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Title

Substrate recognition of the catalytic α-subunit of glucosidase II from *Schizosaccharomyces pombe*

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Running title

Substrate recognition of catalytic subunit of ER GII

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Abstract

The recombinant catalytic α-subunit of *N*-glycan processing glucosidase II from *Schizosaccharomyces pombe* (SpGIIα) was produced in *Escherichia coli*. The recombinant SpGIIα exhibited quite low stability, with a reduction in activity to < 40% after 2-days preservation at 4°C, but the presence of 10% (v/v) glycerol prevented this loss of activity. SpGIIα, a member of the glycoside hydrolase family 31 (GH31), displayed the typical substrate specificity of GH31 α-glucosidases. The enzyme hydrolyzed not only α-(1→3)- but also α-(1→2)-, α-(1→4)- and α-(1→6)-glucosidic linkages, and *p*-nitrophenyl α-glucoside. SpGIIα displayed most catalytic properties of glucosidase II. Hydrolytic activity of the terminal α-glucosidic residue of Glc_2Man_3-Dansyl was faster than that of Glc_1Man_3-Dansyl. This catalytic α-subunit also removed terminal glucose residues from native *N*-glycans ($Glc_2Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$) although the activity was low.

Key words: catalytic α-subunit of ER glucosidase II; heterologous expression; glycoside hydrolase family 31; substrate specificity

Abbreviations

cleavage-1, conversion of G2M9 to G1M9; cleavage-2, conversion of G1M9 to M9; BSA, bovine serum albumin; ER, endoplasmic reticulum; GH31, glycoside hydrolase family 31; GI, glucosidase I; GII, glucosidase II; GIIα, α-subunit of glucosidase II; GIIβ, β-subunit of glucosidase II; G1M3, Glc_1Man_3; G2M3, Glc_2Man_3; G1M9, $Glc_1Man_9GlcNAc_2$; G1M9-PA, pyridylaminated G1M9; G2M9, $Glc_2Man_9GlcNAc_2$; G3M9, $Glc_3Man_9GlcNAc_2$; G3M9-PA, pyridylaminated G1M9; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-thiogalactopyranoside; M9, $Man_9GlcNAc_2$; pNPG, *p*-nitrophenyl α-glucopyranoside; ScGI, glucosidase I of *Saccharomyces cerevisiae*; SpGIIα, α-subunit of glucosidase II of *Schizosaccharomyces pombe*.

Introduction

Secretory proteins of eukaryotic cells are *N*-glycosylated in the endoplasmic reticulum (ER). *N*-Glycosylation is initiated with the transfer of the precursor glycan, $Glc_3Man_9GlcNAc_2$ (G3M9), to an Asn residue in the motif Asn-Xaa-Ser/Thr of nascent polypeptides. Mannoses in G3M9 are organized in a 3′-trimannosyl branch (arm A) and a 6′-petamannosyl branch (composed of arms B and C), with the former type capped by three glucose residues. While still in ER, terminal glucoses in arm A and mannose in arm B are removed by glycosidases. The removal of glucose residues is catalyzed by glucosidases I and II (GI and GII, respectively), and the removal of the mannose residue is catalyzed by ER mannosidase. GI specifically trims the outermost α-(1→2)-linked glucose residue of G3M9 to produce $Glc_2Man_9GlcNAc_2$ (G2M9). Subsequently, GII removes a further two α-(1→3)-linked glucose residues in succession: cleavage-1, conversion from G2M9 to $Glc_1Man_9GlcNAc_2$ (G1M9); and cleavage-2, conversion from G1M9 to $Man_9GlcNAc_2$ (M9). These cleavage processes by GII have important implications for folding and quality control of nascent glycoproteins.[1)]

GII is a heterodimeric protein composed of tightly bound α- and β-subunits (GIIα and GIIβ, respectively). GIIα is the catalytic subunit and displays significant amino acid sequence similarity to glycoside hydrolase family 31 (GH31) α-glucosidases. GH31 α-glucosidases are widespread in many organisms and are believed to be involved in the degradation of starch and α-glucooligosaccharides (e.g., maltooligosaccharides and α-glucobioses). The catalytic domain of GH31 α-glucosidase adopts the $(\beta/\alpha)_8$-barrel fold and the active site is located in a pocket at the C-terminus of the inner β-barrel.[2–6)] Recently, the three-dimensional structures of GIIαs from *Chaetomium thermophilum* and murine were determined.[7,8)] The structures are almost identical to other GH31 α-glucosidases, except for an N-terminal segment. The structure of murine GIIα contains a portion of the GIIβ.[8)] This structure reveals that several salt bridges and hydrogen bonds are associated with the formation of the GII heterodimer. GIIβ is necessary for solubility, stability, activity and localization of GII.[9,10] GIIβ is involved in the identification of the substrate because it recognizes the arms B and C by its mannose 6-phosphate receptor homolog domain (MRH domain).[11,12] Structural analysis of the MRH domain of *Schizosaccharomyces pombe* GIIβ indicates that a tyrosine residue is closely associated with the binding of the mannose residue.[13]

GIIα without GIIβ exhibits hydrolysis activity toward chromogenic substrates such as *p*-nitrophenyl α-glucopyranoside (pNPG), but not the glucosidic residues in the physiological glycans, G2M9 and G1M9. Moreover, there are a few reports that GIIα can trim glucose residues in native glycans without GIIβ. GIIα from *Saccharomyces cerevisiae* can catalyze cleavage-1, but not cleavage-2 without GIIβ.[14] *S. pombe* GIIα (SpGIIα) exhibits limited cleavage-1 activity *in vivo*, but not *in vitro*.[12] *Bombyx mori* GIIα, a purified recombinant protein, shows weak cleavage-2 activity.[15] However, the detailed catalytic specificity of GIIα toward the natural *N*-glycan remains unresolved.

In this study, we revealed the substrate recognition of SpGIIα for the first time by successfully obtaining soluble recombinant SpGIIα, and the stability of recombinant SpGIIα was improved by coexistence with glycerol. Substrates used in this study were: i) substrates (maltooligosaccharides, α-glucobioses, and PNPG) of GH31 α-glucosidases, which share the common ancestral protein with GIIα; ii) dansyl substrates (G2M3-Dansyl and G1M3-Dansyl) mimicking arm A of *N*-glycan; and iii) natural *N*-glycan substrates (G2M9 and G1M9). SpGIIα hydrolyzes the substrates of GH31 α-glucosidase with broad specificity. Dansyl substrates and natural *N*-glycan substrates are also cleaved even without GIIβ. However, the hydrolysis rate on *N*-glycan substrates is slow and this observation suggests that the assistance of GIIβ is required for proper activity.

Material and methods

Materials. Bacto yeast extract, bacto peptone and bacto yeast nitrogen base without amino acids and ammonium sulfate were purchased from BD biosciences (Sparks, MD, USA). α,α-Trehalose, pNPG and glycerol were from Nacalai Tesque (Kyoto, Japan). The maltose used was manufactured by Nihon Shokuhin Kako, Tokyo, Japan. Kojibiose, nigerose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were purchased from Wako Pure Chemical Industries (Osaka, Japan). Isomaltose was from Tokyo Chemical Industry (Tokyo, Japan). Pyridylaminated G1M9 and G3M9 (G1M9-PA and G3M9-PA) were purchased from Masuda Chemical industries Co., Ltd. (Kagawa, Japan). All chemicals used were of analytical grade unless otherwise noted.

Strains and vectors. *Escherichia coli* (*E. coli*) strain DH5α was used for cloning, whereas BL21-CodonPlus (DE3)-RIL and -RP (Stratagene, La Jolla, CA, USA), and Rosetta ™ (DE3) (Novagen-Merck Millipore, Billerica, MA, USA) were used for recombinant protein expression. *E. coli* expression vectors, pCold I DNA and pET vectors (pET23d and pET41a), were purchased from Takara Bio (Otsu, Japan) and Novagen-Merck Millipore, respectively. *S. cerevisiae* NBRC 1136 (S288C) was from the National Institute of Technology and Evaluation Biological Resource Center, Kisarazu, Japan, and *Pichia pastoris* GS115 and the pPICZαA plasmid vector were from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA, USA). *S. pombe* AHU 3179 was kindly supplied by the Applied Microbiology Laboratory, Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan.

Cloning of the gls2 gene and construction of its expression plasmid. The *gls2* gene (SPAC1002.03c) encoding SpGIIα was amplified by PCR using genomic *S. pombe* DNA as the template using the two primers (sense_1 of 5′-TGTAACTTCTTCCCGGGAAAGATTCC-

3′ and antisence_1 of 5′-TTTCTCCATAAACGTTAAATATTGG-3′) and thermostable KOD-plus-DNA polymerase (Toyobo, Osaka, Japan). The PCR product was cloned into pBluescript II SK(+) (Stratagene) to obtain SK-gls2. The recombinant SpGIIα was produced as a fusion protein with a His$_6$-affinity tag at the C-terminus as follows. DNA encoding the predicted mature SpGIIα (Ala26 to Val923) was amplified by PCR using SK-gls2 as the template with primers: sense_2 (5'-TGCCCCATGGCATTTCGACATCAATTTAAA-3', the *Nco*I site is underlined) and antisense_2 (5'-AATCTCGAGAACCAAAAAAAGTTGTGGATT-3', the *Xho*I site is underlined). The presence and location of the signal peptide cleavage site was predicted by the Signal P server (http://www.cbs.dtu.dk/services/SignalP/).[16] The PCR product was digested with *Nco*I and *Xho*I, and then introduced into the *Nco*I–*Xho*I sites of pET23d to generate the pET23d-gls2 plasmid for recombinant SpGIIα production.

Cloning of the CWH41 gene and construction of its expression plasmid. The expression vector of the CWH41 gene (YGL027C) encoding *S. cerevisiae* processing glucosidase I (ScGI) was constructed according to previous reports.[17,18] The enzyme was a truncated form (Glu33 to Phe833) with the N-terminal *S. cerevisiae* α-factor secretion signal and a C-terminal His$_6$-affinity tag. ScGI was produced in *P. pastoris* using pPICZαA as follows. The CWH41 gene was amplified by PCR using genomic *S. cerevisiae* DNA as the template with primers: CWH41_F (5′-AAGTAGTGGATAATAACGGTTCAGG-3′) and CWH41_R (5′-CTTACTAGTAAGCGTCCAAGGATGTTGAC-3′, the *Spe*I site is underlined). The insertion of *Spe*I resulted in a substitution of an amino acid residue, Phe833→Leu. Primestar Max DNA polymerase (Takara Bio) was used as the thermostable DNA polymerase. The PCR product was cloned into pBluescript II SK(+) to obtain SK-CWH41. SK-CWH41 was digested by *Eco*RI and *Spe*I, cloned into the *Eco*RI–*Xho*I sites of pPICZαA, and designated as pPICZαA-CWH41 for the production of ScGI.

Production and purification of SpGIIα. E. coli BL21-CodonPlus (DE3)-RIL cells were transformed by pET23d-gls2. Transformants were selected on an LB agar plate (5 g/L NaCl, 10 g/L bacto tryptone, 5 g/L bacto yeast extract and 15 g/L agar), supplemented with 50 mg/L ampicillin and 30 mg/L chloramphenicol. A transformant was grown in 40 mL of LB liquid medium with 50 mg/L ampicillin and 30 mg/L chloramphenicol at 30°C overnight. The resultant 30 mL of overnight LB-culture was inoculated into TB medium (3 L; 12 g/L bacto tryptone, 24 g/L bacto yeast extract, 0.4% (v/v) glycerol, 170 mM KH_2PO_4 and 720 mM K_2HPO_4) supplemented with 50 mg/L ampicillin and cell culturing was continued at 37°C. When the OD_{600} reached 0.7, the culture broth was cooled on ice for 30 min. Induction of the culture was achieved at 12°C for 48 h without the addition of isopropyl β-thiogalactopyranoside (IPTG). Bacterial cells, harvested by centrifugation (11,600 × *g*, 4°C, 10 min), were suspended in 150 mL of 20 mM sodium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol (buffer-A) and disrupted by sonication. The cell-free extract, obtained by centrifugation (9,600 × *g*, 4°C, 10 min), was applied to a Co-chelating Sepharose Fast Flow column (1.5 cm I.D. × 4 cm, GE Healthcare, Buckinghamshire, United Kingdom) equilibrated with buffer-A containing 0.5 M NaCl (buffer-B). After thorough washing with buffer-B and 10 mM imidazole-containing buffer-B in this order, the adsorbed protein was eluted with a linear gradient of imidazole from 10 to 200 mM in buffer-B. The active fractions were loaded on to a Bio-Gel P6 fine column (3 cm I.D. × 28 cm, Bio-Rad, Richmond, CA) equilibrated with buffer-A. The desalted sample was then loaded onto a DEAE-Sepharose Fast Flow column (2.6 cm I.D. × 8.5 cm, GE Healthcare) equilibrated with buffer-A. After washing with buffer-A, the adsorbed protein was eluted with a linear gradient of NaCl from 0 to 1.0 M in buffer-A. The active fractions, desalted by the aforementioned procedure using Bio-Gel P6, were subjected to a second DEAE-Sepharose Fast Flow column chromatography (1.5 cm I.D.

× 6 cm) step. The adsorbed protein was eluted isocratically with 65 mM NaCl in buffer-A. The active fractions were pooled and desalted by Bio-Gel P6 using buffer-A. The purity of the protein was analyzed by SDS-PAGE. The concentration of the purified protein was estimated by amino acid analysis of the protein hydrolysate (6 M HCl, 110°C, 24 h) using JLC-500/V (JOEL, Tokyo, Japan) equipped with a ninhydrin-detection system. The extinction coefficient of 1 mg/mL purified SpGIIα at 280 nm was 2.27.

Production and purification of ScGI. Transformation of *P. pastoris* GS115 was performed as described by Lin-Cereghino *et al.*[19] pPICZαA-CWH41A was linearized with *Sac*I and introduced into *P. pastoris* cells by electroporation. Transformants were selected on YPDSZ agar plates (10 g/L bacto yeast extract, 20 g/L bacto peptone, 2 g/L glucose, 1 M sorbitol, 100 mg/L Zeocin and 15 g/L agar). The selected transformant was grown in BMGY medium [3L; 10 g/L bacto yeast extract, 20 g/L bacto peptone, 3.4 g/L yeast nitrogen base without amino acids and ammonium sulfate, 10 g/L ammonium sulfate, 0.1 M potassium phosphate buffer (pH 6.0), 10 g/L glycerol and 0.4 mg/L biotin] at 30°C overnight. The cells collected by centrifugation at 4°C for 10 min (3,000 × *g*) were resuspended in 500 mL of BMMY medium that contains 1% (v/v) methanol instead of the glycerol of BMGY and incubated at 22°C for 96 h under vigorous shaking. One hundredth of the culture volume of methanol was added every 24 h. The supernatant was removed by centrifugation (11,600 × *g*, 4°C, 10 min) and its pH was adjusted to 7.5 by addition of 1 M NaOH with stirring. Debris were removed by centrifugation (11,600 × *g*, 4°C, 10 min) and the recovered supernatant was loaded onto a Ni-chelating Sepharose Fast Flow column (1.5 cm I.D. × 5 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.5) containing 0.5 M NaCl (buffer-C). After washing with buffer-C and then 5 mM imidazole-containing buffer-C, the adsorbed protein was eluted with 200 mM imidazole in buffer-C. The fractions containing purified ScGI, which was determined by SDS-

PAGE, were dialyzed against 20 mM potassium phosphate buffer (pH 6.8) containing 0.1 M NaCl and concentrated using Centriprep YM-50 centrifugal filter units (Novagen-Merck Millipore).

Standard enzyme assay. The activity of SpGIIα was determined by measuring the increase of *p*-nitrophenol during hydrolysis of pNPG. A reaction mixture (50 μL) consisting of an appropriate concentration of SpGIIα, 2 mM pNPG, 40 mM MES-NaOH buffer (pH 6.5), 2% (v/v) glycerol and 0.2 mg/mL bovine serum albumin (BSA) was incubated at 30°C for 10 min. The reaction was stopped by mixing with two volumes of 1 M sodium carbonate. The amount of *p*-nitrophenol released was measured by absorption at 400 nm in a 1-cm cuvette, using a molar extinction coefficient of 5,560 M^{-1} cm^{-1}. One unit of SpGIIα activity was defined as the amount of enzyme that produced 1 μmol *p*-nitrophenol per min under these conditions.

Optimum pH and stability to pH and heat. The optimum pH of the catalytic reaction was determined by measuring the hydrolytic rate at various pH values. The SpGIIα concentration and buffer used were 8.2 μg/mL and 80 mM Britton–Robinson buffer (pH 2.7–11.5), respectively. The other reaction conditions were the same as those of the standard assay method. For measurement of pH stability, SpGIIα (82 μg/mL) was incubated in 10-fold-diluted Britton–Robinson buffer (pH 3.3 to 10.5) containing 0.1% BSA and 10% (v/v) glycerol at 4°C for 24 h, followed by measurement of the residual activity under the standard assay conditions. The stable region was defined as the pH range exhibiting residual activity of > 90%. For measurement of thermal stability, SpGIIα (2.7 μg/mL) in 67 mM MES-NaOH buffer (pH 6.5) containing 0.1% BSA and 10% (v/v) glycerol was kept at 26 to 55°C for 15 min, followed by measurement of their residual activities under the standard assay conditions.

The stable region was defined as the temperature range exhibiting residual activity of > 90%.

Effect of additives on enzyme stability. SpGIIα in 20 mM HEPES-NaOH (pH 7.5) was incubated with NaCl (0.05, 0.2, or 0.5 M), $CaCl_2$ (0.05 or 0.2 M), EDTA·2Na (0.1, 0.5, or 1 mM), glycerol [2, 10, or 20% (v/v)], or Triton X-100 [0.5 or 1% (v/v)] at 4°C. At the indicated time, an aliquot of the mixture was taken and diluted with 40 mM MES-NaOH (pH 6.5) containing 0.1% BSA and 10% (v/v) glycerol. The residual activity was evaluated by hydrolysis of 0.2% maltose at 30°C. The hydrolysis reaction was terminated by mixing with two volumes of 2 M Tris-HCl buffer (pH 7.0) and liberated glucose was measured with the Glucose C II-Test Wako (Wako Pure Chemical Industries).

Effect of various salts on the enzyme reaction. The enzyme reactions were performed in 40 mM MES-NaOH (pH 6.5) containing 1.6 μg/mL SpGIIα, 0.2% maltose, 2% (v/v) glycerol and 0.2 mg/mL BSA at 30°C for 10 min by adding salt (0–40 mM KCl, $MgCl_2$, or various sodium salts). The concentration of liberated glucose was measured, as described above.

Kinetic parameters for hydrolysis of various substrates. Kinetic parameters for hydrolysis of pNPG, nigerose, kojibiose and a series of maltooligosaccharides were calculated from the initial rates at various substrate concentrations by fitting to the Michaelis–Menten equation using KaleidaGraph 3.6J (Synergy Software, Reading, PA, USA). Substrate concentrations were as follows: 0.4–15 mM for pNPG, 0.5–10 mM for nigerose, 1–80 mM for kojibiose, 3.2–80 mM for maltose, 2.0–64 mM for maltotriose, 2.0–80 mM for maltotetraose and maltopentaose, 2.0–40 mM for maltohexaose and 0.8–40 mM for maltoheptaose. Since the K_m value for isomaltose was so large, its k_{cat}/K_m value was determined from the slope of Lineweaver-Burk plots at concentrations from 16 to 80 mM.

The amount of *p*-nitrophenol released from pNPG was measured by absorption at 400 nm, as described above. For other substrates, the liberated glucose was measured as described above.

Measurement of the hydrolytic rates of G2M3-Dansyl and G1M3-Dansyl. The hydrolytic rates for G2M3-Dansyl and G1M3-Dansyl, both of which were prepared as described previously,[20,21] and nigerose were determined by measuring the liberated glucose concentration. A reaction mixture (180 μL) consisting of SpGIIα (0.35 μg/mL for nigerose and G2M3-Dansyl, 1.8 μg/mL for G1M3-Dansyl), 0.4 mM substrate, 40 mM MES-NaOH buffer (pH 6.5), 2% (v/v) glycerol and 0.2 mg/mL BSA was incubated at 30°C. At 5, 10, 15 and 20 min, an aliquot of the reaction mixture was taken and heated at 100°C for 3 min to terminate the reaction. The glucose concentration was measured by high-performance anion exchange chromatography [Dionex ICS-3000 system with pulsed amperometric detection (Dionex/Thermo Fisher Scientific, Idstein, Germany)]. The analytical column (CarboPac PA1, 4 mm I.D. × 250 mm, Dionex/Thermo Fisher Scientific) was equilibrated by 100 mM NaOH at a flow speed of 0.8 mL min^{-1}. Separations were performed with the 6 min-linear gradient of 100–640 mM NaOH for nigerose and with the 42 min-linear gradient of 0–600 mM sodium acetate in 100 mM NaOH for dansyl oligosaccharides. The NaOH solution was prepared from super special grade 50% NaOH (Wako Pure Chemical Industries). Standards and samples contained 0.1 mM fructose as an internal reference. Peak areas and retention times of eluted carbohydrates were evaluated with the Chromeleon software (Dionex/Thermo Fisher Scientific). The concentration of glucose in each sample was calculated from its peak area using a calibration curve prepared from standard glucose and internal fructose.

Detection of deglucosylation of pyridylaminated oligosaccharides. A reaction mixture (100 μL) consisting of SpGIIα (1.04 μg/mL), 0.4 μM substrate (G1M9-PA or G3M9-PA), 40 mM MES-NaOH buffer (pH 6.5), 2% (v/v) glycerol and 0.2 mg/mL BSA was incubated at 30°C. For ScGI-catalyzed hydrolysis of the outermost glucose residue of G3M9-PA, 2.1 μg/mL ScGI was used under the same reaction conditions as SpGIIα. The reaction was terminated by incubation at 95°C for 5 min. The oligosaccharides were separated by high-performance liquid chromatography (HPLC) using an Asahipack NH2-50-4E column (4.6 mm I.D. × 250 mm, Shodex, Tokyo, Japan) at 40°C. The elution was done at a flow speed of 0.8 mL min^{-1} with a linear gradient of acetonitrile from 68 to 34% in 0.3% ammonium acetate buffer (pH 7.0). Pyridylaminated oligosaccharides were monitored by the fluorescence signal (excitation wave length, 310 nm; emission wavelength, 380 nm) using a fluorescence detector FP-2020 Plus (Jasco, Tokyo, Japan).

Results and discussion

Production and characterization of SpGIIα

The expression conditions for obtaining soluble recombinant SpGIIα in *E. coli* were examined. The DNA coding for the mature SpGIIα was in-frame inserted into different expression plasmids (pET23d, pCold I, or pET41a) and enzyme production was tested using three different *E. coli* strains [Rosetta (DE3), BL21-CodonPlus (DE3)-RIL, or -RP]. In most expression systems tested, recombinant SpGIIα was produced as an insoluble form; however, the BL21-CodonPlus (DE3)-RIL strain transformed with the pET23d was the best combination for soluble protein production. Furthermore, cultivation in TB medium supplemented with 50 μg/mL ampicillin at 12°C without IPTG induction was also effective for production of soluble SpGIIα.

The induced cells cultivated under the optimum conditions for 48 h were disrupted in 20 mM sodium phosphate buffer (pH 7.5) including 10% glycerol, and the crude extract obtained contained 11.5 U per 1 L culture. The presence of glycerol in both the purification and storage buffers was essential for preventing SpGIIα from inactivation, as described below. Recombinant SpGIIα produced by *E. coli* was purified using cobalt-affinity and anion-exchange chromatographies. The final desalting column step yielded 1.8 mg of electrophoretically homogeneous SpGIIα (Fig. 1) from 3 L of culture with specific activity of 4.0 U/mg.

Figure 2 shows the effects of various additives on the stability of SpGIIα. The residual activity of SpGIIα without any additive decreased to < 40% of the initial activity after 2 d (Fig. 2A), and to < 1% after 23 d (Figs. 2B, C). Inhibitory effect was observed when the protein was incubated with NaCl, $CaCl_2$ and Triton X-100 (Fig. 2A). In particular, addition of $CaCl_2$ and Triton X-100 induced substantially activity loss. Both glycerol and EDTA as additives improved the stability of recombinant SpGIIα (Figs. 2B, C). It is hard to understand how EDTA contributes to the stability of SpGIIα. It is anticipated that EDTA removes contaminating divalent ions such as the calcium ion, which adversely affects the SpGIIα stability. The addition of ≥ 10% (v/v) glycerol was more effective in stabilizing SpGIIα than EDTA, with the enzyme maintaining > 90% activity after 92 d (Fig. 2C). Glycerol is known to induce protein compaction, reduce protein flexibility, stabilize specific partially unfolded intermediates and affect protein aggregation,[22)] resulting in its wide use as a protein stabilizer. Based on the effect of glycerol on protein stability, Vangenende *et al*. proposed that glycerol prevents protein aggregation thorough preferential interaction with hydrophobic surface regions.[22)] In nature, GIIβ forms a heterodimer with GIIα. Thus, glycerol was anticipated to affect the exposed dimer interface of GIIβ lacking the GIIα subunit, because generally an oligomer interface is hydrophobic. However, the three-dimensional structure of murine GIIα complexed with an N-terminal portion of GIIβ showed that the interface area is not that hydrophobic,[8)] suggesting less hydrophobicity of the equivalent regions of SpGIIα. Therefore, it is possible that SpGIIα has other surface exposed hydrophobic regions where glycerol interacts to prevent instability of the protein. Nonetheless, stabilization conditions found in this study allow us to perform purification and characterization of SpGIIα.

The effects of various salts on the activity were investigated by measuring the initial hydrolytic velocity on maltose using 2% (v/v) glycerol. NaCl, NaH_2PO_4, Na_2SO_4, $NaNO_3$, KCl, $MgCl_2$, or EDTA·2Na with concentrations up to 40 mM did not affect activity, while the addition of 40 mM $CaCl_2$ decreased to 63% of the original initial velocity. The calcium ion should be harmful to both activity and stability of SpGIIα. As shown in Fig. 2A, 50 mM $CaCl_2$ causes its inactivation within 2 d, which shows a negative effect of this salt on its stability.

The effects of pH and temperature on the activity were examined in the presence of 10% (v/v) glycerol. The pH optimum was 6.5 and SpGIIα was stable between pH 6.2 and 9.1. SpGIIα was thermally stable to 40°C with complete inactivation observed at 55°C (15 min treatment). In the absence of glycerol, this thermal stability was reduced to < 30% of the initial activity observed when incubated in the presence of glycerol for 15 min at 40°C.

Substrate specificity of SpGIIα: substrates for GH31 α-glucosidase

Substrate specificity of GIIα is of interest, because this protein has a common ancestor with GH31 α-glucosidases, which can hydrolyze α-(1→2)-, α-(1→3)-, α-(1→4)- and α-(1→6)-glucosidic linkages.[23–27)] By primarily exhibiting specificity to α-(1→4)-glucosidic linkage, GH31 α-glucosidases are mostly linked with metabolic pathways of starch and maltooligosaccharides degradation.[28)] While GIIα plays a different role in biological processes when compared with that of GH31 α-glucosidases, both enzymes might display similar substrate specificities, because catalytic function of a protein is generally conserved during molecular evolution. Substrate specificity of SpGIIα towards common substrates of α-glucosidases was evaluated by determining kinetic parameters (Table 1). SpGIIα hydrolyzed all substrates tested, proving broader specificity than ER-resident processing GI, which recognizes the outermost α-1,2-glucosy residue in the *N*-glycan so strictly that kojibiose acts as an inhibitor.[29,30] Among the disaccharides, nigerose was the best substrate with the highest k_{cat}/K_m value (Table 1). The K_m values for maltose and kojibiose were around 10 times as large as that for nigerose, and that for isomaltose could not be obtained due to unsaturated curve in $s - v$ plots up to 80 mM isomaltose, indicating that SpGIIα displays weaker binding to α-(1→4)-, α-(1→2)- and α-(1→6)-glucosidic linkages. This specificity is similar to the GH31 α-1,3-glucosidase, which hydrolyzes α-(1→4)-, α-(1→2)- and α-(1→3)-glucosidic linkages together with weak specificity towards the α-(1→6)-glucosidic linkage.[31–33] This enzyme has been found in some microorganisms and is hypothesized to be involved in a metabolic pathway rather than the trimming of sugar chains.[31-33] Hydrolysis of pNPG was characterized by low k_{cat} and K_m values, which are common features of the GH31 α-glucosidases and the α-1,3-glucosidase.[23–25,28,31,32] These results demonstrate that SpGIIα conserves substrate specificity while its localization and physiological role are different. The higher k_{cat}/K_m value for maltose than that for malto-triose, -tetraose and -pentaose (Table 1) indicates that SpGIIα prefers maltose, and this observation is consistent with the structure of the active-site pocket of GIIα being capable of accommodating only disaccharides.[7,8] The k_{cat}/K_m values for malto-hexaose and -heptaose increased 3.0 to 5.2 times over -triose, -tetraose and -pentaose. The GH31 sugar beet α-glucosidase possesses the machinery that accommodates long-chain substrates,[34] but SpGIIα has no equivalent element. Thus, it is difficult to explain the increase in specificity toward longer-chain substrates.

Substrate specificity of SpGIIα: N-*glycan-relating substrates*

We measured the hydrolytic rates on 0.4 mM G2M3-Dansyl and G1M3-Dansyl, which mimic arm A of *N*-glycan, together with 0.4 mM nigerose for comparison. SpGIIα was able to hydrolyze the α-glucosidic linkage in G2M3-Dansyl and G1M3-Dansyl with hydrolytic rates of 1.23 and 0.274 μmol min^{-1} mg^{-1}, respectively, although these values were slower than that of nigerose (2.42 μmol min^{-1} mg^{-1}). The difference in hydrolytic rates between G2M3-Dansyl and G1M3-Dansyl is in agreement with the observation that GII catalyzes the hydrolysis of the α-Glu-(1→3)-Glc linkage (cleavage-1) faster than that of the α-Glu-(1→3)-Man linkage (cleavage-2).[35,36)] The difference in the catalytic rate between both cleavages was accounted for by binding of GIIβ to mannose residues in arms B and C.[35–37)] Alternatively, recent crystal structure analyses proposed that an interaction between OH-2 of a mannose residue and the carboxy group of the acid/base catalyst of GIIα is responsible for the slow reaction rate of cleavage-2.[7,8)] Our SpGIIα, devoid of GIIβ, shows cleavage-2, corroborating the latter explanation.

GIIα without the GIIβ is able to hydrolyze much smaller substrates, such as pNPG, but not able to deglucosylate G2M9 and G1M9. These distinct results are also reported as follows. Stigliano *et al.* demonstrated that SpGIIα was able to degrade G2M9 and G1M9 *in vivo*.[12)] GIIα in *S. cerevisiae* catalyzes cleavage-1 but not cleavage-2.[14)] GIIα from *B. mori* weakly hydrolyzes G1M9 *in vitro*.[15)] According to these observations, GIIα does not seem to require GIIβ to hydrolyze the α-glucosidic linkages in *N*-glycan. We thus examined hydrolysis of G1M9-PA and G3M9-PA using the purified SpGIIα to evaluate its hydrolytic ability toward *N*-glycan. SpGIIα weakly but clearly catalyzed cleavage-2 to generate M9-PA from G1M9-PA (Fig. 3A). After 1 h of the reaction, the peak of M9-PA appeared, and then increased to the same level of G1M9-PA after the reaction had proceeded for 12 h. As shown in Fig. 3B, SpGIIα cannot degrade G3M9-PA (Fig. 3B), which coincides with the general specificity of GII. Conversion from G3M9 to G2M9 is known to be catalyzed by GI, which removes the outermost α-Glc-(1→2)-Glc linkage of G3M9. ScGI rapidly hydrolyzed G3M9-PA to produce G2M9-PA (Fig. 3C). Figure 3D shows the hydrolysis of G3M9-PA in the presence of SpGIIα and ScGI, which demonstrates that G2M9-PA, generated by the rapid reaction of ScGI until

1 h, is degraded to G1M9-PA and M9-PA at 12 h. These results indicate that the purified SpGIIα catalyzes the trimming of glucose residues from G2M9-PA and G1M9-PA without the assistance of GIIβ. Although the purified SpGIIα certainly catalyzes cleavage-1 and -2, their reaction rates are not high (Figs. 3A, 3D). According to the result of Watanabe *et al*.,[37] 50% conversion of 25 μM G1M9 to M9 requires 45 min by a membrane fraction containing GII, even though the accurate concentration of GII was unknown. The present study provides a more quantitative analysis in which 9.6 nM SpGIIα (1.0 μg/mL SpGIIα) and a 12-h reaction yield approximately 50% conversion of 0.4 μM G1M9 (Fig. 3A). The low hydrolysis of G1M9 and G2M9 by SpGIIα is probably because of insufficient binding energy for efficiently hydrolyzing these natural substrates, because SpGIIα can only hold the small terminal moiety of the comparatively large structure of G1M9 or G2M9. The active-site pocket of GIIαs has the gourd-shaped bilocular pocket, which can accommodate only disaccharides,[7,8] and GIIβ would be necessary to stabilize the enzyme-substrate complex. Olson *et al*. demonstrates an importance of the MRH domain of GIIβ to the hydrolytic activity of GIIα through a recognition of arms B and C of the natural *N*-glycan. [13] We need to direct our future studies to understanding the effect of GIIβ on the catalytic properties including substrate recognition.

Author contributions

Conceived and designed the experiments: M. Okuyama. Performed the experiments: M. Okuyama, M. Miyamoto, I. Matsuo, S. Iwamoto, R. Serizawa and M. Tanuma. Contributed reagents/materials/analysis tools: M. Ma, P Klahan and Y. Kumagai. Wrote the paper: M. Okuyama, T. Tagami and A. Kimura.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

1. Parodi AJ. Role of *N*-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. Biochem. J. 2000;348:1–13.

2. Ernst HA, Lo Leggio L, Willemoes M, et al. Structure of the *Sulfolobus solfataricus* α-glucosidase: Implications for domain conservation and substrate recognition in GH31. J. Mol. Biol. 2006;358:1106–1124.

3. Sim L, Quezada-Calvillo R, Sterchi EE, et al. Human intestinal maltase-glucoamylase: crystal structure of the N-terminal catalytic subunit and basis of inhibition and substrate specificity. J. Mol. Biol. 2008;375:782–792.

4. Tan K, Tesar C, Wilton R, et al. Novel α-glucosidase from human gut microbiome: substrate specificities and their switch. FASEB J. 2010;24:3939–3949.

5. Ren L, Qin X, Cao X, et al. Structural insight into substrate specificity of human intestinal maltase-glucoamylase. Protein Cell. 2011;2:827–836.

6. Tagami T, Yamashita K, Okuyama M, et al. Molecular basis for the recognition of long-chain substrates by plant α-glucosidases. J. Biol. Chem. 2013;288:19296–19303.

7. Satoh T, Toshimori T, Yan G, et al. Structural basis for two-step glucose trimming by glucosidase II involved in ER glycoprotein quality control. Sci. Rep. 2016;6:20575.

8. Caputo AT, Alonzi DS, Marti L, et al. Structures of mammalian ER α-glucosidase II capture the binding modes of broad-spectrum iminosugar antivirals. Proc. Natl. Acad. Sci. USA. 2016;113:E4630–E4638.

9. Pelletier MF, Marcil A, Sevigny G, et al. The heterodimeric structure of glucosidase II is required for its activity, solubility, and localization *in vivo*. Glycobiology. 2000;10:815–827.

10. Treml K, Meimaroglou D, Hentges A, et al. The α- and β-subunits are required for expression of catalytic activity in the hetero-dimeric glucosidase II complex from human liver. Glycobiology. 2000;10:493–502.

11. Totani K, Ihara Y, Matsuo I, et al. Substrate specificity analysis of endoplasmic reticulum glucosidase II using synthetic high mannose-type glycans. J. Biol. Chem. 2006;281:31502–31508. .

12. Stigliano ID, Caramelo JJ, Labriola CA, et al. Glucosidase II β subunit modulates *N*-glycan trimming in fission yeasts and mammals. Mol. Biol. Cell. 2009;20:3974–3984. .

13. Olson LJ, Orsi R, Peterson FC, et al. Crystal structure and functional analyses of the lectin domain of glucosidase II: Insights into oligomannose recognition. Biochemistry. 2015;54:4097–4111.

14. Wilkinson BM, Purswani J, Stirling CJ. Yeast GTB1 encodes a subunit of glucosidase II required for glycoprotein processing in the endoplasmic reticulum. J. Biol. Chem. 2006;281:6325–6333.

15. Watanabe S, Kakudo A, Ohta M, et al. Molecular cloning and characterization of the α-glucosidase II from *Bombyx mori* and *Spodoptera frugiperda*. Insect Biochem. Mol. Biol. 2013;43:319–327.

16. Petersen TN, Brunak S, von Heijne G, et al. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods. 2011;8:785–786.

17. Faridmoayer A, Scaman C. Truncations and functional carboxylic acid residues of yeast processing α-glucosidase I. Glycoconj. J. 2007;24:429–437

18. Barker MK, Wilkinson BL, Faridmoayer A, et al. Production and crystallization of processing α-glucosidase I: *Pichia pastoris* expression and a two-step purification toward structural determination. Protein Expr. Purif. 2011;79:96–101.

19. Lin-Cereghino J, Wong WW, Xiong S, et al. Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast *Pichia pastoris*. Biotechniques. 2005;38:44–48.

20. Iino K, Iwamoto S, Kasahara Y,et al. Facile construction of 1,2-cis glucosidic linkage using sequential oxidation–reduction route for synthesis of an ER processing α-glucosidase I substrate, Tetrahedron Lett. 2012;53:4452-4456.

21. Iwamoto S, Kasahara Y, Kamei K, et al. Measurement of endo-α-mannosidase activity using a fluorescently labeled oligosaccharide derivative. Biosci. Biotechnol. Biochem. 2014;78:927–936.

22. Vagenende V, Yap MGS, Trout BL. Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. Biochemistry. 2009;48:11084–11096.

23. Okuyama M, Tanimoto Y, Ito T, et al. Purification and characterization of the hyper-glycosylated extracellular α-glucosidase from *Schizosaccharomyces pombe*. Enzyme Microb. Technol. 2005;37:472–480.

24. Nakai H, Ito T, Hayashi M, Kamiya K, et al. Multiple forms of α-glucosidase in rice seeds (*Oryza sativa* L., var Nipponbare). Biochimie. 2007;89:49–62.

25. Sato F, Okuyama M, Nakai H, et al. Glucoamylase originating from *Schwanniomyces occidentalis* is a typical α-glucosidase. Biosci. Biotechnol. Biochem. 2005;69:1905–1913.

26. Tagami T, Okuyama M, Nakai H,et al. Key aromatic residues at subsites + 2 and + 3 of glycoside hydrolase family 31 α-glucosidase contribute to recognition of long-chain substrates. Biochim. Biophys. Acta. 2013;1834:329–335.

27. Saburi W, Okuyama M, Kumagai Y, et al. Biochemical properties and substrate recognition mechanism of GH31 α-glucosidase from *Bacillus* sp. AHU 2001 with broad substrate specificity. Biochimie. 2015;108:140–148.

28. Frandsen TP, Svensson B. Plant α-glucosidases of the glycoside hydrolase family 31. Molecular properties, substrate specificity, reaction mechanism, and comparison with family members of different origin. Plant. Mol. Biol. 1998;37:1–13.

29. Dhanawansa R, Faridmoayer A, van der Merwe G, et al. Overexpression, purification, and partial characterization of *Saccharomyces cerevisiae* processing α-glucosidase I. Glycobiology. 2002;12:229–234. .

30. Miyazaki T, Matsumoto Y, Matsuda K, et al. Heterologous expression and characterization of processing α-glucosidase I from *Aspergillus brasiliensis* ATCC 9642. Glycoconj. J. 2011;28:563–571.

31. Yamamoto T, Unno T, Watanabe Y, et al. Purification and characterization of *Acremonium implicatum* α-glucosidase having regioselectivity for α-1,3-glucosidic linkage. Biochim. Biophys. Acta. 2004;1700:189–198.

32. Kang M-S, Okuyama M, Mori H, et al. The first α-1,3-glucosidase from bacterial origin belonging to glycoside hydrolase family 31. Biochimie. 209;91:1434–1442.

33. Song KM, Okuyama M, Kobayashi K, et al. Characterization of a glycoside hydrolase family 31 α-glucosidase involved in starch utilization in *Podospora anserina*. Biosci. Biotechnol. Biochem. 2013;77:2117–2124.

34. Tagami T, Yamashita K, Okuyama M, et al. Structural advantage of sugar beet α-glucosidase to stabilize the Michaelis complex with long-chain substrate. J. Biol. Chem. 2015;290:1796–1803.

35. Kaushal GP, Pastuszak I, Hatanaka K, et al. Purification to homogeneity and properties of glucosidase II from mung bean seedlings and suspension-cultured soybean cells. J. Biol. Chem. 1990;265:16271–16279.

36. Totani K, Ihara Y, Matsuo I, et al. Effects of macromolecular crowding on glycoprotein processing enzymes. J. Am. Chem. Soc. 2008;130:2101–2107.

37. Watanabe T, Totani K, Matsuo I, et al. Genetic analysis of glucosidase II β-subunit in trimming of high-mannose-type glycans. Glycobiology. 2009;19:834–840.

38. Deprez P, Gautschi M, Helenius A. More than one glycan is needed for ER glucosidase II to allow entry of glycoproteins into the calnexin/calreticulin cycle. Mol. Cell. 2005;19:183–195.

Table

Table 1. Kinetic parameters of SpGIIα hydrolysis of GH31 α-glucosidase substrates.

Substrate	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} M^{-1})
*p*NPG	3.62 ± 0.02	0.0150 ± 0.0001	4.15
Nigerose	2.13 ± 0.06	0.102 ± 0.001	48.1
Kojibiose	21.0 ± 1.6	0.0575 ± 0.001	2.75
Isomaltose	N.D.	N.D.	0.014 ± 0.007
Maltose	18.0 ± 0.1	0.241 ± 0.007	13.4
Maltotriose	16.0 ± 0.3	0.0343 ± 0.0001	2.15
Maltotetraose	28.5 ± 2.5	0.0477 ± 0.0012	1.68
Maltopentaose	26.7 ± 0.2	0.0367 ± 0.0004	1.38
Maltohexaose	15.3 ± 0.6	0.109 ± 0.001	7.15
Maltoheptaose	15.0 ± 0.2	0.0965 ± 0.0007	6.43

N.D.: Values could not be determined because *s*-*v* plots were not saturated.

Figure captions

Fig. 1. Expression and purification of recombinant SpGIIα.

Notes: Expression and purification of GIIα were analyzed by 10% SDS–PAGE. Lane 1, IPTG-induced whole *E. coli* cells harboring pET23d-gls2; lane 2, supernatant from cell lysate of lane 1; lane 3, eluate from Co^{2+} affinity chromatography; lane 4, Bio-Gel P6 chromatography (1st); lane 5, anion exchange chromatography (1st); lane 6, Bio-Gel P6 chromatography (2nd); lane 7, anion exchange chromatography (2nd); lane 8, Bio-Gel P6 chromatography (3rd); lane M, marker proteins with molecular masses (kDa) shown at the side.

Fig. 2. Effect of additives on long-term stability of SpGIIα.

Notes: (A) Residual activity of GIIα was measured after incubation with various additives at 4 °C for 2 days. (B) Time course of residual activity of GIIα in the presence of 0.1 mM (○), 0.5 mM (■) and 1 mM (□) EDTA·2Na. (C) Time course of residual activity of GIIα in the presence of 2% (○), 10% (■), and 20% (□)glycerol. The residual activity without any additives is represented by closed circles (●) in both (B) and (C).

Fig. 3. HPLC analysis of the hydrolysates of (A) G1M9 by SpGIIα, (B) G3M9 by SpGIIα, (C) G3M9 by ScGI, and (D) G3M9 by ScGI and SpGIIα.

Notes: The reaction mixture samples were taken at 0, 1 and 12 h and were analyzed by HPLC. Symbol used for the structure formulae: ▲, glucose; ○, mannose; and ■, *N*-acetylglucosamine.

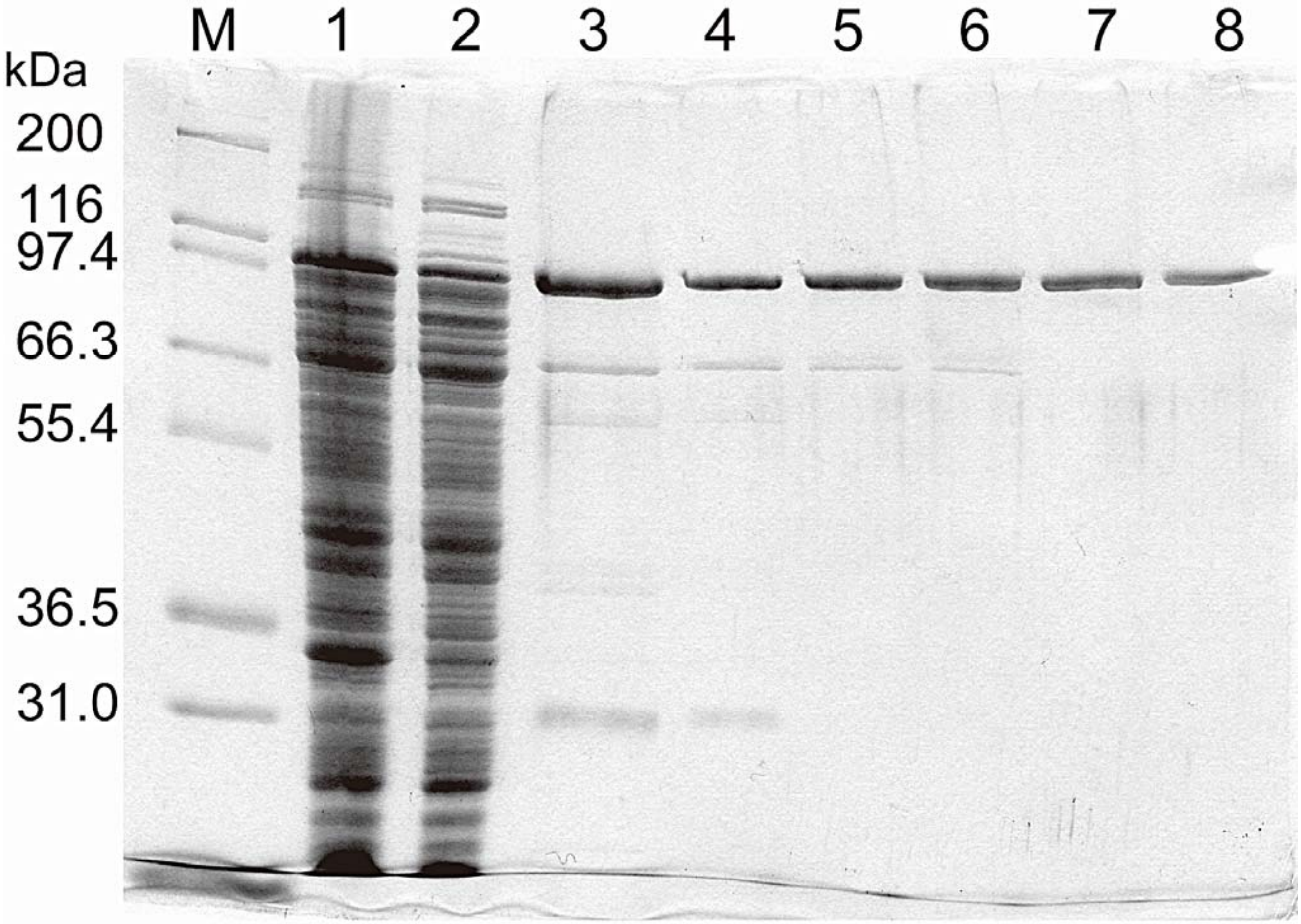

Figure 1. Okuyama et al

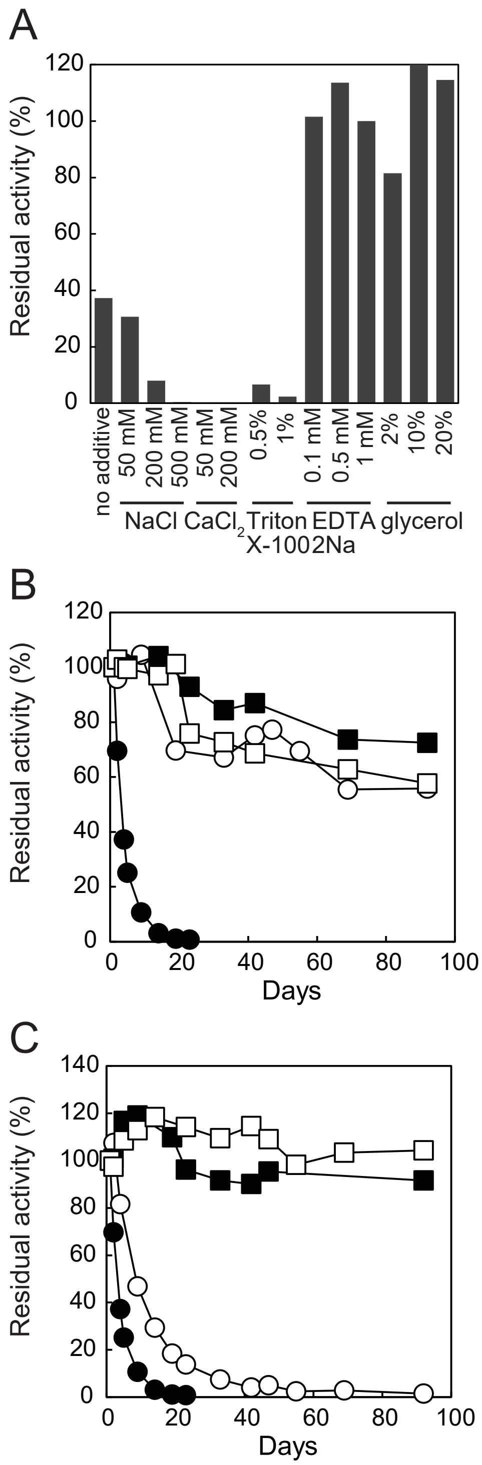

Figure 2. Okuyama et al

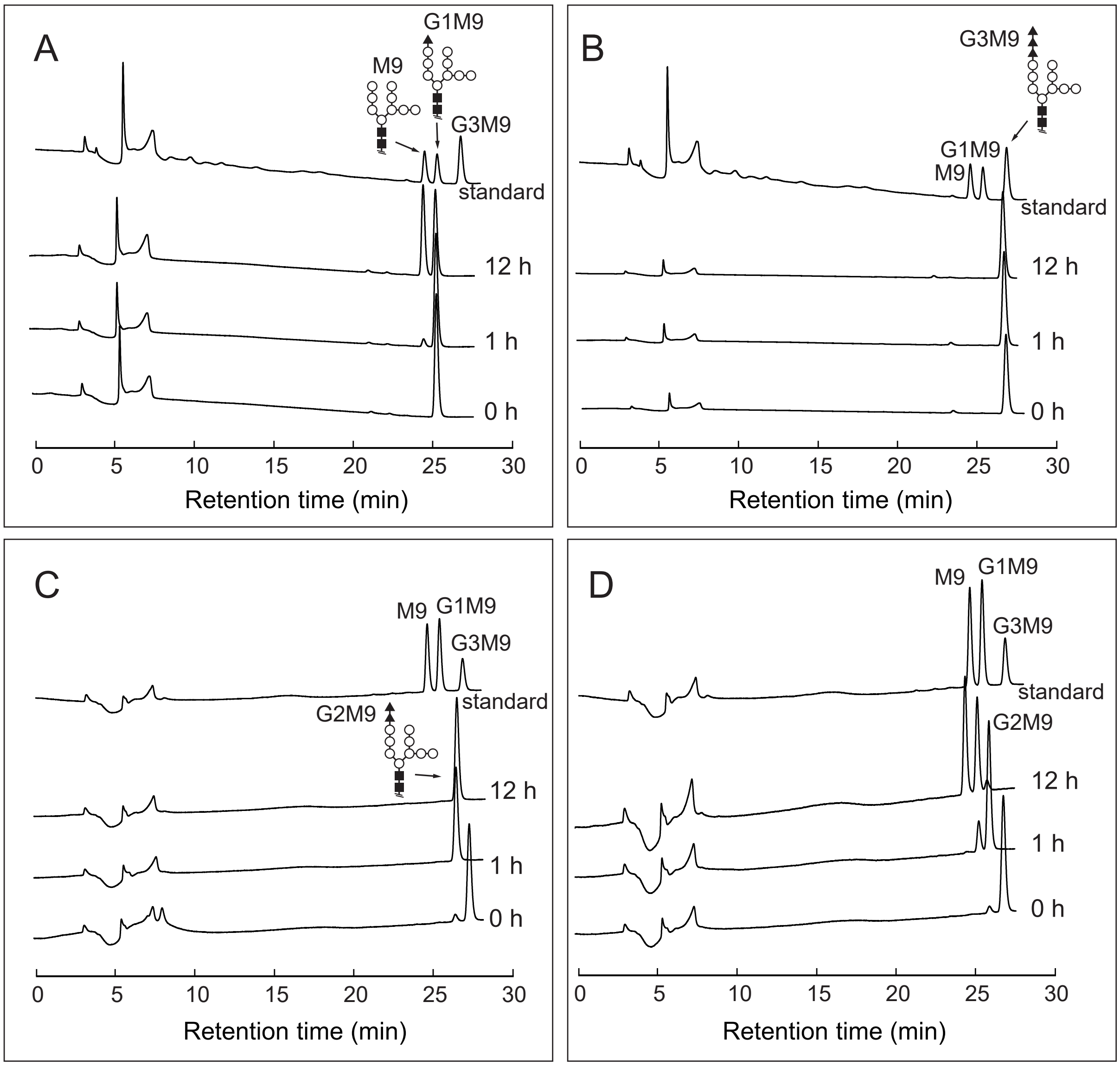

Figure 3. Okuyama et al