Cytokinins in Azotobacter vinelandii Culture Medium

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Azotobacter vinelandii OP was grown to stationary phase in defined medium. The cell-free culture medium was analyzed for cytokinin content by XAD-2 and Sephadex LH-20 chromatography, thin-layer chromatography, tobacco callus bioassay, and enzyme immunoassay. Three cytokinin-active fractions were detected and tentatively identified as trans-zeatin, isopentenyladenosine, and isopentenyladenine. The total cytokinin activity was equivalent to 0.75 μg of kinetin per liter.

Inoculation of plants with Azotobacter spp. influences plant growth and development, sometimes increasing yields (3, 5, 13). Although Azotobacter spp. are capable of fixing nitrogen, the effects on plant growth are often attributed to the synthesis of plant growth substances. Auxins, gibberellins, and cytokinins have been found in the culture media of several Azotobacter species (2, 3, 5), including Azotobacter vinelandii (2, 6, 9). However, in most cases the growth substances were detected by activity in bioassays and specific compounds were not identified.

A number of rhizosphere bacteria have been shown to produce cytokinins. These include the actinomycetes Streptomyces flavescens (7) and Frankia spp. (14). In addition to being found in Azotobacter spp., cytokinins have been found in the culture media of Arthrobacter spp. (4) and Bradyrhizobium spp. (12; D. B. Sturtevant and B. J. Taller, Plant Physiol., in press). The report presented here describes the cytokinins present in A. vinelandii culture medium. Three cytokinin-active substances were found and tentatively identified as trans-zeatin, isopentenyladenosine, and isopentenyladenine.

A. vinelandii OP (from P. E. Bishop, Raleigh, N.C.) was grown under N₂-fixing conditions in Burk medium containing 2% sucrose (NFS medium) as described previously (16). Cells were transferred three times in NFS before use. Then, 10 ml of the culture was inoculated into 500 ml of NFS in 2-liter flasks, and the cultures were grown in an incubator shaker at 300 rpm at 27°C. At stationary phase (60 h), the cells were harvested by centrifugation, and the supernatant was stored at −20°C until cytokinin analysis.

Cytokinins were concentrated from 2.8 liters of culture medium by trace enrichment on Amberlite XAD-2 resin as described by Kim et al. (8). The XAD-2 column was eluted with 70% (vol/vol) ethanol, and the eluate was dried in vacuo at 37°C. The dried sample was dissolved in 2.5 ml of 35% (vol/vol) ethanol and chromatographed on a Sephadex LH-20 column (28.5 g; 1.5 by 66.5 cm) in the same solvent (1). Fractions (6 ml each) were collected at a flow rate of 36 ml/h. The resulting fractions were pooled according to cytokinin standards (Sigma Chemical Co., St. Louis, Mo.) run on the same column after the sample. A total of 7.5 bed volumes of sample eluate were collected and pooled into 12 fractions. The cytokinin activity of all the pooled fractions was determined in the tobacco callus bioassay (10). Because the free base is more active than the corresponding riboside, the samples were acid hydrolyzed (0.1 N HCl, 100°C, 45 min) before bioassay. The test samples were incorporated into 100 ml of medium (10) containing 2 mg of indoleacetic acid per liter, and fivefold serial dilutions were made in the same medium. Four replicate flasks containing 20 ml of medium bioassayed for each concentration tested. A series of standards, containing from 0 to 15 μg of kinetin per liter, was similarly prepared for each assay. Three pieces of cytokinin-dependent tobacco callus were planted in each flask, and the fresh weight yield was determined after 35 days. Curve Fitter software (Interactive Microware, Inc., State College, Pa.) was used to generate a standard curve from the kinetin standards and to determine the cytokinin activity of the test samples by comparing fresh weight yields. Activity is expressed as kinetin equivalents; one kinetin equivalent is defined as the amount of kinetin, in micrograms, required to give the same growth as the test sample.

Pooled fractions from the Sephadex LH-20 column were also examined by enzyme immunoassay. The Phytodetek trans-zeatin riboside immunoassay kit (Idetek, Inc., San Bruno, Calif.) was used for the putative zeatin fraction. Isopentenyladenine and its riboside were tested with polyclonal antibodies prepared and purified as described by MacDonald and Morris (11). The antibodies were then used in an indirect enzyme-linked immunosorbent assay modified for the assay of the antigen isopentenyladenosine and its free base. The samples were developed with goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate, with ortho-phenylenediamine as the substrate. Microdilution plates were read with a computer-controlled Biotek model EL309 microplate reader.

Isomers of zeatin were separated by thin-layer chromatography on silica gel (chromagram sheet, no. 13181, with fluorescent indicator; Eastman Kodak Co., Rochester, N.Y.) in chloroform-glacial acetic acid-methanol (90:7.5:2.5) (15). Chromatograms were divided into zones according to reference standards or UV-absorbing spots. The zones were scraped from the plate, eluted with 95% ethanol, and examined by bioassay.

Bioassay of the pooled fractions from the Sephadex LH-20 fractionation of the culture filtrate produced three distinct peaks of cytokinin activity, corresponding to the elution positions of zeatin, isopentenyladenosine, and isopentenyladenine (Fig. 1). The total cytokinin activity is equivalent to about 0.75 μg of kinetin per liter of culture medium. This represents a minimum value, since it is not corrected for losses during extraction and purification. The cytokinin-active component of fraction 4 was further identified as trans-zeatin by enzyme immunoassay. The occurrence of

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isopentenyladenine and its riboside was also substantiated by immunoassay.

The putative zeatin fraction (fraction 4) was rechromatographed on Sephadex LH-20 eluted with water to remove additional impurities. The fractions corresponding to the elution volume of zeatin were pooled and then fractionated by thin-layer chromatography as described above. Bioassay of zones of the plate showed cytokinin activity in the fraction corresponding to trans-zeatin (0.05 kinetin equivalents per liter) and no activity in the cis-zeatin fraction.

Our results are consistent with the findings of others (2, 6) who reported three cytokinin-like substances in A. vinelandii cultures. The occurrence of zeatin was substantiated by cochromatography with a zeatin standard on Sephadex LH-20 both in 35% ethanol and in water, bioassay activity, cochromatography in a thin-layer chromatography system, and enzyme-linked immunosorbent assay with monoclonal antibodies for trans-ribosylzeatin. Zeatin is the characteristic cytokinin of higher plants, and its synthesis by microorganisms is thought to be limited to bacteria and fungi which infect plants (7). Since trans-zeatin is approximately five times more active than an equal amount of isopentenyladenine in the tobacco bioassay, the amount of zeatin in the culture medium is small.

The total cytokinin activity in the culture medium is similar to that found in cultures of Rhizobium spp. (12) and Frankia spp. (14) but is considerably less than that previously found in Azotobacter cultures. Barea and co-workers reported 20 μg of cytokinin equivalents per liter for Azotobacter paspali (3) and 50 μg/liter for A. vinelandii (2). More recently, it was determined that A. vinelandii culture medium contained 1 mg of cytokinin per liter (6). In all of these previous reports, cytokinins were partially purified by ethyl acetate extraction and paper chromatography and were assayed by the radish cotyledon expansion test and by chlorophyll retention. Since cytokinins are defined in part by their ability to promote plant cell division, these non-cell division assays must be interpreted with caution, particularly when impure compounds are being tested. The tobacco bioassay is sensitive and specific, and it is useful for initial cytokinin screening, since not all available antibodies may have sufficient affinities to detect all cytokinins. Differences in cytokinin production reported in the literature may also be the result of culture conditions or strain selection.

The biological significance of cytokinin production by Azotobacter spp. is not known. As discussed above, the growth-promoting activity of this organism is commonly attributed to its production of plant growth substances. There is considerable commercial interest in plant growth regulators that increase yield, commonly microbial fermentation products which contain cytokinins among their components. The amount of cytokinin in our cultures was low, but cytokinin synthesis in the rhizosphere may be influenced by factors from the plant.

LITERATURE CITED