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Preparation and Properties of RNase T2 Immobilized on Concanavalin A-Sepharose

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ABSTRACT

Partially purified RNase T2 (EC 2.7.7.17) from *Aspergillus oryzae* was bound through its carbohydrate moiety to Concanavalin A-Sepharose. The retention of activity was high, ranging from 70% at low enzyme load to approximately 9% at high enzyme load. Though there was no change in the pH and temperature optima, the pH stability and the K_m decreased after immobilization. Compared to the soluble enzyme, the immobilized RNase T2 showed enhanced temperature stability and more resistance to metal ions. Both soluble and immobilized enzymes were stable to 8 *M* urea. On repeated use, the bound enzyme retained more than 60% of its initial activity after six cycles.

Index Entries: Ribonuclease T2 from *Aspergillus oryzae;* RNase T2 immobilization; RNase T2 immobilization on Concanavalin A-Sepharose; binding through carbohydrate moiety; immobilization of RNase.

INTRODUCTION

Takadiastase from *Aspergillus oryzae* contains two ribonucleases, viz, RNase T1 and RNase T2, in a ratio of approximately 10:1 and these enzymes can be distinguished by their pH optima and isoelectric points (*1*). Like RNase 1-A and RNase T1, RNase T2 also exhibits both RNA digesting and 2′3′ nucleotide hydrolysing activity. RNase T2 is an analytically important enzyme and is used in the base analysis of RNA. For base analysis, digestion with RNase T2 is preferable to alkaline hydrolysis, since

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the latter results in the slight degradation of nucleotide bases, especially minor components. In addition, alkaline hydrolysis gives rise to a mixture of 2′3′ nucleotides that in turn complicates the procedure of base analysis. RNase T2 has also played an important role in the discovery and identification of new minor components in tRNA, namely, 5,6dihydrouridylic acid, uridine-5-oxyacetic acid, N^6-methyl-adenosine and *N*-9-(β-D-ribofuranosyl) purin-6-yl carbamoyl threonine (2). The transferase activity associated with the enzyme has also been exploited for the synthesis of certain nucleotide 3′ phosphates, such as Coenzyme A (3). RNase T2 is a glycoprotein and contains approx 12–15% carbohydrate (4). It has been reported that coupling of glycoproteins to insoluble matrices is manifested with a number of problems owing to the shielding of the reactive groups of amino acid sidechains by carbohydrates. To overcome this difficulty, several glycoprotein enzyme adducts have been prepared in which the carbohydrate sidechains provide the point of attachment between the enzyme and the matrix (5). Such methods of binding either by adsorption or by covalent coupling to solid supports afford high retention of enzyme activity, presumably owing to the fact that the carbohydrate moiety of the enzyme is not essential for its catalytic activity and the protein moiety is therefore free of the restrictions imposed upon it as a result of immobilization. Since the carbohydrate moiety of RNase T2 consists of mannose and glucose residues (6), attempts were made to immobilize it on Concanavalin A-Sepharose, results of which are described in the present communication.

MATERIALS AND METHODS

Yeast RNA (Sisco Research Laboratories, India), DEAE-Cellulose (Whatman, UK), Concanavalin A-Sepharose, and α-methyl D-mannoside (Sigman Chemical Co., USA), bovine serum albumin (Loba-Chemie Indoaustranal Co., India), Takadiastase (Sankyo, Japan), and glutaraldehyde 50% v/v (Fluka AG, Switzerland) were used. All other chemicals used were of analytical grade.

Purification of RNase T2

Partial purification of RNase T2 was carried out essentially according to the procedure of Uchida and Egami (4). Takadiastase powder, after extraction and batchwise treatment with DEAE-Cellulose (pH 7.0) was subjected to heat treatment (78°C for 2 min at pH 1.8–2.0). The resultant enzyme preparation after concentration by ammonium sulfate precipitation was dialysed extensively against acetate buffer pH 4.5 (0.03 *M*) and used for immobilization studies. Though the partially purified RNase T2 preparation was free from contaminating enzymes such as phosphomonoesterase, phosphodiesterase, and DNase, it contained RNase T1 activity. How-

ever, no attempts were made to remove RNase T1, as it automatically gets eliminated during immobilization on Con A-Sepharose, because of its nonglycoprotein nature (*7*).

Immobilization Technique

Binding of partially purified RNase T2 to Con A-Sepharose was carried out as follows: 1 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 U (300 μg protein) of partially purified enzyme at 4°C for 6–8 h with occasional agitation. The supernatant was collected by centrifugation and the matrix was extensively washed, initially with coupling buffer and then with the assay buffer until the washings showed no detectable RNase activity. The amount of enzyme bound was determined by estimating the difference in the enzyme activity before loading on the matrix and after coupling.

Covalent Coupling of RNase T2

Covalent coupling of partially purified RNase T2 to Con A-Sepharose was carried out by incubating 5 mL suspension of Con A-Sepharose-RNase T2 conjugate (36 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. RNase T2 covalently bound to the matrix was determined by incubating the crosslinked enzyme preparation with 0.5 *M* α-methyl D-mannoside for 1 h followed by assaying the solubilized enzyme 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.

Assay of Soluble and Immobilized RNase T2

Soluble and immobilized enzymes were assayed at 37°C in a thermostated shaker water bath (75–100 rpm) by a slightly modified method of Vogt (*8*).

For the soluble enzyme activity, the standard reaction mixture of 1 mL contained 100 μg of yeast RNA, 20 m*M* EDTA in 0.2 *M* sodium acetate buffer pH 4.5, and appropriately diluted enzyme. The reaction was initiated by the addition of RNA, followed by incubation at 37°C for 30 min. After the incubation period, the reaction was terminated by the addition of 1 mL of 10% of TCA and 1 mL of 0.1% BSA. The reaction mixture was kept on ice for 10 min and then centrifuged at 3000 rpm for 10 min to

sediment the precipitate. Acid soluble nucleotides were measured at 280 nm, since TCA absorbs strongly at 260 nm.

The immobilized enzyme was assayed similarly by incubating appropriate amounts of the matrix with 3.0 mL of standard reaction mixture at pH 4.5 and 37°C for 30 min, followed by measuring the acid soluble nucleotides at 280 nm after the precipitaiton of unreacted RNA.

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

Optimum pH and pH Stability

pH activity profiles of both soluble and immobilized RNase T2 were determined by incubating them in series of pH ranging from 3.0–6.0 (acetate buffer 0.03 *M*) and assaying their activity at 37°C.

For the pH stability determination, both soluble and immobilized enzymes were preincubated at different pH (3.0–6.0) for 1 h at room temperature, and their activity was assayed under standard conditions.

Optimum Temperature and Temperature Stability

In the optimum temperature determination, both soluble and immobilized enzymes were assayed in a series of temperatures ranging from 40–75°C at pH 4.5.

Temperature stability experiments were carried out by preincubating appropriate amounts of soluble and immobilized enzymes at different temperatures ranging from 40–75°C at pH 4.5 for 1 h, followed by assaying the residual activity under standard assay conditions.

Stability in Urea

Stability in presence of urea was determined by preincubating both soluble and immobilized RNase T2 with varying concentrations of urea (1–8 *M*) at pH 4.5 and 30°C for 1 h followed by measuring the residual enzyme activity under standard assay conditions.

Effect of Metal Ions

To determine the effect of metal ions, both soluble and immobilized enzymes were incubated with different metal ion solutions (5 m*M*) at pH 4.5 and 30°C for 1 h. After preincubation, the enzyme activity was assayed under standard conditions. However, EDTA was added in the assay mixture without preincubation.

Repeated Usability

The reusability of immobilized enzyme was ascertained by assaying a known amount of immobilized enzyme under standard assay conditions. After every use, the immobilized enzyme was washed free of substrate and products and used for a fresh assay.

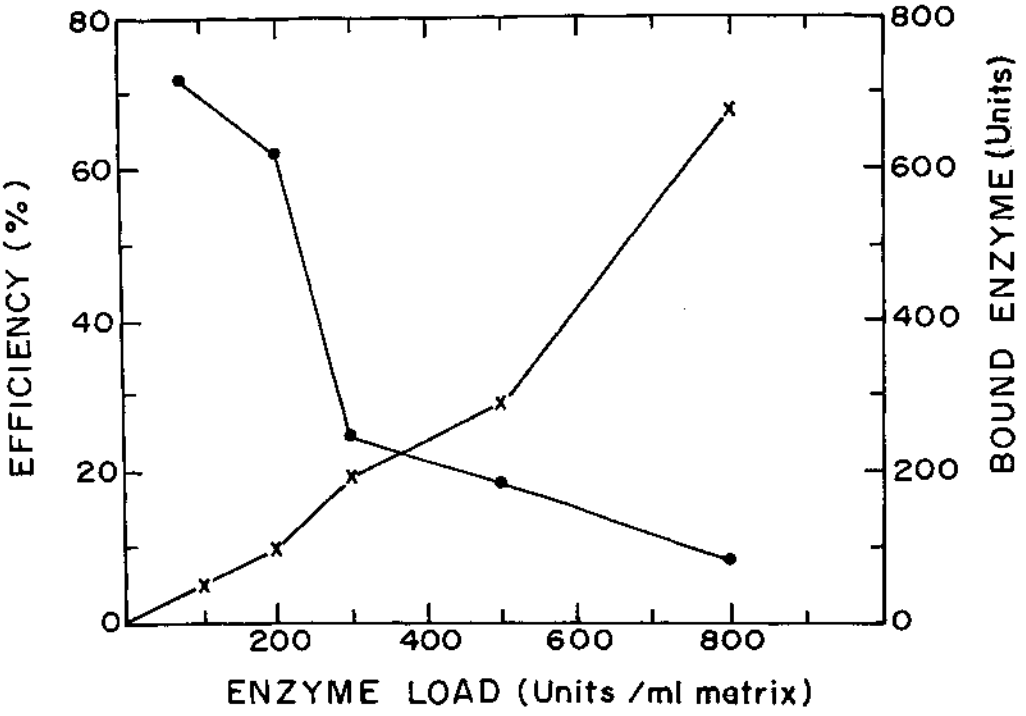

Fig. 1. Effect of enzyme load on the efficiency of the immobilized RNase T2. Efficiency (●) and bound enzyme (x).

Effect of Substrate Concentration

Michaelis-Menten constant (K_m) of both soluble and immobilized RNase T2 was determined in a series of substrate concentrations ranging from 50–200 μg of RNA at pH 4.5 and 37°C. The K_m was determined from Lineweaver Burk plots.

RESULTS AND DISCUSSION

In a typical experiment, when 75 U of partially purified RNase T2 was reacted with 1 mL of Con A-Sepharose, 55 U were bound. The effectiveness factor (η) of the preparation was approximately 0.72, indicating the efficiency of the preparation to be 72%. The bound enzyme did not leach out in presence of 0.5 *M* NaCl (data not shown) indicating the binding to be firm. Increase in the enzyme load on the matrix, though, was accompanied by an increase in the bound enzyme, it also resulted in the progressive decline in the efficiency of the preparation to such an extent that at high enzyme load Con A-Sepharose-RNase T2 conjugate retained only 9% of its activity (Fig. 1). The decrease in the efficiency of the bound

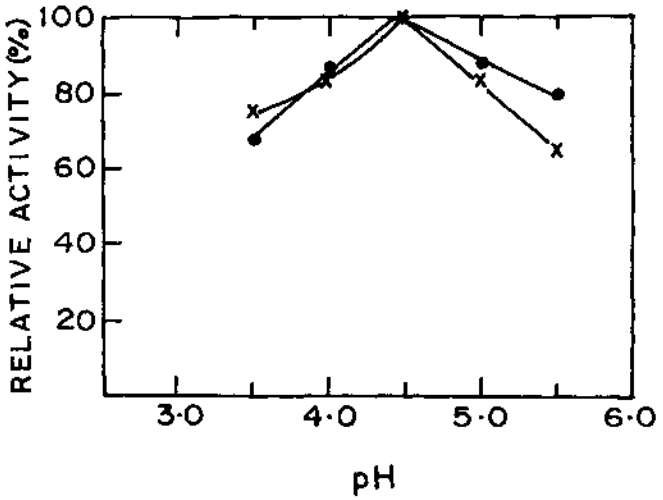

Fig. 2. pH activity profiles of soluble (x) and immobilized (●) RNase T2.

enzyme with increase in enzyme load can be attributed to (a) overcrowding on the matrix, (b) more and more molecules begining to occupy the interior portions of the porous gel matrix and thus may not be readily available for the substrate, and (c) concentration gradient set up in the gel matrix owing to high activity of the surface bound enzyme, in turn resulting in decreased concentrations of the substrate to the internally situated enzyme molecules (*9*). Attempts to covalently bind the enzyme by subjecting the Con A-Sepharose-RNase T2 conjugate to secondary crosslinking was not successful, as the enzyme lost more than 65% of its initial activity after crosslinking. The loss of activity after crosslinking with glutaraldehyde could be due to conformational changes in the enzyme brought about by the multiple attachment of the enzyme to the matrix. This, in turn, indicates that conformational changes is one of the major contributing factors for the loss of activity of immobilized enzymes.

There was no change in optimum pH of the enzyme after immobilization (Fig. 2), indicating the absence of partitioning effects in the microenvironment of the immobilized enzyme. pH stability of immobilized RNase T2 was inferior to that of the soluble enzyme (Fig. 3). Temperature activity profiles of Con A-Sepharose-RNase T2 conjugate showed no shift in the optimum temperature from 65°C for the soluble enzyme (Fig. 4). However, the thermal stability of the enzyme increased on immobilization, as indicated by its inactivation pattern. The soluble enzyme lost its activity completely at 70°C, but the bound enzyme retained 50% of its initial activity (Fig. 5). Enhanced stability of bound enzyme indicates the rigidity of the enzyme structure in the bound form. Immobilized RNase T2 was comparatively more stable to metal ions like Na^+, Zn^{2+}, Cu^{2+}, and Hg^{2+} (Table 1), which are potent inhibitors of the enzyme (*6*). With the exception of Hg^{2+}, the soluble enzyme was also not inhibited to a great

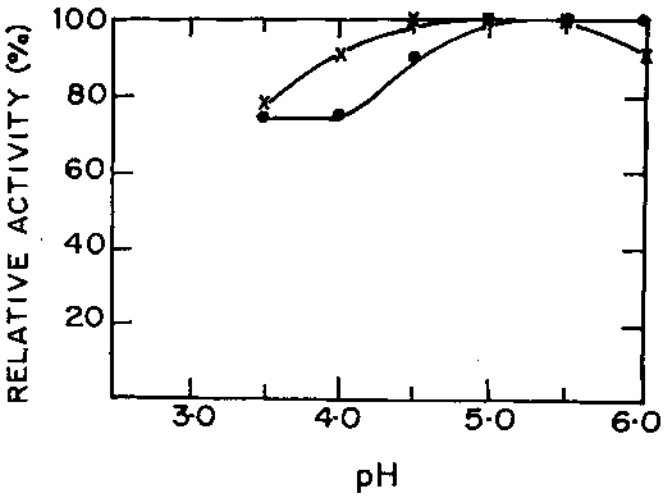

Fig. 3. pH stability of soluble (x) and immobilized (●) RNase T2.

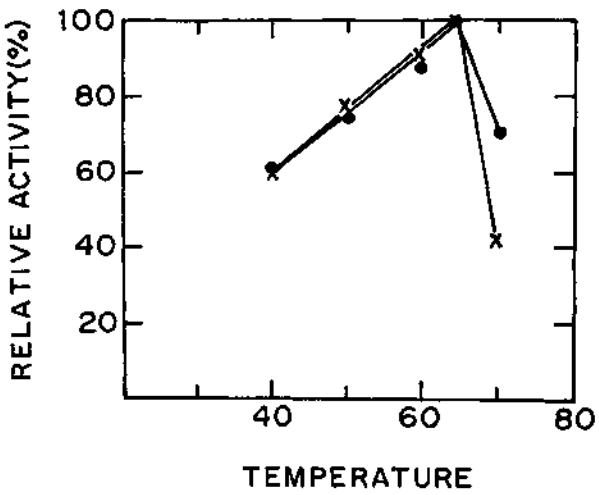

Fig. 4. Temperature activity profiles of soluble (x) and immobilized (●) RNase T2.

extent by these metal ions (as evidenced by the requirement of high concentration of metal ions required to bring about complete inhibition), which may be owing to some protective factor(s) present in the partially purified enzyme. Surprisingly, Cu^{2+} activated the immobilized enzyme (Table 1). Similarly, Osheroff and Guillory (*10*) observed that Mg^{2+} that inhibits the soluble myosin ATPase activates the PAB-cellulose bound enzyme. EDTA which activates RNase T2 (*6*) failed to bring about any significant activation of the immobilized enzyme (Table 1). This observation further substantiates the relative insensitivity of Con A-Sepharose-RNase T2

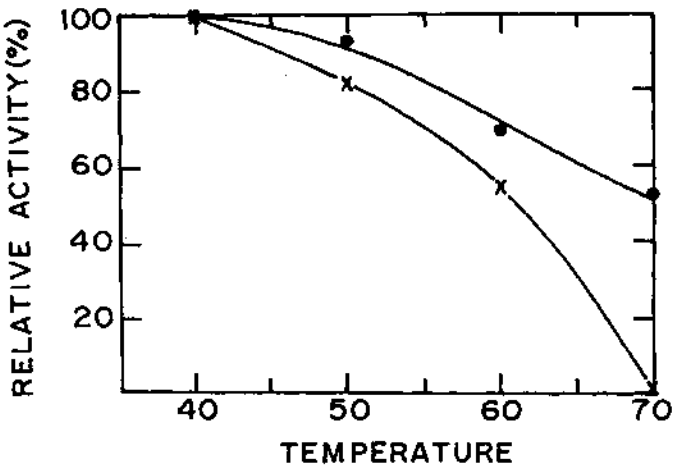

Fig. 5. Temperature stability of soluble (x) and immobilized (●) RNase T2.

Table 1
Effect of Metal Ions and EDTA on the Activity of Soluble and Immobilized RNase T2[a]

Additives	Concentration, mM	Residual activity, %	
		Soluble	Immobilized
NaCl	5	98	95
$ZnSO_4$	5	63	88
$CuSO_4$	5	58	150
$HgCl_2$	5	0	64
EDTA	20	251	116

[a]Activity measured in absence of additives was taken as 100%.

conjugate to toxic metal ions. Both soluble and immobilized RNase T2 retained their activity up to 8 *M* urea (data not shown). On repeated use, Con A-Sepharose-RNase T2 conjugate retained 60% of its initial activity after six cycles (Fig. 6). Some loss of activity could be caused by the loss of gel matrix after every use.

Evaluation of kinetic parameters showed that the bound enzyme follows Michaelis-Menten kinetics and there is a twofold decrease in the apparent K_m (Table 2). Under the assay conditions, Con A-Sepharose is somewhat positively charged and, hence, the decrease in the K_m after immobilization could be owing to the increase in the substrate concentration in the microenvironment of the bound enzyme as a result of attraction between the positively charged matrix and the negatively charged RNA. However, the positive charge on the matrix is not sufficient to bring about any change in the pH optimum. A threefold decrease in the apparent V_{max}

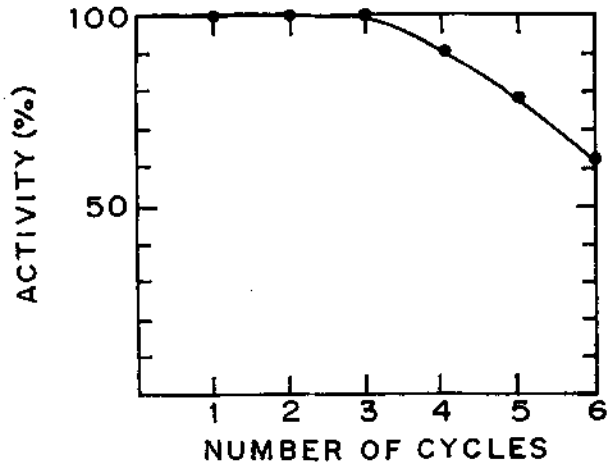

Fig. 6. Effect of number of assay cycles on the activity of immobilized RNase T2.

Table 2
Kinetic Data of Soluble and Immobilized RNase T2

State of the enzyme	K_m, mg/mL	V_{max}, U
Soluble	555	100
Immobilized	266	27

was also observed (Table 2), and this may be owing to the blocking of some active sites during immobilization.

CONCLUSION

A simple method for obtaining highly active and stable immobilized RNase T2 is described. High stability of the immobilized preparation will be useful in its application as a reusable analytical tool. It can also be exploited for the production of 3′ mononucleotides.

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