Cytokinins in Corynebacterium fascians Cultures

ISOLATION AND IDENTIFICATION OF 6-(4-HYDROXY-3-METHYL-CIS-2-BUTENYLAMINO)-2-METHYLTHIOPURINE

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ABSTRACT

In addition to the four cytokinins, 6-(3-methyl-2-butenylamino)purine, 6-methylaminopurine and the cis and trans isomers of 6-(4-hydroxy-3-methyl-2-butenylamino)purine, reported earlier from our laboratories, three cytokinin-active fractions have been obtained from the aqueous medium of 6-day-old Corynebacterium fascians cultures. One of these has been identified as 6-(4-hydroxy-3-methyl-cis-2-butenylamino)-2-methylthiopurine (2-methylthio-cis-zeatin, c-ms6Ade).

The elution volumes of the other two fractions correspond to those of authentic 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine and 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine, indicating the presence of trace amounts of these two ribonucleosides.

Fasciation and modified growth of seedlings closely resembling symptoms of infection with Corynebacterium fascians have been induced in Pismum by soaking the seeds in high concentrations of kinetin (20). That in fact these symptoms of infection by the bacterium might be due to its production of cytokinin was indicated by the ability of extracts of pure cultures to release lateral buds of Pismum from apical dominance in the same manner as kinetin (25). Isolation of cytokinin-active material from aqueous cultures of the same stock was achieved by solvent extraction and chromatography. Three crystalline fractions with cytokinin activity as monitored in the tobacco bioassay were obtained (8, 10). The most active fraction was identified as 6-(3-methyl-2-butenylamino)purine (6Ade) (6), known at that time only as a synthetic, highly active cytokinin (5, 9). The second fraction contained nicotinamide. Inasmuch as synthetic nicotinamide was inactive in the tobacco bioassay, the activity of the isolated material was ascribed to a contaminant. This cytokinin was recently identified as 6-(4-hydroxy-3-methyl-cis-2-butenylamino)purine (cis-zeatin, c-io6Ade) (21). The third active fraction found in 1966 contained 9N-methyladenine, a weakly active cytokinin (12, 23), but in amounts less than required to account for the activity of this fraction in the tobacco bioassay. Hence, another more potent cytokinin was assumed to be present as a contaminant and to be mainly responsible for the cytokinin activity in this fraction. We now report the purification and characterization of this cytokinin from Corynebacterium fascians culture medium as 6-(4-hydroxy-3-methyl-cis-2-butenylamino)-2-methylthiopurine (2-methylthio-cis-zeatin, c-ms6Ade).

MATERIALS AND METHODS

Growth of Corynebacterium fascians Cultures. The origin of the C. fascians strain used in this work and the culture methods employed have been described (21). Cultures were harvested after 6 days growth at about 28 C (about 24 hr after reaching the stationary phase).

Isolation of Cytokinins from the Culture Medium. The preliminary purification and fractionation of the cytokinins present in the medium from C. fascians cultures have been described in detail (21). In summary, the following procedures were used: the cells were separated from 100 liters of stationary phase cultures by centrifugation. The supernatant was acidified, filtered, and fractionated by Dowex 50 chromatography. The NH4OH eluate from the Dowex columns was evaporated to dryness in vacuo and the dry solids were extracted with water-saturated ethyl acetate. The extracts were taken to dryness, redissolved in 35% ethanol, and chromatographed on Sephadex LH-20 columns in 35% ethanol. Three peaks of cytokinin activity were detected in the elution profile from the LH-20 columns. In order of elution, these peaks corresponded to c-io6 Ade (1.4 bed volumes), 6Ade (2.1 bed volumes), and an unidentified cytokinin (2.4 bed volumes). Purification of the latter compound was achieved by a combination of Sephadex LH-20 chromatography and paper chromatography.

Sephadex LH-20 Chromatography. Chromatography on Sephadex LH-20 (Pharmacia) columns was carried out essentially as described by Armstrong et al. (1). Details of chromatographic procedures are given in the legends to the figures. Ethanol was redistilled prior to use, and water was double distilled from a Pyrex distillation apparatus.

Paper Chromatography. Whatman No. 1 filter paper, washed as described (21), was used. Cytokinin samples were dissolved in small volumes of 95% ethanol and applied to the paper as 5-cm streaks. The chromatograms were developed by ascending chromatography over 30 cm. UV-absorbing bands were recovered from the chromatograms in small volumes (about 0.5-1 ml) by

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5 A culture of Corynebacterium fascians was kindly given to us in 1964 by Professor K. V. Thimann.
elution with 95% ethanol. Ethanol was redistilled prior to use, and water was triple distilled.

**Bioassay of Cytokinin Activity.** Cytokinin activity was determined in the tobacco bioassay as described (10, 19) and activity is expressed as kinetin equivalents (KE, the μg of kinetin [6-furfurylaminopurine] required to give the same growth response as the test sample under the specified bioassay conditions).

**Procedures for Identification of Cytokinin in Fraction 7 of Figure 2.** Ultraviolet absorption spectra in absolute methanol were determined with a Cary model 15 spectrophotometer. Mass spectra were obtained with Varian MAT CH-5 and Varian MAT 731 spectrometers. Side chain geometry was determined by high performance liquid chromatography on a column of BioRad Aminex A-5 cation exchange resin as reported by Cole et al. (2).

The syntheses and properties of authentic c-io6Ade, t-io6Ade, c-msio6Ade, and t-msio6Ade used as standards in the identification of cytokinins in this study have been described (17, 27).

**RESULTS**

**Isolation of Cytokinins.** A cytokinin-active peak that eluted after cAde, when partially purified extracts from 100 liters of C. fascians culture medium were fractionated on Sephadex LH-20 columns in 35% ethanol (21), was recovered from the column eluates by evaporation in vacuo at 37°C. The solids were rechromatographed on Sephadex LH-20 in 9.5% ethanol. The elution profile for this column is shown in Figure 1. The cytokinin activity eluted as a single peak.

Repeated attempts to purify the cytokinin further by paper chromatography in aqueous ethanol solutions eliminated some colored material but failed to separate the cytokinin activity from contaminating fluorescent material. Therefore, the cytokinin sample was rechromatographed on a Sephadex LH-20 column in distilled H2O. The elution profile for this column is shown in Figure 2. Two peaks of UV-absorbing compounds were observed, but cytokinin activity was associated with only the second peak. A synthetic sample of the cytokinin 2-methylthiozeatin, applied to the column after the fractionation was completed, eluted at the same position as the cytokinin-active peak.

Further purification of this C. fascians cytokinin was achieved by paper chromatography in 19% ethanol. A single UV-absorbing band was observed at about Rf 0.5. To remove traces of fluorescent material, the compound was chromatographed three times under the above conditions. The UV-absorbing band eluted from the final chromatogram was taken to dryness in a stream of N2. This sample which contained about 1 A590 unit was used for structure determination. Using the extinction coefficient for msip6Ade (λmax 279 nm (ε15,800) and correcting for the fraction of the material used in biosassays, the final yield was equivalent to about 0.001 μmol/l of extracted culture medium.

**Identification of Isolated Cytokinin.** The above dried preparation (about 1 A590 unit) dissolved in absolute methanol had an UV-absorption spectrum practically identical with that of authentic c-msio6Ade. The data in Table I show the congruence of λmax and λmin values of the two compounds and the close agreement in the ratios of their molar absorption values at the absorption maxima. Assuming a molar extinction coefficient of 2500 at 240 nm (28), the UV spectrum indicated that about 10 μg of sample were available for chemical structure determination.

A 20% aliquot was evaporated to dryness in a gold crucible at 50°C and was inserted into the direct inlet of a Varian MAT CH-5 mass spectrometer. The fragmentation patterns derived from the spectra of this sample and from that of authentic c-msio6Ade are shown in Table II. After subtraction of background, the two spectra are identical, but the peak at m/e 265 is very weak in the spectrum of the isolated cytokinin.

The quantity of isolated cytokinin was insufficient for obtaining high resolution mass spectra, but a mass of 265 was confirmed by its field desorption mass spectrum. The dried sample was taken up in 20 μl of absolute methanol. The field emitter wire of a Varian MAT combination electron impact-field desorption ion source was dipped into the solution and the spectrum was determined on a Varian MAT 731 mass spectrometer. At an emitter current of 23 mamp, the oscillographically recorded spectrum consisted of a single peak at m/e 265. This peak was observed at
all emitter current values. Other masses were seen only intermittently at lower temperatures, indicating that complete purification had not been achieved by Sephadex LH-20 chromatography.

To determine the isomeric configuration of the N6-side chain, the remaining sample was subjected to high performance liquid chromatography by methods developed by Cole et al. (2). The elution volumes of authentic cis and trans isomers of ms io6Ade and of the isolated cytokinin together with properties of the column and conditions used are given in Table III. The peak of isolated material which eluted from the Bio-Rad Aminex column at 223 ml, as compared with 222 for authentic c-ms io6Ade, was concentrated to a minimal volume. An aliquot was then freeze-loaded onto an emitter wire by the technique of Olson et al. (15) for determination of the field desorption mass spectrum. The spectrum at 10 mamp emitter current showed a single peak at m/e 266 (protonation due to acidic conditions lead to strong M + H+ peaks), confirming that the high performance liquid chromatography peak was due to ms io6Ade.

The isolated cytokinin was thus proved to be 6-(4-hydroxy-3-methyl-cis-2-butylamino)-2-methylthiopurine.

**Cytokin-in active Ribonucleosides.** In a confirmatory experiment with active material extracted from 200 liters of 6-day-old *C. fascians* culture medium, bioassays revealed two additional peaks of cytokinin activity in the profile from the Sephadex LH-20 column eluted with 35% ethanol. These peaks corresponding to the elution volumes of io6Ade (111 bed volumes) and i6Ade (1.7 bed volumes), and distinct from the elution volumes of io6Ade, i6Ade, and ms io6Ade (1.4, 2.1, and 2.4 bed volumes, respectively), suggest that trace amounts of these two ribonucleosides are also present in free form in the cultures.

**DISCUSSION**

This study brings to five the number of cytokinin-active N6-substituted adenine bases isolated from *C. fascians* cultures, four of which have been chemically characterized: c-ms io6Ade in this report, and i6Ade, c-io6Ade, and the weakly active m4Ade in earlier reports. The presence of i io6Ade was indicated by bioassays of chromatographically separated isomers of isolated io6Ade in our earlier report (21), but the amount of this isomer was not enough for chemical characterization. We now also report indirect evidence for trace amounts of two cytokinin-active ribonucleosides, io6Ade and i6Ade, in the culture medium.

No meaningful quantitative estimate can be made of total cytokinin activity or amounts of various species in the living cells on the basis of the observed biological activities or amounts isolated from the culture medium. The extent to which the above seven cytokinins may reflect the full spectrum of species in *C. fascians* also remains to be determined. The results suggest that this bacterium produces all species of cytokinin which have been found free or as macromolecular constituents in higher plants.

The failure so far to detect ms io6Ade and ms i6Ade may be due merely to the relatively high retention of these methylated purine derivatives on the Sephadex column and their relatively low activity in bioassays, especially of the cis isomers, rather than to their absence or exceptionally low content in the cultures.

As to the origin of free cytokinins in the cultures, they may be nucleic acid degradation products of moribund cells or excreted normal cell metabolites possibly from the same source. Rathbone and Hall (18) have confirmed the presence of i io6Ade in hydrolyzed *C. fascians* tRNA (11) and they found several times more extractable i6Ade in acidic cultures than the approximate 2 μg/ml found in neutral controls. They ascribe the increase in i6Ade to its being released from tRNA in the extraction process. Even this tentative interpretation seems unwarranted on the basis of the methodology and measurements provided, and except by implication, it leaves open the source of the i6Ade in the normal cultures. The presence of both io6Ade and i6Ade in free form as well as the observed preponderance of the cis isomers of both io6Ade (21) and ms io6Ade would be expected if the free cytokinins derive from tRNA, but alternative sources of these ribonucleosides are not excluded by these findings.

Apparently, the symptoms of *C. fascians* pathogenicity (e.g. fascination) may be brought about by bacteria on the plants' surface, i.e. without the need for the microbes to penetrate the epidermis, and can be mimicked by applied cytokinin. The disease, therefore, may be caused by the cytokinins released by the bacteria. The exceptionally high cytokinin activity generated by *C. fascians* is illustrated by the striking growth of cytokinin-dependent tobacco callus or excised pit tissue initiated and sustained by a colony of this bacterium no larger than a pinhead growing on cytokinin-free nutrient agar at distances at least 2 cm away from the plant tissue. Some other plant pathogens (4, 7, 14, 27) and symbionts (3, 13, 16) associated with overgrowth in host tissues similarly have been shown to have relatively high cytokinin contents, although in mycorrhiza at least this relationship may not be obligatory (3). Presumably, other types of microorganisms produce cytokinins of kinds and in quantities ineffective for pathogenicity and not so readily detectable by present assay methods.

Zeatin and its derivatives (cytokinins with one hydroxylated methyl group in the N6-isopentenyl side chain of adenine) have been considered to occur normally in autotrophic plants (22) including algae (24), but generally not in fungi and bacteria, and not in animals (22). Their presence in saprophytic microorganisms has been restricted to pathogens and symbionts or species otherwise associated with overgrowth in plants. However, one apparent exception to such a functional association, the presence of ms io6Ade in *Pseudomonas aeruginosa* tRNA, has been reported (26).

**LITERATURE CITED**


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