Properties of family 79 β-glucuronidases that hydrolyze β-glucuronosyl and 4-*O*-methyl-β-glucuronosyl residues of arabinogalactan-protein

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[‡]The nucleotide sequences reported in this paper have been submitted to the DNA Data Bank of Japan (DDBJ) with accession number An02g11890 for *AnGlcAase* and AB293989 for *NcGlcAase*.

[§]Experimental details concerning cDNA cloning, expression of β-glucuronidase in *Pichia pastoris*, and purification of recombinant enzymes are given in the Supplementary Data.

Abstract

The carbohydrate moieties of arabinogalactan proteins (AGPs), which are mainly composed of Gal, L-Ara, GlcA, and 4-Me-GlcA residues, are essential for the physiological functions of these proteoglycans in higher plants. For this study we have identified two genes encoding family 79 β-glucuronidases, designated *AnGlcAase* and *NcGlcAase*, in *Aspergillus niger* and *Neurospora crassa*, respectively, based on the amino acid sequence of a native β-glucuronidase purified from a commercial pectolytic enzyme preparation from *A. niger*. Although the deduced protein sequences of AnGlcAase and NcGlcAase were highly similar, the recombinant enzymes expressed in *Pichia pastoris* exhibited distinct substrate specificity toward 4-Me-GlcA residues of AGPs: recombinant AnGlcAase (rAnGlcAase) substantially liberated both GlcA and 4-Me-GlcA residues from radish AGPs, whereas recombinant NcGlcAase (rNcGlcAase) activity on the 4-Me-GlcA residues of AGPs was very low. Maximum activity of rAnGlcAase hydrolyzing PNP β-GlcA occurred at pH 3.0 to 4.0, whereas the maximum of rNcGlcAase activity was at pH 6.0. The apparent K_m values of rAnGlcAase were 30.4 μM for PNP β-GlcA and 422 μM for β-GlcA-(1→6)-Gal, and those of rNcGlcAase were 38.3 μM and 378 μM, respectively. Similar to the native enzyme, rAnGlcAase was able to catalyze the transglycosylation of GlcA residues from PNP β-GlcA to various monosaccharide acceptors such as Glc, Gal, and Xyl. We propose that both AnGlcAase and NcGlcAase are instances of a novel type of β-glucuronidase with the capacity to hydrolyze β-GlcA and 4-Me-β-GlcA residues of AGPs, although they differ significantly in their preferences.

Keywords

Arabinogalactan-protein, *Aspergillus niger*, Family 79 glycoside hydrolase, β-Glucuronidase, *Neurospora crassa*.

1. Introduction

Arabinogalactan-proteins (AGPs) are a family of proteoglycans involved in many physiological processes such as cell-to-cell signaling, cell adhesion, cell elongation, cell death, and stress responses in plants.[1-4] AGPs are characterized by large carbohydrate components rich in galactose and L-arabinose, and protein components (core proteins: generally less than 10% of total weight) rich in hydroxyproline, serine, threonine, alanine, and glycine. The carbohydrate moieties of AGPs have a common overall structure of β-(1→3)-linked galactosyl backbones to which side chains of β-(1→6)-linked galactosyl residues are attached through *O*-6. The β-(1→6)-linked galactosyl chains are further substituted with L-arabinose, glucuronic acid, and 4-*O*-methyl-glucuronic acid and lesser amounts of other sugars such as L-rhamnose and L-fucose.[1] In the case of radish root AGPs, uronic acids including β-glucuronic acid and 4-*O*-methyl-β-glucuronic acid constitute more than 4% of total weight of the carbohydrate moieties of AGPs (see Fig. 3 for a schematic rendering of radish AGP structure).[5]

It seems that *in planta,* the carbohydrate moieties of AGPs are structurally modified and degraded through the action of several endogenous glycosidases, such as β-galactosidase (EC 3.2.1.23)[6,7] and α-L-arabinofuranosidase (α-L-Arafase; EC 3.2.1.55).[8,9] Fungi and bacteria appear to have various glycosidases that hydrolyze the carbohydrate moieties of AGPs in

order to utilize the sugars as a carbon source or as nutrients. Examples include exo-β-(1→3)-galactanase (EC 3.2.1.145)[10,11] and endo-β-(1→6)-galactanase (EC 3.2.1.164).[12,13] We have previously purified a β-glucuronidase (EC 3.2.1.31) from a commercial pectolytic enzyme preparation from *Aspergillus niger*. This was the first enzyme shown to hydrolyze the β-glucuronosyl and 4-*O*-methyl-β-glucuronosyl residues substituted at *O*-6 of non-reducing terminals of β-(1→6)-linked galactosyl side chains, which are generally found in AGPs.[14,15]

Glycoside hydrolases are classified into more than 100 families based on amino acid sequence and structural similarity,[16,17] and most β-glucuronidases characterized so far have been classified as glycosyl hydrolase family 2, but their properties and biological functions have not yet been extensively investigated. A small number of β-glucuronidases are categorized as family 1 or 79 glycoside hydrolases. A family 79 β-glucuronidase isolated from skullcap (*Scutellaria baicalensis*) has been shown to hydrolyze baicalein 7-*O*-β-glucuronide (a glycosylated flavone).[18] However, it is unknown if these β-glucuronidases act on AGPs.

For the present study, novel genes encoding β-glucuronidases that may play a role in the metabolism of AGPs were identified in *A. niger* and *Neurospora crassa*. We have characterized the enzymatic properties and investigated the actions on the carbohydrate moieties of AGPs of the corresponding proteins via recombinant enzymes expressed in *Pichia pastoris*.

2. Experimental

Experimental details of cDNA cloning, expression of β-glucuronidase in *P. pastoris*, and purification of recombinant enzymes are given in the Supplementary Data.

2.1. Materials

Aspergillus niger van Tieghem (ATCC 22343) and *Neurospora crassa* OR74A were used for this study. Mycelia of *A. niger* were cultured in liquid medium containing 2% (w/v) malt extract, 2% (w/v) glucose, and 0.1% (w/v) peptone (pH 6.0, MGP medium) at 25 °C for 2 days, then the submerged culture was inoculated into the same medium containing 1.5% (w/v) agar at 25 °C for 5 days. *N. crassa* was cultured in a wheat bran medium containing 2 g of wheat bran and 3 mL of water at 25 °C for 7 days.

The β-(1→3)- and β-(1→6)-linked galactobioses used were prepared from larch wood arabinogalactan[19] and β-(1→4)-galactobiose was prepared from soybean arabinan-galactan.[6] Acacia gum and the sap of the lac tree, *Rhus vernicifera,* were the sources for preparation of following glucuronic acid (GlcA)- or 4-*O*-methyl-glucuronic acid (4-Me-GlcA)-containing acidic oligosaccharides: β-GlcA-(1→6)-Gal, β-GlcA-(1→6)-β-Gal-(1→6)-Gal, 4-Me-β-GlcA-(1→6)-Gal, 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal, and 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→3)-Gal.[14] The preparation of β-GlcA-(1→3)-Gal was made from a polysaccharide produced by cultivation of *Pseudomonas viscogena* TS-1004 as described.[14] Laminaribiose, cellobiose, and heparan sulfate from bovine kidney were purchased from Seikagaku Co. (Tokyo, Japan). Gentiobiose, *p*-nitrophenyl β-glucopyranoside (PNP β-Glc), PNP β-galactopyranoside (PNP β-Gal), PNP α-L-arabinofuranoside (PNP α-L-Ara), PNP β-xylopyranoside (PNP β-Xyl), PNP

β-glucopyranosiduronic acid (PNP β-GlcA), PNP β-galactopyranosiduronic acid (PNP β-GalA), PNP α-L-fucopyranoside (PNP α-L-Fuc), laminarin from *Laminaria digitata*, chitosan from crab shells, guar gum, locust bean gum, and xylan from birchwood were from Sigma (St. Louis, MO, USA). (1→3)(1→4)-β-Glucan from barley, CM-cellulose 4M, and β-(1→4)-galactan from lupin were purchased from Megazyme (Wicklow, Ireland). CM-curdlan was from Wako (Osaka, Japan). Pustulan from *Umbilicaria papullosa* was from Calbiochem (San Diego, CA, USA). AGPs from radish leaves (AGP R-II) and roots (AGP IV) and their α-L-Arafase-treated derivatives were prepared in our laboratories.[5,20]

2.2. Sugar and protein analyses

Total sugar content was determined by the phenol–sulfuric acid method.[21] Total uronic acids in AGPs were determined by a modified carbazole–sulfuric acid method[22] using GlcA as the standard. The concentration of protein was determined by the method of Bradford[23] with BSA as the standard.

Native β-glucuronidase was purified by conventional chromatographic techniques from Pectinex Ultra SP-L, a commercial pectolytic enzyme preparation from *A. niger* (Novo Nordisk Ferment Ltd, Chiba, Japan) as described previously.[14] The purified β-glucuronidase was separated on SDS-PAGE,[24] blotted onto a PVDF-Plus membrane (Osmonics, Moers, Germany) and subjected to N-terminal amino acid analysis with a protein sequencer (HP G1000A, Hewlett Packard, Palo Alto, CA, USA). The N-terminal amino acid sequence of recombinant *A. niger* β-glucuronidase (rAnGlcAase) was determined as well.

2.3. Enzyme assays

Hydrolytic activity was determined in mixtures (200 μL) consisting of the enzyme, 2 mM PNP β-GlcA, and 50 mM sodium acetate buffer, pH 4.0 for rAnGlcAase (32 ng enzyme) or pH 6.0 for recombinant *N. crassa* β-glucuronidase (rNcGlcAase; 39 ng). After incubation at 37 °C, the reaction was terminated by addition of 200 mM sodium carbonate (800 μL) and monitored at 420 nm. One unit of enzyme activity liberates 1 μmole of *p*-nitrophenol per min. Activity toward oligosaccharides was assayed in reaction mixtures (8 μL) containing enzyme (33 ng rAnGlcAase or 63 ng rNcGlcAase), 5 mM oligosaccharide, and 50 mM sodium acetate buffer, pH 4.0 (for rAnGlcAase) or 6.0 (for rNcGlcAase). After incubation at 37 °C for an appropriate reaction time, the liberated GlcA was assayed by the reductometrical method of Milner and Avigad,[25] while the amount of released 4-Me-GlcA was determined reductometrically by the neocuproine assay[26] using GlcA as the standard. One unit of enzyme activity liberates 1 μmole of GlcA or 4-Me-GlcA per min.

Substrate specificity of the recombinant enzymes toward polysaccharides was investigated using reaction mixtures (100 μL) consisting of the enzyme (64 ng rAnGlcAase or 79 ng rNcGlcAase), 0.5% (w/v) polysaccharide, and 50 mM sodium acetate buffer, pH 4.0 or 6.0. After incubation at 37 °C for a suitable reaction period, the amount of reducing sugars was determined by the method of Nelson[27] and Somogyi.[28] The hydrolytic activity toward AGPs was determined for reaction mixtures (20 μL) consisting of the enzyme (12~155 ng rAnGlcAase or 21~436 ng rNcGlcAase), 0.25% (w/v) AGP, and 50 mM sodium acetate buffer, pH 4.0 or 6.0. After reaction at 37 °C, the mixture was heated to 100 °C for 5 min to terminate the reaction, and then centrifuged at 18,000 × *g* for 10 min to remove insoluble

materials. The resulting supernatant was filtered through a membrane filter (0.45 μm; Chromatodisk, Krabou, Osaka, Japan) and was analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex DX-500 liquid chromatograph fitted with a CarboPac PA-1 column (4 × 250 mm; Dionex Japan, Osaka, Japan) and a pulsed amperometric detector as described previously.[29]

2.4. Transglycosylation reaction

Transglycosylation assays of rAnGlcAase used reaction mixtures (20 μL) consisting of the enzyme (67 ng rAnGlcAase), 20 mM PNP β-GlcA, 0.5 M acceptor monosaccharide [Glc, Gal, Xyl, GlcNAc, N-acetylgalactosamine (GalNAc), Man, L-Fuc, L-rhamnose (L-Rha), D-Ara, L-Ara, Rib, or sorbitol], and 7 mM sodium acetate buffer, pH 4.0. This mixture was incubated at 37 °C and the reaction was terminated by heating to 100 °C for 5 min. The amounts of *p*-nitrophenol and GlcA released by transglycosylation and hydrolysis reactions were quantitated colorimetrically as described above. Transglycosylation products in the reaction mixture were analyzed by paper chromatography on Whatman 3MM filter paper using 6:4:3 1-butanol-pyridine-water and 5:2:3 1-butanol-AcOH-water as the solvents. Sugar spots on the paper chromatograms were visualized with an alkaline $AgNO_3$ reagent.

2.5. Linkage analysis of transglycosylation product

In order to identify oligosaccharides formed through transglycosylation by rAnGlcAase, a large-scale reaction (500 μL) with 20 mM PNP β-GlcA and 0.5 M Gal as acceptor substrate as above was incubated at 37 °C for 3 h. The reaction was terminated by heating and the

reaction products (a main product and a minor one) were separated on paper chromatography using Whatman 3MM filter paper with 5:2:3 1-butanol-AcOH-water as the solvent, extracted from the paper, and lyophilized (the main product, yield 5.3 mg).

A part of the transglycosylation product was incubated with rAnGlcAase in 40 μL of a reaction mixture containing 3 mM product (based on the molecular mass obtained by the analysis described below), 7 mM sodium acetate buffer, pH 4.0, and 4 milliunits rAnGlcAase at 37 °C for 10 h. The reaction products were analyzed on HPAEC-PAD.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) of the transglycosylation product was performed with a KOMPACT MALDI IV tDE (Shimadzu, Kyoto, Japan) as described previously.[14] For the mass calibration, cyclomaltoheptaose was used. Structural elucidation of the transglycosylation product was performed by a combination of ^{1}H and ^{13}C NMR spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, with a Bruker DPX-400 spectrometer. The sample was dissolved in D_2O, and MeOD was used as the internal standard (3.3 ppm for ^{1}H and 49.5 ppm for ^{13}C). Ring-carbon assignments in NMR were made by a combination of 1D and 2D NMR experiments. Bruker standard pulse sequences were used for DEPT135, 1D-total correlation spectroscopy, and 2D homonuclear shift correlation spectroscopy experiments.

3. Results and discussion

3.1. Amino acid sequences of β-glucuronidases

The native β-glucuronidase purified from Pectinex Ultra SP-L was partially digested with V8 proteinase and yielded the internal peptide sequence SAGLPVLHPF VSFSIEFVFF PDYAG upon analysis with a protein sequencer. This peptide sequence showed significant similarity to an open reading frame (ORF, An02g11890) of *A. niger* which had not been characterized so far. Based on the internal peptide sequence, we cloned cDNA for An02g11890 by reverse transcriptase-PCR with specific primers, obtaining *AnGlcAase* (*A. niger* β-glucuronidase). The amino acid sequence deduced from *AnGlcAase* did not completely match the peptide sequence determined for the native β-glucuronidase in that four residues differed (the 1st, 3rd, 18th, and 23rd residue among 25 determined for the native enzyme; see Fig. 1A). Because we could find no other highly similar sequence in the genomic database of *A. niger*, we believe that the mismatch in the peptide sequences arises because the strain used for preparation of the purified enzyme and the strain used for the cDNA cloning were different. However, we cannot rule out the possibility that the ORF encodes a closely related enzyme distinct from the native enzyme. Blast search revealed that an ORF (EAA35527) from *N. crassa* was 47% identical to the amino acid sequence deduced from An02g11890, and we thus refer to this ortholog of An02g11890 as *NcGlcAase* (*N. crassa* β-glucuronidase).

AnGlcAase and *NcGlcAase* appeared to encode polypeptides of 541 and 559 amino acids (molecular mass 58,859 and 60,235 Da), respectively (Fig. 1A). Using the SignalP program,[30] putative signal sequences were found in both AnGlcAase and NcGlcAase, indicating that these enzymes are secreted proteins. The calculated molecular masses of the mature proteins of AnGlcAase and NcGlcAase were 56,682 and 58,123 Da. The calculated isoelectric point for mature AnGlcAase was 5.10, while that of NcGlcAase was 6.83. The

cDNA deduced sequences of both proteins have 12 putative N-glycosylation sites; eight of these are conserved between AnGlcAase and NcGlcAase. Indeed, treatment of the native enzyme of *A. niger* with the endo-glycanase Endo-F (Seikagaku Kogyo Co., Tokyo, Japan) reduced the molecular mass of the native enzyme on SDS-PAGE from 68 to 55 kDa (data not shown), confirming that the native enzyme is highly glycosylated.

The amino acid sequences deduced from *AnGlcAase* and *NcGlcAase* were not similar to any known β-glucuronidases that belong to the family 1 or 2 glycoside hydrolases. They did exhibit low but significant similarity to several family 79 enzymes such as β-glucuronidase from skullcap (*Scutellaria baicalensis*, identity, 13%),[18] heparanase from human (*Homo sapiens*, 11–16%),[31] heparanase from mole rat (*Spalax golani*, 10–15%),[32] and heparanase from Norway rat (*Rattus norvegicus*, 14–16%).[33] The β-glucuronidase from skullcap has been shown to possess an acid-base catalysis (Glu residue) and a catalytic nucleophile (Glu residue) in the active site.[18] Although the overall similarity of AnGlcAase and NcGlcAase to family 79 enzymes was low, the catalytic regions of family 79 enzymes appeared to be conserved in AnGlcAase (Glu^{189} and Glu^{324} residues) and NcGlcAase (Glu^{175} and Glu^{312} residues) (Fig. 1A), suggesting that AnGlcAase and NcGlcAase are family 79 glycoside hydrolases. Although the skullcap β-glucuronidase possesses a putative ATP/GTP binding motif and a leucine-zipper motif,[18] neither was found in AnGlcAase or NcGlcAase. Phylogenetic analyses of AnGlcAase and NcGlcAase and other family 79 members indicated that AnGlcAase and NcGlcAase belong to a subfamily of fungal glycosidases. This group is apparently set apart from plant β-glucuronidases, vertebrate heparanases, and bacterial glycosidases (Fig. 1B). However, the functions of the fungal glycosidases, other than

AnGlcAase and NcGlcAase, have not yet been examined. We suspect that other fungal glycosidases will show a pattern of substrate specificity similar to that of AnGlcAase and NcGlcAase described below.

3.2. Properties of rAnGlcAase and rNcGlcAase

The ORFs of *AnGlcAase* and *NcGlcAase*, except for the signal peptides, were fused to a yeast secretion signal sequence (α-factor), and introduced into the methylotrophic yeast *P. pastoris*. Recombinant β-glucuronidases (rAnGlcAase and rNcGlcAase) were induced under the control of the alcohol oxidase promoter and purified from the culture medium by conventional chromatographies (Table S1). The molecular masses of rAnGlcAase (85.7 kDa) and rNcGlcAase (90.1 kDa) determined on SDS-PAGE were larger than expected from the protein sequences (56.7 kDa for AnGlcAase, 58.1 kDa for NcGlcAase). The purified rAnGlcAase specimen was accompanied by a trace amount of impurity with a slightly lower molecular mass, which could not be removed (Fig. S1). It is well known that molecular masses of recombinant proteins expressed in *P. pastoris* with α-factor occasionally increase due to incomplete processing of the α-factor presequence at N-terminals of the expressed proteins.[34] We therefore sequenced the N-terminal amino acid residues of rAnGlcAase with a protein sequencer and found SMNSHPTA. This sequence corresponds to the predicted N-terminal sequence (Fig. 1A) preceded by a 4 residue long extension (SMNS), which derives from the *Cla*I and *Eco*RI sites between the α-factor and the predicted mature cDNA insert (see Methods in the Supplementary Data). This observation indicates that post-translational modification of rAnGlcAase cleaving the α-factor has occurred. This is

likely also the case for rNcGlcAase. We also treated rAnGlcAase and rNcGlcAase with endo-glycosidase Endo-H (Fig. S1) to find that their respective molecular masses were reduced to 60 kDa and 62 kDa by the enzymatic digestion. This indicates that the higher than expected molecular masses of the recombinant enzymes expressed in *P. pastoris* was most likely caused by large high-mannose type glycans attached to the secreted proteins in the yeast.[35]

Activities of rAnGlcAase and rNcGlcAase were examined with PNP β-GlcA as the substrate. While the specific activity (30.6 unit/mg protein) of rAnGlcAase was comparable to that (29.3 unit/mg protein) of the native β-glucuronidase of *A. niger*,[14] the activity (23.4 unit/mg protein) of rNcGlcAase was slightly lower. The maximum activity of rAnGlcAase occurred at pH 3.0 to 4.0, but that of rNcGlcAase at 6.0 when examined in reaction mixtures with the following buffers at final concentrations of 50 mM: glycine-HCl (pH 1.0-3.5); sodium acetate (pH 3.5-6.0); potassium phosphate (pH 6.0-8.0); Tris-HCl (pH 7.0-9.0) (data not shown). We found that rAnGlcAase was stable within the pH range of 1-7 when exposed to various pHs at 4 °C for 24 h, whereas rNcGlcAase was stable between pH 6 and 9, but its activity gradually decreased below pH 6. The optimum reaction temperature was 45 °C for both recombinant enzymes. The thermal stability of both enzymes was also almost identical; both lost 50% of activity when heated to 50 °C for 30 min. Repeated freezing and thawing did not impair rAnGlcAase, whereas rNcGlcAase proved rather unstable and was thus kept at 4 °C after purification.

3.3. Substrate specificity of recombinant enzymes

Substrate specificity of rAnGlcAase and rNcGlcAase toward sugar groups was determined using various PNP glycosides, and oligo- and polysaccharides (Table 1). Both recombinant enzymes specifically acted on PNP β-GlcA and β-GalA; the other PNP glycosides tested did not serve as substrates at all. We examined the hydrolytic activities of rAnGlcAase and rNcGlcAase toward β-GlcA-(1→6)-Gal, β-GlcA-(1→6)-β-Gal-(1→6)-Gal, 4-Me-β-GlcA-(1→6)-Gal, 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal, and 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→3)-Gal, as these occur in acidic side chains of AGPs. Both recombinant enzymes substantially acted on β-GlcA residues substituted at non-reducing terminals of β-galactooligosaccharides, whereas β-GlcA-(1→3)-Gal, a regioisomer of β-GlcA-(1→6)-Gal, was not susceptible to the enzymes. This agrees with observations for the native enzyme of *A. niger*.[14] The recombinant enzymes did, however, exhibit a significant difference in hydrolytic activity toward 4-Me-β-GlcA residues of the oligosaccharides: 4-Me-β-GlcA residues served as a good substrate for rAnGlcAase, but not for rNcGlcAase. Several neutral oligosaccharides tested were not hydrolyzed by the enzymes.

Although AnGlcAase and NcGlcAase are somewhat similar to family 79 endo-β-glucuronidases/heparanases, the enzymes hydrolyzed heparan sulfate only very weakly (Table 1). A likely explanation is that the recombinant enzymes act on β-(1→4)-linked GlcA residues only if they are located at the non-reducing terminals of heparan sulfate. Xylan from birchwood, which contains small amounts of GlcA and 4-Me-GlcA residues attached through α-linkages, and the other polysaccharides tested, did not serve as substrates for rAnGlcAase and rNcGlcAase at all.

3.4. Kinetics of the recombinant enzymes

The effect of substrate concentration on the activity of the recombinant enzymes was examined using PNP β-GlcA, PNP β-GalA, and GlcA- or 4-Me-GlcA-containing acidic oligosaccharides. The resulting K_m, k_{cat}, and catalytic efficiency (k_{cat}/K_m) values are summarized in Table 2. The K_m values of rAnGlcAase (30.4 μM) and rNcGlcAase (38.3 μM) for PNP β-GlcA were almost the same. These values are apparently smaller than that of the native enzyme (230 μM),[14] whereas the values of rAnGlcAase (422 μM) and of rNcGlcAase (378 μM) for β-GlcA-(1→6)-Gal were comparable to that (710 μM) of the native enzyme.[14] Consistent with the weak activity of rAnGlcAase and rNcGlcAase toward PNP β-GalA, the K_m values of the enzymes for PNP β-GalA (9.80 mM and 3.21 mM, respectively) were much higher than those for PNP β-GlcA, giving quite low catalytic efficiency values. Both enzymes exhibited higher affinity for β-GlcA-(1→6)-β-Gal-(1→6)-Gal than for β-GlcA-(1→6)-Gal, consistent with the substrate specificity summarized in Table 1. The K_m values of rAnGlcAase were higher for 4-Me-GlcA-containing oligosaccharides than for GlcA-containing substrates. Consequently, the catalytic efficiency values of rAnGlcAase for 4-Me-GlcA-containing oligosaccharides were much lower than those for GlcA-containing substrates. Among the 4-Me-GlcA-containing oligosaccharides, rAnGlcAase showed the highest affinity toward 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal.

3.5. Action of recombinant enzymes toward arabinogalactan-proteins

The mode of action of rAnGlcAase and rNcGlcAase on AGPs from radish leaves and roots,

and their modified AGPs treated with α-L-Arafase[5,20] was analyzed using HPAEC-PAD (Fig. 2). Initial velocities of recombinant β-glucuronidases releasing 4-Me-GlcA and GlcA residues from radish AGPs were determined (Table 3). As implied by the kinetic values of rAnGlcAase and rNcGlcAase for 4-Me-GlcA- or GlcA-containing oligosaccharides (Tables 1 and 2), rAnGlcAase released both uronosyl groups from native and α-L-Arafase-treated AGPs, while rNcGlcAase acted largely only on GlcA groups. The initial velocity of rAnGlcAase toward both uronic acids of the leaf and root AGPs markedly increased after α-L-Arafase treatment, while that of NcGlcAase did not increase as much following treatment. After exhaustive digestion, rAnGlcAase split 20% and 35% of total uronic acids from α-L-Arafase-treated radish leaf and root AGPs, respectively, whereas rNcGlcAase split only 2.3% and 3.0% from α-L-Arafase-treated leaf and root AGPs, respectively (Fig. 2, Table 3). Recombinant NcGlcAase liberated only small amounts of 4-Me-GlcA residues, while the amounts of GlcA released from the AGPs by rNcGlcAase were comparable to those released by rAnGlcAase. These results indicate the higher capability of rAnGlcAase to release uronosyl residues attached to the carbohydrate moieties of AGPs.

Considering the structure of the sugar moieties of radish AGPs (Fig. 3), it seems highly probable that removal of L-Ara residues attached to longer β-(1→6) galactosyl side chains of AGPs by digestion with α-L-Arafase renders the uronosyl residues more accessible to rAnGlcAase, resulting in effective release of the uronosyl residues. Apparently, the β-glucuronidases could not liberate all uronosyl residues attached to AGPs, even after α-L-Arafase treatment. According to our previous results,[15] the uronosyl residues located at short side chains such as (4-Me-)β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→ and

(4-Me-)β-GlcA-(1→6)-β-Gal-(1→ sequences, and at inner regions of AGPs close to the core peptides may be resistant to the β-glucuronidases due to steric hindrance. Since the peptide sequences of AnGlcAase and NcGlcAase are highly similar (Fig. 1A), we suggest that the distinct substrate specificity toward 4-Me-β-GlcA residues is the result of a difference in a few amino acid residues involved in substrate recognition.

3.6. Transglycosylation activity of rAnGlcAase

The transglycosylation activity of rAnGlcAase was examined in a reaction mixture containing 20 mM PNP β-GlcA as donor and 0.5 M Gal as acceptor. Accumulation of *p*-nitrophenol, GlcA, and transglycosylation products in the reaction mixture were monitored up to 25 h (Fig. 4A). Most of the PNP β-GlcA in the reaction mixture was consumed, and the accumulation of transglycosylation products reached a maximum at 3 h. The transglycosylation products gradually decreased during prolonged incubation, possibly due to hydrolysis of the products by the enzyme. The transglycosylation products were analyzed by paper chromatography (Fig. 4B), which showed the formation of at least two products, a main product (indicated by a *white arrow*) and a minor product (*arrowhead*). The migration of the main product corresponded to β-GlcA-(1→6)-Gal (*S1*) and its structure was confirmed (see below), while that of the minor product corresponded to β-GlcA-(1→3)-Gal (*S2*). There is also the possibility that the minor product was formed by the successive transglycosylation of two GlcA residues onto the acceptor Gal. The kinetic parameters of rAnGlcAase for Gal as acceptor substrate were determined with a reaction mixture consisting of 20 mM PNP β-GlcA and various concentrations of Gal (Fig. 4C). Apparent K_m and k_{cat} values for acceptor Gal

were 391 mM and 51.8 s^{-1}, respectively. The high K_m value of rAnGlcAase for the acceptor Gal indicates that the transglycosylation rarely occurs under natural conditions.

Transglycosylation reactions of rAnGlcAase were performed in reaction mixtures containing various acceptor compounds. After incubation for 3 h, considerable amounts of transglycosylation products, possibly formed by transfer of GlcA residues from PNP β-GlcA onto the monosaccharides, were observed by paper chromatography (data not shown). The formation of transglycosylation products was also confirmed colorimetrically by measuring the reduced content of free GlcA compared with that of the control assay (Fig. 4D). Glc, Gal, Xyl, GlcNAc, and GalNAc were good acceptors, better than Man, L-Fuc, L-Rha, D-Ara, L-Ara, Rib, and sorbitol. It seems that rAnGlcAase prefers saccharides with stereochemistry similar to Gal at C-5 and C-3 positions as acceptors. The transfer of a considerable amount of GlcA to Xyl also suggests that rAnGlcAase can recognize OH groups in positions other than C-6.

In order to identify the transglycosylation products, we performed a large-scale reaction. MALDI-TOFMS of the main transglycosylation product from Gal as acceptor gave a signal at *m/z* 379.4, which matches the mass value (379.2) of the disaccharide GlcA·Gal as a sodium adduct. Incubation of the transglycosylation product with rAnGlcAase resulted in complete digestion of the product into GlcA and Gal, indicating that GlcA residues of the product are linked to the acceptor Gal via β-linkages (Fig. S2A). Further structural analysis of the transglycosylation product by ^{13}C NMR spectroscopy (Fig. S2B) confirmed that it was indeed β-GlcA-(1→6)-Gal.

Glycoside hydrolases are classified as either retaining or inverting enzymes. The

transglycosylation observed for rAnGlcAase implies that the enzyme is a retaining β-glycosidase catalyzed by a double-displacement mechanism.[36] It is well known that various β-glucuronidases prepared from liver, snail, and *Escherichia coli* catalyze transglycosylation reactions in the presence of various glucuronides as donors and various acceptors.[37] A rat liver β-glucuronidase catalyzes the transfer of GlcA residues from phenyl β-GlcA to various acceptor sugars through β-(1→3) linkages.[38] Similarly, a bovine liver β-glucuronidase produces β-GlcA-(1→3)-β-GlcA-PNP and its (1→2) regioisomer, when incubated with PNP β-GlcA alone.[39] Since rAnGlcAase produces β-GlcA-(1→6)-Gal as a major transglycosylation product when incubated with PNP β-GlcA and Gal, we note that fungal β-glucuronidases seem to markedly differ from the β-glucuronidases of other organisms in the specificity of their transglycosylation reactions.

3.7. Conclusion

We have identified two representatives of a novel type of β-glucuronidase in *A. niger* and *N. crassa,* categorized them as family 79 glycoside hydrolases, and established the actions of the recombinant enzymes on β-GlcA and 4-Me-β-GlcA residues of AGPs. The physiological function of AnGlcAase and NcGlcAase is likely the specific hydrolysis of uronosyl residues of AGPs, leading to degradation of the sugar moieties of AGPs under the concerted action of glycosidases and glycanases secreted by the fungi.

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Table 1. Substrate specificity of recombinant β-glucuronidases toward PNP glycosides, oligosaccharides, and polysaccharides

Substrate[a]	Relative activity[b]	
	rAnGlcAase	rNcGlcAase
PNP glycosides		
PNP β-GlcA	100.0 ± 0.0	100.0 ± 0.0
PNP β-GalA	4.7 ± 0.1	3.0 ± 0.3
Acidic oligosaccharides		
β-GlcA-(1→6)-Gal	85.7 ± 11.5	49.9 ± 1.5
β-GlcA-(1→6)-β-Gal-(1→6)-Gal	94.6 ± 11.8	67.4 ± 2.2
4-Me-β-GlcA-(1→6)-Gal	21.4 ± 1.5	3.3 ± 0.5
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal	42.6 ± 0.7	4.8 ± 0.8
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→3)-Gal	35.5 ± 3.0	3.2 ± 0.3
β-GlcA-(1→3)-Gal	7.7 ± 0.1	5.4 ± 0.4
Polysaccharide		
Heparan sulfate	0.25 ± 0.1	0.43 ± 0.1

[a] The enzyme was incubated with substrates at a concentration of 2 mM (PNP glycosides) or 5 mM (oligosaccharide substrates). For polymer substrates a concentration of 0.5% (w/v) was used. Enzyme activity was determined under the optimal conditions for each enzyme.

[b] Activity is expressed in % of that toward PNP β-GlcA (rAnGlcAase, 30.6 unit/mg protein; rNcGlcAase, 23.4 unit/mg protein). The values are averages of three assays performed separately. The following sugars did not serve as substrates at all: PNP glycosides including β-Glc, β-Gal, α-L-Ara, β-Xyl, and α-L-Fuc groups; oligosaccharides including β-(1→3)-, β-(1→4)-, and β-(1→6)-galactobioses, laminaribiose, cellobiose, and gentiobiose; polysaccharides including chitosan from crab shells, CM-cellulose, CM-curdlan, galactomannans from guar and locust bean, β-(1→4)-galactan from lupin, β-(1→3)(1→4)-glucan from barley, laminarin, pustulan, and xylan from birchwood.

Table 2. Kinetics of recombinant β-glucuronidases

Substrate[a]	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
rAnGlcAase			
PNP β-GlcA	30.4	26.9	8.86×10^5
PNP β-GalA	9800	6.70	6.83×10^2
β-GlcA-(1→6)-Gal	422	20.1	4.78×10^4
β-GlcA-(1→6)-β-Gal-(1→6)-Gal	187	32.4	1.73×10^5
4-Me-β-GlcA-(1→6)-Gal	1730	5.35	3.09×10^3
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal	1410	12.9	9.19×10^3
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→3)-Gal	3790	17.9	4.74×10^3
rNcGlcAase			
PNP β-GlcA	38.3	13.5	3.54×10^5
PNP β-GalA	3210	1.14	3.56×10^2
β-GlcA-(1→6)-Gal	378	5.27	1.40×10^4
β-GlcA-(1→6)-β-Gal-(1→6)-Gal	129	6.34	4.90×10^4

[a] Reactions were carried out at various concentrations of substrates (0.025–2.0 mM of PNP β-GlcA, 0.1–25 mM of PNP β-GalA, and 0.06–10 mM of acidic oligosaccharides) under the optimal conditions for each recombinant enzyme. The assays were done three times independently. The K_m and k_{cat} values were calculated by the least squares method from the Hanes–Woolf plot using the obtained activities. Because of the low activity, no kinetic analysis of rNcGlcAase toward 4-Me-GlcA-containing oligosaccharides was performed.

Table 3. Action of recombinant β-glucuronidases on radish AGPs

AGP[a]	Initial velocity[b]		Total uronic acid released from 1 mg AGP[c]		% of hydrolysis[d]
	4-Me-GlcA	GlcA	4-Me-GlcA	GlcA	
	(μmol/min/mg protein)	(μmol/min/mg protein)	(μg)	(μg)	
rAnGlcAase					
Leaf (+)	1.526 ± 0.215	0.287 ± 0.040	18.30 ± 0.26	2.07 ± 0.04	20.2
Leaf (–)	0.010 ± 0.001	0.004 ± 0.000	0.82 ± 0.01	0.31 ± 0.11	1.8
Root (+)	7.455 ± 1.299	0.282 ± 0.053	51.68 ± 0.87	1.80 ± 0.04	35.0
Root (–)	0.669 ± 0.028	0.019 ± 0.003	12.82 ± 0.06	0.77 ± 0.00	10.5
rNcGlcAase					
Leaf (+)	0	0.015 ± 0.002	0.72 ± 0.02	1.63 ± 0.04	2.3
Leaf (–)	0	0.004 ± 0.000	0	0.28 ± 0.06	0.5
Root (+)	0	0.014 ± 0.002	3.25 ± 0.03	1.36 ± 0.03	3.0
Root (–)	0	0.012 ± 0.002	0.23 ± 0.01	0.46 ± 0.01	0.5

[a] Radish leaf and root AGPs treated with α-L-Arafase (+) or untreated (–) were used as substrates.

[b] The enzyme was incubated with AGPs at a concentration of 0.25% (w/v, total 1 mg) and the initial velocity of 4-Me-GlcA and GlcA release was determined. The data are means ± SE of three independent experiments.

[c] The amounts of 4-Me-GlcA and GlcA residues released after exhaustive (24 h) digestion with the recombinant enzymes (0.5 milliunits of each recombinant enzyme) are shown. The data are means ± SE of three independent experiments.

[d] Total uronic acid contents of AGPs are as follows: Leaf (+), 10.1% (w/w; based on total sugar content); Leaf (–), 6.2%; Root (+), 15.3%; Root (–), 13.0%. Values indicate the ratio of released uronic acids to total uronic acids.

Figure legends

Figure 1. Amino acid sequence alignment and phylogenetic analysis of AnGlcAase and NcGlcAase. (A) The amino acid sequences of AnGlcAase and NcGlcAase were aligned using ClustalW. The amino acid residues are numbered from the first methionine. Gaps (–) were introduced to achieve optimal alignment. Identical amino acid residues among these sequences are highlighted in black. The solid line indicates the amino acid sequence corresponding to that determined for the native enzyme purified from Pectinex Ultra SP-L. The *arrowheads* indicate the putative cleavage sites of the signal peptide, and *asterisks* indicate potential N-glycosylation sites. Based on a sequence alignment of AnGlcAase and NcGlcAase with skullcap β-glucuronidase,[18] the potential catalytic acid/base and nucleophile residues conserved among family 79 glycoside hydrolases were identified as indicated by *closed circles*. (B) Phylogenetic relationships of AnGlcAase, NcGlcAase, and other family 79 enzymes were analyzed by using ClustalW. The phylogram is constructed based on the CAZy database (http://www.cazy.oyg). The bar indicates substitutions per site.

Figure 2. Action of recombinant β-glucuronidases on radish AGPs. The enzymes were incubated at 37 °C for 24 h with 0.25% (w/v) α-L-Arafase-treated AGPs prepared from radish roots (A) or leaves (B). Control experiments were done without enzymes. The amount of uronic acids released from the AGPs was determined with HPAEC-PAD. The *arrowheads* indicate the peaks corresponding to 4-Me-GlcA, and the *arrows* indicate the GlcA peaks. The amounts of 4-Me-GlcA and GlcA released from the AGPs are summarized in Table 3.

Figure 3. A schematic model of a partial structure of radish AGPs. Radish leaf and root

AGPs contain L-Fuc, L-Ara, Gal, 4-Me-GlcA, and GlcA in a molar proportion of 5:33:57:4:1, and L-Ara, Gal, 4-Me-GlcA (24:62:14), respectively. This shows that root AGP has a rather simpler architecture, lacking L-Fuc and containing only 4-Me-GlcA as the sole uronic acid constituent.[5,20] Like other AGPs, the carbohydrate moieties of radish AGPs have β-(1→3)-linked galactosyl backbone chains to which β-(1→6)-linked galactosyl side chains are attached through *O*-6. The length of the main chains of radish AGPs is not precisely known, while galactosyl side chains are known to vary in length from single to (at least) 20 consecutive groups.[10] The side chains of radish AGPs are occasionally substituted with uronic acids at their non-reducing terminals, and with single residues and/or short chains of L-Ara residues along the side chains. A distinctive feature of leaf AGP is the presence of L-Fuc residues attached to L-Ara residues through α-(1→2) linkages. Treatment of radish AGPs with α-L-Arafase exposes the uronosyl residues by removing almost all L-Ara residues attached to Gal residues. This renders them more vulnerable to rAnGlcAase and rNcGlcAase.[14,15] The α-L-Fuc-(1→2)-α-L-Ara-(1→ sequences of leaf AGP, however, resist α-L-Arafase digestion.

Figure 4. Transglycosylation reaction of rAnGlcAase. (A) The transglycosylation reaction was examined using 20 mM PNP β-GlcA and 0.5 M Gal for various incubation times. The amount of liberated *p*-nitrophenol (*closed circles*) and GlcA (*open triangles*) were determined. The amount of the transglycosylation product (*open circles*) was calculated by subtraction of the amount of free GlcA from that of free *p*-nitrophenol. The initial amount of PNP β-GlcA (0.4 μmol) is indicated as 100%. The data shown are averages with SE of triplicate assays.

(B) The transglycosylation products catalyzed by rAnGlcAase were separated on paper chromatography. Standard GlcA, PNP β-GlcA, Gal, β-GlcA-(1→6)-Gal (*S1*), β-GlcA-(1→3)-Gal (*S2*), and reaction products at various incubation times (from 0 to 25 h) are shown. The *white broken line* represents the origin of chromatogram. The w*hite arrow* and *arrowhead* indicate the main and minor transglycosylation products, respectively. The *asterisk* indicates the migration position of GlcA. (C) Kinetic parameters of the transglycosylation reaction were determined for rAnGlcAase. Reaction mixtures containing various concentrations of Gal as acceptor and 20 mM of PNP β-GlcA as donor were incubated at 37 °C for 20 min. The inset is the Hanes–Woolf plot of the mean ± SE of three independent experiments, based on which the kinetic parameters were calculated by the least squares method. (D) The specificity of rAnGlcAase transferring GlcA residues onto monosaccharides was examined. Reactions were performed with 20 mM PNP β-GlcA and 0.5 M of each monosaccharide at 37 °C for 3 h. *Open bar* and *hatched bar* indicate the amount of *p*-nitrophenol and GlcA, respectively. The *closed bar* represents the difference of the amount between *p*-nitrophenol and GlcA, which presumably corresponds to the amount of transglycosylation products. The initial amount of PNP β-GlcA (0.4 μmol) is indicated as 100%. Data are averages ± SE of triplicate assays.

A

```
AnGlcAase   MHHHPITLLSLLLGAAQSIAAHPTAHDERRSSSSIGVSASVPDDAALPVLHPFVSFSIEF  60
NcGlcAase   --MKRILGLIAYASVPTVINAVQISVDETAPSN----------PVFDAYVSYSIEF  44

AnGlcAase   AFFPDFAGNLSHPNLFSNQLLDNLADLQGAKPYIRVGGNTQDFALYDPKLRAATNATYIT  120
NcGlcAase   SSFPDYAGNNSRPNTFSENLLDNLGKITGTKPYIRVGGNTQDYALYNASLPYSLNGTIDP  104

AnGlcAase   SISTDYPLILSFGPAFFESYFTWPGTKFIHGFNLGK--NSSSDIELMLESVPLACKALEG  178
NcGlcAase   KRSSDYPTTIFIGPSFFESYNSFKNTRFIHGFNLGLGGNRTSGWQTLLDTVPLACKALGG  164

AnGlcAase   GKLAYWELGNEPDLYKTSAQGIRRPASWTEQDYVDEWLNKTARIEKRLVEACPELAES--  236
NcGlcAase   GKLFAWTYGNEPDLFSTSAQGPVRPPSWNEAEYVDQWLNGTRKIHELLERNCPDLAKNGT  224

AnGlcAase   -KYIAPSFAGVTNSLNPVVTWEKGLDKSRNIALNSEHNYIGGATQPGVTLQNTLMNHTKT  295
NcGlcAase   YGYIAPSFAGVGNKLKAPKAWGEGLNEDKNIKLFATHNYISGATSPGVTLQGTLMNHSMT  284

AnGlcAase   VESVAQQVNVSRILSKDNLTPGIPYILGETNSLYNEGAPGLSNSFGAALWGVDFNLYCAS  355
NcGlcAase   KASVDAHI-VEYNQVKAIDAAAPPLIFGETNSLYNQGRPGLSNTFGAALWGVDFNLYSAS  343

AnGlcAase   QNIRRTHMHQGSNYNYISWQPVGTNRTTIGTKAPYYGNAMVAAMLHGGDDVRIVN-----  410
NcGlcAase   VGFKRVHMHMGTNYRYASWQPIATNKATIGTKAPYYGNIAVASFLAPPPSSPYDSPATSL  403

AnGlcAase   ------LPLAADTEAAYAAYVNETLVRVAVINLVEFNYTSTDSTAEKVES-----RPSAK  459
NcGlcAase   ATVKHLPISSTPFLSAYAAYHSSNLTRLILINLQSYNTTASGEGLAPLPPSSLTPRPSVT  463

AnGlcAase   YTFQLPSSESVYAG----SVSVQRLMANGSNAITGITWDGWSYNYELAQGKPVRLQNVT-  514
NcGlcAase   FNFTLPAAYLLTDGGKEKQVVVKRLMANGSDAITGITWDGWSYNWELDGGRPVRLPNVTR  523

AnGlcAase   --------TGEAISVGDDGVVEIEIPYSSAAILSL-                          541
NcGlcAase   TSESERAWVGEGTSDGGKAGLGVVVEAGSAALVEFV                          559
```

B

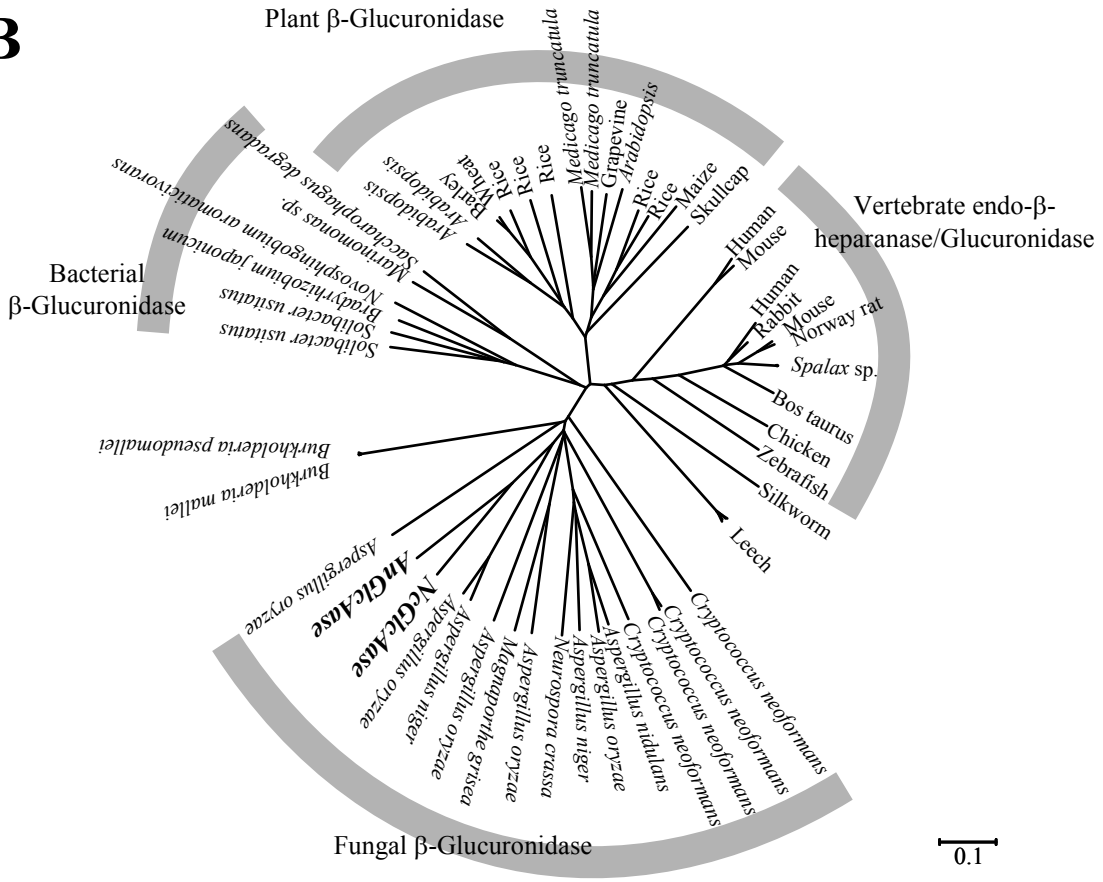
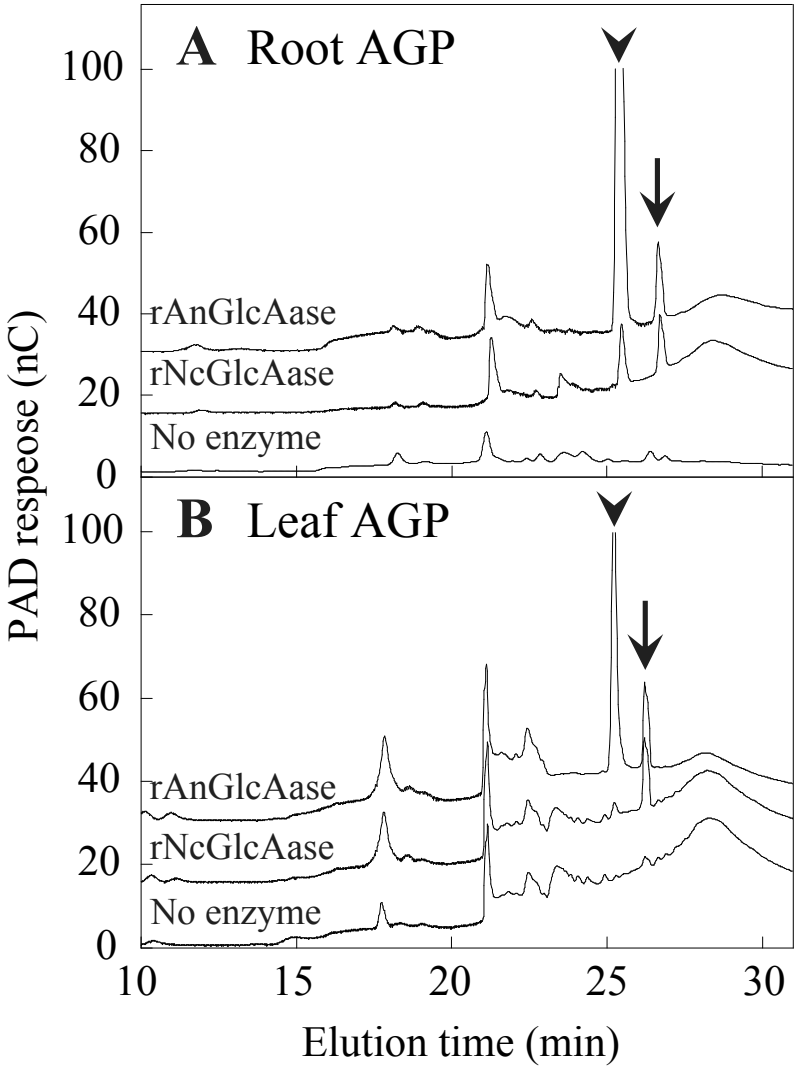


Fig. 1. Konishi et al.



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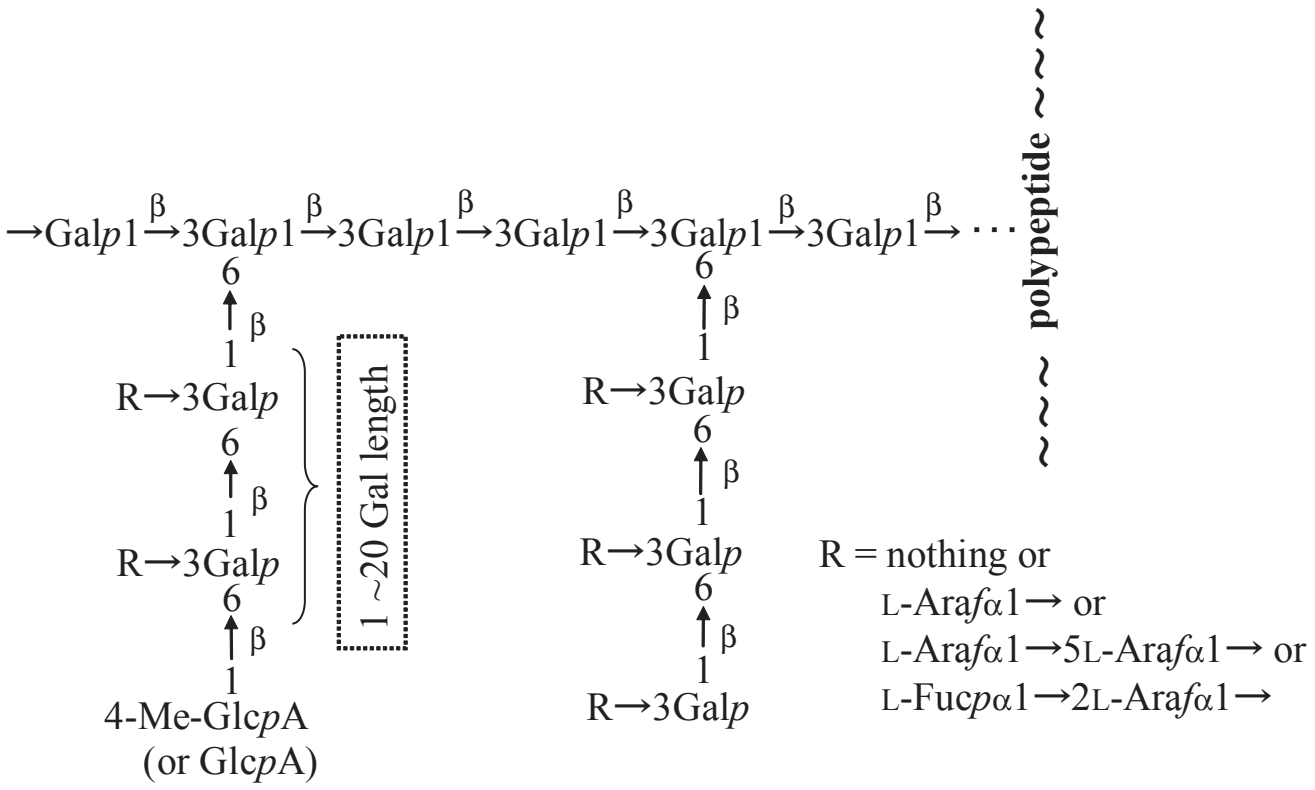

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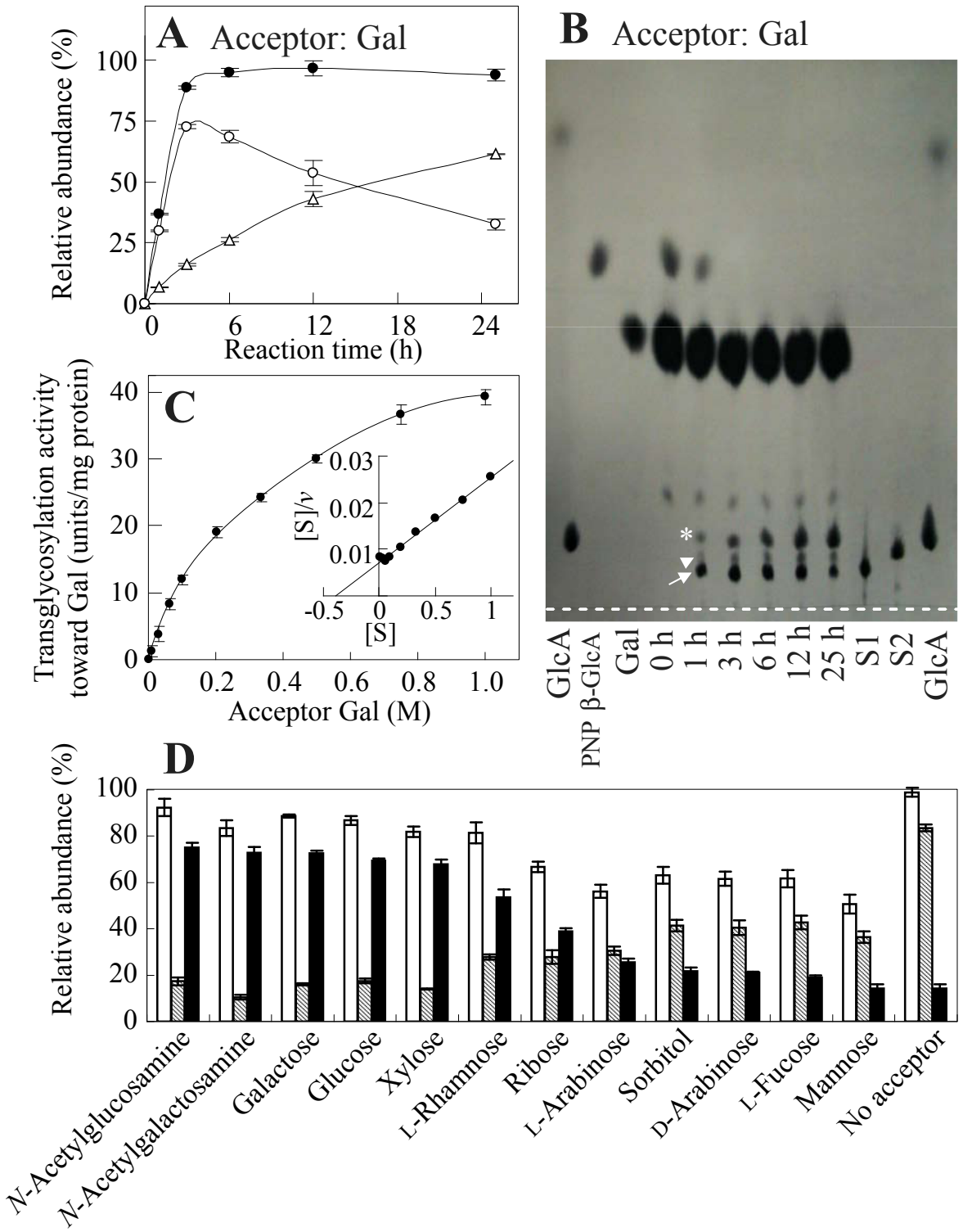

Fig. 4. Konishi et al.

Supplementary Data **for publication online**

Properties of family 79 β-glucuronidases that hydrolyze β-glucuronosyl and 4-*O*-methyl-β-glucuronosyl residues of arabinogalactan-protein

Tomoyuki Konishi, Toshihisa Kotake, Dina Soraya, Koji Matsuoka, Tetsuo Koyama, Satoshi Kaneko, Kiyohiko Igarashi, Masahiro Samejima, Yoichi Tsumuraya

Methods

cDNA cloning

The cDNAs encoding β-glucuronidases were cloned by reverse transcriptase-PCR from conidia of *Aspergillus niger* and *Neurospora crassa*. The conidia were frozen in liquid nitrogen, homogenized with mortar and pestle, and RNA was extracted with an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Single strand cDNA was synthesized from 2 μg of total RNA using a reverse transcriptase, ReverTra Ace-α- (Toyobo, Osaka, Japan) and oligo(dT)$_{12–18}$ primer (Invitrogen, Carlsbad, CA, USA). To amplify cDNA for *A. niger* β-glucuronidase corresponding to a region for the putative mature protein, a set of specific primers, AnF-1 (5′-GAATTCTCATCCGACCGCTCATG-3′) and AnR-1 (5′-CACTAGTCACAGACTGAGGATTGC-3′), was designed based on the genomic database of *A. niger* (DSM, http://www.dsm.com/en_US/html/dfs/genomics_aniger.htm). The PCR was performed with proofreading polymerase (KOD-Plus-, Toyobo), using the primers and the single strand cDNA of *A. niger* as a template under the following conditions: 0.5 min denaturing at 94 °C, 0.5 min annealing at 55 °C and 2.0 min amplification at 68 °C, 35 cycles. To amplify cDNA for *N. crassa* β-glucuronidase corresponding to a region for the putative mature protein, a set of specific primers, NcF-1 (5′-GGATCCCAAATATCCGTCGACGAGAC-3′) and NcR-1 (5′-GAGCTCAAACAAACTCGACCAAAGCC-3′), were designed based on the genomic database of *N. crassa* (University of New Mexico, http://biology.unm.edu/biology/ngp/home.html). The PCR was performed with proofreading polymerase (KOD-Plus-, Toyobo), using the primers and the single strand cDNA of *N. crassa* as a template under the same conditions as for cDNA isolation from *A. niger*. The amplified fragments were treated with rTaq polymerase (Takara, Tokyo, Japan) at 72 °C for 5 min to allow subcloning with a pGEM T-Easy vector (Promega, Madison, WI, USA). The sequences of the cloned cDNAs were confirmed.

Expression of β-glucuronidase in *Pichia pastoris*

The cloned cDNAs were designated *AnGlcAase* (*A. niger* β-glucuronidase) and *NcGlcAase* (*N. crassa* β-glucuronidase). The cDNA fragments corresponding to the predicted mature

enzymes were inserted at the *Eco*RI site that is preceded by yeast α-factor of pPICZαC (Invitrogen). The methylotrophic yeast *P. pastoris* strain KM71 was transformed with the linearized plasmid construct using a multicopy *Pichia* expression kit (Invitrogen). Zeocin resistant colonies were cultured in 1,600 mL YPG medium containing 1% (w/v) yeast extract, 2% (w/v) peptone and 1% (w/v) glycerol at 28 °C with shaking at 100 rpm for 2 days. The cells were harvested by centrifugation (15 min, 5,000 rpm), washed with ice-cold distilled water, then suspended in 50 mL of YPM medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (v/v) methanol. The yeast cells were cultured for another 5 days for *AnGlcAase* or 2 days for *NcGlcAase* at 28 °C, during which time 0.5 mL methanol was added each day, to induce the recombinant enzymes.

Purification of recombinant enzymes

All operations were carried out at 0–4 °C. The culture media of *Pichia* cells treated with methanol for 5 days for recombinant AnGlcAase (rAnGlcAase) or 2 days for recombinant NcGlcAase (rNcGlcAase) were centrifuged (15 min, 8,000 rpm) and the supernatant was collected as a crude enzyme fraction. The crude enzyme fraction of rAnGlcAase was dialyzed against 10 mM sodium acetate buffer, pH 4.3, for 1 day, then applied onto a 2.2 × 15-cm Toyopearl HW-40F (Tosoh, Tokyo, Japan) column. The active fractions passing through the column were collected and adsorbed onto a 1.2 × 10-cm CM-cellulose (CM-32, Whatman, San Diego, CA, USA) column equilibrated with the buffer. The column was eluted with a linear gradient of 0–100 mM KCl in the buffer and again active fractions were collected.

The crude enzyme fraction of rNcGlcAase was dialyzed against 10 mM sodium acetate buffer, pH 4.5, for 1 day, then applied onto a 1.5 × 5.0-cm Toyopearl HW-40F column and the active fraction passing through the column was pooled. This fraction was adsorbed onto a 1.5 × 5.0-cm CM-cellulose column that had been equilibrated with the buffer. The column was eluted with a linear gradient of 0–100 mM KCl in the buffer. The active fractions were collected, dialyzed against 10 mM Tris–HCl buffer, pH 7.5, for 1 day, and applied onto a 1.5 × 3.5-cm DEAE-cellulose (DE-52, Whatman) column that had been equilibrated with the buffer. The column was eluted with a linear gradient of 0–100 mM KCl in the buffer. The active fractions were collected, dialyzed against 10 mM phosphate buffer, pH 7.0 for 1 day, and applied onto a 1.5 × 1.0-cm hydroxylapatite (Bio-Gel HTP, Bio-Rad, Richmond, CA, USA) column equilibrated with the same buffer. The column was first eluted with 400 mM KCl in the buffer, then re-equilibrated with the buffer without KCl, and eluted with a linear gradient of 10–400 mM sodium phosphate buffer, pH 7.0. The active fractions were collected and dialyzed against 10 mM phosphate buffer, pH 6.0, for 1 day.

Table S1. Purification of recombinant β-glucuronidases expressed in *P. pastoris*

	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Purification[a] (-fold)	Yield[a] (%)
rAnGlcAase					
Culture medium	549.0	2,406	4.38	1.0	100.0
CM-Cellulose	36.9	972	26.3	6.0	40.4
rNcGlcAase					
Culture medium	16.4	18.3	1.11	1.0	100.0
CM-Cellulose	0.69	7.96	11.5	10.3	43.4
DEAE-Cellulose	0.68	8.39	12.2	11.0	45.8
Hydroxylapatite	0.28	5.97	21.3	19.2	32.6

[a] Purification is given as the ratio of specific activity after each step to activity of the culture medium. Similarly, yield is the percentage of total activity relative to that of the culture medium.

Figure S1. SDS-PAGE of recombinant β-glucuronidases expressed in *P. pastoris*. The recombinant β-glucuronidases (rAnGlcAase and rNcGlcAase, approximately 1 μg each) purified through conventional chromatography were analyzed by SDS-PAGE. Protein in the gel was stained with Coomasie Brilliant Blue R-250. N-linked glycans attached to the recombinant enzymes were removed by digestion with endoglycosidase Endo-H (New England Biolabs Japan, Tokyo, Japan). *Lanes S*, molecular mass markers; *lane 1*, purified rAnGlcAase; *lane 2*, purified rNcGlcAase; *lane 3*, rAnGlcAase digested with Endo-H; *lane 4*, rNcGlcAase digested with Endo-H. The arrows indicate the purified recombinant enzymes.

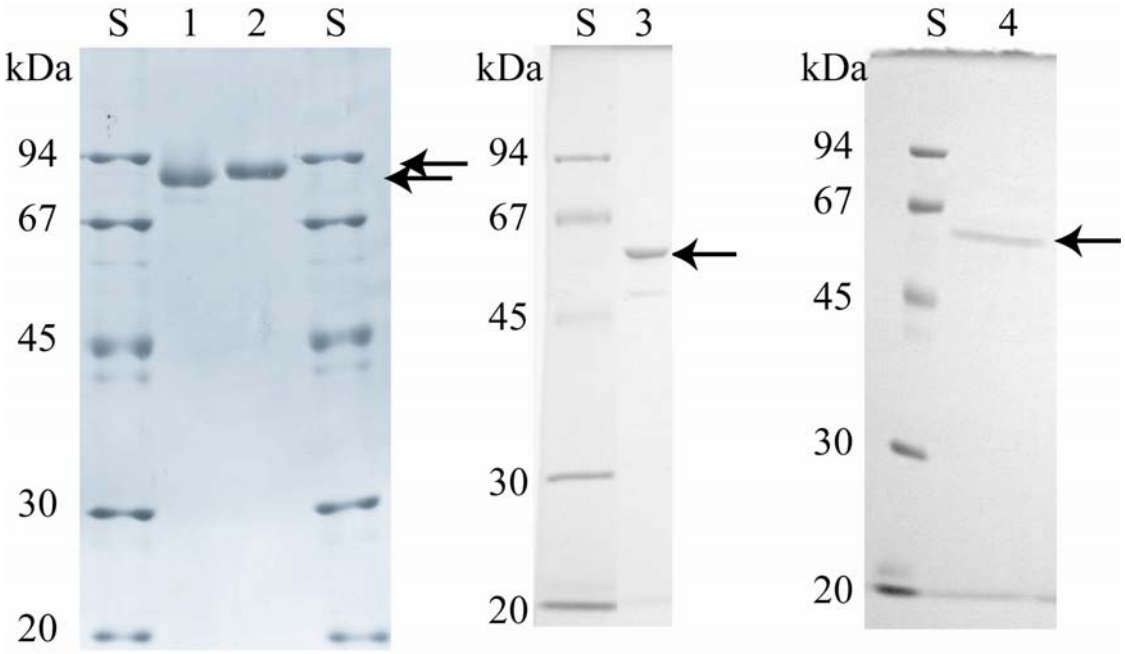

Figure S2. Analyses of the rAnGlcAase transglycosylation product. (A) The transglycosylation product with Gal as the acceptor was incubated with or without rAnGlcAase, and then analyzed by HPAEC-PAD. The peak of the main transglycosylation product emerged at an elution time identical to that of β-GlcA-(1→6)-Gal, which was used as the standard. The product digested with rAnGlcAase coincided with Gal and GlcA. (B) The proton-decoupled ^{13}C NMR spectrum of the main transglycosylation product with Gal as acceptor was recorded. The signals at 70.09 and 69.95 ppm were assigned to the methylene groups at substituted C-6 of respective β- and α-galactosyl residues judged by the results of DEPT135 spectra. In addition, no C-6 signal was observed at around 60 to 65 ppm, where signals for C-6 carbon bearing free OH groups are usually observed. These results, therefore, indicate that the glycosidic bond between GlcA and Gal residue of the main transglycosylation product is a β-(1→6) linkage.