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Heterologous expression and characterization of processing α-glucosidase I from *Aspergillus brasiliensis* ATCC 9642

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Abstract A gene for processing α-glucosidase I from a filamentous fungus, *Aspergillus brasiliensis* (formerly called *Aspergillus niger*) ATCC 9642 was cloned and fused to a glutathione S-transferase tag. The active construct with the highest production level was a truncation mutant deleting the first 16 residues of the hydrophobic N-terminal domain. This fusion enzyme hydrolyzed pyridylaminated (PA-) oligosaccharides $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA and the products were identified as $Glc_2Man_9GlcNAc_2$-PA and Glc_2Man_4-PA, respectively. Saturation curves were obtained for both $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA, and the K_m values for both substrates were estimated in the micromolar range. When 1 μM Glc_3Man_4-PA was used as a substrate, the inhibitors kojibiose and 1-deoxynojirimycin had similar effects on the enzyme; at 20 μM concentration, both inhibitors reduced activity by 50%.

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Abbreviations

GST	glutathione S-transferase
PA	pyridylaminated
*Ab*PGI	*Aspergillus brasiliensis* processing α-glucosidase I
IC_{50}	concentration for 50% inhibition

Introduction

Processing α-glucosidase I (EC 3.2.1.106) cleaves the terminal α(1-2)-glucosidic linkage of $Glc_3Man_9GlcNAc_2$, an oligosaccharide precursor of eukaryotic *N*-linked glycoproteins [1–3]. In the CAZy classification system [4], the enzyme belongs to the glycoside hydrolase family (GH) 63. Processing α-glucosidase I catalyzes the first step of trimming of the *N*-linked oligosaccharide precursor, and is followed by the action of processing α-glucosidase II that removes the two α(1-3)-linked glucose residues. Subsequent modification, catalyzed by various glycosidases and glycosyltransferases, produces wide variety of *N*-linked glycosylated proteins. These reactions are reportedly responsible for folding and quality control of newly formed glycoproteins [5, 6].

To study structure-function relationships in a protein by means of site-directed mutagenesis, a heterologous expression system is indispensable for the efficient production and purification of large quantities of the protein. However, only a few reports are available on the construction of processing α-glucosidase I expression systems. The enzymatic properties of Cwh41p, a processing α-glucosidase I

from *Saccharomyces cerevisiae*, have been intensively studied [7], and expression systems in *S. cerevisiae* AH22 [1] and in *Pichia pastoris* [8] have been constructed. A C-terminal part of *Candida albicans* processing α-glucosidase I has been expressed in *Escherichia coli*, and the recombinant enzyme has shown to hydrolyze a synthetic substrate, 4-methylumbelliferyl-α-D-glucopyranoside [9]. In this paper, a gene for processing α-glucosidase I from a filamentous fungus, *Aspergillus brasiliensis* (formerly called *Aspergillus niger*) ATCC 9642 [10, 11] was cloned and fused to glutathione S-transferase (GST). The enzyme, referred to here as *Ab*PGI, enzyme was affinity purified and its activity was characterized using pyridylaminated (PA-) oligosaccharides $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA as substrates.

Materials and methods

Genomic DNA cloning

E. coli strain JM109 was used for DNA manipulation. All sequence analyses were carried out using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Genomic DNA was extracted from *A. brasiliensis* ATCC 9642 as described [12]. A nested polymerase chain reaction (PCR) was carried out using the genomic DNA as a template. Amino acid sequences of processing α-glucosidase I from fungi, *Aspergillus fumigatus* (DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank, AY461807) [13] and *Aspergillus nidulans* (BN001301) [14], and yeasts, *Schizosaccharomyces pombe* (CU329670) [15] and *S. cerevisiae* (U35669) [16], were aligned using the program ClustalW2 at EMBL-European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Fig. 1). Four conserved sequences, WDEGFHL, EARSKVP, ENYWR(S/G)P, and (H/D)FTGWT(S/A), were identified, and based on these sequences, the degenerate primers, 63-f1, 63-f2, 63-r2, and 63-r1, respectively, were designed. The primer sequences are: 63-f1, 5′-TGG GA(C/T) GA(A/G) GG(A/C/T) TT(C/T) CA(C/T) CT(C/G/T)-3′; 63-f2, 5′-GA(A/G) GC(A/C/G/T) CG(A/C/G/T) TC(A/C/G/T) AA(A/G) GT(C/G/T) CC(C/G/T)-3′; 63-r1, 5′-CA(C/T) TT(C/T) AC(A/C/G/T) GG(A/C/T) TGG AC(A/C/G/T) TC(A/C/G/T)-3′; 63-r2, 5′-GA(A/G) AA(C/T) TA(C/T) TGG CG(A/C/G/T) TC(A/C/G/T) CC(C/G/T)-3′. Primer pairs 63-f1/63-r1 and 63-f2/63-r2 were used for the first and second amplifications, respectively. The PCR products were cloned into a pGEM-T vector (Promega, Madison, WI, USA), followed by sequencing of the PCR product. The full-length open reading frame was obtained by an inverse PCR method. First, PCR amplification was performed on self-ligated *Bam*HI fragments of the genomic DNA with a combination of primers, 5′-AAG GAC CAG TTC TAC GGC AC and 5′-ATT GGA TGG TGA ACT CAG GA-3′. The amplified fragment was cloned into pGEM-T vector but did not cover the N-terminal part of the coding region. Therefore, another inverse PCR amplification was performed on self-ligated *Hin*dIII fragments of the genomic DNA with a pair of primers, 5′-CTG GAT GCG AAG AAG AAC GC-3′ and 5′-ATT GGA TGG TGA ACT CAG GA-3′. The amplified fragment was cloned into pGEM-T vector, and the full-length open reading frame of *Ab*PGI was finally obtained.

cDNA cloning

Total RNA was extracted from the mycelia of *A. brasiliensis* using an ISOGEN RNA Extraction Kit (Nippon Gene, Tokyo, Japan). The first-strand cDNA was synthesized with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting cDNA was subjected to PCR amplification using primers, 5′-ATC ATC CTC AGT CTC TGA GG-3′ and 5′-GCC ATA CAT ACT TCG CCT AC-3′. The PCR product was cloned into pGEM-T vector, resulting in pGEM-T-*Ab*PGI, and the cloned fragment was sequenced. The nucleotide sequence data was deposited in the DDBJ/EMBL/GenBank database under accession number AB665481.

Construction of expression plasmids of recombinant *Ab*PGI

Oligonucleotide-directed mutagenesis was carried out using a QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). To facilitate subcloning of the *Ab*PGI cDNA fragments, *Eco*RI and *Not*I restriction sites in the *Ab*PGI cDNA fragment were removed without changing the amino acid sequence using oligonucleotides, 5′-GAC ATC CGC AAG GGC GGT CGC CAG ACA ATT CAT GAT GC-3′ and 5′-CCG CGG AAT ACA CCG AGT TTT CCA AGT CCA TGC TC-3′, and their complementary oligonucleotides, and the constructed plasmid was used as a template for PCR to construct the expression plasmids. To construct three plasmids that encode GST fused to *Ab*PGI, designated GST-*Ab*PGIΔ0, GST-*Ab*PGIΔ16, and GST-*Ab*PGIΔ23 (see the Results and Discussion for a detailed description), PCR was performed using a forward primer, 5′-GAT GCT TTC GAT ATA AAA AAG AAT TCA TGC ATC TTT CTA AGA TAT C-3′ (for GST-*Ab*PGIΔ0), 5′-CGC CGG TAC TCA ATG CCG AAT TCG CTG CGG TGC TCT CGA G-3′ (for GST-*Ab*PGIΔ16), or 5′-CGG TGC TCT CGA GTC AAG AAT TCG CTC CGG CCG ATG ATC TC-3′ (GST-*Ab*PGIΔ23), and a reverse primer, 5′-ATA CAT ACT TCG CCT ACA GGG AGG CGG CCG CTT ACA GCT CGT

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A.brasiliensis  400 GPKDLFTCVPSRPFFPRGFLWDEGFHLIPVIDWDTDLALEIVRSWLNLMDED-GWIAREQ 458
A.fumigatus     400 GPKDLFTCVPSRPFFPRGFLWDEGFHLIPVIDWDTDLALEIVKSWLNLMDED-GWIAREQ 458
A.nidulans      398 GPKDLFTAVPSRPFFPRGFLWDEGFHLIPILDWDPDLALEIVKSWFSLMDED-GWIAREQ 456
Schizo.pombe    391 IERSLFTIVPSRPHFPRGFYWDEGFHLLPVGLWDNDFSLEILKSWFSLVNED-GWVGREQ 449
S.cerevisiae    404 GPFELFTSVPSRGFFPRGFYWDEGFHLLQIMEYDFDLAFEILASWFEMIEDDSGWIAREI 463

A.brasiliensis  459 ILGSEARSKVPPEFTIQYPHYA-NPPTLFIILEAFIDKLDAKKNASMQTYADSGA----- 512
A.fumigatus     459 ILGAEARSKVPPEFTVQYPHYA-NPPTLFMVLEAFLDKLEASKGAYSQNSGDQEA----- 512
A.nidulans      457 ILGNEARSKVPPEFTVQSTQYG----------------------------------- 478
Schizo.pombe    450 ILGEEARSKVPDEFQTQYPDIAN-PPTLILALKGYIERLQEQQGKLNNRFSGEGEDYSLD 508
S.cerevisiae    464 ILGNEARSKVPQEFQVQNPNIAN-PPTLLLAFSEMLSRAIENIGDFNSDSYHQVMFN-SR 521

A.brasiliensis  513 -TDGLRSIFVDQPELGEAFIRSIYPLIPKHYYWYRSTQKGDIKSYDREAYS--------T 563
A.fumigatus     513 -LDSLRMTYLQKPELGEAFIRSIYPLLKRHYFWYRTTQKGDIKSYDREAFS--------T 563
A.nidulans      479 ----------------------------------------DIKSYDREAFS--------T 490
Schizo.pombe    509 DLEYLRSVSISNPEKSVQFLRDLFPLLLRHYEWFRETQKGDFETWERECFS--------Q 560
S.cerevisiae    522 TAKFMTNNLEANPGLLTEYAKKIYPKLLKHYNWFRKSQTGLIDEYEEILEDEGIWDKIHK 581

A.brasiliensis  564 REAYRWRGRSVQHILTSGLDDYPRPQPPHPGELHVDLMSWMGMMTRALRRIAVTIGETED 623
A.fumigatus     564 KEAYRWRGRSVQHILTSGLDDYPRPQPPHPGELHVDLISWMGMMTRAMRRIAEFVGETED 623
A.nidulans      491 KEAYRWRGRSVQHILTSGLDDYPRAQPPHPGELHVDLISWMGMMTRALRRIAETLGETED 550
Schizo.pombe    561 VEGYRWRGRTYQHCLASGLDDYPRAQPPSTAELHVDLLSWMTSFTRSLHFVAEFLGETEE 620
S.cerevisiae    582 NEVYRWVGRTFTHCLPSGMDDYPRAQPPDVAELNVDALAWVGVMTRSMKQIAHVLKLTQD 641

A.brasiliensis  624 AEVFKTYETAIERNIDDLHWDDDASTYCDATIDEYEE--HVHVCHKGYISIFPFLTGMLG 681
A.fumigatus     624 VEEFRGYETAIERNIDDLHWDEEAQTYCDATIDEFEES--VHVCHKGYISLFPFLTGMLG 681
A.nidulans      551 IEEFKAYETAIERNIDDLHWDADAKTYCDATIDDYEDS--VHVCHKGYVSIAPFLTGIVG 608
Schizo.pombe    621 AEKLAGYENAMLRNLEDNHWDEEVQAYCDSSVDEYDDP--INVCHKGYVTLLPMMLGLLP 678
S.cerevisiae    642 EQRYAQIEQEVVENLDLLHWSENDNCYCDISIDPEDDEIREFVCHEGYVSVLPFALKLIP 701

A.brasiliensis  682 PDSPRLKAILDLIGDPEELWSDYGIRSLSKKDQFYGTAENYWRSPIWVNINYLVLKNLYN 741
A.fumigatus     682 PDSPRLKAVLDLVRDPDELWSDYGIRSLSKKDEFYGTAENYWRSPIWMPINYLVVKNLYD 741
A.nidulans      609 PDSPRLEAILDLIEDPEELWSDYGIRSLSKKDEFYGTAENYWRSPVWININYLVLKNLYN 668
Schizo.pombe    679 ADSGRLTSLLKLIRDENELWSPYGIRSLSMNDVYFGTGENYWRGPIWINMNYLILSSLYQ 738
S.cerevisiae    702 KNSPKLEKVVALMSDPEKIFSDYGLLSLSRQDDYFGKDENYWRGPIWMNINYLCLDAMRY 761

A.brasiliensis  742 ----IAIVSGPHREQARELYSNLRKNLVENVFQEWKKTGFAWEQYNPETGIGRRTQHFTG 797
A.fumigatus     742 ----IAMTSGPHQEQAREMYSSLWKNLVENVFRQWKETGFAWEQYNPETGKGQRTQHFTG 797
A.nidulans      669 ----IALTPGPQQNRARKMYSGLRKNLVENVYREWKNTGFAWEQYNPETGKGQRTQHFTG 724
Schizo.pombe    739 N---YINTPGPNQNLARSIYEELRTNVVNNVFENWRQTGIFWEQYDPTTGKGQRTKDFTG 795
S.cerevisiae    762 YYPEVILDVAGEASNAKKLYQSLKINLSNNIYKVWEEQGYCYENYSPIDGHGTGAEHFTG 821

A.brasiliensis  797 WTSMVVKMMSMPDLPASEQKGHDEL 822
A.fumigatus     797 WTSMVVKIMSMPDLPANKQIGHDEL 822
A.nidulans      724 WTSLVVKMMAMPDLPASEKTGHDEL 749
Schizo.pombe    795 WTSLVVNIMSENY------------ 808
S.cerevisiae    821 WTALVVNILGRF------------- 833
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Fig. 1 Comparison of C-terminal regions of *Ab*PGI and related enzymes. Residues conserved among these proteins are shaded in gray. The degenerate primers were designed based on four conserved motifs highlighted by black-and-white inversion. The carboxylic acid residues (Glu595, Asp599 and Glu804 in *Ab*PGI) expected to be related to enzyme activity are boxed

CAT-3′. The restriction sites of *Eco*RI (underlined in the forward primers) and *Not*I (underlined in the reverse primer) were introduced to facilitate cloning of the PCR products. The PCR products were digested with *Eco*RI and *Not*I and ligated into the multicloning site of the pGEX-5X-1 plasmid (GE Healthcare, Chalfront St Giles, UK). The sequence of the constructs was verified by DNA sequencing.

Expression and purification of GST-*Ab*PGIΔ16

E. coli strain HMS174 was used for overexpression of gene constructs. Cells harboring the desired plasmids were grown at 37°C in 1 L of Luria-Bertani medium containing 50 μg mL^{-1} ampicillin. When the culture reached an optical density of 0.6 measured at 600 nm, it was induced with isopropyl-β-D-thiogalactopyranoside at a final concentration of 10 μM and grown for another 5 h at 25°C. The cells were harvested by centrifugation at 10,000×*g* for 5 min, resuspended in 30 mL of 20 mM Tris-HCl buffer (pH 8.5) containing 50 mM sodium chloride, and disrupted by sonication. The cell lysate was centrifuged at 10,000×*g* for 20 min to remove insoluble material and the supernatant was applied onto a glutathione-agarose (Sigma-Aldrich, St Louis, MO, USA) column equilibrated with 20 mM

Tris-HCl buffer (pH 8.5) containing 50 mM sodium chloride. The elution of the GST-tagged protein was carried out according to the instruction manual of glutathione-agarose. The column was washed with the equilibration buffer containing 1% Triton X-100 and the protein was eluted with 20 mM Tris-HCl (pH 8.5) containing 50 mM sodium chloride and 10 mM reduced glutathione. The purity of the eluted enzyme was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of PA-oligosaccharides

Oligosaccharides $Glc_3Man_9GlcNAc_2$ and Glc_3Man_4 were synthesized as previously described [17]. The preparation of fluorescent PA-oligosaccharides was carried out using GlycoTag (Takara Bio, Otsu, Japan). $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA were purified essentially as described [18] by normal phase high-performance liquid chromatography (HPLC) using an Asahipak NH2P-50-4E column (4.6 mm×250 mm, Shodex, Tokyo, Japan) and a LaChrom Elite L-2100 HPLC system equipped with a fluorescence detector L-2480 (Hitachi, Tokyo, Japan). Solvent A consisted of 97% acetonitrile, 0.3% ammonium acetate buffer (pH 7.0); solvent B consisted of 30% acetonitrile, 0.3% ammonium acetate buffer (pH 7.0). The samples were eluted with a 20 min linear gradient from 30% to 65% solvent B at a flow rate of 0.8 mL min^{-1} and a column temperature of 40°C. The PA-oligosaccharide elutions were monitored via their fluorescence signals (excitation wavelength, 310 nm; emission wavelength, 380 nm) and quantified by comparing their peak areas with a known amount of GlcNAc-PA [19, 20]. The standard PA-glucose oligomers was prepared by labeling Glucose Oligomer 4–20 (Seikagaku, Tokyo, Japan) with 2-aminopyridine.

Measurement of enzymatic activity

The purified GST-*Ab*PGIΔ16 was concentrated with a Centricon Plus-20 (Millipore, Bedford, MA, USA) ultrafiltration device that was pre-rinsed with 5% *N,N*-dimethyldodecylamine *N*-oxide detergent to maximize product yield. Sodium phosphate buffer (20 nM, pH 7.0) was added to the concentrated protein to provide the desired storage and reaction conditions. To examine the effects of pH on the enzyme's activity, reaction mixtures containing 0.3 μg GST-*Ab*PGIΔ16, 10 pmol $Glc_3Man_9GlcNAc_2$-PA and 20 mM sodium phosphate at pHs ranging from 6.0 to 8.0 in a total volume of 10 μL were prepared and incubated for 10 min at 25°C, followed by heat treatment at 95°C for 5 min to stop the enzymatic reaction. For kinetic studies, reaction mixtures containing 0.3 μg GST-*Ab*PGIΔ16, 20–200 pmol PA-sugar, and 20 mM sodium phosphate (pH 7.0) in a total volume of 40 μL were prepared. After 0–30 min of incubation at 25°C, 10 μL of the mixtures was heated at 95°C for 5 min. The amounts of PA-sugar products were measured by HPLC as described in the previous section. To determine the concentration for 50% inhibition (IC_{50}) of kojibiose (Glc-α(1→2)-Glc; Hayashibara Biochemical Laboratories, Okayama, Japan), nigerose (Glc-α(1→3)-Glc; Hayashibara Biochemical Laboratories), and 1-deoxynojirimycin (Wako Pure Chemical Industries), reaction mixtures contained 20 mM sodium phosphate buffer pH 7.0, inhibitor, 0.3 μg GST-*Ab*PGIΔ16, and 10 pmol Glc_3Man_4-PA as a substrate with a final volume of 10 μL. Reactions and product measurements were carried out as described above. Kinetic parameters were calculated by the nonlinear regression analysis using KaleidaGraph (Synergy Software, Reading, PA, USA).

Results and discussion

Molecular cloning and primary structure of *Ab*PGI

The genomic DNA and cDNA of *Ab*PGI were cloned, and their nucleotide sequences were determined. The open reading frame consists of 2,843 bp (including the stop codon) encoding 822 amino acid residues and is interrupted by six introns of 78, 67, 51, 59, 61, and 58 bp. The identified introns had general characteristics of filamentous fungal introns; they contained GT and AG at the exon/intron junctions [21]. Four sequences used for the design of degenerate PCR primers were found as WDEGFHL (amino acid residues 420–426), EARSKVP (463–469), ENYWRSP (720–726), and HFTGWTS (794–800) (Fig. 1). A homology search for the deduced primary structure was performed in the DDBJ database using the BLAST program. *Ab*PGI most resembled a putative processing α-glucosidase I from *Aspergillus niger* CBS

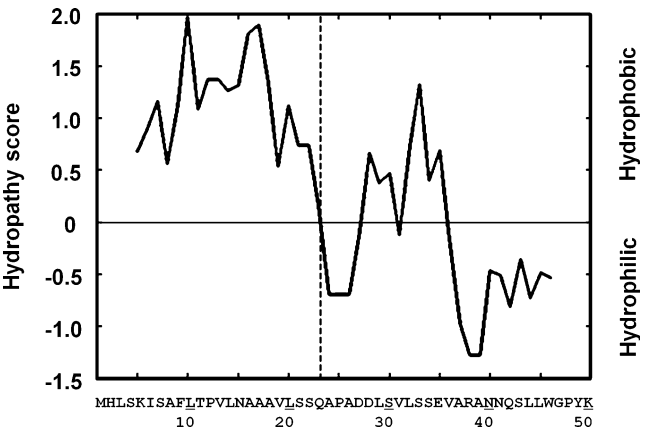

Fig. 2 A hydropathy plot of the N-terminal 50 amino acid residues of *Ab*PGI. The sequence was analyzed by the Kyte-Doolittle algorithm using a Protscale server

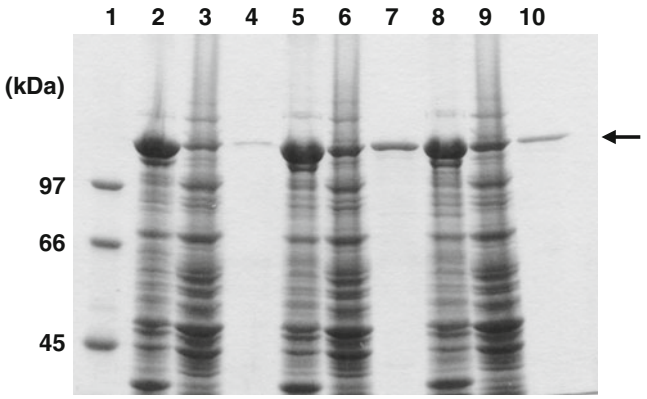

Fig. 3 SDS-PAGE analysis of the GST-fused recombinant enzymes in each purification step. Lane 1, molecular mass markers; lanes 2–4, GST-*Ab*PGIΔ0; lanes 5–7, GST-*Ab*PGIΔ16; and lanes 8–10, GST-*Ab*PGIΔ23. Specifically, in each purification step: lanes 2, 5, and 8, insoluble protein pellet isolated by cell lysate centrifugation; lanes 3, 6, and 9, soluble protein fractions in the supernatant of the cell lysate; lanes 4, 7, and 10, glutathione-agarose chromatography eluate. The arrow indicates the recombinant proteins, GST-*Ab*PGIΔ0 (120 kDa), GST-*Ab*PGIΔ16 (118 kDa) and GST-*Ab*PGIΔ23 (117 kDa)

513.88 (TrEMBL no. A2R4S4, 95% identity) [22]. Among the characterized enzymes, *Ab*PGI resembled a processing α-glucosidase I from *Aspergillus fumigatus* YJ-407, Afcwh41 (TrEMBL no. Q6S9W4, 78% identity) [13]. The determined amino acid sequence was aligned with those from some fungi and yeasts (Fig. 1). Three carboxylic acid residues reported to be critical for enzymatic activity (corresponding to Glu613, Asp617 and Glu804 of *S. cerevisiae* Cwh41p) [8, 23] were found in the *Ab*PGI sequence.

The 5′ upstream region of the *Ab*PGI open reading frame contains several TATA-like sequences at positions 568–571, 715–718, and 760–763 of the deposited sequence (DDBJ/EMBL/GenBank accession number AB665481). However, they do not appear to be the typical TATA box sequences, since their positions are relatively far from the initiation codon as compared with the reported TATA box sequences [24]. Sequence analysis of the mouse processing α-glucosidase I 5' flanking region has been performed and no canonical TATA box was found in this region [25].

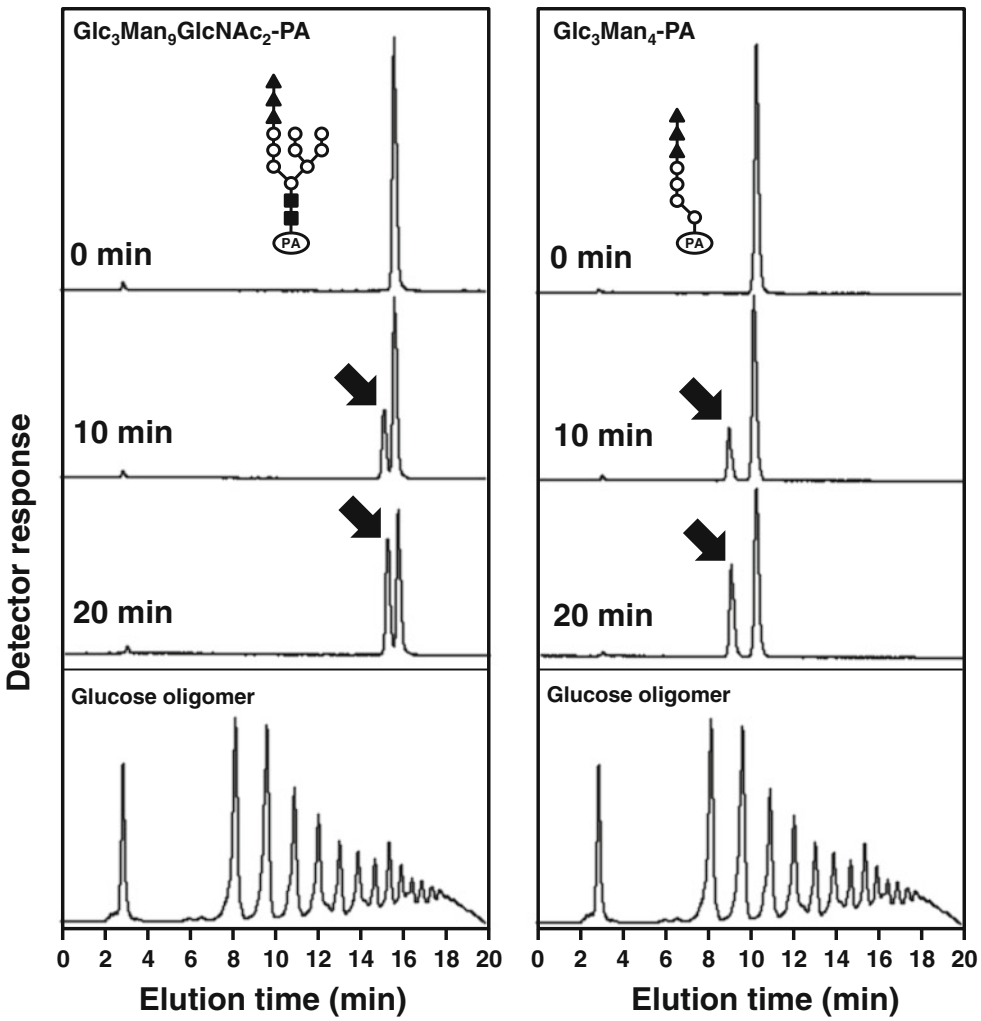

Fig. 4 HPLC analysis of the hydrolysates of $Glc_3Man_9GlcNAc_2$-PA (*left panel*) and Glc_3Man_4-PA (*right panel*) treated with GST-*Ab*PGIΔ16. The reaction mixtures were removed at 10, 20, and 30 min, and analyzed by HPLC. Arrows indicate the elution position of the products. The elution pattern of the standard PA-glucose oligomers is also shown in the bottom row. Symbols used for the structural formulae of substrates: glucose (Glc), ▲; mannose (Man), ○; *N*-acetylglucosamine (GlcNAc), ■; 2-aminopyridine, ellipse labeled "PA"

Three potential *N*-glycosylation sites, Asn-Xaa-Ser/Thr (Xaa is any amino acid residue except Pro) at Asn41, Asn208, and Asn501, were found in the amino acid sequence of *Ab*PGI.

Construction of the expression vector of *Ab*PGI

To construct efficient expression vectors in *E. coli*, removal of the hydrophobic N-terminal part has been reported to result in increased levels of expression [26]. A Kyte-Doolittle hydrophobicity scale of the amino acid sequence was calculated using a Protscale server (http://expasy.org/tools/protscale.html) (Fig. 2), showing that the N-terminal residues 1–23 of *Ab*PGI are highly hydrophobic. Two truncation mutants were therefore designed to remove the first 16 residues and the first 23 residues. Three plasmids were constructed to encode GST fused to each form of *Ab*PGI and the expressed proteins were designated GST-*Ab*PGIΔ0, GST-*Ab*PGIΔ16, and GST-*Ab*PGIΔ23. The production of each of these proteins in *E. coli* was analyzed by SDS-PAGE (Fig. 3). Though most of the expressed proteins accumulated in insoluble inclusion bodies, some soluble species were evident. The soluble fractions were purified to a high degree of homogeneity by affinity

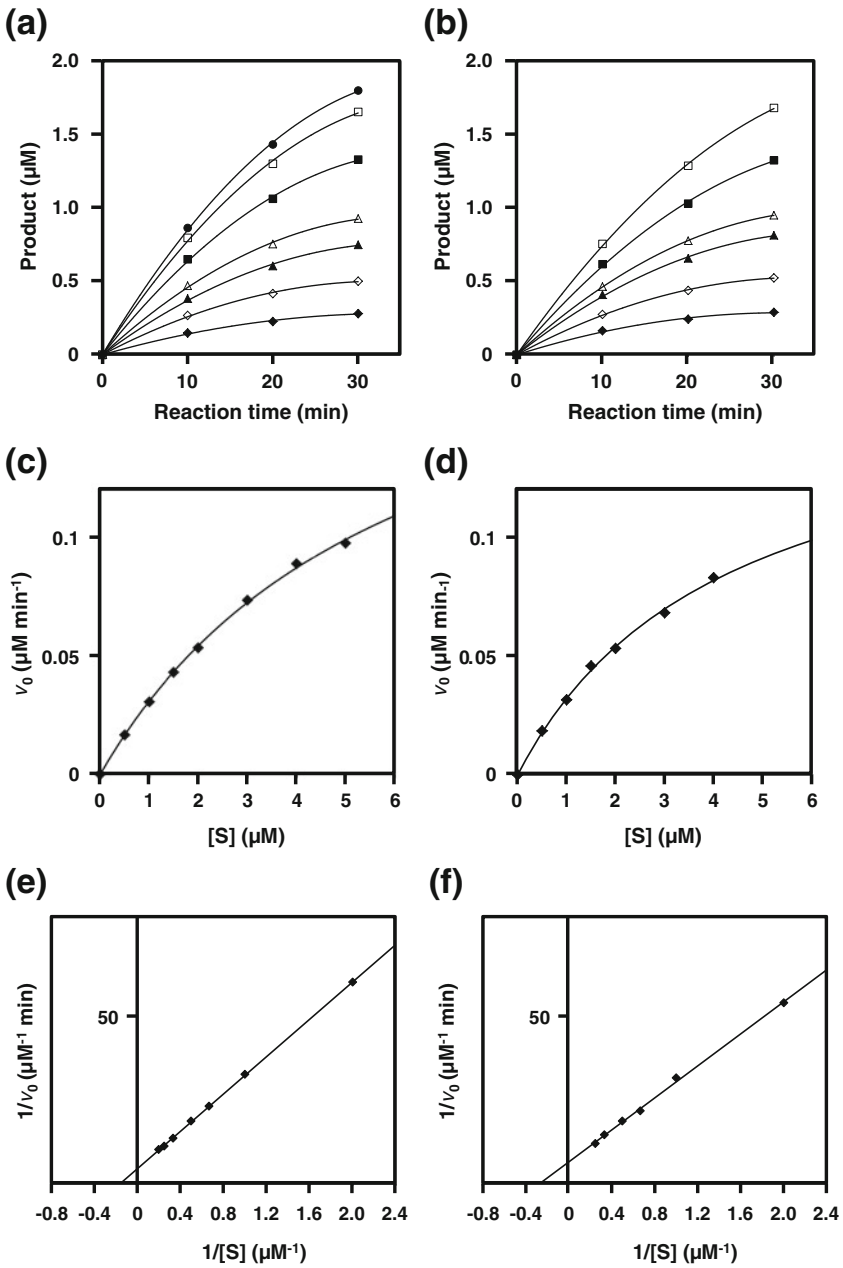

Fig. 5 Kinetic analysis of the GST-*Ab*PGIΔ16 activity for PA-oligosaccharides. First row, reaction time courses for substrates **a** $Glc_3Man_9GlcNAc_2$-PA and **b** Glc_3Man_4-PA. Symbols indicate concentration of substrates: 0.5 μM, ♦; 1.0 μM, ◊; 1.5 μM, ▲; 2.0 μM, Δ ; 3.0 μM, ■; 4.0 μM, □; 5.0 μM, ●. Second row, plots of initial reaction velocities as a function of substrate concentration for **c** $Glc_3Man_9GlcNAc_2$-PA, and **d** Glc_3Man_4-PA. Third row, Lineweaver-Burk plots for hydrolysis of **e** $Glc_3Man_9GlcNAc_2$-PA and **f** Glc_3Man_4-PA

chromatography on glutathione agarose beads. GST-*Ab*PGIΔ16 provided the highest yield and was therefore chosen for further characterization.

Enzymatic activity of GST-*Ab*PGIΔ16

GST-*Ab*PGIΔ16 contains a 220-residue affinity tag. Although removal of the GST portion using a factor Xa protease was attempted, we did not obtain the active enzyme, thus the uncleaved GST-*Ab*PGIΔ16 was used for the enzymatic characterization.

$Glc_3Man_9GlcNAc_2$-PA was incubated with GST-*Ab*PGIΔ16, and the reaction sample was analyzed by HPLC (Fig. 4). The elution profile showed a new peak at 15.5 min. Based on the comparison to the standard PA-glucose oligomers, the difference between the times of the peak at 15.5 min and the original peak corresponded to one glucose residue. The result indicates that the enzyme hydrolyzed $Glc_3Man_9GlcNAc_2$-PA to produce a mixture of $Glc_2Man_9GlcNAc_2$-PA and glucose, as expected. No peak corresponding to hydrolysates of $Glc_2Man_9GlcNAc_2$-PA was detected by further reaction, indicating that the enzyme did not hydrolyze the terminal α(1-2)-linkage of $Glc_2Man_9GlcNAc_2$-PA and was specific for α(1-2)-linkage of $Glc_3Man_9GlcNAc_2$-PA. To measure the time course of $Glc_2Man_9GlcNAc_2$-PA production, the reaction samples were collected at 10-min intervals and analyzed by HPLC (Fig. 4). The plots were not linear, but instead fit to a square regression, probably because the concentration of the substrate was linearly decreased by the hydrolysis (Fig. 5a), and the hydrolytic activity was decelerated due to the low K_m values (described later). The effect of pH on the enzymatic activity was examined with $Glc_3Man_9GlcNAc_2$-PA as a substrate. The optimal pH was 7.0, while the enzyme retained 89% and 94% of maximum activity at pH 6.0 and 8.0, respectively (data not shown).

A similar analysis was performed with Glc_3Man_4-PA. A new peak at 9 min appeared, and was identified as Glc_2Man_4-PA based on comparison to the standard PA-glucose oligomers (Fig. 4). The time course of hydrolysis of Glc_3Man_4-PA was decelerated, as observed in the hydrolysis of $Glc_3Man_9GlcNAc_2$-PA (Fig. 5b).

Kinetic analysis and inhibition assays

The initial velocities, v_o, of the GST-*Ab*PGIΔ16 enzymatic reactions were calculated using regression analysis and the dependence of the v_o values on the concentration of the substrates was plotted. Although substrate was the limiting factor in this reaction system, saturation curves were obtained for both $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA (Fig. 5c and d). The K_m and k_{cat} values were calculated as 6.1±0.4 μM and 1,450±60 s^{-1}, respectively (for $Glc_3Man_9GlcNAc_2$-PA), and 4.2±0.4 μM and 1,100±70 s^{-1}, respectively (for Glc_3Man_4-PA). Good correlations were also observed in the Lineweaver-Burk plots of both substrates (Fig. 5e and f). Therefore, the K_m values for both substrates could be estimated in the micromolar range and the calculated kinetic parameters for $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA appear almost identical.

The activity of GST-*Ab*PGIΔ16 for 1 μM Glc_3Man_4-PA was measured in the presence of various concentrations of 1-deoxynojirimycin, kojibiose, and nigerose (Fig. 6). 1-Deoxynojirimycin has been reported as a strong inhibitor of processing α-glucosidase I [27–32], and the IC_{50} was 20 μM. Interestingly, kojibiose, a part of the non-reducing terminal residues of $Glc_3Man_9GlcNAc_2$, strongly inhibited GST-*Ab*PGIΔ16 and also had an IC_{50} value of 20 μM. Nigerose inhibited the enzymatic activity weakly and its IC_{50} value of 1.9 mM was 95-fold higher than those of 1-deoxynojirimycin and kojibiose.

The enzymatic activity of processing α-glucosidase I has been previously measured by basically two methods: using ^{14}C-labeled oligosaccharides or preparing appropriate synthetic oligosaccharides. With the use of ^{14}C-labeled oligosaccharides, the activities of processing α-glucosidase I enzymes from human hippocampus [31], rat liver [33], calf liver [27], pig liver [29], *S. cerevisiae* [28], and mung bean [32] have been determined. Although the

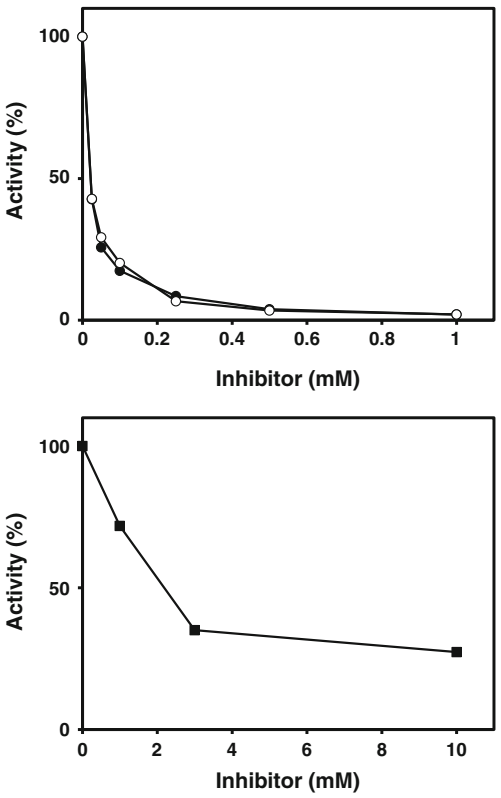

Fig. 6 Effects of inhibitors on the hydrolysis of $Glc_3Man_9GlcNAc_2$-PA. Upper panel, 1-deoxynojirimycin (○) and kojibiose (●). Lower panel, nigerose (■)

K_m values of these enzymes for the ^{14}C-labeled oligosaccharides have not been determined due to the difficulty of quantifying the concentration of the oligosaccharides, the K_i value of *S. cerevisiae* Cwh41p for kojibiose has been reported to be 55 μM [28]. Synthetic oligosaccharides such as Glc_3-O$(CH_2)_8COOCH_3$ and Glc_3ManOMe have also been used for kinetic studies. These demonstrated that the K_m values of *S. cerevisiae* Cwh41p for Glc_3-O$(CH_2)_8COOCH_3$ and Glc_3ManOMe were 1.28 mM and 1.26 mM, respectively [1, 8]. The K_i value of *S. cerevisiae* Cwh41p for kojibiose has been determined to be 0.8 mM when Glc_3-O$(CH_2)_8COOCH_3$ was used as the substrate [1].

In this study, an expression system for GST-*Ab*PGIΔ16 in *E. coli* was constructed, and the enzymatic properties of GST-*Ab*PGIΔ16 were determined. The kinetic studies were carried out using $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA, more natural substrates than the previously used synthetic substrates in other studies, and the K_m values for both $Glc_3Man_9GlcNAc_2$-PA and Glc_3Man_4-PA were estimated in the micromolar range. The IC_{50} value of kojibiose for Glc_3Man_4-PA was 20 μM, which was almost identical to that of 1-deoxynojirimycin. The kinetic values obtained in this study are similar to those of *S. cerevisiae* Cwh41p with ^{14}C-labeled oligosaccharides and markedly different from *S. cerevisiae* Cwh41p with synthetic substrates Glc_3-O$(CH_2)_8COOCH_3$ and Glc_3ManOMe. The reason for this disparity is not clear. The estimated kinetic parameters could include a certain error due to the limited amount of the substrates, as described by the work of Dhanawansa *et al*. that criticized the use of low concentration of ^{14}C-labeled oligosaccharides [1]. However, this does not seem to explain why the IC_{50} values of kojibiose and 1-deoxynojirimycin were almost identical. The activities of these enzymes are likely to be highly dependent on experimental conditions.

The enzymes belonging to GH63 contain an $(\alpha/\alpha)_6$ barrel structure as the catalytic domain [34], and the entire backbone of the $(\alpha/\alpha)_6$ barrel is highly homologous with those of GH37 trehalase, GH15 glucoamylase, and GH15 glucodextranase [35–37]. The K_m values of GH37 trehalase for trehalose and GH15 glucoamylase for maltooligosaccharides have been reported in the millimolar range [35, 38–43]. For example, the K_m and k_{cat} values of *E. coli* trehalase for trehalose have been determined to be 0.41 mM and 199 s^{-1}, respectively [35], and the values of *Aspergillus niger* glucoamylase for maltohexaose have been reported as 0.12 mM and 59.7 s^{-1}, respectively [41]. Enzymes hydrolyzing maltooligosaccharides, such as α-amylases, β-amylases, and α-glucosidases, also show K_m values in the millimolar range [44–49]. On the other hand, the data presented here, together with previous studies using ^{14}C-labeled oligosaccharides, show that processing α-glucosidase I seems to interact with Glc-α(1→2)-Glc structure in the micromolar range of concentration, and the values are unusually low compared to those of other gluco-oligosaccharide hydrolases. Crystallization of *S. cerevisiae* Cwh41p has been reported [8], and it will be intriguing to know the structure-function relationship of processing α-glucosidase I.

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