for single stranded nucleic acids (under the right conditions) (*2*). 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 offers a distinct advantage over soluble enzymes, since the immobilized enzyme can be removed easily from the reaction mixture by physical methods. With this objective in mind, though we could successfully immobilize partially purified S1 nuclease on a glutaraldehyde cross-linked gelatin–alginate composite matrix, the bound enzyme showed only 10% activity of the soluble enzyme (*3*). S1 nuclease is a glycoprotein and contains 18% carbohydrate (*4*). It has been reported that coupling of glycoproteins to insoluble matrices is manifested with a number of problems because of 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 carohydrate side chains provide the point of attachment between the enzyme and the matrix (*5*). Such methods of binding either by adsorption or covalent coupling to solid supports afford high retention of enzyme activity, presumably because the carbohydrate moiety of the enzyme is not essential for the catalytic activity, and its protein moiety is, therefore, free of the restrictions imposed on it as a result of immobilization. It has been shown recently by Shishido and Habuka (*6*) 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 (*4,6*) prompted the present authors to immobilize S1 nuclease through its carbohydrate moiety; the details of which are presented in this communication.

MATERIALS AND METHODS

DEAE-cellulose, CM-cellulose (Whatman, UK), Sepharose 4B (Pharmacia Fine Chemicals, Sweden), Concanavalin A (CSIR Biochemicals, India), 3′AMP, α-methylmannoside (Sigma Chemical Co., USA), Takadiastase (Sankyo, Japan), glutaraldehyde 50% v/v (Fluka AG, Switzerland), and vinyl-sulfone (Aldrich Chemical Co., USA) were used. All other reagents were of analytical grade. High molecular weight DNA was isolated from buffalo liver, according to Mehra and Ranjekar (*7*).

Purification of S1 Nuclease

Partial purification of S1 nuclease was carried out essentially according to the modified procedure of Rushizky et al. (*8*). 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 T1 RNase, T2 RNase, and S1 nuclease are separated. From the S1 nuclease obtained after DEAE-cellulose chromatography, residual T2 RNase and double strand specific DNase contaminants were removed by adsorption to CM-cellulose at pH 4.4 and pH 7.0, respectively (*8*). The partially purified enzyme was assayed for T1 RNase and T2 RNase contaminants, according to Uchida and Egami (*9,10*) and, for double strand specific DNase, according to Rushizky and Whitlock (*11*). The partially purified enzyme was free of the above-mentioned contaminants.

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 (*12*), modified by Mawal et al. (*13*). By this method, quantitative binding of Con A to Sepharose could be achieved. Con A-Sepharose containing 8 mg Con A/mL of Sepharose was obtained 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 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, and 1 m*M* $CaCl_2$ was incubated with 200 units (462 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 the 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 (0.03 *M*) at 30°C for 1 h, with occasional 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* α-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 also subjected to crosslinking under similar conditions.

Assay of Soluble and Immobilized S1 Nuclease

Both soluble and immobilized enzymes were assayed at 37°C in the thermostated shaker water bath (75–100 rpm) by monitoring the increase in the absorbance at 260 nm following the hydrolysis of single stranded DNA.

For the soluble enzyme activity, the standard reaction mixture of 1 mL contained 40 μg of sonicated and heat denatured buffalo liver DNA, 1 m*M* $ZnSO_4$, 0.05 *M* NaCl, and 5% glycerol in 0.03 *M* acetate buffer, pH 4.6, and appropriately diluted enzyme. The reaction was initiated by the addition of the enzyme followed by incubation at 37°C for 30 min. The reaction was terminated by the addition of 1 mL of chilled 10% perchloric acid (PCA) and 1 mL of 0.1% BSA. The mixture was left in ice for 10 min and then centrifuged at 3000 rpm for 10 min to sediment the precipitate. The acid soluble nucleotides in the supernatant were measured at 260 nm.

The immobilized enzyme was assayed similarly by incubating appropriate amount of the bound enzyme with 3 mL of standard reaction mixture at pH 4.6 and 37°C for 30 min followed by measuring the acid soluble nucleotides after the precipitation of 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.

RNase Activity of S1 Nuclease

RNA degrading activity of both soluble and immobilized enzymes was assayed in the same manner as that of single strand DNase activity. However, in place of PCA, 10% TCA was used 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 RNA in 30 min under the assay conditions.

Phosphomonoesterase Activity of S1 Nuclease

Phosphomonoesterase activity of both soluble and immobilized S1 nuclease was assayed using 3'AMP as substrate. The total reaction mixture of 1 mL contained 1 m*M* 3' AMP, 1 m*M* $ZnSO_4$, 0.05 *M* NaCl, and 5% glycerol in 0.03 *M* 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 freshly prepared cold solution of 5% SDS and 0.5 N H_2SO_4. The inorganic phosphate liberated was determined according to Bartlett (*14*).

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

Unless otherwise mentioned routine assays were carried out using single stranded DNA as substrate.

RESULTS

Effect of Lectin Concentration

In a typical experiment, when 200 U of partially purified S1 nuclease was 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 1), it also resulted in a progressive decline in the efficiency of the immobilized preparation to such an extent that the enzyme bound to the "high lectin" matrix (8 mg Con A/mL of Sepharose) showed no detectable activity (Fig. 1). However, it was possible to recover approximately 50% activity on "high lectin" matrix when the immobilization was carried out in presence of glucose (Fig. 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. 3). No precipitation of S1 nuclease-Con A complex was observed in the concentration range tried.

Table 1
Effect of Lectin Concentration on the Bound Activity[a]

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

[a]Activity determinations were carried out using single stranded DNA at pH 4.6 and 37°C.

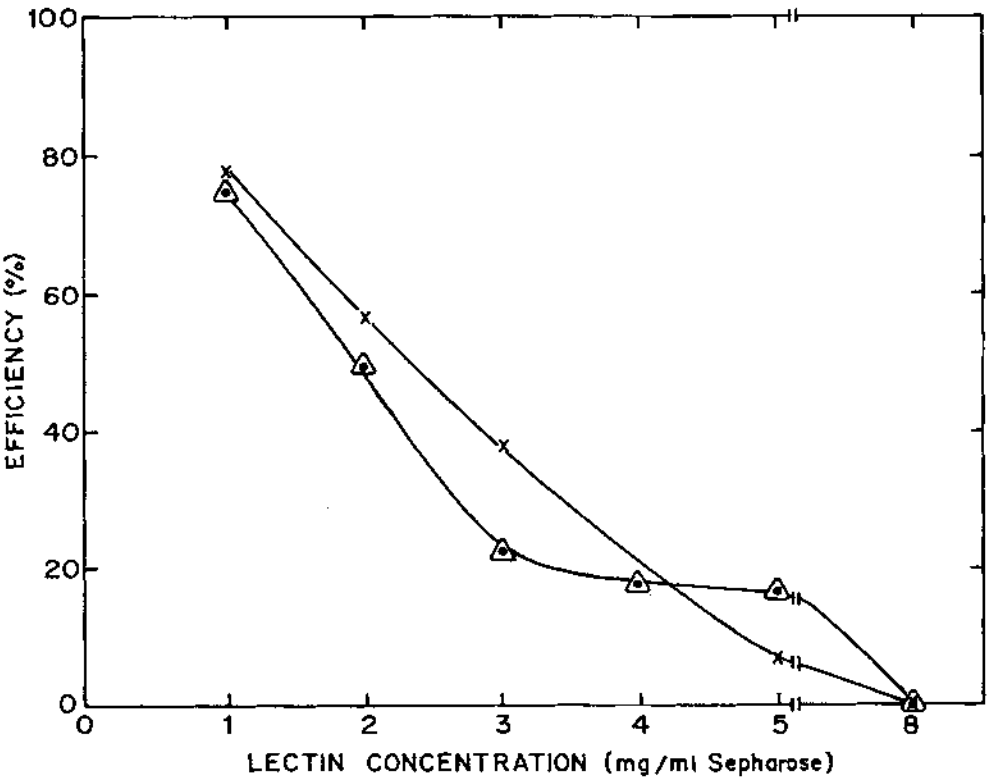

Fig. 1. Effect of lectin concentration on the efficiency of immobilized S1 nuclease. Assays were performed using single stranded DNA (●), RNA (△), and 3'AMP (x) at pH 4.6 and 37°C, as described in Materials and Methods.

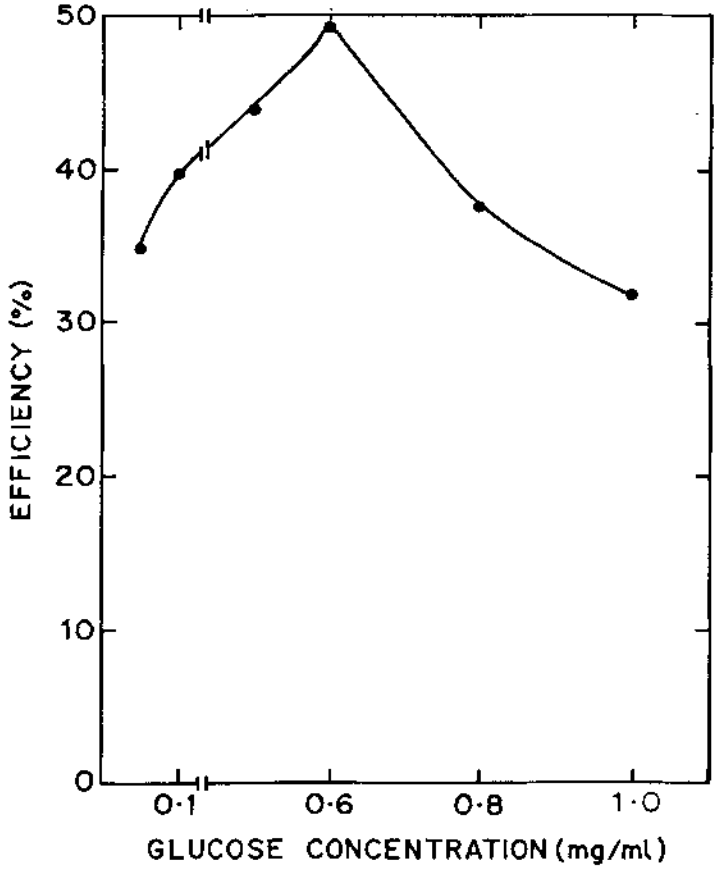

Fig. 2. Effect of glucose concentration on the efficiency of S1 nuclease bound to "high lectin" matrix.

Effect of Glutaraldehyde Crosslinking

Crosslinking 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 soluble conjugates of S1 nuclease-Con A.

Optimum pH and pH Stability

For optimum pH determination, appropriate amounts of both soluble and immobilized enzymes were incubated at different pH ranging from 3.3 to 5.2 (acetate buffer 0.03 *M*) at 37°C followed by assaying their activity. No change in the optimum pH and pH activity profiles were observed as a result of immobilization (Fig. 4).

For pH stability determination, both soluble and immobilized enzymes were preincubated at different pH (3.6–5.2) for 1 h at room temperature

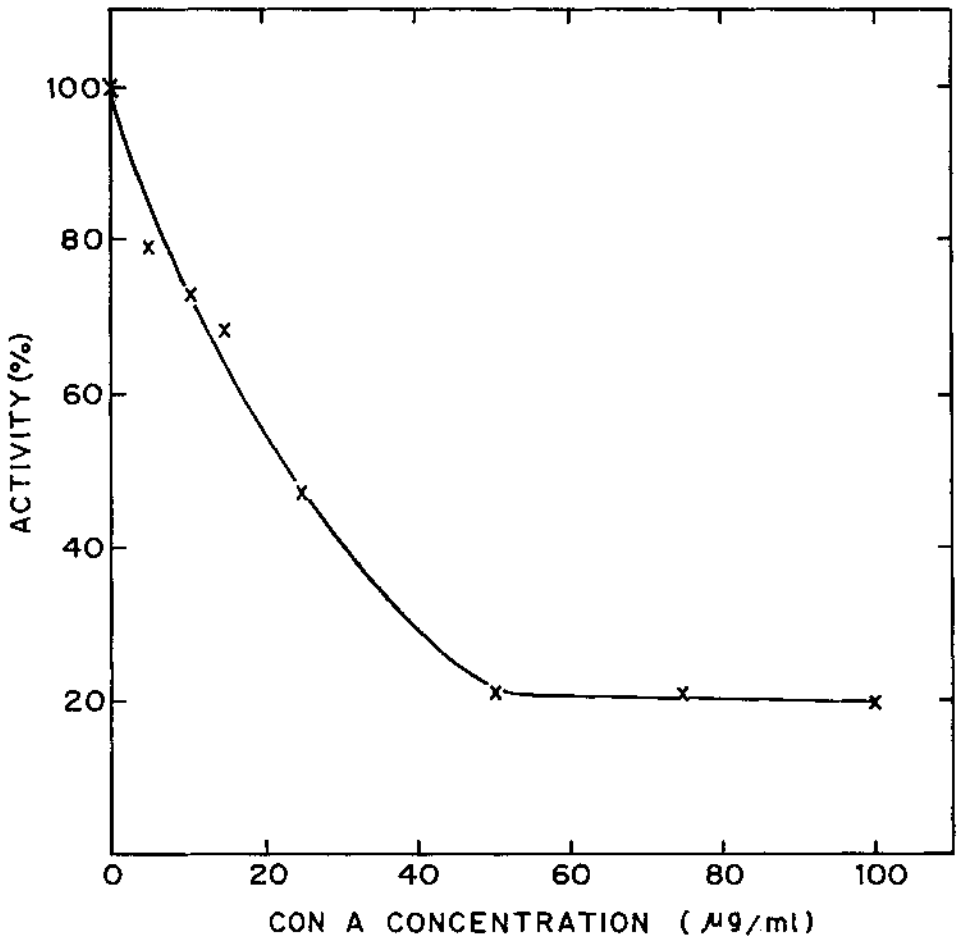

Fig. 3. Effect of Con A concentration on the activity of soluble S1 nuclease.

and then their activities were assayed under standard assay conditions. Immobilization brought about an increase in the pH stability of the enzyme (Fig. 5).

Optimum Temperature and Temperature Stability

In the optimum temperature determination, appropriate amounts of both soluble and immobilized enzymes were assayed in a series of temperatures ranging from 35–75°C at pH 4.6. An increase in the optimum temperature from 55 to 70°C was observed as a result of immobilization. However, no change in the temperature activity curve was observed (Fig. 6).

Temperature stability experiments were carried out by preincubating both soluble and immobilized enzymes at different temperatures, ranging from 40–75°C and pH 4.6 for 1 h followed by assaying the residual activity under standard assay conditions. Immobilized S1 nuclease showed en-

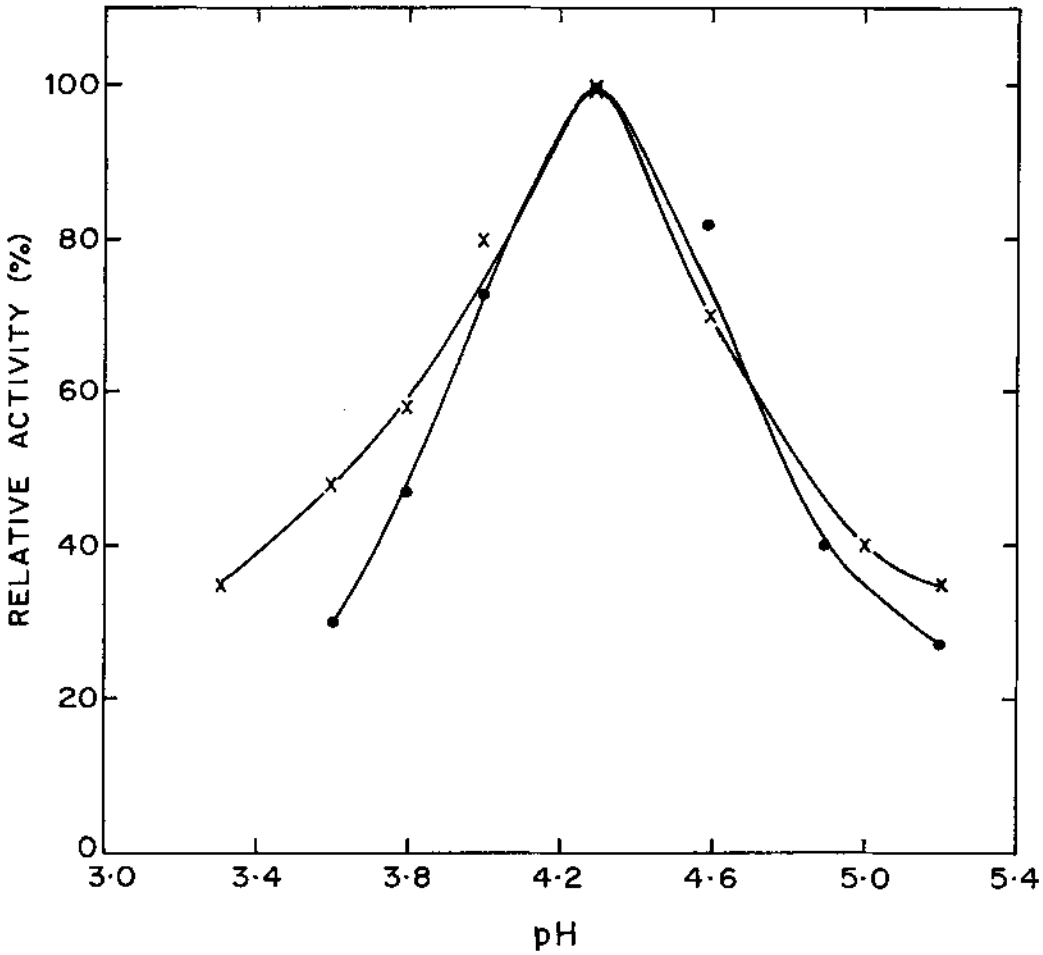

Fig. 4. pH activity profiles of soluble (x) and immobilized (●) S1 nuclease.

hanced temperature stability compared to the soluble enzyme. The soluble enzyme completely lost its activity at 70°C, but the immobilized enzyme retained more than 85% of its activity (Fig. 7).

Stability Toward Denaturants

Stability toward urea was determined by preincubating 15–20 U of both soluble and immobilized S1 nuclease with 8 *M* urea in 0.03 *M* acetate buffer, pH 4.6 at 4 and 30°C, followed by assaying the residual enzyme activity under standard assay conditions. Immobilized preparation showed superior stability toward 8 *M* urea both at 4 and 30°C (Table 2).

Stability to Repeated Use

This was ascertained by assaying a known amount of immobilized preparation under standard conditions. After every use, the immobilized

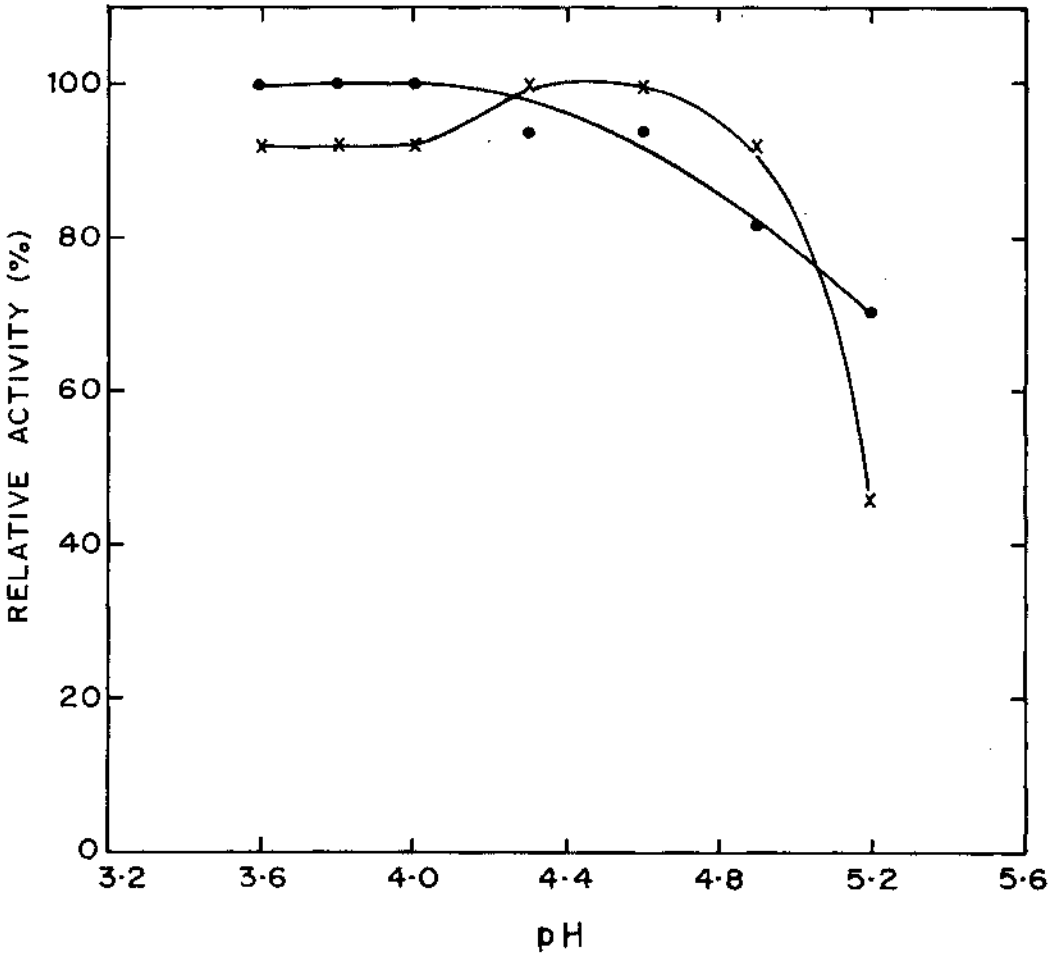

Fig. 5. pH stability of soluble (x) and immobilized (●) S1 nuclease.

preparation was washed free of substrate and products and used for a fresh assay. S1 nuclease-Con A-Sepharose conjugate could be repeatedly used for 6 cycles without any apparent loss in its initial activity (Fig. 8). The slight decrease in the activity was mainly because of the loss of the gel particles after every use rather than enzyme inactivation.

Determination of Michaelis-Menten Constant (K_m) of Soluble and Immobilized S1 Nuclease

Michaelis-Menten constant was determined by assaying appropriate amounts of both soluble and immobilized enzymes in a series of substrate concentrations ranging from 50 to 200 μg of single stranded DNA at pH 4.6 and 37°C. The K_m was determined from Lineweaver Burk plots. There was a marginal increase in the K_m, but an approximately three-fold decrease in the V_{max} was observed on immobilization (Table 3).

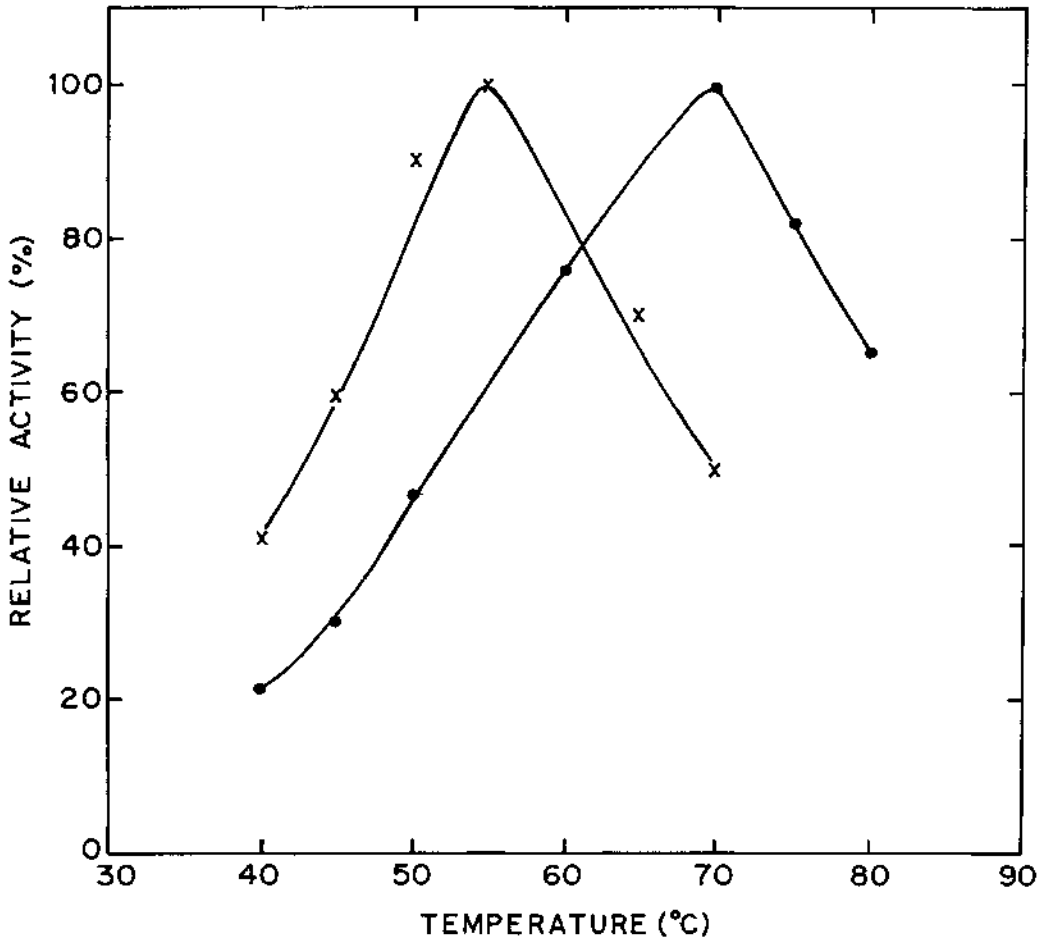

Fig. 6. Temperature activity profiles of soluble (x) and immobilized (●) S1 nuclease.

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 (DNA 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 of the enzyme to the matrix, steric hindrance, partitioning effects, among others. These factors cannot be avoided, but 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

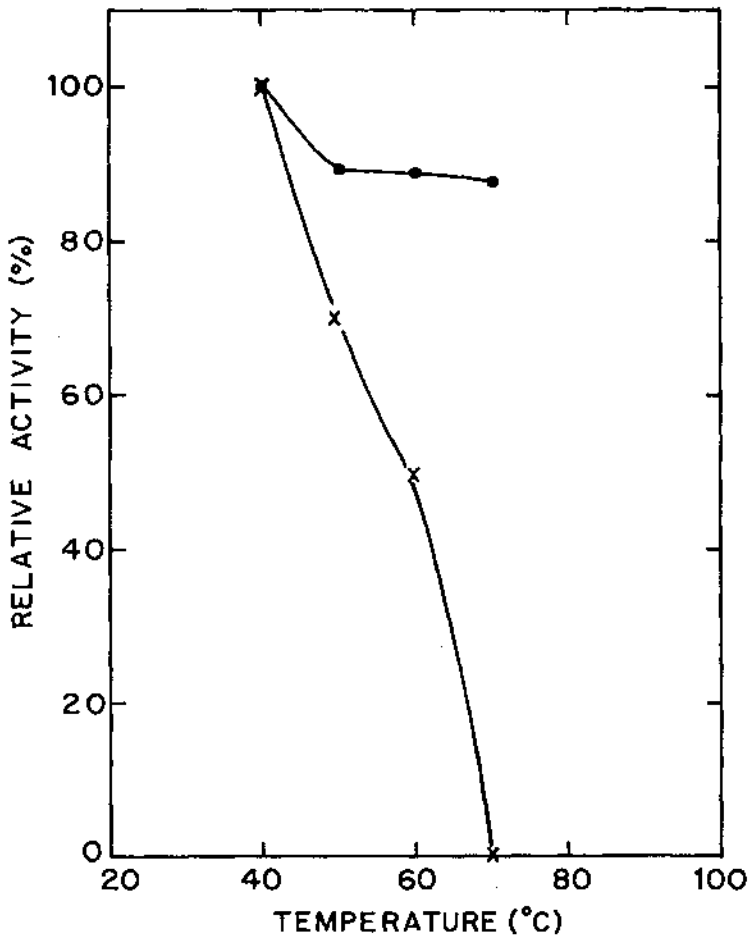

Fig. 7. Temperature stability of soluble (x) and immobilized (●) S1 nuclease.

Table 2
Effect of 8 *M* Urea on the Activity of Soluble and Immobilized S1 Nuclease

Incubation time, min	Residual activity, %[a]			
	Soluble enzyme		Immobilized enzyme	
	4°C	30°C	4°C	30°C
0	100.0	100.0	100.0	100.0
15	77.7	72.2	100.0	100.0
30	72.2	66.5	100.0	100.0
45	77.0	55.5	100.0	100.0
60	77.0	25.0	100.0	100.0
90	72.0	25.0	100.0	100.0

[a]Activity in the absence of 8 *M* urea was taken as 100%.

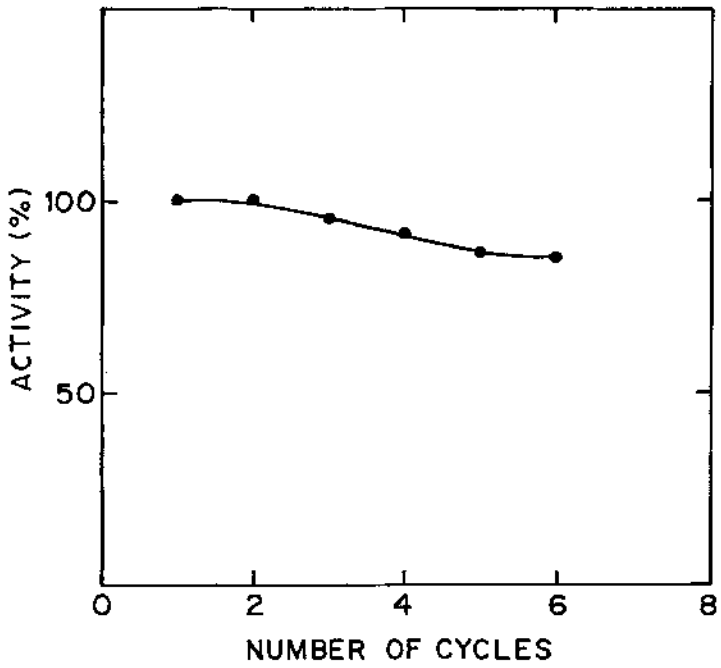

Fig. 8. Effect of number of assay cycles on the activity of immobilized S1 nuclease.

Table 3
Kinetic Data of Soluble and Immobilized S1 Nuclease

State of the enzyme	K_m μg/mL	V_{max}, U
Soluble	266.6	166.6
Immobilized	312.5	60.6

enzyme. On this background, Sepharose was selected as the matrix since it does not have any charged groups responsible for partitioning effects. Second, vinylsulfone activation of the matrix was preferred because during the 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 loss of activity on the "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 owing 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 the "high lectin" matrix (without any detectable decrease in the bound activity) shows that the loss of activity on the "high

lectin'' matrix is owing to the multiple attachment of the enzyme to the matrix rather than overcrowding. The effect of glucose in the restoration of activity can be explained 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. 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 immobilizing the enzyme in the presence of a competing molecule like glucose or mannose. The observed increase in the 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 insensitivity of the soluble enzyme to glutaraldehyde crosslinking further substantiates our view that the loss of activity of the enzyme is caused by conformational changes as a result of multiple attachment of the enzyme to the matrix. Since the enzyme bound to the ''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 the soluble enzyme (Fig. 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 the carbohydrate moiety is not essential for its catalytic activity and it is situated away from the active site of the enzyme (*15*).

Carbohydrate moiety of glycoproteins consists of two main classes of oligosaccharides, such as complex oligosaccharides and high mannose containing oligosaccharides (*16*). 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 a high concentration of α-methylmannoside (0.5 *M*) to elute the bound enzyme substantiates the above explanation. Our results are consistent with those of Shishido and Habuka (*6*), who attributed the strong affinity of S1 nuclease to Con A-Sepharose to its high mannose content.

According to Oleson and Sasakuma (*17*), S1 nuclease catalyzes the hydrolysis of single stranded DNA, RNA, and 3'AMP at relative rates of 100, 52, and 13, respectively. Oleson and Hoganson (*18*) interpreted the ability of 3'AMP to competitively inhibit the hydrolysis of single stranded DNA and vice versa, to the existence of a common catalytic site for both

types of substrates. In the present studies, the similar efficiency pattern obtained using DNA, RNA, and 3'AMP (Fig. 1) also indicates that the hydrolysis of all three substrates is catalyzed by the same active site. If the active sites were different, then it would have resulted in different efficiency patterns.

Action of 8 *M* urea on the soluble enzyme showed that although 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 owing to the flexing of the enzyme molecule 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 the rigidity of the bound form prevents the flexing of the molecule at higher temperatures, it might not prevent 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 toward the rigidity of the conformation. In addition, the repeated usability and absence of leaching in the 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 high efficiency of S1 nuclease, indicates that steric and diffusional limitations may not play 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 a 25–30% decrease in efficiency.

CONCLUSION

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

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