Isolation and Characterization of a Rice Glycoside Hydrolase Family 9 Enzyme

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1. Introduction

Activities of EGases are associated with numerous processes of plant development, such as tissue expansion, fruit ripening, growth of cultured cells and leaf abscission. Many plant EGases belong to GHF9[1)], which comprises some of the most extensive and versatile types of cellulases known in nature[2, 3)]. The GHF9 enzymes are widely distributed among phyla, occurring in numerous bacteria, fungi, slime molds, and various plant and animal species. Plant GHF9 enzymes have been considered a comparatively simple type (with only a catalytic domain), since they do not contain modules specific to microbial GHF9 enzymes such as an immunoglobulin-like domain[2)]. Nevertheless, plant species contain numerous isozymes of GHF9 that modify cell wall structure during tissue development[4, 5)]. Results of genomic research exhibit that rice, a model plant for type II cell wall studies, contains at least 23 GHF9 genes[5, 6)]. The number of GHF9 isozymes in rice is almost the same as that in *Arabidopsis* (22 genes), a model plant for type I cell wall studies. The knowledge-based *Oryza* molecular biological encyclopedia (KOME) full-length database indicates that cDNA clones of GHF9 genes were obtained from shoots, flowers, panicles and cultured cells, suggesting the expression of 20 GHF9 genes during tissue development [6)]. The analysis of gene expression also demonstrated that several GHF9 genes are differentially expressed in a variety of organs including roots of rice[7)]. Although these genomic and DNA studies imply that rice GHF9 gene products, such as xyloglucan endotransglucosylase/hydrolase, are extensively involved in the modification of polysaccharides in the type II cell wall[8, 9)], the biochemical properties of rice GHF9 enzymes including their substrate ranges are not known.

Several GHF9 enzymes or EGases have been purified from higher plants other than rice, and their substrate specificities have been characterized. Most secreted GHF9 enzymes are active against β-1,4-glucosyl linkages such as CM-cellulose, phosphoric acid swollen cellulose, 1,3-1,4-β-glucans and dicot xyloglucan[1, 6)]. Since the primary cell wall of dicots (type I cell wall) contains high amounts of xyloglucan as the principal hemicellulosic polymers that interlock the cellulose microfibrils[10)], the natural substrate of these GHF9 enzymes probably includes xyloglucan, and the integral and peripheral regions of noncrystalline cellulose, particularly the outer layers of cellulose microfibrils where glucan chains are interwoven with xyloglucan chains[11)]. In contrast, the primary cell walls of Gramineae (type II cell wall) contain a low amount of xyloglucan. β-1,3-1,4-glucan, coextensive with low-branched glucuronoarabinoxylan (GAX) and glucomannan, is proposed to tightly cross-link the cellulose microfibrils[12, 13)]. β-1,3-1,4-glucan consists mainly of repeating units, cellotriosyl and cellotetraosyl residues, although it also contains rare and cellulose-like units such as β-$(1,4)_{10}$-glucosyl residues[14)]. Maize EGase recognizes mixed β-glucans mainly at cellulose-like units, releasing 10-15 kDa glucosyl fragments during limited hydrolysis of mixed β-glucan[15)]. Recent studies, however, exhibited that a GHF9 from suspension cultured poplar cells, PopCel2, slightly hydrolyzes β-1,4-xylan[16)]. Glucomannan is also predicted to be another substrate of fruit ripening GHF9 enzymes[11)]. A membrane anchored GHF9 enzyme (korrigan homolog) efficiently hydrolyzes only CM-cellulose and phosphoric acid swollen cellulose but not mixed β-glucan as polymeric substrates, showing its comparatively narrow substrate range[17, 18)]. These enzymatic studies of plant GHF9

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gene products and the recent revelation of a bifunctional GHF3 glycosidase (α-arabinosidase and β-xylosidase) from barley imply that it is difficult to determine the substrate ranges of all GHF enzymes or their biochemical functions in cell wall metabolism based on the annotation of DNA sequences only[19]. This paper summeries the purification and characterization of an EGase (GHF9 isozyme) of *Oryza sativa* from primary root tissues[20] and the isolation of its gene, *OsCel9A* [21].

2. Results and Discussion

2.1 Effect of 2,4-D on the isozyme pattern of endo-β-glucanases in rice root tissues

An auxin analog, 2,4-D stimulated the activity of EGase 6-10-fold over controls after 24 h in primary root tissues of rice. This stimulation was shown to be associated with the formation of lateral root primordia[22]. We compared the isozyme patterns of endo-β-glucanase activity between the extracts from root segments cultured with or without 2,4-D using hydrophobic interaction chromatography (HIC). Before and after the 24 h culturing in the presence and absence of 2,4-D, each of the root extracts was applied to a phenyl Sepharose HP column. As shown in Figure 1A, high activity of EGase was detected only in the HIC profiles of buffer extracts from root tissues cultured with 2,4-D. A single peak eluted from a 75 to 0 mM ammonium sulfate gradient, suggesting that 2,4-D-induced EGase may be a hydrophobic protein, since most of the total protein was eluted from a 200 to 150 mM salt gradient as monitored by 280 nm (total protein in Figure 1A).

Endo-1,3-1,4-β-glucanase activity was detected in unbound fractions of the HIC column independently of culture conditions (Figure 1A, endo-1,3-1,4-β-glucanase). However, endo-1,3-1,4-β-glucanase activity that bound to the HIC column was detected only in extracts from 2,4-D treated root segments. The elution volume of HIC bound endo-1,3-1,4-β-glucanase activity was almost the same as that of 2,4-D-induced EGase activity. There was no significant difference among the culture conditions in the elution patterns of total protein, endo-1,3-β-glucanase activity (Figure 1A) and β-glucosidase activity (not shown) from root extracts, indicating that the induction of enzyme activity by 2,4-D was specific to EGase and endo-1,3-1,4-β-glucanase in the HIC profiles.

The HIC-bound fraction of EGase activity was focused in Ampholine buffers (pH 4-6) with a glass column to determine its isoelectric point(s). As shown in Figure 1B, 2,4-D-induced EGase was attributed to acidic proteins that focused to a major peak (pI: 5.50) and two minor peaks (pI: 5.65 and 5.75) of activity. Thus, the 2,4-D-induced, HIC-bound EGase fraction was composed of at least three acidic proteins.

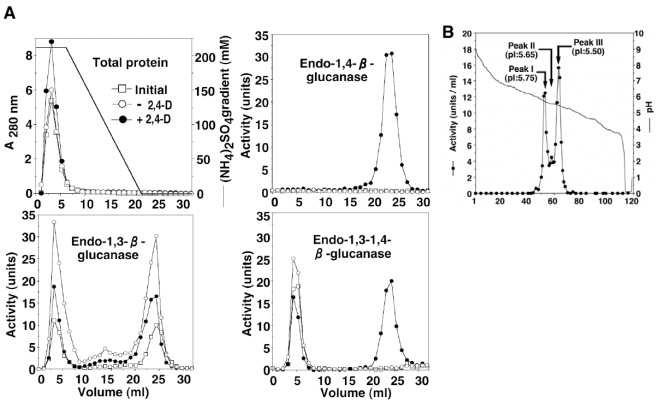


Fig. 1 Effects of 2,4-D on HIC profiles of endo-β-glucanases in crude extracts of root tissues cultured with or without 2,4-D and IEF of 2,4-D-induced EGase[20]. A: Phenyl-Sepharose HP profiles of rice endo-β-glucanases in crude extracts from root tissues cultured with or without 2,4-D. Initial: Extracts from roots before culture. –2,4-D: Extracts from roots cultured for 24 h in the absence of 2,4-D. +2,4-D: Extracts from roots cultured for 24 h in the presence of 13.5 μM 2,4-D. Fractions (1 ml) were assayed for pH (—) and viscometric activity against CM-cellulose (-●-).

2.2 Purification of 2,4-D-induced EGase from rice

2,4-D-induced EGase was extracted from 2.0 kg of aseptically cultured primary root tissues and purified by 4- or 5-step chromatography. The crude enzyme solution (ca. 12 L) containing 8.5 g protein and 91,000 units of EGase, after salting out and resuspension, was put onto a column of phenyl Sepharose HP. The HIC column bound single peak was pooled and subjected to a second step, anion exchange chromatography. As predicted from the results of IEF analysis (Figure 1B), two minor peaks (peaks I and II) and one major peak (peak III) were eluted with 50-100 mM NaCl in 10 mM HEPES buffer (Data not shown) and further purified. The results of the overall purification procedures are summarized in Table 1. The three EGases, designated as peaks I, II and III, were purified 244-, 1,100- and 1,800-fold, respectively (Table 1).

SDS-PAGE and MALDI-TOF MS of the three purifed EGase proteins verified their purity and indicated that each

Table 1 Enzyme yield during purification of EGase (peaks I, II and III) from 2,4-D treated root tissues of *Oryza sativa* L. c.v. Sasanishiki[20)]

Purification step	Volume (ml)	Yield		Specific activity ($U \times mg^{-1}$)	Recovery (%)	Purification factor (fold)
		Protein (mg)	Activity (U)[a]			
Crude homogenate[b]	11500	8486	91028	10.7	100	1
25-50% $(NH4)_2SO_4$	250	3806	84349	22.2	93	2
Phenyl Sepharose HP	400	236	42381	179.5	47	17
Peak I						
Q-Sepharose HP-step	25.5	1.7	1921	1100	2.1	103
Q-Sepharose HP-gradient	13.7	0.146	103	703	0.13	66
Superdex 75 pg	1.74	0.016	40	2594	0.045	244
Peak II / III						
Q-Sepharose HP-step	59	37.1	26250	707	28.8	66
Sephacryl-100	8.33	3.5	6672	1931	7.3	181
Superdex 75 pg	7.3	1.1	4648	4264	5.1	400
Mono Q gradient (Peak II)	2.65	0.018	217	12396	0.24	1164
Mono Q gradient (Peak III)	2.65	0.040	773	19214	0.85	1804

*a: Activity of EGase was assayed viscometrically.
*b: Crude homogenate was extracted from 2 kg of root tissues aseptically cultured with 13.5 μM 2,4-D for 24 h.

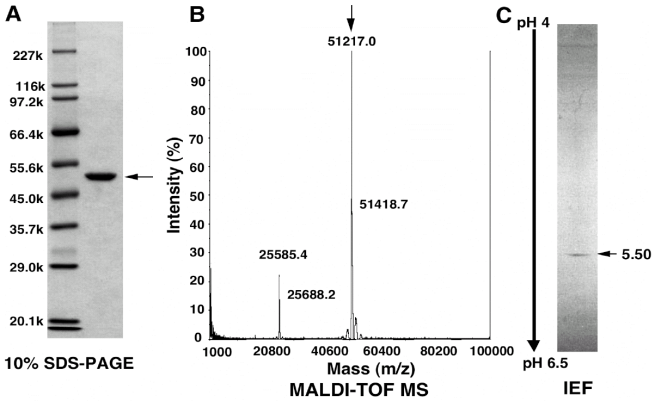


Fig. 2 Purified rice EGase[20)]. A: Ten percent SDS-PAGE of standard molecular marker proteins and 1 μg of purified EGase (peak III) was visualized by Coomassie Blue staining. B: MALDI-TOF MS of purified peak III enzyme. Species from left to right of the spectrum: double-charged molecular ion (m/z = 25,585.4, $(M+2H)^{2+}$), double-charged molecular ion with sinapinic acid (m/z = 25,688.2), single-charged molecular ion indicated by ↓ (m/z = 51,217, $(M+H^+)$), single-charged molecular ion with sinapinic acid (m/z = 51,418.7). Average molecular mass of the purified enzyme was 51,216 Da. C: IEF of purified enzyme (peak III). Ten μg of purified enzyme was loaded on an Ampholine PAGplate (pH 4.0-6.5) for IEF and the gel was stained with Coomassie Blue after electrophoresis.

of the proteins has an average molecular mass of 51,605.1 Da (peak I), 51,391.7 Da (peak II) or 51,216.0 Da (peak III) (Figure 2, only peak III shown). Separation by IEF glass column chromatography (Figure 1B) and anion exchange chromatography on Q Sepharose HP or Mono Q (Table 1) supported a difference in pI among the three proteins. Analytical IEF (Ampholine PAGplate) of the purified proteins also verified their purity (Figure 2, only peak III shown) and confirmed that they show microheterogeneity with pI values of 5.75 (peak I), 5.65 (peak II) and 5.5 (peak III).

2.3 Peptide sequences of purified proteins and the cloning of *OsCel9A* gene encoding 51 kDa rice EGases

Amino acid sequencing of the EGases showed that the 20 amino acid terminal sequence of all three enzymes was identical (Table 2). No secondary sequences were detected, which is an additional indication of their purity. The purified peak III fraction was also in-gel digested with endoproteinase Lys-C (LEP). Eleven peptide fragments from an LEP digest were separated by reversed-phase HPLC and sequenced. More than 200 amino acids were determined and are summarized in Table 2. One peptide sequence, LVGGYYDAGDNVK (LEP53 No. 3 in Table 2), was the conserved region I of the GHF9 enzyme[3)]. A degenerate PCR

Table 2 Summary of sequence results obtained from purified endo-1,4-β-glucanase proteins of rice (peaks I, II, III)[20)]

Designation of peptides	Sequence determined*1
N-terminal peptides of peak I	GGGGHDYGMALSKSILYFEAXRXXVLP
peak II	GGGGHDYGMALSKSILYFEAQRSGXLPXXQ
peak III	GGGGHDYGMALSKSILYFEAQRSG
Internal peptides of peak III*2	
LEP64-R54*3	SILYFEAQRSGVLPGSQRIAWRANSGLADGK
LEP53 No.3	ANXXD**LVGGYYDAGDNVK***4
LEP92,93-R24 or R38	FGLPMAFTVTMMAWSVIEYGEEMAAAGEL GXAVEAIK
LEP53 No.1	WGTDYFAK
LEP75-R48-2	AHPEPNVLYAEVGDGDSDHNXXXRPE
LEP56,57-R41	QLFDFADK
LEP91-1	YRGRYDNSITVARNYYGSFSGYGDELLXAX AXLYQAXDXXRXXXXXXN
LEP53 No.2	YPGVQILAAK
LEP47 No.1	FLLQGK
LEP41,42-R42 No.1	AGEHAGVLQGYRRK
LEP55-R48	ADFFAXSXLGK

*1: Amino acid sequence analyses of the purified EGases (peak I, II or III) were performed in an automatic sequencer (see Materials and Methods). X denotes an unidentified amino acid. *2: Fractions from HPLC (TSKgel ODS-80Ts) after in-gel digestion of the purified protein (peak III) with LEP. *3: Fraction number. *4: The conserved region I of GHF9 is in bold underlined text[3)].

primer set was synthesized based on two peptide sequences (LEP53 No.3 and LEP56, 57-R41 in Table 2) and used for RT-PCR. The full-length cDNA *OsCel9A* (Accession no. AB038510) encoding the purified rice EGase protein was isolated from a 2,4-D-treated root cDNA li brary of rice cv. Sasanishiki using the RT-PCR products. The genomic clone for *OsCel9A* was also isolated from a genomic library of rice cv. Sasanishiki. The cDNA (AK103340) corresponding to *OsCel9A* of Sasanishiki has also been isolated from Nipponbare, another cultivar included in the rice genome projects (http://cdna01.dna.affrc.go.jp/cDNA). The DNA sequence of the genomic clone for *OsCel9A* from Sasanishiki was identical to that from a PAC clone of Nipponbare

(AP002745), which maps to the 6.43-6.57 Mbp region of Chromosome 1 in the rice genome (the ordered locus name of *OsCel9A* : Os Olg 0220 100, http://rapdb_lab.hig.ac.jp./).

The *OsCel9A* gene encodes a 68 kDa EGase, an ortholog of tomato *TomCel8 (SlCelC1)*, which contains acarbohydrate binding module family 49 (distantly related to the microbial CBM 2) at its carboxy terminus[23, 24]. Our amino acid sequencing and MALDI-TOF MS analyses revealed that the 3.3 kDa amino terminal and 14 kDa carboxy terminal regions of *OsCel9A* nascent proteins had been removed in the 51 kDa purified rice EGases. The revelation of structural differences among the three purified enzymes based on posttranslational carboxy terminal cleavage sites in *OsCel9A* is described in the previous paper[21]. The isolation of *OsCel9A* gene from Sasanishiki and a global search of the rice genome database indicated that the purified enzymes (peaks I, II and III) were not isozymes but isoforms that are cognates of a single EGase gene. The isoform of EGase in major peak III was therefore used for subsequent experiments.

2.4 Substrate specificity of the auxin-induced rice EGase

The isolated EGase (peak III isoform) had a broad range of activity on β-1,4-glycans (Table 3). It significantly hydrolyzed not only 1,4-β-glucosyl linkages (CM-cellulose and phosphoric acid swollen cellulose) and mixed 1,3-β- and 1,4-β-glucosyl linkages (1,3-1,4-β-glucan and lichenan) but also 1,4-β-xylosyl linkages (xylan and arabinoxylan) and mixed 1,4-β-glucosyllinkages with a mannosyl backbone (glucomannan). Glucans containing only β-1,3-linkages (laminarin and CM-pachyman) were not hydrolyzed, indicating that the recognition site for β-1,3-1,4-glucan or lichenan was not a sequence of contiguous 1,3-β-linkages. Trace activity on mannan (100% mannose) also indicated that continuous 1,4-β-mannosyl linkages were unlikely to be the recognition site for glucomannan (40% glucose and 60% mannose; the repeated unit has been proposed to be GGMMGMMMMMGGM by Shimahara et al[25]. Thus, the difference in activity of the EGase with these polysaccharides indicates that it prefers either continuous β-1,4-glucosyl or β-1,4-xylosyl backbones, with a comparatively higher preference for birchwood xylan than the activity of PopCel2 against this substrate[16]. The purified rice EGase did not reveal significant hydrolytic activity against lupine galactan, lemon pectin, sugarbeet arabinan, p-nitrophenyl α- or β-glycosides (arabinose, cellobiose, glucose, galactose, mannose and xylose) during the 40 min or 2-24 h reaction periods (data not shown). Several GHF9 enzymes and EGases have been purified from dicots and woody plants and characterized, indicating that their substrates are mainly CM-cellulose, phosphoric acid swollen cellulose, xyloglucan and 1,3-1,4-β-glucan containing β-1,4-glucosyl linkages[1, 6]. The comparatively broader substrate range of the rice GHF9 enzyme, including its ability to hydrolyze phosphoric acid swollen cellulose, wheat endosperm arabinoxylan, barley 1,3-1,4-β-glucan and konjac glucomannan may partly reflect different roles of gramineous and non-gramineous GHF9 enzymes (Table 3).

Table 3 Broad substrate specificity of rice endo-1,4-β-glucanase*1 20)

Substrate (1 mg ml^{-1})	Sugar backbone	Linkage	Specific activity (μmole min^{-1} mg^{-1} protein)	Ratio (%)
CM-cellulose 7MF (0.846*2)	Glucose	β-1,4	2.6	100
CM-cellulose (0.386*2)	Glucose	β-1,4	3.77	144
Microcrystaline cellulose	Glucose	β-1,4	trace	-
Phospho swollen cellulose	Glucose	β-1,4	0.63	25
Tamarind xyloglucan	Glucose, xylose branches	β-1,4	trace	-
Barley 1,3-1,4-β-glucan	Glucose	(β-1,3)(β-1,4)	2.28	87
Lichenan	Glucose	(β-1,3)(β-1,4)	2.11	81
Laminarin	Glucose	β-1,3	n.d.	0
CM-pachyman (0.2*2)	Glucose	β-1,3	n.d.	0
Birch wood xylan	Xylose	β-1,4	0.47	18
Wheat arabinoxylan	Xylose, arabinose branches	β-1,4	0.99	38
Konjac glucomannan	Glucose, mannose	β-1,4	0.35	14
Ivory nut mannan	Mannnose	β-1,4	trace	-

*1: Reactions were performed in 50 mM acetate buffer (pH 5.5) containing 1 mg ml^{-1} substrate, 0.04% NaN_3 and 420 ng ml^{-1} purified enzyme (peak III), and were incubated at 28°C. The reaction volume was 1 ml. Aliquots (0.1 ml) were removed after a 40 min incubation for reducing power determination using a modified Park-Johnson method. Under these conditions, no activity was detected after 24 h towards lupine galactan, lemon pectin or sugarbeet arabinan. *2: DC.

The preference for CM-cellulose with its lower DC and the trace hydrolytic activity against tamarind xyloglucan most likely reflect that this rice EGase prefers a low degree of substitution and small side chains to access the β-1,4-glucan backbone (Table 3). However, this does not seem to be the case for the β-1,4-xylosyl backbone. Wheat flour arabinoxylan consists of 41% arabinose and 59% xylose and the number of side chains per 10 residues of the β-1,4-xylosyl backbone is approximately 6-7. Nevertheless, the rice EGase showed 2-fold higher hydrolytic activity against wheat arabinoxylan than birchwood xylan, reflecting that this enzyme prefers moderate decoration of the xylan backbone with arabinose. Xylan is a β-1,4-linked polymer of D-xylose, a saccharide unit similar to glucose but lacking the

hydroxymethyl group on C-5. This results in subtle differences in side chain effects on the secondary structure of the backbones[26] and might contribute to what appears to be an inconsistent preference of the rice GHF9 isozyme for substituted and unsubstituted β-1,4-linked glycans.

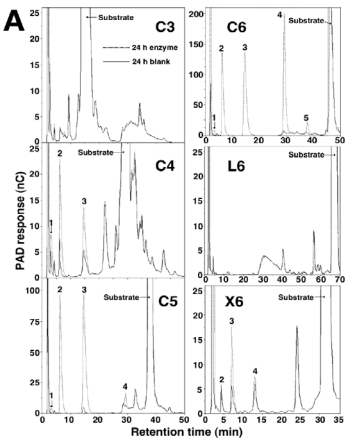
2.5 Activity of rice EGase with oligosaccharides

Products of cellooligosaccharides (DP: 2-6), xylohexaose (DP: 6) or laminarihexaose (DP: 6) after incubation with rice EGase for 24 h were analyzed with an HPAEC-PAD system equipped with a Carbopac PA-1 column (Figure 3). The shortest cellooligosaccharide hydrolyzed by the enzyme was cellotetraose. The enzyme produced about equal amounts of glucose (20%) and cellotriose (25%) or cellobiose (55%) from cellotetraose hydrolysis at a comparatively low rate (C4 in Figure 3B). However, a preference for a cleavage site near the center of cellooligomers by the rice EGase was observed in both cellopentaose (between molecules of cellotriose and cellobiose) and cellohexaose (both between two molecules of cellotrioses and between molecules of cellobiose and cellotetraose) when they were used as substrates (C5 and C6 in Figure 3). Although cellotetraose was hydrolyzed, the rate was about 10 times less than for cellohexaose, indicating an enzyme preference for longer sequences of 1,4-linkages. The rice EGase did not hydrolyze laminarihexaose containing1,3-linkages. No glucosyl transferase activity was detected after reaction with the purified enzyme.

In addition to studies with polymeric substrates, HPAEC profiling of oligosaccharideproducts also indicated hydrolytic activity of the rice EGase against continuous β-1,4-xylosyl linkages. The rice EGase significantly hydrolyzed xylohexaose (X6 in Figure 3), producing detectable amounts of xylobiose and xylotetraose with relatively larger amounts of xylotriose. Xylose and xylopentaose were not detected at significant levels in HPAEC profiles, showing thatthe preferred mode of cleavage of xylohexaose was between two molecules of xylotriose. The preferred mode of cleavage of xylohexaose by this enzyme was similar to that of cellohexaose (i.e., the central region of the oligosaccharide), although the hydrolytic activity against xylohexaose was even lower than that for cellotetraose. This suggests that amino acid residues involved in substrate binding and/or catalysis in the purified rice EGase may tolerate xylose-based polymers.

2.6 HPAEC profiling of hydrolytic products from hemicellulosic polymers by rice EGase

β-1,3-1,4-glucan and arabinoxylan are major components of rice primary cell walls. To investigate endohydrolase activity against these hemicellulosic polymers, a reaction mixture of barley 1,3-1,4-β-glucan or wheat arabinoxylan with the rice enzyme was chromatographed. No signals from monomers or oligomers were detected in the elution profiles in the case of polysaccharides incubated with a buffer blank (24 h blank in Figure 4). As predicted from viscometric and reductometric assays (Data not shown), the rice EGase released oligomeric products in the course of barley 1,3-1,4-β-glucan hydrolysis (24 h enzyme in Figure 4). The higher (DP > 6) oligosaccharides were relatively abundant but lower oligosaccharides were also observed at significant levels, with trace amounts of glucose. The eluted peaks of lower oligosaccharides were relatively broad and their elution times did not match those of standard cellooligosaccharides except for trace amounts of glucose (DP 1), cellobiose (DP 2) and cellotriose (DP 3), indicating that they may be mainly composed of multiple glucosyl oligomers containing mixed 1,3-β- and 1,4-β-linkages and lower amounts of continuous



B

Substrates (1 mg/ml)	Products: nmole・min^{-1}・mg^{-1} protein^{*1} (%)				
	DP1	DP2	DP3	DP4	DP5
Cellobiose(C2)	n.d.	-	-	-	-
Cellotriose (C3)	n.d.	n.d.	-	-	-
Cellotetraose (C4)	4.5 (20)	12.4 (55)	5.8 (25)	-	-
Cellopentaose (C5)	2.5 (2)	67.8 (51)	58.7 (44)	3.9 (3)	-
Cellohexaose (C6)	3.6 (1)	93.5 (35)	86.8 (32)	80.3 (30)	4.9 (2)
Laminarihexaose (L6)	n.d.	n.d.	n.d.	n.d.	n.d.
Xylohexaose (X6)	n.d.	trace	6.3	trace	n.d.

Fig. 3 **A:** HPAEC elution profiles of hydrolysis products released from oligosaccharides from cellotriose (C3) to cellohexaose (C6), xylohexaose (X6) and laminarihexaose (L6) by purified EGase[20]. Numbers on the top of elution peaks indicate the DP of products. **B:** Amounts of products formed after hydrolysis of oligosaccharides, calculated from HPAEC elution profiles (Figure 3A).

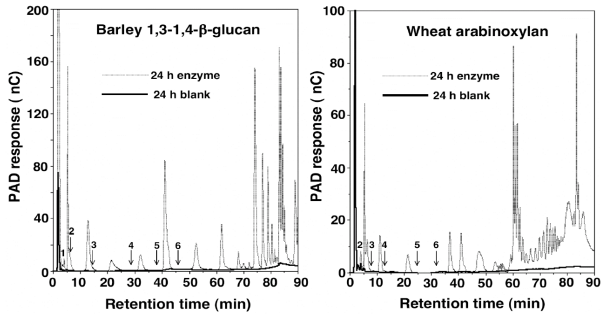


Fig. 4 HPAEC elution profiles of hydrolysis products released from major matrix polysaccharides of a gramineous cell wall by purified rice EGase. Products were analyzed by HPAEC-PAD[20]. Cellooligosaccharides of DP 2-6, xylooligosaccharides of DP 2-6, glucose (DP 1) and xylose (DP 1) were used as standards. Numbers on the top of eluted peaks or arrows in figures indicate the DP of standards.

1,4-β-linkage glucosyl oligomers.

The observation that cellotetraose was the shortest substrate is consistent with the implication that the enzyme can recognize at least cellotetraosyl repeating units of mixed β-glucan. Approximately 90% (w/w) of barley 1,3-1,4-β-glucan consists of mainly cellotriosyl and cellotetraosyl residues separated by single 1,3-linkages. It also contains rare (3-5% w/w) cellulose-like blocks such as five to ten contiguous β-1,4-linked glucosyl residues[14]. The cell wall bound EGase of maize has particularly high activity on the restricted sites (cellulose-like blocks) of barley 1,3-1,4-β-glucan[13]. The limited number of recognition sites within the molecules for the maize enzyme leads to the limited depolymerization of the mixed β-glucan; this observation is based on the release of only high mol wt (10-15,000 Da, DP of 60 to 70) products after long term-incubation[15]. Hence, in contrast to the cell wall bound EGase from maize, there does not appear to be a limited number of recognition sites in mixed β-glucan for the buffer soluble rice EGase, although a possible preference of the rice enzyme for the cellulose-like blocks of this polysaccharide cannot be ruled out.

The elution profile of the hydrolytic products from wheat arabinoxylan treated with the rice EGase was similar to that of 1,3-1,4-β-glucan (Figure 4). The higher oligosaccharides were relatively abundant but lower oligosaccharides were also detected. The elution peaks of lower oligosaccharides were also broad and their elution times did not correspond to those of standard xylooligosaccharides except for trace amounts of xylobiose (DP 2), xylotriose (DP 3) and xylotetraose (DP 4), indicating that they are likely composed mainly of multiple arabinoxylosyl oligomers. Wheat arabinoxylan consists of a xylan backbone that is unsubstituted or 3- or 2,3-substituted with single arabinofuranosyl groups. The number of side chains per 10 residues of the β-1,4-xylose backbone of wheat arabinoxylan is approximately 6-7, although the region of this polysaccharide designated as III_4 contains high amounts of contiguous (6 or more) unsubstituted xylose residues[27] (Izydorczyk and Biliaderis, 1994). Figure 4 also shows that the elution peaks of lower oligomers were broad and most of the elution times did not correspond to those of standard xylooligosaccharides. This indicates that they were mainly composed of multiple arabinoxylosyl oligomers, maybe lower than xylohexaose, implying that the purified enzyme can recognize the highly branched regions of arabinoxylan, although a preference of the rice enzyme for the unsubstituted region cannot be ruled out. Hence, the side group of arabinose along the xylan did not appear to interfere with access of the rice EGase to the β-1,4-xylosyl backbone.

As shown in Figure 4, the HPAEC elution profile of the hydrolytic products from wheat arabinoxylan of rice EGase seems to be different from that of the products of a microbial GHF10 xylanase, CjXyn10C[28]. A GHF10 (endo-1,4-β-xylanase) enzyme from barley endosperm efficiently hydrolyzes both birchwood xylan and wheat arabinoxylan [29]. Another GHF10 enzyme from maize pollen efficiently hydrolyzes oat spelt xylan (sparsely substituted by arabinose) rather than either birchwood xylan or 4-O-methyl-glucuronoxylan[30]. An understanding of the mechanisms by which GHF10 enzymes preferentially bind to decorated xylans is starting to emerge[28, 31]. Although no molecular basis for the catalytic machinery of the rice GHF 9 enzyme that accommodates the decorated β-1,4-xlylosyl backbone can be provided at this stage, our study suggests that the substrate binding site of the purified enzyme may interact with the decorated xylan in a distinct way from microbial GHF10 xylanases.

2.7 Catalytic domain of OsCel9A protein

Substrate binding subsites in glycoside hydrolases are defined as +1, +2…. in the direction of the reducing end and as -1, -2…. toward the nonreducing end of the oligosaccharide with the cleavage site located between -1 and +1[32]. The 3D structure of catalytic domain of OsCel9A was

predicted with homology modeling and superposed on the microbial TfCel9A (figure 5)[21]. TfCel9A from *Thermobifida fusca* is so far the best-characterized cellulase of the E2 subfamily and its three-dimensional structure has been solved[33].

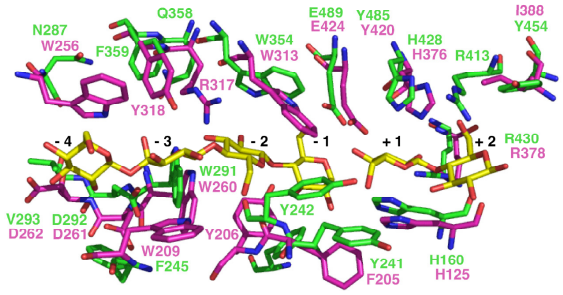


Fig. 5 Catalytic and substrate binding sites of OsCel9A[21]. Superposition of the active site in the experimentally determined structure of TfCel9A (violet) and that of the modeled OsCel9A (green) is according to Fig. 2 of Master et al. (2004) except for two residues, Phe_{205} and Ile_{388} of TfCel9A. Numbering is from the full-length sequence of OsCel9A and the catalytic domain of TfCel9A. Only residues that interact through hydrogen bonds or Trp stacking in TfCel9A, in addition to two other residues, Phe_{205} and Ile_{388} in TfCel9A, are shown. The TfCel9A structure is in a complex with cellotetraose (subsites –4 to –1) and cellobiose molecules (subsites +1 and +2). The reducing end of the binding cleft is to the right, and the nonreducing end is to the left. The image was made with the PyMOL program (Delano 2002). This model was generated by Dr. Satoshi Kaneko of National Food Research Institute.

As noted by Master et al., the tryptophan residues at positions 256, 209 and 313 in TfCel9A, which are crucially involved in binding Glc-4, Glc-3, and Glc-2 of β-1,4-glucans, respectively, are missing in membrane anchored plant GHF9 enzymes[18]. Rice OsCel9A contains a tryptophan corresponding to Trp_{313} (subsite -2) in TfCel9A (Figure 5). The $tryptophan_{209}$ of subsite -3 in TfCel9A is mimicked by Phe_{246} in rice OsCel9A, while a substitution to Thr or Ser is conserved among the membrane anchored GHF9 enzymes. In regard to Tyr_{318} of subsite -3 in TfCel9A, the substitution with phenylalanine is conserved in all CBM2 types of plant GHF9 enzymes. As shown in Figure 5, the modeled structure of OsCel9A reveals that this substitution might leave more room in the substrate binding subsites of this enzyme. In addition to these, the amino acid residue corresponding to Ile_{388} (subsite +2) in TfCel9A is replaced with Tyr or Phe in plant CBM types including OsCel9A but not in membrane anchored plant GHF9 enzymes (Figure 5)[21]. The substrate binding subsites of OsCel9A could be adapted to soluble substrates rather than a crystalline substrate on the grounds that the replacement of Y318F in TfCel9A facilitates enzyme activity against carboxymethyl-cellulose (667% of wild-type enzyme) but lowers the activity on bacterial microcrystalline cellulose (15% of wild-type enzymes, Zhou et al. 2004). Collectively, the substrate binding cleft of rice OsCel9A shows a primitive disposition of an aromatic platform residue (Tyr, Trp or Phe), which is important for hydrophobic stacking interaction with the saccharide ring (Quiocho, 1986), mimicking the catalytic machinery of microbial E2 enzymes. OsCel9A probably contains 5 sugar binding sites; three glycone (-1 to -3) and two aglycone (+1 to +2) subsites based on the constellation of aromatic platform residues. The distinct dispositions of aromatic and hydrophobic platform residues in the substrate binding site of OsCel9A might be involved in the tolerance of this enzyme for relatively hydrophobic xylose-based polymers.

2.8 Endogenous target of auxin-induced rice EGase

A recently revised model of the type II cell wall proposed that a 1,3-1,4-β-glucan-enriched domain, coextensive with GAX of low degrees of side chains and glucomannan, is tightly associated around cellulose microfibrils[12, 13]. This first domain of the type II cell wall may be an endogenous substrate for auxin-induced rice EGase, since the purified enzyme was highly active against both barley 1,3-1,4-β-glucan and lichenan in addition to having relatively lower activities against phosphoric acid swollen cellulose, xylans and glucomannan (Table 3).

The first (1,3-1,4-β-glucan-enriched) domain of the type II cell wall, are interlaced by the GAX with a greater degree of branching by single arabinofuranosyl units[12, 13]. It has also been proposed that an interaction between 1,3-1,4-β-glucan and the interstitial GAX, rather than 1,3-1,4-β-glucan and cellulose, may constitute the biochemical target of growth-altering enzymes[13]. This second complex domain of type II cell walls may be another endogenous substrate of the rice EGase, since the purified enzyme showed a relatively higher activity against wheat endosperm arabinoxylan than birchwood xylan, in addition to its highest activity against mixed β-glucan (Table 3).

3. MATERIALS AND METHODS

All experimental procedures were described in the previous papers[20, 21].

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