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SHORT COMMUNICATION

ISOLATION AND ASSAY OF A CYTOKININ FROM BARLEY

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Abstract—A specific method for the extraction and purification of cytokinins was tested with zeatin and zeatin riboside. For the quantitative determination of cytokinin activity, a bioassay is proposed based upon two different effects of cytokinins on barley during its germination. With the aid of these methods, a barley cytokinin, which resembles zeatin riboside, was isolated and quantitatively determined from 3-day-old barley seedlings.

INTRODUCTION

IN RECENT years, the isolation and characterization of natural cytokinins from higher plants has been the subject of considerable research. Several cytokinins have been characterized,[1] some as free cytokinins, others as constituents of soluble RNA.

Bioassays based upon induction of cell division in callus tissue or on retention of chlorophyll degradation in senescent leaves have mainly been applied for the determination of cytokinin activity. Bioassays are preferred to other techniques, such as GLC or UV absorption, because the latter methods depend on an exact knowledge of the natural cytokinins involved.

In an earlier publication, the action of cytokinin on inhibiting the root growth of barley seedlings was proposed as a bioassay for cytokinin activity;[2] α-amylase formation is also strongly inhibited. After 3 days germination in the presence of cytokinins, a striking decline of α-amylase activity in barley seedlings[3] is observed. Both root growth inhibition and the loss of α-amylase activity are completely independent of each other.[3]

In this paper a further elaboration of the root growth bioassay combined with the specific cytokinin action on α-amylase activity in the same barley seedlings is described as an assay for cytokinins. Together with a specific isolation procedure this bioassay was used for the quantitative determination of cytokinin activity in immature and germinated barley.

RESULTS AND DISCUSSION

The yield of cytokinins obtained using the extraction process was studied using two 'natural' cytokinins. An 80% ethanolic solution of zeatin (Z, 10^{-5} M) and zeatin riboside (Zr. 10^{-5} M) were submitted to the process outlined in Scheme 1. After each purification step

[1] F. SKOOG and D. J. ARMSTRONG, *Ann. Rev. Plant Physiol.* **21**, 359 (1970).
[2] H. A. VAN ONCKELEN, R. VERBEEK and L. MASSART, *Naturwiss.* **52**, 561 (1965).
[3] R. VERBEEK, H. VAN OCKNELEN and TH. GASPAR, *Physiol. Plant.* **22**, 1192 (1969).

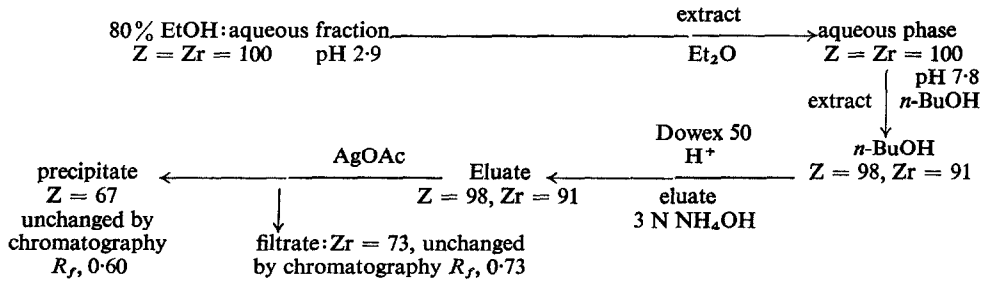
the cytokinin concentration was determined by measuring absorptivity at 267 nm. After ether extraction, both zeatin and zeatin riboside remain 100% in the aqueous phase. Extraction with *n*-butanol causes about a 10% loss of zeatin riboside, while zeatin is almost quantitatively recovered. Purification on a column of Dowex 50 W H^+ ion exchange resin gives no further loss. Silver precipitation results in a separation between zeatin and its riboside. In the precipitate 67% of the initial zeatin concentration is recovered and the filtrate contains 73% of the initial zeatin riboside concentration. Quantitative paper chromatography concludes this purification procedure (Scheme 1) and is effected without further loss, so the total yield of the extraction process for both zeatin and its riboside fluctuates around 70%.

For the determination of the cytokinin activity in a partially purified plant extract, one normally uses a bioassay based upon induction of cell division, upon retardation of chlorophyll degradation or upon other specific cytokinin effects. Most of these bioassays, however, show varying sensitivity to different cytokinins,[4] which is a disadvantage for the quantitative determination of unidentified cytokinins.

The bioassay presented in this paper has the great advantage for quantitative work that the different cytokinins react identically. The test is based upon two different effects of the cytokinins on the germination of barley.

As mentioned earlier, cytokinins strongly reduce root length of barley seedlings. This inhibition of root growth measured after six days of germination is directly proportional to the logarithm of cytokinin concentration in the culture medium (Fig. 1). Zeatin, zeatin riboside, and isopentenyladenine, three 'natural' cytokinins, kinetin and benzyladenine, two 'synthetic' cytokinins, all have an almost identical effect on root length of growing barley seedlings. From 10^{-5} M to 10^{-8} M a linear semi-logarithmic relation between root growth inhibition and cytokinin concentration is observed.

The influence of the same cytokinins on α-amylase activity in 7-day-old barley is shown in Fig. 1. A similar semi-logarithmic relation between decrease in α-amylase activity and cytokinin concentration (10^{-5}–>10^{-7} M) is observed. Both, root growth inhibition and reduced α-amylase activity, being completely independent to each other, are specific for the presence of cytokinins in the culture medium.



SCHEME 1. EXTRACTION SCHEME FOR CYTOKININS.

The cytokinin bioassay based upon these two effects is simple and provides results after a relatively short time. The root length is measured after 6 days of germination, while α-amylase activity is determined one day later in the same barley seedlings. The parallelism of these two effects indicates, better than a single test does, the presence and concentration of cytokinins in the tested solution.

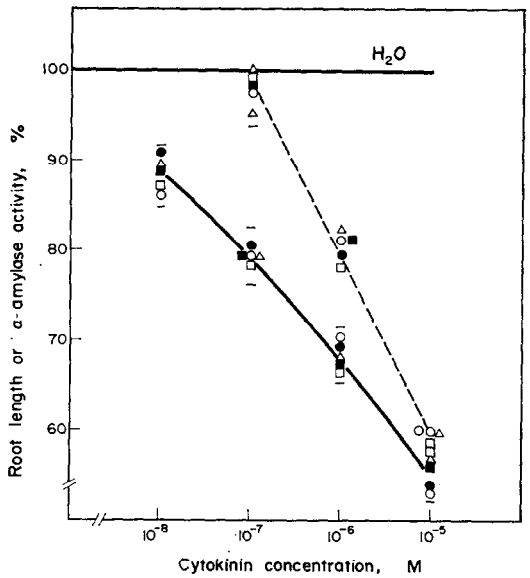


FIG. 1. INFLUENCE OF DIFFERENT CYTOKININS ON THE ROOT LENGTH OF 6-DAY-OLD BARLEY SEEDLINGS, AND α-AMYLASE ACTIVITY OF 7-DAY-OLD SEEDLINGS.
Kinetin (●), benzyladenin (○), zeatin (□), 2iP (△) and zeatinriboside (■). —— root length, ---- α-amylase activity.

The cytokinin extraction and specific bioassay procedures were employed for further investigation on cytokinin activity in barley. 1 kg immature barley grains and 1 kg barley, germinated for 3 days, were submitted to the extraction process. After the final paper chromatographic fractionation the different fractions were tested for cytokinin activity. The results of this bioassay are presented in Table 1.

TABLE 1. ROOT LENGTH AND α-AMYLASE ACTIVITY OF BARLEY GERMINATED ON DIFFERENT FRACTIONS OBTAINED FROM THE EXTRACTION OF 3 DAY GERMINATED BARLEY*

Fraction†	Root length mm	%	α-Amylase activity IDC	%
H_2O	88	100	82	100
Ag^+ filtrate				
R_f 0·05–0·3	88	100	80	100
R_f 0·3–0·5	66	73	70	85
R_f 0·5–front	88	100	83	100

* No activity was found in any fractions from immature barley.
† No activity was found in the silver precipitate fractions.

The germinated barley extract contains one active fraction, while no cytokinin activity could be demonstrated in the immature-barley extract. The active fraction causes a 27%

[4] S. M. HECHT, N. J. LEONARD, R. Y. SCHMITZ and F. SKOOG, *Phytochem.* **6**, 1907 (1970).

root growth inhibition and a 15% decrease in α-amylase activity. Both results point to the same 2×10^{-7} M cytokinin concentration in the tested solution (Fig. 1). This active fraction, found in the silver filtrate and located between R_f 0·3 and 0·5, corresponds with the zone of zeatin riboside, which is present in the silver filtrate at R_f 0·4 after identical extraction and purification process.

For this reason the cytokinin activity in the germinated barley could be expressed as an 'equivalent' zeatin riboside. Taking in account the 70% extraction yield, the 2×10^{-7} M cytokinin concentration in the tested solution (200 ml) corresponds with 16 μg 'equivalent' zeatin riboside. In conclusion, these results show that the barley cytokinin is formed during germination and although it resembles zeatin riboside, further experiments are needed to elucidate its structure completely.

EXPERIMENTAL

Materials. Barley (*Hordeum vulgare* var. Mosane) used throughout all the experiments was disinfected by treatment for 2 hr in H_2SO_4 (50%). The husks were removed and the seeds rinsed in flowing H_2O until neutral. After drying the seeds are stored at 4°.

Germination. Barley seeds were germinated in glass containers (40 × 25 cm) with the ventral side in the culture medium in such a way that they were moistened but not completely immersed, thus providing free respiration and normal growth. Germination was carried out at 20° in the dark.

Cytokinin extraction. The crude 80% EtOH extract was evaporated *in vacuo* (45°) and its pH adjusted to 2·9. This solution was extracted 5× with 1/5 vol. Et_2O. The aqueous layer, brought to pH 7·8 was then extracted with *n*-BuOH (5 × 1/5 vol.). The *n*-BuOH extract was evaporated *in vacuo* and taken up into 10 ml H_2O at pH 2. It was then percolated through a 2·5 × 40 cm column Dowex 50 W H^+ ion exchange resin and eluted with 500 ml of 3 N NH_4OH.

The eluate was evaporated *in vacuo* and taken up into 15 ml H_2SO_4 (0·05 N): 9 ml of a warm saturated Ag OAc (2·5 g Ag acetate in 100 ml H_2O at 80°) was added. After 3 hr the precipitate was centrifuged (15 min; 15 000 rpm). In the supernatant the Ag^+ ions were removed with 0·1 N NaCl. The silver precipitate was treated 12 times with 10 ml 0·1 N HCl in order to resolubilize the Ag–purine complex. Both supernatant and filtrate were freeze-dried and taken up into a minimal quantity of absolute EtOH. After centrifugation, descending paper chromatography was carried out on Whatman 3MM, previously washed with the solvent system, 0·04 M $Na_2B_4O_7$ saturated *n*-BuOH. The spots were revealed under UV light (Philips TUV-GW; Filtre type U.G.5 Jena and Schott 250–300 nm), and eluted with H_2O. The fractions were diluted to 200 ml in order for bioassay of cytokinin activity.

Bioassay. 100 barley seedlings are placed in 100 ml of the tested solution which is renewed after 3 days of germination. After 6 days the main root of each barley seedling was measured and the mean value expressed as a % of the blank value derived from barley germinated on H_2O alone. One day later, the barley seedlings are homogenized in 100 ml 0·2% $CaCl_2$ with a Virtis '40' homogenizer (1 min; 40 000 rpm), and after centrifugation, the α-amylase activity is determined in the supernatant by the method of Briggs[5,6] and expressed in iodine dextrine colour units (IDC).

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[5] D. E. BRIGGS, *J. Inst. Brew.* **67**, 427 (1961).
[6] D. E. BRIGGS, *Enzymol.* **24**, 97 (1962).

Key Word Index—Hordeum vulgare; Gramineae; cytokinin extraction; cytokinins; assay.