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PREPARATION AND PROPERTIES OF WATER-INSOLUBLE DERIVATIVES OF RIBONUCLEASE T_1

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SUMMARY

Several enzymatically active water-insoluble ribonuclease T_1 (ribonucleate guaninenucleotide-2′-transferase (cyclizing), EC 2.7.7.26) derivatives were prepared. One of these, Sepharose T_1, which was prepared by chemically coupling ribonuclease T_1 to Sepharose, was further characterized. The enzyme derivative was stable and had no detectable residual soluble enzymatic activity. Substrate specificity of the enzyme derivative remained unaltered. Kinetic values were similar to the free, native enzyme.

INTRODUCTION

Enzymes and some biologically active proteins can be immobilized to insoluble polymers while retaining their activities[1–6]. Such immobilized enzymes offer several advantages. Such enzyme derivatives can be packed into a column and used as an enzyme "reactor". The enzyme and substrate can be separated readily at the termination of the reaction. Furthermore, in most cases, such enzyme derivatives are stable and can be used continuously for fairly long periods of time.

In the present work, the chemical coupling of ribonuclease T_1 (ribonucleate guanine nucleotide-2′-transferase (cyclizing), EC 2.7.7.26) to various polymers is exploited. There are several methods for linking proteins to different polymers. Most of them involve the preparation of activated intermediates, such as diazotized aminobenzyl derivatives of cellulose[7] and CM-cellulose azide[8]. Recently, cyanogen halides have been used to prepare a reactive polysaccharide intermediate which can couple with protein in aqueous medium[9,10]. This method of activation and coupling seems gentle and has been used for the preparation of several proteolytic enzyme derivatives[2,3,11].

The aim of the present work was to prepare water-insoluble derivatives of ribonuclease T_1 that would retain its native enzymatic activity so that they might be applied to the structure studies of ribonucleic acids.

EXPERIMENTAL PROCEDURES

Materials

Ribonuclease T_1 was purchased from Calbiochem, Los Angeles, California. *p*-Aminobenzyl cellulose (Bio-Rad, 0.13 mequiv/g) was obtained from Bio-Rad Laboratories, Richmond, Calif. The Sephadex G-200 and Sepharose 2B were purchased from Pharmacia Fine Chemicals, Inc. Dinucleoside monophosphates and *Escherichia coli* tRNA were obtained from Mann Research Lab., and General Biochemicals respectively.

Preparation of cyclic phosphate

Guanosine 2',3'-cyclic phosphate was prepared by the reaction of guanosine 2'(3')-phosphate with 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)-carbodiimide metho-*p*-toluenesulphonate. The final product was purified by paper chromatography in isopropanol–conc. ammonia–water (7:1:2, by vol.).

Preparation of ribonuclease T_1 derivative coupled to p-aminobenzyl cellulose (PAB-T_1)

Ribonuclease T_1 was linked to diazotized *p*-aminobenzyl cellulose according to a modified process of KURIYAMA AND EGAMI[12]. The aminobenzyl cellulose was diazotized in the cold by reacting with $NaNO_2$. The product was washed with cold sodium acetate–urea (5% each) followed by cold distilled water. The diazotized cellulose (10 mg) was added to a phosphate solution (1 ml, 0.05 M, pH 8.0) containing ribonuclease T_1 (1 mg). The coupling reaction was allowed to proceed at 4° for 24 h, followed by heating at 37° for 1 h to remove unreacted diazotized groups. The product was packed into a small glass column and washed with cold distilled water (100 ml), $NaHCO_3$ solution (0.1 M, 100 ml), HCl (10^{-3} M, 100 ml) and NaCl solution (0.5 M, 100 ml).

Preparation of ribonuclease T_1 derivative coupled to Sephadex (Sephadex-T_1)

Ribonuclease T_1 was coupled to Sephadex G-200 by the method of AXIN *et al.*[9] in the preparation of Sephadex–chymotrypsin derivative. Sephadex G-200 (200 mg) was activated by the treatment with cyanogen bromide as described. The product was then stirred gently in a sodium bicarbonate solution (0.1 M, 5 ml) containing ribonuclease T_1 (4000 units). The coupling reaction was allowed to proceed for 48 h at 4°. The product was then packed in a small glass column and washed with cold distilled water (100 ml), $NaHCO_3$ solution (0.1 M, 100 ml), HCl (10^{-3} M, 100 ml) and NaCl solution (0.5 M, 100 ml).

Preparation of ribonuclease T_1 derivative coupled to Sepharose (Sepharose-T_1)

This enzyme derivative was prepared by the coupling of ribonuclease T_1 with Sepharose 2B according to a modified procedure of PORATH[10]. Sepharose 2B (200 mg wet wt. in 5 ml) pre-washed with cold distilled water, was treated with cyanogen bromide (25 mg/ml, 2 ml) for 6 min while the pH of the reaction mixture was maintained at 11 with the addition of NaOH (2 M). The product was immediately transferred to a glass filter and washed with cold distilled water (20 ml) and $NaHCO_3$ solution (0.1 M, 20 ml). The activated polymer was suspended in $NaHCO_3$ solution (0.1 M, 2 ml). Ribonuclease T_1 (10 000 units) was added. The coupling reaction was

allowed to proceed at 4° for 20 h with gentle stirring. The reaction product was packed into a small column and washed.

Assay of enzyme activity

(A) The enzyme activity of ribonuclease T_1 and its derivatives was determined by measuring the absorbance at 260 mμ of the acid-soluble products of RNA similar to the procedure of UCHIDE AND EGAMI[13]. The unit of enzyme activity is defined as follows: an increase of 0.1 $A_{260\ m\mu}$ unit in 15 min is 0.1 unit of enzyme activity[13].

(B) The enzyme activity using guanosine 2',3'-cyclic phosphate as substrate was determined by the production of guanosine monophosphate which was separated from the substrate by paper chromatography in the isopropanol-conc. ammonia water solvent system described above. The ultraviolet-absorbing spot was cut out, eluted and quantitated spectrophotometrically.

Paper electrophoresis of dinucleoside monophosphates and their degradation products

Nucleotide materials were analyzed on washed Whatman 3MM paper in ammonium formate (0.05 M, pH 3.0) at 40 V/cm for 2 h. Their identities were determined by spectral analysis and electrophoretic mobilities as compared with standard compounds.

RESULTS AND DISCUSSION

The enzymatic activity per unit weight of the solid support of the various enzymatic derivatives are shown in Table I. Ribonuclease T_1 bound to Sephadex and Sepharose showed considerable activity toward *E. coli* ribosomal RNA. All subsequent studies were done with Sepharose-T_1. The possibility that this enzymatic

TABLE I

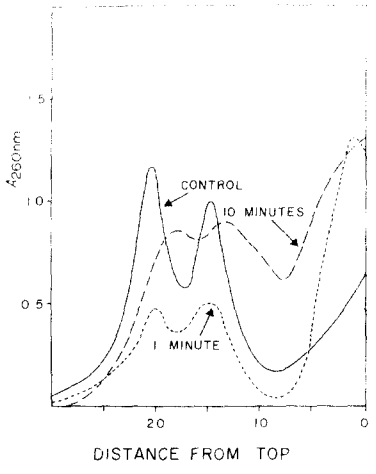
ENZYMATIC ACTIVITY OF THE VARIOUS ENZYMATIC DERIVATIVES

The values given are calculated on the basis of an increase in absorption at 260 mμ of the acid-soluble products of high molecular weight *E. coli* ribosomal RNA per mg (wet wt.) of the solid supporting material.

Enzyme derivative	*Enzymatic activity (units/mg)*
PAB-T_1	0.001
Sephadex-T_1	0.05
Sepharose-T_1	0.06

activity might be due to residual soluble enzymatic activity was eliminated as shown below. Further incubation of ribosomal RNA after removal of the enzyme derivative showed no detectable increase in acid soluble material. Moreover, the sedimentation profile of ribosomal RNA in sucrose density gradient was not altered upon further incubation of the RNA sample after the enzyme derivative was removed.

Fig. 1 shows the sedimentation profile of *E. coli* rRNA after digestion with Sepharose-T_1. The profile of the starting material is also shown. After a short period



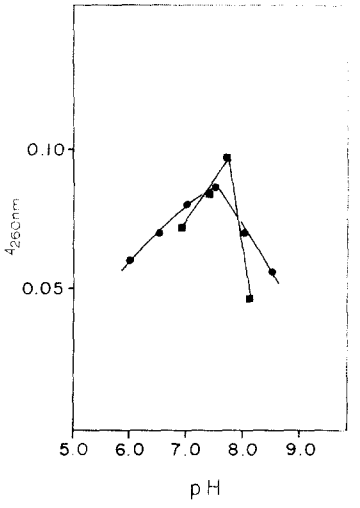


Fig. 1. Sedimentation profile of *E. coli* rRNA in sucrose gradient. Samples of 0.1 ml were layered over 5–20% sucrose gradients (4 ml) and centrifuged at 25 000 rev./min in a SW 50.1 rotor (Spinco) for 16 h at 25°. Samples were then analyzed with the use of a Gilford spectrophotometer equipped with a flow cell.

Fig. 2. Enzymatic activity as a function of pH. Assays as described in the text were used in buffer adjusted to the appropriate pH. ●—●, native enzyme; ■—■, Sepharose-T_1.

of digestion (1 min), the higher molecular weight species of rRNA appears to be preferentially degraded into smaller molecular weight RNA molecules. With a somewhat longer period of digestion (10 min), both species of rRNA seem to be degraded to yield smaller RNA products.

The enzymatic activity as a function of pH of ribonuclease T_1, the free enzyme, and the coupled enzyme, Sepharose-T_1 using *E. coli* ribosomal RNA as substrate are shown in Fig. 2. The activity of the enzyme derivative is similar to that of ribonuclease T_1 in the pH range investigated. However, the optimal pH of Sepharose-T_1 is slightly shifted from pH 7.5 for ribonuclease T_1 to pH 7.7. This slight change in pH optimum seems to indicate little interaction between the enzyme and the solid support.

In order to examine the substrate specificity of the enzyme derivative as compared to that of the native enzyme, model compounds, GpA, ApA, CpA and UpA were incubated with the enzyme derivative. At the end of 2 h at 37°, the nucleotide

TABLE II

SUBSTRATE SPECIFICITY OF SEPHAROSE -T_1

Substrate ($A_{260\ m\mu}$ *units*)		*Products* ($A_{260\ m\mu}$ *units*)	
GpA	(2.0)	GpA	(<0.02)
		Gp	(0.8)
		A	(1.1)
ApA	(2.0)	ApA	(1.8)
CpA	(2.0)	CpA	(1.9)
UpA	(2.0)	UpA	(1.8)

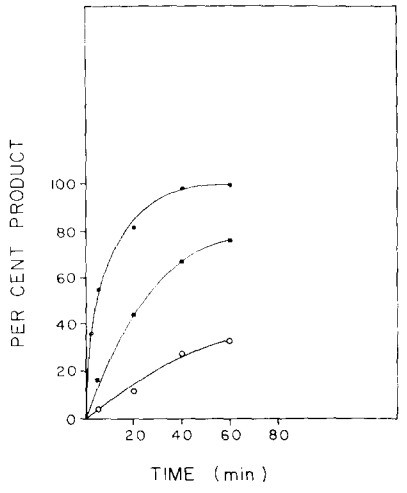


Fig. 3. Enzymatic activity of Sepharose-T_1 on various substrates. Assays were carried out in duplicates and were as described in the text. Substrate used: ● —●, guanosine 2′,3′-cyclic monophosphate; ■ —■, *E. coli* tRNA; ▲ —▲, rRNA.

products were analyzed using paper electrophoresis with standards. Only GpA was completely degraded into its components, Gp and A (Table II). Results with native T_1 with these substrates are similar. Although one cannot use these data to determine the relative rate of hydrolyses of the substrate by the native and derivatized enzyme, it is clear that the substrate specificity of the derivatized enzyme remained the same as the native enzyme.

The results in Fig. 3 show the enzymatic activity of Sepharose-T_1 using different

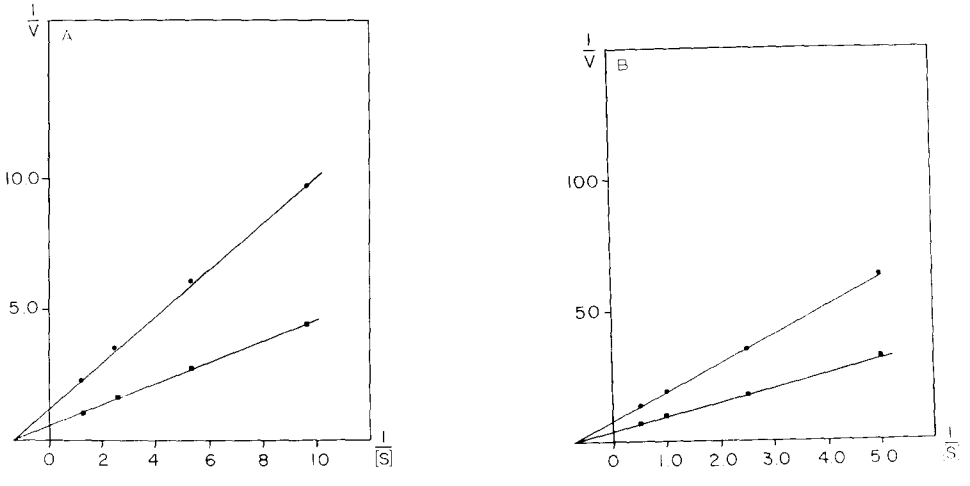


Fig. 4. Lineweaver–Burk plots. Enzymatic activity of free and coupled enzymes were determined as described in the text at various substrate concentrations. Substrate concentrations, *s*, are expressed as $A_{252\ m\mu}$ units/ml for guanosine 2′,3′-cyclic phosphates and mg/ml for tRNA. Reaction velocity, *v*, is expressed as $A_{252\ m\mu}$ units guanosine 3′-phosphate produced per 10 min and $A_{260\ m\mu}$ units per 10 min for the respective reaction conditions. ■ —■, ribonuclease T_1; ● —●, Sepharose-T_1. (A) Guanosine 2′,3′-cyclic phosphate. (B) *E. coli* tRNA.

substrates. Guanosine 2',3'-cyclic phosphate was readily hydrolyzed, whereas *E. coli* ribosomal RNA was hydrolyzed least readily. One possible explanation for this discriminating effect on the enzymatic activity towards substrates of different molecular size may be steric hinderance and/or charge-charge interactions between the active site of the coupled enzyme and these substrates. However, there seem to be negligible interactions between the substrate molecules and the solid support. This is based on the observations that ribonuclease T_1, free and attached to Sepharose shows no change in apparent K_m (Fig. 4) using either guanosine 2',3'-cyclic phosphate or *E. coli* tRNA as substrates. HORNBY *et al.*[4] had shown that the apparent K_m is particularly sensitive to the nature of the microenvironment of the enzyme generated by the support.

TABLE III

ENZYMATIC ACTIVITY OF SEPHAROSE-T_1 WITH TIME

Time (days)	*Enzymatic activity ($\Delta A_{260\ m\mu}$ units/10 min)*	*Percent original activity remaining*
0	0.75	100
5	0.72	96
20	0.70	94
35	0.65	87

It has been reported that ribonuclease T_1 possesses two cysteine residues and that reductive cleavage of these disulphide bonds by thioglycolate or mercaptoethanol inactivated enzymatic activity[13]. Thus, it was of interest to examine whether the enzyme derivative remained dependent on the integrity of the disulphide bonds. We found that the enzyme derivative was inactive when 0.06 M β-mercaptoethanol was included in the assay mixture.

The stability of the insolubilized enzyme when stored in sodium acetate (0.1 M, pH 6.0) at 4° was studied for five consecutive weeks. The data (Table III) indicate that relatively little enzymatic activity had been lost for at least 5 weeks. In addition, the enzyme derivative retained its activity even after several uses.

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