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DETERMINATION OF QUINOLINIC ACID PHOSPHORIBOSYLTRANSFERASE IN TOBACCO

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; quinolinic acid phosphoribosyltransferase; HPLC.

Abstract—A fast procedure was developed for the extraction and assay of quinolinic acid (QA) phosphoribosyl (PR) transferase based on gel filtration through a prepacked disposable PD 10 column and on reversed-phase HPLC. This makes the use of radiolabelled QA unnecessary. The specific activities determined in different organs of tobacco and in cell suspension cultures were determined; they indicate that this enzyme is probably involved in the regulation of nicotine biosynthesis. With a preparation from the roots of *Nicotiana tabacum* var. Samsun, the K_m values for QA and PR-pyrophosphate were 5.1 and 21 μM, respectively.

INTRODUCTION

Quinolinic acid phosphoribosyltransferase (QAPRT) is the anaplerotic enzyme of the pyridine nucleotide cycle which provides not only NAD but also components for the biosynthesis of alkaloids as ricinine, nicotine and anabasine [1], but also *N*-methylnicotinic acid (trigonelline), which is believed to have phytohormone properties [2, 3].

In an investigation on the regulation of the nicotine pathway in differentiated and undifferentiated tobacco cells, we chose QAPRT because there are indications that this enzyme has regulatory functions. It has been reported that QAPRT is enhanced in tobacco roots relative to the leaves and to the roots of non-nicotine-producing plants [4].

QAPRT has been purified from *Ricinus* seedlings [5], from bovine liver [6, 7] and from bacteria [8, 9]; however, it has not been thoroughly described from tobacco. In the present paper, a fast method for the extraction and assay of QAPRT is described and data are presented concerning the properties of the tobacco enzyme and its activities in differentiated tissue and suspension cultured cells.

RESULTS AND DISCUSSION

In their classical work on QAPRT of castor beans, Mann and Byerrum [5] determined $^{14}CO_2$ released during the decarboxylation of labelled QA which had to be synthesized. Figure 1 shows that HPLC offers a rapid and simple method for the direct determination of the enzymatic product nicotinate mononucleotide. As this compound is negatively charged, tetrabutylammonium ions were used in the isocratic procedure to enhance the retention time slightly in the reversed-phase column. Recently, an isocratic procedure using 25 mM $(NH_4)_3PO_4$ as the mobile phase was described for the determination of nicotinate PR-transferase from bakers yeast [10].

For the extraction of enzyme activity from the roots, best results were obtained using for homogenization a glass homogenizer (Potter–Elvehjem). After centrifugation, the supernatant was either dialysed overnight to eliminate low MW substances, or applied onto the recently available prepacked disposable PD 10 column, which is filled with Sephadex G 25. Table 1 shows that the latter procedure gave the best results with the higher specific activity; furthermore, it is very rapid and reproducible. The whole procedure can easily be scaled down; for one assay 40 μl was used, which corresponds in the case of roots to *ca* 12 mg fresh plant material.

The enzyme is stable in the treated extract for at least 2

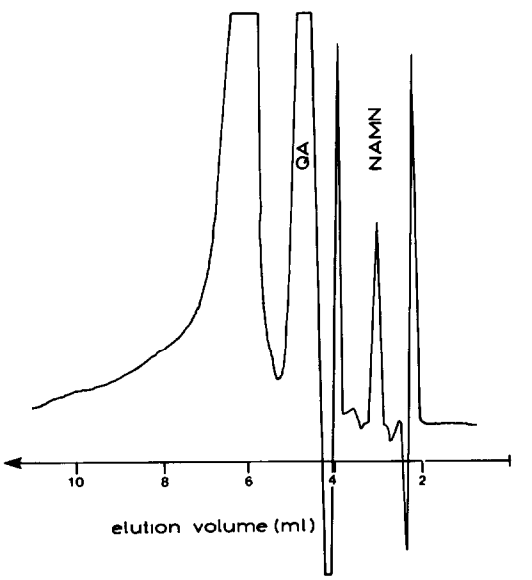

Fig. 1. Elution profile on an RP-18 column for the determination of the product after the enzymatic reaction (NAMN, nicotinic acid mononucleotide; QA, quinolinic acid). 30 μl sample injection, flow rate 1 ml/min, scale 10 m OD, eluant (isocratic) 0 2 M $(NH_4)_2HPO_4$ of pH 5.1 and 10 mM tetrabutylammonium hydrogen sulphate.

Table 1. Specific activity of quinolinic acid phosphoribosyltransferase in tobacco plant tissue and suspension cultured cells

Probe (method)*	Amount of tissue (g (fr. wt))	Amount of protein (mg)	Total activity (nmol/min)	Activity per g fr. wt (nmol/min per g)	Specific activity (nmol/min per mg)
Root					
(dialysis)	2.8	3.7	6.9	2.5	1.9
(PD 10)	2.8	4.0	10.6	3.8	2.7
Leaf lamina	1.4	11	1.14	0.81	0.10
Stem	1.4	1.0	0.70	0.50	0 70
Suspension cultured cells					
8 days old	1.5	5.1	0.72	0.48	0.14
21 days old	3.5	9.5	1.3	0.37	0 14

*With the exception of an aliquot of the root, all the other probes were applied on a prepacked disposable Sephadex G25 column (PD 10) for gel filtration. The probes from the suspension cultured cells were taken at days 8 and 21, respectively, after passage into fresh medium.

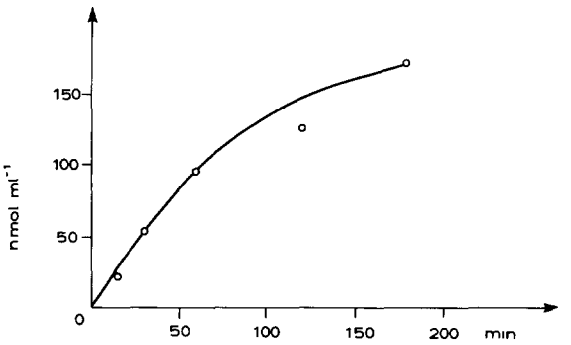

Fig. 2. Kinetics of the enzymatic reaction. For each point, 40 μl of the PD 10 treated enzyme fraction from tobacco roots was brought to a total volume of 150 μl under the conditions of buffer B and incubated at 30°. After the time indicated, the probe was heated for 1 min in a boiling water bath and 25 μl was injected for the determination of NAMN as shown in Fig. 1. The amount of NAMN formed related to 1 ml of the extract is plotted vs time.

days in the cold; however, it can also be frozen and stored at −20° without significant loss of activity. Figure 2 shows the kinetics of the enzymatic action and indicates that under the conditions applied the reaction is linear at least for 60 min.

The HPLC procedure allows an easy determination of K_m values. For QA and PR-pyrophosphate, values of 5.1 and 21 μM were obtained; they are in the magnitude of those reported for the enzyme from other sources. A K_m value for QA of 12 μM was found for the enzyme from *Ricinus* seedlings [5], whereas for bovine liver enzyme values of 7 [6] and 56 μM [7] were reported, and a value of 120 μM for the pseudomonad enzyme [9]. For PR-pyrophosphate, the K_m values for QAPRT from *Ricinus* seedlings was found to be 45 [5], 50 and 74 μM for the liver [7] and the pseudomonad [9] enzyme, respectively. These values may not only reflect different active sites of the enzymes from different sources, but also depend on the different assay conditions applied, such as variations in the pH, ionic strength and Mg^{2+} ion concentrations.

The specific activity found for the extract from *N. tabacum* root of 2.7 nmol/min per mg is rather high when compared to a value of 0.4 nmol/min per mg reported by Mann and Byerrum [4] for an extract of *N. rustica* roots. The reason for this discrepancy may arise from an improved extraction procedure or lie in differences of the varieties. The same authors reported specific activities of 1.5 and 0.03 nmol/min per mg for extracts from castor bean endosperm and leaves, respectively. Table 1 shows a value of 0.1 nmol/min per mg for the tobacco leaves and, interestingly, a significantly higher specific activity for the extract of the stem. The dedifferentiated suspension cultured cells are known to turn off their secondary metabolism. The 20-fold reduction in activity of QAPRT indicated in Table 1 may demonstrate that the suspension cultured cells have activities necessary for the primary metabolism only. This culture produces only very little nicotine, a value of 7.7 μg/g dry wt was determined, whereas the nicotine content of the roots from *N. tabacum* var. Samsun was found to vary from 2500 to 4000 μg/g dry wt. This confirms the suggestion that the high level of QAPRT activity found in the tobacco root is a prerequisite for effective nicotine production in this organ. It is a great challenge to explore how this enzyme is regulated, which is connected both to primary and secondary metabolism.

EXPERIMENTAL

Chemicals. Tetrabutylammonium hydrogen sulphate was purchased from Merck, QA, nicotinic acid mononucleotide, dithioerythritol and 3-(*N*-morpholino)propane sulphonic acid (MOPS) from Sigma; and PR-pyrophosphate from Serva; the PD 10 prepacked disposable column was from Pharmacia.

Buffers. (A) 100 mM MOPS–NaOH (pH 7.4), 5 mM $MgCl_2$, 0.1 mM EDTA and 10 mM DTE. (B) 115 mM KPi (pH 7), 12.5 mM $MgCl_2$, 5 mM DTE, 0.2 mM QA and 0.4 mM PRPP.

Plant material. Nicotiana tabacum var. Samsun plants were grown in a glasshouse with natural light conditions. The suspension culture was obtained from seedlings of the same plant and was maintained in Murashige and Skoog medium [11] with 3% sucrose, 0.2 ppm kinetin and 2 ppm α-naphthyl acetic acid.

Enzyme extraction. The roots of *ca* 12-week-old plants were carefully washed, dried with filter paper, and weighed. All the following operations were performed at 4°. Roots (3 g) were

suspended in 6 ml buffer A and homogenized in a glass homogenizer (Potter–Elvehjem) After centrifugation at 27 000 *g*, 2.5 ml of the supernatant was applied onto a Sephadex G 25 prepacked PD 10 column equilibrated and eluted with buffer A. The protein peak and the activity were contained within 3.5 ml of the eluate.

Enzyme assay. The reaction was started by the addition of the enzyme soln to a total vol. of 150 μl adjusted to the conditions of buffer B. For the determination of the K_m values, the incubation time was 30 min, otherwise 1 hr at 30°. The reaction was stopped by heating in a boiling water bath for 1 min and thereafter frozen for the determination of the product.

HPLC chromatography. The product was separated on a 5 μm Merck Lichrosorb RP-18 column (25 cm × 4 mm); the HPLC system consisted further of a LDC constametric II pump, Rheodyne probe injector and Kontron Uvikon 722 LC. The isocratic mobile phase was 200 mM $(NH_4)_2HPO_4$ of pH 5.1 and 10 mM tetrabutylammonium hydrogen sulphate filtered through ultrafilter RC 55, 0.45 μm of Schleicher and Schüll. The detection was at 254 nm; the amount of product was determined from the peak height or area.

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