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Immobilization of Single-Strand Specific Nuclease (S1 Nuclease) from *Aspergillus oryzae*

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ABSTRACT

S1 nuclease from *Aspergillus oryzae* (EC 3.1.30.1) 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, optimum temperature increased to 65°C after immobilization. Bound S1 nuclease showed increased pH and temperature stabilities. Immobilization brought about a twofold decrease in the Michaelis-Menton constant (K_m).

Index Entries: Single-strand specific nuclease, from *Aspergillus oryzae;* S1 nuclease, immobilization; S1 nuclease, immobilization on crosslinked gelatin–alginate composite matrix; immobilization, in presence of a coprotein; immobilization, of nuclease.

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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 because of this property several enzymes have been immobilized for their potential application in routine biochemical and clinical analyses (*1,2*). Single-strand, specific nucleases, by their ability to selectively hydrolyze single-strand nucleic acids, have found extensive application as an analytical tool in molecular biology research (*3*). Among the several single-strand, specific nucleases, S1 nuclease from *Aspergillus oryzae* has extensively been used because it can be prepared easily in large amounts from commercially available 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 (*4*). Though single-strand specific nuclease P1 from *Penicillium citrinum* has been immobilized on several supports (*5–7*), no information is available on the immobilization of S1 nuclease. The present communication describes the preparation and properties of S1 nuclease immobilized on glutaraldehyde crosslinked gelatin–alginate composite matrix.

MATERIALS AND METHODS

Yeast RNA (Sisco Labs, Bombay), DEAE-cellulose and CM cellulose (Whatman, UK), glutaraldehyde (Fluka AG, Switzerland), sodium alginate and bovine serum albumin (Loba Chemie Indoaustranal, Co., Bombay), and takadiastase (Unisankyo, Hyderabad) were used. Gelatin was obtained from the local market. All other chemicals were of analytical grade. High-molecular-weight DNA from buffalo liver was isolated according to Mehra and Ranjekar (*8*).

Purification of S1 Nuclease

S1 nuclease from takadiastase was purified essentially according to the modified procedure of Rushizsky et al. (*9*). Takadiastase powder, after extraction and heat treatment, was subjected to ammonium sulfate precipitation. The fraction containing S1 nuclease was chromatographed successively on DEAE cellulose at pH 5.0 and CM cellulose at pH 4.4. Finally, double-strand DNAse contamination was removed by adsorption to CM cellulose at pH 7.0, a procedure in which S1 is not bound (*10*). In the partially purified S1 nuclease, T_1 RNAse and T_2 RNAse contamination was checked according to Egami (*11,12*) and double-strand DNAse contamination according to Rushizsky and Whitlock (*10*). The partially purified preparation was free from the above contaminating nucleases and was used for immobilization.

Immobilization Technique

Immobilization of S1 nuclease on glutaraldehyde crosslinked gelatin–alginate composite matrix using the residual-free aldehyde groups on the surface of the crosslinked matrix was carried out by the slightly modified method of Kennedy et al. (*13*). The gelatin–alginate composite beads were prepared according to Brodelius and Nilsson (*14*).

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 (*15*) into a 3% solution of $CaCl_2$. The microbeads obtained were left in $CaCl_2$ 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 washed free of excess glutaraldehyde with distilled water and immediately used for coupling.

In a typical experiment, 1 g (wet wt) of crosslinked beads was incubated with 150 U of 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 occasional 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 enzymatic activity before loading on the matrix and after coupling.

Assay of Soluble and Immobilized S1 Nuclease

Both soluble and immobilized enzymes were assayed at 37°C in a thermostated shaker water bath (75 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 assay mixture of 1 mL contained 40 μg of sonicated and heat-denatured buffalo liver DNA, 0.1 m*M* $ZnSO_4$, 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 the addition of the enzyme followed by incubation at 37°C for 30 min. The reaction was terminated by the addition of 1 mL 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. Acid soluble nucleotides in the supernatent were measured at 260 nm.

The immobilized enzyme was assayed similarly by incubating 1 g (wet wt) of the matrix with 3 mL of the standard reaction mixture at 37°C for an appropriate time (10–15 min) and then measuring the acid soluble nucleotides 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-strand DNA in 30 min under the assay conditions.

Optimum pH and pH Stability

pH activity profiles of both soluble and immobilized S1 nuclease were determined by incubating them at different pH, ranging from 3.6 to 5.2, and assaying their activity.

For the pH stability determination, both soluble and immobilized enzymes were preincubated at different pH (3.6–5.2) for 3 h at room temperature, and then their activity was assayed.

Optimum Temperature and Temperature Stability

In the optimum temperature determination, the activities of both soluble and immobilized enzymes were determined in a series of temperatures ranging from 35–75°C at pH 4.6.

For temperature stability, the experimental setup was similar to that of optimum temperature determination, except that both soluble and immobilized enzymes were preincubated at different temperatures ranging from 35–70°C for 1 h before assaying their activity.

Determination of Michaelis-Menton Constant of Soluble and Immobilized S1 Nuclease

Michaelis-Menton constant (K_m) of both soluble and immobilized S1 nuclease was determined in a series of substrate concentrations ranging from 50–200 μg of single-strand DNA at pH 4.6 and 37°C. The K_m was determined from the Lineweaver Burk plots.

RESULTS AND DISCUSSION

Crude S1 nuclease (enzyme preparation after ammonium sulfate fractionation) when coupled to gelatin–alginate composite matrix, retained approximately 10% activity of the soluble enzyme. With increasing glutaraldehyde concentration used for crosslinking, the efficiency decreased significantly (Fig. 1). Since 1% glutaraldehyde crosslinked beads gave optimum results with respect to efficiency and stability of the matrix, further experiments were carried out with 1% glutaraldehyde crosslinked matrix. It was also observed that efficiency decreased with increase in the bound activity, which could be related to overcrowding on the matrix.

When partially purified S1 nuclease was coupled to glutaraldehyde crosslinked matrix, the immobilized preparation did not show any detectable enzyme activity. However, the activity could be restored when the immobilization was carried out in the presence of a coprotein (BSA) or the substrate. The loss in activity in the absence of coprotein could be

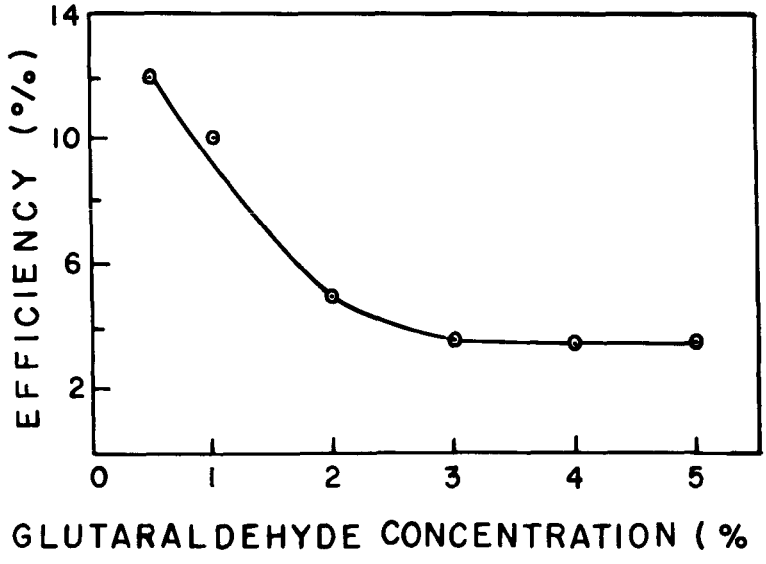

Fig. 1. Effect of glutaraldehyde concentration on the efficiency of immobilized S1 nuclease.

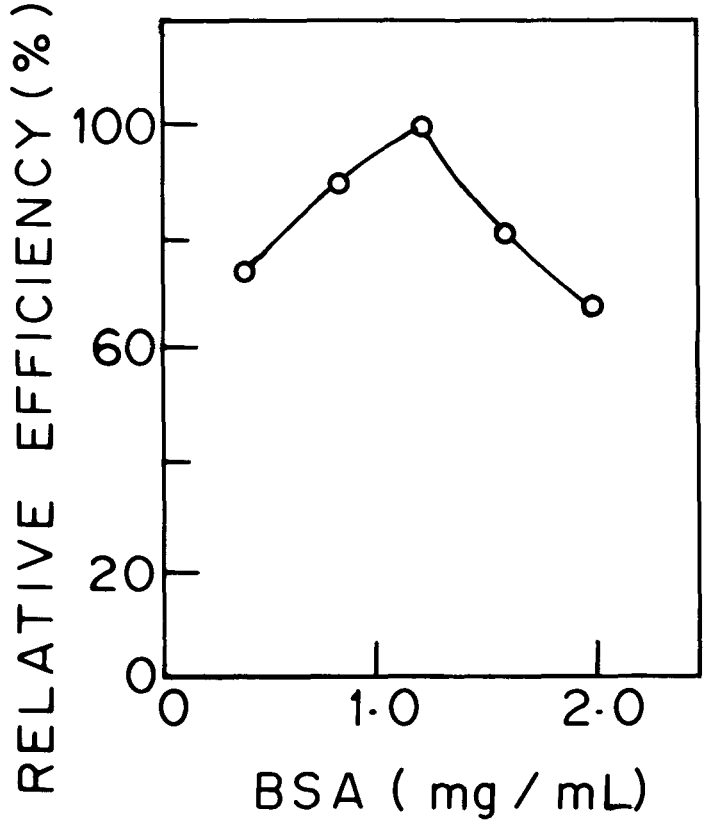

Fig. 2. Effect of BSA concentration on the efficiency of bound S1 nuclease.

caused by conformational changes as a result of multiple attachment of the enzyme to the matrix, rather than diffusional restrictions, since S1 nuclease bound to Con A Sepharose under similar conditions (data not shown) retained more than 70% activity of the soluble enzyme. The decrease in efficiency with increasing glutaraldehyde concentration in the case of crude enzyme (Fig. 1) supports the above speculation. The effect of BSA and single-stranded DNA in the restoration of activity can be explained on the basis that both these macromolecules must be competing with S1 nuclease for free aldehyde groups on the matrix, thus preventing the multiple attachment of the enzyme to the support. The effect of increasing BSA concentration on the efficiency (Fig. 2) supports this explanation. Figure 2 shows that initially efficiency increases with increasing 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.

The optimum pH of the immobilized S1 nuclease shifted to 3.8 from 4.3 for the soluble enzyme (Fig. 3). This implies that the carrier surface is somewhat positively charged either because of charged residual amino

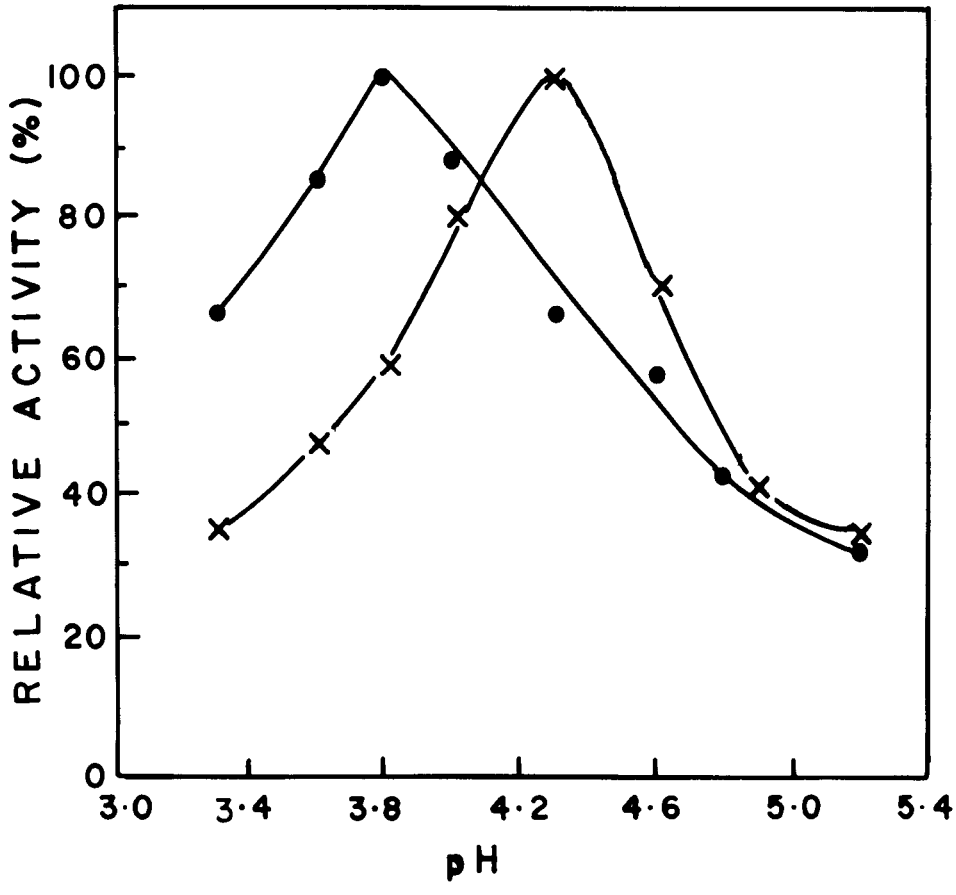

Fig. 3. pH activity profiles of soluble (x) and immobilized (•) S1 nuclease.

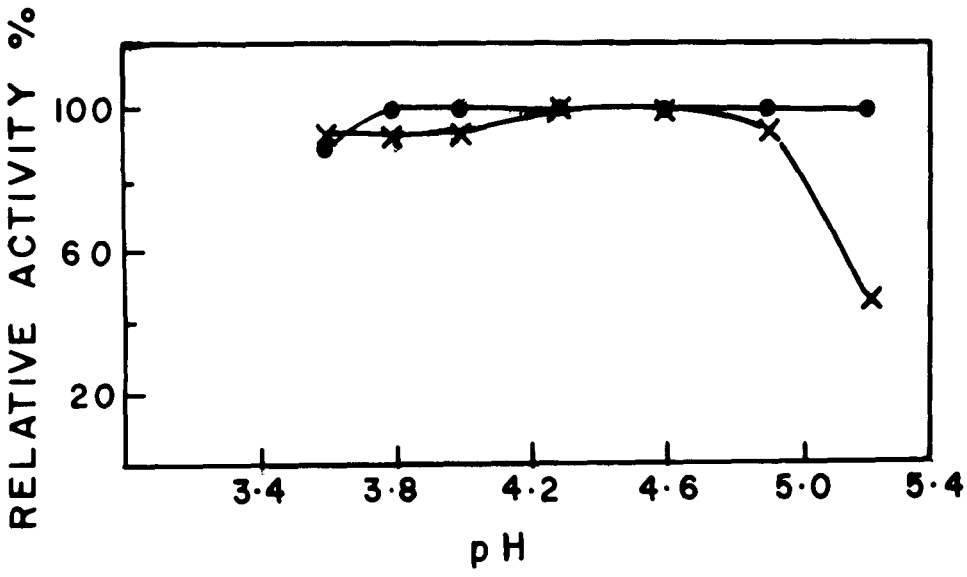

Fig. 4. pH stability of soluble (x) and immobilized (•) S1 nuclease.

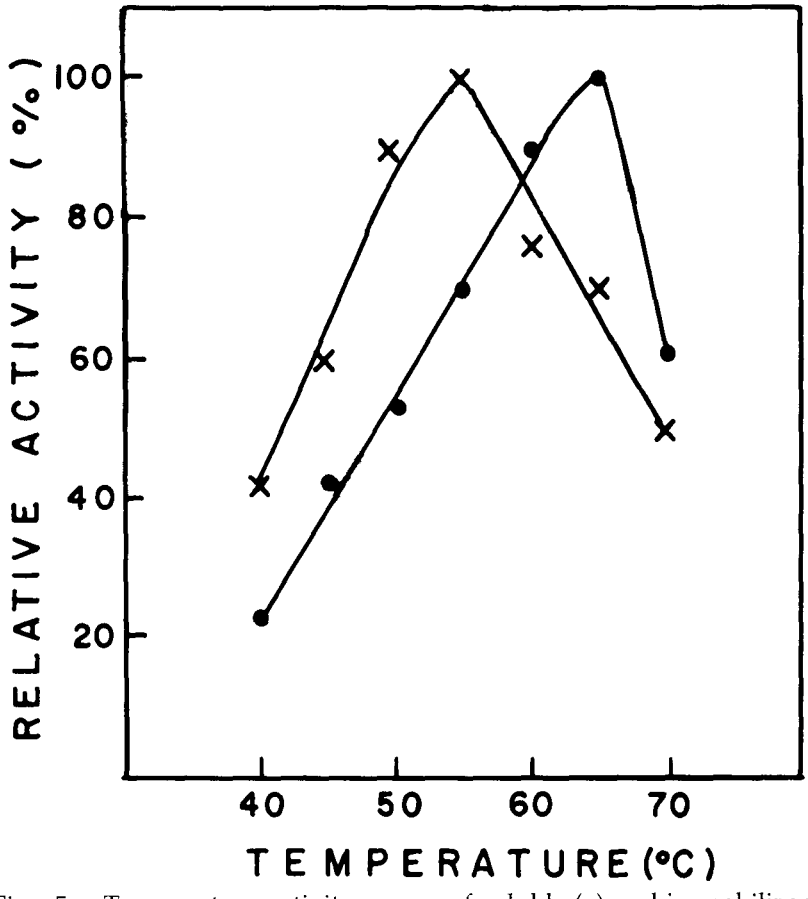

Fig. 5. Temperature activity curves of soluble (x) and immobilized (•) S1 nuclease.

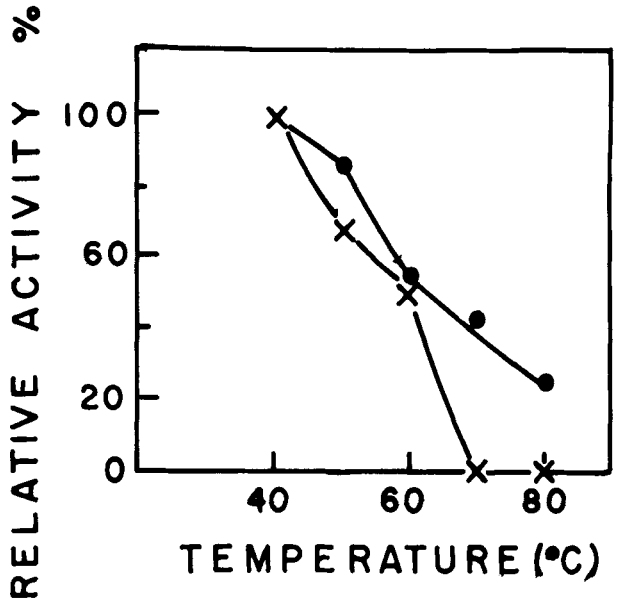

Fig. 6. Temperature stability of soluble (x) and immobilized (•) S1 nuclease.

groups or metal cations (Ca^{2+} in the present case). The immobilized enzyme also showed comparatively higher pH stability (Fig. 4). Temperature activity profiles of the immobilized enzyme showed a shift in the optimum temperature to 65°C from 55°C for the soluble enzyme (Fig. 5). The thermal stability of the enzyme increased on immobilization as indicated by the inactivation pattern. Although the free enzyme completely lost its activity at 70°C, the immobilized enzyme retained more than 40% of its initial activity (Fig. 6).

Evaluation of the kinetic parameters showed more than 2-fold decrease in the $K_{m_{app}}$ after immobilization (Fig. 7, Table 1). The decrease in the $K_{m_{app}}$ 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 (single-stranded DNA) toward the positively charged matrix.

CONCLUSION

A relatively simple method 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.

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TABLE 1
Kinetic Data of Soluble and Immobilized S1 Nuclease

State of the enzyme	K_m, μg/mL	V_{max}, U
Soluble	266	200
Immobilized	114	58

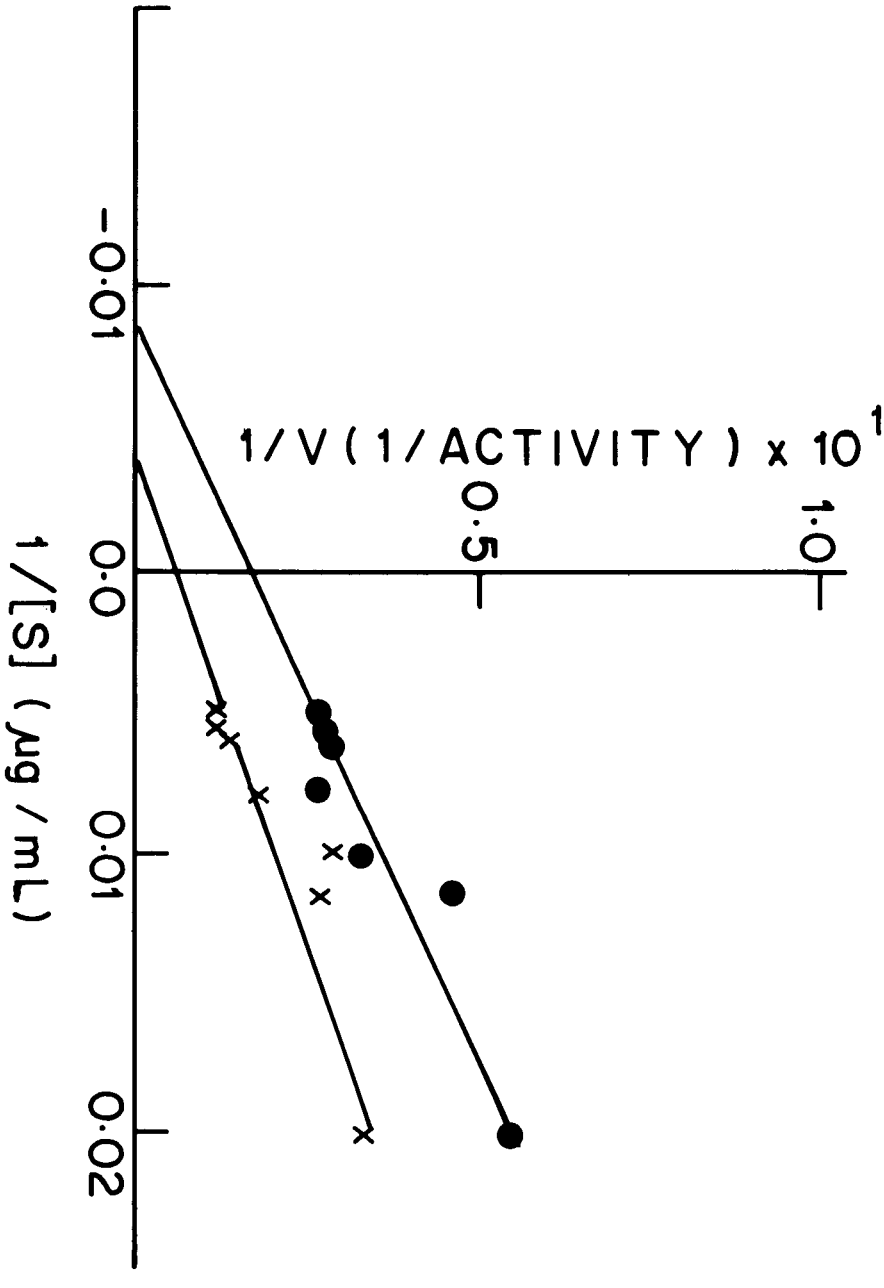

Fig. 7. Lineweaver-Burk plots for soluble (x) and immobilized (●) S1 nuclease.

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