COMPUTERISED

PARTIAL PURIFICATION AND IMMOBILIZATION OF RNase T1

A DISSERTATION
SUBMITTED TO THE
UNIVERSITY OF POONA
IN PARTIAL FULFILMENT
FOR THE DEGREE OF
MASTER OF PHILOSOPHY
(IN BIOTECHNOLOGY)

577·152·277(043)
GIT

By

SADANAND GITE
DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE - 411 008

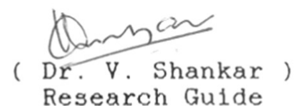
July 1990

C O N T E N T S

	Pages
CERTIFICATE	
ACKNOWLEDGEMENTS	
SUMMARY	1
INTRODUCTION	2-20
MATERIALS AND METHODS	21-26
RESULTS AND DISCUSSION	27-48
REFERENCES	49-53

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 T1 " submitted by Mr. S. U. Gite 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

I would like to take this opportunity to express my gratitude to :

Dr. V. Shankar, for his guidance, encouragement and sustained interest during the course of this investigation.

The Head and members of the Biochemical Sciences Division, for their cooperation and support.

The Director, National Chemical Laboratory, for his permission to submit this work in the form of a thesis and Council of Scientific and Industrial Research, New Delhi, for the financial support.

(Sadanand Gite)

SUMMARY

A simple and rapid procedure, involving heat treatment followed by chromatography on Bio-Gel P-10, was developed for the partial purification of RNase T1, with an overall yield of approximately 63 %.

The partially purified enzyme, when coupled to glutaraldehyde activated aminoethyl Bio-Gel P-2 (AE Bio-Gel P-2), retained 22-24% activity of the soluble enzyme. Optimization of coupling conditions showed that the most active immobilized preparations are obtained when 1 ml (packed volume) of 2% (v/v) glutaraldehyde activated AE Bio-Gel P-2 is reacted with approximately 100 U of RNase T1 at pH 6.0 and 4°C for 15 h. Immobilization resulted in an increase in optimum temperature and temperature stability, but it did not affect the pH optimum. However, the pH stability of bound enzyme was inferior compared to the soluble enzyme. Immobilization brought about a slight decrease in the Michaelis-Menten constant (K_m) and a five fold decrease in V_{max}. On repeated use, the bound enzyme retained 80% of its initial activity after 6 cycles. The immobilized RNase T1 also showed good storage stability and could be stored in wet state (in 0.2 M Tris-HCl buffer, pH 7.5) for more than 50 days without any apparent loss in its initial activity.

INTRODUCTION

1. RIBONUCLEASE T1 (RNase T1)

The presence of ribonucleases in *Aspergillus oryzae* was first reported by Kuninaka (1954, 1955a, 1955b) and Saruno (1956). They studied the various characteristics of these enzymes and found that Takadiastase (a commercial product of *A. oryzae*) contains atleast three different RNA degrading enzymes and one of them is a thermostable enzyme similar to pancreatic ribonuclease (RNase I-A).

Subsequent studies on the substrate specificity of the major enzymatic component (now known as RNase T1) revealed that, unlike RNase I-A, this enzyme splits the phosphodiester bonds of 3′-guanylic acid, while the minor enzymatic component (now known as RNase T2), preferentially 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, which is a multifunctional enzyme and exhibits ssDNase, RNase and phosphomonoesterase activities (Ando, 1966).

Since RNase T1 and RNase T2 show base specificity, they are of great interest due to their application in the structural studies of RNAs. In addition, RNase T1, due to its low molecular weight and high stability, attracted considerable attention and extensive studies have been carried out on this enzyme to determine its chemical nature

and structure-function relationship.

1.1 PURIFICATION

Sato and Egami (1957) described the partial purification of RNase T1 (approximately 360 fold) using the following procedure : water extraction of the Takadiastase powder, ammonium sulfate fractionation, adsorption on calcium phosphate gel and treatment with activated charcoal. Though, this preparation was used for the determination of several enzymatic properties, purification to homogeneity was essential to study its molecular properties. Takahashi (1962) first described the extensive purification of RNase T1 and obtained a homogeneous protein in the form of fine spherical crystals. The steps involved were : heat treatment of the crude extract of Takadiastase powder at acidic pH, ammonium sulfate fractionation, adsorption of the colour impurities on acid clay, successive chromatography on DEAE-cellulose, readsorption of the impurities on acid clay and finally crystallization by dialysis against 60 % saturated ammonium sulfate solution. In this purification procedure, DEAE-cellulose was found to be very effective since RNase T1, due to its acidic nature (pI = 2.9), bound very strongly to this exchanger and eluted at a high salt concentration. This step also helped in separating RNase T1 from RNase T2 and

other uncharactarized RNases and phosphodiestarases. Based on DEAE-cellulose chromatographic studies, the author concluded that 80-90% of the total RNase activity found in Takadiastase is due to RNase T1. Subsequently, Rushizky and Sober (1962) described an alternate purification procedure for RNase T1 consisting of water extraction of Takadiastase powder at pH 2.6, concentration with 66% acetone and repeated chromatography (4 times) on DEAE-cellulose. Based on A_{280}-A_{260} absorbtion ratio, the RNase T1 preparation was comparatively less pure than the one obtained with Takahashi´s (1962) procedure. Uchida (1965) simplified the purification procedure of RNase T1 with increase in both yield and purity. The method essentially involved batchwise treatment of crude extract with DEAE-cellulose, heat treatment at pH 1.5-1.8 for 3 min at 80°C, acid clay treatment, ammonium sulfate fractionation and finally, chromatography on DEAE-cellulose at pH 7.0-7.5. This method gave an enzyme preparation having a specific activity 500-700, with an overall yield of 29-38%.

Minato *et al.* (1966) developed a protocol for the large scale purification of RNase T1 for commercial purposes. The method consisted of chromatography on Duolite A-2 (twice), DEAE-Sephadex A-25 (twice) and finally crystallization. By this procedure, they obtained 62 mg of crystalline enzyme

from 2 Kg of Takadiastase powder, having a specific activity of 8250. However, the purification procedure described by Fields *et al.* (1971) involved only repeated DEAE-cellulose chromatography. Though the overall yield was high (48%), Walz *et al.* (1979) subsequently noted that phenol extraction is necessary to remove the minor impurities from this enzyme preparation.

Affinity chromatography has been used for the purification of RNase T1 by several investigators. Jervis and Pettit (1974) employed buffer extraction, gel filtration on Sephadex G-100 and finally, affinity purification on a column of 5′ (4-aminophenyl phosphoryl) guanosine 2′(3′)-monophosphate-glass. Other affinity adsorbents used include, acylated phosphocellulose containing guanosine 2′(3′)-phosphate (Waki *et al.*, 1974), NADP-agarose (Janski and Oleson, 1976), guanylyl (2′,5′)-guanosine bound to aminohexyl-Sepharose (Ishiwata and Yoshida, 1978) and 5′-GMP coupled to aminohexyl-Sepharose 4B (Kanaya and Uchida, 1981).

1.2 PROPERTIES

1.2.1 Assay procedure : RNase T1 is assayed at pH 7.5 and 37°C by measuring the absorbance of acid soluble nucleotides at 260 nm, following the hydrolysis of RNA. One unit of enzyme is defined as an increase in 1 OD in 15 min.

1.2.2 Physical properties : The molecular weight of the enzyme derived from sedimentation coefficient was found to be 11,000 daltons. However, Fields *et al.* (1971) obtained a value of 18,000 daltons from SDS-polyacrylamide electrophoresis, though the amino acid composition data was not significantly different from that obtained by Takahashi (1962). This discrepancy in the molecular weights was attributed to anomalous electrophoretic behavior of small molecular weight proteins in SDS-gels. RNase T1 is an acidic protein having pI 2.9 (Uchida and Egami, 1967a).

1.2.3 Catalytic properties : RNase T1 is active in a broad range of pH i.e. pH 4.0-8.5. The enzyme shows an optimum pH of 7.5 for RNA hydrolysis (Sato and Egami, 1957) and 7.2 for the hydrolysis 3′-cyclic GMP (Irie *et al.*, 1970). At pH 5.5 and 8.0, the enzyme shows 50 % of its maximum activity. However, according to Rushizky and Sober (1962) optimum pH of RNase T1 is 7.3 and the enzyme exhibits 50% of its maximum activity at pH 5.5 and 7.9.

RNase T1 is a very stable protein and at pH 6.0 it can be stored frozen for several months or in solution at 4°C for several weeks without appreciable loss in activity. The enzyme (at 0.1% protein concentration) retains its full activity in 0.2 M Tris buffer, pH 7.5, at 25° C for more

than 40h. It is fairly resistant to heat (100°C for 10 min at pH 6.0) and acid. In 0.1 N HCl at room temperature, the enzyme retains its full activity for 20 h (Egami *et al.*, 1964) and in 0.4 N HCl at 4°C, it is stable for 48h (Rushizky and Sober, 1962). At higher pH values, it is somewhat unstable and loses its activity rapidly above pH 9.0. In 0.1 N NaOH at room temperature, the enzyme lost 85 % of its initial activity within 28 h. RNase T1 retained its full activity after exposure to 8 M urea at room temperature for 25 h (Egami *et al.*, 1964).

RNase T1 shows significant inhibition in presence of metal ions like Ag^{+}, Hg^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+} and Cu^{2+}. Histidine (1mM) activates RNase T1 and can eliminate the inhibitory effect of Zn^{2+}. This effect is not observed in presence of EDTA, suggesting that the activation in presence of histidine is mainly due to its metal chelating action. EDTA stimulates the enzyme activity probably by eliminating the inhibitory effect of metal ions. Heparin, which inhibits RNase I-A, does not inhibit RNase T1 and this has been attributed to the acidic nature of the enzyme. In addition, neither natural RNase I-A inhibitor from rat liver (Shortman, 1962) nor *A. oryzae* nuclease inhibitor (Uozumi *et al.*, 1969) has any effect on RNase T1 activity.

1.3 STRUCTURE

RNase T1 consists of a single polypeptide chain of 104 amino acids with two disulfide bonds (Takahashi, 1965). The primary structure of RNase T1 is shown in Fig. 1. Based on UV absorbtion, circular dichroism and optical rotatory dispersion studies Yamamoto and Tanaka (1970) suggested that RNase T1 consists of 33% α-helix, 24% β-sheet and 43% random coil.

Reductive cleavage of the disulfide bonds destroys enzymatic activity but reoxidation with air regenerates both the native conformation as well as enzymatic activity, suggesting that disulfide bonds are essential for the proper folding of the enzyme molecule required for it´s catalytic activity (Kasai, 1965). Recently, Nishikawa *et al.* (1990) using cassette mutagenesis reported the synthesis of RNase T1 having three disulfide bonds. Though the mutant RNase T1 was more thermostable than the native enzyme, it was totally and irreversibly inactivated when heated at 100°C for 15 min.

1.4 ACTIVE SITE

By gel filtration studies and by differential spectrum of the mixture of RNase T1 and 2´-guanylate (a strong inhibitor of RNase T1), it was shown that one molecule of RNase T1 binds to one molecule of 2´-guanylate. Based on these results, Sato and Egami (1965) suggested that RNase T1

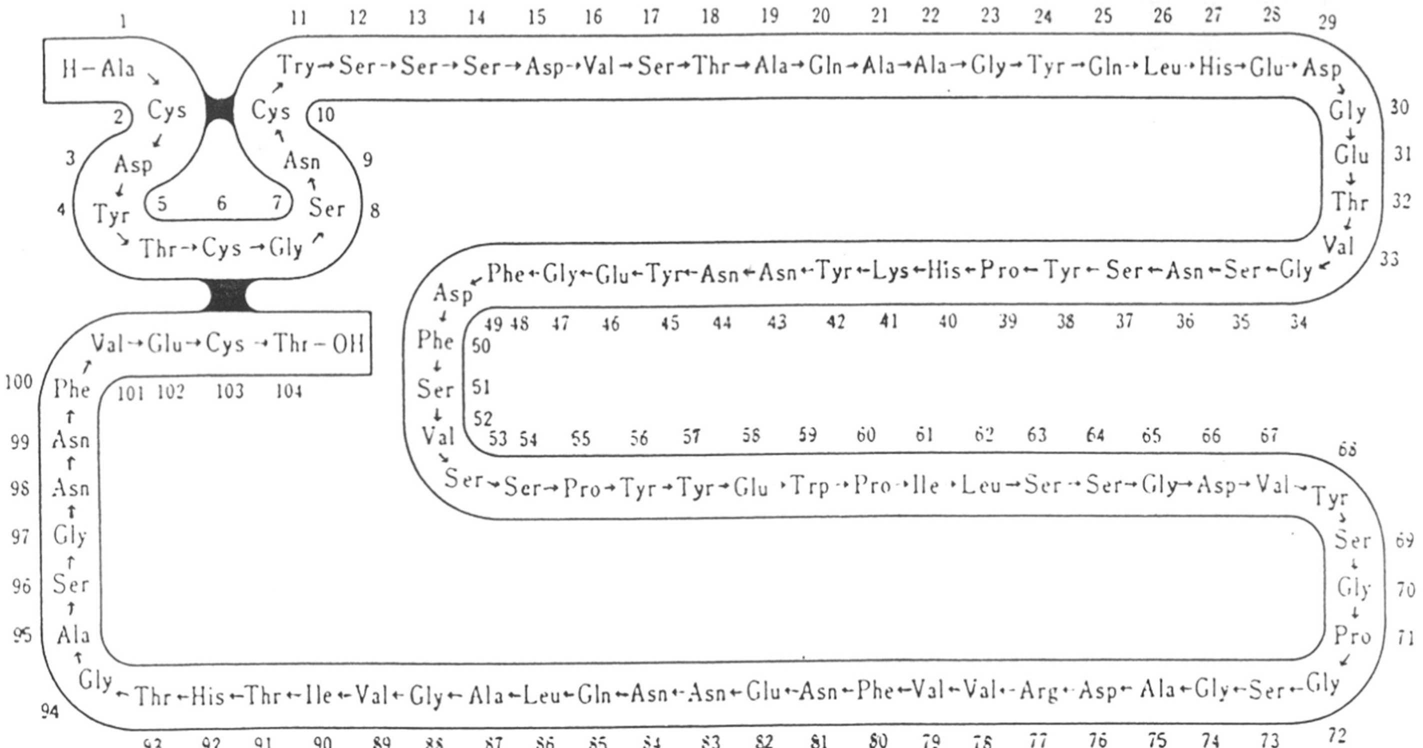


FIG. 1. The primary structure of RNase T_1. From Takahashi (1965).

may have only one active center for the specific binding of the substrate(s).

Treatment of RNase T1 with iodoacetate at pH 5.5 led to the inactivation of the enzyme. Since this involves the specific esterification of glutamic acid 58, it was concluded that γ-COOH of this amino acid is essential for activity. The carboxymethylated enzyme could bind 3´-GMP in a ratio 1:1, thus indicating that glutamic acid 58 is more directly involved in the catalysis than in substrate binding. Through inactivation studies with iodoacetamide, which does not react with glutamic acid 58, Takahashi *et al.*, (1967) showed that the loss of activity towards RNA proceeds in parallel with the loss of 2´histidine residues. The pH rate profile of this inactivation implicated residues with pK_a´s in the range of 7.5-8.0 (Takahashi, 1973, 1976). It was noted that histidine 92 and histidine 40 react with iodoacetamide very readily and at similar rates, while histidine 27 is least reactive. Alkylation of histidine 92 occurs at a much slower rate when glutamic acid 58 in carboxymethylated enzyme is treated with iodoacetamide. In addition, alkylation of histidine 40 slowed down considerably in presence of GMP. Based on these results, it was concluded that histidine 92 and histidine 40 are involved in the active site.

Involvement of histidine residues was also shown by methylene blue and rose bengal catalyzed photooxidation studies (Waku and Nakazawa, 1970; Irie, 1970; Takahashi, 1971).

Modification of arginine 77 by phenylglyoxal resulted in parallel loss of activity towards RNA and 2′,3′-cyclic GMP indicating that this residue is present at or near the active site (Takahashi, 1972). When the two amino groups in RNase T1 (i.e. α-NH_2 of N terminal alanine and ϵ-NH_2 of lysine 41) were deaminated with nitrous acid, the enzyme retained appreciable activity (Tamaoki *et al.*, 1976). Based on these results it was concluded that neither of these two amino acids are directly involved in the catalysis. On the other hand, acetylation of amino groups with several dicarboxylic acid anhydrides like succinic, maleic, citraconic and *cis*-aconitic anhydride led to extensive inactivation of the enzyme. This inactivation was attributed to the modification of ϵ-NH_2 of lysine 41, which is adjacent to histidine 40 (Takahashi, 1977).

Modification of tryptophan and tyrosine residues showed that they are not involved in catalysis. However, it was shown that the indole ring of the enzyme is relatively inaccessible to modification but its integrity is essential for the folding of the chain into the active conformation (Takahashi, 1972).

1.5 SPECIFICITY AND MODE OF ACTION

RNase T1 is considered to be a guanyloribonuclease [EC 2.7.7.26, ribonucleate guaninenucleotido-2′-transferase (cyclizing)]. It splits the internucleotide bonds between 3′-guanylic acid and 5′-hydroxyl group of the adjacent *nucleotide with the intermediate formation of guanosine* 2′,3′-cyclic monophosphate. The mechanism of action of RNase T1 was deduced from the following experimental evidences: (a) the enzyme digests RNA and produces only 3′-guanylic acids as the mononucleotide (b) the terminal residue of the oligonucleotides produced contains only guanylic acid and (c) guanosine 2′,3′-cyclic monophosphate is obtained as an intermediate product and it is further hydrolyzed to 3′-guanylic acid. The first step of the reaction i.e. transphosphorylation is reversible but the final step (hydrolysis of phosphate ester) is practically irreversible (Sato-Asano, 1959). The mode of action of RNase T1 is depicted in Fig. 2.

RNase T1 does not split the secondary phosphate ester bonds of adenosine 3′-phosphate and hence the specificity seems to depend on the substituents in the purine ring. Further studies revealed that RNase T1 can digest deaminated RNA by hydrolyzing the secondary phosphate ester bonds in

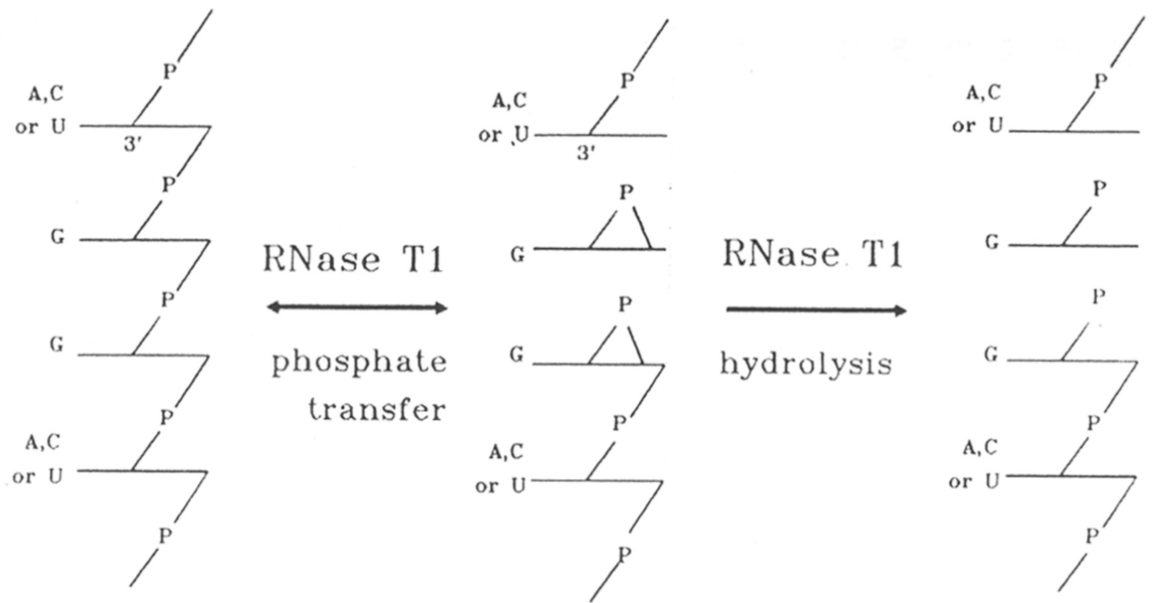


Fig. 2 : Mode of action of RNase T1.

both inosine 3′-phosphate and xanthosine 3′-phosphate with the formation of intermediate 2′,3′-cyclic phosphates (Sato-Asano and Fujii, 1960). McCully and Cantoni (1961) observed that methylated guanylyl residues found in certain RNA′s are resistant to RNase T1. Azegami and Iwai (1964) observed that RNA trinitrophenylated at the guanine residues is resistant to hydrolysis. Results from base specificity studies showed that the essential requirement for the preferred substrates by RNase T1 must have a keto group at position 6 and trivalent nitrogen at position 7 of the purine ring. The presence of proton at position 4, as in the case of guanine, further increases the susceptibility. Thus, these groups may be regarded as the specific binding sites of the purine base with the enzyme. This consideration may explain the apparent resistance of double stranded RNA (Billeter *et al.*, 1966) and ribopolynucleotides highly rich in guanylyl residues (Itagaki *et al.*, 1965) to the action of RNase T1. RNase T1 is sugar specific and requires 2′-hydroxyl group in the substrate because DNA is resistant to this enzyme.

Podder and Tinoco (1969) noted that RNase T1 can synthesize G-(2′,5′)-G bond from cyclic-GMP. Further investigation by Egami and Inone revealed that unlike at 36°C, at 100°C, G-(2′,5′)-$G_{3'-p}$ is split to produce $G_{3'-p}$. In addition, at 100°C,G-(3′,5′)-$G_{3'-p}$ is attacked in a very

different manner. This observation was correlated to the altered action of partly heat denatured enzyme (Takahashi and Moore, 1982).

1.6 APPLICATIONS

Since 1962, several investigators have used RNase T1 as an analytical tool for the nucleotide sequence analysis of RNAs, especially of highly purified tRNAs. Finally, in 1965, Holley *et al.* successfully elucidated the structure of alanine specific tRNA from yeast, using RNase T1 as the main analytical tool. Since then, this enzyme has been used extensively for the determination of nucleotide sequence of various RNAs.

RNase T1 has also been used for the synthesis of guanylyl nucleosides, oligoguanylate and other guanosine containing oligonucleotides with (3′-5′)-phosphate bonds (Uchida and Egami, 1971).

1.7 OTHER GUANINE SPECIFIC RNases

Several guanine specific RNases comparable to RNase T1 have been isolated from *Ustilago sphaerogena* (RNase U1), *Neurospora crassa* (RNase N1), *Streptomyces erythreus* (RNase St), *Chalaropsis sp.* (RNase Ch), *A. fumigatus* RNase, *Fusarium moniliforme* (RNase F1), *A. clavatus* (RNase C2), an intracellular RNase from *A. clavatus* (RNase II), RNase from

Actinomyces aureoverticillatus, *Penicillium chrysogenum* (RNase Pch1) and RNase from *Penicillium brevicompactum*. Like RNase T1, most of these enzymes are small molecular weight (MW 10,000-15,000), thermostable proteins and they specifically cleave the 3′-phosphodiester bonds *via* the corresponding 2′,3′-cyclic phosphates. Majority of these enzymes appear to be homologous to RNase T1, with a similar active site, characterized by the presence of a carboxylic group (probably a specific glutamic acid residue) and hence may function in a similar manner (Takahashi and Moore, 1982).

1.8 IMMOBILIZED RNase T1

One of the most important applications of immobilized enzyme technology is its use as probes in analytical systems. High sensitivity and specificity of enzymes make them excellent analytical tools and due to this property several enzymes have been immobilized for their application in routine biochemical and clinical analysis (Guilbault, 1982; Sundaram, 1982). Additionally, immobilization (a) enables the enzyme to process a large amount of substrate, since it can be easily removed from the mixture of substrate and product, thus allowing the enzyme to be reused (b) in general imparts greater stability to the enzyme, so that it can be used for the development of continuous process (c) permits the use of multienzyme systems (d) allows the use of

enzyme(s) from non-GRAS organism(s) (e) enables greater control of the catalytic process (f) helps to obtain enzyme free products and (g) permits the economical utilization of an otherwise cost prohibitive enzyme.

RNase T1, due to its unique substrate specificity i.e. cleaving phosphodiester bond involving 3′-guanylic acid, has found wide application as a reagent in the study of chemical structure of RNA. This enzyme has also been utilized for preparative purposes such as, preparation of nucleoside 2′,3′-cyclic phosphates and oligonucleotides containing guanosine residue (Takahashi and Moore, 1982).

Kuriyama and Egami (1966) for the first time reported the preparation of water insoluble derivatives of RNase T1, where the enzyme was covalently coupled to carboxymethyl cellulose or p-aminobenzyl cellulose. The carboxymethyl cellulose-bound enzyme retained only 2% of the original activity towards yeast RNA, while the activity of p-aminobenzyl cellulose-bound enzyme was much lower. Subsequently, Lee (1971) linked RNase T1 to diazotized p-aminobenzyl cellulose, cyanogen bromide-activated Sephadex G-200 and Sepharose 2B and studied the properties of the Sepharose bound enzyme. No change was observed, in the K_m and specificity of the enzyme, as a result of immobilization.

577.152.277(043)
GIT
TH-656

Glitz *et al.* (1974) bound four guanyloribonucleases, namely RNase T1, *A. fumigatus* RNase, RNase U1 and RNase N1, covalently to Sepharose and noted that the electrophoretic band pattern of RNA digest on polyacrylamide gels was comparable to those obtained with their soluble counterpart, suggesting the utility of these immobilized enzymes in RNA sequence analysis. RNase T1 bound to Enzacryl AH by acid azide method showed 44% and 77% activity towards RNA and 2′,3′-cyclic GMP, respectively. The bound enzyme was active towards RNA above pH 9.0 at 37°C or above 60°C at pH 7.5, whereas the soluble enzyme was inactive. In addition, the immobilized RNase T1 retained a significant amount of its activity when assayed at 37°C after incubation in the pH range of 1.0-10.0 at 37°C or after heating at 100°C at pH 7.5, where the soluble enzyme lost much of its activity. The immobilized enzyme could be repeatedly recovered and reused without much loss in its activity (Ito *et al.*, 1977a). Similarly, RNase T1 bound to copolymers of acrylamide and divinyl benzene by acid azide method retained most of the activity of the soluble enzyme. The bound enzyme showed superior temperature and pH stabilities (Ito *et al.*, 1977b).

Zhenodarova *et al.* (1987) bound guanylospecific nucleases like, RNases from *A. clavatus*, RNase T1 from *A. oryzae* and nuclease from *Bacillus intermedius* and used the

immobilized systems for the preparation of tri-, tetra- and penta-nucleotides having guanylic acid as the 5′ terminal. The choice of the immobilized system depended upon the nucleotide sequence required. The immobilized preparations showed high stability to storage and repeated use.

PRESENT INVESTIGATION

RNase T1 from *A. oryzae* is an analytically important enzyme and is used extensively in the structural determination of RNA. Takadiastase (a commercial product of *A. oryzae*), apart from RNase T1, contains other RNA degrading activities like RNase T2 and S1 nuclease. Secondly, it has been observed that after the nuclease treatment of nucleic acid samples, removal of residual enzyme activity from the reaction mixture is essential and requires repeated extractions with phenol which in turn results in the loss of nucleic acid samples. In such circumstances, use of immobilized enzyme offers a distinct advantage over the soluble enzyme, as it can easily be removed from the reaction mixture by physical methods. Hence the present investigation was carried out to develop (a) a simple and rapid procedure for the partial purification of RNase T1, free from the above contaminating enzymes and (b) an immobilization procedure to obtain a highly active and stable immobilized preparation suitable for routine analytical purposes.

MATERIALS & METHODS

MATERIALS

Takadiastase (Sankyo, Japan); RNA and ethylenediamine (Sisco Research Laboratories, India); glutaraldehyde (50% v/v) (Fluka AG, Switzerland); Bio-Gel P-2 and P-10 (BioRad, USA); bovine serum albumin, (Sigma Chemical Co., USA); uranyl acetate (Loba Chemie Indoaustranal Co., India); perchloric acid (S. D. Fine-Chem Pvt. Ltd., India) were used. All other chemicals used were of analytical grade.

METHODS

Assay of RNase T1 : RNase T1 activity determination was carried out according to the procedure of Uchida and Egami (1967a). 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 (Fig. 3). The amount of acid soluble nucleotides were calculated by assuming a molar extinction

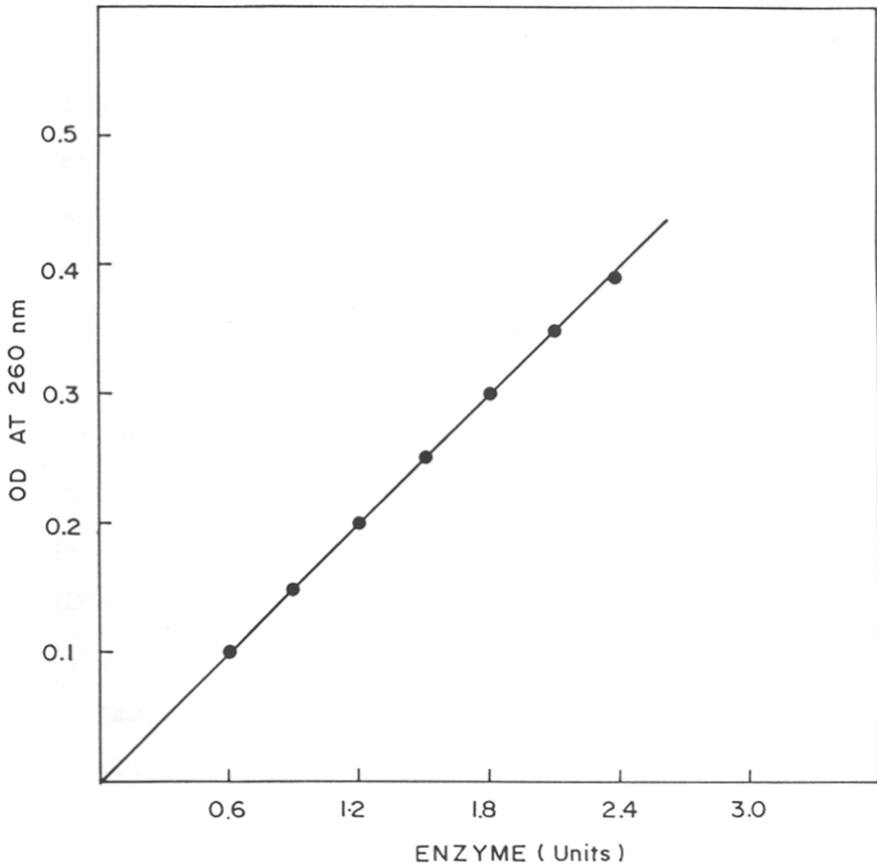


FIG. 3: Calibration curve for the determination of RNase Tl activity.

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 the standard assay conditions.

Assay of RNase T2 : The assay procedure was similar to that of RNase T1 except the pH of assay mixture was 4.5 (Uchida and Egami, 1967b).

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

Purification of RNase T1

Unless otherwise stated, all the operations were carried out at 4°C.

Heat treatment : Ten g 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. Subsequently, the pH of the solution was brought to 4.8 with 1 N NaOH and the heavy precipitate formed was removed by centrifugation (6800 g, 10

min). The clear supernatant was then brought to pH 6.0 and stored frozen until further use.

Chromatography on Bio-Gel P-10 : Two ml of sample obtained from the above step was loaded on a Bio-Gel P-10 column (1 cm x 100 cm, equilibrated with 0.05 M phosphate buffer, pH 6.0) at the rate of 7 ml/h. Fractions (1.7-1.8 ml) were collected and those fractions having specific activity higher than 700 were pooled and stored at 4°C until further use. No loss of activity was observed when the enzyme was stored under these conditions. The specific activity of partially purified RNase T1 which ranged from 900-950 was used for immobilization studies.

Preparation of aminoethyl Bio-Gel P-2 (AE Bio-Gel P-2): The amination of Bio-Gel P-2 was carried out essentially according to the procedure of Inman and Dintzis (1969). In a typical experiment, 100 ml ethylenediamine was preheated under mild stirring in an oil bath maintained at 90°C, kept in a fume hood. Subsequently, 10 g of dry Bio-Gel P-2 beads were gradually added and the reaction was allowed to proceed at 90°C for 6 h. At the end of incubation time, the reaction mixture was cooled in 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 to remove excess ethylenediamine (as indicated by a negative colour test with

2, 4, 6-trinitrobenzene sulphonic acid (TNBS); the positive test is indicated by the formation of an orange colour on addition of few drops of 3% TNBS prepared in saturated sodium tetraborate) followed by 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-2) was activated by incubating with 5 ml of 2% (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 and used immediately for coupling.

b. Coupling of the enzyme to the activated matrix : In a typical experiment, 90-100 U of partially purified RNase T1 (Sp. activity 900) in 2 ml of phosphate buffer (0.05 M, pH 6.0) was incubated with 1 ml (packed volume) of the activated matrix, at 4°C for 15 h, under mild agitation. The supernatant was decanted and the matrix was washed successively with the coupling buffer, 1 M NaCl in the coupling buffer and finally with the assay buffer (0.2 M Tris-HCl buffer, pH 7.5) till the washings showed no RNase T1 activity. The amount of enzyme/protein bound to the matrix was determined by estimating the difference in enzyme

activity/protein before loading on the matrix and after coupling.

Assay of immobilized RNase T1 : 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 thermostated shaker water bath (100-125 rpm), for 15 min followed by measuring the acid soluble nucleotides at 260 nm, after precipitation of the unreacted RNA.

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 T1 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

RESULTS

PARTIAL PURIFICATION OF RNase T1

The results of a typical procedure for the partial purification of RNase T1 are given in Table 1.

Table 1. Partial purification of RNase T1.

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Recovery (%)
Crude	36	57,000	1500	38	1	100
Heat treatment	48	45,300	520	87.11	2.29	79.5
Bio-Gel P-10 Chromatography	288	36,000	38.8	927.8	24.4	63.1

The enzyme was purified approximately 25-fold with an overall yield of 63 %. Though the enzyme obtained after the heat treatment was free from S1 nuclease activity, it did contain significant amount of RNase T2 activity. However, chromatography on Bio-Gel P-10 yielded a RNase T2 free RNase T1 preparation (Fig. 4).

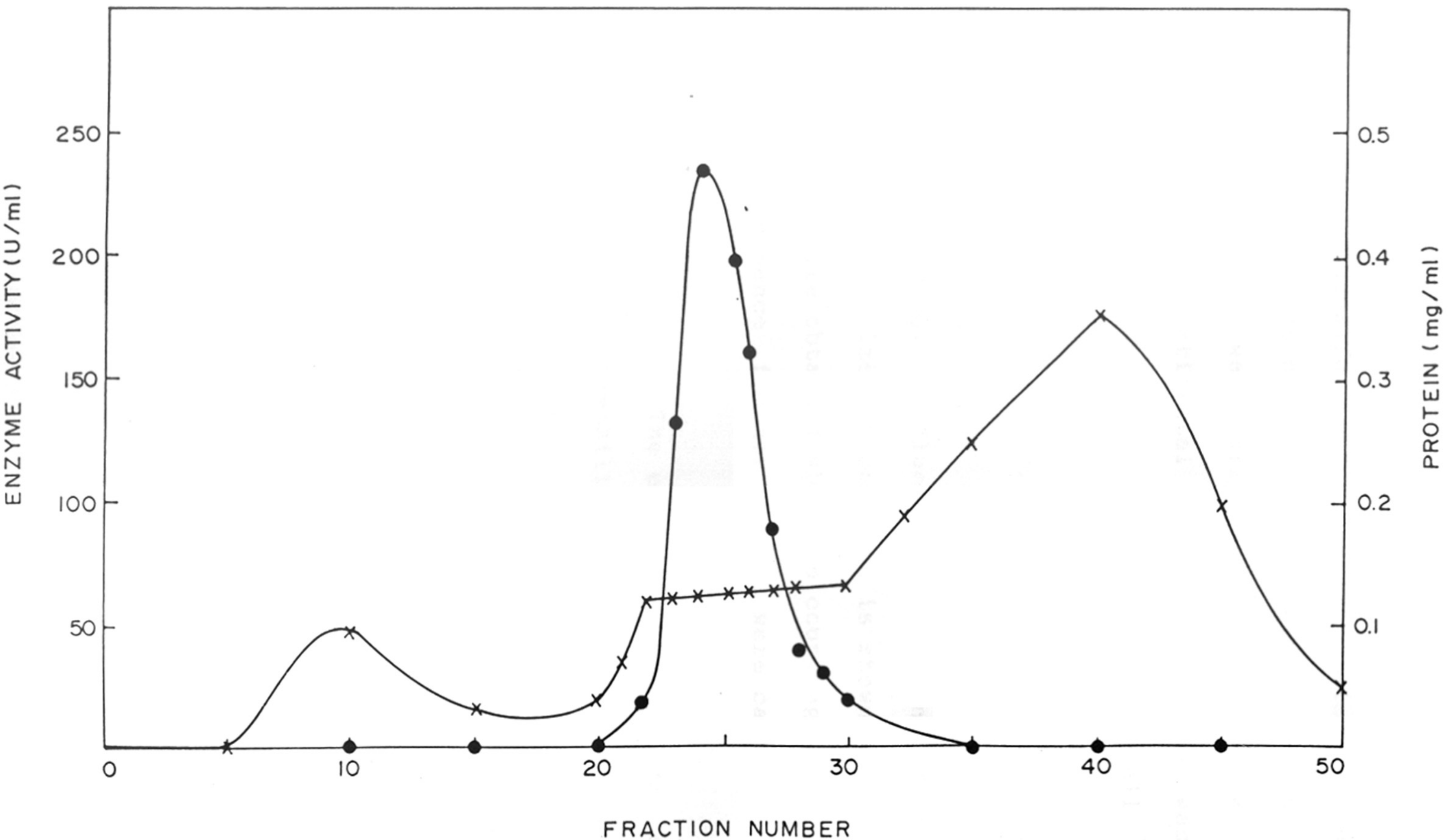


FIG. 4 : Chromatographic profiles of RNase T1 on Bio-Gel P-10. Enzyme activity (●) and protein (X). (1.7 - 1.8 ml fractions were collected at a flow rate of 7 ml/h).

IMMOBILIZATION OF RNase T1

Since the successful immobilization of any enzyme depends on factors like coupling pH, matrix to enzyme ratio etc, initial studies were carried out to optimize these parameters. The experimental strategy adopted was to optimize only one parameter at a time, keeping all other variables constant. Once the parameter in question was found to be optimal, it was incorporated while optimizing the subsequent parameter.

Effect of coupling pH: Influence of coupling pH on the activity of the immobilized preparation is shown in Fig. 5. Optimal results were obtained when the coupling was carried out at pH 6.0 and hence further studies were carried out at this pH.

Effect of enzyme load : The effect of matrix to enzyme ratio on the activity of immobilized preparation showed that after an initial increase, the efficiency decreased with the increase in enzyme load and maximum efficiency was obtained when approximately 100 U of enzyme were reacted with 1 ml (packed volume) of activated matrix at pH 6.0 (Fig. 6).

The results of a typical immobilization procedure of partially purified RNase T1 on glutaraldehyde activated AE Bio-Gel P-2, under optimized conditions, are given in Table 2.

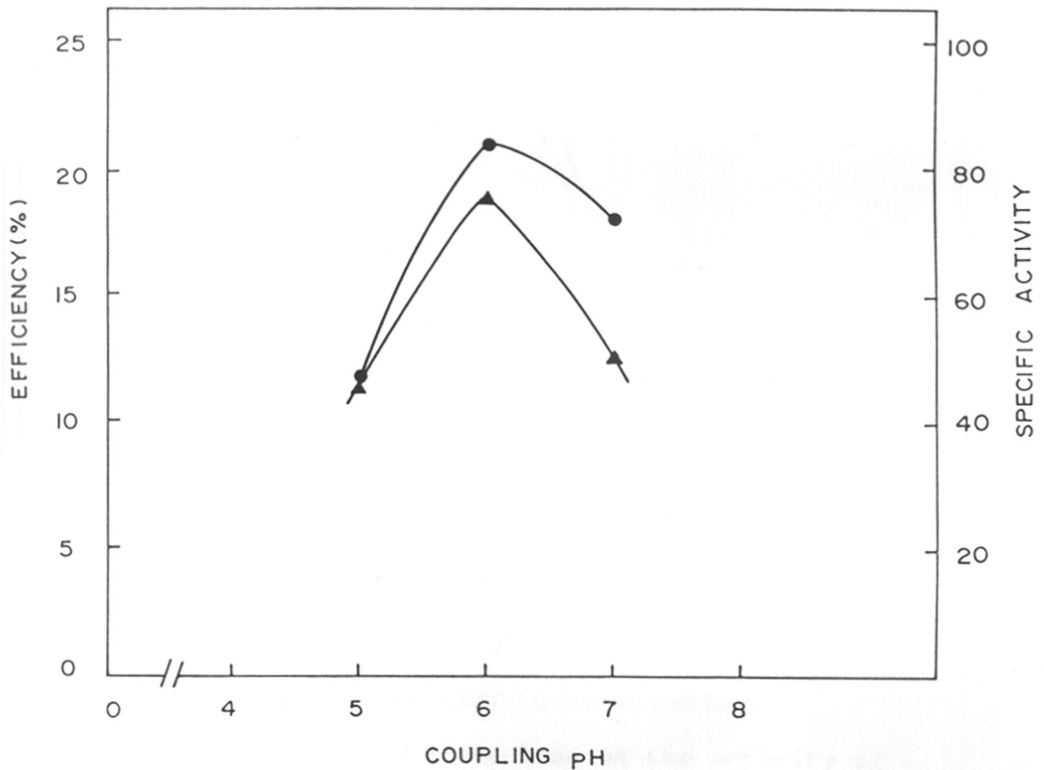


FIG. 5: Effect of coupling pH on the activity of AE Bio-Gel P-2 bound RNase Tl. Efficiency (●) and specific activity (▲).

One ml (packed volume) of 2% glutaraldehyde activated matrix was incubated with approximately 100 U of partially purified RNase Tl at different pH (5.0 - 7.0) and at 4°C for 15 h and efficiency of the immobilized enzyme was determined as described under Methods.

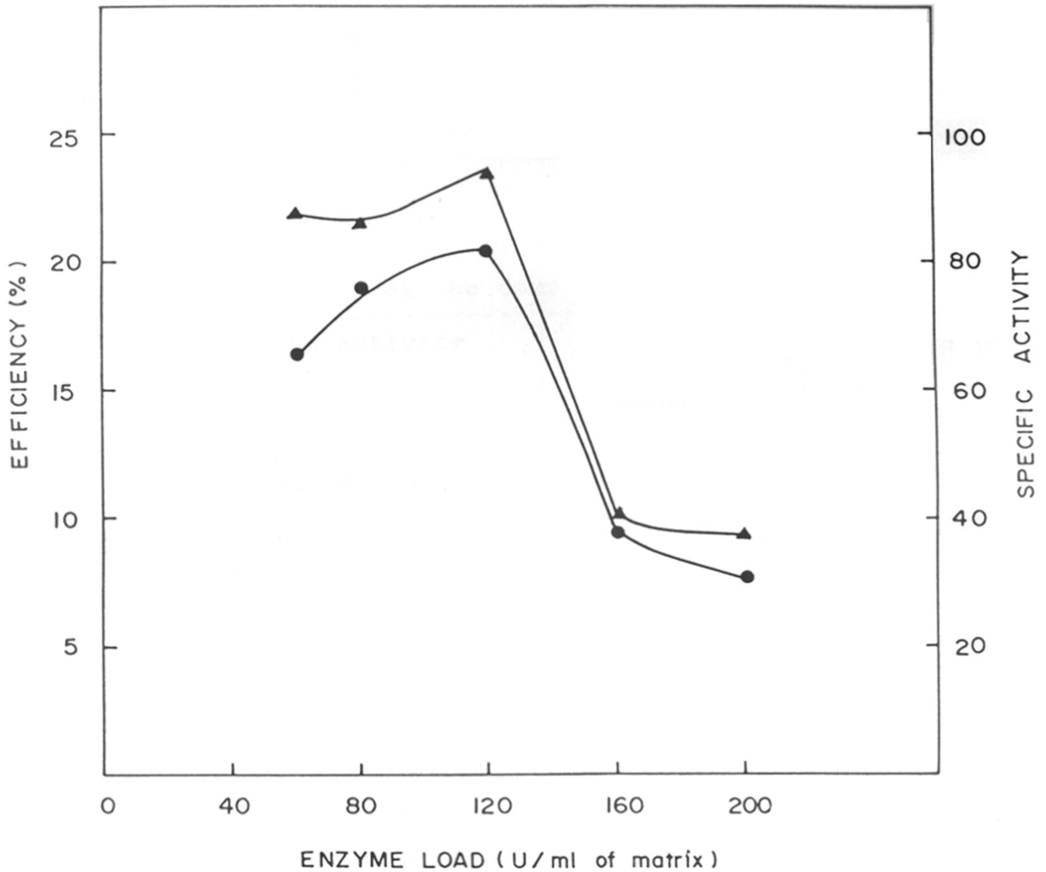


FIG. 6: Effect of enzyme load on the activity of AE Bio-Gel P-2 bound RNase T1. Efficiency (●) and specific activity (▲).

One ml of 2% glutaraldehyde activated matrix was incubated with varying amounts of the enzyme (60-200 U) at pH 6.0 and at 4°C for 15 h and efficiency of the immobilized enzyme was determined as described under Methods.

Table 2. **Immobilization of RNase T1 on gluteraldehyde activated AE Bio-Gel P-2.**

Enzyme Loaded		Enzyme Bound		Activity of the complex (Units expressed)	Efficiency*
U	Protein (mg)	U	Protein (mg)		
250	0.275	50	0.140	11.9	23.8%

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

When 250 U of partially purified RNase T1 were reacted with 2.5 ml (packed volume) of 2% (v/v) glutaraldehyde activated AE Bio-Gel P-2 at pH 6.0, 50 U were bound. The effectiveness factor (η) of the preparation was approximately 0.24 indicating the efficiency of immobilized system to be 24 % (Table 2).

Effect of pH on reaction rate : Comparison of pH activity profiles of soluble and immobilized RNase T1 showed no change in the optimum pH of the soluble enzyme as a result of immobilization. However, there was a slight change in the pH activity profiles (Fig. 7).

Effect of temperature on reaction rate : Determination of temperature activity profiles of soluble and immobilized

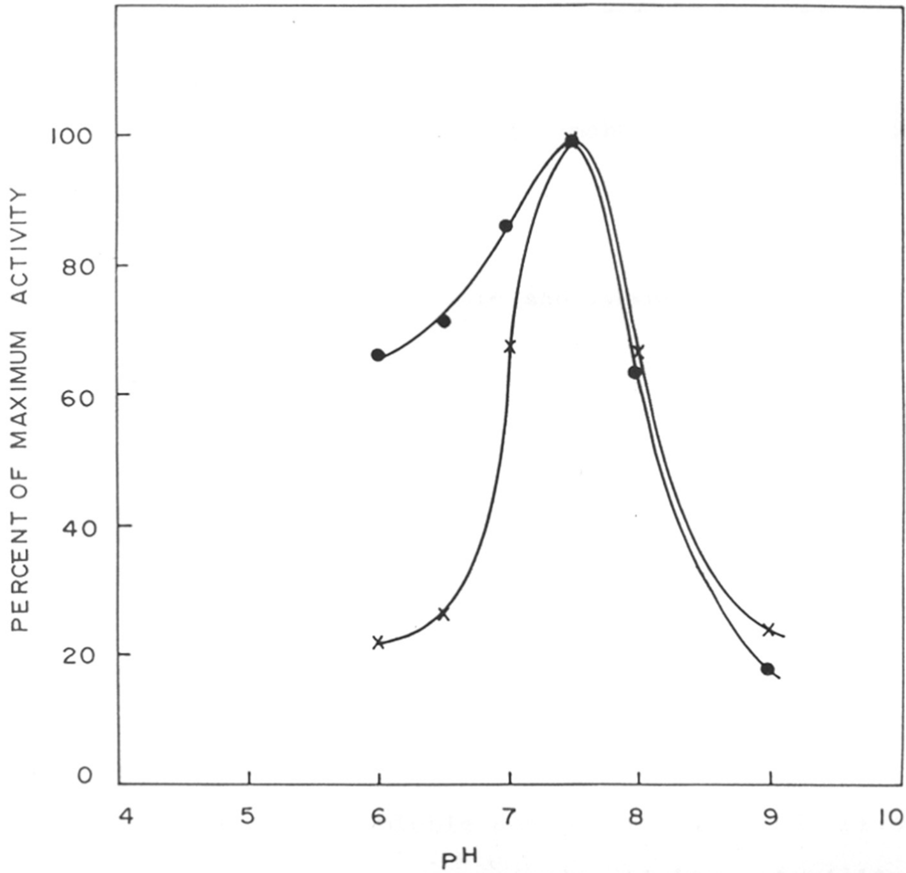


FIG. 7: pH activity profiles of soluble (●) and immobilized (X) RNase Tl.

Both soluble and immobilized enzymes (1.2 - 1.5 U) were assayed in a series of pH (6.0 - 9.0) at 37°C as described under Methods.

RNase T1 showed an increase in optimum temperature to 50°C from 45°C for the soluble enzyme. However, there was no change in the temperature activity profiles (Fig. 8).

Effect of substrate concentration : Determination of kinetic parameters of soluble and immobilized enzymes showed a slight decrease in K_m and approximately 5 - fold decrease in V_{max}, as a result of immobilization (Fig. 9, Table 3).

Table 3. Kinetic data of soluble and immobilized RNase T1.

State of the enzyme	K_m (mg/ml)	V_{max} (U/ml)
Soluble	20.0	100
Immobilized	14.2	20

pH stability : pH stability of both soluble and immobilized RNase T1 is shown in Fig. 10. The immobilized enzyme was more labile than the soluble enzyme at acidic pH (i.e. pH 4.0-5.0) but showed comparatively higher stability at alkaline pH (i.e. pH 8.0-10.0). At pH 10.0, the native enzyme showed 57% of its initial activity , while the bound enzyme retained 65% of its activity.

Temperature stability : Comparison of temperature stabilities of both soluble and immobilized enzymes showed

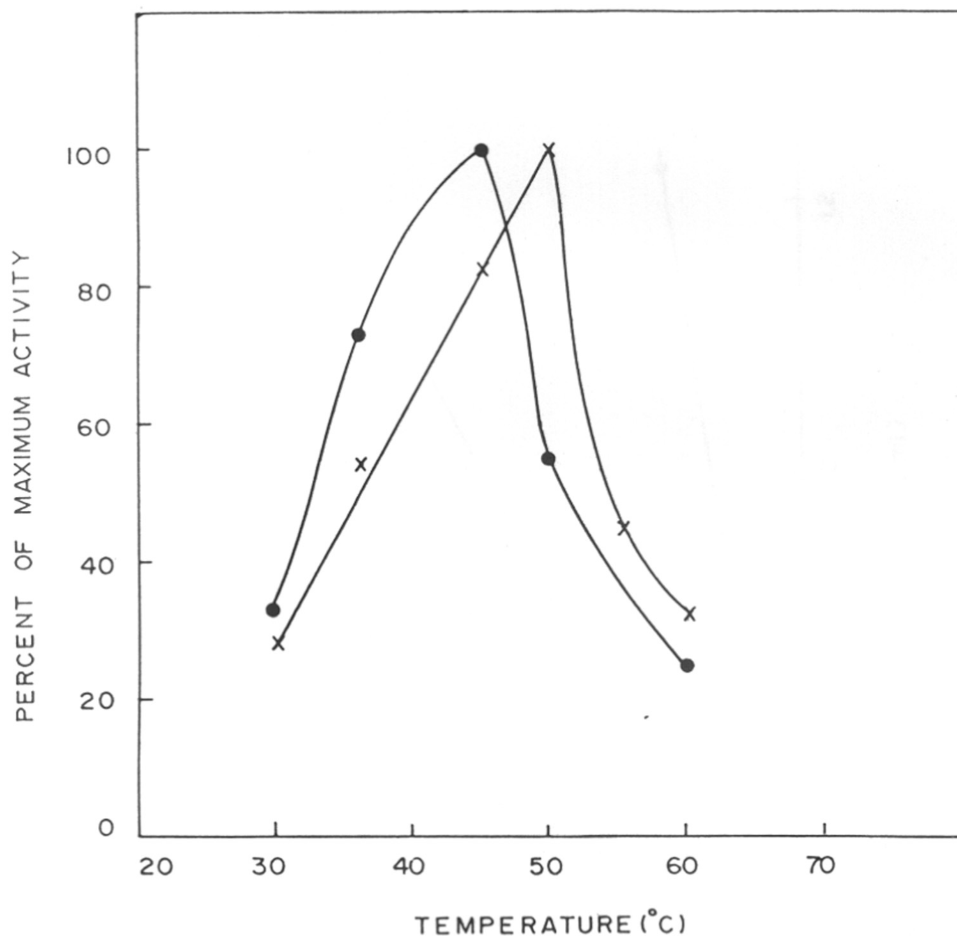


FIG. 8: Temperature activity profiles of soluble (●) and immobilized (X) RNase T1.

Appropriate amounts of soluble and immobilized enzymes (1.2 - 1.5 U) were incubated in a series of temperatures (30 - 60°C) at pH 7.5 and their activities were determined as described under Methods.

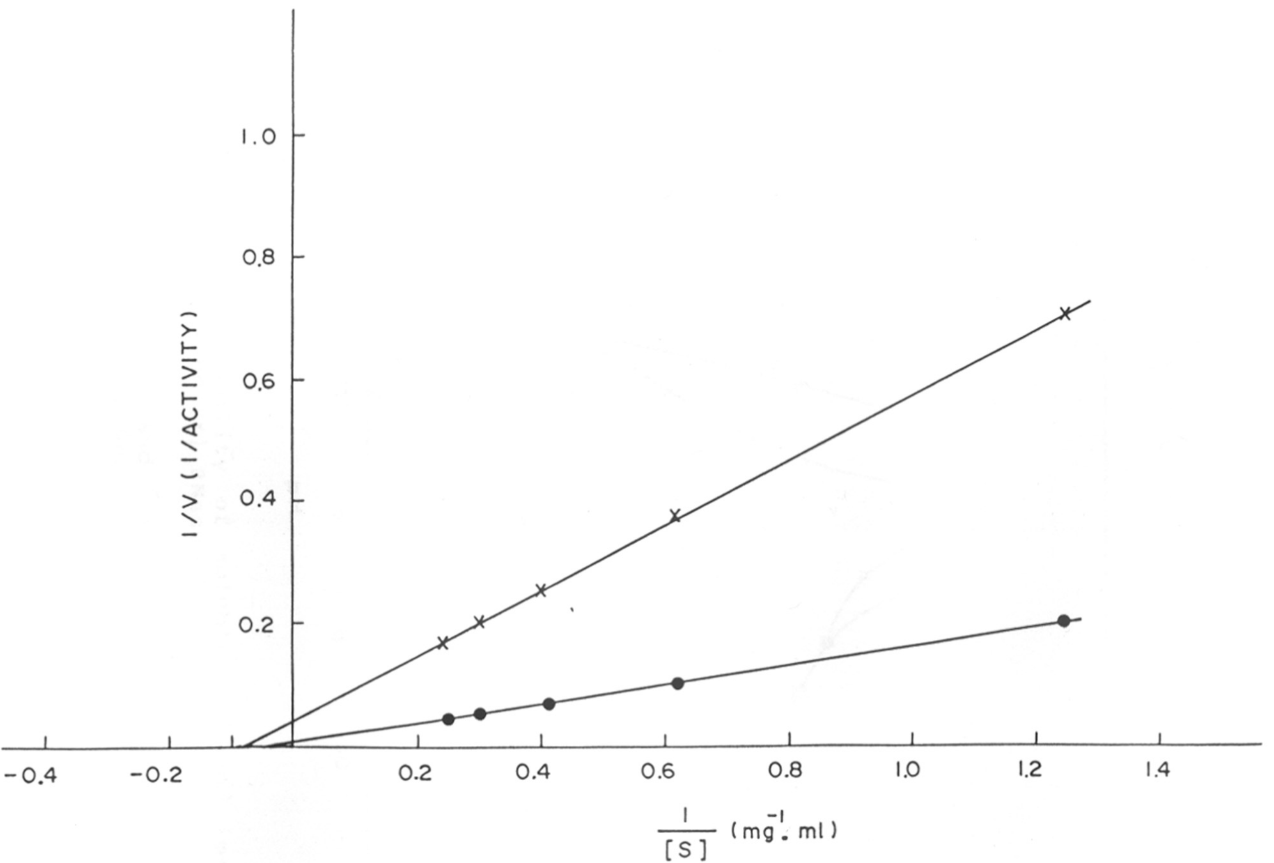


FIG. 9: Lineweaver-Burk plots for soluble (●) and immobilized (X) RNase Tl.

Soluble and immobilized enzymes (1.2 - 1.5 U) were assayed in a series of substrate concentration (0.8 - 4.0 mg/ml) at pH 7.5 and 37°C.

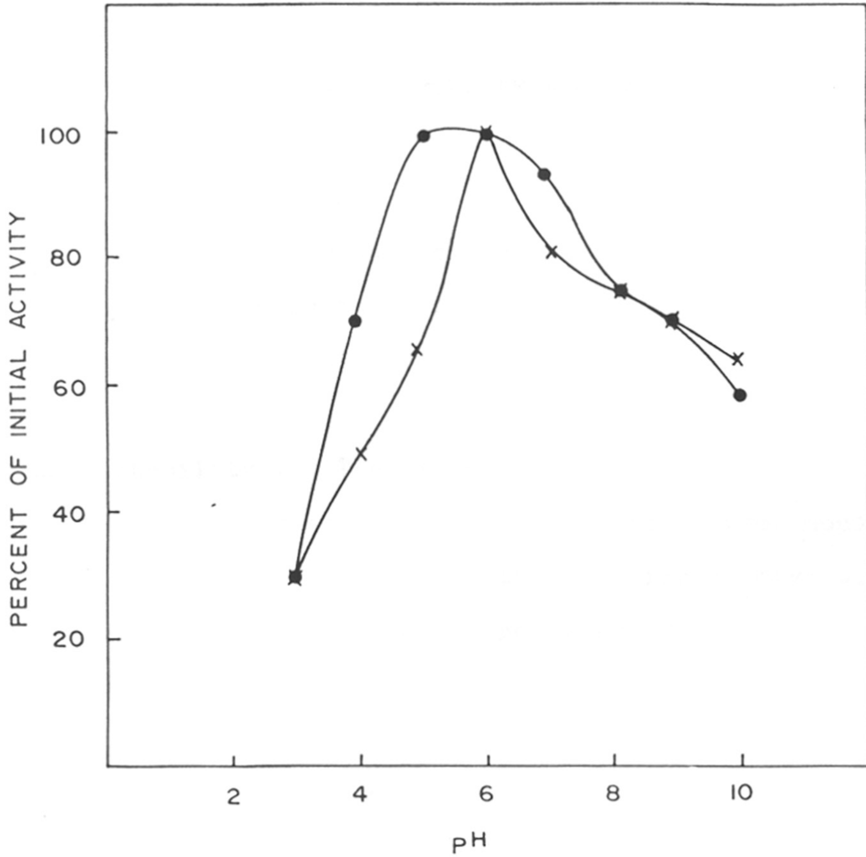


FIG. 10: pH stability of soluble (●) and immobilized (X) RNase T1.

Soluble and immobilized enzymes (1.2 - 1.5 U) were preincubated at different pH (3.0 - 10.0) for 1 h at 37°C and their activities were determined under standard assay conditions.

the immobilized enzyme to be more stable. While the soluble enzyme lost 60% of its initial activity at 80°C, the immobilized enzyme retained 66% of its original activity (Fig. 11).

Stability to repeated use : The AE Bio-Gel P-2 bound enzyme could be used repeatedly upto 6 cycles without any significant loss in its initial activity. In the absence of any detectable leaching (evidenced by the absence of enzymatic activity in the supernatant), the decrease in the activity can be correlated to slight inactivation of the enzyme (Fig. 12).

Storage stability : The storage stability of immobilized RNase T1 was determined at 4°C. The bound enzyme could be stored in 0.2 M Tris-HCl buffer, pH 7.5 for 50 days without any apparent loss in its initial activity (Fig. 13).

DISCUSSION

Takadiastase powder is known to contain four nucleic acid hydrolyzing enzymes namely, RNase T1, RNase T2, S1 nuclease and double strand specific nuclease. Hence, our initial attempts were directed towards the development of a suitable procedure for obtaining a RNase T1 preparation free from the above contaminating enzymes. Heat treatment of the crude extract at 80°C (pH 2.0) though resulted in the

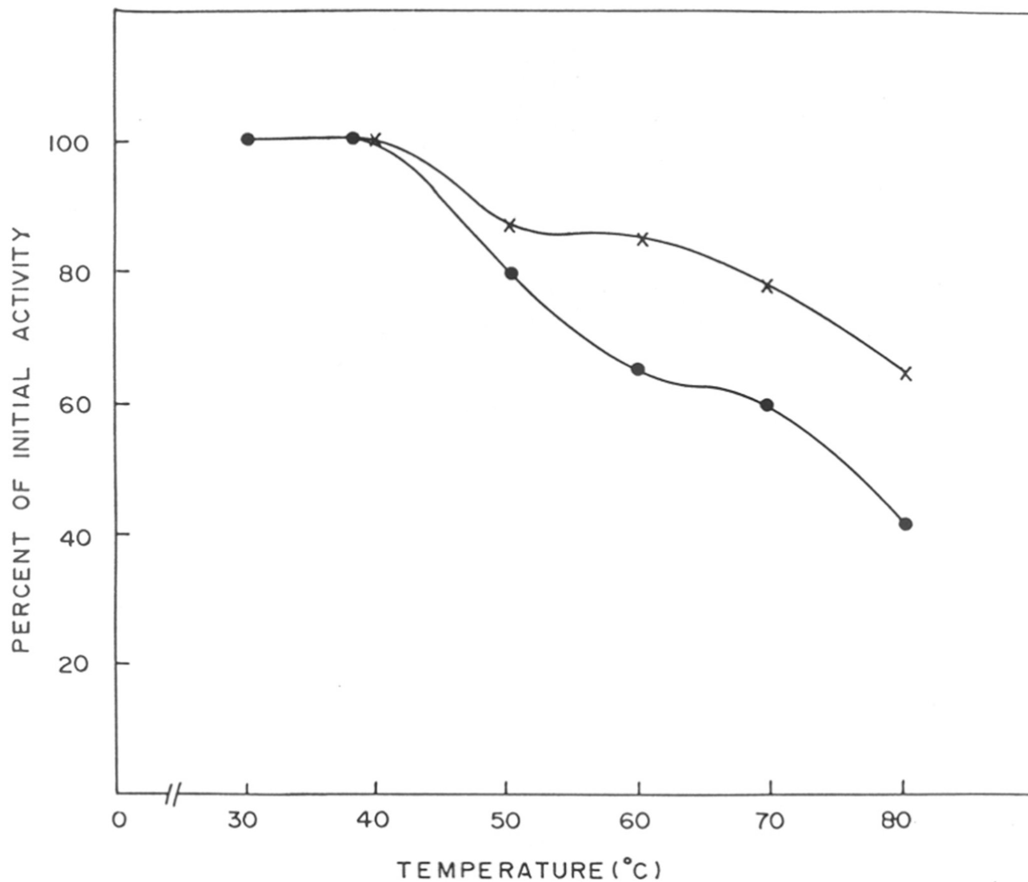


FIG. 11: Temperature stability of soluble (●) and immobilized (X) RNase T1.

Soluble and immobilized enzymes (1.2 - 1.5 U) were preincubated at different temperatures (30 - 80°C) for 1 h at pH 7.5 and their activities were determined as described underMethods.

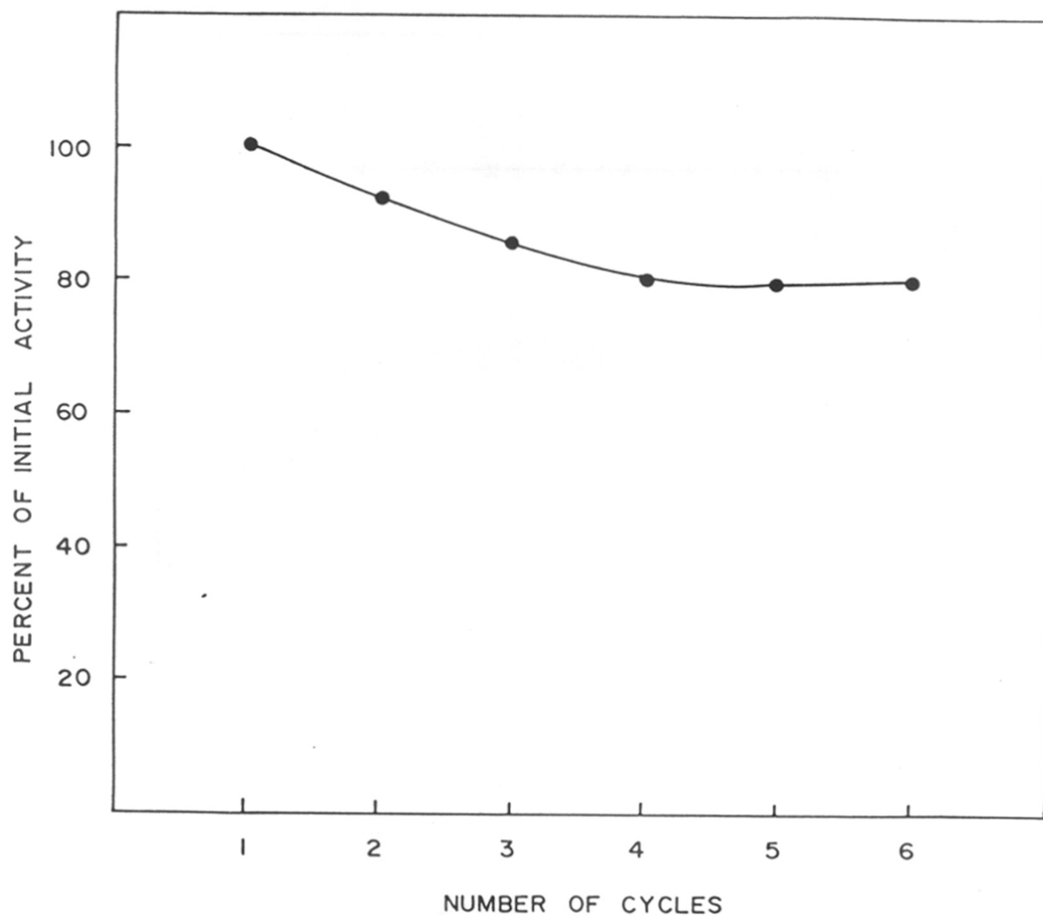


FIG. 12: Effect of number of assay cycles on the activity of immobilized RNase T1.

0.5 ml (1.2 U) of the immobilized preparation was assayed at pH 7.5 and 37°C. After every cycle, the immobilized enzyme was washed free of substrate and products and used for the next assay.

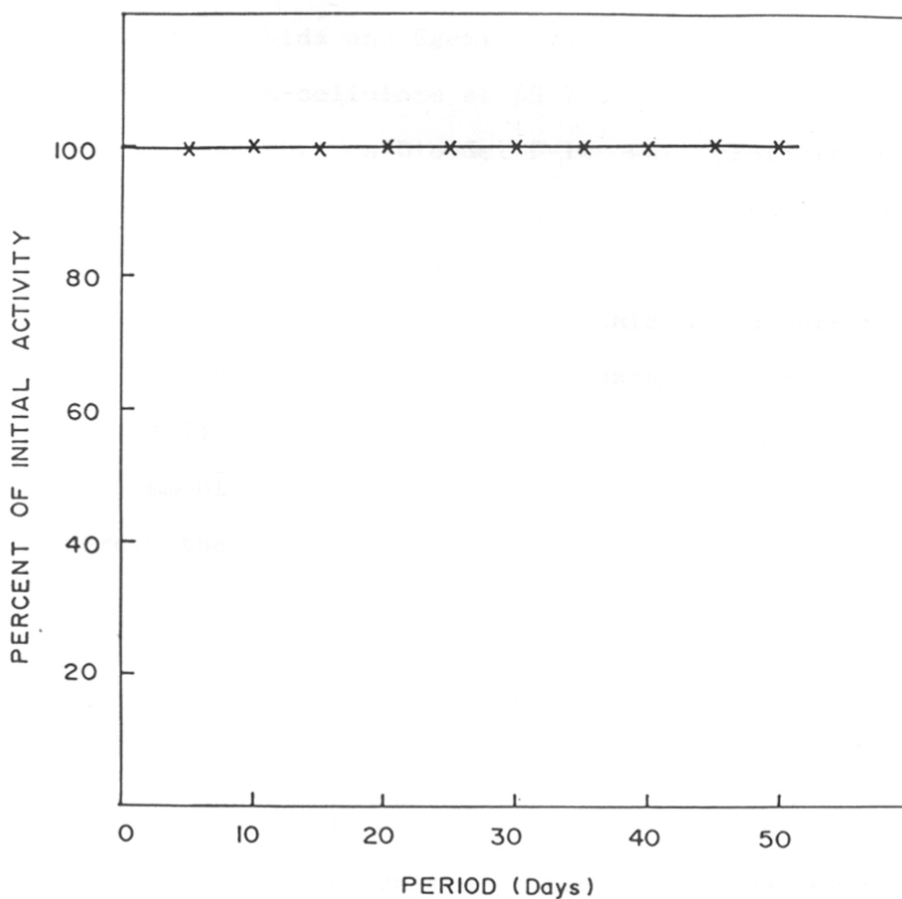


FIG. 13 : Effect of storage on the activity of immobilized RNase T1.

The immobilized preparation was stored in 0.2 M Tris-HCl buffer (pH 7.5) at 4°C. Fresh aliquots of 0.5 ml matrix (1.2 - 1.5 U) were removed and assayed for its activity under standard assay conditions at an interval of five days.

inactivation of both S1 nuclease and double strand specific nuclease, it did contain significant amount of RNase T2 activity. Though, Uchida and Egami (1967a) separated RNase T1 and RNase T2 on DEAE-cellulose at pH 7.5, in the present studies, chromatography on Bio-Gel P-10 was preferred as this helps to remove RNase T2 (MW 32,000) contamination from RNase T1 (MW 11,000) preparations, due to the vast difference in their molecular weights (Fig. 4). This procedure, in fact, gave a RNase T2-free RNase T1 preparation with high recovery (Table 1).

In any immobilized enzyme system the major components involved are, the enzyme, the carrier and the mode of attachment of the enzyme to the carrier. Apart from the enzyme, the other most important contributing component which determines the performance of immobilized enzyme system is the carrier. In other words, a judicious selection of the carrier is essential, as it not only affects the operational stability but also the performance of the immobilized enzyme. In the present investigation Bio-Gel P-2 was selected, since polyacrylamide based matrices are more resistant to chemical and microbial degradation, have a lower tendency to physically adsorb proteins, can be easily derivatized and are commercially available. Though the low porosity of Bio-Gel P-2 (exclusion limit 1800 daltons) limits the surface

area available for enzyme binding, it will reduce the diffusional constraints with respect to the high molecular weight substrate, since the enzyme is immobilized only on the external surface of the carrier.

In the present studies, immobilization of RNase T1 through its amino groups was attempted, since it has been reported that amino groups can be used for the binding of enzyme to solid supports (Takahashi and Moore, 1982). Among 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 (Marty, 1985). Though alkaline pH is favoured for glutaraldehyde activation of matrix, the subsequent coupling of the enzyme can be achieved depending on the stability of the enzyme in question. During the activation step, 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 coupling of the enzyme (Onyezili, 1987). Thus, glutaraldehyde will also provide a spacer arm for binding the enzyme away from the matrix.

Optimization of coupling conditions showed that, most active preparations are obtained when 1 ml (packed volume) of

2% (v/v) glutaraldehyde activated matrix is reacted with approximately 100 U of RNase T1 at pH 6.0 and 4°C for 15 h (Fig. 5, 6). Studies on matrix to enzyme ratio on the efficiency of AE Bio-Gel P-2-RNase T1 conjugate showed that after an initial increase, the efficiency decreased steadily. The decrease in efficiency at higher enzyme load (> 120 U) can be attributed to overcrowding of the enzyme on the matrix, as there was a decrease in specific activity of the bound enzyme (Fig. 6). Under optimum coupling conditions, the partially purified RNase T1 bound to glutaraldehyde activated AE Bio-Gel P-2 retained 22-24% activity of the soluble enzyme. Our efficiency values are higher compared to CM-cellulose bound enzyme (2%) by acid azide method (Kuriyama and Egami, 1966) but it is lower than the values reported for the enzyme bound to Enzycryl AH (45%) by similar method (Ito *et al.*,1977a). However, an effective comparison of the efficiency values of different preparations is not feasible, as the efficiency determination of CM-cellulose and Enzycryl AH bound enzymes were carried out by different methods.

Since the enzyme was coupled to glutaraldehyde activated AE Bio-gel P-2, only amino groups i.e. α-amino group of N-terminal alanine and ε-amino group of lysine 41 might be involved in the reaction. Though the amino groups are reported to be not essential for the catalytic activity of

RNase T1, modification of ϵ-amino group of lysine is known to lower the activity towards RNA to varying extent, depending on the nature and the size of the group introduced (Takahashi and Moore, 1982). When an enzyme is bound to an insoluble matrix via glutaraldehyde, primarily ϵ-amino group of lysine is involved in the binding. Hence, the low efficiency observed in the present case could be due to the involvement of lysine 41, as it is situated next to histidine 40, which in turn has been shown to be a part of the active site of the enzyme (Fig. 1, page 8). Our efforts to bind the enzyme in presence of the substrate (i.e. freezing the enzyme in its native conformation), so as to protect the active site, failed to improve the efficiency. In addition, an increase in the exposure of the bound enzyme to the substrate (i.e. by increasing the incubation period) failed to bring about any increase in the efficiency of the immobilized enzyme, indicating that the low efficiencies observed in the present case cannot be correlated to the extent of exposure of the bound enzyme to the substrate.

Changes in the enzymatic properties caused as a result of binding enzymes to insoluble supports are not only useful in assessing the potential of the immobilized systems but also in elucidating the structure-function relationship and

the mechanism of enzyme action. Changes in the properties of 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 characteristic of the matrix. Modification of the enzymatic properties caused by the matrix are due to (a) steric hindrance (b) electrostatic interactions between carrier and substrate and (c) formation of 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).

Comparison of pH activity profiles showed no change in the optimum pH of the enzyme as a result of immobilization (Fig. 7) indicating the absence of partitioning effects in the microenvironment of the immobilized enzyme. However, immobilization resulted in an increase in the optimum temperature to 50°C from 45°C for the soluble enzyme (Fig. 8).

Immobilization of enzymes to solid supports can also cause alteration in the conformation of the enzyme, resulting in changes in affinity of the enzyme to the substrate (Chibata, 1978). In addition to the effect of pH and temperature on the reaction rate, determination of kinetic parameters like K_m and V_{max} are important in determining the

operating conditions in any immobilized system. Evaluation of kinetic parameters of immobilized RNase T1 showed that the bound enzyme followed Michaelis-Menten's kinetics and there was slight decrease in the K_m. This observation indicates that, steric and diffusional limitations may not have a role. However, there was a five fold decrease in V_{max}, which could be due to blocking or masking of some of the active sites during immobilization. As mentioned earlier, the immobilized RNase T1 retained only 22-24% activity of the soluble enzyme.

It has been observed in many cases that, immobilization increases the stability of enzyme to pH and temperature. Such increase in the stability of an immobilized preparation is very important since, it helps in evaluating its potential applications. pH stability of AE Bio-Gel P-2-RNase T1 conjugate was inferior to that of soluble enzyme, especially in the acidic range (Fig. 10). The immobilized enzyme showed higher temperature stability compared to its soluble counterpart, as indicated by its inactivation pattern (Fig. 11). Increase in the temperature stability of the bound enzyme can be correlated to its rigid conformation in the bound form. In addition, stability of the immobilized enzyme to repeated use and storage, is suggestive of its high stability.

A simple and rapid procedure for the partial purification and immobilization of RNase T1 is described. Comparatively high activity and improved stability makes AE Bio-Gel P-2-RNase T1 conjugate a potentially useful system for routine analytical purposes.

REFERENCES

Ando, T. (1966) *Biochim. Biophys. Acta* **114**, 158-168.

Azegami, M. and Iwai, K. (1964) *J. Biochem. (Tokyo)* **55**, 346-348.

Billeter, M. A., Weissmann, C. and Warner, R. C. (1966) *J. Mol. Biol.* **17**, 145-173.

Chibata, I. (1978) In *Immobilized Enzymes : Research and Development* (Chibata, I. Ed) A. Halsted Press Book, Kodansha Ltd, Tokyo, pp. 108-147.

Curtis, P. J., Burdon, M. G. and Smellie, R. S. (1966) *Biochem. J.* **98**, 813-817.

Egami, F., Takahashi, K. and Uchida, T. (1964) In *Progress in Nucleic Acid Research and Molecular Biology* (J. N. Davidson and W. E. Cohn eds) Vol. 3, Academic Press, New York and London, pp. 59-101.

Fields, R., Dixon, H.B.F. and Law, G.R. (1971) *Biochem. J.* **121**, 591-596.

Glitz, G.D., Eichler, D.C. and Angel, L. (1974) *Anal. Biochem.* **62**, 552-567.

Guibault, G. G. (1982) *Appl. Biochem. Biotechnol.* **7**, 85-98.

Holley, R. W., Apgar, J., Everett, G.A., Madison J.T., Marquisee, M., Merrill, S. H., Penswick, J.R. and Zamir, A. (1965) *Science* **147**, 1462-1465.

Inman, J. and Dintzis, H. (1969) *Biochemistry* 8, 4074-4082.

Irie, M. (1970) *J. Biochem. (Tokyo)* 68, 69-79.

Irie, S., Itoh, T., Ueda, T. and Egami, F. (1970) *J. Biochem. (Tokyo)* **68**, 163-170.

Ishiwata, K. and Yoshida, H. (1978) *J. Biochem. (Tokyo)* 83, 783-788.

Itagaki, K., Kuriyama, Y., Shiobara, Y. Hayashi, H., Tamagata, T. and Egami, F. (1965) *Seikagaku* **37**, 217-225 (Chem. Abstr. **63**, 10338d).

Ito, H., Hagiwara, M., Takahashi, K. and Ichikizaki, I. (1977a) *J. Biochem. (Tokyo)* **82**, 877-883.

Ito, H., Hagiwara, M., Ichikizaki, I. and Takahashi, K. (1977b) *Chem. Letts.* **3**, 215-216.

Janski, A.M. and Oleson, A.E. (1976) *Anal. Biochem.* **71**, 471-480.

Jervis, L. and Pettit, N.M. (1974) *J. Chromatogr.* **97**, 33-38.

Kanaya, S. and Uchida, T. (1981) *J. Biochem. (Tokyo)* **89**, 591-597.

Kasai, K. (1965) *J. Biochem. (Tokyo)* **57**, 372-379.

Kuninaka, A. (1954) *J. Agr. Chem. Soc. Japan* **28**, 282-287.

Kuninaka, A. (1955a) *J. Agr. Chem. Soc. Japan* **29**, 52-57.

Kuninaka, A. (1955b) *J. Agr. Chem. Soc. Japan* **29**, 797-800.

Kuriyama, Y. and Egami, F. (1966) *Seikagaku* **38**, 735-740 (Chem. Abstr. **66**, 72694q).

Lee, J.C. (1971) *Biochim. Biophys. Acta* **235**, 435-444.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275.

Marty, J. (1985) *Appl. Microbiol. Biotechnol.* 22, 88-91.

McCully, K.S. and Cantoni, G.L. (1961) *Biochim. Biophys. Acta* **51**, 190-194.

Minato, S., Tagawa, T. and Nakanishi, K.(1966) *J. Biochem. (Tokyo)* **59**, 443-448.

Nishikawa, S., Adiwinata, J., Morioka, H., Fugimura, T., Tanaka, T., Uesugi, S., Hakoshima, T., Tomita, K., Nakagawa, S. and Ikehara, M. (1990) *Protein Engg.* **3**, 443-448.

Onyezili, F. (1987) *Biotechnol. Bioeng.* **29**, 399-402.

Podder, S.K. and Tinoco, I. Jr. (1969) *Biochem. Biophys. Res. Commun.* **34**, 569-574.

Rushizky, G.W. and Sober, H.A. (1962) *J. Biol. Chem.* **237**, 834-840.

Saruno, R. (1956) *Bull. Agr. Chem. Soc. Japan* **20**, 57-58.

Sato, K. and Egami, F. (1957) *J. Biochem. (Tokyo)* **44**, 753-767.

Sato-Asano, K. (1959) *J. Biochem. (Tokyo)* **46**, 31-37.

Sato-Asano, K. and Fujii, Y. (1960) *J. Biochem. (Tokyo)* **47**, 608-615.

Sato, S. and Egami, F. (1965) *Biochem. Z.* **342**, 437-448.

Shortman, K. (1962) *Biochim. Biophys. Acta* **55**, 88-96.

Sundaram, P.V. (1982) *Enzyme Microb. Technol.* **4**, 290-298.

Takahashi, K. (1962) *J. Biochem. (Tokyo)* **51**, 95-108.

Takahashi, K. (1965) *J. Biol. Chem.* **240**, 4117-4119.

Takahashi, K., Stein, W.H. and Moore, S. (1967) *J. Biol. Chem.* **242**, 4682-4690.

Takahashi, K. (1971) *J. Biochem. (Tokyo)* **69**, 331-338.

Takahashi, K. (1972) In *Protein : Structure and Function* (M. Fanatsu, K. Hirosji, K. Imahori, T. Murachi and K. Narita eds.) Vol. 1, Kodansha, Tokyo; Wiley, New York, pp. 285.

Takahashi, K. (1973) *J. Biochem. (Tokyo)* **74**, 1279-1282.

Takahashi, K. (1976) *J. Biochem. (Tokyo)* **80**, 1267-1275.

Takahashi, K. (1977) *J. Biochem. (Tokyo)* **81**, 641-646.

Takahashi, K. and Moore, S. (1982) In *The Enzymes* (Boyer, P. D. ed.) Vol. 15, Academic Press New York and London, pp. 435-468.

Tamaoki, H., Sakiyama, F. and Narita, K. (1976) *J. Biochem. (Tokyo)* **79**, 579-589.

Uchida, T. (1965) *J. Biochem. (Tokyo)* **57**, 547-553.

Uchida, T. and Egami, F. (1967a) *Methods Enzymol.* **12A**, 228-238.

Uchida, T. and Egami, F. (1967b) *Methods Enzymol.* **12A**, 239-247.

Uchida, T. and Egami, F. (1971) In *The Enzymes* (Boyer, P. D. ed.) Vol. 4, Academic Press New York and London, pp. 205-250.

Uozumi, T., Tamura, G. and Arima, K. (1969) *Agric. Biol. Chem.* **33**, 639-644.

Waki, M., Mitsuyasu, N., Terada, S., Matsuura, S., Kato, T. and Izumiya, N. (1974) *Biochem. Biophys. Res. Commun.* **61**, 576-582.

Waku, K. and Nakazawa, Y. (1970) *J. Biochem. (Tokyo)* **68**, 63-67.

Walz, F.G.Jr., Osterman, H.L. and Libertin, C. (1979) *Arch. Biochem. Biophys.* **195**, 95-102.

Yamamoto, Y. and Tanaka, J. (1970) *Biochim. Biophys. Acta.* **207**, 522-531.

Zhenodarova, S.M., Smolyaninova, O.A., Soboleva, I.A. and Khabarova, M.I. (1987) *Bioorg. Khim.* **13**, 1023-1030 (Chem. Abstr. **108**, 164357w).