Note

A Highly Sensitive *Amaranthus* Betacyanin Assay for Cytokinins

Shiro NISHIKAWA, Zenzaburo KUMAZAWA
and Naoki KASHIMURA

Laboratory of Biofunctional Chemistry,
Faculty of Bioresources, Mie University,
Tsu, Mie 514, Japan

Received April 7, 1989

Both natural and synthetic cytokinins have various biological effects on intact or cultured plant tissues and their cytokinin activities have been evaluated by many assay methods. Among them, a tobacco callus assay is the most sensitive¹⁾ and widely used, although it has some limitations: long incubation period (about one month) and laborious work under aseptic conditions. On the other hand, an *Amaranthus* betacyanin assay developed by Köhler and Conrad²⁾ and later modified by Biddington and Thomas³⁾ is a convenient and reliable method for the measurement of cytokinin activity⁴⁾ because it is less laborious, more accurate than the callus assay and not so time-consuming. However, it is several orders of magnitude less sensitive than the former assay. If we could raise the sensitivity of the *Amaranthus* betacyanin assay up to

that of the callus assay, it would be one of the most attractive cytokinin assays.

During the course of studies on the synthetic cytokinins, we found that the use of 3-(3,4-dihydroxyphenyl)-L-alanine (L-dopa) instead of L-tyrosine, a betacyanin precursor in the *Amaranthus* betacyanin assay, made it possible to detect cytokinin activity of 10^{-9} M of *N*⁶-benzyladenine (BA), almost comparable to the minimum detectable concentration of BA (10^{-9} M)⁵⁾ in the tobacco callus assay. This paper describes the results of this highly sensitive *Amaranthus* betacyanin assay.

Biosynthesis of betacyanins from L-tyrosine through L-dopa in young cactus fruits has been established;⁶⁾ two molecules of L-tyrosine are incorporated into betanidine and betanine. A similar biosynthesis of the betacyanins has also been observed in *Amaranthus* sp. Molten Fire.⁷⁾ However, the possibility of the use of L-dopa for an *Amaranthus* betacyanin assay has not been studied. Since L-dopa is the first metabolite of L-tyrosine in the biosynthesis, we expected that administration of L-dopa to the seedlings of *Amaranthus caudatus* L. might give a better yield of betacyanin than L-tyrosine, as exemplified in the cactus fruits.⁶⁾

Seeds of *Amaranthus caudatus* L., which were kindly presented by Dr. H. Hayashi (Osaka Prefectural University), cultivated and harvested, were used. The reported betacyanin assay⁴⁾ was slightly modified for this study. Thus, ten seedlings of *Amaranthus caudatus* L., which were grown for 48~60 hr at 27.5°C on a moistened filter paper in a plastic box (18×25×8.5 cm) in the dark and derooted, were soaked in a solution (1.0 ml, total volume) containing potassium phosphate buffer (pH 6.3, 6.5 mM), betacyanin precursors (0.5 mg/ml) and BA

Fig. 1. Effects on Betacyanin Biosynthesis of L-Tyrosine, L-Dopa, and D-Dopa in the Presence of BA.

L-Tyrosine, ●—●; L-dopa, ▲—▲; D-dopa, △—△. Broken (---) and dashed lines (--- and ---) denote controls for L-tyrosine, D-dopa, and L-dopa, respectively. The solid line is the mean of the amount of betacyanin ($n=6$) incubated in a solution containing potassium phosphate buffer, L-tyrosine, and $0.1 \mu\text{M}$ BA.

(0.0001~100 μM) in a vial ($\phi 2.5\text{ cm} \times 6\text{ cm}$). After incubation at 27.5°C for 24 hr, the seedlings were transferred to a glass tube ($\phi 1.5\text{ cm} \times 10.5\text{ cm}$) filled with deionized water (3 ml), frozen at -20°C overnight (lapping with a sheet of polyethylene film can avoid breakdown of the glass tube during freezing), and thawed. The differential absorbances between 542 nm and 620 nm of the solutions for two duplicates were measured and averaged. L-, D-Dopa and L-tyrosine (products of Nakarai Chemicals Co.) were used as available. For control experiments, the seedlings were incubated in the absence of BA.

Incubation in L-dopa resulted in a significant increase in the yield of betacyanin at concentrations above 0.004 μM of BA; the maximum yield at 10 μM induced by L-dopa was twice as high as that induced by L-tyrosine (Fig. 1). To confirm the minimum detectable concentration of BA in this test, the same assay was repeated in ten runs for each concentration below 0.01 μM . Although the average yield at 0.0004 μM (0.043 ± 0.003 , 95% confidence) was substantially the same as that of the control for L-dopa (0.039 ± 0.002) within experimental error, the yield at 0.001 μM (0.050 ± 0.003) was higher than the control. Thus, BA was detectable above 0.001 μM in the new assay; the yields at 0.004 μM and 0.01 μM were 0.056 ± 0.007 and 0.066 ± 0.003 , respectively. Controls for L- and D-dopa were slightly higher than that for L-tyrosine.

Sensitivity for BA was compared between the ordinary betacyanin assay using L-tyrosine and our method using L-dopa in terms of $C_{0.1\mu\text{MBA}}$, concentration at which the sample gives the same amount of betacyanin as 0.1 μM BA.²⁾ The yield at 0.004 μM of BA in this assay was equal to that at 0.1 μM of BA in the ordinary assay. Thus, the use of L-dopa resulted in a 25-fold increase in sensitivity. D-Dopa was one hundred times less effective than L-tyrosine, as expected.

L-Dopa, having a catechol structure, is easily autooxidized and its solution darkens. Although the darkening of

the seedlings and test solutions was observed during the assay, it was not very serious because the results were reproducible. To minimize the degradation of L-dopa, it is desirable to freshly prepare test solutions just before the assay.

Our *Amaranthus* betacyanin assay using L-dopa showed a marked increase in sensitivity to become almost comparable to that of tobacco callus assay. Therefore, the new assay method has high sensitivity as well as a short incubation period, convenience, and reproducibility. This assay can be applicable to the evaluation of cytokinin activity of both purine and non-purine cytokinins including 4-styrylpyrimidines.⁸⁾

References

- 1) F. Skoog, H. Q. Hamzi, A. M. Szweykowska, N. J. Leonard, K. L. Carraway, T. Fujii, J. P. Helgeson and R. N. Loeppky, *Phytochemistry*, **6**, 1169 (1967).
- 2) K. H. Köhler and K. Conrad, *Biol. Rund.*, **4**, 36 (1966).
- 3) N. L. Biddington and T. H. Thomas, *Planta*, **111**, 183 (1973).
- 4) S. Koyama, Z. Kumazawa, N. Kashimura and R. Nishida, *Agric. Biol. Chem.*, **49**, 1859 (1985).
- 5) T. R. Henderson, C. R. Frihart and N. J. Leonard, *Phytochemistry*, **14**, 1687 (1975).
- 6) N. Fisher and A. Dreiding, *Helv. Chim. Acta*, **55**, 649 (1972).
- 7) A. S. Garay and G. H. N. Towers, *Canad. J. Botany*, **44**, 231 (1966).
- 8) S. Nishikawa, Z. Kumazawa and N. Kashimura, Abstracts of Papers, Japan-U.S. Congress of Pharmaceutical Sciences, Honolulu, December, 1987, s223.