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CONVERSION OF FUROSTANOL GLYCOSIDE TO SPIROSTANOL GLYCOSIDE BY β-GLUCOSIDASE IN *COSTUS SPECIOSUS*

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Key Word Index—*Costus speciosus*; Zingiberaceae; post-harvest hydrolysis; β-glucosidase; furostanol glycoside; HPLC; *in vitro* cultured plantlets; rhizomes; spirostanol glycoside.

Abstract—A diosgenin-rich plant, *Costus speciosus* (Koenig) Smith was found to contain β-glucosidase which converts a furostanol glycoside, protogracillin, to a spirostanol glycoside, gracillin. It was demonstrated that the enzyme was present in rhizomes of plants grown in a greenhouse and whole parts of plantlets cultured *in vitro*. The soluble enzyme from the cultivated rhizomes showed pH optima of 5.0 and the apparent *Km* for protogracillin of 0.10 mM. Activity of the enzyme was inhibited by typical β-glucosidase inhibitors, such as D-glucono-1,5-lactone and conduritol-β-epoxide.

INTRODUCTION

In plants, spirostanol glycosides are formed from furostanol glycosides during post-harvest treatment and storage [1]. Although the biological activities of these glycosides have been well described [2, 3], little is known about their physiological roles in intact plants. In 1969 Joly and his co-workers demonstrated conversion of [4-^{14}C]protodioscin to dioscin by a *Dioscorea floribunda* homogenate [4], but no further reports on this conversion have been published. Gurielidze and co-workers detected an oligofurostanoside-specific β-glucosidase activity in leaves of *D. deltoidea* in 1986 [5]. They estimated enzyme activity by the decrease of oligofurostanosides using a color reaction with *p*-dimethylaminobenzaldehyde, where more than half of the activity was found in the chloroplast fraction. However, the enzymatic formation of spirostanol glycosides has not been determined.

In order to elucidate the physiological roles of furostanol and spirostanol glycosides, we are studying the enzyme responsible for the post-harvest conversion of these glycosides. In this publication, we report the presence of a novel β-glucosidase in various organs of *Costus speciosus*, a plant known to contain diosgenin [6, 7], furostanol and spirostanol glycosides [8–12].

RESULTS AND DISCUSSION

One of the predominant furostanol glycosides of *C. speciosus*, protogracillin [12], was used as a substrate for the β-glucosidase assay. Protein extracts prepared from rhizomes of plants grown in a greenhouse were incubated with protogracillin and the reaction was monitored by HPLC (Fig. 1). The increase in gracillin during the incubation correlated well with a decrease of the furostanol glycoside. Formation of furostanol and spirostanol glycosides other than protogracillin and gracillin was not detected in the reaction mixture. In controls using boiled protein solution, neither production of gracillin nor reduction of protogracillin could be detected. The stoichiometry of the reaction was established by quantification of the released D-glucose (data not shown) as shown in the following equation:

$$\text{Protogracillin} \rightarrow \text{Gracillin} + \text{D-Glucose}$$

In the following experiments, β-glucosidase activity was estimated by determination of gracillin by HPLC because of its high sensitivity and convenience.

Protogracillin 26-*O*-β-glucosidase activity in different organs of cultivated and *in vitro* cultured *C. speciosus* was studied (Table 1). Rhizomes of cultivated plants, which are known to accumulate furostanol and spirostanol glycosides [13], were found to contain this β-glucosidase activity. On the other hand, other parts of the plants which do not accumulate the glycosides [13] did not show any detectable enzyme activity. Whole parts of *in vitro* cultured plantlets of *C. speciosus*, as well as the rhizomes of cultivated plants, have been found to contain mainly furostanol glycosides, such as protodioscin and protogracillin, if they are analysed while fresh (Inoue, K. and Ebizuka, Y., unpublished results). They all showed almost the same level of activity as that of cultivated rhizomes (Table 1).

For preliminary purification and characterization of the enzyme, the rhizomes of the cultivated plants were

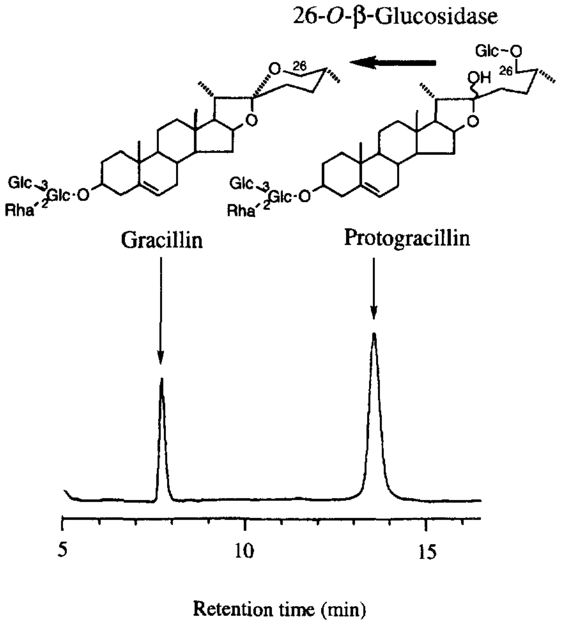

Fig. 1. HPLC profile of n-BuOH soln of the assay mixture. For conditions, see Experimental.

Table 1. Protogracillin 26-O-β-glucosidase activity in different parts of *C. speciosus*

Parts		Specific activity (nkat mg^{-1} protein)
In vitro cultured plantlets	Roots	36.5
	Stems	47.7
	Leaves	16.4
Field grown plants	Rhizomes	62.6
	Roots	n.d.
	Stems	n.d.
	Leaves	n.d.

n.d., not detectable.

used as the starting material because of their availability and relatively high specific activity (Table 1). The β-glucosidase activity was found in both the supernatant and pellet fractions in a ratio of 2:3 after 107000 g centrifugation of the post-mitochondrial fraction. Attempts to solubilize the latter fraction with KCl and detergents such as Triton X-100 were unsuccessful, so the enzyme in the 107000 g supernatant fraction was characterized.

The pH optimum of the soluble enzyme was 5.0 (50% activity at pH 3.5 and 6.5, respectively). The apparent Km for protogracillin, calculated by Lineweaver–Burk plots, was 0.10 mM. Reducing reagents such as 2-ME and DTT (5–20 mM) and two detergents, Triton X-100 (0.05, 0.10%) and Tween 20 (0.1 and 0.5%), had no influence on the β-glucosidase activity. D-Glucono-1,5-lactone and conduritol-β-epoxide, well-known inhibitors for β-glucosidases [14, 15], caused 50% inhibition of the reaction at concentrations of 0.5 mM and 10 mM, respectively.

A β-glucosidase activity on the 26-O-glucoside bond of a furostanol glycoside has been demonstrated to be localized in the organs which accumulate furostanol glycosides in *C. speciosus*. The co-occurrence of the glucoside and the enzyme involved in its degradation has been also reported for a chamomile plant which contains apigenin 7-O-glucoside [16]. Purification of the protogracillin 26-O-β-glucosidase in *C. speciosus* is now in progress using the HPLC assay method devised in the present work. The purified enzyme would serve for revealing the organization and regulation of this 'post-harvest' conversion leading to the elucidation of physiological significance of furostanol and spirostanol glycosides in plants.

EXPERIMENTAL

Plant materials. *Costus speciosus* used for experiments was cultivated in a greenhouse of the Experiment Station for Medicinal Plant Studies of The University of Tokyo in Chiba prefecture, Japan, where the voucher specimen is deposited.

In vitro *culture.* The medium used for the experiments was half strength Murashige-Skoog (MS) [17] basal medium containing 30 g l^{-1} sucrose and adjusted to pH 5.7 before autoclaving at 121° for 15 min, solidified with 0.3% Gelrite. The cultures were maintained at 25° under 16 hr per day light (70 μE m^{-2} s^{-1}). Shoots excised from aerial parts of *C. speciosus* were sterilized by 75% ethanol and 2% NaOCl containing Tween 20 (1 drop per 50 ml). About 1 mm height of shoot tip was cut off aseptically and placed on the medium supplemented with 0.5 mg l^{-1} indole acetic acid and 0.1 mg l^{-1} kinetin. About four months later, regenerated plants were obtained and subcultured further on the same medium without phytohormones at 6–8 week intervals to obtain sufficient materials for further experiments.

Enzyme extraction. Each part of the cultivated plant was sepd, frozen in dry ice–acetone and ground with a grater. *In vitro* cultured plantlets, cultured for 2–3 months, were harvested and divided into each part, frozen in liquid nitrogen and ground in a mortar. Ground material was homogenized in a blender with ice-cold 0.10 M K-Pi buffer (pH 7.0), 2 ml per g fr. wt material. After centrifugation at 12000 g for 30 min and cotton filtration, a post-mitochondrial fraction was obtained. The experiment was carried out three times. Protein was quantified by Bradford's method and a calibration curve obtained with bovine-serum albumin [18].

Standard enzyme assay. Protogracillin and gracillin, isolated from the rhizomes of *C. speciosus* [12], were used as a standard substrate and a product, respectively. The reaction soln containing 125 μl of 50 mM K-Pi buffer (pH 6.0) and 10 μl of 5 mM protogracillin in EGME was preincubated at 35° for 5 min, and then 15 μl of appropriately diluted protein soln was added. For determination of gracillin by HPLC, the reaction was stopped after

10 min incubation by addition of 150 μl *n*-BuOH satd with H_2O. After centrifugation (2000 *g* for 2 min), 20 μl of the upper layer was subjected directly to HPLC as following: column, TSK gel Amide-80 (5 μm, Tosoh, Japan); column temp, 40°; gradient elution, solvent A (H_2O) and solvent B (CH_3CN) (gradient: 0–1 min, 85–75% B; 1–13 min, 75% B; 13–14 min, 75–85% B; 14–20 min, 85% B); flow rate, 0.8 ml min^{-1}; detection, UV203 nm. Gracillin concentrations were determined using a calibration curve that was over a concentration range of 1.3×10^{-2} nmol to 1.7×10 nmol per 20 μl *n*-BuOH ext. For determination of D-glucose, the reaction was stopped after 10 min incubation by boiling in a water bath for 3 min. The released D-glucose was estimated with the glucose oxidase–peroxidase–*o*-dianisidine assay system [19]. The amount of protein in the reaction mixture was adjusted to keep the reaction velocity linear with time up to 30 min.

Partial characterization of the soluble β-glucosidase. Fractionation by ultracentrifugation (107000 g, 1 hr) was repeated to avoid cross contamination of the supernatant and pellet fractions. The reaction mixture contained 1.0 μg soluble protein. Fifty mM K-Pi buffers in pH range 6.0–8.0 and 50 mM sodium citrate buffers of pH 3.0–6.0 were used to determine the pH optimum. The *Km*-value was obtained by varying the protogracillin concentration from 1.1×10^{-2} to 1.5 mM.

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