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Note

Single-step high-performance liquid chromatographic analysis of nucleoside monophosphates for ribonuclease specificity

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In previous studies it was shown that a lysosomal fraction of tomato fruits[1] and isolated vacuoles of a tomato cell suspension culture[2,3] harbour RNA-splitting activity. Using cultured tomato cells, this activity was purified to near homogeneity, characterized as a cycling ribonuclease of the RNase I type and classified as E.C. 3.1.27.1[4]. The enzyme splits RNA endonucleolytically by a phosphotransferase reaction yielding 2′:3′-cyclic nucleoside monophosphates and, to minor extents, 3′(2′) nucleoside monophosphates. To compare this vacuolar tomato enzyme with other characterized plant ribonucleases it was necessary to study in more detail the base specificity of the splitting reaction. This can be done by using yeast RNA as a substrate and by analyzing quantitatively the appearance of monomeric end products.

In this paper we present a reversed-phase high-performance liquid chromatographic (HPLC) method which allows the measurement of the formation of 2′:3′-cyclic nucleoside monophosphates and 2′(3′)-nucleoside monophosphates resulting from the enzymatic hydrolysis of yeast RNA.

EXPERIMENTAL

Materials

RNA (yeast) was obtained from Boehringer (Mannheim, F.R.G.) and nucleoside monophosphates from Serva Feinbiochemica (Heidelberg, F.R.G.). All other solvents and chemicals were of analytical reagent grade.

Apparatus

The HPLC equipment included a pump (Liquochrom 307, Budapest, Hungary), a variable-wavelength UV detector (Model OE-308; Labormim, Budapest, Hungary) and a recorder (Model OH-814/1; Radelkis, Budapest, Hungary).

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Sample preparation

The cultivation of tomato (*Lycopersicon esculentum*) cells[5], purification and routine assay of vacuolar ribonuclease with yeast RNA as a substrate[3] have been described. Prior to use, RNA was chromatographed on Sephadex G-25 and the high-molecular-weight fraction was employed.

For enzymatic RNA hydrolysis, reaction mixtures contained in a total volume of 2 ml 50 m*M* acetate buffer (pH 5.6), 10 mg yeast RNA as a substrate and 0.4 Wilson units[3] of purified ribonuclease (specific activity 68 400 Wilson units per mg protein). After an appropriate time, 100 μl were withdrawn and pipetted into 900 μl 96% (v/v) ethanol. After chilling (−20°C, 12 h) and centrifugation (11 000 *g*, 15 min), the supernatants were evaporated. Samples were solubilized using the HPLC eluent (see below), and aliquots (20 μl) were injected.

HPLC conditions

The HPLC column was a standard prepacked octadecyl-silica 100 Polyol (10 μm) column (250 mm × 4.6 mm), from Serva Feinbiochemica. Chromatographic experiments were performed isocratically, using 0.02 *M* ammonium dihydrogen-phosphate (pH 6.2) as the mobile phase at a flow-rate of 1.5 ml/min and were followed at 254 nm. Before elution, the column was equilibrated with the mobile phase for 60 min. The column was cleaned at the end of the day with methanol–water (30:70).

RESULTS AND DISCUSSION

For this study it was necessary to separate all possible 2′:3′-cyclic nucleoside monophosphates and 3′(2′)-nucleoside monophosphates from each other under isocratic conditions in a single step. Inspection of the literature revealed that no such method was available. In earlier studies, either insufficient resolution of these compounds was achieved[6,7] or cyclic nucleoside monophosphates were not included[8].

Using phosphate buffer of low ionic strength under isocratic conditions, all the respective standard substances were separated (Table I). Compared with the results of Nguyen *et al.*[9], a better resolution of pyrimidine nucleoside monophosphates was

TABLE I

RETENTION TIMES OF RIBONUCLEOSIDE MONOPHOSPHATES

Separation conditions as in Fig. 1.

Nucleotide	*Retention time (min)*	*k′*
3′(2′)-CMP	3.4	0.6
2′:3′-cCMP	3.8	0.8
3′(2′)-UMP	4.1	0.9
2′:3′-cUMP	4.6	1.2
3′(2′)-GMP	6.1	1.9
2′:3′-cGMP	14.0	5.6
3′(2′)-AMP	17.0	7.0
2′:3′-cAMP	44.0	19.7

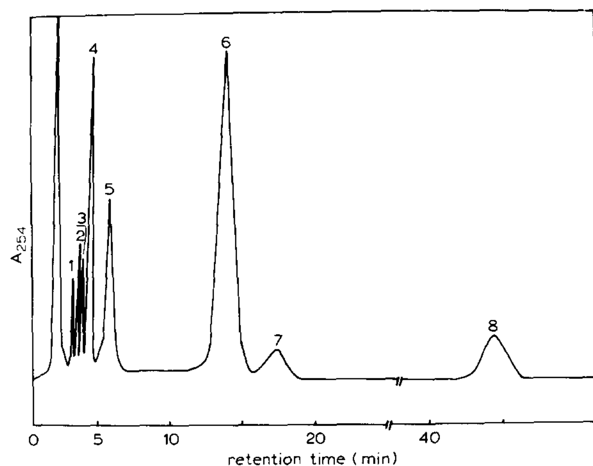

Fig. 1. Elution profile of ribonucleoside monophosphates after hydrolysis of yeast RNA with a purified plant ribonuclease. Incubation time: 30 min. For assay conditions see Experimental. Peaks: 1 = 3′(2′)-CMP; 2 = 2′:3′-cCMP; 3 = 3′(2′)-UMP; 4 = 2′:3′-cUMP; 5 = 3′(2′)-GMP; 6 = 2′:3′-cGMP; 7 = 3′(2′)-AMP; 8 = 2′:3′-cAMP.

achieved. Moreover, we succeeded in separating both cyclic pyrimidine nucleoside monophosphates.

An elution profile of the monomeric end products of ribonuclease acting on yeast RNA as a substrate is shown in Fig. 1. The results obtained are in accord with the generally accepted mechanism for this type of RNase[10] which suggests the initial formation of 2′:3′-cyclic nucleoside monophosphates. These products are subsequently hydrolyzed in a slower reaction to the corresponding 3′(2′)-nucleotides. More importantly, from the elution profile a preferential release of guanosine nucleotides during RNA hydrolysis can be seen. From this result, cleavage specificity of the ribonuclease adjacent to this purine base must be deduced.

Application of the method described to kinetic studies and to the analysis of the hydrolytic activity toward different RNA types will further advance our knowledge of the specificity of such enzymes.

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