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AFFINITY CHROMATOGRAPHY OF *NICOTIANA TABACUM* RIBONUCLEASES

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; ribonuclease; affinity chromatography.

Abstract—The purification of tobacco ribonuclease by affinity chromatography is described. 5′-(4-aminophenylphosphoryl)-guanosine 2′, (3′) phosphate, a ribonuclease inhibitor, has been synthesized and insolubilized onto agarose beads. The resulting adsorbent binds tobacco and some other plant ribonucleases strongly but reversibly at pH 5·4. The bound enzyme can be eluted by changing the pH or ionic strength of the eluting buffer, or by specific elution with substrate or inhibitor. Binding is not due to simple ion-exchange properties of the adsorbent.

INTRODUCTION

THE TECHNIQUE of affinity chromatography has been the subject of a number of recent reviews.[1–3] The principles of the technique, and the main requirements for its use have been well covered in these reviews.

Plant ribonucleases (RNases) have been classified into two main groups.[4] Both groups have acid pH optima and hydrolyse ribonucleic acid (RNA) to the 2′,3′-cyclic ribonucleoside monophosphates as the primary products. RNases from group 1 then hydrolyse the purine cyclic nucleotides to the 3′-monophosphates, but do not hydrolyse the pyrimidine cyclic nucleotides. Enzymes from group 2 hydrolyse both purine and pyrimidine cyclic nucleotides. A number of plant RNases have been shown to be subject to inhibition by the end products of RNA hydrolysis,[5,6] and 2′,(3′)-guanosine monophosphate (GMP) is a particularly potent inhibitor of *Avena* and tobacco RNases.

This paper describes the synthesis of an insoluble RNase inhibitor based on GMP, and its use in the purification of some plant RNases. Some of the work described here has been reported in a preliminary communication.[7]

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RESULTS AND DISCUSSION

Behaviour of adsorbents

The inhibitor used here is similar to those used for purifying staphylococcal nuclease,[8] and bovine pancreatic RNase A.[9] The amino phenyl phosphoryl spacer group was introduced into the GMP molecule to overcome the problem of steric hinderance that is frequently encountered in affinity chromatography.[1-3] Behaviour of the adsorbents was examined using RNase already purified by standard techniques.[6] Adsorbents made by attaching GMP to Sepharose by the purine amine group (GMP-Sepharose) did not bind RNase. In contrast adsorbents prepared by attaching APP-GMP to Sepharose (APP-GMP-Sepharose), presumably by the phenyl amine group, did bind RNase at pH 5·4 under conditions of low ionic strength. The bound enzyme could be eluted by raising the pH to 9 with Tris-HCl, but not by lowering the pH to 3 with acetic acid. Elution of the bound RNase could also be achieved by raising the ionic strength of the solution passing through the column with KCl (Fig. 1). The concentration of KCl required to elute the enzyme was about 150 mM. At this concentration, the activity of the enzyme was severely inhibited, so it seems unlikely that the enzyme is bound to the column by non-specific ion exchange. This was confirmed by eluting the column with GMP solutions of different concentrations in 20 mM NH_4 acetate–KCl pH 5·4. At concentrations as low as 10^{-6} M. GMP solutions eluted all the bound RNase from APP-GMP-Sepharose adsorbents (Fig. 2a). Solutions of RNA in starting buffer (2 mg RNA/ml) would also elute the enzyme from the column (Fig. 2b). In addition to elution of the bound enzyme by substrate and inhibitor, the column of APP-GMP Sepharose would bind all RNase even in the presence of high concentrations (2 mg/ml) of contaminating protein (Fig. 2c). All these results indicate that APP-GMP-Sepharose does bind tobacco RNase by specific affinity rather than by non-specific ion exchange.

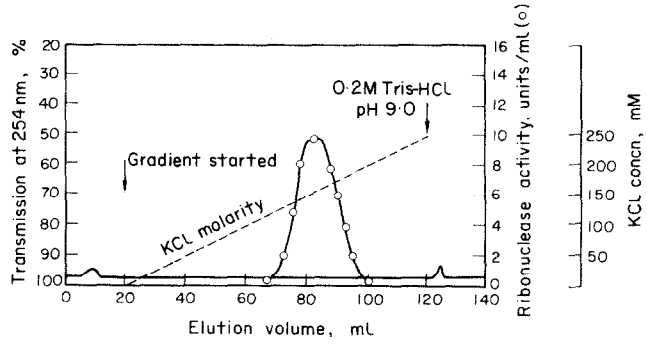

FIG. 1. EFFECT OF KCl CONCENTRATION ON THE ADSORPTION OF TOBACCO (RNASE).
A sample of purified tobacco RNase was applied to an APP-GMP column in 20 mM NH_4 acetate pH 5·4. The column was washed with 20 ml of this buffer and then eluted with a linear gradient 0–500 mM KCl in 20 mM NH_4 acetate KCl (total vol. 100 ml).

Affinity chromatography of crude leaf RNase

Crude leaf extracts would be expected to contain, in addition to contaminating proteins, nucleic acid breakdown products. As these inhibit RNase, competition would be expected

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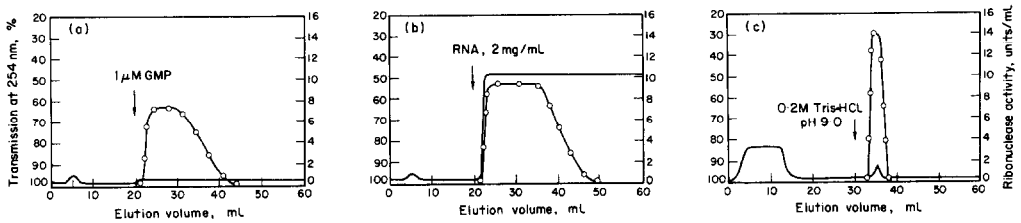

FIG. 2. SPECIFIC ELUTION OF RNase FROM AFFINITY COLUMNS.
Figs 2(a) and (b). Samples of RNase were applied to APP-GMP-Sepharose columns in 20 mM NH_4 acetate–KCl pH 5·4 buffer. The columns were then washed with 20 ml of the same buffer and then eluted with either 1 μM GMP or 2 mg/ml RNA in starting buffer. Fig. 2(c). A sample of RNase was mixed with 10 ml of a solution of soya bean trypsin inhibitor (2 mg/ml) and applied to an APP-GMP-Sepharose column. After washing with 20 ml of starting buffer, the enzyme was eluted with 200 mM pH 9·0 Tris-HCl.

between the soluble nucleotides and the insolubilized GMP, for the RNase. The effectiveness of 10^{-6} M GMP in eluting RNase from APP-GMP-Sepharose indicates this. When crude leaf preparations were applied to affinity columns (Fig. 3), competition was in fact observed. Only a fraction of the total RNase was bound by the column, the rest of the enzyme passed through unretarded.

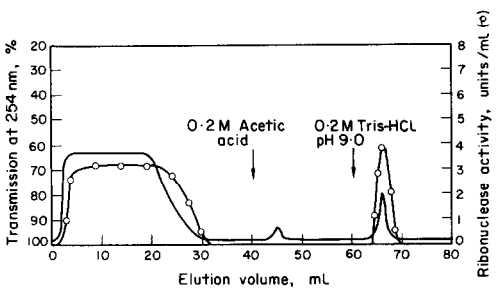

FIG. 3. CHROMATOGRAPHY OF CRUDE TOBACCO LEAF RNase ON AFFINITY COLUMNS.
A sample of crude tobacco leaf extract (20 ml) was applied to a column of APP-GMP-Sepharose in 20 mM acetate–KCl buffer pH 5·4. The column was then washed with 20 ml of the same buffer followed by 20 ml pH 3 acetic acid and 20 ml 200 mM Tris-HCl pH 9·0.

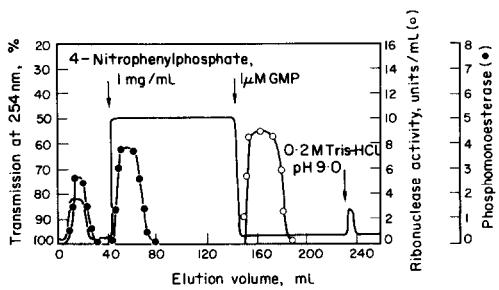

FIG. 4. SPECIFIC ELUTION OF PHOSPHOMONOESTERASE FROM AFFINITY COLUMNS.
A sample of partially purified RNase was applied to an APP-GMP-Sepharose column. The column was washed with starting buffer and then eluted with 100 ml of 4-nitrophenylphosphate (1 mg/ml) in starting buffer, followed by 100 ml 1 μM GMP in starting buffer.

Another problem was encountered when crude leaf preparations were applied to affinity columns. It was found that, after a crude leaf extract had been purified on an affinity column, the column lost most of its ability to bind purified RNase. This was shown to be due to the presence, in crude leaf extracts, of an enzyme that could hydrolyse the insolubilized inhibitor. This enzyme was a phosphodiesterase with a MW of about 55000.[10] It could be removed from RNase preparations by gel filtration, hence partial purification of all leaf extracts by ammonium sulphate precipitation and gel filtration was adopted as a necessary preliminary to affinity chromatography on the adsorbent described here.

[10] JERVIS, L., unpublished results.

Affinity chromatography of partially purified RNase

When samples of partially purified RNase were subjected to affinity chromatography on APP-GMP-Sepharose, excellent purification was achieved with KCl elution or GMP elution. Elution with pH 9 Tris-HCl was less satisfactory because this buffer also eluted a considerable amount of polyphenolic material bound to the adsorbent by non-specific ion exchange. All methods of elution gave RNase that was contaminated with phosphomonoesterase activity. This activity was best removed from affinity columns before elution of the RNase and washing the column with a solution of 4-nitrophenyl phosphate in starting buffer (1 mg/ml) was effective in doing this (Fig. 4). The nitrophenyl phosphate did not elute RNase and this could be released later as desired.

Purification

Excellent purification of tobacco RNase was achieved by affinity chromatography on APP-GMP-Sepharose adsorbents. The specific activity of the purified enzyme varied with the method of elution used (Table 1). The elution procedure adopted was chosen with regard to the future use of the enzyme. Elution with either a KCl gradient or with 10^{-6} M GMP gives the enzyme in a relatively large volume (*ca* 40–50 ml) whereas elution with pH 9 Tris-HCl releases all the enzyme in less than 5 ml. Because Tris-HCl elution gives a preparation contaminated with polyphenols, if enzyme is required in a small volume it is usually convenient to elute the enzymes with KCl, dialyse away the KCl, reapply the enzyme to another affinity column and elute with Tris-HCl.

TABLE 1. PURIFICATION OF TOBACCO RNase BY AFFINITY CHROMATOGRAPHY

Purification stage	Sp. act. (RNase units/mg protein)	Fold purification
Crude leaf extract	20	1
Partially purified RNase	1400	70
Tris-eluted RNase	56 000	2800
KCl-eluted RNase	83 000	4150
GMP-eluted RNase	117 000	5850

The capacity of adsorbents varies with the preparation but usually a 0·8 × 10 cm column would purify all the RNase extracted from 1 kg of tobacco leaves.

Many plants appear to contain RNases that are relatively purine specific and the adsorbent described here may prove generally useful for the purification of this type of RNase. It has been successfully used to purify RNase from pea leaf extracts and to remove RNase contamination from commercial preparations of soya bean trypsin inhibitor.

EXPERIMENTAL

Materials. Guanosine 2′,(3′)-monophosphoric acid was from Sigma Chemicals Ltd., London. Highly polymerized yeast RNA, sodium 4-nitrophenyl phosphate and dicyclohexylcarbodi-imide were from B.D.H. Ltd. Cyanogen bromide was from Koch-Light Ltd.

Synthesis of 5′(4-aminophenylphosphoryl)-guanosine 2′,(3′)-phosphate (APP-GMP). Guanosine 2′,(3′)-monophosphoric acid (1 mmol) was converted to the 2′,3′-cyclic phosphate, tributylamine salt, using dicyclohexylcarbodiimide (DCC) as the dehydrating agent.[11] The nucleotide was dried azeotropically from anhyd. dimethylformamide-pyridine (3:2). The dry nucleotide was then dissolved in 10 ml of the solvent mixture. The Na salt of 4-nitrophenyl phosphoric acid (NPP) (2 mmol) was converted to the pyridine salt with Zeo-Karb 225 (pyridine

[11] SHUGAR, D. (1967) *Meth. Enzymol.* **12**, 131.

form). The salt was dried as described above and dissolved in the nucleotide soln. DCC (10 mmol) was added and the mixture was warmed until homogeneous. The mixture was then incubated in the dark at room temp. for 5 days with exclusion of H_2O. After incubation, 1 ml H_2O was added and after 24 hr a further 50 ml H_2O was added. The mixture was shaken vigorously and filtered to remove insoluble dicyclohexylurea. The insoluble residue was extracted another 3 × with 20 ml portions of H_2O. The aq. extracts were combined and washed 3 × with Et_2O. The aq. soln was passed through a Zeo-Karb 225 (H^+) column and then adjusted to pH 6·3 with NH_4HCO_3 and applied to a 4 × 20 cm column of DEAE-cellulose (HCO_3^{2-}), (Whatman DE 32) equilibrated with 10 mM NH_4HCO_3. The column was then developed with a linear gradient 10–400 mM NH_4HCO_3 (4 l.). The effluent was monitored at 254 nm, and the absorption spectrum from 220 to 350 nm of each peak was examined. 5′(4-Nitrophenyl phosphoryl)-guanosine 2′,(3′)-phosphate was detected in the fraction eluted between 2450 and 3150 ml. This fraction was collected and concentrated to dryness. The dry product was kept under vacuum for 24 hr to ensure complete volatilization of NH_4HCO_3. Incubation of the product with snake venom phosphodiesterase released 1 mol of 4-nitrophenol per mol of purified product, indicating that the 4-nitrophenol group was attached to the 5′ position of the GMP molecule. The product was converted to the amino derivative by hydrogenation over 10% Pal/C. The hydrogenation was carried out in water for 24 hr[12] and was quantitative. The catalyst was removed by filtration through celite and the filtrate was collected and lyophilized.

Preparation of adsorbents. Both APP-GMP and 2′,(3′)-GMP were covalently attached to Sepharose 2B by the cyanogen bromide procedure.[13] Coupling was carried out in 100 mM pH 9·5 $NaHCO_3$ buffer for 24 hr at 4°. After coupling, adsorbents were packed into small columns and washed with 1 l. of each of the following: 200 mM HOAC containing 500 mM KCl; 200 mM Tris-HCl pH 9·0 containing 500 mM KCl; 50 mM NH_4 acetate pH 5·4 containing 500 mM KCl; 20 mM NH_4 acetate containing 20 mM KCl. Adsorbents usually contained 2–3 μmol of APP-GMP or 0·5–1·0 μmol 2′,(3′)-GMP per ml of gel.

Enzyme preparations. Purified tobacco RNase 1 was prepared as described elsewhere.[6] Crude tobacco leaf RNase was prepared by macerating fresh or frozen tobacco leaves in 50 mM pH 5·4 NH_4 acetate buffer. The resulting pulp was filtered through muslin and the filtrate was centrifuged for 30 min at 5000 *g* to remove insoluble material. The extract was diluted to 20 mM NH_4 acetate and readjusted to pH 5·4 if necessary. Partially purified tobacco leaf RNase was prepared by extracting leaves as above and precipitating RNase at pH 5·5 by making the extract 90% saturated with respect to $(NH_4)_2SO_4$. The precipitated material was dissolved in 20 mM pH 5·4 Na acetate buffer containing 20 mM KCl and the soluble material was applied to a 4 × 90 cm column of Sephadex G100 equilibrated and eluted with the same buffer. Fractions containing RNase were combined and used as partly purified RNase.

Enzyme assay. Ribonuclease was assayed at pH 5·4 using highly polymerized yeast RNA as substrate,[14] by measuring the increase in acid-soluble hydrolysis products. One unit of activity was defined as the amount of enzyme causing an increase of 0·1 absorbance units at 260 nm in 1 hr. Phosphomonoesterase was assayed at pH 5·4 using 4-nitrophenyl phosphate as substrate.[15] Phosphodiesterases were assayed at pH 5·4 and at 8·9 using bis-*p*-nitrophenylphosphate as substrate.[16] In addition, enzyme preparations were assayed for their ability to hydrolyse 5′-(4 nitrophenylphosphenyl)-guanosine 2′(3′)-phosphate.

Protein estimation. Protein was estimated by the Lowry method,[17] using bovine serum albumin as the standard protein.

Column operation. Adsorbents were packed into small columns (0·8 × 10 cm) and washed with 20 mM acetate–KCl pH 5·4. Enzyme preparations were applied to columns of the same buffer. After application of the enzyme, columns were washed with starting buffer until the UV absorption at 254 nm fell to its original value. Columns were eluted as described in the text.

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