Revised Methods for Purification of Ribosyl-trans-zeatin from Vinca rosea L. Crown Gall Tumor Tissue

Carlos O. Miller
Department of Plant Sciences, Indiana University, Bloomington, Indiana 47401

Abstract

Ribosyl-trans-zeatin has been purified from Vinca rosea L. crown gall tumor tissue by two new sequences of isolation procedures. Identification of the compound has been established by mass spectrometry, ultraviolet absorbancy spectra, chromatographic values, and growth activity. The isolation sequences eliminate the exposures to pH extremes and the strong cation-exchange resin used in the purification reported earlier. The initial isolation procedures have been designed so as to prevent enzymatic alteration or production of active material and to prevent the inclusion in the extracts of nucleic acids which might serve as sources of the small active compounds. The production of ribosylzeatin by the tumor tissue is confirmed and the validity of isolation steps such as the use of cation exchangers is supported.

The production of ribosyl-trans-zeatin by Vinca rosea L. crown gall tumor tissue induced by an A6 strain of Agrobacterium tumefaciens (Smith and Townsend) Conn has been reported (3). Precautions were employed in that investigation to avoid alteration of the cell division factors produced by the tumor tissue and to prevent the formation of any active materials during purification. Furthermore, evidence was presented that such changes did not occur. Nevertheless, one may wonder about possible effects of certain features of the isolation procedure. These features include a fairly long extraction period in ethanol, exposure to a strong cation-exchange resin (Dowex 50) with conceivably some catalytic action and a momentarily low pH at the resin surface, and exposure to a high pH during and for a while after elution from the resin. Therefore, I have devised two new isolation sequences which avoid these features and which have led once again to the conclusion that the crown gall tumor tissue synthesizes a free cytokinin in the form of ribosyl-trans-zeatin. The sequences have been devised specifically for the purpose of examining for the presence of the ribosylzeatin but will, perhaps, be of use for other related compounds.

Materials and Methods

Tissues and Bioassays. The A6 line of Vinca rosea tumor tissue was kindly supplied by H. Wood of The Rockefeller University and was cultured as already reported (3). The bioassays of cytokinin activity were performed with soybean callus tissue as previously outlined (3).

Purification, Method 1. A total of 1500 g of frozen tumor tissue was extracted in a Waring Blender by grinding 100 g at a time in 375 ml of cold 95% ethanol. This gave a calculated ethanol concentration of 75% assuming all of the tissue to be water. Each small batch was filtered through a layer of cotton immediately after grinding. Filtration was achieved in less than 1 min after the start of blending. The clear filtrate had a temperature of about 4°C and a similar preparation yielded no precipitate when allowed to stand in a refrigerator for one week. A small sample of the filtrate was streaked onto Whatman No. 1 filter paper and the chromatogram was developed with water-saturated 2-propanol. Bioassay of the chromatogram indicated by far the greatest cytokinin activity around Rf 0.8, but a little activity around Rf 0.2. The combined filtrates from the 1500 g of tissue were reduced to a volume of 580 ml by use of an air stream, the temperature never rising above that of the room. The remaining aqueous solution was extracted four times using 200 ml of cold ethyl acetate for each extraction. The combined ethyl acetate layers were washed with 100 ml of H2O which was then added to the other H2O layer. Bioassays indicated only traces of activity in the ethyl acetate fraction—which therefore was discarded—and a high level of activity in the H2O layer. The aqueous solution, which had a pH of 5.2, was next reduced as above to a volume of 280 ml. Twenty-eight grams of KH2PO4 were dissolved in the solution (now pH 4.5), and this was followed by the addition of 196 g of K2HPO4 (final pH 8.2). The extract was cooled in a bucket of ice as the salts were added slowly so that the temperature never rose above that of the room. The solution was then extracted eight times with 250 ml of ethyl acetate being used for each extraction. The combined ethyl acetate layers, which contained most but not all of the activity, were reduced to dryness and the H2O layer, which had only very little activity, was discarded. The residue from the ethyl acetate preparation was stirred thoroughly into 5 ml of H2O. The mixture was added to a 2 x 42 cm column of insoluble PVP (commercial Polyclar AT) prepared in 0.1 M KH2PO4. The column was developed as described by others (2) with 0.1 M KH2PO4 and the eluent was collected in 4-ml fractions. These fractions were bioassayed after pooling them into groups of 10 each. Cytokinin activity was detected mainly in two groups of fractions from tubes 61 to 70 and 71 to 80, each group having about the same level of activity. A little activity was noted in groups 51 to 60 and 81 to 90; none was found in any other fractions although a total of 512 was collected. After elution was completed, synthetic ribosyl-trans-zeatin was applied to and developed on the same column; maximal elution occurred in tube 72. The fractions obtained from the tumor
tissue and appearing in tubes 61 to 80 were dried down, and the active material, along with other unknown compounds, was removed from the salt with a small amount of ethanol. The ethanol solution was streaked onto sheets of Whatman No. 1 filter paper and the chromatograms were developed with water-saturated sec-butanol. Activity was restricted to a zone centered at Rf 0.85 and a material quenching in UV was apparent at this position. This was also the exact position to which synthetic ribosylzeatin migrated. The material was eluted with ethanol and then applied to a sheet of thoroughly washed filter paper. This sheet was developed with distilled H2O. A quenching band at Rf 0.72 contained the active material and coincided with the position of synthetic ribosylzeatin. The compound was eluted with redistilled ethanol.

**Purification, Method 2.** Fifteen hundred grams of frozen tissue were added as small pieces to a mixture of 1500 ml of cold ethyl acetate and a blend of 150 g of KH2PO4 and 1050 g of K2HPO4. The mixture was stirred very thoroughly and, as the tissue gradually broke up and melted, the salts dissolved. The temperature when this process was completed was 7 C. The ethyl acetate layer was removed and the remaining aqueous layer was extracted further with seven successive 500-ml volumes of cold ethyl acetate. The ethyl acetate layers were reduced to a single aqueous layer of 60 ml. This layer was centrifuged to remove solids and further evaporated to 12 ml. The liquid was directly applied to a PVP column exactly as described for Method 1 and all subsequent steps were as given for the first method.

**Mass Spectra.** Mass spectral data of solid materials were obtained with a computerized Varian MAT CH-7 mass spectrometer at various probe temperatures in the range 137 to 181 C.

**RESULTS**

Both methods of isolation of the cytokinin yielded materials which had UV absorption spectra identical to those of the previously isolated factor and to those of synthetic ribosyl-trans-zeatin (3). With both materials, maxima were observed at 268 nm in 95% ethanol, at 268 and 217 nm in 0.1 N NaOH, and at 264 nm in 0.1 N HCl. The migration values of 0.85 and 0.72 on paper chromatograms developed with water-saturated sec-butanol or water, respectively, were essentially the same as those reported earlier and exactly the same as those obtained with the synthetic compound. The factors from each method showed essentially the mass spectrum obtained previously (3) with major peaks at 351, 334, 320, 262, 248, 219, 202, 201, 199, 188, 186, 164, 160, 149, 148, 136, 135, 119, and 108. There were no other significant or unexpected m/e peaks at values higher than any of these. These peaks are precisely those already reported and those obtained with ribosyl-trans-zeatin (3). Both of the isolated compounds migrated with the trans (Rf 0.14) rather than the cis (Rf 0.20) form of ribosylzeatin when developed with a 9:1 mixture of chloroform and methanol on thin layer silica gel plates (4). The two materials were active in the soybean callus assay at levels at least as low as 10-10 M (estimated from UV absorbancy).

Assuming a molar extinction coefficient of 19,000 at 268 nm when the compound is dissolved in 95% ethanol, the yield of ribosylzeatin was 0.27 mg for Method 1 and 0.30 for Method 2. Since the total beginning activity for the tumor tissue used was estimated to be equal to about 0.7 mg of ribosylzeatin/kg of tissue, these values indicate recoveries of roughly 26% of total beginning activity for Method 1 and 28% for Method 2.

**DISCUSSION**

The extraction from small batches of tumor tissue and the subsequent filtering in Method 1 were accomplished in less than one minute. The temperature remained low during the extraction, the final ethanol concentration was at least 75%, the pH of the filtrate when freed of ethanol was 5.2, and no precipitate was obtained by prolonged cooling of the filtrate. The possibility of enzymatic activity altering or producing active compounds therefore seems very unlikely. Furthermore, the possibility of the presence of macromolecules such as nucleic acids in the extract from which the active compound could be released seems to be very slight. Also, considering that with both procedures the temperature never was allowed to rise above that of the room, that the pH was maintained in the range 4.5 to 8.2, that chromatographic values were altered by neither procedure, and that the results are the same as those obtained in earlier work (3), the conclusion that ribosyl-trans-zeatin exists in the tumor tissue in substantial amounts seems warranted. A logical extension of this conclusion is that the cytokinin plays a role in the capability of the tumor tissue for continued cell division. This does not rule out, of course, the involvement of additional active compounds. Indeed, in the earlier work the presence of two other compounds which fit the chromatographic patterns expected of free zeatin and zeatin ribonucleotide was noted (3). Nevertheless, the ribosylzeatin seems to be contributing a very large share of the total activity. A study of the pathway of synthesis of ribosylzeatin and closely related compounds in the tumor tissue and a comparison of particular enzymatic steps involved with those in normal tissue may be instructive not only as to the nature of the tumorous condition but also as to the control of cytokinin synthesis in the normal plant.

The two methods presented herein have yielded the same result as obtained with the earlier sequence in which Dowex 50 cation exchange resin was employed. Therefore, the reliability of using the resin in cytokinin isolation work is confirmed. The precautions to prevent localized heating should, of course, be observed (1). The two new sequences have not been as efficient in recovery of activity as is desirable. This apparent low efficiency may be misleading since I have made various cuts from chromatograms rather narrow so as to achieve a high degree of purity, and certainly some of the activity is attributable to other compounds. The use of PVP columns, as suggested by others (2), seems to be very promising in cytokinin work. The 'salting out' of cytokinins into ethyl acetate also is a powerful step but seems to work best when volumes are small.

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**LITERATURE CITED**