Cytokinins in Pisum Transfer Ribonucleic Acid

HENDRIK J. VREMAN and FOLKE SKOOG
Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706

CHARLES R. FRIHART and NELSON J. LEONARD
School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Five cytokinin-active ribonucleosides have been isolated from the transfer RNA of 7-day-old green pea shoots (Pisum sativum L. var. Alaska). Ultraviolet spectroscopy and mass spectrometry have been used to identify 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosyluridine, 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosyluridine, and 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosyluridine. The latter was separated into the cis- and trans-isomers by thin layer chromatography. The fifth cytokinin is indicated to be 6-(3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosyluridine on the basis of its chromatographic properties.

Cytokinin-active ribonucleosides have been found in tRNA hydrolysates from a wide variety of organisms (4, 7, 10, 15, 19). The number and proportions of different cytokinins depend on the source of the tRNA (19). In tRNA from Escherichia coli, ms-2iPA predominates and, with 2iPA, accounts for the bulk of the observed cytokinin activity (1, 5, 8), whereas yeast (3, 6) and Staphylococcus epidermidis (2) tRNA appear to contain mainly 2iPA. In tRNA from higher plants (spinach, pea, and corn) ribosyl-cis-zeatin has been reported to be present together with 2iPA in the first two species, but 2iPA was not found in corn (11, 12). Ribosyl-cis-zeatin is also the predominant cytokinin in tRNA from wheat germ (7, 14, 18) and from tobacco callus tissue, grown on a medium containing 6-benzylaminopurine (9), but in both these preparations from nongreen tissues, ms-ribosylzeatin and 2iPA have also been found. In wheat tRNA, a fourth cytokinin-active constituent was identified as ms-2iPA.

This report deals with the isolation and identification of five cytokinins from tRNA of young, green pea shoots.

MATERIALS AND METHODS

Pea seeds, Pisum sativum L. var. Alaska, (13.5 kg) were soaked in aerated tap water for 16 hr and were then planted on one-fourth inch mesh wire screens, 4 cm deep in a 10 cm layer of vermiculite and grown in flats in a greenhouse for 7 days. The daytime temperature ranged from 28 to 35 C. At harvest the screens with the plants attached were lifted from the flats, and the vermiculite was removed by inverting and gently shaking the wire screens. The young shoots, 7 to 10 cm long, were cut off just above the cotyledons, rinsed in ice water, and stored at -20 C (16 days). The total yield of fresh weight was 13.2 kg.

The tRNA was isolated essentially by the method used by Burrows et al. (9) for tobacco callus tRNA with the following major modifications. The frozen tissue (13.2 kg) was homogenized in batches of 150 g, each with 200 ml of a medium containing 0.1 m tris-HCl buffer, pH 7.6, and 2% sodium lauryl sulfate. After stirring for 30 min at 25 C and defoaming with drops of diethyl ether, the homogenate was centrifuged. The nucleic acids were precipitated with 1.5, instead of the specified 2.5, volumes of 95% ethanol. A phenol extraction was performed after, instead of before, the treatment with 3 m NaCl. To lower the carbohydrate content, the crude tRNA was passed twice through a DEAE-cellulose column loaded to provide 1 g of cellulose for each 150 g fresh weight of the tissue. The cytokinin-active ribonucleosides in the tRNA (24,600 A260 units or 1.36 g) were extracted and purified by procedures described by Burrows et al. (9). All fractions were tested for cytokinin activity by the tobacco bioassay (17) as adapted for this purpose (20). The medium contained the mineral salts and organic constituents specified in Table VI, part A and B, of reference 17.

Ultraviolet-absorbing regions on the paper chromatograms, obtained by the described procedure (9), were eluted with 95% (v/v) ethanol. Before the eluates were dried and subjected to spectral analyses, 5% aliquots were removed for thin layer chromatography and subsequent bioassays. The thin layer chromatography was performed with cellulose powder (MN 300 F254, Merck, Nagel and Co., 516 Düren, Germany) and 19% (v/v) ethanol. The plates were divided so as to separate UV absorbing and/or fluorescent spots. Each spot and the areas in between were bioassayed.

Each of the cytokinin-active eluates was taken to dryness and then transferred to a 0.1 cm UV cell with three washings of 100 μl of absolute methanol. The UV spectra were determined.
RESULTS

The ethyl acetate-soluble ribonucleosides from the tRNA hydrolysate, fractionated on a Sephadex LH-20 column with 33% (v/v) ethanol, separated as shown in Figure 1. The cytokinin activity was distributed as follows: fractions 5, 6, and 7 corresponded to the elution volume of ribosylezatin (cis- and/or trans-isomers). Fractions 10 and 12 corresponded to the elution volumes of 2iPA and ms-ribosylezatin respectively. Fraction 11 apparently contained some of each of the latter two compounds. Fraction 19, which had barely detectable cytokinin activity in the bioassy of a 5% aliquot, represented the elution volume of ms-2iPA. All other fractions had less than detectable cytokinin activity.

Fractions 5, 6, and 7 (Fig. 1) were combined, lyophilized, dissolved in distilled water, and fractionated on a Sephadex G-10 column with distilled water as solvent. Cytokinin activity was detected in fractions 7', 8', 9', and 10' (Fig. 2), and the elution volume of this peak corresponded to that of ribosylezatin (cis- and/or trans-isomers). These fractions were pooled, lyophilized, and further purified by ascending paper chromatography on acid-washed Whatman No. 1 paper with 19% (v/v) ethanol as solvent. An ultraviolet-absorbing spot with Rf 0.60 was eluted with 95% (v/v) ethanol. This eluate was evaporated to dryness, and the active material was identified as ribosylezatin by comparisons of UV and mass spectra with those of the synthetic product. The solid residue, when dissolved in methanol, exhibited a UV maximum at 269 nm, typical of an N8-substituted adenine, and the absorbance corresponded to 110 µg ± 5 µg. The low resolution mass spectrum at 70 eV, probe temperature 210 C, gave prominent peaks at m/z (%Σ) 351(0.20), 334(0.46), 320(0.31), 262(0.29), 248(0.35), 228(0.28), 219(0.77), 202(3.51), 188(3.22), 160 (2.10), 148(1.52), 136(3.76), and 135(3.48, base peak), inter alia, typical of 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine. Separation and identification of cis- and trans-isomers were then achieved by thin layer chromatography on silica gel with a mixture of chloroform and methanol (9:1,
v/v) as the solvent system (16, 18). Visual estimates of UV absorption of the two spots of ribosylzeatin on the thin layer plates in comparisons with standards of known composition indicated that the amounts of the cis- and trans-isomers were of the same order. Both spots were active in the tobacco bioassay.

Fractions 10, 11, and 12 (Fig. 1) were pooled, lyophilized, and fractionated with distilled water on a Sephadex LH-20 column (Fig. 3). Cytokinin activity was found in fractions 3’ and 5’, corresponding to the elution volumes of 2iPA and ms-ribosylzeatin respectively. These two fractions were further purified by ascending paper chromatography. The chromatogram of fraction 3’, developed with 9.5% (v/v) ethanol, displayed an ultraviolet-absorbing spot at Rf 0.63 which was eluted with 95% (v/v) ethanol, evaporated to dryness, and the active material was identified as 2iPA by its UV and mass spectra. The solid residue, when dissolved in methanol, exhibited a broad UV maximum in the range of 258 nm (synthetic 2iPA has a peak at 268 nm) and the absorbance corresponded to 25 μg ± 5 μg of material (the concentration was too small to obtain an accurate UV absorbance). The mass spectrum at 70 eV, probe temperature 220°C, gave prominent peaks at m/e (%S) 335(0.12), 292(0.11), 246(0.15), 232(0.17), 203(0.83), 188(1.03), 160(3.17, base peak), 149(1.32), and 135(2.23), inter alia, typical of 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpyrwin.

The chromatogram of fraction 5’, developed with 19% (v/v) ethanol, had an ultraviolet-absorbing spot at Rf 0.64, which was eluted, and the active material was identified as ms-ribosylzeatin by its UV and mass spectra. When dissolved in methanol, the solid residue had UV maxima at 243 and 276 nm, and the absorbance indicated 90 μg ± 5 μg of material. The mass spectrum at 70 eV and probe temperature 200°C gave prominent peaks at m/e (%S) 397(0.08), 382(0.29), 380(0.10), 377(0.23), 313(0.11), 294(0.21), 288(0.12), 274(0.24), 265(0.73), 250(0.73), 246(0.91), 245(1.12), 232(1.53), 206(0.80), 194(0.65), 181(2.03), 165(0.92), 151(1.34) and 135(2.07), inter alia, typical of 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylypyrwin (side chain stereochemistry not established).

Fraction 19 (Fig. 1) was lyophilized, and the entire remaining sample was used for the bioassay. The results definitely established the presence of cytokinin activity in the elution volume corresponding to that of ms-2iPA, but the active compound was not further characterized.

DISCUSSION AND CONCLUSIONS

The data provide evidence for the occurrence of five cytokinin-active ribonucleosides in Pisum sativum tRNA: ribosyl-trans-zeatin, ribosyl-cis-zeatin, ms-ribosylzeatin, 2iPA, and ms-2iPA. The presence of ms-2iPA was indicated only by the biological activity of fraction 19 from the LH-20 (33% [v/v] ethanol) column (Fig. 1). This is the first report of the occurrence of ribosyl-trans-zeatin in tRNA. Ribosylzeatin preparations isolated from wheat germ (7, 18) and a nongreen tobacco callus tissue, which had been cultured on a medium with 6-benzylaminopurine, did not contain detectable amounts of the trans-isomer (9, 18). Whether or not the presence of ribosyl-trans-zeatin in the tRNA from Pisum shoots is related to the abundance of chloroplasts in the starting material has not been determined, but it is of interest that only the cis-isomer was found in tRNA from Pisum roots (4).

The results of bioassays performed at three successive stages of the purification procedures gave values of ribosylzeatin and ms-ribosylzeatin content in good agreement with the values computed from UV absorption data for the isolated products. The bioassays indicated a relatively higher content of 2iPA than of the other cytokinins (4, 11), but this was not confirmed by the UV absorption measurements. From the cytokinin activities of all fractions determined at three stages in the isolation procedure and from the ultraviolet absorption data of the isolated fractions, values for a total cytokinin tRNA mole ratio of between 0.02 and 0.06 were derived. These estimates are subject to errors from many sources, but appear to give reasonable minimum values as judged by available information from other organisms (3–5, 11, 21).

It is concluded that except for the presence of ribosyl-trans-zeatin in Pisum tRNA, which is a new finding, the tRNA from vegetative, green shoots of Pisum contain qualitatively the same cytokinins as earlier found in wheat germ tRNA (7).

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LITERATURE CITED


