Biochimica et Biophysica Acta, 527 (1978) 497—500

BBA Report

BBA 61346

A RAPID MICRO SCALE METHOD FOR THE DETECTION OF LYSOPINE AND NOPALINE DEHYDROGENASE ACTIVITIES

LEON A.B.M. OTTEN and ROBBERT A. SCHILPEROORT

Department of Biochemistry, State University of Leiden, Wassenaarseweg 64, Leiden (The Netherlands)

(Received July 11th, 1978)

Summary

A rapid and sensitive method has been developed to determine lysopine dehydrogenase (EC 1.5.1.-) and nopaline dehydrogenase activities in crown gall tumour tissues. By this method, enzyme activities as low as 0.2 μmol octopine or nopaline per h per g fresh weight tumour tissue can still be detected. In non-infected young pea seedlings, no lysopine dehydrogenase activity was detected.

Crown gall is a neoplastic disease of dicotyledonous plants, caused by *Agrobacterium tumefaciens*. In Crown gall cells a number of unusual amino acid derivatives are found belonging to three classes, the so-called octopine class, nopaline class, and a recently discovered class, containing an addition product of α-ketoglutaric acid and glutamic acid [1]. The enzymes, synthesizing these compounds D-(+)-lysopine dehydrogenase (EC 1.5.1.-) [2], nopaline dehydrogenase and the enzyme responsible for the formation of the α-ketoglutaric acid-glutamic acid addition product, only occur in crown gall tumours [1,3—6]. Their synthesis in the tumours is strain specific [7] and is now known to be controlled by *Agrobacterium* plasmid genes [3,8,9].

Recently, it has been demonstrated that the tumorous state is characterized by the presence of multiple copies of an *Agrobacterium* plasmid fragment [10], which is transcribed into RNA [11,12]. This makes it likely that plant cells are transformed by plasmid DNA, transferred during tumour induction. The enzymes mentioned above are probably encoded by one of the plasmid genes. Therefore, the presence of these enzymes can be considered as a good marker for cells transformed by *Agrobacterium*.

In fact, testing for lysopine or nopaline dehydrogenase activity represents a method which is superior to tests aimed at detecting the mere presence of unusual amino acid derivatives. Based on the phenanthrenequinone staining for

guanidine compounds [13,14], a test was developed to permit fast screening of plant material for the presence of lysopine or nopaline dehydrogenase activity.

L-Arginine, α-ketoglutaric acid, sodium pyruvate were obtained from Merck; NADH from Boehringer and D-(+)-octopine from Sigma. Nopaline was synthesized and kindly provided by H. Kester (Leiden).

Nicotiana tabacum var. White Burley leaf tumours were initiated by infection with *Agrobacterium tumefaciens* strains B6S3 (LBA2) or C58 (LBA201) and cultured in vitro on a hormone-free medium as described earlier [3].

The extraction buffer used was 0.1 M Tris-HCl, 0.5 M sucrose, 0.1% (w/v) ascorbic acid, 0.1% (w/v) cysteine-HCl (pH 8.0). To a small amount of callus tissue or spun down cells (e.g. 10 mg) 1 vol. cold extraction buffer was added and the tissue ground with a small glass rod. The extract was centrifuged in an Eppendorf 3200 centrifuge for 2 min and the supernatant was used immediately for the enzyme assay.

Lysopine dehydrogenase activity was assayed with the following incubation mixture: 30 mM L-arginine, 75 mM pyruvate, 20 mM NADH, dissolved in 0.2 M sodium phosphate (pH 7.0). The arginine stock solution was neutralized with HCl before adding to the mixture. Nopaline dehydrogenase activity was tested with the following mixture: 60 mM L-arginine, 60 mM α-ketoglutaric acid, 16 mM NADH, dissolved in 0.2 M sodium phosphate (pH 6.8). Arginine and α-ketoglutaric acid were neutralized before adding to the mixture. 1 vol. incubation mixture was added to 1 vol. extract and incubated for 1 h at room temperature.

At the start and end of the reaction period 2 μl reaction mixture was spotted at the anodal site of a 20 × 25 cm MN 214 paper sheet and dried. 2 μl 0.5 μg/ml octopine or nopaline was used as a marker, while aqueous ethanol methylene green can be used as a visual marker, which migrates just behind arginine. Electrophoresis was performed in formic acid/acetic acid/water (5 : 15 : 80, v/v) for 1 h at 400 V. The paper was stained in 1 vol. 0.02% (w/v) phenanthrenequinone/ethanol, 1 vol. 10% (w/v) NaOH/60% (v/v) ethanol [13]. After drying, the spots were visualized under a long-wave ultraviolet lamp (366 nm). Guanidine compounds, yellowish at first, turned blue after about 2 days.

A representative chromatogram is shown in Fig. 1. It shows the formation of octopine and nopaline-type callus, respectively. It also shows that with the electrophoretic method used, a good separation of octopine and nopaline can be achieved. Since in the crude extract of the nopaline-type tumour nopaline is visibly present, this nopaline was eliminated by passing the crude extract over a Sephadex G-25 column.

Fig. 2 shows the result of an analysis of a number of calli derived from a transformation experiment in which *Nicotiana tabacum* protoplasts were infected with *Agrobacterium tumefaciens* and selected for growth on phytohormone-less medium [14].

Since the discovery of unusual amino acid derivatives in crown gall cells [16], a number of investigators have reported the occurrence of octopine in normal plant cells [17,18]. These reports were subsequently shown to be erroneous by Bomhoff in our laboratory [3] and also by others [5,19], and attributed to a misinterpretation of blue fluorescing spots which are especially

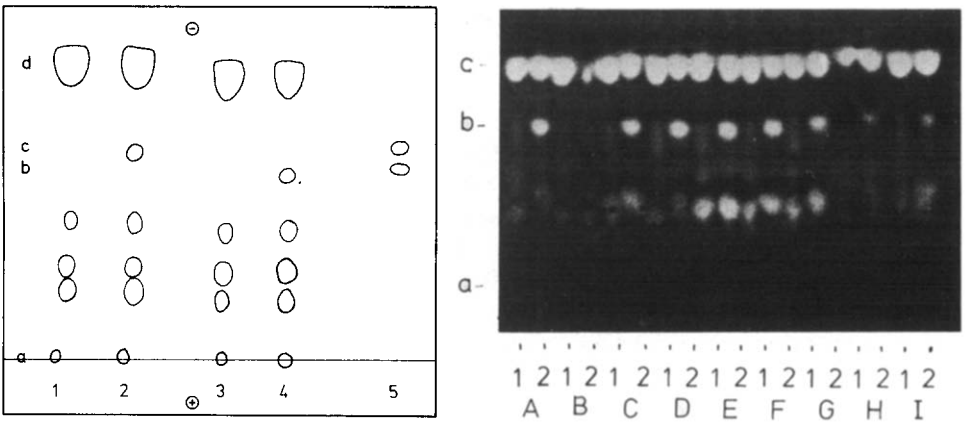

Fig. 1. Lysopine and nopaline dehydrogenase activities in octopine-type callus and nopaline-type callus respectively. 1. octopine test, t = 0 min; 2. octopine test, t = 60 min; 3. nopaline test, t = 0 min; 4. nopaline test, t = 60 min; 5. octopine and nopaline reference spots. a. start; b. nopaline; c. octopine; d. arginine.

Fig. 2. Analysis of lysopine dehydrogenase activity in tobacco calli after infection of tobacco protoplasts with *Agrobacterium tumefaciens*. 1. octopine test, t = 0 min; 2. octopine test, t = 60 min; A to I: different calli. a. start; b. octopine; c. arginine.

found in leguminous plants. A callus tissue containing octopine which was presumed to be normal, was shown to contain plasmid DNA sequences (Merlo, D.J., unpublished data). Enzyme tests will show the presence of lysopine dehydrogenase or nopaline dehydrogenase unambiguously by the increase of octopine or nopaline spots respectively. Octopine formation was tested with the method described in the supposedly octopine containing young seedlings of *Phaseolus vulgaris* [17], but were all negative (Otten, L.A.B.M., unpublished data). Up to now, there have been no other reports of octopine or nopaline in plant cells other than crown gall tissues. By use of the present enzyme assay, lysopine dehydrogenase and nopaline dehydrogenase activities have been detected in crown gall tumours induced on *Kalanchoe daigremontiana* by an *Agrobacterium* strain, which harbours a co-integrate of an octopine and nopaline TI-plasmid (Hooykaas, P., unpublished data). The crude extracts, however, only showed the presence of nopaline. The absence of octopine might reflect differing affinities and hence competition of both enzymes towards their common substrates, arginine and reduced cofactor in the tumour cells. Although lysopine dehydrogenase and nopaline dehydrogenase preferentially use NADPH as a cofactor [2,6,20], we used NADH in the enzyme tests, since contrary to NADH, NADPH was found to be inhibitory at concentrations higher than 100 μM. Since crude extracts of callus material contain a high NADPH and NADH oxidizing activity, a high NADPH concentration would be needed in the assay, but this would prevent a sufficient concentration of octopine or nopaline to be build up within a reasonable time. Because of NADPH and NADH oxidizing activities, it is also impossible to use the decrease in absorbance at 340 nm to measure enzyme activity. To do so, substantial purification is needed [2].

The lysopine dehydrogenase and nopaline dehydrogenase activities vary little in the temperature range of 20°C—30°C, but the enzymes become rapidly

unstable at temperatures above 30°C (Otten, L.A.B.M., unpublished data). The detection limit of octopine or nopaline is about 0.2 μM after paper electrophoresis. Most callus tissues in our laboratory contain lysopine dehydrogenase activities of about 20—100 nmol per h in the assay described, amounting to concentrations of 2—10 μM. Suspension cultures generally contain even more activity. Extraction with sand added, or homogenizing cells with a Polytron apparatus did not result in improved enzyme recoveries. Up to now, no nopaline tumours were found, which did not contain easily detectable quantities of endogenous nopaline. With the development of in vitro crown gall transformation systems, the enzyme test described provides a rapid screening for transformants containing lysopine dehydrogenase or nopaline dehydrogenase, with only small quantities of material needed: 5 mg callus tissue or pelleted suspension cells can still be handled easily.

References

1 Chang, C-C. and Lin, B-Y. (1978) Plant Physiol. 61, 4, suppl. 73
2 Otten, L.A.B.M., Vreugdenhil, D. and Schilperoort, R.A. (1977) Biochim. Biophys. Acta 485, 268—277
3 Bomhoff, G. (1974) Ph.D. Thesis, Leiden
4 Lejeune, B. (1973) Ph.D. Thesis, Paris
5 Gordon, M.P., Farrand, S.K., Sciaky, D., Montoya, A., Chilton, M-D. and Nester, E.W. (1977) in Molecular Biology of Plants (Rubinstein, I., ed.), Academic Press, New York, in the press
6 Hack, E. and Kemp, J.D. (1977) Biochem. Biophys. Res. Commun. 78, 2, 785—791
7 Petit, A., Delhaye, S., Tempé, J. and Morel, G. (1970) Physiol. Vég. 8, 205—213
8 Watson, B., Currier, T.C., Gordon, M.P., Chilton, M-D. and Nester, E.W. (1975) J. Bacteriol. 123, 255—264
9 Bomhoff, G., Klapwijk, P.M., Kester, H.C.M., Schilperoort, R.A., Hernalsteens, J.P. and Schell, J. (1976) Mol. Gen. Genet. 145, 177—181
10 Chilton, M-D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P. and Nester, E.W. (1977) Cell 11, 263—271
11 Drummond, M.H., Gordon, M.P., Nester, E.W. and Chilton, M-D. (1977) Nature 269, 535—536
12 Ledeboer, A.M. (1978) Ph.D. Thesis, Leiden
13 Yamada, S. and Itano, H.A. (1966) Biochim. Biophys. Acta 130, 538
14 Márton, L., Wullems, G.J., Molendijk, L. and Schilperoort, R.A., Nature, in the press
15 Itano, H.A. and Yamada, S. (1972) Anal. Biochem. 48, 483
16 Lioret, C. (1956) Bull. Soc. Fr. Physiol. Vég. 2, 76
17 Wendt-Gallitelli, M.F. and Dobrigkeit, I. (1973) Z. Naturforsch. 28c, 768
18 Johnson, R., Guderian, R.H., Eden, F., Chilton, M-D., Gordon, M.P. and Nester, E.W. (1974) Proc. Natl. Acad. Sci. U.S. 71, 536
19 Holderbach, E. and Beiderbeck, R. (1976) Phytochemistry 15, 955—956
20 Goldman, A. (1977) Plant Sci. Lett. 10, 49—58