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β-D-GLUCOSIDASE ACTIVITY IN DEVELOPING LEAVES OF *NICOTIANA TABACUM**

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Abstract—Tobacco (*Nicotiana tabacum* L. cv. Burley 21) leaves were assayed for β-D-glucosidase activity, using esculin as substrate. The enzymatically produced esculetin was silylated and quantitatively measured by GLC, using a tritium foil electron-capture detector. In field-grown plants, the activity in mid-stalk leaves increased with plant maturation; conversely, the activity in the top leaves decreased.

INTRODUCTION

β-D-Glucosidase [E.C. 3.2.1.21] catalyzes the hydrolysis of phenolic glucosides to their corresponding aglycones and glucose [1]. Some of these aglycones are substrates of peroxidase and polyphenoloxidase [2]. Among the products of the latter enzyme-catalyzed reactions are lignins, lignans and brown pigments, which are of importance to tobacco physiology and quality [2–4].

Existing methods for β-D-glucosidase use colorimetric [5–7] and fluorometric [8, 9] techniques. Esculin was a satisfactory substrate for a colorimetric β-glucosidase assay in yeasts [7]. Gas chromatographic (GLC) methods have recently been used to separate and detect nanogram amounts of phenols [10, 11]. A GLC technique for L-phenylalanine ammonia-lyase [E.C. 4.3.1.5] activity in plants was developed [12], which provided the means for the analysis of tissues low in that enzyme. The objectives of the present investigation were to develop a rapid, sensitive GLC assay for β-D-glucosidase activity in leaves of tobacco (*Nicotiana tabacum* L.) during growth and development.

RESULTS AND DISCUSSION

A GLC-electron-capture detection method was developed for measurement of β-D-glucosidase activity using esculin as substrate. In tests it proved possible to detect esculetin (the hydrolysis product) at concentrations as low as 15 ng when esculin was incubated with a partially purified form of the enzyme from tobacco. Extracts from leaves of actively growing tobacco like potato [5] had enzyme activities that were linear with incubation time from 0–6 hr. The range of enzyme activities in the tobacco tissues studied was such that 3 mg esculin provided sufficient excess of substrate for an assay.

To determine precision, assays were performed on four separate extracts of a pooled leaf sample. The mean activity was 26·00 ± 0·88 units/mg dry wt. Extracts were dialyzed and carried through the complete analytical procedure without substrates, and no esculetin was produced. Also, esculin without enzyme extract was taken through that part of the method following the dialysis step, and no esculetin was formed in the absence of the enzyme. The method thus provides the sensitivity and precision necessary to analyze plant tissue low in glucosidase activity.

The β-D-glucosidase activities in tobacco leaves were undetectable at time of transplant, i.e. 8 weeks after seed germination. In field-grown plants, leaves harvested from the middle of the stalk had enzyme activities that steadily increased as the plants grew and matured (7–85 units from the 3 to 13-week stage). On the other hand, upper leaves from these plants had peak activity (129 units) much earlier, i.e. 3 weeks post-transplant.

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After 3 weeks, activities in upper leaves dropped to 27–32 units from 7–13 weeks after transplant. This plateau coincided with the rapid growth of the plants and early development of the floral buds.

In greenhouse-grown plants, enzyme activities in leaves from the middle position of the stalk, harvested at the time of transplant and at 7 and 13 weeks post-transplant, ranged from 0 to 50 units. The activities did not appear to increase steadily, as they did in field-grown leaves. However, samples represented only three harvest dates. Upper leaves of greenhouse-grown plants had less activity at 7 and 13 weeks post-transplant than similar leaves from plants grown in the field. It was shown earlier that polyphenoloxidase activity was higher in leaves of field-grown than in those of greenhouse-grown plants [13]. These differences have been attributed to differences in temperature and light quality in the two environments. The near-ultraviolet light present in the field is absent in the greenhouse [14, 15]. Leaf position had a significant effect on levels of the plant phenolics, chlorogenic acid, scopoletin, rutin, and enzyme activities that are involved in the biosynthesis, oxidation, and polymerization of these compounds [13–16].

Flowers from greenhouse-grown plants at full bloom had a glucosidase activity of 159 units, as compared to 14 units in the top leaves taken from the same plants. Therefore, floral tissue had higher activity than any of the leaf samples assayed.

EXPERIMENTAL

Reagents. All chemicals were reagent grade. Acetonitrile, bis-(trimethylsilyl)trifluoroacetamide (BSTFA), esculin, esculetin, and caffeic acid were obtained and handled as previously described [10, 11]. Caffeic acid *n*-butyl ester was synthesized as before [17]. β-D-Glucosidase (emulsin) was obtained from a commercial source.

Analytical procedure. A 100-mg sample of plant tissue (freeze-dried) homogenized with 10 ml 0·01 M phosphate buffer (pH 6·80). The homogenate was centrifuged for 10 min at 11000 *g*. The supernatant was brought to 60% saturation with solid $(NH_4)_2 SO_4$. The mixture was centrifuged at 16000 *g* for 10 min, and the supernatant discarded. The ppt. was dissolved in 5 ml 0·20 M phosphate buffer (pH 6·80), the solution adjusted to pH 4·40, and, the solution dialyzed overnight against H_2O. All steps were performed at 5°. The dialyzed protein solution plus 3·0 mg esculin was shaken for 4 hr at 22° and the solution was immediately taken to dryness. For GLC, an internal standard (caffeic acid *n*-butyl ester, *ca* 0·25 mg) was added and the mixture dissolved in 0·80 ml acetonitrile and silylated with 0·20 ml BSTFA. The silylation and GLC conditions used were as before, except that about 6 μl was injected [10]. The peak areas of TMS-esculetin and TMS-caffeic acid *n*-butyl ester were used to determine esculetin contents in terms of a wt/unit response [18]. An enzyme unit was defined as the amount of enzyme that produced 1·0 nmol esculetin/hr.

Plant growth and sampling conditions. Nitociana tabacum L. cv. Burley 21 seedlings were grown to transplant size under high-intensity cool-white fluorescent light [15]. When plants were about 10 cm, they were transplanted to field or greenhouse. Normal cultural procedures were used during the post-transplant period, except that the floral buds were not removed. Leaves were collected (34 plants) from upper- and mid-stalk positions at various intervals during the growth period. Midribs were discarded, and the lamina tissue pooled and freeze-dried, and stored *in vacuo*. After the last collection of leaves, 13 weeks after transplant, flowers of greenhouse-grown plants at the stage of anther dehiscence were harvested, pooled, frozen in dry ice, and freeze-dried. Enzyme assays were performed within 3–5 days after leaves and flowers were collected from plants.

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