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PURIFICATION AND CHARACTERIZATION OF A RIBONUCLEASE FROM BARLEY ROOTS*

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Key Word Index—*Hordeum vulgare*; Graminae; barley; ribonuclease; enzyme purification; enzyme characterization.

Abstract—A ribonuclease isolated from barley malt roots exhibited characteristics that conformed to those of RNase I (EC 3.1.27.1). It differed from RNase I from barley leaves and barley seeds in its action on polynucleotides and on 3′,5′-dinucleoside monophosphates, and from barley seed RNase I in its optimum pH. Gel electrophoresis indicated that the enzyme was present in the embryo, roots, shoot and endosperm of germinating barley. The enzyme showed pH optimum at 5.0, isoelectric pH at 4.5, a thermal optimum of 50°, and an apparent molecular weight of 19 000.

INTRODUCTION

Plant ribonucleases are classified into three categories [1]. Nuclease I catalyses the hydrolysis ofboth DNA and RNA to yield 5′-deoxyribonucleotides or 5′-ribonucleotides respectively. RNase I reacts only with RNA to produce cyclic 2′,3′-purine and pyrimidine ribonucleotides, the former being acted upon further to yield 3′-purine ribonuclotides. The enzyme does not react with the cyclic pyrimidine nucleotides. RNase II acts similaly to RNase I upon RNA to form 2′,3′-cyclic purine and pyrimidine nucleotides but, unlike RNase I, it reacts with both types of cyclic 2′,3′-nucleotides to form either 3′-purine ribonucleotides or 3′-pyrimidine ribonucleotides.

Lantero and Klosterman [2] have characterized the main ribonuclease from barley leaves which had an M_r of 25 000 and appeared to be the RNase I type. Johnson *et al.* [3] and Kenefick *et al.* [4] reported that shoot tissue from less winter hardy barley has more of an RNase (believed to be RNase I) than did the shoot tissue from winter hardy barley. In addition, qualitative differences in RNases between hardy and less hardy barley types appear to be present. For example, RNase I activity of hardy types experiences a greater activity loss when exposed to high ionic strength than does the enzyme from the less hardy varieties. Skoczek [5] has indicated up to nine uncharacterized nuclease and/or RNase isozymes in crude extracts of barley roots. RNases I and II and nuclease I have been isolated from whole barley seeds [6].

We report here the characterization of an RNase from barley roots which appears to have the characteristics of RNase I but differs from similar enzymes reported from barley tissues [2, 6].

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RESULTS

Purification

Figure 1 shows the electrofocusing of DNase and RNase from malted barley roots (see Experimental). The data for these activities from all tissues of the germinated kernels showed essentially the same pattern, i.e. DNase and most of the RNase are isoelectric at about pH 4.5, with a small amount of RNase activity isoelectric at pH 5.5.

Table 1 shows a typical purification of RNase from malted barley roots.

The final specific activity was 35×10^3 which rep-

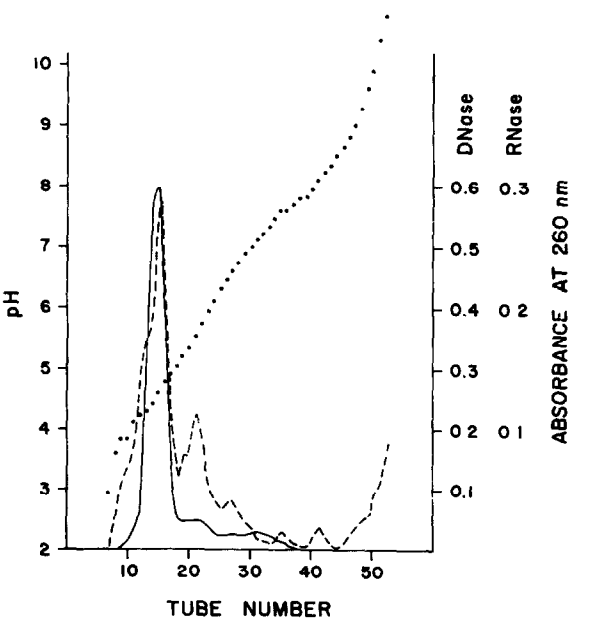

Fig. 1 Electrofocus pattern for malt root DNase and RNase. RNase, – – –; DNase, ——; pH, . . .

Table 1. Purification of RNase

Purification step	Total protein mg	Total RNase units	Specific activity units/mg protein	% Recovery	Purification factor*
Crude extract	5970	1730	0.29	100	1
First					
80% $(NH_4)_2SO_4$ precipitate	4310	1720	0.40	99	1.4
Dialysis *vs* 0.005 M acetate, pH 6.0	1500	1110	0.74	64	2.6
DEAE eluate, pH 6.0	630	705	1.1	41	3 8
Second					
80% $(NH_4)_2SO_4$ precipitate	384	525	1.4	30	4.8
Dialysis *vs* 0.12 M NaCl	341	375	1.1	22	3.8
First					
Bio-Gel P-30 chromatography	9.55	255	27	15	92
Third					
80% $(NH_4)_2SO_4$ precipitate	7 0	230	33	13	113
Second					
Bio-Gel P-30 chromatography	2.56	173	68	10	233
Affinity chromatography with 5'-adenosine phosphate-AH-Sepharose 4B	4.2×10^{-3}†	147	35×10^3	8	120×10^3

* $\frac{\text{Specific activity of product}}{\text{Specific activity of crude extract}}$.

† Protein determined by silver stain

resented an overall purification factor of 120×10^3. This overall purification is based on the total RNase units present in the original extract. But in the original extract several RNase activities were present and the enzyme we have purified originally represented only a fraction of this total which would make the purification factor for this RNase somewhat higher. For example, a nuclease (DNase) which has both DNase and RNase activity was present in the original extract and was removed by the Bio-Gel P-30 filtration (Fig. 2). The elution pattern for the adenosine 5'-phosphate aminohexyl Sepharose 4B column is shown in Fig. 3. Most of the contaminating protein was eluted at low ionic strength while the affinity-bound RNase was removed at high sodium chloride concentrations.

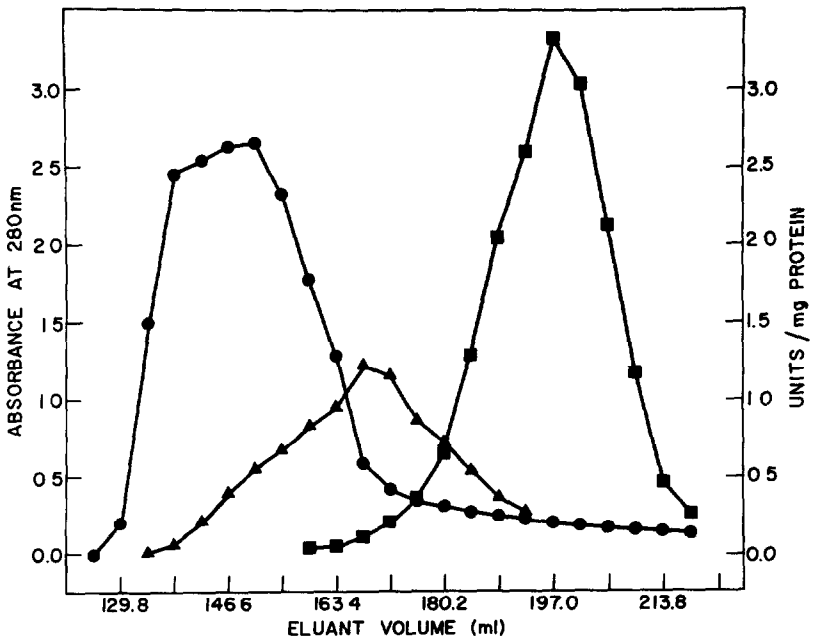

Fig. 2. Gel filtration (Bio Gel P-30) of DNase and RNase from malt roots. DNase, ▲——▲; RNase, ■——■; A_{280nm}, ●——●.

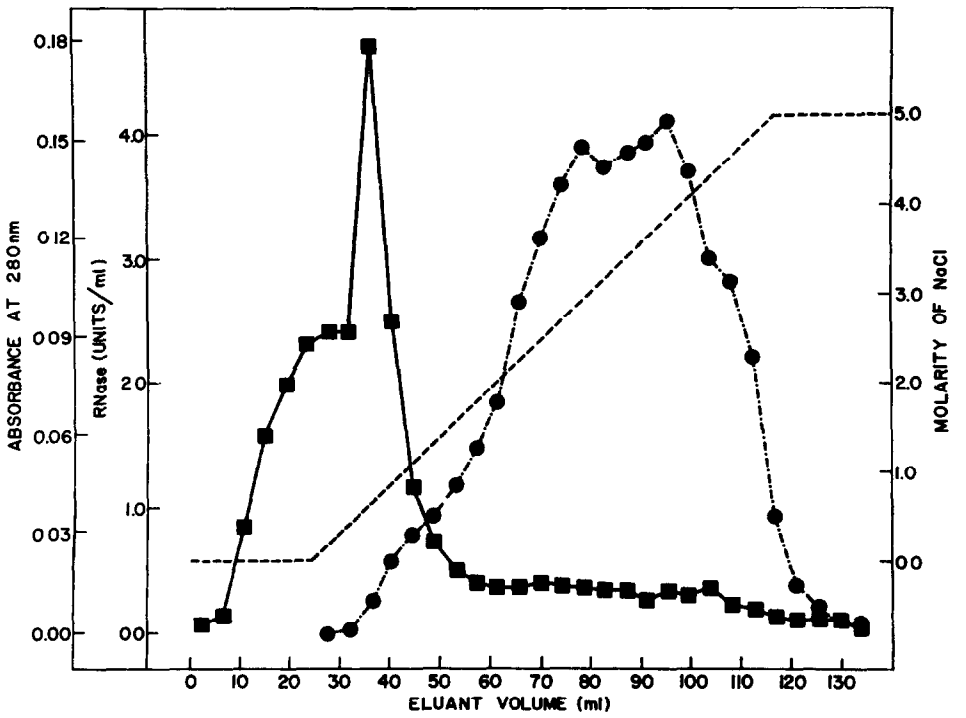

Fig 3. Affinity chromatography of malt root RNase on adenosine AH-Sepharose-4B. A_{280nm}, ■——■; RNase, ●——●; molarity of NaCl, – – – –.

Reaction with RNA

The products from the reaction with yeast RNA were the 2',3'-cyclic nucleoside phosphates shown in Table 2. After 60 min reaction time about 72% of the substrate had been converted to 2',3'-cyclic phosphates of adenosine, cytidine, uridine, and guanosine. No 3'-nucleoside phosphates were observed, nor were any seen after 4 hr reaction time. This lack of 3'-phosphates may have been caused by too low a concentration of the cyclic phosphates.

Reaction with polynucleotides

The choice of adenosine-5'-phosphate for coupling with the Sepharose 4-B was made upon consideration of the reactivity of the enzyme with the polynucleotides poly A, poly U, poly C and poly G. The specific activities of the RNase with these substrates were 14, 1.5, 1.0 and 0 respectively, a sequence common for plant nucleases [1]

Table 2. Reaction products from yeast RNA*

	Reaction time (min)		
	10	30	60
Products	% of substrate		
2',3'-CMP	12	18	22
2',3'-AMP	9	11	12
2',3'-UMP	9	14	16
2',3'-GMP	19	24	22
Total	49	67	72

*See Experimental for reaction conditions.

Reaction with dinucleoside phosphates

Table 3 shows the reactivity of the enzyme with the dinucleoside phosphates. It was extremely active with ApG but much less so with the remaining substrates. However, it showed appreciable affinity for GpU, ApC, ApU, UpU and CpG. The product in every case was the 2',3'-cyclic nucleoside monophosphate and the nucleoside released from the 5' ester bond.

Reaction with nucleoside phosphates

The enzyme did not react with any of the noncyclic nucleoside monophosphates, the nucleoside di- and tri-

Table 3. Reaction with dinucleoside phosphates

Substrate	% Hydrolysis	Substrate	% Hydrolysis
ApG	100*	CpA	33
ApA	35	CpG	73
ApC	80	CpC	22
ApU	79	CpU	58
GpA	43	UpA	32
GpG	39	UpG	66
GpC	64	UpC	35
GpU	91	UpU	75

*Substrate was completely hydrolysed in 5 min.

phosphates, or with the *p*-nitrophenyl substrates. When 12 μg of enzyme (but not when 1.2 μg of enzyme) was used in the reaction for 15, 30, 60, 120 and 240 min at 30° with the cyclic nucleoside phosphates 2',3'-cyclic AMP and 2',3'-cyclic GMP, the 3'-nucleoside phosphates formed as percent of the substrates at the above reaction times were as follows: for 2',3'-cyclic AMP; 3, 4, 5, 7 and 10% respectively; for 2',3'-cyclic GMP; 3, 3, 6, 13 and 18% respectively. There was no reaction with 2',3'-cyclic CMP or 2',3'-cyclic UMP. The lower enzyme level corresponded to the quantity that was effective in hydrolysing RNA (Table 2). Since 10 times this amount of enzyme was required for appreciable hydrolysis of the cyclic compounds, the enzyme appears to have much less affinity for the cyclic nucleotides than for RNA at the substrate concentrations used (0.1 mg RNA/ml; 1.8 mg cyclic nucleotide/ml).

pH optimum and stability

The optimum pH is 5.0 for RNA and 6.0 for poly A. In both cases activity decreased sharply above and below these values. When kept at 4° under a pH range of 4–10, the enzyme was stable from pH 6.0 to 9.5, but below pH 6.0 there was a marked loss of activity.

Thermal optimum and stability

The enzyme was stable at temperatures up to 50° for 2 hr but was inactivated above 50°. The thermal optimum was 50° since reaction rate doubled for each 10° increase in reaction temperature over the range 20–50°.

Electrophoresis

The purity of the enzyme is indicated from the electrophoretic patterns of Fig. 4. A major band and three minor ones were revealed by each detection method, i.e. by silver stain (Fig. 4a), and by location of RNase activity (Fig. 4c). This indicates that little, if any, contaminating non-enzymic protein is present. Based on the colour intensity by the two methods 95–97% of the protein and RNase activity are associated with the broad band, and about 1% each for the other bands. There appears to be aggregation under the electrophoretic conditions which may be why more than a single band is present. The main band was present also in extracts of tissue (embryo, shoots, roots and endosperm) from germinated barley. These crude extracts and the crude malt root extract (Fig. 4b) showed 6–10 RNases to be present.

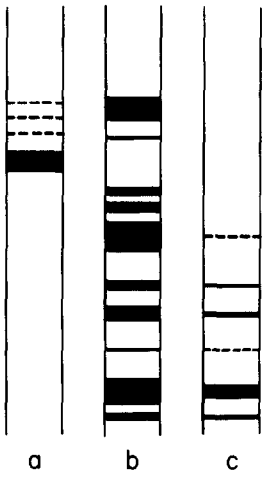

Fig. 4. Electrophoresis of RNase. (a) RNase after affinity chromatography Silver stain. (b) RNase enzymes in crude extract of malt roots. Toluidine blue stain (c) RNase after affinity chromatography. Toluidine blue stain

Molecular weight estimations

The apparent molecular weight of the major component as estimated by electrophoresis on gels of varying polyacrylamide concentrations was 33 000. The enzyme was eluted from the Bio-Gel P-30 column at an elution volume of 197 ml (Fig. 2) as was the myoglobin molecular weight standard, which indicated an M_r of about 19 000. However, when the enzyme was electrophoresed after treatment with SDS and dithiothreitol the main band showed an M_r of 20 000.

Effect of cations and sulfhydryl reagents

The reaction rates (as % of control) for the enzyme in the presence of EDTA and cations (10^{-3} M) were: EDTA, 96; Ca, 95; Mg, 69; Zn, 51; Fe, 48; Ba, 47; Cu, 8. Maleimide and N-acetylimidazole (10^{-3} M) did not affect the reaction rate, but iodoacetic acid lowered the rate to 77% of the control. These effects were similar to those observed by Lantero and Klosterman for the barley leaf enzyme [2].

DISCUSSION

With the polynucleotides the enzyme showed the preference poly A ≫ poly U = poly C ≫ poly G. Rice bran RNase, however, preferred poly A > poly C > poly U [7]. RNase I from whole barley seeds reacted in the sequence poly U > poly A > poly C > poly G [6] and the wheat leaf enzyme poly U > poly A > poly C = poly G [8]. The affinity for poly A was substantiated by its activity with 3 of the 4 adenylyl dinucleosides (Table 2) and the affinity shown for the AMP-Sepharose 4B column (Fig. 3). Presumably the preferential bond attacked in RNA is the adenylyl one. The newly isolated enzyme appeared to differ from the one from barley leaf tissue described by Lantero and Klosterman [2] which showed greatest affinity for GpG.

The enzyme we describe showed affinity for 2',3'-cyclic phosphates of adenosine and guanosine, but none for the 2',3'-cyclic phosphates of uridine and cytidine, and thus appears to conform to Wilson's plant RNase I. It also appears to have pH requirements similar to plant RNase I [1]. Its pH optimum of 5.0 seems to be common among comparable enzymes from rice bran [7], spinach [9], wheat leaves [10], corn [11], and barley leaves [2], but is lower than the pH 5.7 reported for RNase I from barley seeds [6]. There are plant RNases, however, which have pH optima 7.0–7.6 similar to the mammalian enzymes, i.e. RNase from *Vicia faba* roots which has an optimal pH of 7.2 [12] and RNase from wheat leaf chromatin which is optimum at pH 7.0–7.2 [8]. RNase II commonly shows a pH optimum of 6 [12].

The thermal stability of the enzyme appears to be somewhat lower than that of spinach RNase [9] which

lost only 50% of its activity after 10 min at 100°, and of rice bran RNase which retained 75% of its activity after 16 min at 100°, but comparable to the enzyme from wheat leaf chromatin which lost 75% of its activity after 10 min at 60°.

The molecular weight of the enzyme is in the low range commonly reported for plant RNases; e.g. for the rice bran enzymes, 14 500 and 35 000 [7]; corn RNase I, 23 000 [11]; barley leaf RNase I, 25 000 [2]; and barley seed RNase I, 19 000 [6].

EXPERIMENTAL

Preparation of tissues from germinating barley. Barley seeds (*Hordeum vulgare* var Larker) were germinated on filter paper in petri dishes (50 kernels per dish) with 4 ml water at 15° for 96 hr. Seedlings were separated into root, shoot, embryo, and degermed caryopsis tissues. All tissues were freeze-dried.

Preparation of malt roots. Barley seeds (*Hordeum vulgare* var Larker and Klages) were malted by the conventional procedure [13]: (a) the grain was steeped in water at 15° until the moisture content was 45%; (b) the drained barley was germinated in the dark for 5 days at 15° and 100% relative humidity; and (c) the germinated kernels were dried by kilning at 35° for 9 hr, 45° for 7 hr, 55° for 5 hr, 65° for 5 hr, 75° for 2 hr and 85° for 2 hr. Malt moisture was normally 4% The brittle roots were removed from the malt

Extraction of tissue

Degermed caryopsis. The freeze dried material was ground in a Udy Cyclone Mill (Udy Corp., Boulder, CO, U.S.A.). 4 g was stirred at 4° for 2.5 hr with 20 ml 0.1 M Tris–chloride buffer, pH 7.0. The suspension was centrifuged at 80 000 *g* for 30 min at 4. The supernatant was exhaustively dialysed against distilled water at 4° and freeze dried.

Embryo, shoots and roots. Each tissue (0.2 g) was ground in a mortar with sand and then stirred with 15 ml of the above buffer at 4° for 2 hr. Suspensions were centrifuged and dialysed as above and freeze dried.

Malt roots. 400 g of malt roots were milled with a Udy Cyclone Mill and extracted with 3.2 l of the above buffer at 4°. The suspension was stirred for 2.5 hr at 4°, then filtered through 4 layers of cheese cloth, and centrifuged (1200 *g*) at 4° in 250 ml bottles. The supernatant was then exhaustively dialysed against deionized water at 4° and frozen.

Assay for protein and enzyme activity

Protein assay. Protein was assayed by the Warburg–Christian method [14] except for enzyme solutions from the affinity chromatography. The protein in the final product from affinity chromatography was too low to be measured by this method. An estimation was made by electrophoresing 0.1–0.4 μg of bovine serum albumin (Schwarz-Mann Co., Orangeburg, NJ, U.S.A.) next to an aliquot of the sample and then staining the developed gel with silver as described in the section for electrophoresis. The quantity of sample electrophoresed was estimated by the intensity of the band relative to the bovine serum albumin band.

DNase and RNase assays. DNase was determined by the method of Liao [15] and RNase by the method of Prentice and Heisel [16]. A unit of activity is a unit change in absorbance at 260 nm per min. Specific activity is the units per mg of protein.

Electrofocusing

Tissues of germinated barley. The LKB 110 ml electrofocusing column, ampholytes and power supply (LKB Instruments, Inc., Rockville, MD, U.S.A.) were used according to the manufacturer's instructions. Extracts of tissues (0.3 g roots, 0.6 g shoots, 4.0 g degermed caryopses, and 0.4 g embryo) were exhaustively dialysed against 1% glycine at 4° before electrofocusing at 300 V for 72 hr. 2 ml fractions were drained from the focused column and assayed for RNase and DNase.

Malt roots. The extract of 20 g of roots was dialysed against 1% glycine at 4° and electrofocused with the LKB 440 ml column. Solutions with approximately 440 mg N were electrofocused at 500 V for 72 hr. 5 ml fractions were drained from the focused column and assayed for RNase and DNase activities.

Treatment of malt root extracts

Ammonium sulphate precipitation The frozen malt root extract was thawed and centrifuged at 55 000 *g* for 20 min. The supernatant was brought to 30% saturation with solid ammonium sulphate at 4° and centrifuged for 20 min at 55 000 *g*. The precipitate was discarded and the supernatant raised to 80% ammonium sulphate saturation and centrifuged as above. The 80% supernatant was then discarded and the precipitate was dissolved in 360 ml 0.005 M acetate at pH 6.0 and dialysed against 2 × 1300 ml of the buffer. All operations were done at 4°.

Treatment with diethylamino ethyl cellulase (DEAE). 50 ml of the solution from the 80% ammonium sulphate precipitation was diluted to 250 ml with 0.005 M acetate at pH 6.0 and added to 510 g moist DEAE which had been equilibrated with this buffer. The suspension was stirred for 30 min and filtered through a coarse sintered glass funnel. The filtrate was retained and the DEAE was washed 3 times with 250 ml portions of the buffer. The DEAE was eluted with four 250 ml portions of 0.1 M acetate, pH 6.0, four 250 ml portions of 0.25 M acetate, pH 6.0, and finally with four 250 ml portions of 0.5 M acetate, pH 6.0.

The 0.25 M and 0.5 M acetate elutions were combined, made 80% saturated with ammonium sulphate, and centrifuged at 14 000 *g*. The pellet was dissolved in 0.5 M NaCl and centrifuged at 140 000 *g* to remove DEAE fines. The supernatant solution was dialysed against 0.12 M NaCl and stored frozen. All operations were done at 4°.

Gel filtration. A column (2.6 cm diameter × 89 cm in length) of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, CA 94804, U.S.A.) was equilibrated with 0.12 M NaCl according to the manufacturer's instructions. Sample volumes of 25 ml from the DEAE treatment which contained up to 100 mg of protein in 0.12 M NaCl were applied to the column by upward displacement. The column void volume was 125 ml. The column was eluted with 0.12 M NaCl at 18–22 ml per hr, the eluant was monitored for A280 nm with an ultraviolet spectrophotometer (Model UA-5, ISCO Co., Lincoln, NE 68505, U.S.A.), and fractions were collected with a Model 1200 ISCO fraction collector. All operations were done at 4–12°.

Determination of molecular weight by gel filtration. Molecular weight was determined by gel filtration of enzyme and standards in unbuffered 0.12 M NaCl with a 2.5 × 30 cm column of Bio-Gel P-30 (Bio-Rad Corp., Richmond, CA, U.S.A.) equilibrated with 0.12 M NaCl The standard proteins (Schwarz-Mann, Orangeburg, New York, U.S.A.) were myoglobin, ovalbumin, bovine serum albumin, and human gamma globulin with M_rs 17 800, 45 000, 67 000 and 160 000 respectively. The mol. wt of the enzyme was determined by reference to a standard curve of log mol. wt. *vs* K_{av} for the standard proteins, where $K_{av} = (V_E - V_0)/(V_t - V_0)$, V_0 = void vol., ml, V_t = total column vol., ml, and V_E = elution vol., ml

Affinity chromatography with 5'-adeonsine phosphate-AH-Sepharose 4B. The 5'-adenosine phosphate-AH-sepharose 4B was prepared according to Kanaya and Uchida [17]. 1 mmole of 5'-AMP was stirred with 5 ml 0.2 M $NaIO_4$ for 30 min at room temperature. To this was added 10 g A–H Sepharase 4B (Pharmacia Fine Chemicals, Inc., Piscataway, New York, U.S.A.) dissolved in 30 ml 0.1 M $Na_4B_4O_7$. Stirring was continued for 2 hr. Solid $NaBH_4$ (3 mmole) was added gradually and the mixture was stirred 30 min at 4°. The resulting gel was washed successively with 5 M NaCl, water and 0.1 M $Na_4B_4O_7$ until the washings had $A_{260\,nm} < 0.07$. The gel was then suspended in 0.025 M acetate, pH 5.3 which was 0.1 M NaCl.

To determine the capacity of the gel 0.2686 g of dry gel (dried at 50–80°) was hydrolysed at 100° with 1.5 ml 1 N H_2SO_4 in a sealed tube for 1 hr. The solution was neutralized with $Ba(OH)_2$. 10 μl of the neutralized solution was injected on a Beckman (Beckman Instruments, Lincolnwood, IL 60646, U.S.A.) C-18 column and chromatographed for adenine by the method of Qureshi *et al.* [18]. The dry gel was found to contain 0.95% adenine.

A column (1 cm diameter and 7 cm height) was prepared and washed with 0.025 M acetate, pH 5.3. 24 ml of enzyme solution (4.6 mg protein) from the P-30 gel filtration was applied to the top of the column at 4°. The column was developed at 21.2 ml per hr with a linear NaCl gradient (0–5 M). Fractions of 4.25 ml per tube were collected.

Reaction with RNA. The substrate was 0.1 mg yeast RNA Type III (Sigma Chemical Co., St. Louis, MO, U.S.A.) per ml 0.1 M sodium acetate, pH 5.0. To 2.94 ml of substrate solution 60 μl of aqueous enzyme solution (18 μg protein, specific activity 10.8) was added and the reaction was conducted at 30°. At 10, 30 and 60 min, 50 μl portions of the reaction mixture were assayed chromatographically for nucleotide products [18]. The detector was a Perkin–Elmer LC75 scanning spectrophotometer (Perkin–Elmer Corp., Norwalk, CT, U.S.A.) with which the products were identified by absorbance ratios at selected wavelengths from 240 to 300 nm. Reference compounds obtained from the Sigma Chemical Co. were chromatographed and scanned similarly. Identification of the reaction products was confirmed by the TLC procedure of Ranerath [19].

Reaction with polynucleotide substrates. Substrates (poly A, poly G, poly C, and poly U) (Sigma Chemical Co., St. Louis, MO, U.S.A.) were prepared at 0.1 mg/ml in 0.1 M acetate, pH 5.9. To 1 ml substrate 2 μg of enzyme (specific activity 7.7) or 0.9 μg of enzyme (specific activity 22) was added in 25 μl of solution. The increase in absorbance at 260 nm was followed as for the RNase assay

For the determination of optimal pH, the poly A substrate was prepared in 0.1 M acetate buffer at pH 4.5, 5.0, 5.5, 6.0 and 6.5; and in 0.1 M Tris chloride buffer at pH 6.5, 7.0, and 7.5. From 2 to 8 μg of the enzyme (specific activity 7.7) per ml of substrate solution were used for the activity assay.

Reaction with dinucleoside monophosphates. The dinucleoside monophosphates (Sigma Chemical Co., St. Louis, MO, U.S.A.) were prepared at 5 mM in 0.1 M acetate buffer at pH 5.0. 100 μl of the substrates which contained 6 μg of enzyme (specific activity 10.8) were incubated 15 min at 30°. The adenylyl (3' → 5') guanosine solution contained only 0.6 μg of enzyme in one experiment. After incubation the reaction solutions and appropriate blanks were frozen. As required the solutions were thawed and 10 μl aliquots were assayed for substrate and products by the HPLC method of Qureshi *et al.* [18]. The dinucleoside phosphates used were guanylyl (3' → 5') adeonsine (GPA) and the others shown in Table 3.

Reaction with mono-, di- and triphosphonucleosides, p-nitrophenylphosphate, bis-p-nitrophenylphosphate, and thymidine 5'-p-nitrophenylphosphate. The phosphonucleosides (Sigma Chemical Co.) were: 5'-ATP, 5'-GTP, 5'-CTP, 5'-UTP, 5'-ADP, 5'-UDP, 5'-CDP, 5'-GDP, 5'-TMP, 5'-IMP, 5'-CMP, 5'-UMP, 3'-TMP, 3'-IMP, 3'-CMP, 3'-AMP, 3'-UMP, 3'-GMP; 2'-CMP, 2'-AMP, 2'-UMP, 2'-GMP, 2',3'-cyclic AMP; 2',3'-cyclic GMP; 2',3'-cyclic UMP; 2',3'-cyclic CMP.

The cyclic monophosphates were prepared at 5 mM in 0.1 M acetate, pH 5.0 and 200 μl were incubated with 4 or 40 μl (1.2 or 12 μg, specific activity 10.8) of enzyme solution for up to 4 hr at 30°. Products of the reaction were sought by injecting 10 μl of reaction mixture (diluted 1:10 with H_2O) on the C-18 reverse phase HPLC system of Qureshi *et al.* [18].

The remaining nucleoside phosphates (2 mmole) were dissolved in 5 ml of the buffer. Each substrate solution (0.5 ml) was incubated with 10 μl (3.0 μg protein, specific activity 10.8) of enzyme at 30° for 30 min. Products of the reaction were determined as for the cyclic monophosphate reactions.

The reactions with the *p*-nitrophenyl phosphate substrates were performed as described previously [13]. The quantity of enzyme of each assay was 10 μl (3.0 μg, specific activity 10.8).

In all assays appropriate enzyme and substrate blanks were included.

pH Optimum. The RNA substrate (0.1 mg/ml) was prepared in 0.1 M acetate at pH 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. The assays with 1.7 μg of enzyme (specific activity 16.0) were done by the standard procedure with appropriate blanks.

pH Stability. Enzyme was incubated in H_2O (control), in 0.1 M acetate pH 4.5 to 6.5, and in 0.1 M Tris–chloride pH 7.0 to 9.5 at 4° overnight. Activities of the treated enzymes were measured with 60 μg of enzyme protein (original specific activity 10.8).

Thermal optimum. Reactions with appropriate controls were carried out at temperatures of 20–60°. For temperatures of 20–35° 1.5 μg of enzyme (specific activity 8.6) was used: for 40°, 0.9 μg; for 45 and 50°, 0.6 μg; and for 55 and 60°, 0.3 μg.

Thermal stability. For each temperature (4, 25, 30, 40, 50, 60 and 70°) 250 μl of enzyme (15.5 μg protein, specific activity 8.6) in water was incubated for 1 hr. After incubation the enzyme solutions were cooled in ice and the following quantities were used in the activity determination: 4°, 1.22 μg; 20°, 0.92 μg; 30°, 40° and 50°, 1.53 μg; 60°, 3 1 μg; 70°, 6.1 μg.

Slab gel electrophoresis The equipment was the 16 cm Bio Rad Protean (Bio Rad Laboratories, Richmond, CA, 94804, U.S.A.) and was operated according to the manufacturer's instructions. For the SDS procedure (11% gel) the method of Laemmli [20] was used as outlined in the Sigma Bulletin MWS-877L. For the M_r determination the standards were egg albumin, carbonic anhydrase, trypsinogen, beta-lactoglobulin and lysozyme The quantities of standards electrophoresed were 5–8 μg.

For the electrophoresis of the nondenatured enzyme the procedure of the Sigma Bulletin MKR-137 was followed. Gels of 8, 10 and 15% polyacrylamide were used for electrophoresis of 1.3–5 μg of the M_r standards; alpha lactoglobulin, myoglobin, carbonic anhydrase, egg albumin, and bovine serum albumin. The quantitites of enzyme electrophoresed ranged from 0.1 to 0.43 μg for each procedure.

Slabs from both electrophoretic procedures were stained by the silver stain method (Bio Rad Laboratories Bulletin 1089). The nondenatured electrophoresed enzymes were also located by the method of Wilson [21], in which the slabs are incubated in RNA solution, then stained with toluidine blue. The RNase locations where RNA has been hydrolysed show white against the blue RNA-toluidine background.

Effect of cations and sulfhydryl reagents. Ca, Ba, Cu, Mg, Zn and Fe chlorides, maleimide, N-acetylamidazole, and iodoacetic

acid were incorporated separately in the substrate solution at 10^{-3} M, and activities were determined with 25 μl of enzyme solution (3.5 μg protein, specific activity 10.8).

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