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High-performance liquid chromatography of deoxyribonucleoside di- and triphosphates in tomato roots

INDRANI DUTTA*, PROBIR K. DUTTA, DON W. SMITH and GERARD A. O'DONOVAN
Department of Biological Sciences, University of North Texas, Denton, TX 76203 (U.S.A.)

ABSTRACT

The levels of endogenous deoxyribonucleotides in plants are greatly affected by the absence of the micronutrient boron. Described here is a high-performance liquid chromatographic assay for quantitating deoxyribonucleoside di- and triphosphates in tomato root tip tissues under boron-sufficient and boron-deficient conditions. The extraction procedure consists of (a) removing phenols from the tomato root tips by grinding frozen tissue in 6% trichloroacetic acid containing polyvinylpolypyrrolidone, (b) neutralizing the extract with freon-amine, (c) selective degradation of ribonucleotides with periodate, followed by (d) treatment with rhamnose and methylamine prior to injection of the deoxyribonucleotides into an anion-exchange column. Results indicate that the eight deoxyribonucleoside di- and triphosphates can be reproducibly resolved and quantified. By this extraction procedure, column life was extended to more than 70 runs per cartridge. This assay technique can be used with less than 500 mg of fresh root tip tissue and allows quantitation of plant deoxyribonucleotides in the picomole range.

INTRODUCTION

Boron is required as an essential micronutrient for the growth of vascular plants [1,2] and influences nucleic acid metabolism in all plants [3–5]. One way to monitor changes in nucleic acid metabolism is to compare the concentrations of ribo- and deoxyribonucleotides in boron-sufficient and boron-deficient plants. We have reported earlier on the effects of boron deficiency on the ribonucleotide pools [3] as determined by high-performance liquid chromatography (HPLC). Results of that study have led us to believe that the level of the deoxyribonucleotide pools in the boron-deficient plants would be simultaneously affected, since deoxyribonucleotides are required in the micromolar range for DNA replication and cellular nucleotide metabolism [3]. HPLC is the method of choice for monitoring such deoxyribonucleotide pool changes in animal systems [4,5]. Although HPLC can be readily used for quantifying plant ribonucleotides in boron-sufficient plants [3,6–9], there have been no published reports on deoxyribonucleotide pools in plant cells. It is therefore important to address some of the existing problems in analyzing deoxyribonucleotides in plant cells. These problems include: (a) There is a large excess of ribonucleoside triphosphates (rNTPs) over deoxyribonucleoside triphosphates (dNTPs). As a result, several methods of selective degradation of ribonucleotides in cell extracts and subsequent analysis by HPLC have been reported [4,5,10–12]. (b) The chromato-

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graphic separation and UV determination of deoxyribonucleotides in plant extracts are greatly hindered in tomato roots by the interference of phenols [3,13,14,16].

In the present experiments, we report an improved method for absorbing phenols by water-insoluble polymers having hydrogen-bonding capability, such as polyvinylpolypyrrolidone (PVPP) [3,14,15], and a modified selective degradation procedure of ribonucleotides from boron-deficient root tissues. Herein, we describe such a method, which affords detection of nanomolar concentrations of deoxyribonucleotides from 500 mg tomato root tips by anion-exchange HPLC.

EXPERIMENTAL

Chemicals and reagents

Nucleotides, trichloroacetic acid (TCA), PVPP, tri-*n*-octylamine, sodium periodate, methylamine and rhamnose were from Sigma (St. Louis, MO, U.S.A.); 1,1,2-trichloro-1,2,2-trifluoroethane (freon) and monobasic ammonium phosphate were from Mallinckrodt (Paris, KY, U.S.A.) and potassium chloride was from Eastman Kodak (Rochester, NY, U.S.A.). All other chemicals were of analytical grade and were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Plant material

Tomato seeds (*Lycopersicon esculentum* Mill., cv. Improved Summertime) were obtained from Texas A & M University Experiment Research Station (College Station, TX, U.S.A.). Seeds were germinated in plastic Petri plates (9 cm in diameter) after a disc of Whatman No. 1 filter paper had been inserted into each dish. The filter paper was moistened with water, which had been deionized with a Milli-Q water system (Millipore, Bedford, MA, U.S.A.). Petri dishes were incubated for 5–7 days under UV radiation at 26 $\pm$ 2°C until the first true leaf pairs emerged. Seedlings about 5 cm in length with 2 or 3 small leaves, were transplanted to an aerated, full-strength, complete nutrient solution [17]. The pH of the nutrient solution was adjusted to 4.7 by either 0.1 *M* sodium hydroxide or 0.1 *M* hydrochloric acid. Two plants were mounted in a two-holed No. 6 rubber stopper. Fresh nutrient solutions were used after every 5 days. When the plants were 20 cm in height (measured from the cotyledons to the apical meristems), they were used as experimental materials. At the desired time interval of treatment, the roots were measured and the tips were harvested for nucleotide analysis or returned to the solution for later measurement and analyses.

Extraction of deoxyribonucleotides

Excised root tips were washed, weighed and frozen in liquid nitrogen. The frozen root tissue was stored at −80°C until ready for purification, when it was powdered in a mortar precooled by liquid nitrogen. The powdered root tissue was homogenized in chilled 6% (w/v) TCA, containing PVPP (0.5 g/10 ml), and incubated on ice in a gyrating shaker (New Brunswick Sci. Co., Edison, NJ, U.S.A.) for 30 min before centrifugation at 14 000 *g* for 12 min. The acid extract, containing nucleotides, was neutralized with an equal volume of ice-cold freon-amine [18] solution. The freon-amine mixture was agitated on a vortex mixer for 1 min and allowed to separate while standing at 4°C for 10 min. The top aqueous layer, which contained the deoxyribonucleotides, was removed with a 5-ml syringe and frozen at −80°C until ready for use.

Modified selective degradation of ribonucleotides [4,5]

To 160 μl of neutralized extract, 40 μl of 0.25 *M* $NaIO_4$ were added and incubated for 5 min at 37°C. The mixture was then centrifuged at 8000 *g* for 2 min. Next, 60 μl of 4 *M* methylamine and 14 μl of 1 *M* rhamnose were added before incubation on ice for 2 min. The mixture was then incubated for 30 min at 37°C before centrifugation at 8000 *g* for 2 min. This extract was then filtered through a 0.45-μm ACRO LC3A filter (Gelman, Ann Arbor, MI, U.S.A.) and kept frozen at −20°C until used.

Chromatographic apparatus and conditions

The HPLC equipment (Waters, Milford, MA, U.S.A.) consisted of two Model 510 pumps, a Model 680 automated gradient controller, a U6K injector and a Model 481 LC spectrophotometer. Deoxyribonucleotides were detected by monitoring the column effluent at 254 nm with the sensitivity fixed at 1 V a.u.f.s. (absorbance unit full scale). Separations were performed on a Beckman Ultrasil AX (10 μm) column (25 cm × 4.6 mm I.D.). The elution buffer system used consisted of eluent A, 7 m*M* $NH_4H_2PO_4$ (pH 3.8), and eluent B, 250 m*M* $NH_4H_2PO_4$ (pH 4.5) with 500 m*M* potassium chloride [3].

Deoxyribonucleotide samples (100 μl) obtained from plant root tips were injected into the column. A linear gradient of eluent A to eluent B was applied for 40 min, followed by an isocratic period of 20 min with eluent B. The column was re-equilibrated by washing with 30 ml of eluent A. The flow-rate was maintained at 1 ml/min. Analyses were performed at ambient temperature. Peaks were integrated either manually on a Cole-Palmer (Chicago, IL, U.S.A.) strip-chart recorder or on a Waters 740 data module.

Individual components of the deoxyribonucleotide pool mixture were identified on the basis of their retention time as compared with standards and by injecting known internal standards. The recoveries of deoxyribonucleotides from solutions of deoxyribonucleotide standards were determined by measuring peak heights before and after the extraction procedures. The percentage recoveries of added deoxyribonucleoside diphosphate (dNDP) and dNTP were [mean of two experiments ± standard error of the mean (S.E.M.)]: dTDP, 95 ± 2.1; dCDP, 97 ± 2.8; dADP, 97 ± 4.1; dGDP, 95 ± 1.8; dTTP, 96 ± 6.1; dCTP, 89 ± 2.4; dATP, 98 ± 3.1 and dGTP, 98 ± 1.8.

The concentration of the sample was calculated by comparing its peak height to the standard, for which the concentration was known (1 m*M*). The deoxyribonucleotide concentration in all samples, expressed as nanomoles per gram fresh weight was computed as follows:

$$\frac{S_a}{S_t} C \frac{V_{st}}{V_{sa}} \frac{V}{FW}$$

where S_a is the peak height of the sample, S_t the peak height of the standard, C is the amount (gram) of compound in the standard divided by the molecular weight of the compound, V is the total volume of the sample, V_{sa} the volume of injected sample, V_{st} the volume of injected standard and FW the fresh weight (gram) of the sample.

RESULTS AND DISCUSSION

A major problem associated with determining the level of ribo- and deoxyribonucleotides in plant tissue arises from the severe interference by low-molecular-weight material, such as phenolic compounds and precursors or degradation products of chlorophyll, which are extracted along with the nucleotides [6]. In this research, the small quantity of available root tissue, typically less than 500 mg, was an additional obstacle. As was the case for the ribonucleotides [3], experiments in our laboratory with various extraction techniques have shown that 6% (w/v) TCA produced the most reproducible results. Grinding of the frozen tissue in a precooled mortar proved to be a very useful as well as convenient method of homogenization for the extraction of the deoxyribonucleotides. This was the case also with ribonucleotides [3,8].

Tomato plants are especially rich in phenols. This makes nucleotide determinations very difficult under any circumstance [3]. This was further complicated by the fact that in these experiments the phenol content of the tomato plant tissues increased enormously when they were starved for boron [19]. In order to remove phenol, PVPP was added to the 6% (w/v) TCA solution and used in the homogenization buffer. It was critical that after extraction the samples be shaken on ice for at least 30 min before centrifugation. In this procedure all the phenols are adsorbed and this results in much greater yield of deoxyribonucleotides. Samples prepared by this technique did not affect column life. Of vital importance here was the fact that after this preparation, it was possible to quantitate more than 70 samples on the same Ultrasil AX column without any loss of sensitivity.

There is a previous report [8] which shows that freon-amine neutralization led to lower recovery. In our case, neutralization with potassium hydroxide, followed by removal of the potassium chloride precipitate by centrifugation resulted in a distortion of peaks and decreased column life. Accordingly, the freon-amine neutralization step [18] was used throughout the experiments despite the minor loss (3%) in recovery.

There are some limitations to the measurement of deoxyribonucleotides, which are present in very low concentrations. In order to overcome these limitations, selective degradation of ribonucleotides prior to HPLC has been devised. Using three previously described methods [4,5,19], total nucleotide pool extracts were treated to destroy the ribonucleotides in the sample. All three methods had to be modified so that HPLC could be used for tomato root tips. Analysis of the ribonucleotide standards showed that these methods effectively destroyed the ribonucleotides. Analysis of mixed deoxyribonucleotide standards, treated in the same way, showed that the recovery of deoxyribonucleotides was not reduced and that the chromatography of these compounds was not altered. Treatment of sample labelled with ^{3}H-dTTP showed that the retention of this compound was not altered.

Because previous methods [4,5] appeared to reduce the resolving power of the column after about 10 runs, we devised a new technique, based on an earlier method [5] which proved to be very successful for plant dNDP and dNTP. This is described under *Modified Selective Degradation of Ribonucleotides*, above. We applied this method to a standard mixture of the eight ribonucleotides and the eight deoxyribonucleotides, using concentrations typical of those found in plant tissue extracts. All ribonucleotides were degraded completely, and this gave highly reproducible and accurate results (data not shown). In the present method, the nucleotide pool extract was incubated for 5 min

before methylamine and rhamnose were added. All solutions were added without altering the pH of any mixtures.

Fig. 1A shows the chromatogram of a mixture of the eight dNDP and dNTP standards. In order to avoid errors in identification due to any variation in retention times, a mixed standard was injected between every sample run. Regardless of individual variation, retention times always fell in the pattern: diphosphates < triphosphates. As seen in Fig. 1A, all eight deoxyribonucleoside di- and triphosphates were separated. The quantitative HPLC data are presented in Table I. Extracts were prepared from the roots of boron-sufficient (B^+, positive control) and boron-deficient (B^-, negative control) tomato plants. As can be seen from Table I, there was a significant decrease in the levels of all deoxyribonucleotides in the boron-deficient plants. When tomato plants were starved of boron for longer than two days, their roots did not recover and the plants died. When boron was provided to plants after they had been starved of boron for two days, recovery, as reflected in the nucleotide levels, was not at all certain (see Table I, column 3). Though the plants seemed to survive, they were unable to replenish their nucleotide pools to the boron-sufficient levels. It is noteworthy that addition of the pyrimidine base, uracil, to boron-deficient nutrient

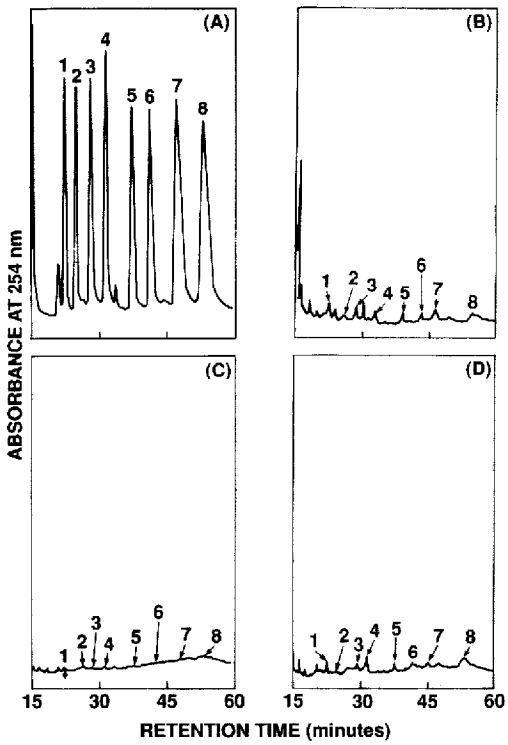

Fig. 1. Representative anion-exchange chromatograms of deoxyribonucleotides on a Beckmans Ultrasil AX column. (A) Standard dNDP and dNTP mixture (100 μl), consisting of 10^{-6} M of each deoxyribonucleotide. (B) Sample (100 μl) from tomato plant root tip tissue grown in boron-sufficient, (C) boron-deficient, (D) uracil-containing boron-deficient media, respectively. Peaks: 1 = dTDP; 2 = dCDP; 3 = dADP; 4 = dGDP; 5 = dTTP; 6 = dCTP; 7 = dATP and 8 = dGTP.

TABLE I

CONCENTRATIONS OF DEOXYRIBONUCLEOSIDE DI- AND TRIPHOSPHATES FROM TOMATO ROOT TIPS (nmol/g FRESH WEIGHT)

All values are the averages of two separate analyses.

	+B[a]	−B[b]	−B +B[c]	−B +U[d]	−B +U[e]
dTDP	3.12	0.83	1.01	1.04	1.02
dCDP	0.97	0.41	0.21	0.83	0.39
dADP	2.67	0.46	0.91	0.36	1.09
dGDP	0.68	0.28	0.51	0.29	0.61
dTTP	2.81	0.61	0.55	0.44	1.87
dCTP	4.31	0.49	0.67	0.97	2.95
dATP	5.40	0.13	4.10	6.01	9.72
dGTP	8.41	0.64	5.31	7.33	8.42

[a] +B = Positive control, boron present at all times.
[b] −B = Negative control, plants grown in boron-free medium for 2 days.
[c] Plants, grown without boron for 2 days, were given boron for an additional 2 days.
[d] Plants, grown without boron for 2 days, were given uracil (0.1 m*M*) for an additional 2 days.
[e] Plants, grown without boron for 2 days, were given uracil (0.1 m*M*) at the time boron was removed.

solution [16] restored the nucleotide pools close to their boron-sufficient level (Fig. 1D and Table I).

Data in Table I demonstrate the following points: (a) Boron starvation depletes the dCTP and dTTP pools much more than it does the dATP and dGTP pools. Indeed, dGTP levels seem almost resistant to boron depletion. This suggests that pyrimidine nucleotides are more susceptible to boron starvation than purine nucleotides. (b) It is not possible to restore the boron-sufficient levels by the re-addition of boron after two days of starvation. This suggests that cell permeability might have been impaired. (c) It is also not possible to restore the boron-sufficient levels by adding uracil after two days of boron starvation. This suggests that the cells can no longer concentrate uridine nucleotides. (d) The inclusion of uracil as soon as boron was removed allowed the restoration of the deoxyribonucleotides to their boron-sufficient levels. This implicates uridine compounds in restoring nucleotide levels to their boron-sufficient state.

The method of selective destruction of the ribonucleotides described here was based on the reactivity of 2′,3′-*cis*-diol of ribonucleotides with periodate. After the inactivation of periodate with rhamnose, the reaction of methylamine caused N-glycosyl bond cleavage with β-elimination of phosphate [20,21]. During the above treatment, ribonucleotides were completely (>99.99%) destroyed to give the bases, while the deoxyribonucleotides were not affected (data not shown). The reaction mixture could then be injected directly into the anion-exchange Ultrasil AX column for analysis. All peaks were identified except one which appeared between dATP and dGTP. So far we have not been able to identify this peak.

The following general statements are made regarding deoxyribonucleotide measurements from the roots of tomato plants: (1) dNDP and dNTP pool levels always paralleled the root elongation data. (2) The four deoxyribonucleoside diphosphates were quantitated in the boron-sufficient samples. (3) Their levels

appeared to be particularly responsive to boron deprivation. (4) The levels of the four dNTPs were higher than those for the dNDPs, regardless of how the samples were prepared (data not shown). (5) As has typically been observed for other systems [22], the dGTP level was the greatest of the four dNTPs though its value in the root was only 8.41 nmol/g fresh weight. (6) This assay procedure could be used to quantitate deoxyribonucleosides at nanomolar concentrations. (7) This method is simple and rapid when compared to other current methods of quantifying deoxyribonucleotides such as the *in vitro* polymerizing system [23] and $^{32}PO_4$ label in thin-layer chromatography [10]. We believe that our method provides a direct and unambiguous measurement of the deoxyribonucleotide concentrations without the necessity of working with ^{32}P. It is rapid and simple. This technique should be of great value in plant physiology experiments where deoxyribonucleotide concentrations need to be measured.

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REFERENCES

1 W. M. Dugger, *Adv. Chem. Ser.*, 123 (1973) 112.
2 D. T. Clarkson and J. B. Hanson, *Annu. Rev. Plant. Physiol.*, 31 (1980) 239.
3 I. Dutta, G. A. O'Donovan and D. W. Smith, *J. Chromatogr.*, 444 (1988) 183.
4 C. Garrett and D. V. Santi, *Anal. Biochem.*, 99 (1979) 268.
5 K. Tanaka, A. Yoshioka, S. Tanaka and Y. Wataya, *Anal. Biochem.*, 139 (1984) 35.
6 R. H. Nieman, D. L. Pap and R. A. Clark, *J. Chromatogr.*, 161 (1978) 137.
7 R. H. Nieman and R. A. Clark, *J. Chromatogr.*, 317 (1984) 271.
8 R. Meyer and K. G. Wagner, *Anal. Biochem.*, 148 (1985) 269.
9 R. Meyer and K. G. Wagner, *Physiol. Plant.*, 67 (1986) 666.
10 C. D. Yegian, *Anal. Biochem.*, 58 (1974) 231.
11 B. Ullman, L. J. Gudas, S. M. Clift and D. W. Martin, Jr., *Proc. Natl. Acad. Sci. USA*, 76 (1979) 1074.
12 E. J. Ritter and L. M. Bruce, *Biochem. Med.*, 21 (1979) 16.
13 R. M. Reeve, *Am. J. Bot.*, 46 (1959) 210.
14 R. Watanabe, W. J. McIlrath, J. Skok, W. Chorney and S. H. Wender, *Arch. Biochem. Biophys.*, 94 (1961) 241.
15 W. D. Loomis and J. Battaile, *Phytochemistry*, 5 (1966) 423.
16 R. A. Anderson and J. A. Sowers, *Phytochemistry*, 7 (1968) 293.
17 D. I. Arnon and D. R. Hoagland, *Soil Sci.*, 50 (1940) 463.
18 J. X. Khym, *Clin. Chem. (Winston-Salem, N.C.)*, 21 (1975) 1245.
19 H. J. Perkins and S. Aronoff, *Arch. Biochem. Biophys.*, 64 (1956) 506.
20 J. X. Khym and W. E. Cohn, *J. Biol. Chem.*, 236 (1961) PC9.
21 D. H. Rammler, *Biochemistry*, 10 (1971) 4699.
22 R. L. Hopkins and M. F. Goodman, *J. Biol. Chem.*, 260 (1985) 6618.
23 U. Lindberg and L. Skoog, *Anal. Biochem.*, 34 (1970) 152.