Purification and characterization of furostanol glycoside 26-*O*-β-glucosidase from *Costus speciosus* rhizomes

Kentaro Inoue, Yutaka Ebizuka*

Department of Pharmacognosy and Phytochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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Abstract **In plants, spirostanol glycosides (steroid saponins) are known to be formed from furostanol glycosides during postharvest treatment and storage. Furostanol glycoside 26-*O*-β-glucosidase (F26G) involved in this conversion was purified to apparent homogeneity for the first time from *Costus speciosus* rhizomes which accumulate these glycosides. The enzyme was highly specific for cleavage of the C-26-bound glucose moiety of furostanol glycosides showing K_m for protogracillin of 50 μM. Glucono-1,5-lactone, a typical β-glucosidase inhibitor, and diosgenin, an aglycone of spirostanol glycosides, strongly inhibited the enzyme activity. The purified F26G is dimeric with a native apparent molecular weight of 110,000 consisting of subunits of 54,000 and 58,000. The N-terminal sequence of the 54,000 protein has a high similarity to the sequences found in N-terminal regions of known plant β-glucosidases.**

Key words: Furostanol glycoside; Spirostanol glycoside; β-Glucosidase; Postharvest hydrolysis; F26G; *Costus speciosus* Sm

1. Introduction

Spirostanol glycosides showing typical saponin characteristics such as antifungal and haemolytic activities are formed from furostanol glycosides which do not show any of these activities (Scheme 1) [1–3]. This conversion proceeds during postharvest treatment and storage of the plant material [2]. Many plant β-glucosidases have been found to be specific for their native substrates [4,5]. Thus, we use the term 'furostanol glycoside 26-*O*-β-glucosidase (F26G)' for the enzyme involved in the above conversion since it is also expected to be specific for removal of the C-26-bound glucose of furostanol glycosides.

There have been only a few reports on the F26G. In 1969, Joly et al. demonstrated conversion of [4-^{14}C]protodioscin to dioscin by a *Dioscorea floribunda* homogenate [6]. Several Russian groups have detected the oligofurostanoside-specific β-glucosidase activity in three plants which contain furostanol and spirostanol glycosides [7–9]. They estimated the enzyme activity by determining decrease of oligofurostanosides monitored by their specific color reaction with *p*-dimethylaminobenzaldehyde. In leaves of *D. deltoidea*, more than half of the activity was found in the chloroplast fraction [7]. In leaves of *Yucca gloriosa*, the activity was manifested to be in mesophyll tissue [8]. The enzyme in inflorescences of *Allium erubescens* showed K_m for deltoside, a furostanol glycoside of this plant, of 23 mM [9]. However, the enzyme responsible for the formation of spirostanol glycosides has not been purified nor characterized from any sources yet.

Although the biological activities of spirostanol and furostanol glycosides have been well-described [1,10], little is known about their functions in intact plants. In order to elucidate their physiological roles, we have been studying the F26G in *Costus speciosus*, a Gingiberaceous tropical plant, known to contain furostanol and spirostanol glycosides [11–15]. We have established a highly sensitive F26G assay method using HPLC and successfully detected the activity in the rhizomes which accumulate furostanol glycosides [16]. In this publication, we report the first purification and characterization of F26G from *C. speciosus* rhizomes.

2. Materials and methods

2.1. Materials

C. speciosus used for experiments was cultivated in a greenhouse of the Experiment Station for Medicinal Plant Studies of University of Tokyo in Chiba prefecture, Japan. Furostanol and spirostanol glycosides were isolated and identified from rhizomes of *C. speciosus* [15]. β-Sitosterol was isolated from soybean. All other reagents were of analytical grade. Protein was quantified by Bradford's method and a calibration curve obtained with bovine-serum albumin [17].

2.2. Purification of furostanol glycoside 26-*O*-β-glucosidase (F26G)

Rhizomes of *C. speciosus* free from other organs were sliced into small pieces and shock-frozen in dry ice/acetone. The frozen materials (36 g) were homogenized with a Waring blender in 110 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing Polyclar AT (3%, w/v) and sodium isoascorbate (0.3%, w/v). Unless otherwise stated, purification of F26G was performed at 4°C. After centrifugation at 12,000 × *g* for 30 min, the supernatant (crude extract) was subjected to ammonium sulfate fractionation. The precipitates formed between 30 to 60% saturation of ammonium sulfate were dissolved in 5 ml of buffer A (20 mM potassium phosphate buffer, pH 7.0) and then passed through PD-10 columns (Pharmacia) equilibrated with buffer A to remove ammonium sulfate and other low molecular weight compounds. Protein containing fractions were pooled and loaded onto a DEAE-cellulose column (Whatmann DE52, 16 × 40 mm) equilibrated with buffer A. After washing the column with 80 ml of buffer A, the protein with F26G activity was eluted with 40 ml of buffer A containing 0.5 M KCl. The pooled active fraction was precipitated with ammonium sulfate (60% saturation), dissolved in 2.5 ml of buffer A and applied to a PD-10 column equilibrated with buffer A. Solid ammonium sulfate was added up to a final concentration of 1 M and insoluble materials were removed by Micropure-0.22 (Amicon). Then the clear protein solution was subjected to HPLC using a TSKgel Ether-5PW column (Tosoh; 7.5 × 75 mm) equilibrated with buffer A containing 1 M ammonium sulfate at room temperature. Bound protein was eluted by linearly decreasing the

*Corresponding author. Fax: (81) (3) 5689 2962.
E-mail: yebiz@mol.f.u-tokyo.ac.jp

Abbreviations: EGME, ethylene glycol monomethyl ether; F26G, Furostanol glycoside 26-*O*-β-glucosidase; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibition concentration; pNP, *p*-nitrophenol; pNPG, *p*-nitrophenyl β-D-glucoside; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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ammonium sulfate concentration to 0 M. Buffer of the pooled active fractions was exchanged to buffer A by ultrafiltration using Centricon 30 (Amicon) and the resultant concentrated protein solution was subsequently subjected to HPLC using a TSKgel DEAE-5PW column (Tosoh; 7.5 × 75 mm). F26G was eluted at room temperature with a linear gradient of 0.1–0.5 mM KCl in buffer A and either used directly for further studies or stored at 4°C. No significant loss of activity was observed at least for 3 weeks under this condition.

2.3. Enzyme assays

The F26G assay mixture contained 0.4 mM protogracillin, 10 μl of EGME, 15 μl of appropriately diluted enzyme solution and 50 mM sodium acetate buffer, pH 5.0, in a total volume of 150 μl. Reaction was initiated by adding the enzyme solution. After being incubated for 10 min at 40°C, produced gracillin was extracted with 150 μl *n*-BuOH saturated with H_2O and determined by HPLC [16]. For the substrate specificity study, the concentration of glycosidic substrate was adjusted to 1 mM. Reaction was terminated by boiling for 3 min and glucose production was measured by glucose oxidase procedure [18]. When pNPG was used as a substrate, the reaction was stopped by adding 0.3 M Na_2CO_3 and liberated pNP was determined by absorbance at 400 nm. The amount of protein in the reaction mixture was adjusted to keep the reaction velocity linear with time up to 30 min. When furostanol glycoside, protogracillin or protodioscin, was used as a substrate, the amount of released D-glucose was in good accordance with that of produced spirostanol glycoside, gracillin or dioscin, respectively. All assays were performed in duplicate and values shown represent the mean of both trials.

2.4. Analytical gel filtration

Gel filtration HPLC was performed using columns of $G4000SW_{XL}$ (Tosoh, 7.8 × 300 mm) and $G3000SW_{XL}$ (Tosoh, 7.8 × 300 mm) connected in series (eluent, 0.1 M potassium phosphate buffer, pH 7.0, containing 0.2 M KCl; flow rate, 0.4 ml/min; detection, UV280 nm) at room temperature. The column was calibrated with proteins of known M_r. Cytochrome *c* (12,400); Adenylate kinase (32,000); Enolase (67,000); Lactate dehydrogenase (142,000); Glutamate dehydrogenase (290,000); and gave a linear relationship between the logarithm of M_r and retention time.

2.5. Protein sequencing

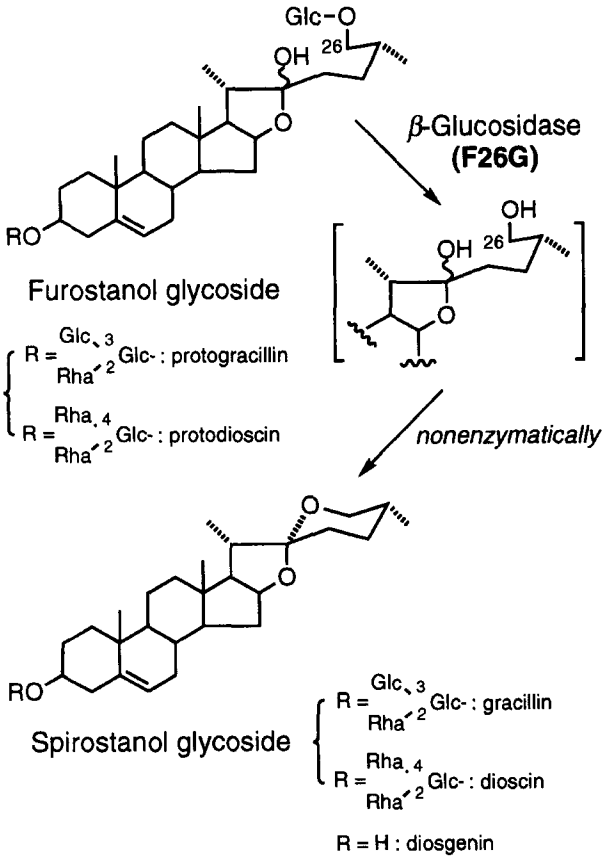
The purified enzyme was subjected to 7.5% SDS-PAGE followed by electroblotting onto polyvinylidenedifluoride membrane (ProBlott, Applied Biosystems). The 54,000 and 58,000 protein bands were excised separately and subjected to N-terminal sequence analysis by using an automated protein sequencer (Applied Biosystems 473A).

2.6. Preparation of antibodies against the synthetic peptide

The sequence of amino acid residues 3 to 13 of the 54,000 protein was used to direct synthesis of a 12-amino acid-long peptide [^{3}Pro-Ala-Glu-Val-Val-Leu-Gly-Arg-Ser-Ser-^{13}Phe-(Lys)] using a MilliGen 9020 Pep-Synthesizer. The synthetic peptide was coupled to keyhole-limpet hemocyanin using glutaraldehyde [19]. Polyclonal antibodies to this conjugate were raised in rabbits by injecting 3 × with 1 mg of the conjugate every 2 weeks. Blood samples were obtained 1 week after the last injection.

3. Results

When the rhizomes were extracted with 0.1 M potassium phosphate buffer, pH 7.0, the F26G activity was separated into soluble and insoluble fractions in a ratio of 2:3 by centrifugation at 107,000 × *g* for 60 min [16]. Several attempts to solubilize the insoluble fraction by KCl or detergents, such as Triton X-100, were unsuccessful. However, almost all the F26G activity was found in the 107,000 × *g* soluble fraction when the rhizomes were homogenized with Polyclar AT and sodium isoascorbate. Therefore, this extraction method was used in the following experiments.



Scheme 1

A four-step purification procedure afforded a 25-fold purification of F26G with 15% recovery of activity (Table 1). The resultant DEAE-5PW fraction gave only two bands with M_r of 54,000 and 58,000 on SDS-PAGE (Fig. 1) and eluted as a single symmetrical peak associated with all the F26G activity from an HPLC gel filtration column (Fig. 2). The apparent native M_r of F26G was determined to be 110,000 after calibration with marker proteins. Since this peak fraction also gave only two bands on SDS-PAGE which were stained equally with

Table 1
Purification of F26G from *C. speciosus* rhizomes

Purification step	Total protein (mg)	Total activity (μkat)	Specific activity (nkat/mg)	Yield (%)	Purification (*x*-fold)
Crude extract	59.3	13.1	221	100	1.0
30–60% $(NH_4)_2SO_4$	35.3	7.8	221	60	1.0
DE 52	24.6	6.5	264	50	1.2
Ether-5PW	0.43	1.8	4190	14	19.0
DEAE-5PW	0.37	2.0	5420	15	24.5

Fig. 1. Purification of F26G monitored by electrophoretic analysis. SDS-PAGE (9.6%) was performed as described by Laemmli [26] and the gel was silver stained. Lane 1, fraction eluted from HPLC on DEAE-5PW (0.8 μg). Lane 2, protein markers: bovine serum albumin (66,000); ovalbumin (45,000); carbonic anhydrase (29,000); trypsinogen (24,000).

Coomassie brilliant blue (data not shown), the native F26G appears to be a heterodimeric protein.

The optimum pH of the purified enzyme was determined between 5.0 and 5.5 when protogracillin was used as a substrate. The purified F26G showed saturation kinetics and the K_m for protogracillin was calculated to be 50 μM by Lineweaver–Burk plots. This value was $4 \times 10^2 \times$ smaller than that of oligofurostanoside-specific β-glucosidase in *A. erubescens* for deltoside [9].

The β-glucosidase activity of the purified F26G on several β-glucosides is summarized in Table 2. Potential substrates tested included two major furostanol glycosides of *C. speciosus* rhizomes (protogracillin and protodioscin) [15] and three commercially available β-glucosides (amygdalin, gentiobiose and octyl β-glucoside), all of which produce primary alcohols on hydrolysis. Protodioscin was as good a substrate as protogracillin, but other β-glucosides examined were not. On the other hand, the furostanol glycosides were found to be quite poor substrates for three commercially available enzymes (β-glucosidase from almond, cellulases from *Tricoderma viride* and *Aspergillus niger*, data not shown). These results suggest that the F26G is highly specific for the cleavage of C-26-bound glucose moiety of furostanol glycosides.

Two typical β-glucosidase inhibitors were tested for their effects on the purified F26G (0.3 to 10 mM). Glucono-1,5-lactone [20] was effective in inhibiting F26G, showing 50% inhibition at 250 μM, while conduritol β-epoxide [21], was less effective (IC_{50} was 2.4 mM). Three typical sterols were also investigated for their effects (0.01 to 5 mM) on the purified F26G. Diosgenin, an aglycone of spirostanol glycosides, inhibited 50% of the F26G activity at 79 μM, near the K_m of the F26G for protogracillin. By contrast, β-sitosterol and cholesterol showed little influence on the activity (IC_{50} were 1.4 mM and 2.8 mM, respectively).

Table 2
Substrate specificity of purified F26G

Substrate	nmol D-glucose or pNP released
Protogracillin	53
Protodioscin	47
Amygdalin	<0.1*
Cellobiose	<0.1*
Gentiobiose	<0.1*
Octyl β-D-glucoside	<0.1*
Prunasin	<0.1*
pNPG	0.93

*Amount of produced D-glucose was under detection limit.

The purified F26G gave two bands which M_r of 54,000 and 58,000 on SDS-PAGE (Fig. 1). The 58,000 protein appeared to be N-terminal blocked since it did not give a signal during automated sequencing. On the other hand, the 54,000 protein yielded a 20-amino acid sequence quantitatively. It contained a well-conserved plant β-glucosidase N-terminal region motif Arg-X-X-Phe-Pro-X-X-Phe-X-Phe-Gly (Fig. 3). Arg-10 to Gly-20 of the F26G 54,000 protein is identical to Arg-37 to Gly-47 of a β-glucosidase from *Trifolium repens* except for one amino acid residue (Fig. 3).

A polyclonal antibody directed against the synthetic N-terminal peptide of the 54,000 protein has been prepared. On the western blot of SDS-PAGE gels, this antiserum reacted not only with the 54,000 protein but also with the 58,000 one (data not shown). This result suggests that these two proteins are very similar, at least in their N-terminal sequences. A similar situation was reported for a β-glucosidase from lodgepole pine xylem, which showed two immunologically similar proteins differing in M_r (24,000 and 28,000) on SDS-PAGE and the smaller one is presumed to be a processing or degradation product of the larger one [22]. The 54,000 protein of F26G might be also derived from the 58,000 protein.

4. Discussion

We have been studying furostanol 26-*O*-β-glucosidase (F26G) involved in the postharvest conversion of furostanol glycosides to spirostanol glycosides in *C. speciosus*. The F26G in rhizomes may easily aggregate to become an insoluble form

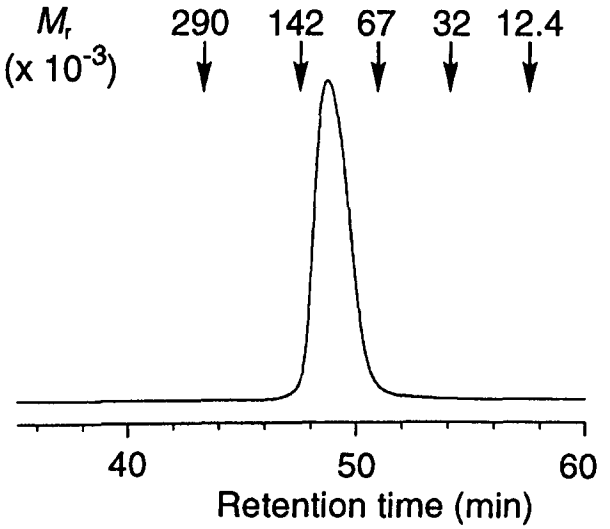


Fig. 2. Gel filtration HPLC of the purified F26G. The DEAE-5PW fraction was applied to the gel filtration HPLC as described in materials and methods. M_r values are given on upper ordinate from calibration with marker proteins.

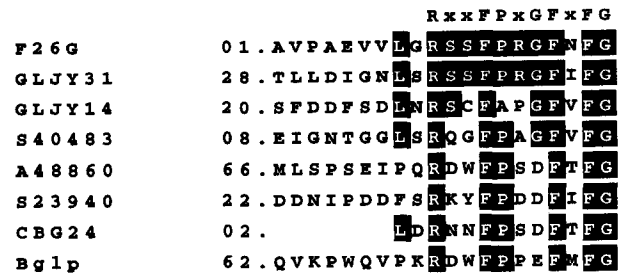


Fig. 3. Optimal N-terminal amino acid sequence alignment of the F26G (54,000 subunit) and other plant β-glucosidases. Amino acid sequence comparisons were carried out with BLASTP v. 1.3.11 on Protein Identification Resource release 44.0 and reference [22]. Sequences are aligned to maximize similarity. The 'plant β-glucosidase N-terminal motif' is shown in the top line. Identical residues are shown as white character on a black background. The number to the left of the sequence represents the first residues in each line. GLJY31 and GLJY14, *Trifolium repens* β-glucosidases; S40483, *Hordeum vulgare* β-glucosidase; A48860, *Zea mays* β-glucosidase; S23940, *Manihot esculenta* β-glucosidase; CBG24, *Pinus contorta* β-glucosidase; Bglp, *Avena sativa* β-glucosidase.

in the presence of phenolic compounds without the addition of Polyclar AT and sodium isoascorbate to the extraction buffer.

We have obtained highly purified F26G protein as judged by SDS-PAGE followed by silver staining (Fig. 1) and gel filtration HPLC (Fig. 2) although only about 25-fold purification could be achieved (Table 1). Since the yield is 15%, it suggests that the F26G may be a predominant protein constituting almost 4% of the total protein in the rhizomes of *C. speciosus*, where furostanol glycosides, the native substrates of F26G, are accumulated [14]. Enzymatic reaction proceeds only after plants are harvested. Under normal physiological conditions, the enzyme and its substrate might be compartmented spatially and/or chemically. The co-occurrence of glucosides and enzymes involved in their degradation has been reported in several cyanogenic plants [23,24] and chamomile plant which contains flavone glucoside [25]. Study on the subcellular localization of F26G and its substrate would clarify the reason why spirostanol glycosides are not formed under normal physiological conditions.

The purified F26G showed high specificity for the removal of C-26-bound glucose moiety of furostanol glycosides and was strongly inhibited by diosgenin. These results suggest that the enzyme recognizes spirostanol and furostanol skeletons. We have already obtained several internal peptide fragments of the purified F26G. Based on the amino acid sequences of these peptides, oligonucleotide primers have been designed to be used for isolation of its cDNA clone by polymerase chain reactions. Molecular characterization of the F26G is now in progress and the results would give some clues to elucidate mechanism of its specific hydrolytic action and reveal whether the 54,000 protein is a degradation product of the 58,000 protein or not.

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