β-Glucosidase, exo-β-glucanase and pyridoxine transglucosylase activities of rice BGlu1

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The bglu1 cDNA for a β-glucosidase cloned from rice (*Oryza sativa* L.) seedlings was expressed as a soluble and active protein in *Escherichia coli* and designated BGlu1. This enzyme hydrolysed β-1,4-linked oligosaccharides with increasing catalytic efficiency (k_{cat}/K_m) values as the DP (degree of polymerization) increased from 2 to 6. In contrast, hydrolysis of β-1,3-linked oligosaccharides decreased from DP 2 to 3, and polymers with a DP greater than 3 were not hydrolysed. The enzyme also hydrolysed *p*-nitrophenyl β-D-glycosides and some natural glucosides but with lower catalytic efficiency than β-linked oligosaccharides. Pyridoxine 5′-*O*-β-D-glucoside was the most efficiently hydrolysed natural glycoside tested. BGlu1 also had high transglucosylation activity towards pyridoxine, producing pyridoxine 5′-*O*-β-D-glucopyranoside in the presence of the glucose donor *p*-nitrophenyl β-D-glucoside.

Key words: β-glucosidase, pyridoxine glucoside, rice, subsite mapping, transglycosylation.

INTRODUCTION

Plant β-glucosidases (EC 3.2.1.21) in GH (glycosyl hydrolase) family 1 catalyse transfer of a non-reducing glucosyl group from a glycoside or carbohydrate to water (hydrolysis) or another alcohol (transglycosylation) via a retaining mechanism [1,2]. This mechanism requires two carboxylic acid residues on β-strands 4 and 7 of the $(\beta/\alpha)_8$ barrel structure of this family, which act as the general acid-base catalyst and the nucleophile respectively [3–5]. Although these enzymes share the same general structure and catalytic mechanism, they vary greatly in their substrate specificity. Czjzek et al. [6,7] have shown that in maize β-glucosidase certain residues in the funnel-shaped pocket appear to contact the sugar and aglycone residues, thus providing a basis for determining substrate specificity. However, other positions lining the pocket have been suggested to play this role for other β-glucosidases [8], and some have been demonstrated to specifically affect substrate binding and hydrolysis by site-directed mutagenesis of cassava linamarase [9]. Additionally, Hrmova and colleagues [10] have demonstrated a much more extensive binding site including six glucose residues for barley β-glucosidase/exo-β-glucanase, which is also in GH family 1.

In rice (*Oryza sativa* L.), purified β-glucosidases have been shown to hydrolyse gibberellin glucosides [11] and oligosaccharides derived from cell-wall β-glucans [12]. Recently, two rice seedling β-glucosidase cDNAs, bglu1 and bglu2, were isolated and sequenced [13]. Both *bglu1* and *bglu2* genes are highly expressed in shoots during germination, but only *bglu1* is expressed in flowers. In addition, a BLAST search of the expressed sequence tag database showed that the bglu1 mRNA was the most highly represented GH family 1 gene transcript in rice, with approximately 6 times more expressed sequence tags than any other GH family 1 gene.

Previously, we expressed the bglu1 cDNA as a recombinant protein (BGlu1) in *Escherichia coli*, and preliminary analyses showed that it possessed hydrolytic activity on various glucoside substrates and also showed transglucosylation activity towards β-linked oligosaccharides [13]. This activity appeared to be somewhat similar to that of the barley β-glucosidase with which it shared the highest sequence identity [10,14]. In the present study, kinetic analysis of the enzyme is used to evaluate its substrate specificity and demonstrate differences in the oligosaccharide binding and hydrolysis by rice BGlu1 and barley β-glucosidase. The deduced protein sequences of the rice and barley enzymes are compared in relation to their substrate specificities. In addition, we report the ability of this enzyme to transfer glucose from *p*NPG (*p*-nitrophenyl β-D-glucoside) to pyridoxine and characterized the hydrolytic activity of this enzyme towards pyridoxine glucoside.

EXPERIMENTAL

Materials

The cDNA (accession number U28047) encoding the mature protein product of rice β-glucosidase gene bglu1 was cloned into pET32a(+) and expressed in *E. coli*, and the soluble active recombinant BGlu1 protein was purified as described previously [13]. Cellobiose, gentiobiose, sophorose, *p*NPG, other *p*NP (*p*-nitrophenyl) glycosides, pyridoxine (vitamin B_6), other glucosides and peroxidase/glucose oxidase glucose assay reagents were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Pyridoxine 5′-*O*-β-D-glucoside was provided by Professor J. F. Gregory of the University of Florida, Gainesville, FL, U.S.A. Torvoside A was purified as described by Arthan et al. [15]. Cello-oligosaccharides of DP (degree of polymerization) 3–6 and

Abbreviations used: A_i, subsite affinity; DP, degree of polymerization; GH, glycosyl hydrolase; *p*NP, *p*-nitrophenyl; *p*NPG, *p*-nitrophenyl β-D-glucoside; H,H-COSY, two-dimensional homonuclear correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation.

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laminari-oligosaccharides of DP 2–5 were from Seikagaku Kogyo Co. (Tokyo, Japan). Silica gel 60 F_{254} aluminium sheet (0.2 mm) and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Chloroform, NH_4OH and methanol were purchased from Carlo ERBA (Rodano Milano, Italy).

β-Glucosidase assays and kinetic analysis

Kinetic parameters were calculated from triplicate assays done at 30 °C in 50 mM sodium acetate buffer, pH 5.0. The β-glucosidase activity for various substrates was assayed by spectrophotometric measurement of either (i) *p*NP liberated from the *p*NP derivatives of monosaccharides and disaccharides or (ii) glucose released from natural or artificial glucoside substrates by the peroxidase/glucose oxidase assay, as described previously [16]. HPLC was used to quantify the amount of pyridoxine glucoside hydrolysed. Solutions of 0.01–0.16 μmol per 20 μl of pyridoxine 5′-*O*-β-D-glucoside were prepared as standards. Pyridoxine 5′-*O*-β-D-glucoside was incubated with 0.01 nmol of purified BGlu1 in 50 mM sodium acetate, pH 5.0, in 50 μl reactions at 30 °C for 15 min. The reaction was terminated by heating at 100 °C for 5 min. The products were separated by an Eclipse XDB-C-18 column (Agilent Corp, Palo Alto, CA, U.S.A.) on an HP 1100 series HPLC (Hewlett Packard, Waldbronn, Germany) with detection by absorbance at 290 nm. A gradient of methanol in 0.1 % trifluoroacetic acid in water was used to separate the product compounds as follows: 0–10 % over 20 min, 10–20 % over 5 min and 20–80 % over 5 min. One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 μmol of product/min. Protein assays were performed by using a protein assay kit (Bio-Rad, Richmond, CA, U.S.A.) with BSA as a standard.

Kinetics

Kinetic parameters, K_m and V_{max} (at pH 5.0 and 30 °C), were calculated according to Lineweaver and Burk using the Enzfitter computer program (Elsevier Biosoft, Cambridge, U.K.). Subsite affinities for binding of cello-oligosaccharides at subsites +2 to +5 were calculated as described by Hrmova et al. [10], based on the differences of $RT\ln(k_{cat}/K_m)$ for oligosaccharides of different DP values. The values of A_{+1} and A_{-1} were calculated from the equation $1/K_{m,n} = \Sigma K_{n,j}$, where $K_{n,j} = (0.018)\exp(\Sigma A_i/RT)$ (where A_i is subsite affinity) for all subsites *i* covered by an oligosaccharide of length *n* starting from site *j* at the non-reducing end, by simultaneous solution of the equations for the five substrates [17]. The k_{int} was calculated from the equation $k_{int} = (k_{cat}/K_m)_n/K_{n,1}$ for all cello-oligosaccharides tested [17].

Transglucosylation reactions

Freshly prepared 5, 10, 20 and 40 mM *p*NPG and 1, 5, 10 and 20 mM pyridoxine in 50 mM sodium acetate, pH 5.0, were incubated with 0.01–0.02 nmol of purified rice BGlu1 in a 100 μl reaction mixture. The reaction mixtures were incubated at 30 °C for 1–24 h. The reactions were stopped by heating at 100 °C for 5 min before analysis by TLC. For each reaction, controls without enzyme and without pyridoxine were included in the assay. An aliquot (5 μl) of the reaction solution and standards were spotted on to a F_{254} 0.2 mm silica-coated aluminium plate (10 cm × 10 cm). The plate was chromatographed vertically at room temperature in chloroform/methanol/NH_4OH (7:2.8:0.2, by vol.) for 40 min. The products were visualized under UV light at 366 nm. The TLC plates were scanned with a Fluor-S™ MultiImager and the intensities of the signals from the compounds were quantified with Quantity One software (Bio-Rad, Richmond, CA, U.S.A.).

For structural analysis of the putative pyridoxine glucoside product resulting from transglucosylation, the reactions were scaled up to 15 ml and the pyridoxine glucoside product was purified by HPLC. The reaction mixture was separated on a 7.6 mm × 250 mm Inert ODS-3 C_{18} column (GL Sciences, Tokyo, Japan) with the HP Series 1100 HPLC with the same methanol gradient as described for analysis of pyridoxine glucoside hydrolysis. Products were detected by absorbance at a wavelength of 290 nm.

Table 1 Kinetic parameters of rice BGlu1 during the hydrolysis of oligosaccharides

ND, not detectable.

Substrate	DP	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Cello-oligosaccharides	2	31.5 ± 1.6	1.52 ± 0.13	0.05 ± 0.002
	3	0.72 ± 0.02	18.13 ± 0.35	25.4 ± 0.4
	4	0.28 ± 0.01	17.34 ± 0.63	61.1 ± 0.4
	5	0.24 ± 0.01	16.90 ± 0.06	71.5 ± 2.2
	6	0.11 ± 0.01	16.93 ± 0.32	152.9 ± 0.5
Laminari-oligosaccharides	2	2.05 ± 0.1	31.9 ± 3.1	15.7 ± 1.9
	3	1.92 ± 0.04	21.2 ± 0.2	11.0 ± 0.2
	4	ND	ND	ND
	5	ND	ND	ND
Sophorose		13.89 ± 0.92	5.87 ± 0.21	0.42 ± 0.02
Gentiobiose		38.3 ± 4.1	0.99 ± 0.08	0.03 ± 0.003

NMR analysis

NMR spectra were recorded on a Varian UNITY INOVA-300 MHz (Varian, Darmstadt, Germany), operating at 299.9 MHz for protons and 75.4 MHz for carbon. All samples for NMR analysis were dissolved in ^2H_2O. Determination of the anomeric configuration was based on observed chemical shifts, and the $J_{1,2}$ coupling constants were measured from one-dimensional ^1H spectra. H,H-COSY (two-dimensional homonuclear correlation spectroscopy), HMQC (heteronuclear multiple quantum coherence) and HMBC (heteronuclear multiple bond correlation) experiments were done in order to assign the respective proton and carbon signals.

RESULTS

Substrate specificity of rice BGlu1

BGlu1 hydrolysed the disaccharides sophorose β-(1 → 2), laminaribiose β-(1 → 3), cellobiose β-(1 → 4), and gentiobiose β-(1 → 6). It also hydrolysed laminaritriose and cello-oligosaccharides with DP of 3–6. As shown in Table 1, BGlu1 hydrolysed laminaribiose with a relatively low K_m and high k_{cat}, but hydrolysed cellobiose, sophorose and gentiobiose with high K_m and low k_{cat} values. For cello-oligosaccharides, the K_m decreased with increasing chain length of the substrate, while k_{cat} values appear to be relatively independent of DP above a DP of 2. Catalytic efficiency, as judged by k_{cat}/K_m values, increased with increasing chain length of the cello-oligosaccharides so that the k_{cat}/K_m of cellohexaose was 3000-fold higher than that of cellobiose. In contrast, laminaribiose showed slightly higher k_{cat}, and k_{cat}/K_m values compared with laminaritriose, while no products could be observed with laminaritetraose and laminaripentaose.

Table 2 Binding affinities of rice BGlu1 and barley β-glucosidase isozyme βII for cello-oligosaccharide glucose residues

The subsite affinities were calculated from the differences in affinities of different-length oligosaccharides with the equation $A_n = RT[\ln(k_{cat}/K_m)_{n+1} - \ln(k_{cat}/K_m)_n]$, where n is the subsite number and number of glucose residues in the oligosaccharide, as described by Hrmova et al. [10]. Affinity of the first two subsites was determined by solving for $A_{-1} + A_{+1}$ in the equation relating K_m to binding constants described by Hiromi et al. [17] as follows: $A_{-1,+1} = RT\ln\{[1/(0.018)K_m]_n - \Sigma K_{n,j}\} - \Sigma A_i$ for $1 < i < n$ and $1 < j < 7$, where i is the subsite number and $K_{n,j}$ is the binding constant for an oligosaccharide of length n starting from subsite j. For initial estimates, A_{+1} was assumed to be 0, which caused insignificant errors in the value of $A_{-1} + A_{+1}$ for oligosaccharides with $n > 3$. Values for barley βII A_i were from Hrmova et al. [10].

Subsite I	Sugar residue in productive complex	Rice BGlu1 A_i	Barley βII A_i
−1	1	13.51 ± 0.20	4.34 ± 0.53
+1	2	−0.73 ± 0.05	20.50 ± 0.07
+2	3	15.69 ± 0.05	−1.99 ± 0.03
+3	4	2.21 ± 0.03	4.14 ± 0.01
+4	5	0.40 ± 0.06	2.70 ± 0.03
+5	6	1.92 ± 0.07	0.92 ± 0.03

Subsite affinities of BGlu1 β-glucosidase for glucose residues in cello-oligosaccharides were calculated from the equations of Hiromi et al. [17], as described by Hrmova et al. [10] for barley βII β-glucosidase. However, due to the strong binding at subsite 3, the assumption that almost all substrate molecules bound starting from sites −1 and +1, which was used to simplify the equation relating the K_m to the binding constants, was not valid for cellobiose (which would bind most strongly at subsites +2 and +3). Therefore, the full equation (see the Experimental section) was solved to generate the values of A_{+1} and A_{-1}. These calculations showed productive binding at glucose residues 1 and 3–6 from the non-reducing end (binding subsites −1, +2 to +5), as shown in Table 2. With these values, the k_{int} of BGlu1 was calculated from the data of each oligomer to give a mean value of 17.6 ± 0.7 s^{-1}. Highest affinity was seen at the +2 subsite (corresponding to glucose residue 3 from the non-reducing end in productive binding), which had an affinity of 15.7 ± 0.05 kJ/mol, compared with −1.99 ± 0.03 kJ/mol for this residue in barley βII β-glucosidase. In contrast, the +1 subsite, which has highest affinity in barley βII β-glucosidase (+20.5 ± 0.07 kJ/mol), had negative binding affinity in BGlu1 (−0.76 ± 0.05 kJ/mol).

The purified BGlu1 hydrolysed several *p*NP β-glycosides with varying efficiency (Table 3). BGlu1 did not hydrolyse *p*NP α-D-glucoside or the *p*NP β-glycosides of L-fucose, D-thioglucoside or D-thiofucoside. The enzyme also hydrolysed the *p*NP α-glycoside of L-arabinoside but not β-L-arabinoside. Kinetic studies of rice BGlu1 enzyme were performed to determine the K_m and the k_{cat} values of the enzyme for various *p*NP β-D-glycosides (Table 3). The enzyme had both low K_m and high k_{cat} values for both *p*NP β-D-glucoside and *p*NP β-D-fucoside. BGlu1 hydrolysed *p*NP β-D-galactopyranoside, *p*NP β-D-mannoside, *p*NP β-D-xylose, *p*NP α-L-arabinoside and *p*NP β-D-cellobioside with much lower catalytic efficiency than *p*NPG (Table 3).

Several available natural and artificial glucosides were hydrolysed poorly relative to *p*NPG or not at all (Table 3). Those that were hydrolysed had relatively low k_{cat} values (below 2 s^{-1}), although some also had relatively low K_m values. Pyridoxine 5′-*O*-β-D-glucoside was the most efficiently hydrolysed natural glycoside with a K_m of 0.71 mM and k_{cat} of 1.64 s^{-1}. BGlu1 also hydrolysed the steroid glucoside Torvoside A, but, again, with low efficiency compared with *p*NPG and the better oligosaccharide substrates. Prunasin, amygdalin and dhurrin were hydrolysed with lower efficiency, while linamarin and DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-glucoside were not hydrolysed. BGlu1 did not hydrolyse the phenolic glycosides tested (phenyl-β-D-glucoside, arbutin, salicin), a thioglucoside (sinigrin), a hydroxy coumarin glucoside (esculin), a flavonoid glucoside (naringin), magniferin-C-glucoside or an α-D-disaccharides (nigerose). BGlu1 hydrolysed 4-methylumberiferyl glucoside with a slightly higher K_m and lower k_{cat} than *p*NPG, while for *o*-nitrophenyl β-D-glucoside both the K_m and k_{cat} were slightly higher. Alkyl glucosides were hydrolysed poorly or not hydrolysed, but longer straight-chain alkyl glucosides appeared to be hydrolysed better. BGlu1 hydrolysed methyl, *n*-heptyl and *n*-octyl β-D-glucosides, but did not hydrolyse *n*-amyl β-D-glucoside.

Table 3 Kinetic parameters of rice BGlu1 during the hydrolysis of *p*NP glycosides, alkyl glucosides and natural glucoside substrates

Substrate	K_m (mM)	k_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
*p*NP β-D-glucoside	0.23 ± 0.02	7.93 ± 0.37	34.7 ± 1.4
*p*NP β-D-fucoside	0.23 ± 0.01	13.34 ± 0.17	57.5 ± 2.1
*p*NP β-D-galactoside	3.16 ± 0.16	3.40 ± 0.18	1.08 ± 0.01
*p*NP β-D-mannoside	1.27 ± 0.10	1.32 ± 0.05	1.01 ± 0.02
*p*NP β-D-xyloside	1.29 ± 0.11	1.14 ± 0.01	0.88 ± 0.07
*p*NP α-L-arabinoside	1.21 ± 0.13	1.24 ± 0.19	1.03 ± 0.05
*p*NP β-D-cellobioside	0.78 ± 0.04	2.48 ± 0.04	3.18 ± 0.13
o-Nitrophenyl β-D-glucoside	0.43 ± 0.02	15.95 ± 0.18	37.6 ± 0.9
Methylumberiferyl β-D-glucoside	0.28 ± 0.02	5.01 ± 0.23	17.98 ± 0.90
Methyl β-D-glucoside	49.2 ± 5.1	0.65 ± 0.08	0.013 ± 0.0003
n-Heptyl β-D-glucoside	1.41 ± 0.27	0.87 ± 0.22	0.62 ± 0.02
n-Octyl β-D-glucoside	0.63 ± 0.01	0.78 ± 0.01	1.25 ± 0.03
Amygdalin	8.20 ± 0.44	0.44 ± 0.05	0.05 ± 0.004
Prunasin	5.83 ± 0.53	1.41 ± 0.09	0.24 ± 0.01
Dhurrin	0.62 ± 0.10	0.06 ± 0.006	0.09 ± 0.004
Torvoside A	0.99 ± 0.11	0.67 ± 0.05	0.68 ± 0.02
Pyridoxine 5′-*O*-β-D-glucoside	0.71 ± 0.10	1.64 ± 0.19	2.30 ± 0.09

Transglucosylation activity of rice BGlu1

BGlu1 was able to transfer glucose from *p*NPG to pyridoxine (vitamin B_6). There were five major transglucosylation products visible on the TLC (Figure 1). As expected, four of these spots were *p*NP derivatives, which had the same R_f values as products of the control containing only enzyme and *p*NPG. These transfer products were compared with the known standard from Sigma, one product had an R_f value equal to that of *p*NP β-D-cellobioside, and another had an R_f equal to that of *p*NP β-D-cellotrioside (Figure 1). There was one outstanding purple fluorescent spot in addition to the spot of pyridoxine, which was not seen in the control reaction. This purple fluorescent spot had an R_f value equal to that of the pyridoxine 5′-*O*-β-D-glucoside standard. Both the unique spot and the standard fluoresced purple under 366 nm UV light. The transglucosylation products were also analysed by HPLC and the retention time of the product was the same as that of the pyridoxine 5′-*O*-β-D-glucoside standard (Figure 2).

The yield of pyridoxine glucoside product was optimized by varying enzyme concentration, time of incubation, concentration of *p*NPG glycosyl donor and pyridoxine acceptor. The amount of pyridoxine glucoside produced increased in proportion to the concentrations of the pyridoxine and *p*NPG substrates. The yield of products increased up to 6 h (Figure 1), then remained constant up to 24 h (results not shown). Hydrolysis of transglucosylation products occurred at higher concentrations of enzyme and longer incubation times (results not shown). Thus maximum yields

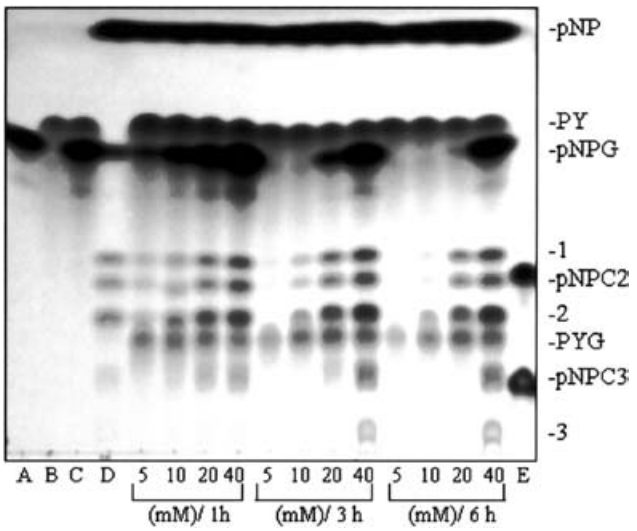

Figure 1 Transglucosylation of 20 mM pyridoxine using rice β-glucosidase after incubation with various concentrations of *p*NPG in 50 mM sodium acetate buffer, pH 5.0, at 30 °C for 1–6 h

The numbers 5, 10, 20 and 40 under each lane represent the concentration of *p*NPG in mM. The incubation times for each reaction set were 1, 3 and 6 h. Reactions were analysed by TLC on silica gel 60 F_{254}. Lane A, *p*NPG standard marker; lane B, pyridoxine standard marker; lane C, blank without enzyme; lane D, control containing enzyme and 10 mM *p*NPG; lane E, *p*NP β-D-cellobiose and *p*NP β-D-cellotriose. PY, pyridoxine; PYG, pyridoxine 5′-*O*-β-D-glucoside; pNPC2, *p*NP β-D-cellobioside; pNPC3, *p*NP β-D-cellotrioside. 1, 2 and 3 represent unknown compounds.

were produced with 0.1 μM enzyme incubated 6 h in 20 mM pyridoxine and 20 mM *p*NPG.

The pyridoxine glucoside product was purified by reversed-phase HPLC to greater than 95 % purity and ^{1}H- and ^{13}C-COSY, HMBC and HMQC NMR techniques were used to identify it as pyridoxine 5′-*O*-β-D-glucopyranoside, which was previously reported by Yasumoto et al. [18].

DISCUSSION

The preference of rice BGlu1 for 1,4-β-oligosaccharides of increasing chain length suggested that it has an extensive subsite-binding region that is dependent on substrate geometry, as has been described for barley βII β-glucosidase [10,19,20]. The subsite-binding region of polysaccharide hydrolases consists of an array of tandemly arranged subsites, where each subsite binds a single glycosyl residue of the polymers [17,21]. The increased hydrolytic rate with oligosaccharide length is a characteristic often observed with polysaccharide exohydrolases, unlike the rates of hydrolysis of oligomeric substrates by β-glucosidases which tend to remain approximately constant or decrease with increasing DP [22]. Therefore, as pointed out for barley βII β-glucosidase by Hrmova et al. [19], rice BGlu1 may be considered an exo-1,4-β-glucanase (EC 3.2.1.74).

In hydrolysis of gluco-oligosaccharides, opposing trends were seen for 1,3- and 1,4-linked oligosaccharides. The k_{cat} for β-1,3-linked oligosaccharides (laminaribiose and laminaritriose) was greater than that of cello-oligosaccharides, which suggests that this linkage may have a better geometry for hydrolysis.

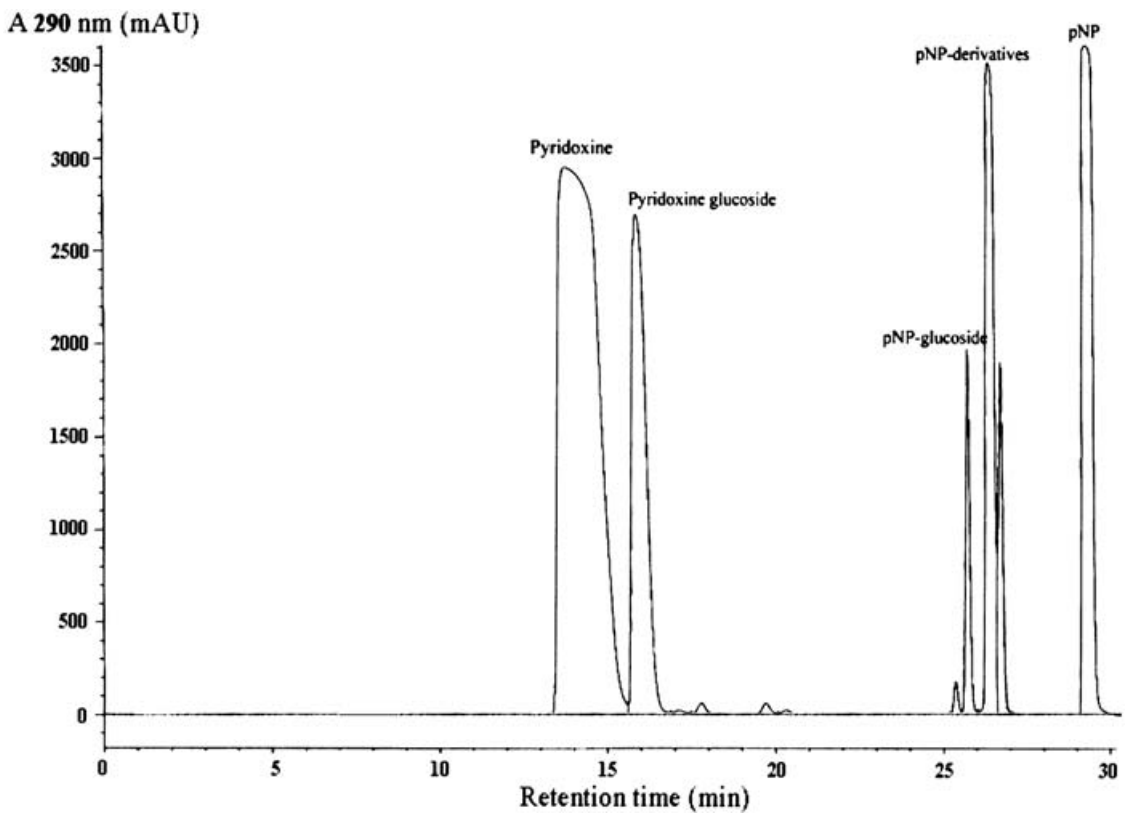

Figure 2 HPLC elution profiles of the transglucosylation products of pyridoxine using rice BGlu1, *p*NPG and pyridoxine in the reaction

The enzyme (0.01 nmol) was incubated with 20 mM pyridoxine and 20 mM *p*NPG in 50 mM sodium acetate buffer (pH 5.0) in a 100 μl reaction at 30 °C for 6 h. The reaction products were subjected to HPLC analysis.

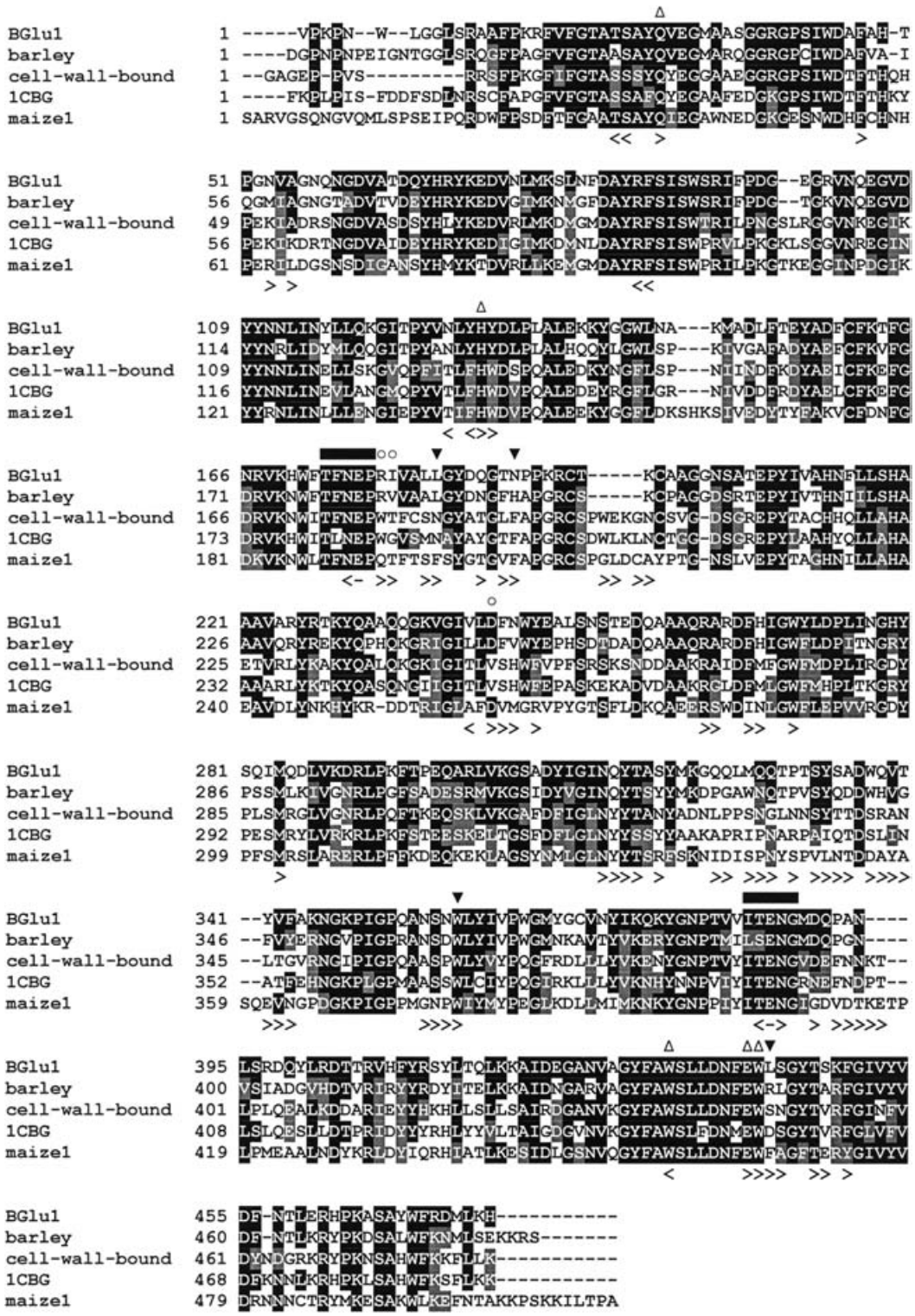

Figure 3 Alignment of the predicted mature protein sequences of rice BGlu1 with related β-glucosidases

Rice cDNA derived sequences are labelled as BGlu1; maize1 is maize β-glucosidase 1 (accession no. U33816); 1CBG is white clover linamarase (P26205); barley is deduced from barley BGQ60 nucleotide sequence (AAA87339), which was determined previously to be the same as βII β-glucosidase [14,10]; and cell-wall-bound was predicted rice cell-wall-bound β-glucosidase from the GenBank accession no. AL731582 genomic sequence nucleotides 48978–43967, which matched the N-terminal amino acid sequence of Akiyama et al. [12]. Sequences corresponding to the catalytic acid/base and nucleophile consensus sequences are marked by a thick line above the sequences. Residues shown by Czjzek et al. [6] to be in contact with the DIMBOA aglycone in the maize β-glucosidase are indicated by ▼; other residues thought to affect aglycone specificity in other β-glucosidase are marked by ○ [8,9]; those conserved residues making contacts with the sugar are marked with △ above the column. Residues lining the active site of the 1CBG structure are marked with a < or > beneath the column in the alignment for residues appearing to reside inside the two catalytic carboxylic acids and those appearing to be outside these residues respectively. The alignment was generated using the Clustal X implementation of Clustal W [27,28], analysed and manually adjusted by Genedoc (http://www.psc.edu/biomed/genedoc/), and shaded for residues conserved with BGlu1 using the BOXSHADE 3.21 program of K. Hoffman and M.D. Baron (http://www.ch.embnet.org/software/BOX_form.html).

However, the k_{cat} decreased when the DP increased from 2 to 3, suggesting that longer 1,3-linked oligosaccharides may not maintain this binding geometry. Rice BGlu1 was shown to have binding sites for six 1,4-linked glucose residues, as in barley βII β-glucosidase, although substantial differences were seen in the affinity of binding at different subsites. Modelling of the barley [10] and rice BGlu1 (results not shown) active sites indicated that each enzyme has room for six 1,4-linked glucose residues in the active-site cleft. Barley β-glucosidase has a relatively low K_m (2.67 mM) and a k_{cat} (11.58 s^{-1}) near its intrinsic k_{cat} (14.10 s^{-1}) for cellobiose, but cellotriose has a nearly 6-fold lower k_{cat} [10]. In contrast, the k_{cat} of BGlu1 increases 10 fold as the DP increases from 2 to 3. The difference corresponded to a high affinity of subsite $+2$ for glucose residue 3 in BGlu1, while a negative interaction was seen at this site in the barley enzyme. Conversely, the binding affinity at site $+1$ is much lower for BGlu1, consistent with its high K_m and low k_{cat} for cellobiose. Thus despite their 66% amino acid sequence identity and common hydrolysis of oligosaccharides, rice BGlu1 and barley β-glucosidases may have somewhat different active-site geometries.

The maize β-glucosidase residues Trp-378, Phe-198, Phe-205 and Phe-466 that line the active-site cleft interact directly with the substrate aglycone [6]. Therefore it was suggested that these residues are critical for determining substrate specificity. The last two of these residues are different in BGlu1 and barley βII, with Asn for BGlu1 and His for barley at the maize Phe-205 position, and Leu for BGlu1 and Arg for barley at the Phe-466 position (Figure 3). These and other differences in residues that line the active-site cleft might help explain their different substrate-binding properties.

Similar to maize β-glucosidase [16], but unlike barley β-glucosidase [14] and rice cell-wall-bound β-glucosidase [12], the sugar moiety had less influence on the K_m of the rice BGlu1 enzyme than the aglycone moiety. As seen in Figure 3, the amino acids identified by Czjzek et al. [6] as critical for glucose binding (Gln-38, His-142, Glu-191, Glu-406, Glu-464 and Trp-465 in maize Bglu1) are conserved in rice BGlu1, rice cell-wall-bound and barley β-glucosidases, so differences in their glycone specificities may reside elsewhere.

Rice β-glucosidase BGlu1 hydrolysed pyridoxine 5′-*O*-β-D-glucoside and could be used to synthesize this glucoside *in vitro* efficiently by transglucosylation. Though the physiological significance of the hydrolysis is unclear, the transglycosylation reaction implies that the enzyme may play a role in the glycosylation of pyridoxine seen when germinating rice, rice callus or mixed rice bran β-glucosidases were incubated with pyridoxine [23–25]. However, these crude systems produced both 5′-*O*-β-glucoside and 4′-*O*-β-glucoside, while transglucosylation with BGlu1 was highly specific for pyridoxine 5′-*O*-β-glucoside.

In summary, the recombinant rice BGlu1 β-glucosidase has highest protein sequence similarity to barley β-glucosidase and hydrolyses similar oligosaccharide substrates. However, some pronounced differences between the two enzymes were seen in hydrolysis of 1,3- and 1,4-β-oligoglucosides of different lengths, which reflect differences in the binding of these substrates to each enzyme. In addition to the hydrolysis of oligosaccharides, the enzyme also hydrolyses pyridoxine 5′-*O*-β-D-glucoside and transglycosylates the coenzyme pyridoxine to form this product. Since the protein can be efficiently produced in a recombinant system, rice BGlu1 is a good candidate for structural and mutational studies to investigate the determinants of oligosaccharide binding [26].

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