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PARTIAL PURIFICATION AND IMMOBILIZATION OF RNase T2

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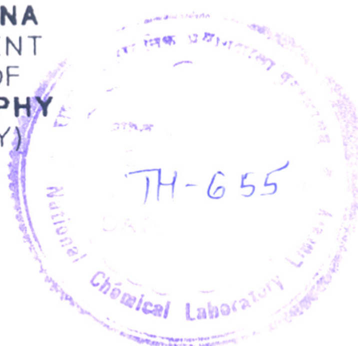

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C E R T I F I C A T E

Certified that the work incorporated in the thesis entitled: " Partial Purification and Immobilization of RNase T2 " submitted by Daphne Leon was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. V. Shankar)

Research Guide

A C K N O W L E D G E M E N T S

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Daphne Leon

(Daphne Leon)

SUMMARY

A simple procedure for the partial purification of RNase T2 from commercially available Takadiastase powder is described. The steps involved were : water extraction, heat treatment at pH 2.0, negative adsorption on DEAE-cellulose at pH 5.0 and affinity chromatography on Concanavalin A-Sepharose. The enzyme was purified approximately 19-fold with an overall yield of 56%.

The partially purified enzyme when coupled to glutaraldehyde activated aminoethyl Bio-Gel P-60 (AE Bio-Gel P-60), retained 12-16% activity of the soluble enzyme. Optimization of coupling conditions showed that most active preparations are obtained when 1 ml (packed volume) of 1% (v/v) glutaraldehyde activated AE Bio-Gel P-60 is reacted with approximately 40-45 U of RNase T2 at pH 6.0 and 4°C for 15 h. Though there was no change in the pH and temperature optima, the pH stability decreased after immobilization. The temperature stability of immobilized RNase T2 was comparable with its soluble counterpart. On repeated use, the bound enzyme retained approximately 55% of its activity after 6 cycles. Immobilized RNase T2 when stored in a wet state (0.2 M acetate buffer, pH 4.5) retained 50% of its initial activity after 25 days.

INTRODUCTION

1. RIBONUCLEASE T2 (RNase T2)

The existence of ribonucleases in *Aspergillus oryzae* was reported for the first time by Kuninaka (1954, 1955a, 1955b) and Saruno (1956), who studied the various characteristics of these enzymes and found that Takadiastase (a commercial product of *A. oryzae*) contains at least three RNA degrading enzymes. Further studies on the substrate specificity of the major enzymatic component (now known as RNase T1) showed that this enzyme splits the phosphodiester bonds of 3′-guanylic acid while the minor enzymatic component (now known as RNase T2) splits the phosphodiester bonds of 3′-adenylic acid in RNA (Sato and Egami, 1957). The third RNA degrading activity was found to be associated with S1 nuclease, a multifunctional enzyme, which exhibits ssDNase, RNase and phosphomonoesterase activities (Ando,1966). Since the partially purified RNase T2 preferentially attacked the phosphodiester bonds of adenosine - 3′- phosphates in RNA, initially it was assumed that it could be an adenine-specific enzyme (Naoi-Tada *et al.*, 1959). However, subsequent studies (Rushizky and Sober, 1963; Egami *et al.*, 1964 and Uchida, 1966) with sufficiently purified enzyme revealed that RNase T2 has no absolute base specificity and splits all the phosphodiester bonds in RNA with a preference for adenylic

acid bonds. Thus, this enzyme attracted considerable attention as a potential tool in the base composition analysis of RNA. In addition, extensive studies are being carried out on this enzyme to determine its chemical nature and structure - function relationships.

1.1 PURIFICATION

Naoi-Tada *et al.* (1959), for the first time, reported the partial purification of RNase T2 involving the following steps : water extraction of the Takadiastase powder, heat treatment, ammonium sulfate fractionation, adsorption on calcium phosphate gel and finally DEAE-cellulose chromatography. Uchida and Egami (1967c) purified RNase T2 to homogeneity by removing most of the RNase T1, from the crude extract, by a batchwise treatment with DEAE- cellulose at pH 7.0. The RNase T2-rich fraction was then subjected to heat treatment, ammonium sulfate fractionation and DEAE-cellulose column chromatography at pH 7.0, when RNase T1, RNase T2-A and RNase T2-B were separated. Further purification of RNase T2-A and RNase T2-B was achieved by ethanol fractionation and DEAE-cellulose column chromatography with a milder gradient. Though RNase T2 separated into two fractions (RNase T2-A and RNase T2-B), no differences were observed in their properties. The purified enzyme appeared homogeneous in paper or zone electrophoresis and sedimentation analysis.

Rushizky and Sober (1963) described an alternate procedure for the purification of RNase T2 consisting of water extraction of Takadiastase powder acidified to pH 2.6, acetone fractionation, followed by chromatography on DEAE-cellulose (twice) and CM-cellulose (twice).

Affinity chromatography has also been employed for the purification of RNase T2. Janski and Oleson (1976) observed that RNase T2 showed 100% binding on NADP-agarose through a biospecific interaction between the bound nucleotide and the enzyme. The bound enzyme could be eluted with 3′-AMP. Based on the above observation and the preference of RNase T2 for adenylic acid bonds (Sato *et al.*, 1966; Imazawa *et al.*, 1968), Kanaya and Uchida (1981) purified RNase T2 on 5′-adenylate-aminohexyl-Sepharose 4B (pA- AH-Sepharose 4B) at pH 4.5. The initial purification steps were similar to that of Uchida and Egami (1967c). The enzyme obtained after the affinity chromatographic step, when subjected to gel filtration on Sephadex G-25, resolved into two forms, namely, RNase T2 and RNase T2-L. The protein moiety of both forms were the same but they differed in their carbohydrate contents and hence in their molecular weights. It was also observed that RNase T2-L was a heterogeneous mixture of, at least five forms of the enzyme differing only in their carbohydrate content.

1.2 PROPERTIES

1.2.1 Assay procedure : The enzyme is usually assayed at pH 4.5 and 37°C, by measuring the acid soluble nucleotides at 260 nm following the hydrolysis of RNA. One unit of the enzyme is defined as the increase in 1 OD in 15 min.

1.2.2 Physical properties :

Size : Based on sedimentation equilibrium studies, Uchida (1966) determined the molecular weight of RNase T2 to be 36,200 daltons. However, Rushizky and Sober (1963) obtained a value of 30,500 daltons by the same method. Kanaya and Uchida (1981) calculated the molecular weight of RNase T2 by component analysis and obtained a value of 36,000 daltons. In addition, they found that the five forms of RNase T2-L had molecular weights in the range of 42,000-92,000 daltons. Recently, Kawata *et al.* (1988) determined the molecular weight of RNase T2 on the basis of (a) primary structure and sugar content and (b) sedimentation equilibrium and obtained a value of 29,155 and 29,200 daltons, respectively. Based on these studies, the above authors suggested that the higher value reported by Uchida (1966) may have been due to the higher sugar content of their enzyme preparation.

Isoelectric point : Uchida and Egami (1967c) determined the isoelectric point of RNase T2 and reported the pI as 5.0. However, Kanaya *et al.* (1981) found the pI of RNase T2 to be

4.65. Though their RNase T2-L preparations gave some peaks on isoelectric focusing, the isoelectric points were below 4.65. The authors also suspected some interactions between RNase T2-L and the carrier ampholyte.

Carbohydrate content : RNase T2 contains approximately 12-15% carbohydrate (Uchida and Egami, 1971). RNase T2-A and RNase T2-B can be distinguished by the nature of their sugar components i.e. RNase T2-A contains mannose and glucose whereas RNase T2-B contains mannose and galactose (Uchida, 1966a). Klemer *et al.* (1981) reported that the carbohydrate moiety of RNase T2 is made up of 4 mannose, 6 glucosamine, 8 glucose, 6 fucose, 1 xylose and 4 galactose residues. Kanaya and Uchida (1981) reported two forms of RNase T2 *viz.* RNase T2, a homogeneous glycoprotein containing 10.2% carbohydrate and RNase T2-L, also a glycoprotein, containing approximately 50% carbohydrate. The latter was a markedly heterogeneous fraction which on further purification by gel filtration resolved into five fractions of RNase T2-L, each differing from the other in its carbohydrate content and molecular weight. The authors opined that each RNase T2-L fraction could still be heterogeneous. However, recently, Kawata et al. (1988), while carrying out the sequencing of RNase T2 found that it is comprised of 5 glucosamine and 7-8

residues of mannose. Further, the glycosylation sites and amino acids involved were found to be aspargines 15, 76 and 239.

1.2.3 Catalytic properties

pH optimum : RNase T2 exhibits maximum hydrolytic activity at pH 4.5 for RNA and homopolymers, while it hydrolyzes various nucleoside's 2',3'- cyclic phosphates at pH 6.0-6.3. Fifty percent of the maximal activity remains at pH 3.8 and 5.7. Uchida (1966) reported that at pH 7.5 (the optimum pH of RNase T1), RNase T2 exhibits 20% of its maximal activity but according to Rushizky and Sober (1963), the activity is only 5-7 %.

Stability : RNase T2 is somewhat less stable compared to RNase T1 but it is still fairly stable as compared to most other enzymes. It is most stable at neutral pH but gradually loses its activity on standing. The enzyme loses approximately 20% of its initial activity when frozen at pH 5.5 for 20 days or when dialyzed against cold distilled water for 3 days (Uchida, 1966). Partially purified RNase T2 when heated at 80°C for 5 min at pH 6.0 retained its full activity. In alkaline medium at room temperature, it is most stable but in acidic medium it is less stable than RNase T1. RNase T2 appears to be somewhat less stable than RNase T1 to lyophilization and loses approximately 15% of its initial

activity when lyophilized at pH 6.0.

Activators and inhibitors : RNase T2 shows complete inhibition in presence of metal ions like Ag^{+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+} and Fe^{2+}. Though partly purified enzyme preparations are activated by EDTA, it has practically no effect on the purified enzyme (Naoi-Tada *et al.*, 1959).

1.3 SPECIFICITY AND MODE OF ACTION

RNase T2 (ribonucleate nucleotido-2´-transferase (cyclizing), EC 2.7.7.17) is a non-specific endoribonuclease and preferentially splits the internucleotide bonds between 3´-adenylic acid and 5´-hydroxyl group of adjacent nucleotides in RNA, with the intermediate formation of adenosine 2´, 3´-cyclic phosphates. Subsequently, it splits all secondary phosphate ester bonds of other nucleotides *via* the nucleotide´s 2´, 3´-cyclic phosphates (Fig. 1). The mode of action on various RNAs and synthetic homopolymers, investigated by 3´-terminal analysis of the digestion products have shown that exhaustive digestion of RNA with the enzyme results in the production of 3´-nucleotides (Uchida and Egami, 1967a). However, when RNA or tRNA mixtures are subjected to partial digestion, it results in : (a) the formation of large fragments (approximately 20%) terminating in adenylyl residues in the initial stages, with

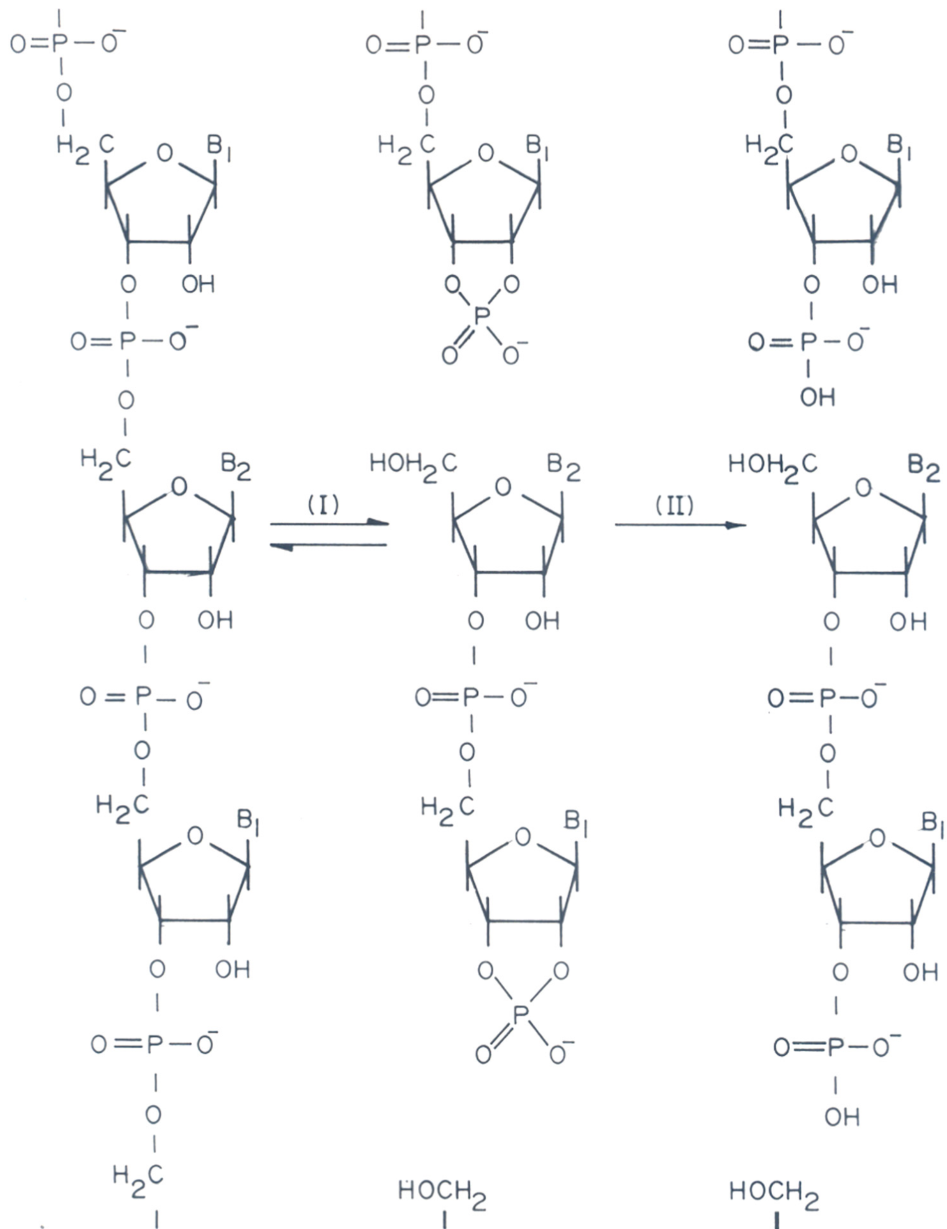

Fig. 1 Mode of action of RNase T2.

no increase in the acid soluble products (b) a rapid accumulation of adenylic acid residues initially, followed by the appearance of other mononucleotides after a short lag. The rate of appearance of mononucleotides was in the order of A>>U>G>C (c) the absence of di- or trinucleotides with 3′-terminal Ap formation throughout the process of digestion except for 3′- terminal Gp or Up and (d) negligible formation of adenosine 2′, 3′- cyclic phosphate. All these results suggest that the action of RNase T2 on RNA occurs by the cooperative action of an adenylic acid-specific endonuclease and a nonspecific exonuclease activity, releasing mononucleotides from the 3′- terminus.

The susceptibility of various RNAs and homopolymers to RNase T2 depends on their nature (Uchida and Egami, 1967a). While poly A and poly C are fairly resistant to RNase T2 (despite the preference of RNase T2 for adenylic acid linkages), poly U is more susceptible to hydrolysis. The digestion pattern of poly U is very similar to that of RNA i.e. significant accumulation of the 3′- end in the initial stages (25% of the total residues) followed by the accumulation of UpUp (uridine phosphate dimer) or 2′, 3′-cyclic Up and after a short lag, a rapid appearance of Up, indicating that RNase T2 behaves like an endonuclease for poly U as for RNA. In the digestion of poly A or poly C, the

initial endonuclease activity seems markedly reduced (approximately 4% of total residues) as evidenced by the small increase in the 3′- terminus in the initial stages. Therefore, only 3′- mononucleotides are detected as the digestion product. This could be because poly A forms a rigid double stranded helical structure in acidic medium while poly U has no secondary structure and is easily digested. In 40% methanol, part of the double stranded structure of poly A is destroyed, making it now more susceptible to RNase T2 and the mode of action is very similar to that on RNA and poly U. The susceptibilities amongst various RNAs to RNase T2 are in the order: commercial yeast RNA (low molecular weight) > high molecular weight yeast RNA > tRNA (which contains more secondary structure than other RNAs). This observation further confirms the resistance of ordered structure in the substrate to enzymatic attack.

Though RNase T2 has no absolute specificity, β- methyl ribofuranoside 2′,3′-cyclic phosphate is not cleaved (Uchida and Egami, 1967a), indicating that at least a part of the base structure is of significance for the enzymic action. It has also been found that RNase T2 completely cleaves phosphodiester bonds of 4-thio-uridylate in tRNA (Saneyoshi

et al., 1969) and 6-thio-guanylyl-(3′,5′)-cytidine (Irie *et al.*, 1970). This shows that the substitution of sulfur at position 4 of the pyrimidine base or at position 6 of the purine base does not effect the enzyme action. Furthermore, the phosphodiester bonds of the nucleotides with a large substituent at position 6 of the purine base such as N^6-isopentenyladenosine (Hashimoto *et al.*, 1969), 2-methyl thio-N^6-(Δ^2-isopentenyl) adenosine (Harada *et al.*, 1968) or N-9-(β-D-ribofuranosyl) purin-6-yl carbamoyl threonine (Ishikura *et al.*, 1969; Takemura *et al.*, 1969) were hydrolyzed to produce the corresponding 2′,3′- cyclic phosphates.

Madison and Holley (1965) showed that RNase T2 can split the phosphodiester bonds of pseudouridylic acid and 5,6-dihydrouridylic acid. Saneyoshi *et al.* (1969) detected a new minor constituent, Vp with 5-methyl cytidine 3′-phosphate and other mononucleotides, on exhaustive digestion of *Escherichia coli*. $tRNA^{val}$ with RNase T2. Vp was later characterized as uridine-5-oxyacetic acid (Murao *et al.*, 1970).

Tada (1966) noted that the phosphodiester bonds of dimethyl guanylic acid and 1-methyl guanylic acid are fairly resistant to RNase T2. These observations show that blocking at N-1 of the base with a substituent group results in increased resistance to RNase T2. Hence, N-1 in the base is

of significance for RNase T2 action.

Sato *et al.* (1966) studied the action of RNase T2 on various nucleoside 2′,3′- cyclic phosphates and determined the K_m and V_{max} at the optimum pH. Although the K_m value for adenosine 2′,3′-cyclic phosphate was a little less than that of others, no significant differences were observed between the K_m and V_{max} values of adenosine 2′,3′-cyclic phosphate and with other cyclic nucleotides. The result perhaps reflects the lack of base specificity of RNase T2. The apparent preference for adenylic acid bonds might have been due to a base specific affinity of RNase T2 to adenylic acid. Imazawa *et al.* (1968) also measured the K_m and V_{max} values at pH 6.0 with various dinucleoside monophosphates (XpY) and noted that when either X or Y were adenylyl residues, their K_m values were less than those of others. This finding also supports the above speculation.

1.4 AMINO ACID COMPOSITION

Egami *et al.* (1964) and Uchida (1966) showed that RNase T2 contains 325 amino acids. However recently, Kawata *et al.* (1988) based on sequence analysis, reported that it contains only 239 amino acid residues. The amino acid content obtained by both groups is shown in Table 1.

Table 1 : Amino acid composition of RNase T2.

Amino acid	Egami *et al.* (1964)	Kawata *et al.* (1988)
Lys	23	19
His	6	4
Arg	4	3
Asp	39	17
Asn	-	12
Thr	25	14
Ser	32	24
Glu	39	17
Gln	-	9
Pro	23	16
Gly	28	18
Ala	19	12
Cys (1/2)	11	10
Val	7	4
Met	1	1
Ile	19	15
Leu	18	14
Tyr	14	15
Phe	10	8
Trp	7	7
Total	325	239

1.5 STRUCTURE

RNase T2 consists of a single polypeptide chain of 239 amino acid residues, cross-linked by 5 disulfide bonds. Two of the established S-S linkages occur between Cys 68-Cys 118 and Cys 191-Cys 225. The N-terminal residue is glutamic acid while the C-terminal residue is aspargine. RNase T2 is a glycoprotein and contains sugar moieties attached to aspargines 15, 76 and 239 (Kawata *et al.*,1988). The sequence of RNase T2 is given in Fig. 2.

1.6 ACTIVE SITE

Recently, using chemical modification, proton-NMR titration and photo-chemically induced dynamic nuclear polarization studies, Kawata *et al.* (1990) demonstrated that 2 histidine residues, namely, His 53 and His 115, were involved in the active center of RNase T2. Earlier studies on sequence comparison analysis of RNase T1 and RNase T2 (Kawata *et al.*, 1988), had anticipated the involvement of Glu 111 of RNase T2 as a catalytic residue in its active site, corresponding to the Glu 58 of RNase T1. However, peptide mapping studies showed no modification of the Glu 111, indicating that this residue is not involved in catalysis. The disulfide bond between Cys 68 and Cys 118 is of importance since it brings the 2 peptide segments, containing His 53 and His 115, closer in the tertiary structure.

1
Glu - Phe - Pro - Ser - Cys - Pro - **Lys** - Asp - Ile - Pro - Phe - Ser - Cys - Gln - Asn -

16
Ser - Thr - Ala - Val - Ala - Asp - Ser - Cys - Cys - Phe - Asn - Ser - Pro - Gly - Gly -

31
Ala - Leu - Leu - Gln - Thr - Gln - Phe - Trp - Asp - Thr - Asn - Pro - Pro - Ser - Gly -

46 (53 under Trp)
Pro - Ser - Asp - Ser - Trp - Thr - Ile - His - Gly - Leu - Trp - Pro - Asp - Asn - Cys -

61
Asp - Gly - Ser - Tyr - Gly - Gln - Phe - Cys - Asp - **Lys** - Ser - **Arg** - Glu - Tyr - Ser -

76
Asn - Ile - Thr - Ala - Ile - Leu - Gln - Glu - Gln - Gly - **Arg** - Thr - Glu - Leu - Leu -

91
Ser - **Tyr** - **Met** - **Lys** - **Lys** - Tyr - Trp - Pro - Asn - Tyr - Glu - Gly - Asp - Asp - Glu -

106 (115 under Tyr)
Glu - Phe - Trp - Glu - His - Glu - Trp - Asn - **Lys** - His - Gly - Thr - Cys - Ile - Asn -

121
Thr - Ile - Glu - Pro - Ser - Cys - Tyr - **Lys** - Asp - Tyr - Ser - Pro - Gln - **Lys** - Glu -

136
Val - Gly - Asp - Tyr - Leu - Gln - **Lys** - Thr - Val - Asp - Leu - Phe - **Lys** - Gly - Leu -

151
Asp - Ser - Tyr - **Lys** - Ala - Leu - Ala - **Lys** - Ala - Gly - Ile - Val - Pro - Asp - Ser -

166
Ser - **Lys** - Thr - Tyr - **Lys** - Arg - Ser - Glu - Ile - Glu - Ser - Ala - Leu - Ala - Ala -

181
Ile - His - Asp - Gly - **Lys** - **Lys** - Pro - Tyr - Ile - Ser - Cys - Glu - Asp - Gly - Ala -

196
Leu - Asn - Glu - Ile - Trp - Tyr - Phe - Tyr - Asn - Ile - **Lys** - Gly - Asn - Ala - Ile -

211
Thr - Gly - Glu - Tyr - Gln - Pro - Ile - Asp - Thr - Leu - Thr - Ser - Pro - Gly - **Cys** -

226 (239 under Gly)
Ser - Thr - Ser - Gly - Ile - **Lys** - Tyr - Leu - Pro - **Lys** - **Lys** - Ser - Glu - Asn

Fig. 2 : Sequence of RNase T2 (Kawata *et al.*, 1988).

1.7 SEQUENCE COMPARISON OF RNase T2 WITH OTHER RNases

It is evident that, although RNase T1 and RNase T2 are produced by the same fungus, they differ from each other markedly with respect to their molecular size, substrate specificity and amino acid residues at the active center. Now that the amino acid sequence of RNase T2 is available (Kawata *et al.*, 1988), and two histidine residues at the active site have been deciphered (Kawata *et al.*, 1990), a comparison of the primary structure of the two RNases is possible. Even though the sequences show low similarity, sequence comparison has shown several interesting features. Results of sequence comparison analyses of RNase T1 and RNase T2 and a few other RNases are summarized below:

1) Presence of similar sequence between Ser 91-Asp 104 in RNase T2 and Ser 37-Asp 49 in RNase T1. In this region, 9 out of 14 amino acids are identical.

2) This local but high similarity was also observed between RNase T2 and other fungal RNases, *viz.* RNase Ms (*Aspergillus saitoi*), RNase C2 (*Aspergillus clavatus*), RNase F1 (*Fusarium moniliforme*) and RNase Pch1 (*Penicillium chrysogenum*152A).

3) Bacterial RNases, *viz.* RNase St (*Streptomyces erythraeus*), RNase Sa (*Streptomyces aureofaciens*), RNase Bi (*Bacillus intermedius*) and RNase Ba (*Bacillus amyloliquefaciens*) show less similarity even in this region.

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1.8 APPLICATIONS

RNase T2 is an analytically important enzyme and is used in the base analysis of RNA. For base analysis, digestion with RNase T2 is preferred to alkaline hydrolysis as the latter results in slight degradation of nucleotide bases, especially minor components. RNase T2 has also played an important role in the discovery and identification of new minor components in tRNA, namely, 5,6-dihydrouridylic acid, uridine-5-oxyacetic acid, N^6-methyl-adenosine and N-9-(β-D-ribofuranosyl) purin-6-yl carbamoyl threonine (Uchida and Egami, 1971). The transferase activity associated with the enzyme has also been exploited for the synthesis of certain nucleotide 3′ phosphates, such as Coenzyme A (Michelson, 1964).

1.9 IMMOBILIZED RNase T2

The advent of immobilized enzyme technology has led to increasing efforts to replace conventional enzymatic processes with immobilized systems since immobilization (a) enables the enzyme to process large amounts of substrate as it can easily be separated from the mixture of substrate and product and hence can be reused (b) in general, imparts greater stability to the enzyme so that they can be used for the development of continuous processes (c) affords the possibility of obtaining enzyme-free endproducts (d) allows

the use of enzyme(s) from non-GRAS (generally regarded as safe) cleared organisms (e) enables greater control of the catalytic process and (f) permits the economical utilization of an otherwise cost prohibitive enzyme.

RNase T2 is an analytically important enzyme and has extensively been used as an analytical tool for the base analysis of RNA. The synthetic activity associated with the enzyme has been exploited for the preparation of oligonucleotides. Hence, attempts have been made to immobilize RNase T2 (a) to assess its potential application as a reusable analytical tool (b) for the synthesis of oligonucleotides and (c) as a model system for studying immobilized enzymes.

Soboleva *et al.* (1985) bound RNase T1 and RNase T2 on CM-cellulose for studying the influence of immobilization on their synthetic activities and noted that immobilization did not significantly alter their synthetic activities. It was interesting to note that inspite of its higher lysine content (19 residues), RNase T2 reacted poorly with the CM-cellulose azide matrix, whereas, RNase T1, which has only one lysine residue, bound most efficiently. When RNase T2 was bound to Con 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, immobilized RNase T2 showed enhanced stability towards temperature and metal ions (Reddy and Shankar, 1989).

PRESENT INVESTIGATION

RNase T2 is a non-specific ribonuclease which shows preference towards adenylic acid linkages in RNA. Due to this property, it has been extensively used as an analytical tool in the structural determination of RNA. It has been observed that after nuclease treatment of RNA containing samples, the removal of residual enzyme activity is obligatory and involves repeated extractions with phenol, which in turn results in the loss of nucleic acid samples. In such circumstances, use of immobilized enzyme offers a distinct advantage over the soluble enzyme, since it can easily be removed from the reaction mixture by physical methods. Takadiastase, apart from RNase T2, contains other RNA degrading enzymes like RNase T1 and S1 nuclease. Hence, the present investigation with RNase T2 was undertaken (a) to develop a simple and rapid procedure to obtain a contamination-free partially purified RNase T2 suitable for immobilization and (b) to design an immobilization procedure to obtain a highly active and stable immobilized RNase T2, suitable for analytical purposes.

MATERIALS & METHODS

MATERIALS

Takadiastase (Sankyo, Japan); RNA (Sisco Research Laboratories, India); glutaraldehyde (50 % v/v) (Fluka AG, Switzerland); Bio-Gel P-60 (BioRad, USA); bovine serum albumin and methyl α-D-mannopyranoside (Sigma chemical Co., USA); uranyl acetate (Loba-Chemie Indoaustronal Co., India); perchloric acid (SD Fine Chemicals Pvt. Ltd., India); Concanavalin A (CSIR Biochemicals, India); DEAE-cellulose (Whatman, UK); Sepharose 4B (Pharmacia Fine Chemicals, Sweden), divinyl sulphone (Aldrich Chemical Co., USA); ethylenediamine (Wilson Laboratories, India) were used. All other chemicals used were of analytical grade.

METHODS

Assay of RNase T2 : RNase T2 activity determination was carried out according to the procedure of Uchida and Egami (1967c). The standard reaction mixture of 0.75 ml contained 0.25 ml of RNA (12 mg/ml), 0.3 ml of 0.2 M acetate buffer, pH 4.5, 0.1 ml of 20 mM EDTA and 0.1 ml of appropriately diluted enzyme. The reaction was initiated by the addition of RNA, followed by incubation at 37°C for 15 min. The reaction was then terminated by the addition of 0.25 ml of chilled uranyl reagent (0.75 % uranyl acetate in 25 % perchloric acid) and the precipitate obtained was removed immediately by centrifugation. Subsequently, 0.1 ml of the supernatant

was diluted with 2.5 ml of distilled water and the acid soluble nucleotides were measured at 260 nm (Fig. 3). The amount of acid soluble nucleotides were calculated by assuming a molar extinction coefficient of 10,600 for the ribonucleotide mixture (Curtis *et al.*, 1966).

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

Assay of RNase T1 : RNase T1 activity determination was carried out according to the procedure of Uchida and Egami (1967b). The standard reaction mixture of 0.75 ml contained 0.25 ml of RNA (12 mg/ml), 0.3 ml of 0.2 M Tris - HCl buffer pH 7.5 , 0.1 ml of 20 mM EDTA and 0.1 ml of appropriately diluted enzyme. The reaction was initiated by the addition of RNA, followed by incubation at 37°C for 15 min. After the incubation period, the reaction was terminated by the addition of 0.25 ml of chilled uranyl reagent (0.75 % uranyl acetate in 25 % perchloric acid) and the resulting precipitate was immediately removed by centrifugation. Subsequently, 0.1 ml of the supernatant was diluted with 2.5 ml of distilled water and the acid soluble nucleotides were measured at 260 nm. The amount of acid soluble nucleotides were calculated by assuming a molar extinction coefficient

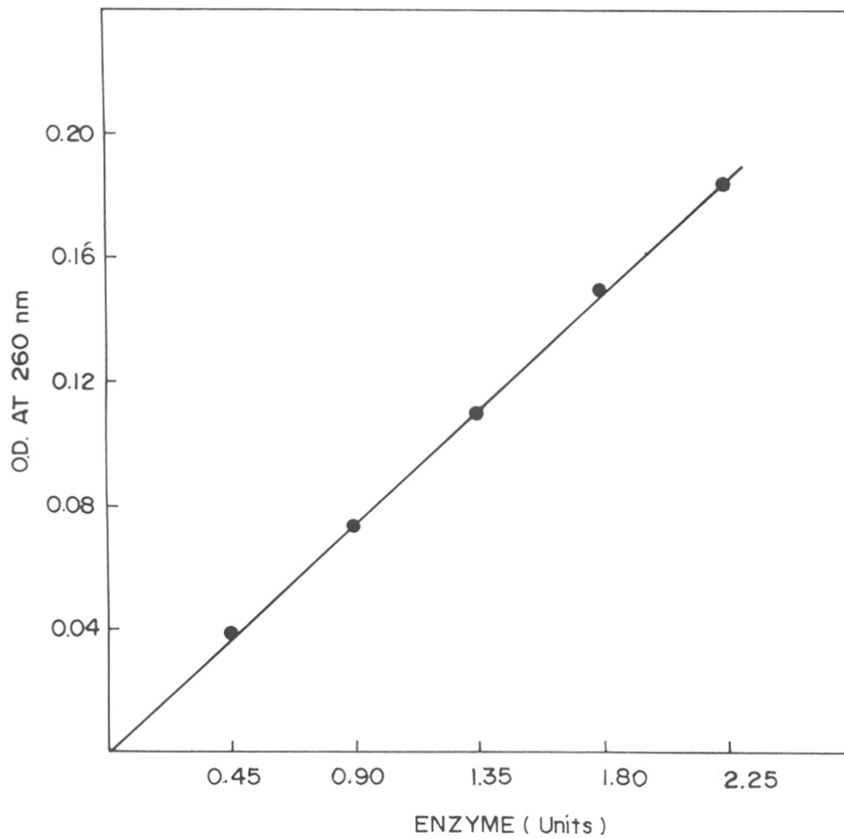

Fig. 3 Calibration curve for the determination of RNase T2 activity.

10,600 for the ribonucleotide mixture (Curtis *et al.*, 1966).

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

Determination of protein : Protein was estimated according to the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard. The blue colour developed after the addition of Folin & Ciocalteau's phenol reagent was read at 660 nm.

Preparation of Con A - Sepharose: Concanvalin A-Sepharose was prepared by the vinyl sulphone 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 at pH 11.0 and 2 ml of divinyl sulphone (DVS) and the mixture was incubated for approximately 90 min, under gentle agitation. From the reaction mixture, excess DVS was removed, by washing extensively with 0.3 M $NaHCO_3$, pH 10.0. Coupling of Con A to the activated Sepharose was carried out by reacting 8 mg of the lectin, (dialyzed previously against 0.3 M $NaHCO_3$ buffer, pH 10.0) with the matrix at pH 10.0, for 6-8 h, at room temperature. After the incubation period, the matrix was washed successively with

0.3 M Na_2CO_3, 0.3 M NaCl, 0.3 M glycine and 0.03 M sodium acetate buffer, pH 5.0. The Con A-Sepharose obtained by this method contained 6 mg Con A per ml of Sepharose and was used for affinity purification of RNase T2.

Partial purification of RNase T2

Unless otherwise mentioned, all the operations were carried out at 4°C. Since RNase T1 shows considerable activity at pH 4.5, the ratio of RNase T2 : RNase T1 activity was monitored throughout the purification steps.

Heat treatment : Ten grams of Takadiastase powder was suspended in 30 ml of glass distilled water and stirred for 1 h. The pH of the dark brown solution was then adjusted to 2.0 with 2 N HCl and heated at 80°C for 3 min with vigorous swirling and chilled immediately on ice. The pH of the solution was then brought to 4.9 with 1 N NaOH and the heavy precipitate formed was removed by centrifugation at 6800 g for 10 min. The clear supernatant was dialyzed extensively against 0.03 M acetate buffer, pH 4.9 and was used for further purification steps.

DEAE - Cellulose chromatography : The dialyzed enzyme solution was then passed through a DEAE - cellulose column (15 cm X 2 cm) previously equilibrated with 0.03 M acetate buffer (pH 4.9), at a rate of 15 ml/h. The flow - through

fractions, which contained considerable amount of RNase T2 activity were collected and used for affinity purification.

Affinity chromatography on Con A - Sepharose : The enzyme preparation obtained after DEAE-cellulose chromatography was adsorbed at a rate of 15 ml/h, onto a Con A-Sepharose column (10 cm X 2 cm) previously equilibrated with 0.03 M acetate buffer, pH 5.0 containing 1mM $MgCl_2$, 1mM $CaCl_2$ and 1mM $MnCl_2$. The column was then washed with the above buffer till the effluent showed no detectable RNase activity. Subsequently, the bound enzyme was eluted with 0.5 M α- methylmannoside prepared in the above buffer. The eluted enzyme was dialyzed extensively against 0.03 M acetate buffer, pH 6.0 and was stored frozen. No loss of activity was observed when the enzyme was stored under these conditions. The specific activity of the partly purified RNase T2 ranged from 360 - 380 and this was used for immobilization studies.

Preparation of Aminoethyl Bio-Gel P-60 (AE Bio-Gel P-60) : The amination of the Bio-Gel P-60 was carried out essentially according to the procedure of Inman and Dintzis (1969). 100 ml of anhydrous ethylenediamine was preheated to 90°C in an oil bath, kept in a fume hood. Subsequently, 10 g of dry Bio-Gel P-60 was gradually added using a funnel and the reaction was allowed to proceed for 6 h under mild agitation. At the end of the reaction time, the reaction

mixture was cooled on ice and the contents were mixed with an equal volume of crushed ice. The aminated gel was then extensively washed with 0.1 M NaCl till the supernatant was free of ethylenediamine, as indicated by a negative colour test with 2, 4, 6 trinitrobenzene sulphonic acid (TNBS); (A positive test is indicated by the formation of an orange colour, on addition of a few drops of 3% TNBS solution prepared in saturated sodium tetraborate). Finally, the matrix was washed with acetate buffer (0.03 M, pH 5.0) and stored in the same buffer at 4°C till further use.

Immobilization technique

a. Activation of matrix : One ml (packed volume) of the matrix (AE Bio-Gel P-60) was activated by incubating it with 5 ml of 1 % (v/v) glutaraldehyde in 0.05 M phosphate buffer, pH 7.5, at room temperature for 3 h, under mild agitation. The matrix was then washed free of excess glutaraldehyde with distilled water and used immediately for coupling.

b. Coupling of the enzyme to the activated matrix : One ml (packed volume) of the activated matrix was incubated with 40 U of partially purified RNase T2 (approximately 100 μg protein) in 2 ml of 0.03 M acetate buffer, pH 6.0, at 4°C for 15 h under mild agitation. The supernatant was decanted and the matrix was washed successively with coupling buffer, 1 M

NaCl in assay buffer and finally with the assay buffer (0.2 M acetate buffer, pH 4.5) till there was no activity in the washings. The amount of enzyme/protein bound to the matrix was determined by estimating the difference in the enzyme activity/protein before loading on the matrix and after coupling.

Assay of immobilized RNase T2 : The immobilized enzyme was assayed by incubating appropriate amounts of the bound enzyme with 2.25 ml of standard reaction mixture at 37°C, in a thermostated shaker water bath (100 - 120 rpm), for 30 min followed by measuring the acid soluble nucleotides at 260 nm as described before.

One unit of the enzyme is defined as the amount of enzyme required to liberate 1.0 µmole of acid soluble nucleotides per min under standard assay conditions. Specific activity is defined as measured activity / mg protein / ml of matrix.

Determination of efficiency : Efficiency of the immobilized RNase T2 was determined by assaying appropriate amounts of immobilized preparation under standard assay conditions followed by calculating the ratio of measured activity to bound activity.

RESULTS & DISCUSSION

Purification of RNase T2:

Takadiastase from *A. 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, i.e. 7.5 for RNase T1 and 4.5 for RNase T2; and isoelectric points i.e. 2.9 for RNase T1 and 5.0 for RNase T2 (Rushizky and Sober, 1963). RNase T2 exhibits very less activity at pH 7.5 whereas RNase T1 shows considerable activity at pH 4.5. Hence, the RNase activity in a crude extract of Takadiastase powder, at pH 4.5, represents the combined activities of both RNase T1 and RNase T2. While developing a suitable purification procedure for RNase T2, the ratio of RNase T2 : RNase T1 activity (at their respective optimum pH) was monitored at each step, to ensure the removal of RNase T1 contamination. The increasing T2 : T1 ratio indicated the removal of RNase T1 at each step. The results of a typical purification procedure are given in Table 1.

The enzyme was purified 19-fold with a yield of 5.5%. In this procedure, dialysis was carried out after heat treatment, not only to remove excess salts but also to remove RNase T1 contamination, which due to its low molecular weight (MW 11,000) dialyses out. The procedure in fact, did remove considerable amount of RNase T1, as evidenced by the increase in the T2 : T1 ratio.

Table 1. Partial purification of RNase T2

Step	Volume (ml)	Total activity	Protein (mg)	Specific activity	Fold purification	Recovery %	T2 : T1 activity ratio
Crude	36	29,700	1499	19.8	1.0	100	1 : 1.9
Heat treatment and dialysis	72	13,800	165.6	83.3	4.15	46.5	1 : 1
DEAE-cellulose chromatography (pH 4.9)	75	2,850	75	38.0	1.91	9.6	1 : 0.2
Con A-Sepharose chromatography (pH 6.0)	75	1,650	4.5	366.6	18.4	5.5	1 : 0

It should be noted that, though the enzyme obtained after this step showed high specific activity, it did contain considerable amount of RNase T1 activity as indicated by the RNase T2 : RNase T1 ratio. Though Egami *et al.* (1964) and Uchida (1966) separated RNase T2 from RNase T1 by DEAE-cellulose chromatography at pH 7.0, we chose to chromatograph the enzyme near its isoelectric point (4.9) as the enzyme shows very little binding to DEAE-cellulose at this pH. The flow-through fraction obtained after this step contained a significant amount of RNase T2 activity as evidenced by the T2 : T1 ratio. The drastic reduction in RNase T1 activity in this fraction explains the decrease in specific activity and fold purification. Finally, from the enzyme preparation obtained from the above step, residual RNase T1 contamination was removed by chromatography on Con A-Sepharose, a step where RNase T1 does not bind to the matrix due to its non-glycoprotein nature. The partially purified RNase T2 thus obtained was completely free from RNase T1 contamination as indicated by the T2 : T1 ratio (Table 1). Though the overall yield seems to be low, in fact, it is much higher if we consider the fact that RNase T2 activity constitutes only 10% of the total RNase activity in Takadiastase powder, i.e. in the present case 2970 U out of the total 29,700 U. On this basis, the recovery of RNase T2 obtained by this procedure is

approximately 56%. The specific activity of the partially purified enzyme, which ranged from 360-380, was used for immobilization studies.

Immobilization of RNase T2 :

The major components considered in an immobilized system are : the enzyme, the matrix and the mode of attachment of the enzyme to the matrix. Besides the enzyme, the other vital contributing factor that determines the performance of immobilized enzyme systems, is the matrix. Hence, a careful selection of the matrix is highly essential, as it not only affects the operational stability but also the performance of the immobilized system. In the present studies, Bio-Gel P-60 (exclusion limit 60,000) was preferred, as polyacrylamide-based matrices show more resistance to chemical and microbial degradation, have a lower tendency to physically adsorb proteins, can be easily derivatized and are commercially available. Porous matrices however suffer from a major disadvantage in that, the enzyme binds into the pore´s internal structure leading to diffusional constraints. An additional problem that often arises in carriers with broad pore distributions is that, they have only a limited number of pores large enough to accommodate both the enzyme and substrate. Hence, only a small portion of the total available surface area is utilized. Nevertheless, the advantages in

using porous matrices are, availability of high surface areas for immobilization and protection of the enzyme bound into the internal surfaces, from the harsh external environment.

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 broad range of pH. Though alkaline pH is preferred for glutaraldehyde activation of the matrix, the subsequent coupling of the enzyme can be achieved depending on the pH stability of the enzyme in question. During activation, the proximal aldehyde group of each glutaraldehyde molecule will form Schiff´s base with the primary amino group of the matrix, leaving the distal aldehyde group free for the coupling of the enzyme (Onyezili, 1987). Thus, glutaraldehyde additionally will provide a spacer arm for binding the enzyme away from the matrix. When an enzyme is bound to an insoluble matrix *via* glutaraldehyde, primarily the ϵ-amino group of lysine is involved in the binding. Hence, in the present investigation, attempts were made to bind RNase T2 to glutaraldehyde - activated - AE Bio-Gel P-60 due to its high lysine content (19 residues).

The partially purified RNase T2 coupled to glutaraldehyde activated AE Bio-Gel P-60 retained 12-16% activity of the soluble enzyme. Effect of coupling pH on the

efficiency of immobilized RNase T2 showed that most active preparations are obtained when the coupling is carried out at pH 6.0. Though the increase in the efficiency values obtained at pH 6.0 was marginally higher than the value obtained at pH 5.0, a significant decrease in the efficiency was observed at pH 7.0. This can be correlated to overcrowding of the enzyme on the matrix, as reflected by the decrease in specific activity of the bound enzyme at pH 7.0 (Fig. 4). The results of a typical immobilization procedure of partially purified RNase T2 on glutaraldehyde activated AE Bio-Gel P-60, under optimized conditions, are given in Table 2.

Table 2. Immobilization of RNase T2 on glutaraldehyde activated AE Bio-Gel P-60.

Enzyme Loaded		Enzyme Bound		Activity of the complex (Units expressed)	Efficiency*
U	Protein (mg)	U	Protein (mg)		
800	2.2	241	0.66	30.75	12.76

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

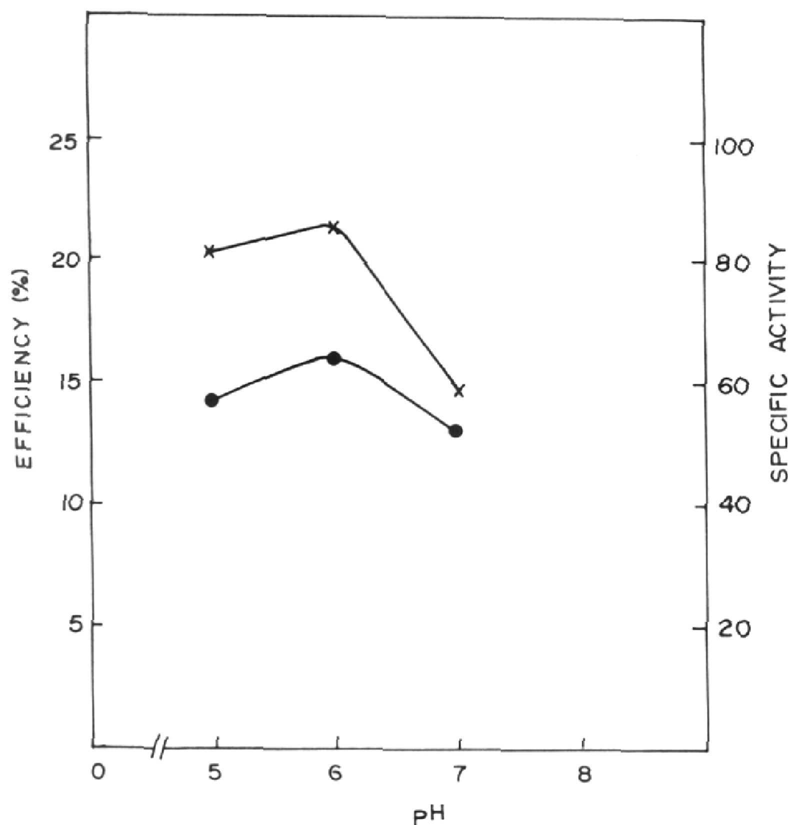

Fig. 4 Effect of coupling pH on the activity of AE Bio-Gel P-60 bound RNase T2. Efficiency (●), specific activity (X).

One ml (packed volume) of 1% glutaraldehyde activated matrix was incubated with approximately 38 U of partly purified RNase T2 at different pH (5.0 - 7.0) and $4^{o}C$ for 15 h and efficiency of the immobilized enzyme was determined as described under Methods.

When 800 U (2.2 mg protein) of partially purified RNase T2 was reacted with 20 ml (packed volume) of 1% (v/v) glutaraldehyde -activated - AE Bio-Gel P-60 at pH 6.0 and 4°C for 15 h, 241 U were bound. The effectiveness factor (η) of the preparation was approximately 0.13 indicating the efficiency of immobilized system to be 13% (Table 2).

Changes caused as a result of binding of enzymes to insoluble supports are not only useful in assessing the potential of immobilized systems but also in elucidating the structure-function relationship and mechanism of enzyme action. Changes in the properties of an enzyme on immobilization can be caused by changes in the enzyme conformation as a result of modification of amino acids or due to physico-chemical characteristics of the matrix. Modification of enzymatic properties brought about by the matrix are due to (a) steric hindrance (b) electrostatic interaction between carrier and substrate and (c) formation of a diffusional layer around the bound enzyme. The observed changes in the properties of the enzyme on immobilization are the result of complicated interactions of these factors (Chibata, 1978).

AE Bio-Gel P-60-RNase T2 conjugate retained only 13% of its initial activity. The loss of activity could be due to

multiple attachment of the enzyme to the matrix since lysine (Lys) residues are distributed evenly throughout the primary sequence of the enzyme (Kawata *et al.*,1988). Though, till now, Lys residues have not been implicated in the catalytic activity of the enzyme, one Lys residue, *viz.* Lys 114, is situated adjacent to histidine (His) 115, which in turn has been implicated in catalysis (Kawata *et al.*, 1989). In case of RNase T1, modification of the ε- amino group of Lys 41, which is situated adjacent to His 40 (implicated in catalysis), lowered the activity of the enzyme towards RNA to a varying extent, depending on the nature and size of the group introduced (Takahashi and Moore, 1982). In the present studies, a similar phenomenon may be occurring. In addition, a few Lys residues namely, Lys 94, 95, 128 and 134 are situated adjacent to His 115 while Lys 70 is close to His 53 (also implicated in catalysis, Kawata *et al.*, 1989). The probable involvement of some of these residues, may be preventing the accessibility of the substrate to the enzyme. This speculation is supported by our observation that, binding the enzyme by freezing it in its native conformation (i.e. in presence of substrate) so as to protect the active site region, failed to improve the efficiency. Further, increase in the exposure of the bound enzyme to the substrate (i.e. by increasing the incubation period) did not bring

about an increase in the efficiency of the bound enzyme, indicating that the low efficiency values observed in the present case cannot be attributed to the extent of exposure of the bound enzyme to the substrate.

Comparison of pH activity profiles of soluble and immobilized RNase T2 showed no change in the optimum pH (Fig.5) indicating the absence of partitioning effects in the microenvironment of the immobilized enzyme. Similarly, no changes were observed in the optimum temperature of the soluble enzyme as a result of immobilization (Fig. 6).

It has been reported in many cases that immobilization affects the stability of the enzyme to pH and temperature. Such studies are of importance to evaluate the potential applications of the immobilized system. The pH stability of the immobilized RNase T2 was inferior to that of the soluble enzyme (Fig. 7). The temperature stability of the AE Bio-Gel P-60-RNase T2 conjugate was fairly comparable to its soluble counterpart (Fig. 8). This indicates the lack of rigidity of the enzyme structure in the bound form. On repeated use, the bound enzyme retained 55% of its initial activity after 6 cycles (Fig. 9). In the absence of any detectable leaching (as evidenced by the absence of enzymatic activity in the supernatants), the decrease in activity can be correlated to

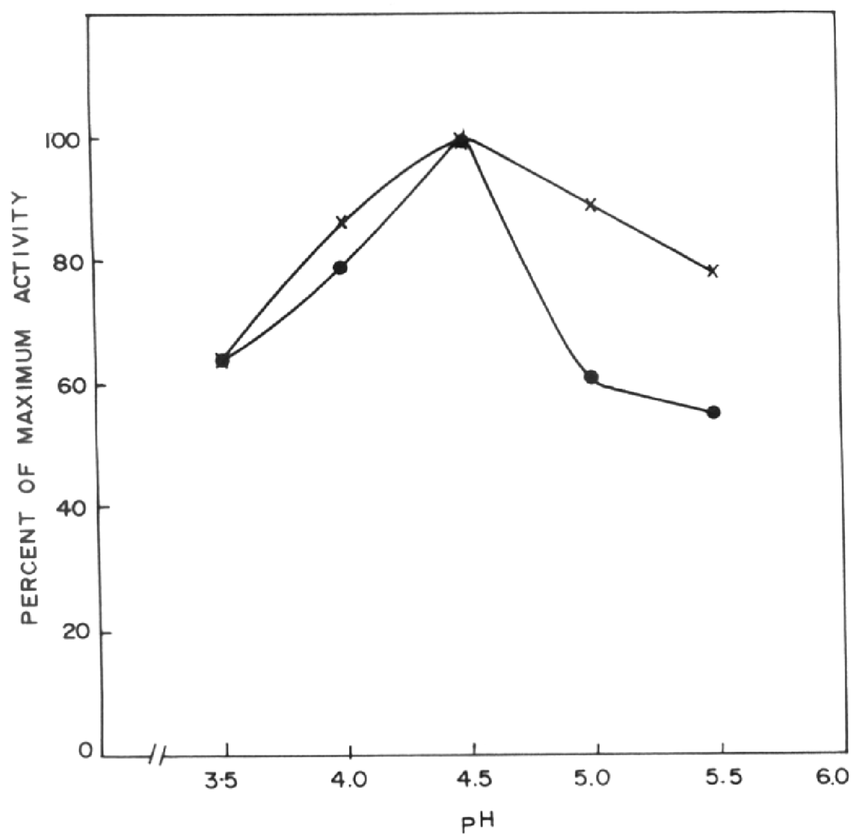

Fig. 5 pH activity profiles of soluble (x) and immobized (●) RNase T2.
Both soluble and immobilized enzymes (1.2 - 1.5U) were assayed in a series of pH (3.5 - 5.5) at 37°C as described under Methods.

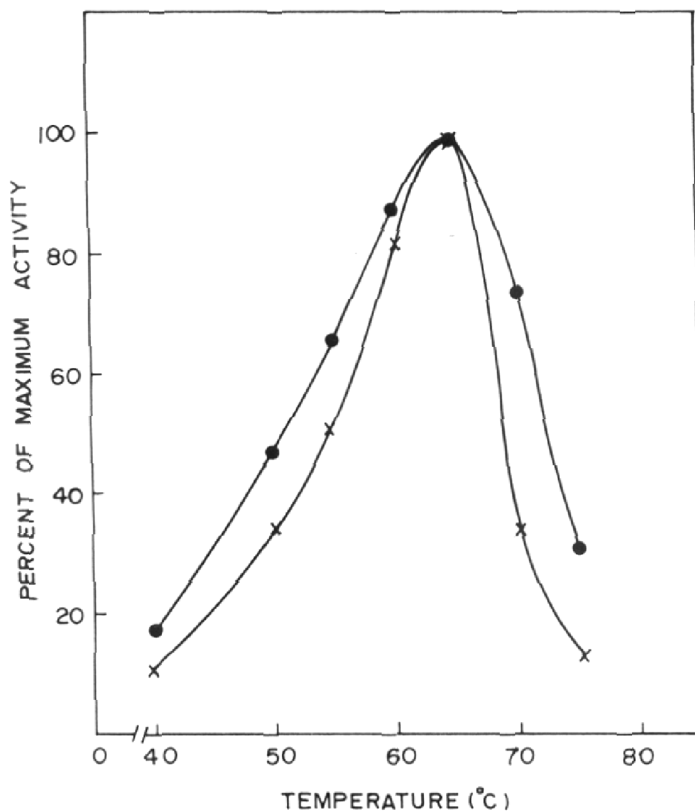

Fig. 6 Temperature activity profiles of soluble (x) *and immobilized* (●) *RNase* T2.

Appropriate amounts of soluble and immobilized enzymes (1.2 - 1.5U) were incubated in a series of temperatures (40 - 70 °C) at *pH* 4.5 *and their* activities *were* determined as described under Methods.

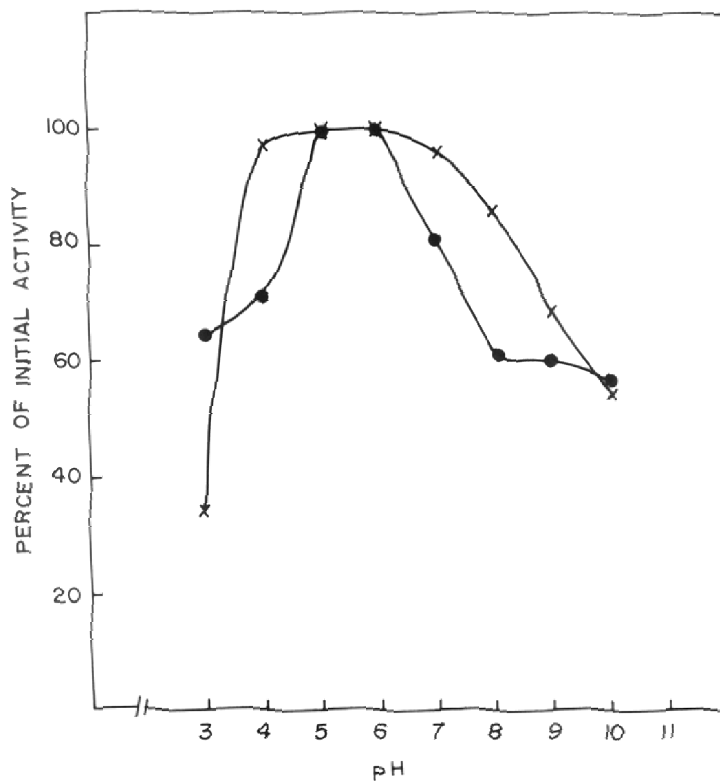

Fig. 7 pH stability of soluble (x) and immobilized (●) RNase T2.

Soluble and immobilized enzymes (1.2 - 1.5U) were preincubated at different pH (3 - 10) for 1 h at 37°C and their activities were determined under standard assay conditions.

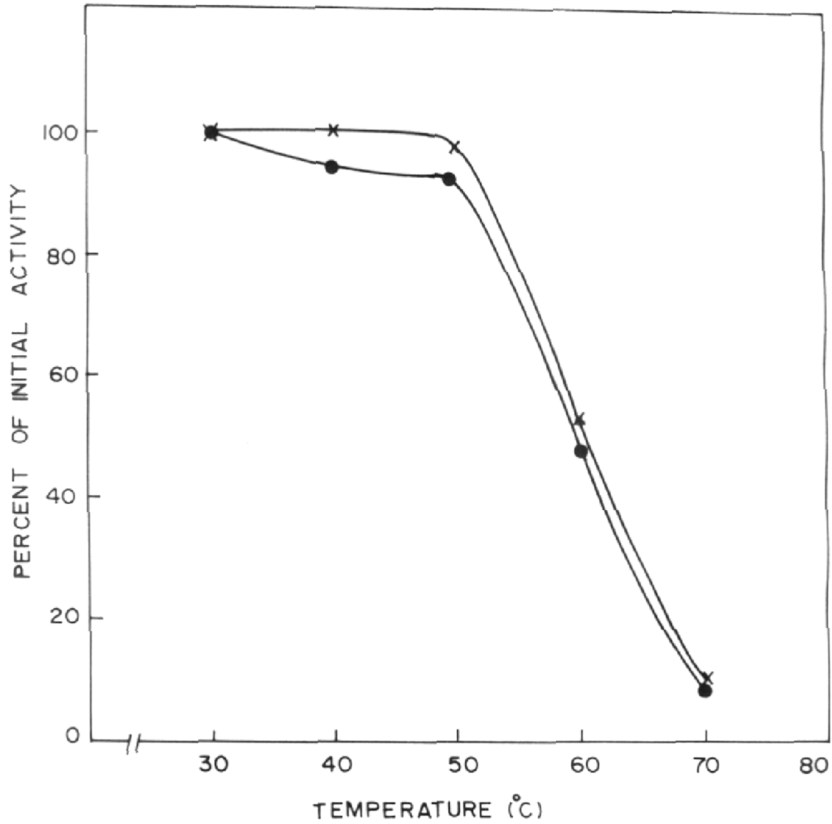

Fig. 8 Temperature stability of soluble (x) and immobilized (●) RNase T2.

Both soluble and immobilized enzymes (1.2 - 1.5U) were preincubated at different temperatures (30 - 70°C) for 1 h at pH 4.5 and their activities were determined as described under Methods.

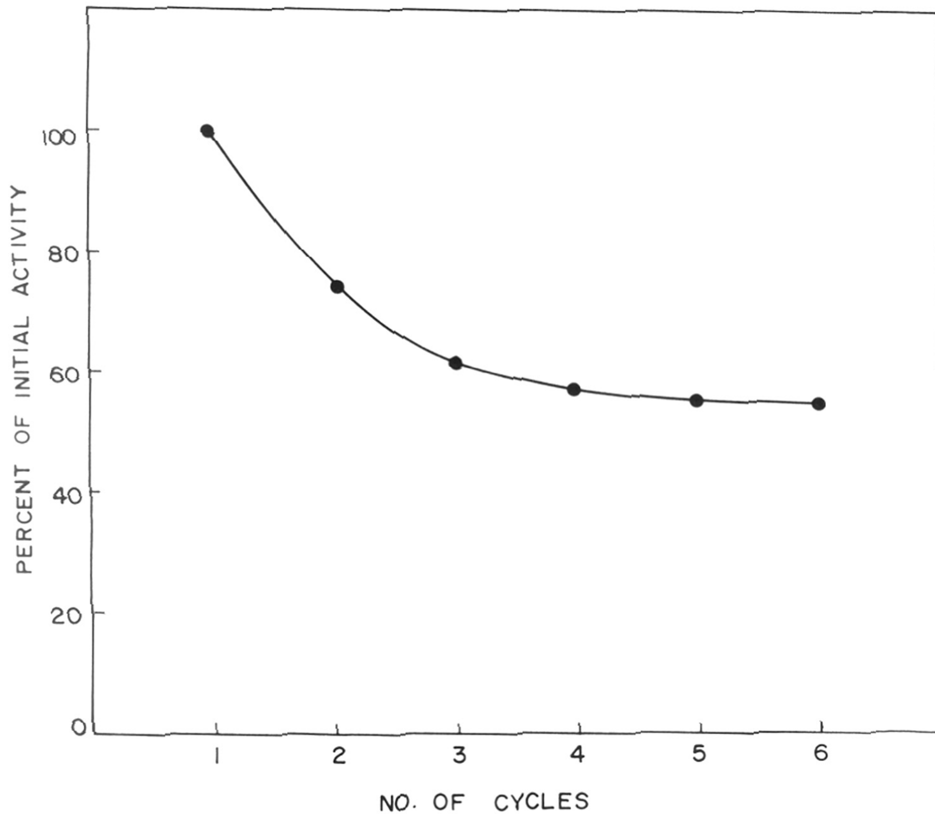

Fig. 9 Effect of number of assay cycles on the activity of immobilized RNase T2.

1.2 ml (approximately 1.5U) of immobilized RNase T2 was assayed at pH 4.5 and 37^{0}C. After every cycle, the immobilized enzyme was washed free of substrate and products and used for a fresh assay.

the inactivation of the enzyme after every use. The bound RNase T2 showed decreased storage stability and lost 50% of its initial activity after 25 days when stored in 0.2 M acetate buffer, pH 4.5 at 4°C (Fig. 10).

Koch-Schmidt and Mosbach (1977) while studying the immobilization of RNase A and α- chymotrypsin to cyanogen bromide activated-Sepharose noted that, increasing the number of points of attachment between the enzyme and support (by activating the matrix with different amounts of cyanogen bromide) gives immobilized preparations with lower specific activity. Transition temperature studies showed that, while the weakly bound enzyme (less number of attachment points) showed a similar behaviour to that of the soluble enzyme, the strongly bound enzyme (more number of attachment points) exhibited higher temperature stability.

The decreased temperature stability of AE Bio-Gel bound RNase T2 also points towards the lack of rigidity of the enzyme structure in the bound form. These observations support our view that the low efficiency obtained with AE Bio-Gel P-60 bound RNase T2, can be correlated to the involvement of Lys residues situated near the catalytic active site rather than multiple attachment of the enzyme to the matrix. Had the enzyme been bound through multi-point attachment, it would have resulted in a more rigid

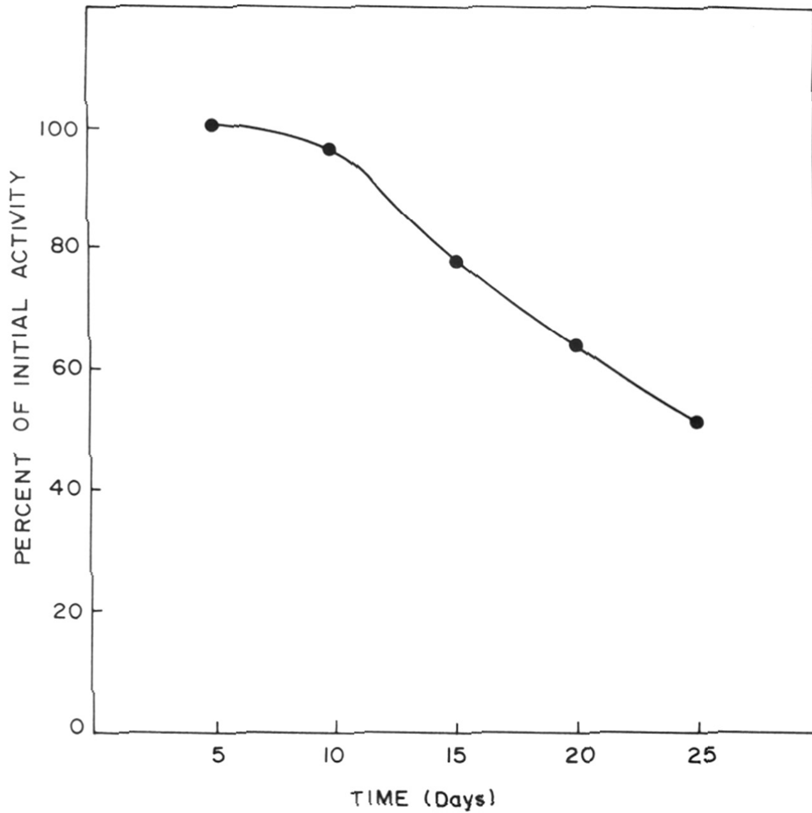

Fig. 10 Effect of storage on the activity of immobilized RNase T2.

The immobilized preparation was stored in 0.2M acetate buffer (pH 4.5) at 4°C. Fresh aliquots of 1.2 ml matrix (1.5U) were removed and assayed for its activity under standard assay conditions at an interval of five days.

conformation and hence, the immobilized RNase T2, in general, would have exhibited a higher stability. However, further studies like chemical modification of the amino groups (especially lysine residues) are required to substantiate the above speculation.

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