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A CELL WALL-BOUND β-GLUCOSIDASE FROM GERMINATED RICE: PURIFICATION AND PROPERTIES

TAKASHI AKIYAMA,* HANAE KAKU† and NAOTO SHIBUYA†

National Hokkaido Agricultural Experiment Station, 1 Hitsujigaoka, Toyohira, Sapporo 062, Japan;
†Department of Cell Biology, National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan

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Key Word Index—β-Glucosidase; *Oryza sativa*; Gramineae rice; germination; cell wall-bound; purification and characterization; amino acid sequence.

Abstract—A large portion of β-glucosidase (EC 3.2.1.21) in germinating rice seeds, which appears to be ionically bound to cell walls, can be solubilized with 1 M NaCl. Its activity increased more than eight-fold within five days of germination. It was purified to electrophoretic homogeneity from the extracts of germinated rice seeds by fractionation with $(NH_4)_2SO_4$ followed by CM-Sepharose, Polybuffer exchanger 118, Concanavalin A-Sepharose and Bio-Gel P-100. The Mr of the purified enzyme, estimated by SDS-PAGE, was 56,000 and the isoelectric point was >10.0. Its N-terminal amino acid sequence (44 residues) exhibited high homology to those of β-glucosidases from other plants, such as barley and white clover. Its activity was optimal at pH 4.5 and 50°, and it was strongly inhibited by glucono-1,5-lactone. The enzyme showed hydrolytic as well as transglycosylation activity towards (1 → 3)-β- and (1 → 4)-β-linked oligosaccharides with degree of polymerization of 2–4. The results suggest that the β-glucosidase is probably involved not only in hydrolysis but also in modification of oligosaccharides in cell walls of germinating rice seeds.

INTRODUCTION

β-Glucosidases occur widely among microorganisms, animals and plants. In higher plants, they have been classified into several groups based on their putative roles in biological processes, assessed mainly on the basis of specificity towards their substrates. Some have been implicated in degradation of oligosaccharides produced by the action of endo-type β-glucanases on cell wall β-glucans [1–4]. A few have been related to chemical defense against plant pathogens and herbivores due to their role in releasing hydrogen cyanide from cyanogenic glucosides [5–7]. Others have been implicated in regulating the amount of plant hormones due to their action in releasing active forms from inactive hormone-glucoside conjugates [8, 9]. It was previously reported that β-glucosidase activity in dwarf rice increases with germination time [10]. In our preliminary experiments, it was found that β-glucosidase activity also increases in normal rice during germination, and that the enzyme activity solubilized from the cell wall fraction with high salt was much higher than that obtained from the buffer soluble fraction. However, cell wall-bound β-glucosidase has not been purified and characterized from germinating rice seeds and its physiological function is unknown.

The present work describes, for the first time to our knowledge, the purification, characterization and N-terminal amino acid sequencing of cell wall-bound β-glucosidase from germinated rice seeds, and its possible involvement in cell wall metabolism.

RESULTS AND DISCUSSION

Changes in β-glucosidase specific activity monitored with *p*-nitrophenyl-β-D-glucopyranoside (PNPG) in germinating rice seeds are shown in Fig. 1. Activity of β-glucosidase in the cell wall-bound fraction, which can be solubilized with a buffer containing 1 M NaCl, began to increase rapidly about three days after germination to reach more than eight-fold within five days, while the enzyme activity in the soluble fraction only slowly increased. A similar increase in activity of the buffer-soluble β-glucosidase activity was reported in germinating seeds of dwarf rice [10]. The activity of β-glucosidase in the cell wall-bound fraction (70–80% of the total activity) is much higher than that of the buffer-soluble fraction, regard-

*Author to whom correspondence should be addressed. Fax: +81 11 859 2178. E-mail: takiyama@cryo.affrc.go.jp.

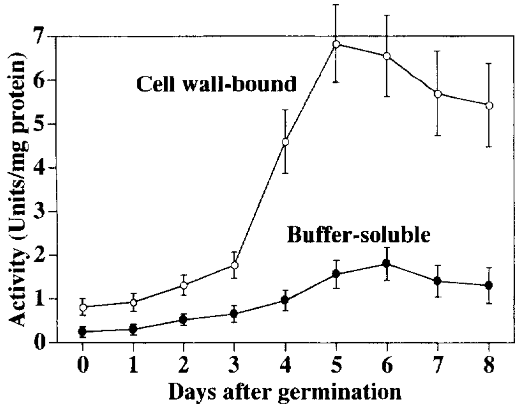

Fig. 1. Changes in β-glucosidase activity in germinating rice seeds. β-Glucosidase activity in the cell wall-bound (○) and the buffer-soluble (●) fractions from germinating seeds was assayed using PNPG as the substrate. Values indicate mean $\pm$se of two experiments each with $n = 4$.

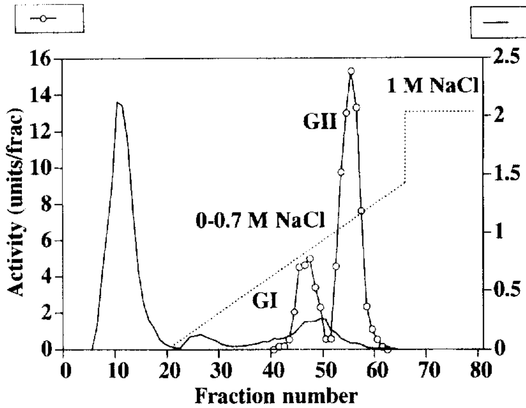

Fig. 2. CM-Sepharose chromatography of the rice β-glucosidase after $(NH_4)_2SO_4$ fractionation.

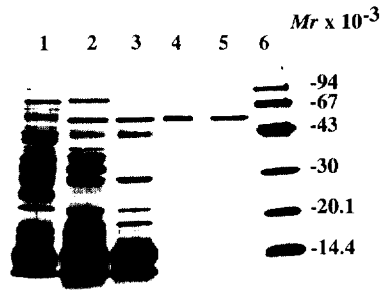

Fig. 3. SDS-PAGE of proteins at each purification step of the rice β-glucosidase. Lane 1, 50–80% $(NH_4)_2SO_4$ fration; lane 2, CM-Sepharose GII fraction; lane 3, PBE 118 fraction; lane 4, Con A-Sepharose fraction; lane 5, Bio-Gel P-100 fraction; lane 6, M_r markers (Pharmacia): phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400).

less of the stage of germination. The considerable amount of β-glucosidase present in cell walls suggests that the enzyme is probably involved in metabolism of the cell walls in germinating rice seeds.

The overall purification protocol for the cell wall-bound β-glucosidase is summarized in Table 1. It was extracted from rice seeds germinated for five days with 1 M NaCl, and fractionated with $(NH_4)_2SO_4$ (50–80%). The β-glucosidase activity in the $(NH_4)_2SO_4$ fraction was separated into two peaks, GI and GII, by ion-exchange chromatography on CM-Sepharose (Fig. 2). The predominant peak, GII, which is eluted at 0.6–0.65 M NaCl and accounts for approximately 80% of the total activity yielded from CM-Sepharose chromatography, was further purified by chromatofocusing on Polybuffer exchanger (PBE) 118 and affinity chromatography on Concanavalin A (Con A)-Sepharose followed by gel filtration on Bio-Gel P-100. The cell wall-bound β-glucosidase was absorbed on Con A-Sepharose and eluted with a buffer containing 500 mM methyl-α-D-mannopyranoside, suggesting that the enzyme may be glycosylated. Finally, a 383-fold purification was achieved (yield 14%) (Table 1).

Proteins at each purification step were analysed by SDS-PAGE (Fig. 3). Although an affinity chromatography on Con A-Sepharose was efficient to increase the purity of rice β-glucosidase, the enzyme preparation still contained trace amounts of impurities (Fig. 3, lane 4). After gel-filtration on Bio-Gel P-100, rice β-glucosidase migrated as a single band on SDS-PAGE and its apparent M_r was estimated to be 56,000 (Fig. 3, lane 5). The M_r of the purified rice β-glucosidase, which was determined by gel-filtration on Bio-Gel P-100 (data not shown), was 55,000–57,000. These data support the idea that rice β-glucosidase is

Table 1. Purification protocol of the cell wall-bound β-glucosidase from germinated rice seeds

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
1 M NaCl extract	143	1699	11.9	100	1
50–80% $(NH_4)_2SO_4$	28.4	863	30.4	51	2.6
CM-Sepharose	9.60	748	77.9	44	6.5
PBE 118	2.36	396	168	23	14.1
Con A-Sepharose	0.193	285	1477	17	124
Bio-Gel P-100	0.052	237	4558	14	383

a monomeric protein. The barley and maize β-glucosidases which have hydrolytic activity towards PNPG have M_r's ranging 53,000 to 62,000 [2–4, 11], and the M_r (56,000) of the purified rice enzyme is within this range. Furthermore, chromatofocusing of the purified rice β-glucosidase on Polybuffer exchanger (PBE) 118 revealed that the enzyme has a pI higher than 10.0 (data not shown). This basic pI of the purified rice β-glucosidase is similar to that described for the same enzyme purified from germinated barley (pI 8.9–9.5) [2, 4].

An optimal alignment of the N-terminal amino acid sequence of the purified rice β-glucosidase (44 residues) with the known N-terminal sequences of other plant β-glucosidase is shown in Fig. 4. Homology among these N-terminal amino acid sequences lies primarily within the sequence represented by 37 residues between 8 and 44 of the rice β-glucosidase. When homology was compared in the limited 37-residues, the amino acid sequence of the rice β-glucosidase exhibited high homology to those of the barley β-glucosidase (70%) [2–4] and the white clover cyanogenic β-glucosidase (68%) [6]. The sequence of the rice β-glucosidase also had considerable homology to those of the white mustard myrosinase (41%) [24] and the maize β-glucosidase which preferentially acts on cytokinin-*O*-glucosides (51%) [9].

The substrate specificity of the purified rice β-glucosidase towards natural oligosaccharides with β-glucosidic linkages and PNPG is summarized in Table 2. The purified rice β-glucosidase was most active at pH 4.5 and 50° (data not shown). The purified rice β-glucosidase hydrolyzed all natural oligosaccharides examined and PNPG, but at varying rates. The purified rice β-glucosidase preferentially hydrolyzed disaccharides, such as laminaribiose (1,3-β-), gentiobiose (1,6-β-), cellobiose (1,4-β-) and sophorose (1,2-β-); laminaribiose was the most preferred substrate, while Leah [3] reported that the barley β-glucosidase preferentially hydrolyzed disaccharides, such as laminaribiose, cellobiose, sophorose, but not gentiobiose. The purified rice β-glucosidase hydrolyzed laminari-oligosaccharides with degree of polymerization (DP) of 3–7 and cello-oligosaccharides of DP 3–5 at considerably higher rate. However, the rates of hydrolysis of oligosaccharides were much slower compared to those of disaccharides. The purified enzyme also cleaved salicin and methyl-β-D-glucopyranoside at slower rates. The purified rice β-glucosidase did not hydrolyze polysaccharide substrates. This suggests that the purified enzyme is distinct from a β-glucan exohydrolase which can hydrolyze both β-glucan substrates and PNPG [4]. It showed no activity towards the remaining *p*-nitrophenyl derivatives of monosaccharides with α-glucosidic linkage and/or glycones excluding glucose. This result indicates that the purified rice β-glucosidase is specific not only to β-glycosidic linkages but also to the glucose moiety of the glycone portion of artificial glucosides.

The inhibitory effects of sugar analogs towards β-glucosidase substrates are shown in Table 3. D-Glucono-1,5-lactone, a strong inhibitor of β-glucosidases

Table 2. Substrate specificity of the purified rice β-glucosidase

Compound	Relative activity[a] (%)
PNP-β-D-glucopyranoside	100
Gentiobiose	97
Sophorose	70
Laminaribiose	131
Laminaritriose	44
Laminaritetraose	48
Laminaripentaose	54
Laminarihexaose	56
Laminariheptaose	59
Cellobiose	78
Cellotriose	36
Cellotetraose	38
Salicin	15
Methyl-β-D-glucopyranoside	2
Laminarin (*Laminaria digitata*)	0
Barley β-glucan	0
Lichenin (*Cetralia islandica*)	0
CM-cellulose	0
PNP-α-D-glucopyranoside	0
PNP-α-D-galactopyranoside	0
PNP-β-D-galactoypranoside	0
PNP-α-D-mannopyranoside	0
PNP-β-D-mannopyranoside	0
PNP-α-D-xylopyranoside	0
PNP-β-D-xylopyranoside	0

[a]Percent activity relative to glucose or *p*-nitrophenol released from *p*-nitrophenyl (PNP)-β-D-glucopyranoside.

		10	20	30	40
Rice (this work)		GAGEPVVSRR	SFPKGFIFGT	ASSAYQYEGX	AAEGGRGPSI WDTF
Barley[a]		DGPNPNPEIGNTGGL**Q	G**A**V***	*A****V**M	*RQ*****X* **A*
White clover[b]		FKPLPISFDDFSDLN*S	C*AP**V***	****FC***A	*F*D*K**** ****
Maize[c]		SARVGSQNGVQMLSPSEIPQ*D	W**SD*T**A	*T****I**A	WN*D*K*E*N **HF
White mustard[d]		DEEITCEENEPFTCSNTDIL*SK	N*GKD****V	******I**G	RGR*VNVWDG FSHR

Fig. 4. Optimal alignment of the N-terminal amino acid sequence of the purified rice β-glucosidase with those of other plant β-glucosidases. × S indicate unidentified amino acid residues. Asterisks represent identical amino acid residues to those of the rice β-glucosidase. References are: [a][2–4], [b][6], [c][9, 25], [d][24].

Table 3. Effect of inhibitors on activity of the purified rice β-glucosidase

Inhibitor	Concentration (mM)	Relative activity (%)
None*		100
D-Glucono-1,5-lactone	10	0
	1	3
	0.1	15
	0.01	61
1-Amino-1-deoxy-β-D-glucose	10	0
	1	5
	0.1	31
	0.01	72
Deoxynojirimycin	10	3
	1	23
	0.1	67
	0.01	98
Galactono-1,4-lactone	10	58
p-Aminophenyl 1-thio-β-D-glucopyranoside	10	69

*The standard assay mixture contained 2 mM PNPG, 50 mM NaOAc (pH 5.0) and enzyme.

[13, 14], showed a potent inhibitory effect on the rice β-glucosidase. Legler [15] reported that the almond β-glucosidase was strongly inhibited by both β-glucosylamine and its derivatives. A glycosylamine, 1-amino-1-deoxy-β-D-glucose, inhibited the rice β-glucosidase as strongly as D-glucono-1,5-lactone. 1-Deoxynojirimycin, a reduction product of nojirimycin, also inhibited the purified rice β-glucosidase, but its inhibitory effect was less than that of glucono-1,5-lactone. These results are in conformity with those reported for the apricot β-glucosidase [16]. D-Galactono-1,4-lactone and p-aminophenyl 1-thio-β-D-glucopyranoside also showed some inhibitory effect, but the degree of inhibition was much less.

It is known that many glycosyl hydrolases have transglycosylation as well as hydrolytic activity towards the substrates [17–19]. In order to investigate the translgycosylation activity of the purified rice β-glucosidase towards the oligosaccharide substrates, the reaction products were analysed by TLC (Fig. 5). Of the oligosaccharide substrates tested, laminaribiose appeared to be hydrolyzed more rapidly than the other oligosaccharides. Indeed a large amount of glucose was detected after the reaction with laminaribiose (Fig. 5, lane 3). In contrast, the purified rice β-glucosidase showed more transglycosylation activity towards oligosaccharide substrates with DP of 3 and 4. In these reactions, a large amount of transglycosylation products with DP higher than that of oligosaccharides used as the substrate were detected, along with oligosaccharides of DP less than that of the substrates and a small amount of glucose (Fig. 5, lanes 5, 7, 11 and 13). It was reported that the almond α-galactosidase catalyzed transglycosylation only in the presence of considerably high concentration of acceptor substrate (0.5–2 M) [20]. However, the purified rice β-glucosidase showed transglycosylation activity towards oligosaccharides with DP of 2–4 at relatively low substrate concentration (2 mM) in this study.

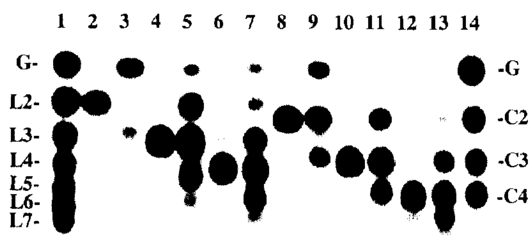

Fig. 5. TLC of products of hydrolysis and transglycosylation of oligosaccharide substrates by the purified rice β-glucosidase. Lane 1, markers: glucose (G) and laminarioligosaccharides of DP 2–7 (L2–L7); lanes 2 and 3, laminaribiose; lanes 4 and 5, laminaritriose; lanes 6 and 7, laminaritetraose; lanes 8 and 9, cellobiose; lanes 10 and 11, cellotriose; lanes 12 and 13, cellotetraose (lanes with even numbers, before reaction; lanes with odd numbers, after reacting for 1 h at 37°); lane 14, markers: glucose (G) and cello-oligosaccharides of DP 2–4 (C2–C4).

In summary, a cell wall-bound β-glucosidase has been purified to homogeneity from germinated rice seeds and characterized. The N-terminal amino acid sequence of the purified rice β-glucosidase exhibited considerable homology to those of β-glucosidases from other plant species. The activity of β-glucosidase increased more than eight-fold within five days of germination, suggesting that the enzyme is likely to play an important role in the germination process. Since the purified rice β-glucosidase had a relatively broad substrate specificity towards β-linked small oligosaccharides, one possible role for the enzyme might be further hydrolysis of oligosaccharides released from cell wall β-glucans during germination. The rice β-glucosidase also showed considerably high transglycosylation activity towards short chain cello- and laminari-oligosaccharides at relatively low concentration. This indicates another possible function of the enzyme in regenerating new oligosaccharides with different β-linkages from oligosaccharides in germinating rice seeds. However, further studies on the localization and the stage- and tissue-specific expression of the rice β-glucosidase seem necessary for a clear understanding of its function.

EXPERIMENTAL

Plant material

Rice (*Oryza sativa* L. cv. Kirara 397) seeds were germinated in the dark at 28° under sterile conditions on a pad of four Whatman 3MM chromatography papers moistened with deionized and distilled H_2O.

Enzyme assay

β-Glucosidase activity for various substrates was assayed by determining either (a) *p*-nitrophenol (PNP) liberated from the *p*-nitrophenyl derivatives of monosaccharides including *p*-nitrophenyl-β-D-glucopyranoside (PNPG), or (b) glucose released from natural oligosaccharide substrates. In assays with the artificial chromogenic substrate, the assay mixt. of 250 μl containing 2 mM substrate, 100 mM NaOAc (pH 5.0), and enzyme, was incubated for 10 min at 37°. The reaction was terminated by adding an equal vol. of 100 mM NaOH. The released PNP was determined immediately at 405 nm. In assays with the natural oligosaccharide substrate, the reaction mixture of 200 μl containing 2 mM substrate, 100 mM NaOAc (pH 5.0), and enzyme was incubated for 10 to 30 min at 37°. The reaction was terminated by boiling (5 min). Glucose released from the substrate was determined enzymatically by the coupled reaction of hexokinase and glucose 6-phosphate dehydrogenase (Boehringer). The amount of β-NADPH formed was measured at 340 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of PNP or glucose from the substrate per min.

Enzyme extraction and purification

For the time course analysis of enzyme activity, 10 seeds were harvested at each stage of germination, and ground with 10 ml of 50 mM NaOAc (pH 5.0) containing 10 mM NaN_3, 10 mM EDTA, 3 mM β-mercaptoethanol and 3 mM PMSF (buffer A) in a chilled mortar. The homogenate was centrifuged for 20 min at 20,000 g at 4°. The ppt. was extracted two more times with buffer A, and all supernatants were pooled as the buffer-soluble fr. Then the ppt. was extracted with 10 ml of buffer A containing 1 M NaCl three times and the supernatants were combined as the cell wall-bound fr.

All procedures for enzyme purification were performed at 4°. Rice seeds germinated for five days (ca 100 g by fr. wt) were homogenized in a Waring blender in 500 ml of buffer A, and the homogenate was squeezed through four layers of nylon gauze (100 mesh). The cell wall material remained on the nylon gauze was collected and washed twice with buffer A, then extracted with 500 ml of buffer A containing 1 M NaCl in a Waring blender. The resulting suspension was squeezed through four layers of nylon gauze. The filtrate was centrifuged for 20 min at 20,000 g, and the supernatant was dialysed against buffer A for 16 h. The dialysed soln was centrifuged for 20 min at 20,000 g, and the clarified supernatant was fractionated with $(NH_4)_2SO_4$ (50–80%). The resulting ppt. was collected by centrifugation, resuspended in 50 mM NaOAc (pH 5.0), and dialysed against the same buffer. The dialysed soln was centrifuged for 20 min at 20,000 g and the supernatant was applied to a CM-Sepharose (Pharmacia) column (5 × 12 cm) equilibrated with 50 mM NaOAc (pH 5.0). The bound material was eluted with a linear gradient of NaCl (0–0.7 M) in the same buffer. The active frs were pooled and concd by ultrafiltration on a Diaflo YM10 membrane (Amicon). The concd soln was dialysed against 25 mM triethylamine-HCl (pH 10.4), and applied to a Polybuffer exchanger (PBE) 118 (Pharmacia) chromatofocusing column (1 × 10 cm) equilibrated in the same buffer. Active frs yielded in the unbound frs were concd and its buffer was replaced to 50 mM Tris-HCl (pH 6.8) by ultrafiltration as described above. The soln was applied to a Concanavalin A-Sepharose (Pharmacia) column (1 × 4 cm) equilibrated in 50 mM Tris-HCl (pH 6.8) and bound material was eluted with the same buffer containing 500 mM methyl-α-D-mannopyranoside. Active frs were pooled, concd and applied to a Bio-Gel P-100 (Bio-Rad) column (2.5 × 70 cm) equilibrated in 50 mM NaOAc (pH 5.0) containing 200 mM NaCl, and eluted in the same buffer (flow rate: 6 ml h^{-1}).

Protein assay

Protein was determined by the method of Bradford [21] using bovine serum albumin as the standard.

SDS-PAGE

Proteins from each purification step were analysed by SDS-PAGE on 11% gel containing 6% stacking gel [22], and stained with Coomassie Brilliant Blue R-250.

TLC

The assay mixture of 100 μl comprising 2 mM laminari- and/or cello-oligosaccharides, 50 mM NaOAc (pH 5.0), and enzyme was incubated for 1 h at 37°. The reaction was terminated by boiling for 10 min. The aliquots of reaction mixtures were applied on a Kieselgel 60 TLC plate (Merck). After developing for 2 h in the soln of EtOAc–HOAc–H_2O (2 : 1 : 1), the presence of sugars was detected by orcinol spray as described previously [23].

Effects of pH and temperature

The effect of pH was measured in 50 mM NaOAc (pH 3.0–6.5) and 50 mM Na–Pi (pH 6.5–8.0) for PNPG. The effects of temperatures from 0 to 90° were determined in 50 mM NaOAc (pH 4.5) for PNPG.

N-terminal protein sequencing

The N-terminal amino acid sequencing of protein electroblotted to PVDF membrane was performed using the routine 3.1 PVDF method on the HP G1005A N-terminal protein sequencing system (Hewlett Packard).

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