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Phytochemistry 67 (2006) 27–33
PHYTOCHEMISTRY
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Furostanol glycoside 26-*O*-β-glucosidase from the leaves of *Solanum torvum*

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Received 3 June 2005; received in revised form 24 August 2005
Available online 11 November 2005

Abstract

A β-glucosidase (torvosidase) was purified to homogeneity from the young leaves of *Solanum torvum*. The enzyme was highly specific for cleavage of the glucose unit attached to the C-26 hydroxyl of furostanol glycosides from the same plant, namely torvosides A and H. Purified torvosidase is a monomeric glycoprotein, with a native molecular weight of 87 kDa by gel filtration and a p*I* of 8.8 by native agarose IEF. Optimum pH of the enzyme for *p*-nitrophenyl-β-glucoside and torvoside H was 5.0. Kinetic studies showed that K_m values for torvoside A (0.063 mM) and torvoside H (0.068 mM) were much lower than those for synthetic substrates, *p*NP-β-glucoside (1.03 mM) and 4-methylumbelliferyl-β-glucoside (0.78 mM). The enzyme showed strict specificity for the β-D-glucosyl bond when tested for glycone specificity. Torvosidase hydrolyses only torvosides and dalcochinin-8′-β-glucoside, which is the natural substrate of Thai rosewood β-glucosidase, but does not hydrolyse other natural substrates of the GH1 β-glucosidases or of the GH3 β-glucosidase families. Torvosidase also hydrolyses C_5–C_{10} alkyl-β-glucosides, with a rate of hydrolysis increasing with longer alkyl chain length. The internal peptide sequence of *Solanum* β-glucosidase shows high similarity to the sequences of family GH3 glycosyl hydrolases.

Keywords: β-Glucosidase; *Solanum torvum*; Solanaceae; Torvoside A; Torvoside H

1. Introduction

β-Glucosidases (EC 3.2.1.21) catalyse the hydrolysis of β-*O*-glucosidic linkages formed between D-glucose and an aglycone or another sugar. Plant β-glucosidases play important roles in many biological processes such as phytohormone activation, lignin synthesis, cell wall degradation, and defense mechanisms (Esen, 1993). There are many plant β-glucosidases which have specificity for various natural substrates including cyanogenic glucosides (Pocsi et al., 1989; Poulton, 1990), hydroxamic acid glucosides (Sue et al., 2000), β-linked oligoglucosides (Hrmova et al., 1996; Akiyama et al., 1998), isoflavonoid glucoside (Svasti et al., 1999) and furostanol glycosides (Nisius, 1988; Gus-Mayer et al., 1994; Inoue and Ebizuka, 1996). In this paper, we report the purification and characterization of a β-glucosidase, which specifically cleaves steroid glycosides. Most steroid glycosides have spirostane derivatives as their skeleton, while the sugar moieties are oligosaccharides containing 2–4 types of sugar, usually attached to the C-3 hydroxyl group (Mahato et al., 1982). The precursors of spirostane glycosides are furostanol glycosides, containing a glucose unit attached to the C-26 hydroxyl of the aglycone, which is cleaved by a β-glucosidase enzyme to form the E/F spiro rings. So far, there have been few reports on the purification and characterization of β-glucosidases which hydrolyse furostanol glycosides. Such β-glucosidases and their specific natural substrates have been extracted from both *Avena sativa*

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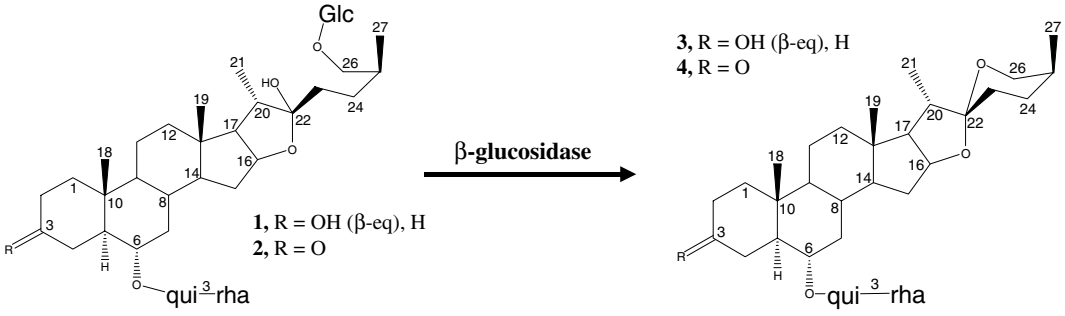
doi:10.1016/j.phytochem.2005.09.035

and *Costus speciosus*, which are monotyledonous plants. Furostanol glycoside 26-*O*-β-glucosidase, purified from the rhizome of *C. speciosus*, converts the furostanol glycosides, namely protodioscin and protogacillin, to the corresponding spirostane glycosides by specific cleavage at the 26-*O*-β-glucosidic linkage (Inoue and Ebizuka, 1996). Avenacoside A and B are furostanol glycosides (also known as saponins) extracted from leaves of *A. sativa* (oat) that consist of the aglycone nuatigenin attached to two sugar moieties, namely an oligoglucoside at C-3 and glucose at C-26. Nuatigenin glycosides have an F-ring with the five membered furan ring, instead of a six membered pyran ring as in spirostane. Aveconasidase, an enzyme extracted from etiolated oat seedlings, catalyses the hydrolysis of the natural substrates avenacoside A and B to form the monodesmosides (26-degluco-avenacosides) and glucose (Nisius, 1988; Gus-Mayer et al., 1994). Recently, furostanol glycosides (Arthan et al., 2002) have been isolated and characterized in our laboratory from the fruits of *Solanum torvum*, a dicotyledonous plant. The furostanol glycosides, namely, torvoside A (**1**) and torvoside H (**2**), contain the aglycone furostane, which is converted to furostanol glycoside by attachment of two sugar moieties, glucose at the C-26 hydroxyl and oligosacharides at the C-6 hydroxyl, so that they differ in structure from other furostanol glycosides described. Since the furostanol glycosides extracted from the fruits of *S. torvum* have unique structures, we have purified and characterized the β-glucosidase enzyme which specifically hydrolyses torvoside A (**1**) and H (**2**) to form the 26-degluco-torvoside A (**3**) and H (**4**) (Scheme 1). This is the first report of the purification and characterization of a furostanol glycoside 26-*O*-β-glucosidase from a dicotyledonous plant. Preliminary data indicate that the substrates are located in fruits, whereas the β-glucosidase is present in petioles and leaves, but not in fruits and other tissues. Here, we report the purification of *Solanum* β-glucosidase from leaves, and compare its properties to avenacosidase from *A. sativa* and furostanol glycoside 26-*O*-β-glucosidase from *C. speciosus*.

2. Results and discussion

Young leaves (up to 3 cm length) provided a convenient source for the purification of β-glucosidase (torvosidase), because of their high activity and low content of phenolic compounds and chlorophyll, compared to other tissues or other leaf development stages. The purification of torvosidase from young leaves required the following chromatographic steps, butyl-toyopearl, Con A-Sepharose, CM-Accell, Sephacryl S-300 HR and butyl-Sepharose FF chromatography, respectively. The results of purification of torvosidase are summarized in Table 1. The final cumulative yield and fold purification of torvosidase, obtained at the last step of purification, were 12% and 170-fold, respectively. The enzyme shows only one protein band when stained with Coomassie blue R-250 on SDS–PAGE (Fig. 1(a), lane 1). As well, a single protein band was found on native agarose IEF (Fig. 1(b), lane 1). Therefore, the purified β-glucosidase appears to be homogeneous, by SDS–PAGE and native agarose IEF.

Since torvosidase was tightly bound by Con A-Sepharose, the enzyme is probably glycosylated, as is the case for many β-glucosidases (Hughes et al., 1992; Akiyama et al., 1998; Varghese et al., 1999; Ketudat-Cairns et al.,



Scheme 1. Hydrolysis of torvoside A (**1**) and torvoside H (**2**) by β-glucosidase from *Solanum torvum* to form 26-degluco-torvoside A (**3**) and 26-degluco-torvoside H (**4**).

Table 1
Purification of β-glucosidase from young leaves (40 g) of *Solarium torvum* Sw

Step	Total activity (nkat)[a]	Total protein (mg)	Specific activity (nkat/mg)	Yield (%)	Purification (fold)
Crude extract	119	288	0.42	100	1
30% $(NH_4)_2SO_4$	112	89.0	1.26	95	3
Butyl-Toyopearl	103	17.4	5.92	87	14
Con A-Sepharose	156	5.69	27.4	132	65
CM-Accell	106	2.03	52.2	89	124
Sephacryl S-300 HR	45	1.02	44.1	38	105
Butyl-Sepharose FF	15	0.210	72.4	12	170

[a] 1 nkat represents the amount of enzyme releasing 1 nmol glucose per sec from 2 mM torvoside A (**1**) at 37 °C and pH 5.0.

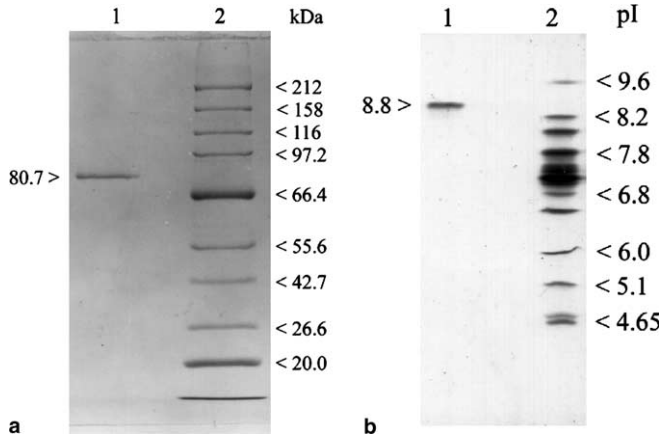


Fig. 1. Electrophoresis of purified β-glucosidase from it Solanum torvum. (a) SDS–PAGE with protein stain: lane 1; purified enzyme (4 μg): lane 2, molecular weight markers; (b) agarose IEF of purified enzyme: lane 1; purified enzyme (4 μg): lane 2; p*I* markers: stained for protein with Coomassie blue R-250.

2000). Torvosidase consists of a single peptide chain of subunit M_r of 80.7 kDa by SDS–PAGE (Fig. 1(a)), and has native M_r of 87 kDa on Sephacryl S-300 HR chromatography, so the enzyme is likely to be monomeric. Torvosidase also had a p*I* of 8.8 by native agarose IEF. The basic p*I* of torvosidase is similar to that of the β-glucosidases purified from barley (Leah et al., 1995; Hrmova et al., 1996), rice (Akiyama et al., 1998), walnut (Duroux et al., 1998) and maize (Kim et al., 2000). These results suggested that the purified β-glucosidase is a monomeric, basic, glycoprotein.

Optimum activity of β-glucosidase was observed at pH 5.0, when *p*NP-β-glucoside and torvoside A (**1**) were used as substrates. Studies of the substrate specificity for the glycone moiety showed that the β-glucosidase hydrolyses only *p*-nitrophenyl (*p*NP)-β-glucoside, but not the other *p*NP-β-glycosides including *p*NP-β-D-fucoside, *p*NP-β-L-fucoside, *p*NP-β-D-xyloside, *p*NP-β-D-galactoside, *p*NP-β-D-NAc-glucosamine, and *p*NP-β-D-mannoside or any of the *p*NP-α-glycosides tested. These results suggest that the purified enzyme is highly specific for β-glucopyranosides.

Kinetic parameters of β-glucosidase were determined for the natural substrates and compared to selected synthetic substrates (Table 2). Kinetic constants showed that K_m values for torvoside A (**1**) (63 μM) and torvoside H (**2**) (68 μM) were similar and lower than the K_m values for synthetic substrates, *p*NP-β-glucoside (1.03 mM) and 4-methylumbelliferyl (MU)-β-glucoside (0.78 mM). However, the k_{cat} values for all four substrates were similar. Thus, the catalytic efficiency (k_{cat}/K_m) of the natural substrates was also much higher than the synthetic substrates, indicating that *S. torvum* β-glucosidase preferred its own natural substrates to synthetic substrates.

When the hydrolytic activity of β-glucosidase towards various natural substrates was tested to study aglycone specificity (Table 3), β-glucosidase was able to hydrolyse torvoside A (**1**). But it showed little hydrolysis of dalcochinin-8′-β-glucoside, which is the natural substrate of Thai rosewood β-glucosidase, and did not hydrolyse the other natural substrates of β-glucosidases so far tested. Furthermore, *Solanum* β-glucosidase did not hydrolyse oligosaccharides containing (1→3)-, (1→4)-, and (1→6)-β-linked glucosyl residues, the natural substrates of family GH3 β-glucosidases. *Solanum* β-glucosidase also hydrolysed C_5–C_{10} alkyl-β-glucosides (1-pentyl-β-glucoside, 1-hexyl-β-glucoside, 1-heptyl-β-glucoside, 1-octyl-β-glucoside, and 1-decyl-β-glucoside) but did not hydrolyse methyl-β-glucoside. Additionally, the rate of hydrolysis increased with longer alkyl chain length. These results suggested that the enzyme showed rather narrow substrate specificity. This is similar to *Podophyllum peltatum* β-glucosidase, which is very specific for its lignin natural substrate podophyllotoxin-4-β-D-glucoside, and cannot hydrolyse pNP-β-D-glycosides, but has low activity toward β-linked oligosaccharides (Dayan et al., 2003). Since, *Solanum* β-glucosidase hydrolysed torvoside A, torvoside H and dalcochinin-8′-β-glucoside (albeit with low efficiency), which have bulky hydrophobic aglycones, this suggests that the enzyme probably has a large hydrophobic site for binding of the aglycone moiety.

The effect of various substances, such as metal ions, EDTA and D-glucono-1,5-lactone was investigated (Table 4). Most metal ions and EDTA showed little effect on

Table 2
Kinetic parameters of *Solanum* β-glucosidase comparing various natural substrates and synthetic substrates

Substrates	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Torvoside A (**1**)	0.063	8.6	136,500
Torvoside H (**2**)	0.068	7.7	113,200
*p*NP-β-glucoside	1.03	9.1	8800
4-MU-β-glucoside	0.780	8.3	10,700

Assays were performed in 0.1 M NaOAc buffer, pH 5.0, at 37 °C, and glucose release was measured. k_{cat} was estimated assuming M_r of 80.7 kDa.

Table 3
Hydrolysis of natural substrates and alkyl-β-glucosides by β-glucosidase from *Solanum torvum* compared to hydrolysis of the natural substrate, torvoside A

Substrates	Relative activity (%)
Torvoside A (**1**)	100[a]
Dalcochinin-8′-β-glucoside	24
1-Decyl-β-glucoside	68
1-Octyl-β-glucoside	53
1-Heptyl-β-glucoside	29
1-Hexyl-β-glucoside	22
1-Pentyl-β-glucoside	13

Reactions employed 2 mM substrates in 0.1 M NaOAc buffer, pH 5.0, at 37 °C for 30 min, and released glucose was measured.

[a] 100% activity was equivalent to 116 nkat/mg when torvoside A (**1**) was used as substrate. No hydrolysis was observed with cyanogenic glucosides (amygdalin, laminarin and prunasin), glucosinolate (sinigrin), aromatic glucosides (arbutin, salicin), indoxyl-β-glucoside, and methyl-β-glucoside. When natural substrates of family GH3 β-glucosidases were tested, torvosidase could not hydrolyse β-linked oligosacccharides, namely cellobiose, cellotriose, cellotetraose, laminaribiose, laminaritriose, laminaritetraose and gentiobiose.

Table 4
Effect of various substances on *Solanum* β-glucosidase activity

Substances	Final concentration (mM)	Relative activity (%)
Control	–	100[a]
$FeCl_3$	1	101
$MgCl_2$	1	90
$ZnCl_2$	1	88
$HgCl_2$	1	12
EDTA	1	110
D-Glucono-1,5-lactone	1	56
D-Glucono-1,5-lactone	10	9

Solanum β-glucosidase was preincubated with each compound for 15 min before being assayed with 2.5 mM *p*NP-β-glucoside in 0.1 M NaOAc buffer, pH 5.0, at 37 °C, and the released *p*NP was then measured.

[a] 100% activity was equivalent to 116 nkat/mg when torvoside A was used as substrate.

β-glucosidase. The sensitivity of β-glucosidase to inhibition by Hg^{2+} may indicate that Hg^{2+} may react with sulfhydryl groups of cysteines required for activity (Duroux et al., 1998) or chelate active site acidic amino acids (Srisomsap et al., 1996). D-Glucono-l,5-lactone, which is a transition state analogue of glucose, is a specific inhibitor of many β-glucosidases (Srisomsap et al., 1996; Inoue and Ebizuka, 1996; Hrmova et al., 1996; Duroux et al., 1998; Akiyama et al., 1998) inhibits torvosidase by 44% and 91% at 1 and 10 mM D-glucono-l,5-lactone, respectively.

The partial internal sequence analysis of the purified enzyme was obtained by digestion with trypsin and sequence analysis by LC–MS/MS. X is used to represent leucine or isoleucine, since LC–MS/MS cannot separate these amino acids because of their identical mass. Alignment of *Solanum* β-glucosidase sequence with sequences available in protein and nucleic acid databases using BLAST (from the website of the National Center for Biotechnology Information, URL: http://www.ncbi.nlm.nih.gov/BLAST/) (Fig. 2) showed sequence similarity with family GH3 β-glucosidase from plants, including β-D-glucan exohydrolases from *Hordeum vulgare* subsp. vulgare (barley, AAD23382), and *Zea mays* (maize, AAQ97669), *Arabidopsis thaliana* (NP 680141), *Nicotiana tabacum* (tobacco, BAA33065), *Tropheolum majus* (nasturtium, CAA07070) and *Oryza sativa* (rice, AAL58976). Seven peptides covering some 60 residues in *Solanum* β-glucosidase show good similarity with the other family GH3 β-glucosidases. Interestingly, the GFXXSDSQGXDR motif found in the internal sequence of *Solanum* β-glucosidase is similar to the conserved GFVISDW putative catalytic nucleophile motif of family GH3 glycoside hydrolases from

Peptides	#	MTXEEK	#	IGQMSQXDAR	#	RXGAATAXEVR
Bgx_N	46	MTLEEK	52	IGQMTQIE--	149	RIGAATALEVR
Bgx_O	97	MTLAEK	103	IGQMTQIE--	200	RIGAATALEVR
Bgx_T	47	MTLEEK	53	IGQMTQIE--	150	RIGEATALECR
Bgx_A	48	MTLEEK	54	IGQMVQID--	147	RIGAATAVEVR
Bgx_Z	52	MTLAEK	58	VGQMTQIE--	155	RIGAATALEVR
Bgx_H	48	MTLAEK	54	IGQMTQIE--	151	RIGEATALEVR

Peptides	#	GFXXSDSQGXDR	#	HNXXPMSR	#	XDDAVSR	#	KSXVXXK
Bgx_N	303	GFVISDWQGIDR	358	-NIIPMSR	365	IDDAVKR	410	KSLVLLK
Bgx_O	354	GFVISDWQGIDR	410	--IIPMSR	416	IDDAVTR	461	KSLVLLK
Bgx_T	304	GFVISDWEGIDR	359	-NIIPMSR	366	IDDAVKR	411	KSLVLLK
Bgx_A	305	GFVISDWQGIDR	---	--------	367	IDDAVRR	412	KSLVLLK
Bgx_Z	309	GFVISDWEGIDR	366	---IPMSR	371	IDDAVTR	416	KSLVLLK
Bgx_H	305	GFVISDWEGIDR	361	--VIPMSR	367	IDDAVTR	412	KSLVLLK

Fig. 2. Alignment of *Solanum* β-glucosidase peptides with the amino acid sequences of family GH3 glycoside hydrolases: Bgx; β-D-glucan exohydrolase, from: *Hordeum vulgare* subsp. vulgare (barley, AAD23382), *Zea mays* (maize, AAQ97669), *Arabidopsis thaliana* (NP 680141), *Nicotiana tabacum* (tobacco, BAA33065), *Tropheolum majus* (nasturtium, CAA07070) and *Oryza sativa* (rice, AAL58976), X represents Ile or Leu, – represents unmatched amino acid, and bold letters represent the identical amino acids to those of *Solanum* β-glucosidase peptide.

many plants (Varghese et al., 1999; Harvey et al., 2000; Hrmova et al., 2002) except for substitution of Val by Leu/Ile and Trp by Ser. Further studies will be required to determine the complete sequence of *Solanum* β-glucosidase to confirm whether the above peptide is indeed derived from the catalytic nucleophile sequence. However, from the data available, it appears that *Solanum* β-glucosidase, which shows high specificity for furostanol glycosides, should be classified in the family GH3 of the glycosyl hydrolases according to the Henrissat classification (Henrissat and Bairoch, 1993; Henrissat and Davies, 1997). To our knowledge, this is the first report that a plant family GH3 β-glucosidase specifically hydrolyses the furostanol glycosides.

The properties of *Solanum* β-glucosidase are summarized in comparison to family GH1 β-glucosidases from *A. sativa* and *C. speciosus*, which share similar furostanol glycosides as natural substrates. Although, *Costus* and *Avena* β-glucosidases show similar K_m values and pH optima, they differ in some biochemical properties. *Costus* β-glucosidase and *Avena* β-glucosidases are dimeric and oligomeric, respectively, whereas *Solanum* β-glucosidase is a monomeric protein. Furthermore, the amino acid sequences of *Costus* and *Avena* β-glucosidases share similarity with family GH1 β-glucosidases whereas *Solanum* β-glucosidase is similar to family GH3 β-glucosidase. Therefore, the biochemical properties of *Solanum* β-glucosidase clearly differ from those of *Avena* and *Costus* β-glucosidases (Table 5).

It is likely that the function of this glucosidase is to hydrolyse furostanol glucosides in the plant. Interestingly, TLC analysis of methanolic extracts indicated that the substrate was present in the fruit but not in the leaf or petiole, while incubation of purified torvoside with aqueous extracts of the fruit, leaf or petiole, showed that enzyme was present in the leaf and petiole but not in the fruit (unpublished data). Since the β-glucoside substrates appear to have their primary location in the fruit, while the enzyme appears to be primarily found in the leaves, especially young leaves, and petioles, translocation of enzyme or substrate may be required for the two components to meet and react. Physical separation of β-glucosidase enzymes from their substrates by differences in subcellular or tissue location are well known in plants, especially with the cyanogenic and hydroxamic acid β-glucosidases. The white clover β-glucosidase, linamarase, is located in the cell wall, separated from the substrate linamarin in the vacuole, prevents enzymatic release of the toxic hydrogen cyanide, until the tissue is damaged (Kakes, 1985). Translocation of β-glucosides between tissues has been reported in many plants. For example, dhurrin-6′-glucoside, a cyanogenic diglucoside from *Sorghum bicolor*, is found in seedlings and is transported to leaves, where it is hydrolysed by a specific β-glucosidase to form dhurrin (Selmar et al., 1996). With steroid glucosides, oligofurostanosides in *Dioscorea caucasica* (Lipsky) are located in the leaves, while the specific β-glucosidase enzyme resides in the rhizome (Gurielidze et al., 2004). This difference in tissue localization of substrates and enzyme prevents the furostanol structure from being cleaved during its transport from leaves to rhizomes.

Since the chemical structures of torvoside A (**1**) and H (**2**) are similar to the saponins, such as avenacoside, it is possible that the torvoside–torvosidase combination may also act as a defense mechanism in *S. torvum* by releasing a toxic aglycone to deter pests and/or predators. In this case, the differential localization of the torvoside substrates from the enzyme torvosidase would prevent premature cleavage of the furostanol substrate in the fruits. Then, translocation of the torvosides to leaves would allow cleavage by their specific degrading β-glucosidase to release the aglycone. However, such a hypothesis requires further studies for verification.

Table 5
Comparison of the biochemical properties of the β-glucosidases from *Avena sativa*, *Costus speciosus* and *Solarium torvum*

Properties	β-Glucosidases		
	Avena sativa	*Costus speciosus*	*Solanum torvum*
Natural substrates	Avenacoside A	Protogracillin	Torvoside A
Optimum pH	6.0	5.0–5.5	5.0
K_m for natural substrate	12 μM	50 μM	63 μM
Glycoprotein	No	No	Yes
Native M_r	Oligomer	110 kDa	87 kDa
Subunit M_r	60	54, 58 kDa	80.7 kDa
Amino acid sequence similarity	Family GH1	Family GH1	Family GH3

3. Experimental

3.1. Plant material and isolation of furostanol glycosides

Young leaves (up to 3 cm length) of *S. torvum* Sw were obtained from Angthong, Thailand, freeze-dried, and then stored at −20 °C. Isolation of furostanol glycosides, natural substrates of β-glucosidase, from *S. torvum* have been described previously (Arthan et al., 2002).

3.2. Enzyme extraction and purification

To remove phenolic compounds, pigments, and lipids, the freeze-dried leaves (40 g) were extracted with 20 mM potassium phosphate buffer, pH 7.2, containing 70% saturated $(NH_4)_2SO_4$, 10 mM ascorbic acid, 25 mM β-mercaptoethanol by grinding with sand in a mortar and centrifuging at 17,400*g* for 20 min. Then, the pellet was extracted three times with the same solution and the remaining residue was ground in extraction buffer (20 mM potassium phosphate buffer, pH 7.2, containing 10 mM ascorbic acid, 25 mM β-mercaptoethanol, 1 mM PMSF, 4 mM EDTA, and 0.2 M NaCl), filtered, and then centrifuged at 17,400*g* for 30 min. The supernatant was

collected for measurement of protein and β-glucosidase activity. $(NH_4)_2SO_4$ was added to a final concentration of 30%. The supernatant obtained by centrifugation at 17,400*g* for 30 min was then loaded on to the butyl-toyopearl column (TosoHaas, 1.5 × 25 cm) equilibrated with 30% saturated $(NH_4)_2SO_4$ in 20 mM potassium phosphate buffer, pH 7.2. The bound components were eluted by applying a linear gradient of 30–0% saturation of $(NH_4)_2SO_4$ in the same buffer. The active fractions were pooled and applied on to a Con A-Sepharose column (Pharmacia, 1 × 6 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.2, containing 0.5 M NaCl. The column was then eluted with a linear gradient of 0–0.3 M methyl-α-mannoside (5 + 5 bed volumes) in the same buffer. The active fractions were pooled and dialyzed against 20 mM potassium phosphate buffer, pH 7.2. The dialyzed enzyme was then applied onto a CM-Accell column (Waters, 1.8 × 20 cm) equilibrated with the same buffer. The column was first washed with 5 bed volumes of starting buffer to remove unbound components. To elute bound components, a gradient of 0–1.0 M NaCl (5 + 5 bed volumes) in 20 mM potassium phosphate buffer, pH 7.2, was applied. Fractions possessing β-glucosidase activity were pooled and concentrated by ultrafiltration through a PM 30 membrane (Amicon). The concentrated β-glucosidase pool was loaded on a Sephacryl S-300 HR column (Pharmacia, 1.5 × 90 cm) equilibrated with 20 mM potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl. The active fractions were pooled, and then added with $(NH_4)_2SO_4$ to a concentration of 30%. The enzyme solution was then applied onto a butyl-Sepharose FF column (Pharmacia, 1 × 6 cm) equilibrated with 30% $(NH_4)_2SO_4$ in 20 mM potassium phosphate buffer, pH 7.2. The bound components were then eluted with a linear gradient of 30–0% $(NH_4)_2SO_4$ (20 + 20 bed volumes) in 20 mM potassium phosphate buffer, pH 7.2.

3.3. Enzyme assays and protein determination

Two assays for β-glucosidase were performed. The first assay, the *p*NP-glycoside assay was used to study the sugar specificity of glycosides. In this assay, 70 μl of the appropriate dilution of the purified enzyme was placed into a microplate. Next, 70 μl of 5 mM *p*-nitrophenyl glycoside in 0.2 M NaOAc buffer, pH 5.0 was added to initiate the reaction. After incubation at 37 °C for 15 min, 70 μl of 0.4 M Na_2CO_3 was added to stop the reaction. The released *p*NP was measured at 410 nm using a microplate reader (Dynatech).

β-Glucosidase activity was also measured by glucose release. Cleavage of the β-glucoside was performed in a reaction mixture of 80 μl, containing appropriately diluted enzyme and 2 mM of final concentration of substrate in 0.1 M NaOAc buffer, pH 5.0. The reaction was incubated for 15 min at 37 °C, and then stopped by boiling for 2 min. Then, 50 μl of each reaction was aliquoted into the microplate, and 50 μl of ABTS (2,2′-azonobis-3-ethylbenzthiazolinesulfonic acid) (Roche) and 100 μl of glucose oxidase/peroxidase (Sigma) solution (2mg/ml) were added. The reaction was further incubated at 37 °C for 15 min. The absorbance at 410 nm was then measured. One nkat of enzyme activity is defined as the amount of enzyme releasing 1 nmol of pNP or glucose from the substrate per sec at 37 °C, pH 5.0. Kinetic parameters were estimated by using Prism 3 program (Graphpad software). Protein concentration was determined by the Bio-Rad protein assay kit (BioRad) using BSA as standard.

3.4. Analytical gel electrophoresis

The purified protein was analyzed by SDS–PAGE on a 10% separating gel and a 4% stacking gel, according to the procedure of Laemmli (1970). p*I* of the purified enzyme was determined by native agarose IEF using a standard p*I* marker kit from BioRad. The protein band was stained with Coomassie blue R-250.

3.5. Protein internal sequencing

The partial internal sequence analysis of the purified enzyme was performed by trypsin digestion, followed by sequence analysis with LC–MS/MS in the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia.

Acknowledgments

J.S. is a Senior Research Scholar of the Thailand Research Fund. We thank Dr. Nicholas E. Sherman at the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia for analysis of internal peptide sequences of purified protein. D.A. received a scholarship from the Chulabhorn Research Institute and National Science and Technology Development Agency (NSTDA). Thanks are due to Dr. Tadashi Kishimoto and Dr. Leonel Verdoucq for their help.

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