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In vitro mannose trimming property of human ER α-1,2 mannosidase I

Jun-ichi Aikawa · Ichiro Matsuo · Yukishige Ito

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Abstract Endoplasmic reticulum α-1,2 mannosidase I (ERManI) is an enzyme, which removes α(1-2) linked mannoses from asparagine-linked oligosaccharides on glycoproteins in the endoplasmic reticulum (ER). ERManI preferentially removes one α(1-2) linked mannose from B-chain of $Man_9GlcNAc_2$. When glycoproteins fail to achieve properly folding, increased removal of α(1-2) linked mannoses on their oligosaccharides is induced and leads them to be disposed and degraded by ER-associated degradation pathway. However, it is still inconclusive whether accelerated removal of α(1-2) linked mannoses on those glycoproteins is catalyzed by the α-1,2 mannosidase I, proteins similar to mannosidase I [*e.g.* ER degradation-enhancing α-1,2 mannosidase-like protein (EDEM)], or both of them. Therefore, to approach this issue, we have investigated its *in vitro* activities using various oligosaccharides and glycoproteins as substrates. A recombinant form of human ERManI (hERManI) was prepared by using *Escherichia coli*. First, the enzyme generated $Man_6GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA from 100 μM $Man_9GlcNAc_2$-PA after a one-hour reaction. Second, we have exposed bovine thyroglobulin and soybean agglutinin to denaturing conditions, *e.g.* 8 M urea, and used those glycoproteins as substrates. Sugar moieties were released from the reactant by PNGase F and their structures and amounts were elucidated by HPLC analysis. Intriguingly, the enzyme was shown to remove mannoses from bovine thyroglobulin and soybean agglutinin to larger extents when they were exposed to a denaturant. Therefore, our results suggested that hERManI could recognize tertiary and/or quaternary structures of glycoproteins and remove more α-1,2 linked mannoses from misfolded glycoproteins in living cells.

Keywords *N*-glycosylation · Mannose · Pyridylamine · Glycoprotein · Denatured · HPLC

Abbreviations

bTg	Bovine thyroglobulin
EDEM	Endoplasmic reticulum degradation-enhancing α-1,2 mannosidase-like protein
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ERManI	Endoplasmic reticulum α-1,2 mannosidase I
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
hERManI	Human endoplasmic reticulum α-1,2 mannosidase I
Man	Mannose
PA	Pyridylamine
SBA	Soybean agglutinin
UGGT	UDP-glucose:glycoprotein glucosyltransferase

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J.-i. Aikawa (✉) · Y. Ito
RIKEN Advanced Science Institute,
2-1 Hirosawa,
Wako, Saitama 351-0198, Japan
e-mail: aikawa@riken.jp

I. Matsuo
Graduate School of Engineering, Gunma University,
1-5-1 Tenjin-cho,
Kiryu, Gunma 376-8515, Japan

Y. Ito
ITO Glycotrilogy Project, ERATO, JST,
2-1 Hirosawa,
Wako, Saitama 351-0198, Japan

Introduction

In eukaryotes, asparagine-linked glycosylation (*N*-glycosylation) of polypeptides plays significant roles in various biological events by modulation of protein properties *e.g.,* activity and stability [1]. The *N*-glycosylation is introduced co-translationally by *en bloc* transfer of a tetradecasaccharide (Glucose $(Glc)_3$-Mannose $(Man)_9$-*N*-acetylglucosamine $(GlcNAc)_2$) (Fig. 1a) from dolicholpyrophosphate to an asparagine residue of consensus triad Asn-X-Thr/Ser or rarely Asn-X-Cys (X; any amino acid except Pro) of nascent polypeptides in the ER [2–4]. Subsequent trimming of three α-linked glucose residues is then conducted by the action of α-glucosidases, giving $Man_9GlcNAc_2$ in a stepwise manner (pathway A in Fig. 1b). A series of studies concluded that an α-1,2 linked mannose on the B-chain of $Man_9GlcNAc_2$ is selectively removed by ERManI (pathway B in Fig. 1b). In a majority of cases, thus formed $Man_{8B}GlcNAc_2$ carrying glycoproteins (Fig. 1a) are transported to Golgi apparatus, where they will be further modified by glycosidases and glycosyltransferases (pathway C in Fig. 1b). If glycoproteins harboring two α-1,2 linked mannoses on A-chain of *N*-linked sugar chain are folded improperly or remain unfolded (pathway D in Fig. 1b), so-called "calnexin/calreticulin cycle" assists them in achieving correct folding (pathway E in Fig. 1b) [5]. Failure to achieve this even after repeated engagement in this process will force proteins to be either (1) disposed to the cytosol after removal of α-1,2 linked mannoses and further degraded by ERAD pathway or (2) accumulated in the ER (pathway F in Fig. 1b) [6, 7].

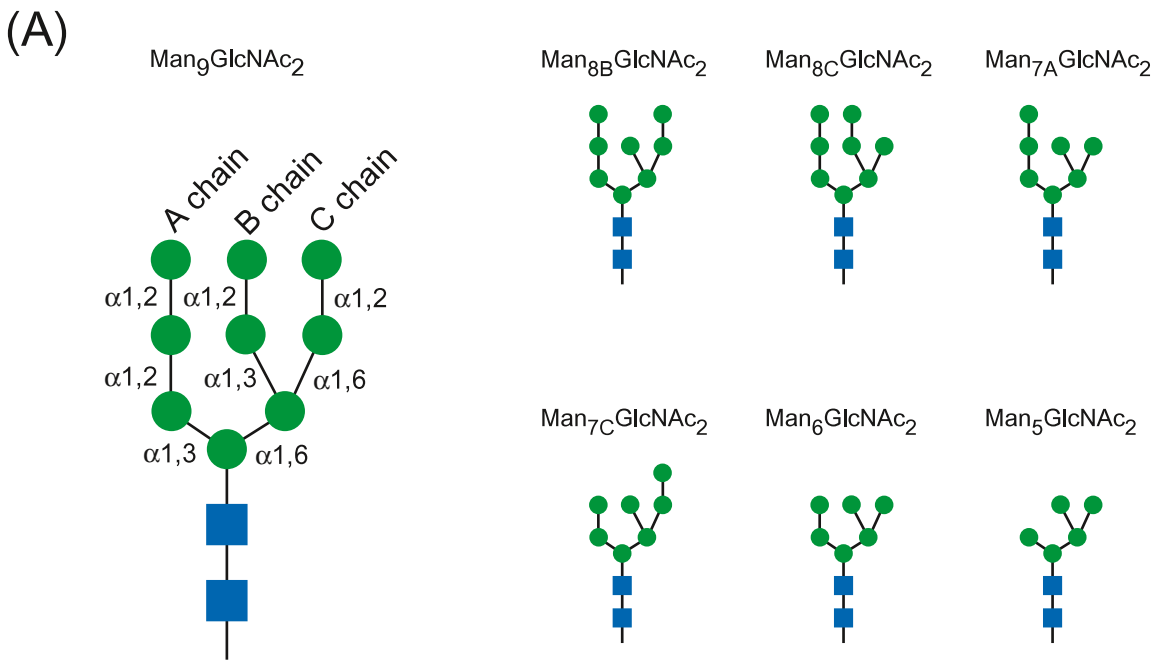
In total, there are four α(1-2) linked mannose residues in fully mannosylated glycans, such as $Man_9GlcNAc_2$ (Fig. 1a). Removal of them in the ER is catalyzed by α-1,2 mannosidases that belong to glycosylhydrolase family 47 [8]. In mammals, seven members of α-1,2 mannosidase have been known; ERManI, Golgi α-1,2 mannosidase IA, IB & IC, and EDEM1, 2 & 3. However, it is still controversial if EDEMs are *bona fide* mannosidases [9, 10].

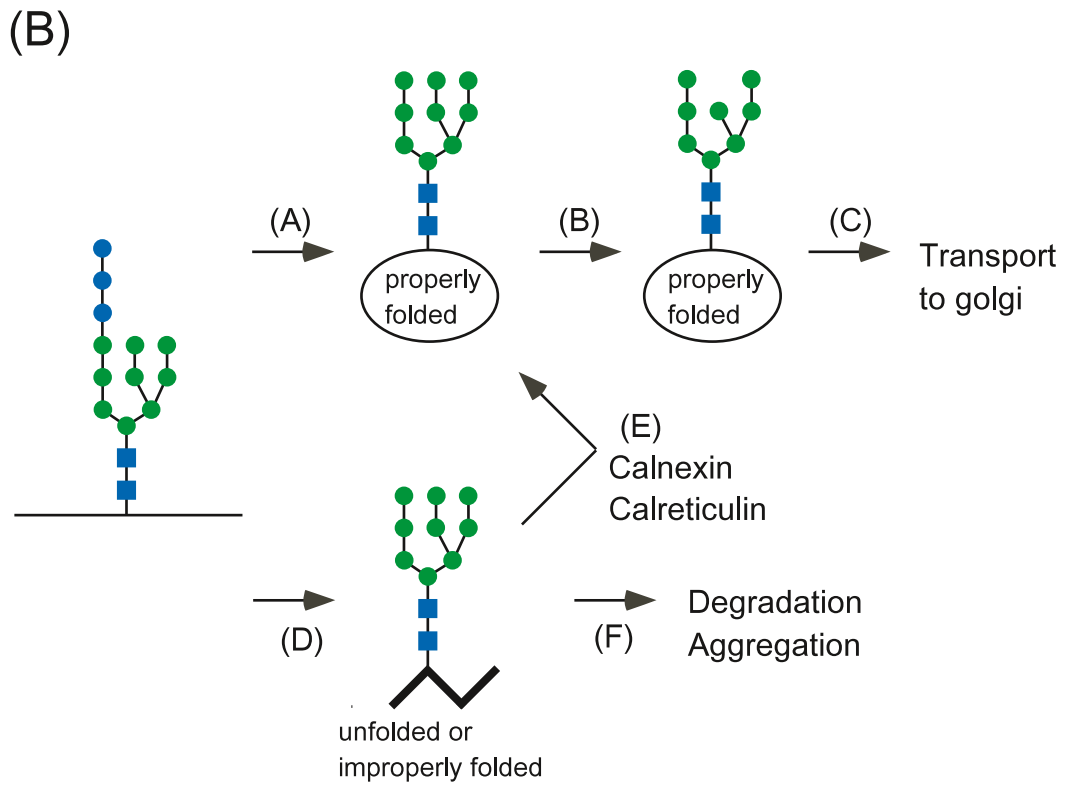
Although ERManI has been shown to preferentially remove one α(1-2) linked mannose from the B-chain of $Man_9GlcNAc_2$ (pathway B in Fig. 1b) [11, 12], recent studies provided an indication that its specificity is not entirely strict. In particular, misfolded glycoproteins are quite susceptible to demamnosylation, and two or more mannose residues are removed from each single *N*-linked oligosaccharide in the ER [13–15]. However, mechanistic rationale behind this observation is still unclear, because the genuine entity of the enzyme(s) responsible for *in vitro* demannosylation has not been well defined. Meanwhile, increasing amount of EDEMs has revealed to accelerate the demannosylation in tissue cultured cells. However, all attempts to prove α-1,2 mannosidase activities of recombinant EDEM proteins have been unsuccessful [16–18]. On the contrary, *in vitro*, a recombinant ERManI of both human and yeast origin was reported to generate $Man_{6-5}GlcNAc$ from $Man_9GlcNAc$, *albeit* with low velocity [19]. Precise *in vitro* analysis of ERManI using unfolded or improperly folded glycoproteins, in comparison with native glycoproteins, has yet to be conducted.

Fig. 1 A schematic representation for structures of high-mannose type oligosaccharides in a biosynthetic pathway. Colored symbols indicating mannose (Man), *N*-acetylglucosamine (GlcNAc) and glucose (Glc) and a line indicating a chemical bond were drawn according to Varki *et al.* [3]. **a** The structure of $Man_9GlcNAc_2$ An α-linked bond between two mannoses was indicated beside the corresponding linkage. Three chains in $Man_9GlcNAc_2$ are shown as A, B & C-chain. Structures for $Man_{8B}GlcNAc_2$, $Man_{8C}GlcNAc_2$, $Man_{7A}GlcNAc_2$, $Man_{7C}GlcNAc_2$, $Man_6GlcNAc_2$, and $Man_5GlcNAc_2$ were also depicted. **b** Processing of high-mannose type oligosaccharides on proteins in ER Oligosaccharides in properly folded glycoproteins are trimmed to $Man_9GlcNAc_2$ (pathway A) and then $Man_{8B}GlcNAc_2$ (pathway B). Those glycoproteins carrying $Man_{8B}GlcNAc_2$ are ready to be transported into Golgi apparatus (pathway C). If glycoproteins harboring $Man_9GlcNAc_2$ are improperly folded or remain to be unfolded during the process (pathway D), those glycoproteins entered the "calnexin/calreticulin cycle" (pathway E). When glycoproteins failed to be properly folded after futile interactions with those chaperons, those glycoproteins are susceptible to be demannosylated and further degraded by ERAD pathway or aggregated in ER (pathway F)

Previous studies in our group achieved the synthesis of various high-mannose type oligosaccharides by a convergent strategy and used them for biochemical analysis of proteins involved in the ER quality control [20, 21]. Utilizations of those oligosaccharides as substrates of UGGT, glucosidase II and calreticulin gave us quantitative estimates of their substrate specificities [22–24]. In this study, we aimed to clarify biochemical characteristics of ERManI, by using a variety of substrates, including synthetic oligosaccharides, pyridylamine (PA)-labeled glycans, and naturally occurring glycoproteins.

To initiate the study, we set out to establish conditions to produce recombinant hERManI in a soluble form using *Escherichia coli*. Its enzymatic property was examined using synthetic oligosaccharides and *N*-linked glycan containing proteins. According to our results, a fluorescently labeled mannotriose was reactive as a substrate of hERManI. Subsequent experiments using a high-mannose-type glycan substrate showed that the enzyme was able to remove multiple mannose residues to produce $Man_{6-5}GlcNAc_2$ from $Man_9GlcNAc_2$. In addition, we revealed that the hERManI was able to cleave mannose residues from bTg and SBA to a larger extent when they were denatured. Our results provide an indication that hERManI has an ability to discriminate folding states of glycoproteins, preferentially removing α(1-2) linked mannoses from unfolded or improperly folded glycoproteins.





Materials and methods

Chemicals

Most of the chemicals, otherwise mentioned, were purchased from Nacalai-Tesque, Inc. (Japan) or Wako Pure Chemical Industries, Ltd. Inc. (Japan). Plasmid DNAs, pColdIII & pTf16 were purchased from Takara Bio Inc. (Japan). PA-labeled oligosaccharides were purchased from Takara Bio Inc. (Japan), Masuda Chemical Industries Co, LTD. (Japan) or Glyence Co. Ltd. (Japan). A cDNA library of MCF7 cells was purchased from Clontech (USA). An

Escherichia coli origami B strain(F^- *ompT hsd*S_B(r_B^- m_B^-) *gal dcm lacY1 ahpC gor522*::Tn*10 trxB* (Kan^R, Tet^R) was purchased from Novagen Inc. (USA). A complete-mini EDTA-free protease inhibitor cocktail and a PNGase F were purchased from Roche Diagnostics GmbH (Germany). A Ni-NTA agarose was purchased from Qiagen Inc. (USA). A 1-(3-Sulfopropyl)pyridinium Hydroxide Inner Salt was purchased from Tokyo Kasei Chemicals (Japan). An Amicon Ultra-4 ultrafiltration device (10,000 molecular weight cut-off) was purchased from Millipore (USA). A Precision Plus Protein™ Unstained Standards, a Protein Assay Dye Reagent Concentrate and an Oriole Fluorescent Gel Stain were purchased from Bio-Rad Laboratories (USA). A bovine serum albumin, an Immobilized D-Galactose Gel and a Slide-A-Lyzer dialysis cassette (10,000 molecular weight cut-off) were purchased from Thermo Scientific Pierce (USA). A bTg and a soybean flour were purchased from Sigma Aldrich Corp. (USA). A Cellulose Tubing (12,000~14,000 molecular weight cut-off) was purchased from Viskase Companies Inc. (USA). A MonoFas I spin column was purchased from GL Sciences Inc. (JAPAN).

Production of hERManI in *Escherichia coli*

General techniques to handle DNA were performed essentially according to Sambrook *et al.* [25]. A DNA fragment for hERManI was prepared from a cDNA library of MCF7 cells. Nucleotides encoding 226th to 699th amino acid residue of the enzyme were then prepared by PCR using hMAN1B1-NDE-HIS-226SOL-F (5′-CACCCATATGCATCATCATCATCATCATGCAGAAGTGCCCACCAAGCCTCCCCTG-3′) and hMAN1B1-XHOR (5′-GCCACTCGAGCTAGGCAGGGGTCCAGATAGGCAGAG-3′) as primers. The PCR product was digested with NdeI and XhoI, recovered and ligated with NdeI&XhoI-digested pColdIII, giving a plasmid DNA, lg100517-1#2. Nucleotide sequences of a portion of hERManI in the plasmid DNA were analyzed at the Support Unit for Bio-material Analysis in RIKEN BSI Research Resources Center.

Expression of a recombinant form of hERManI in *Escherichia coli* was performed essentially according to the manufacture's instruction. Briefly, *E. coli* origami B cells carrying pTf16 and lg100517-1#2 or pColdIII were established. Expression of the protein was induced at 16°C. The bacteria cells from an 800 ml of culture were frozen at −80°C until they were thawed at 37°C. A cell pellet was suspended in 12 ml of buffer A (50 mM Tris-Cl (pH 7.4), 50 mM NaCl) containing one tablet of complete-mini EDTA-free protease inhibitor cocktail and then disrupted by a sonication of 2 min in total. A soluble fraction was recovered after centrifugations and applied to Ni-NTA agarose essentially according to the manufacture's instruction. The bound proteins were recovered with 250 mM imidazole (pH 7.4) in buffer A.

Proteins after Ni-NTA agarose were further concentrated using an ultrafiltration device essentially according to Karaveg *et al.* [26]. Briefly, the eluted hERManI in a solution was mixed with an equal volume of 1.5 M 1-(3-Sulfopropyl) pyridinium Hydroxide Inner Salt in buffer A, applied to Amicon Ultra-4 and concentrated. Using the same ultrafiltration device, the concentration of 1-(3-Sulfopropyl)pyridinium Hydroxide Inner Salt was lowered to 0.25 M in buffer A. Protein concentration of hERManI in the solution was measured by a Bradford's method using a Protein Assay Dye Reagent Concentrate [27]. A bovine serum albumin was used as a standard. N-terminal amino acid sequence of hERManI was analyzed at the Biomolecular Characterization Team in RIKEN ASI.

Reaction of hERManI with Manα(1-2)Manα(1-2) Man-O-$(CH_2)_3$-dansyl

Preparation of Manα(1-2)Manα(1-2)Man-O-$(CH_2)_3$-dansyl will be described elsewhere (Matsuo, I. *et al.*). Twenty μl of solutions containing the enzyme (4.6 μg) and 100 μM Manα(1-2)Manα(1-2)Man-O-$(CH_2)_3$-dansyl in 20 mM MES (pH 7.0), 150 mM NaCl and 5 mM $CaCl_2$ were incubated for 1, 2 and 4 h at 37°C. A heat-inactivated enzyme was prepared by a treatment at 100°C for 5 min and was used as a negative control. To detect compounds on HPLC, 40 μl of acetonitrile was added to the reaction mixture and a portion of the solution was separated on an Amide 80 column (4.6 mmI.D.×15 cm, Tosoh Corp., Japan) using HPLC e2695 apparatus (Waters Corp., USA). A solvent containing CH_3CN 65% in 0.3% AcOH-Et_3Naq. (pH 7.3) was used at 1 ml/min for 12 min. Fluorescence of dansyl-sugars excited at 372 nm was detected at 518 nm using a 2475 fluorescence detector (Waters Corp.). For mass-spectrum analysis, the enzyme (2.3 μg) and 63 μM Manα(1-2) Manα(1-2)Man-O-$(CH_2)_3$-dansyl were used in 10 μl of reaction mixtures. Then, 0.5 μl of the reaction mixture was mixed with 0.5 μl of α-cyano-4-hydroxycinnamic acid and directly applied to a MALDI-TOF mass analyzer (AXIMA-LTR, Shimadzu, Japan).

Reaction of hERManI with PA-labeled sugar chains

An oligosaccharide, $Man_9GlcNAc_2$ was prepared as described previously [28] and modified with 2-aminopyridine essentially according to Kondo *et al.* [29]. Briefly, 1 mg of the oligosaccharide was dissolved in 20 μl of 2-aminopyridine solution and kept for 1.5 h at 80°C. Then, 20 μl of borane dimethylamine complex was added to the solution. The mixture was incubated for one hour at 80°C. PA-labeled $Man_9GlcNAc_2$ ($Man_9GlcNAc_2$-PA) was puri-

fied on an Amide 80 column (7.8 mmI.D.×30 cm, Tosoh Corp.) essentially according to Totani *et al.* [22], evaporated and dissolved in ultrapure water. A concentration of the oligosaccharide in the solution was calculated using a commercial $Man_9GlcNAc_2$-PA (Takara Bio Inc., Japan) as a standard.

Solutions (10 μl) containing the enzyme (4.7 μg in 7.1 μl or 3.7 μg in 5.6 μl) and 100 or 200 μM $Man_9GlcNAc_2$-PA in 20 mM MES (pH 7.0), 150 mM NaCl and 5 mM $CaCl_2$ were incubated for 1 h at 37°C, respectively. At the end of reactions, 40 μl of ultrapure water and 100 μl of acetonitrile were added to the solution in order. The mixtures were separated on an Amide 80 column (4.6 mmI.D.×25 cm, Tosoh Corp.) using a linear gradient of CH_3CN 65% to 59% in 0.3% AcOH-Et_3Naq. (pH 7.3), 30 min, 1 ml/min. Fluorescence of PA-labeled oligosaccharides excited at 320 nm was detected at 400 nm using the fluorescence detector.

Preparation and urea-treatment of glycoproteins

SBA was prepared from soybean flour essentially according to Evers *et al.* [30]. The protein was further purified using an immobilized galactose column essentially according to Franco-Fraguas *et al.* [31], dialyzed against ultrapure water and lyophilized.

Urea-treatment of bTg and SBA was performed essentially according to Trombetta *et al.* [32]. Briefly, glycoproteins were dissolved in 25 mM Tris-Cl (pH 7.0) and dialyzed against 8 M urea in 25 mM Tris-Cl (pH 7.0) for 5 h at 4°C using a Slide-A-Lyzer dialysis cassette. The protein solution was further dialyzed against ultrapure water for 4 followed by 12 h at 4°C and then recovered. To measure concentrations of the glycoproteins, they were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained by the Oriole fluorescent dye stain. The fluorescence corresponding to the protein was measured by a fluorescence imager, LAS4000mini (FUJIFILM Corp., Japan) and used to calculate its concentration. Proteins without any treatment were used as standards for the calculation.

Enzymatic reaction of hERManI with glycoproteins

Solutions of 20 μl containing the enzyme (2.0 μg) and bTg (36 μg) or SBA (12 μg) in 20 mM MES (pH 7.0), 150 mM NaCl and 5 mM $CaCl_2$ were incubated for 1 h at 37°C. A heat-inactivated enzyme was prepared as described above and used as a negative control. After the reaction, the solutions were left on ice for 5 min. Releases of oligosaccharides from glycoproteins by PNGaseF were performed according to the manufacture's instruction. Briefly, 2 μl of 10% SDS solution was added to the reaction mixture. The mixture was heated for 5 min at 100° C and cooled to a room temperature for 5 min. Subsequently, 2.2 μl of 10% Nonidet P-40 solution and 0.5 units of PNGaseF were added to the reaction mixtures in order. The mixtures were incubated for more than 12 h at 37°C. After the deglycosylation reaction, 98.8 μl of ethanol was added to the reaction mixture. The mixtures were kept for 1 h at 4°C and centrifuged for 5 min at 15,000 rpm at 4°C. The supernatants were recovered and evaporated *in vacuo* using a centrifugal concentrator (CC-105, TOMY Co. Ltd., Japan). The dried substances were used as oligosaccharides to be labeled.

Labeling of oligosaccharides by 2-aminopyridine was performed as described above. The mixture of 40 μl of the reaction solution containing PA-labeled oligosaccharides and 360 μl of acetonitrile was applied to a silica-based MonoFas I spin column. The columns were washed three times with 100% acetonitrile and subsequently the same times with 95% acetonitrile. The PA-labeled oligosaccharides were eluted with ultrapure water and evaporated *in vacuo*. Then, one volume of ultrapure water and two volumes of acetonitrile were added to those oligosaccharides in order. A portion of the solutions was subjected to an Amide 80 column (4.6 mmI.D.×15 or 25 cm, Tosoh Corp.) on the HPLC apparatus. ApexTrack method equipped in Empower^{TM}2 software (Waters Corp.) was used to detect each peak and measure its area. Averages and standard deviations of area were calculated from duplicate mannosidase-reactions.

Results

Expression of hERManI in *Escherichia coli*

Nucleotide sequence of hERManI cDNA in our preparation from the cultured cell was the same as those reported by Tremblay & Herscovics [12]. A cold expression system of *Escherichia coli* [33] was employed to prepare a polypeptide containing of 226th to 699th amino acid residue of hERManI (Fig. 2a), and inclusion of a Trigger factor in the system was intended to increase the solubility of the enzyme. In a soluble fraction separated after sonications of the bacterial cells, a protein reacting with polyclonal anti-hERManI was detected near the possible size of 56 kDa by a Western blotting technique (data not shown). The protein was finally purified using a Ni-NTA immobilized resin (Fig. 2b). More than 1 mg of the protein was routinely obtained from one liter culture of the bacteria. An analysis of the N-terminal amino acid sequence revealed that polypeptides starting from both 1st methionine and 2nd asparagine (Fig. 2a) existed in our preparation.

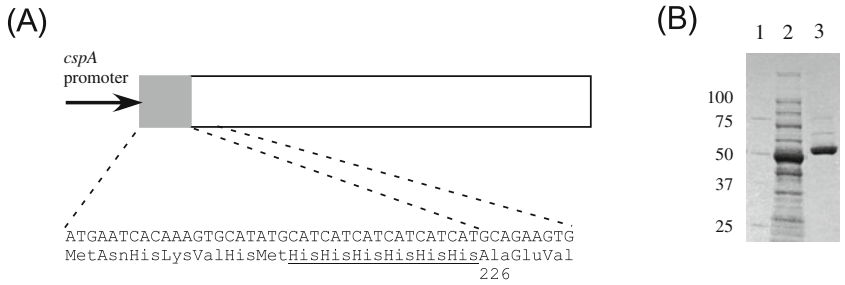


Fig. 2 Expression of hERManI in *Escherichia coli.* **a** A schematic representation of the plasmid DNA, lg100517-1#2 to express a soluble form of the enzyme in this study (*upper*) *A main part of the plasmid DNA* Nucleotides coding 226th to 699th amino acid of hERManI preceded by 13 additional amino acids were transcribed under the control of *cspA* promoter. (*lower*) *Nucleotides and the deduced amino acids at an N-terminal portion of histidine-tagged hERManI* The poly histidine sequence was underlined. **b** Detection of the purified enzyme after Ni-NTA agarose by CBB-staining (lane 1) *molecular weight marker* Actual molecular weight was indicated on left side of the lane. (lane 2) *sonicated sample of Escherichia coli lysate* The bacterial cells were disrupted by sonication. (lane 3) *elution fraction from Ni-NTA agarose* Histidine-tagged hERManI was eluted using 250 mM imidazole

Enzymatic reactivity of hERManI on Manα(1-2) Manα(1-2)Man-O-$(CH_2)_3$-dansyl

We first examined the activity of hERManI toward Manα(1-2) Manα(1-2)Man to test whether a simple mannotriose can be utilized as a substrate of the enzyme. To facilitate the detection, a dansyl-label was introduced, giving Manα(1-2)Manα(1-2) Man-O-$(CH_2)_3$-dansyl. Analysis with an amide column revealed a single peak (data not shown), which remained unchanged by incubation with exclusion of the enzyme, with an imidazole-eluting fraction generated from *E. coli* lysate harboring an empty vector DNA, pColdIII (data not shown), or with a heat-inactivated enzyme (peak **3** in Fig. 3a). When 100 μM of Manα(1-2)Manα(1-2)Man-O-$(CH_2)_3$-dansyl was mixed with 4.6 μg of the enzyme at 37°C for 1, 2 and 4 h, two faster eluting peaks (**1** and **2**) prior to the mannotriose (peak **3**) were detected (Fig. 3b-d). While the peak **2** was the most intense after 2 h reaction, the fastest eluting peak **1** was rich after 4 h reaction. To assign their structures, reaction mixtures were directly applied to a mass-spectrum analysis. The mixture after 2 h incubation revealed the presence of molecules possessing a mass of 818.2, 655.7 and 493.4, which were in good agreement with values calculated for sodium salts of Manα(1-2)Manα(1-2)Man-O-$(CH_2)_3$-dansyl (817.8), Manα(1-2)Man-O-$(CH_2)_3$-dansyl (655.7) and Man-O-$(CH_2)_3$-dansyl (493.5), respectively. Consequently, it is clear that the enzyme accepts Manα(1-2)Manα(1-2)Man-O-$(CH_2)_3$-dansyl as a substrate, from which two α(1-2) linked mannoses are removed sequentially. Since no significant accumulation of the peak **2** was observed, there seems to be little difference between velocities of two cleavage steps.

Processing of pyridylamine-labeled $Man_9GlcNAc_2$ by hERManI

Subsequently, *in vitro* activity of hERManI toward high-mannose type oligosaccharides was examined. For this purpose, we used PA-labeled oligosaccharides. When $Man_9GlcNAc_2$-PA at a concentration of 1 μM was treated with hERManI at 37°C for 1 h, $Man_{8B}GlcNAc_2$-PA and $Man_{7A}GlcNAc_2$-PA were produced (data not shown). Likewise, $Man_{7A}GlcNAc_2$-PA produced $Man_6GlcNAc_2$-

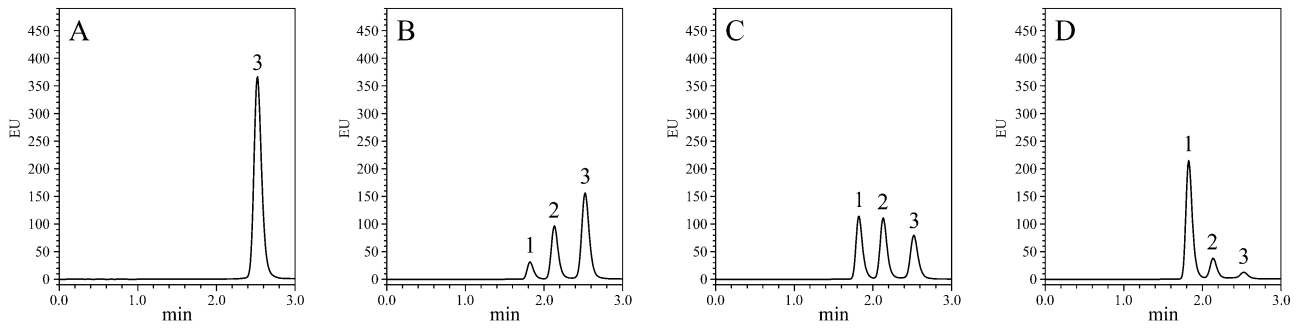


Fig. 3 Removal of α(1-2) linked mannoses from Manα(1-2)Manα(1-2) Man-O-$(CH_2)_3$-dansyl by hERManI. Mixture containing **a** heat-inactivated or **b**, **c**, **d** intact hERManI and Manα(1-2)Manα(1-2)Man-O-$(CH_2)_3$-dansyl after 1 (**a**, **b**), 2 (**c**) and 4 (**d**) hour reaction was separated on an Amide-80 column. Peak 3 derived from Man(α-1,2) Manα(1-2)Man-O-$(CH_2)_3$-dansyl. Peak 1 and 2 were as Man-O-$(CH_2)_3$-dansyl and Manα(1-2)Man-O-$(CH_2)_3$-dansyl, respectively, estimated from changes of profiles in HPLC peaks and molecular masses dependent on reaction times

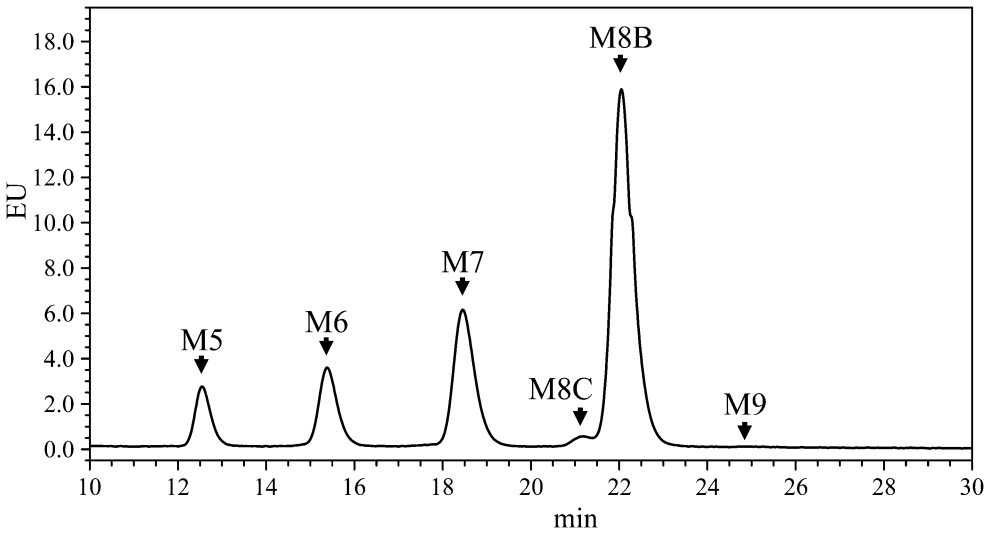


Fig. 4 Removal of α(1-2) linked mannoses from pyridylaminated $Man_9GlcNAc_2$ by hERManI. Reaction mixture containing the enzyme (4.7 μg) and 100 μM $Man_9GlcNAc_2$-PA was separated on an Amide-80 column. M9, M8B, M8C, M7, M6 and M5 indicated the elution time for $Man_9GlcNAc_2$-PA, $Man_{8B}GlcNAc_2$-PA, $Man_{8\text{-}CGlcNAc2}$-PA, $Man_{7A\&\text{-}CGlcNAc2}$-PA, $Man_6GlcNAc_2$-PA, and $Man_5GlcNAc_2$-PA in a separate run, respectively

PA and $Man_5GlcNAc_2$-PA under the same conditions. In order to obtain more rigorous confirmation, higher concentrations (100 or 200 μM) of $Man_9GlcNAc_2$-PA were incubated with 4.7 or 3.7 μg of the enzyme in 10 μl reaction volume. In the reaction mixture, four new and definite peaks were eluted from an amide column equipped to an HPLC apparatus (Fig.4 and S1). Comparison of retention times with standard oligosaccharides revealed that the mixture was composed of $Man_5GlcNAc_2$-PA, $Man_6GlcNAc_2$-PA, $Man_7GlcNAc_2$-PA and $Man_{8B}GlcNAc_2$-PA (M5, M6, M7 and M8B in Fig. 4, respectively). Fractions corresponding to $Man_7GlcNAc_2$-PA contained both $Man_{7A}GlcNAc_2$-PA and $Man_{7C}GlcNAc_2$-PA (data not shown). In addition, a small but discernable shoulder-like peak prior to $Man_{8B}GlcNAc_2$-PA was observed, whose elution time was identical to that of $Man_{8C}GlcNAc_2$-PA. This interpretation was confirmed by re-chromatography of fractions containing this area (data not shown).

Enzymatic reaction of hERManI with native and urea-treated glycoproteins

Since hERManI usually digests *de novo* synthesized glycoproteins, naturally occurring *N*-linked glycoproteins were tested as substrates. We used bTg and SBA as model glycoproteins, because both of them are rich in high-mannose type oligosaccharides, including $Man_9GlcNAc_2$. They were further converted to denatured forms by soaking in 8 M urea solution. In fact, urea-treated thyroglobulin has been used as a benchmark substrate of UGGT, which functions as a folding sensor enzyme in the ER [32]. To analyze *N*-linked sugar moieties in glycoproteins after incubation, oligosaccharides were released by PNGaseF, labeled with 2-aminopyridine and separated with HPLC equipped with an amide column as described in the previous section.

When intact bTg was incubated with heat-inactivated hERManI, PA-labeling of oligosaccharide mixture revealed the presence of more than ten species (Fig. 5a). Our analysis assigned that four of them were derived from high-mannose type oligosaccharides, $Man_6GlcNAc_2$-PA, $Man_7GlcNAc_2$-PA, $Man_{8B}GlcNAc_2$-PA and $Man_9GlcNAc_2$-PA (M6, M7, M8B and M9 in Fig. 5a, respectively). Subsequently, the same protein was treated with hERManI at 37°C for 1 h and the profile of *N*-linked sugar chains was analyzed (Fig. 5b). While a proportion of $Man_9GlcNAc_2$-PA was slightly reduced (23.9±2.1% and 20.8±0.9% occupancy of sugar chains detected in 10–30 min on HPLC for heat-inactivated and intact hERManI, respectively), a peak corresponding to $Man_{8B}GlcNAc_2$-PA was increased proportionally (6.7±0.8% and 9.0±0.5% for heat-inactivated and intact hERManI, respectively). This result indicated that bTg was marginally reactive as a substrate for hERManI. Subsequently, a reaction of urea-treated bTg was examined, whose *N*-linked sugar chain profile was compared to that of native bTg (Fig. 5c, d and S2). While a proportion of $Man_9GlcNAc_2$-PA was dramatically decreased (1.7±0.2% and 24.8±0.8%), contents of smaller oligosaccharides were increased ($Man_{8B}GlcNAc_2$-PA: 17.7±0.2% and 6.6±0.4%, $Man_7GlcNAc_2$-PA: 8.2±0.2% and 1.8±0.2%, $Man_6GlcNAc_2$-PA: 4.0±0.5% and 0.7±0.1%). A similar HPLC profile was obtained from heat-treated bTg (data not shown). All of these results suggest that hERManI removes more mannoses from *N*-linked sugar chains in bTg when they are exposed to conditions that cause denaturation.

To substantiate the above interpretation, a similar set of experiments was conducted by using SBA. When intact SBA was incubated with heat-inactivated hERManI, major species corresponding to $Man_9GlcNAc_2$-PA and additional species were detected as minor peaks (Fig. 6a). The HPLC profile exhibited nearly identical peak ratio, when active

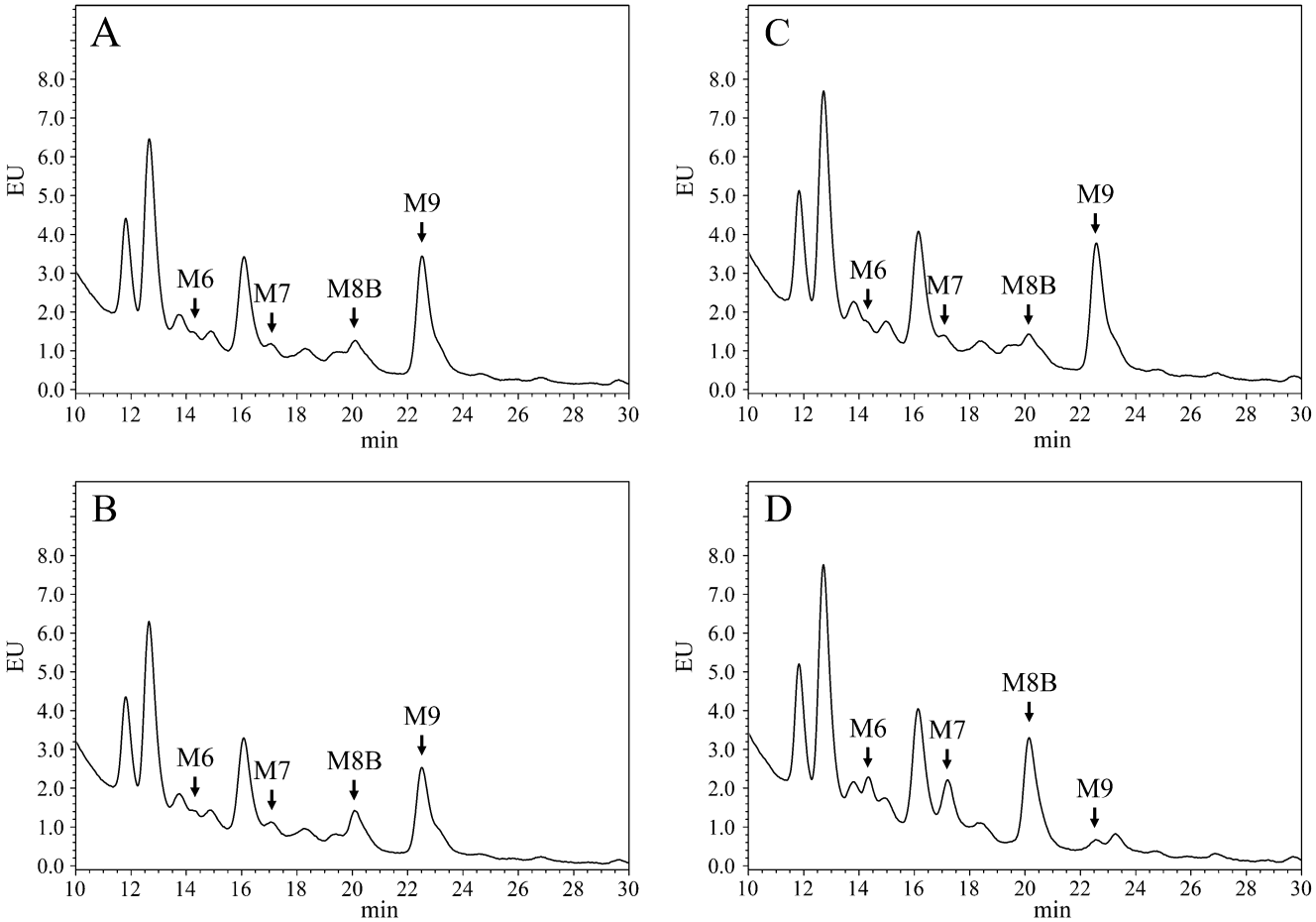


Fig. 5 Removal of α(1-2) linked mannoses from *N*-linked oligosaccharides in bTg by hERManI. BTg (**a**, **b**) before or (**c**, **d**) after urea treatment were reacted with (**a**, **c**) heat-inactivated or (**b**, **d**) intact hERManI. Their oligosaccharides were released from the glycoproteins and labeled with 2-aminopyridine. Labeled oligosaccharides were separated by Amide-80 column (4.6mmI.D.×25 cm), 40°C, CH_3CN 65% to 56% in 0.3% AcOH-Et_3Naq. (pH 7.3), 30 min, at a flow rate of 1 ml/min. M9, M8B, M7, and M6 indicated the elution time for $Man_9GlcNAc_2$-PA, $Man_{8B}GlcNAc_2$-PA, $Man_{7A\&C}GlcNAc_2$-PA, and $Man_6GlcNAc_2$-PA in a separate run, respectively. Relative contents of $Man_9GlcNAc_2$-PA, $Man_{8B}GlcNAc_2$-PA, $Man_{7A\&C}GlcNAc_2$-PA, and $Man_6GlcNAc_2$-PA from duplicate reactions of urea-treated bTg with heat-inactivated or intact hERManI were calculated and presented in Fig. S2

enzyme was used (Fig. 6b). This result suggested that native SBA is highly resistant to hERManI. By contrast, when urea-treated SBA was subjected to the same conditions, the proportion of $Man_9GlcNAc_2$-PA dramatically decreased. In this case, four molecular species eluted prior to $Man_9GlcNAc_2$-PA appeared, which were assigned as $Man_5GlcNAc_2$-PA, $Man_6GlcNAc_2$-PA, $Man_7GlcNAc_2$-PA and $Man_{8B}GlcNAc_2$-PA (M5, M6, M7 and M8B in Fig. 6c, d, respectively). A similar result was obtained from heat-denatured SBA (data not shown). These results clearly indicate that hERManI is able to remove more mannose residues from SBA when it is denatured.

Discussion

In this study, we prepared a recombinant form of hERManI and examined its *in vitro* enzymatic activities using various substrates. hERManI is composed of 699 amino acids and belongs to a family of type II membrane proteins in which a catalytic portion is localized at the C-terminus. We expressed a polypeptide containing 226th to 699th amino acid residue of the enzyme (Fig. 2a), because this region exhibits a high extent of similarity to members of mammalian glycosylhydrolase family 47 [8]. As a matter of fact, the expressed protein exhibited the α-1,2 mannosidase activity [34]. More than one milligram quantity of the polypeptide was routinely obtained from soluble fractions of one liter of *E. coli* culture. Expression of the protein at 16°C in the presence of Trigger factor has proven effective in producing the active protein with effective folding.

In order to detect its activity, we first synthesized a fluorescently labeled trisaccharide, Man(α-1,2)Man(α-1,2)Man-O-$(CH_2)_3$-dansyl, which corresponds to the A-chain of $Man_9GlcNAc_2$ (Fig. 1a). Although being smaller than any

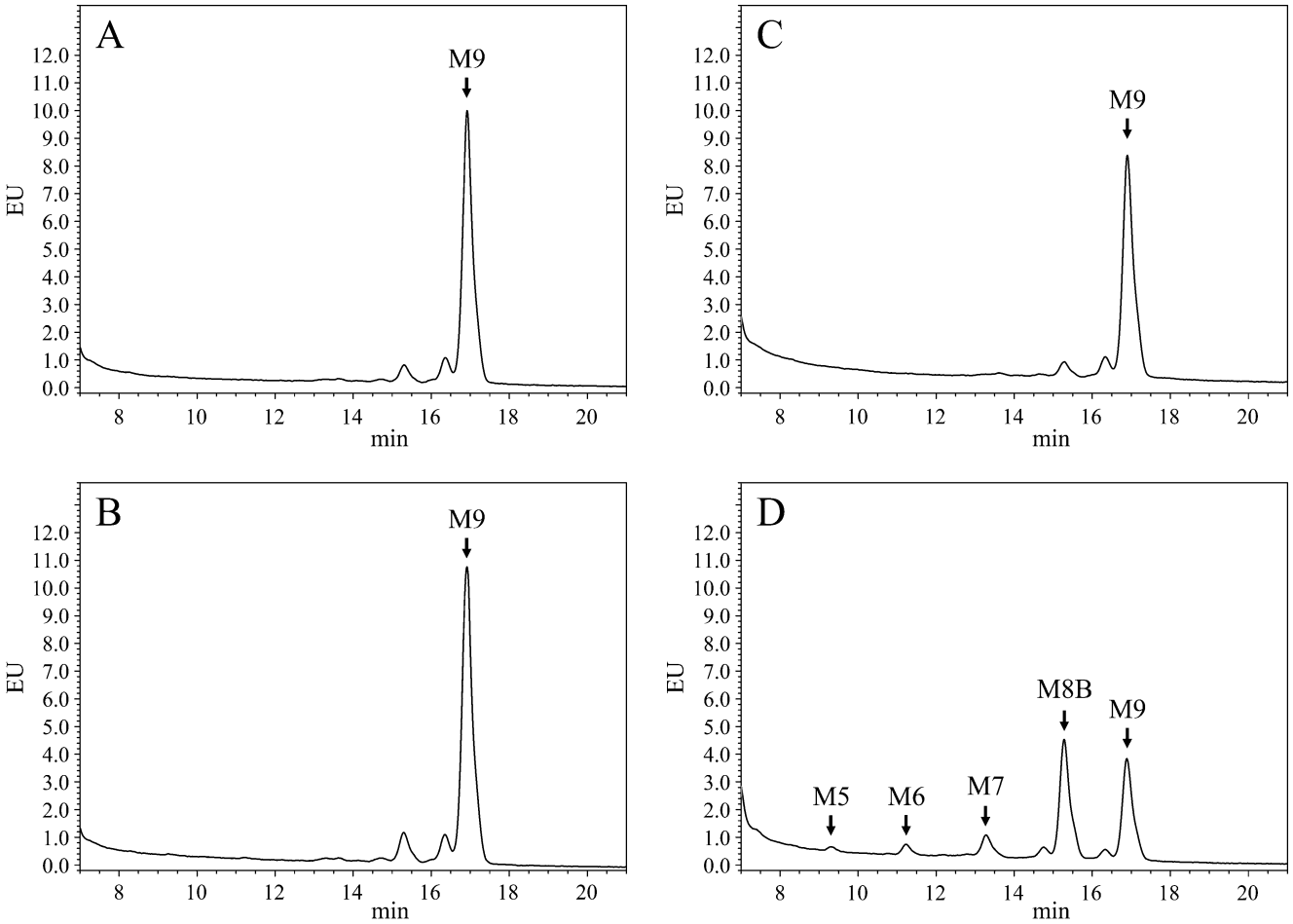


Fig. 6 Removal of α(1-2) linked mannoses from *N*-linked oligosaccharides in SBA by hERManI SBA (**a**, **b**) before or (**c**, **d**) after urea treatment were reacted with (**a**, **c**) heat-inactivated or (**b**, **d**) intact hERManI. Their oligosaccharides were released from the glycoproteins and labeled with 2-aminopyridine. Labeled oligosaccharides were separated by HPLC (Amide-80 4.6 mm I.D.×15 cm, 40°C, CH_3CN 65% to 54.5% in 0.3% AcOH-Et_3Naq. (pH 7.3), 21 min, 1 ml/min). M9, M8B, M7, M6 and M5 indicated the elution time for $Man_9GlcNAc_2$-PA, $Man_{8B}GlcNAc_2$-PA, $Man_{7A\&C}GlcNAc_2$-PA, $Man_6GlcNAc_2$-PA, and $Man_5GlcNAc_2$-PA in a separate run, respectively

glycan reported as substrate of hERManI, this compound was shown to be trimmed quite smoothly. While formation of the disaccharide Manα(1-2)Man-O-$(CH_2)_3$-dansyl was confirmed by mass spectrometric analysis, further digestion proceeded to give Man-O-$(CH_2)_3$-dansyl, indicating that even a simple disaccharide may well be a good substrate of hERManI. More detailed kinetic study using those compounds is currently under investigations.

When $Man_9GlcNAc_2$-PA was employed as a substrate, we detected the formation of quite extensively digested products, such as $Man_6GlcNAc_2$-PA and $Man_5GlcNAc_2$-PA. Herscovics *et al*. observed the generation of [^{3}H-]$Man_{7-6}GlcNAc$ by incubating [^{3}H-]$Man_9GlcNAc$ with 5 μg of crude hERManI in 170 μl of reaction mixture for 24 h [19]. In our study, trimming of $Man_9GlcNAc_2$-PA to $Man_5GlcNAc_2$-PA proceeded with markedly higher efficiency; since 4.7 μg of purified hERManI in 10 μl of reaction mixture gave rise to a high degree of demannosylation after 1 h. By employing highly active enzyme preparation and using the substrate completely labeled with the fluorophore at the reducing end, this study clearly demonstrated that hERManI removes essentially all α(1-2) linked mannose residues from $Man_9GlcNAc_2$ in shorter time. The enzyme concentrations adopted in our experiments may well be in a range of physiological relevance, considering that the protein concentration in ER is estimated to be more than 100 mg/ml [35] and about 1,500 proteins are detected in ER by proteomic research [36].

Rather unexpectedly, formation of a small fraction of $Man_{8C}GlcNAc_2$-PA from $Man_9GlcNAc_2$-PA was observed, providing the first indication that octamannose oligosaccharide other than $Man_{8B}GlcNAc_2$ may be generated by hERManI. According to recent studies, removal of a mannose residue in C-chain by α-1,2 mannosidases would be required in order for improperly folded glycoproteins to be recognized by OS-9, a protein involved in the ERAD pathway [37]. Intriguingly, Hosokawa *et al*. reported that larger amount of $GlcMan_{8C}GlcNAc_2$ was

detected by enhanced expression of EDEM1 in human tissue cultured cells [38], while an active-site mutant of EDEM1 did not induce the above effect. Although the contribution of hERManI to that phenomenon has been still unclear, it would be possible that substrate specificity of hERManI to an outermost mannose in either B or C-chain is modulated by EDEM through unknown mechanisms to produce increased amount of $Man_{8C}GlcNAc_2$ under such a circumstance.

To study the behavior of glycoproteins, bTg and SBA were used as model substrates. Among them, however, proportions of $Man_9GlcNAc_2$ in total *N*-linked sugar chains are vastly different. Namely, nearly 90% of *N*-linked sugar chains are $Man_9GlcNAc_2$ and located on the single asparagine residue in SBA [39]. On the other hand, glycan composition of bTg is more diverse, containing only ca. 20% of $Man_9GlcNAc_2$[40]. While hERManI slowly removed α(1-2) linked mannoses from $Man_9GlcNAc_2$ of intact bTg, SBA was highly resistant to the enzyme. This indicates that the activity to hERManI is dependent on the nature of glycoproteins. Petrescu *et al.* analyzed surface geometry and relative accessibility of asparagine residue on occupied *N*-glycosylation sites [41]. Among them, 33% were situated on a convex surface, suggesting that α(1-2) linked mannoses on those sites were easily accessible by hERManI. On the contrary, 8% of them were in deep recesses of proteins. Mannoses on these sites might well be far less susceptible to the action of the enzyme. Differences in local environments near *N*-glycosylation sites would affect susceptibility of mannose residues in *N*-glycosyl moieties to hERManI.

Obviously, hERManI removes a larger amount of α(1-2) linked mannoses from denatured proteins. One possible way to rationalize this phenomenon is that hERManI may have an ability to recognize a specific property of denatured glycoproteins. This observation is reminiscent of the characteristic of UGGT [32], a key enzyme in ER quality control system, which preferentially accepts incompletely folded glycoproteins as substrates. The unique property of UGGT has been explained by hypothesizing its ability to recognize surface-exposed hydrophobic patches of glycoproteins [42]. A crystal structure of hERManI [34] revealed the presence of some aromatic amino acid residues, such as Trp284 and Trp389, located on the entry site of a substrate-binding cleft (Fig. S3). Those residues are likely to be distant from the catalytic center and might have a function to interact with hydrophobic regions, instead of glycan chains, of glycoproteins, which are exposed on the surface by denaturant-treatment. An alternative possibility would be that hERManI is sensitive to steric hindrance and preferentially accesses to mannose residues of *N*-linked sugar chains linked to denatured glycoproteins. Flexibilities of high-mannose type oligosaccharides would be affected by structures of polypeptides [43, 44]. For example, $Man_5GlcNAc_2$ at asparagine 34 of bovine ribonuclease B was more mobile when amino acid residue 1–20 was removed by a protease [45]. Therefore, denaturation of glycoproteins might enhance movements of the oligosaccharide which causes more chances to be attacked by hERManI.

Very recently, Pan *et al.* reported endogenous hERManI was accumulated in the Golgi apparatus of HeLa cells [46]. Now there is a question whether removal of α(1-2) linked mannose from glycoproteins catalyzed by hERManI is proceeding in the ER or/and in Golgi. In any case, however, the activity of hERManI is strongly dependent on glycoproteins and their fold-status.

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