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Identification of the Catalytic Acid–Base Residue of *Arthrobacter* Endo-β-*N*-Acetylglucosaminidase by Chemical Rescue of an Inactive Mutant

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Arthrobacter **endo-β-*N*-acetylglucosaminidase (Endo-A), a member of glycoside hydrolase (GH) family 85, catalyses the hydrolysis and transglycosylation of asparagine-linked oligosaccharides of glycoproteins with retention of anomeric configuration. Glu-173 of Endo-A is a catalytically essential amino acid residue, and the corresponding residue is conserved in all GH family 85 members. The catalytic activity of Endo-A E173A mutant was rescued by the addition of sodium azide or sodium formate. Furthermore, the produced β-glycosyl azide ($Man_5GlcNAc$-β-N_3) retained the anomeric configuration, indicating that Glu-173 is the catalytic acid–base residue of Endo-A. This is the first identification of the catalytic residue for GH family 85 endo-β-*N*-acetylglucosaminidases.**

Key words: azide, catalytic acid–base residue, chemical rescue, endo-β-*N*-acetylglucosaminidase, inactive mutant.

Abbreviations: Endo-A, endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae*; Endo-H, endo-β-*N*-acetylglucosaminidase from *Streptococcus plicatus*; Endo-M, endo-β-*N*-acetylglucosaminidase from *Mucor hiemalis*; GH, glycoside hydrolase; GlcNAc, *N*-acetylglucosamine; Man, mannose.

Endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) catalyses the hydrolysis of the *N*,*N*′-diacetylchitobiose moiety of asparagine-linked oligosaccharides of various glycoproteins. The enzymes are classified into the glycoside hydrolase (GH) family 18 and 85. GH family 85 endo-β-*N*-acetylglucosaminidases are widely distributed in mammal (*1*), plant (*2*), nematode (*3*), fungus (*4*) and bacteria (*5*–*7*). Among them, Endo-A from *Arthrobacter protophormiae* and Endo-M from *Mucor hiemalis* have been used for the synthesis of neoglycoconjugates, neoglycopeptides and neoglycoproteins by means of their transglycosylation activities (*8*, *9*). These enzymes can stereo- and regioselectively exchange various oligosaccharides on glycoproteins without damaging them. However, the 3D structure of GH family 85 endo-β-*N*-acetylglucosaminidase has not been resolved, and the catalytic residue is still unclear. In our previous study (*10*), we identified a catalytically essential glutamate residue at position 173 in Endo-A by mutagenesis experiments. The corresponding amino acid residue was also reported to be catalytically essential in several other GH family 85 members (*4*, *5*, *11*). The catalytic residues of retaining glycosidases have been identified by chemical rescue experiments in combination with site-directed mutagenesis (*12*). In the present study, we attempted to determine a catalytic residue of Endo-A by chemical rescue using an alanine-substitution mutant of Glu-173 (E173A mutant).

MATERIALS AND METHODS

Expression and Purification of Wild-Type and Mutant Enzymes—A previously constructed pET32a-Endo-A plasmid (*10*) was digested with *Nco*I and *Not*I, and the Endo-A ORF fragment was inserted into the corresponding sites of pET-23d (+) (Novagen). pET23d-Endo-A E173A mutant was constructed by QuikChange site-directed mutagenesis kit (Stratagene) using the mutagenic forward primer (5′-GGTTTATTAACCAAGCAACA GAAGGGG-3′, the position of the mutated sequence is underlined) and the complementary primer. The desired mutation was confirmed by DNA sequencing. The resulting plasmids were introduced into *E. coli* BL21 (λDE3), and the cells were grown in LB medium containing ampicillin (150 μg/ml) at 37°C. After OD_{600} reached 0.4, isopropyl β-D-thiogalactoside (IPTG) was added at the final concentration of 0.4 mM. The culture was then incubated for a further 3 h at 37°C before harvesting the cells by centrifugation. The cell pellet was resuspended in BugBuster reagent (Novagen) to generate the soluble protein extract. The recombinant protein was then purified by DEAE-Toyopearl 650M column chromatography as described previously (*6*).

Preparation of Substrate and Measurement of Enzymatic Activity—$Man_5GlcNAc_2Asn$ was prepared

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from ovalbumin glycopeptides by the method of Huang *et al*. (*13*). Endo-β-*N*-acetylglucosaminidase activity was assayed with dansylated $Man_5GlcNAc_2Asn$. The standard assay of endo-β-*N*-acetylglucosaminidase activity was performed with 40 nmol of $Man_5GlcNAc_2Asn$-dansyl in 20 μl of 100 mM sodium acetate buffer (pH 6.0) at 37°C for 5 min. The reaction mixture terminated by the addition of trichloroacetic acid was directly analysed by HPLC as described previously (*14*). One unit of enzymatic activity was defined as the amount of enzyme yielding 1 μmol of GlcNAc-Asn-dansyl per minute at 37°C. The activity of the E173A mutant rescued by azide was measured at 37°C for 30 min in 20 μl of 100 mM sodium acetate buffer (pH 6.0), sodium azide at the concentrations indicated, 7.5 μg of E173A mutant and 20 nmol of $Man_5GlcNAc_2Asn$-dansyl. The formate rescue analysis was performed at 37°C for 30 min in 20 μl of sodium formate buffer (pH 4.0) at the concentrations indicated, 7.5 μg of E173A mutant and 20 nmol of $Man_5GlcNAc_2Asn$-dansyl. The activities were measured as described earlier for the standard assay.

Enzymatic Synthesis of Glycosyl Azide—One millimolar $Man_5GlcNAc_2Asn$ was incubated with 17.8 μg of the E173A mutant in 0.32 ml of 100 mM acetate buffer (pH 6.0) in the presence of 2 M sodium azide. After incubation for 24 h at 37°C, the reaction mixture was concentrated by lyophilization and then subjected to HPLC.

TLC and HPLC Analyses—The reaction product was analysed by TLC and HPLC. TLC analysis was carried out using silica gel 60 plates (Merck Art. 5626) in a solvent system of *n*-propanol/acetic acid/water, 3/3/2 (v/v/v), and the oligosaccharides were visualized by orcinol-H_2SO_4 reagent. HPLC was carried out using a Hitachi D-7500 chromatograph system equipped with a L-7420 UV-VIS detector. The reaction mixture was concentrated by lyophilization and then fractionated on a TSKgel Amide-80 column (4.6 × 250 mm, Tosoh) under isocratic conditions using 55% acetonitrile at a flow rate of 1.0 ml/min at 40°C. The eluate was monitored for absorbance of the *N*-acetyl group at 214 nm.

Mass Spectrometry and ^1H NMR Spectroscopy—Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry was performed with a Voyager DE-STR mass spectrometer (Applied Biosystems) using 2,5-dihydroxybenzoic acid as the matrix. ^1H NMR spectra were recorded on a JEOL ECA-600 instrument. $Man_5GlcNAc\text{-}N_3$ was lyophilized three times in D_2O prior to NMR experiments. The structure was also confirmed by ^1H-^1H COSY NMR analysis.

RESULTS

Chemical Rescue of Endo-A E173A Mutant by Sodium Formate and Sodium Azide—Recombinant wild-type Endo-A and the E173A mutant were engineered for heterologous expression in *E. coli* BL21(λDE3). The recombinant proteins were each purified to homogeneity from the respective cell lysates in a single-step purification using anion exchange chromatography (Fig. 1). Kinetic parameters for wild-type Endo-A were determined; K_m 0.207 mM and k_{cat} 365.0 s^{-1}. Although the precise kinetic parameters could not be established, Endo-A E173A mutant enzyme displayed at least 10^5-fold lower activity than that of the wild-type enzyme. The effect of external nucleophiles on the activity of the E173A mutant was further investigated by using different concentrations of sodium azide and formate. As shown in Fig. 2, the activity of the mutant was rescued by the addition of azide or formate. In the presence of 2 M sodium azide and 250 mM sodium formate, the enzymatic activities were 127- and 40-fold greater than in the absence of external nucleophiles, respectively. The kinetic parameters of Endo-A E173A mutant in the presence of 2 M sodium azide were calculated; K_m 0.876 mM and k_{cat} 0.0458 s^{-1}. However, the k_{cat}/K_m value (0.0523 $s^{-1}mM^{-1}$) was 30,000-fold lower than that of wild-type Endo-A (1760 $s^{-1}mM^{-1}$), indicating that the enzymatic activity was only partially rescued by the external nucleophiles.

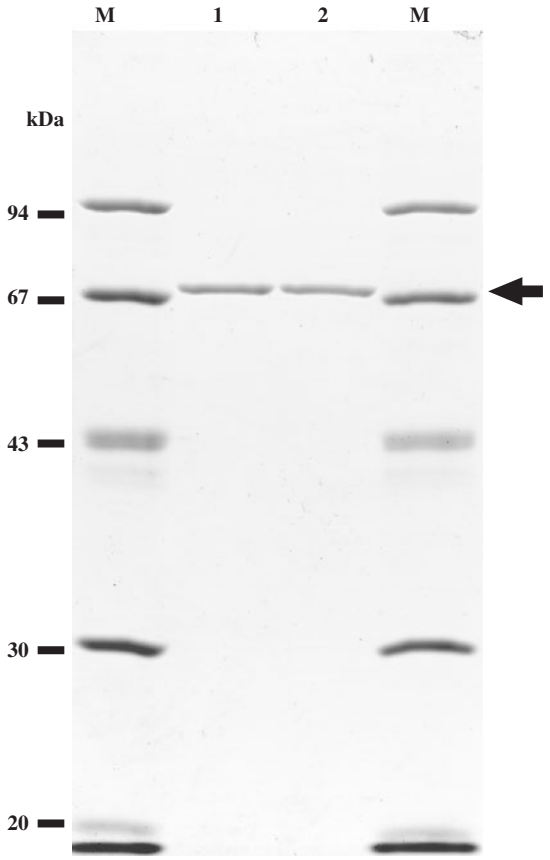

Fig. 1. **SDS–PAGE analysis of the recombinant wild-type Endo-A and the E173A mutant.** The proteins were subjected to SDS–PAGE using a 10% polyacrylamide gel, and visualized by Coomassie Brilliant Blue R-250 staining. Lane 1, purified recombinant Endo-A; lane 2, purified Endo-A E173A mutant; lane M, molecular size markers. The arrow indicates the position of the recombinant Endo-A.

Analysis of Glycosyl Azide—We examined the formation of the glycosyl azide by Endo-A E173A mutant using $Man_5GlcNAc_2Asn$ as the substrate in the presence of 1 M sodium azide as described in 'Materials and methods' section. The reaction mixture produced was separated

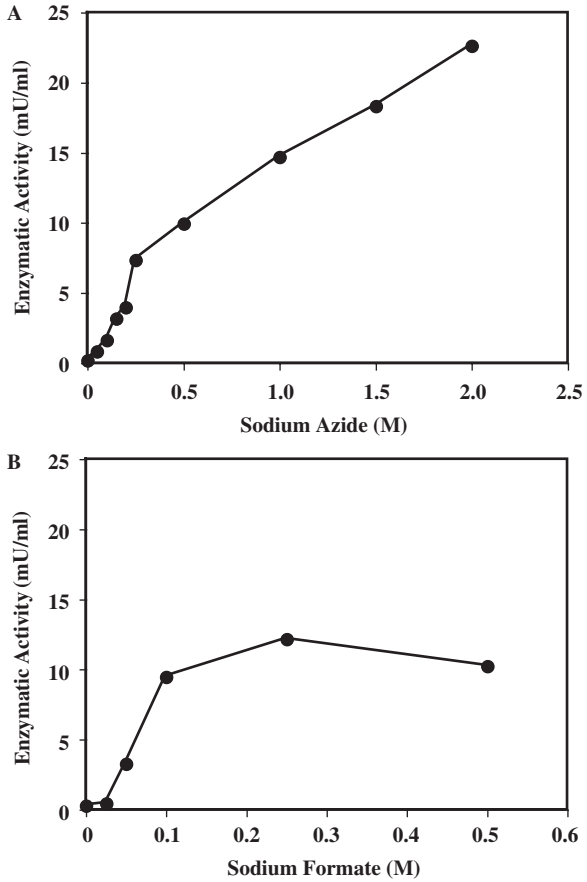

Fig. 2. **(A) Enzymatic activity of the Endo-A E173A mutant in the presence of sodium azide and sodium formate.** (B) Assays were performed at 37°C for 30 min using $Man_5GlcNAc_2Asn$-dansyl as described in the text.

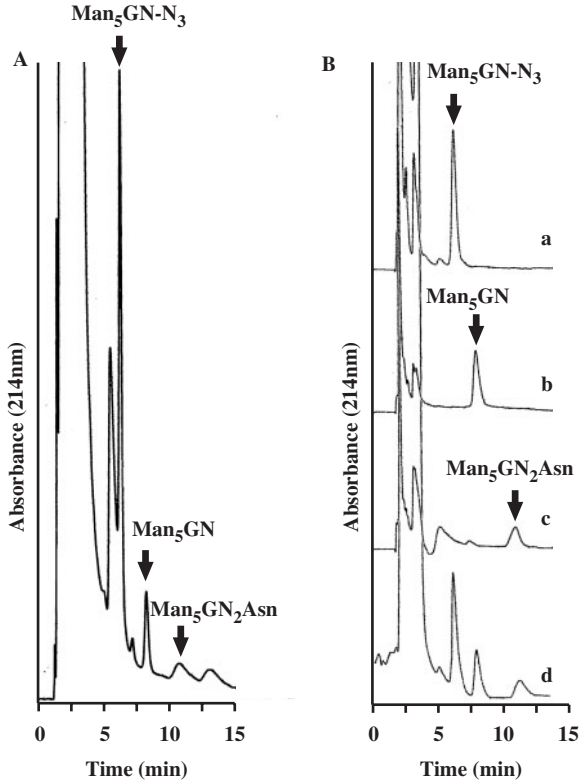

Fig. 3. **(A) HPLC analysis of the reaction mixtures incubated with $Man_5GlcNAc_2Asn$ and Endo-A E173A mutant in the presence of sodium azide.** (B) HPLC profile of the purified glycosyl azide (a), $Man_5GlcNAc$ (b), $Man_5GlcNAc_2Asn$ (c) and the sample mixture (d).

by HPLC (Fig. 3A), and the newly generated peak fractions were collected. The reaction product was confirmed in the peak fraction of 5.9 min by TLC analysis, but the peak fractions of 3.0 and 5.5 min were not visible on TLC (data not shown). Furthermore, an aliquot of the peak fraction of 5.9 min was analysed by HPLC. The retention time (Fig. 3B-a, 6.21 min) was separated from that of $Man_5GlcNAc$ (Fig. 3B-b, 7.95 min) and $Man_5GlcNAc_2Asn$ (Fig. 3B-c, 11.0 min). We then used the peak fraction of 5.9 min for MALDI TOF-MS and proton NMR measurements. MALDI TOF-MS analysis gave a molecular ion at *m/z* 1079.48, corresponding to the sodium adduct of glycosyl azide $Man_5GlcNAc\text{-}N_3$ (calculated for $C_{38}H_{64}N_4O_{30}Na$ $[M+Na]^+$, 1079.35). The anomeric configuration of glycosyl azide was identified by proton NMR analysis (Fig. 4). The chemical shift, δ4.68, and the coupling constant, $J = 8.9$ Hz, of reducing terminal proton were quite similar to the doublet of axial anomeric hydrogen of β-*N*-acetylglucosaminyl azide (δ4.74, $J = 9.0$ Hz) (*15*), which indicates that a β-glycosyl azide ($Man_5GlcNAc\text{-}β\text{-}N_3$) was formed.

Furthermore, the glycosyl azide products formed during the reaction were analysed by TLC (Fig. 5A). Aliquots of the reaction mixture were taken at various time points and immediately boiled prior to TLC analysis. A spot appeared after 1-h incubation with sodium azide (Fig. 5A-b lane 2), which was not visible when the reaction was performed in the absence of external nucleophile (Fig. 5A-a lane 2). The purified $Man_5GlcNAc\text{-}N_3$ was also hydrolysed by Endo-A E173A mutant (Fig. 5B) and wild-type Endo-A (data not shown), which supported that the newly generated glycosyl azide was gradually hydrolysed on additional incubation (Fig. 5A-b lanes 7–9).

DISCUSSION

The catalytic acid–base residue of GH family 18 enzymes has been identified (e.g. Glu-132 in Endo-H from *Streptococcus plicatus*) (*16–18*). GH family 18 and 85 members have a consensus motif, (LIVMFY)(DNEH)G(LIVMFW)(DNLF)(LIVMF)(DN)XE, in which the glutamic acid underlined was Glu-132 in Endo-H and Glu-173 in Endo-A (*4*). However, the consensus motif provides no direct evidence for the catalytic rescue of GH family 85 enzymes. In the present study, inactive E173A mutant of Endo-A was rescued in the presence of sodium azide and produced a β-glycosyl azide from $Man_5GlcNAc_2Asn$. Because the transglycosylation products of Endo-A retain β-anomeric configuration (*19*, *20*), the reaction mechanism of this enzyme is presumably retaining. The acid–base catalyst mutants

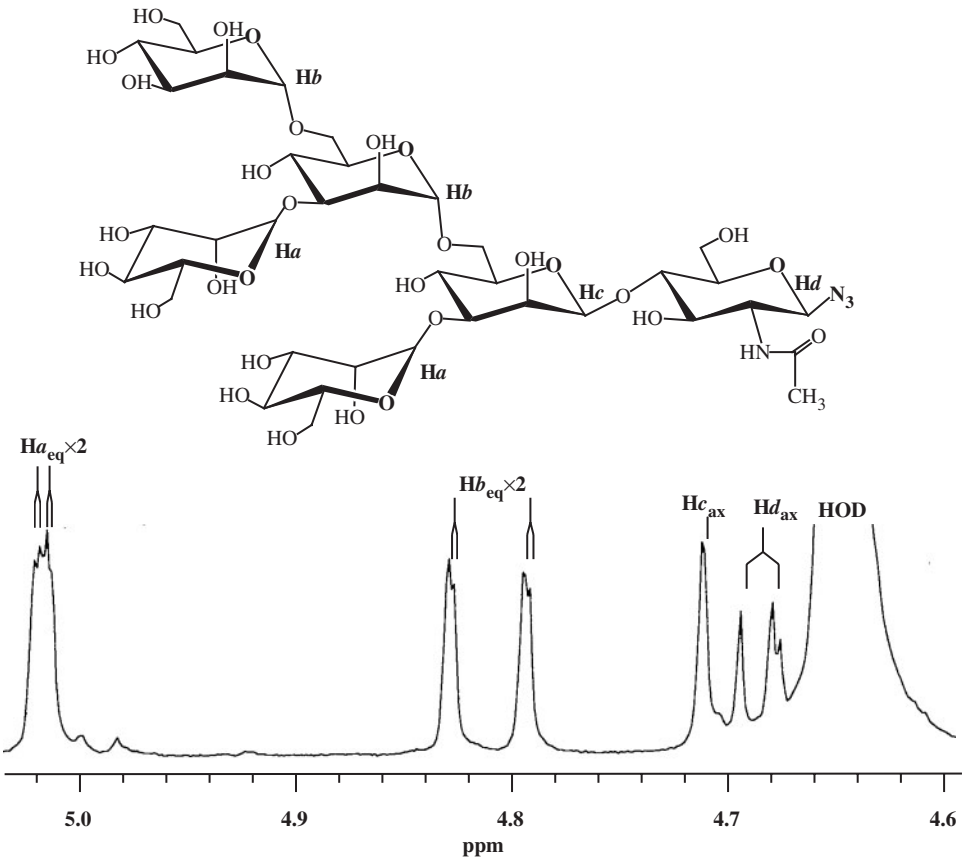

Fig. 4. **^{1}H NMR spectra of the purified glycosyl azide.** ^{1}H NMR signals for the region of the anomeric protons (4.59–5.04 ppm) are shown. The signal for the anomeric proton of Man$_5$GlcNAc-azide: α1,3 Man residues (Ha_{eq}; δ 5.02), α1,6 Man residues (Hb_{eq}; δ 4.79, $J = 2.1$ Hz and δ 4.83, $J = 1.4$ Hz), β1,4 Man (Hc_{ax}; δ 4.71) and GlcNAc-azide (Hd_{ax}; δ 4.68, $J = 8.9$ Hz). The large signal around δ 4.64 is from HDO.

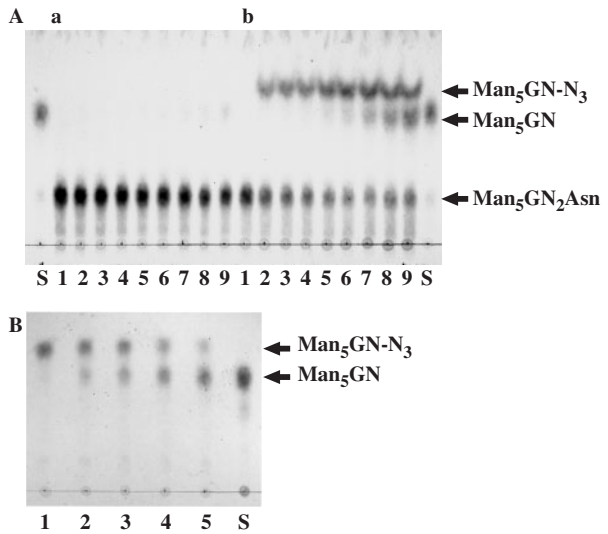

Fig. 5. **(A) TLC analysis of the reaction mixtures incubated with Man$_5$GlcNAc$_2$Asn and Endo-A E173A mutant in the presence of sodium azide.** 0.4 mg/ml of Endo-A E173A mutant was incubated with 1.0 mM Man$_5$GlcNAc$_2$Asn in the absence (a) or presence (b) of 1 M sodium azide at 37°C for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5), 12 h (lane 6), 24 h (lane 7), 48 h (lane 8) and 72 h (lane 9). Lane S, Man$_5$GlcNAc. (B) TLC analysis of hydrolysis of purified glycosyl azide by Endo-A E173A mutant. 0.4 mg/ml of Endo-A E173A mutant was incubated with 1.0 mM of purified Man$_5$GlcNAc-N$_3$ at 37°C for 0 min (lane 1), 10 min (lane 2), 20 min (lane 3), 40 min (lane 4) and 80 min (lane 5). Lane S, Man$_5$GlcNAc.

of β-retaining glycosidases are known to produce β-glycosyl azide in the azide rescue studies (*12*). These data indicate that the Glu-173 residue acts as the catalytic acid–base residue of Endo-A. Furthermore, it has been proposed that the carbonyl oxygen of the 2-acetamide group in the substrate of GH family 18 chitinases (*21*) and endo-β-*N*-acetylglucosaminidases (*18*), family 20 hexsosaminidases (*22*), family 56 hyaluronidases (*23*) and family 84 *O*-GlcNAc-ases (*24*) acts as the nucleophile to form an oxazolinium intermediate. In the case of GH family 85 endo-β-*N*-acetylglucosaminidase, synthetic oligosaccharide oxazolines were used as the donor for the Endo-A and Endo-M transglycosylation reaction (*25–27*). These facts suggest that Endo-A possesses a substrate-assisted catalytic mechanism in which Glu-173 acts as the catalytic acid–base (Fig. 6A). Therefore, the reaction mechanism of the catalytic mutant is suggested that azide act as base instead of Glu-173 (Fig. 6B).

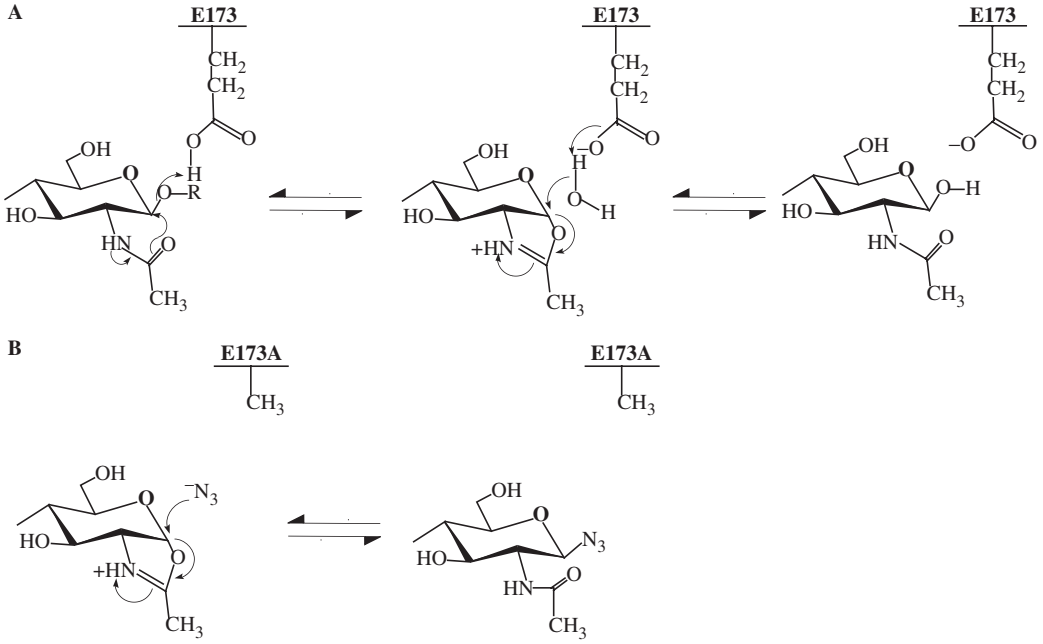

Fig. 6. **Schematic drawing of reaction mechanism of Endo-A.** (A) Proposed hydrolysis mechanism of sodium azide. (B) Reaction mechanism of chemical rescue by

GH family 85 enzymes possess a potential to synthesize neoglycoproteins by utilizing the transglycosylation activity (*8*, *9*). We previously identified a critical residue for the transglycosylation activity, but the residue had no effect on the hydrolysis activity [e.g. W216R for the Endo-A (*10*) and W228R for Endo-M (*4*)]. GH families 85 and 18 enzymes possess no sequence homology except for the consensus motif, and any GH family 18 endo-β-*N*-acetylglucosaminidases have not been reported to exhibit transglycosylation activity (*8*). In the present study, we identified the catalytic acid–base residue of Endo-A. This is the first identification of the catalytic acid–base residue for GH family 85 endo-β-*N*-acetylglucosaminidases. This finding will help to elucidate the transglycosylation mechanism of GH family 85 enzymes.

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