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One-Pot Enzymatic Glycan Remodeling of a Therapeutic Monoclonal Antibody by Endoglycosidase S (Endo-S) From *Streptococcus pyogenes*

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Abstract

A facile, one-pot enzymatic glycan remodeling of antibody rituximab to produce homogeneous high-mannose and hybrid type antibody glycoforms is described. This method was based on the unique substrate specificity of the endoglycosidase S (Endo-S) from *Streptococcus pyogenes*. While Endo-S efficiently hydrolyzes the bi-antennary complex type IgG Fc N-glycans, we found that Endo-S did not hydrolyze the “ground state” high-mannose or hybrid glycoforms, and only slowly hydrolyzed the highly activated high-mannose or hybrid N-glycan oxazolines. Moreover, we found that wild type Endo-S could efficiently use high-mannose or hybrid glycan oxazolines for transglycosylation without product hydrolysis. The combination of the remarkable difference in substrate specificity of Endo-S allows the deglycosylation of heterogeneous rituximab and the transglycosylation with glycan oxazoline to take place in one-pot without the need of isolating the deglycosylated intermediate or changing the enzyme to afford the high-mannose type, hybrid type, and some selectively modified truncated form of antibody glycoforms.

Graphical abstract

Keywords

Monoclonal antibody; Fc glycosylation; Glycoengineering; Endoglycosidase; Transglycosylation; Endo S; Chemoenzymatic synthesis; rituximab

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1. Introduction

Therapeutic monoclonal antibodies are an important class of therapeutic proteins that are widely used for the treatment of human diseases, including cancer, inflammatory disorders, and infectious diseases^{1,2}. All types of antibodies (IgG, IgA, IgD, IgM, and IgE) are glycosylated at the Fc domain. In the case of the IgG type antibodies, the Fc glycosylation at the conserved N297 glycosylation site has been shown to play a crucial role in modulating antibody's effector functions³⁻⁶. Moreover, the fine structures of the Fc glycans can sometimes dictate the fate of a given antibody^{3,7,8}. For example, it has been well documented that removal of the core fucose from the Fc glycans of rituximab, a therapeutic monoclonal antibody, significantly enhances its antibody-dependent cellular cytotoxicity (ADCC), mainly through an enhanced binding to the Fc γ IIIa receptor on the effector cells, resulting in its improved therapeutic efficacy in anti-cancer therapy^{9,10}. On the other hand, the terminal α -2,6-sialylation of intravenous immunoglobulin (IVIG) has been shown to be important for IVIG's anti-inflammatory activity¹¹⁻¹⁴. Thus controlling Fc glycosylation to the correct state is critically important for producing effective therapeutic antibodies. Nevertheless, recombinant antibodies are usually produced as mixtures of heterogeneous glycoforms that have the same protein backbone but differ in the Fc glycans attached, from which pure homogeneous glycoforms are difficult to separate.

To address this problem, we have attempted to develop an *in vitro* chemoenzymatic glycan remodeling approach for engineering Fc glycosylation, which involves two key steps: an endoglycosidase-catalyzed deglycosylation to remove the heterogeneous Fc N-glycans, and a subsequent enzymatic transfer of a pre-assembled N-glycan from the activated N-glycan oxazoline to constitute the desired homogeneous antibody glycoforms^{15,16}. Two bacterial endoglycosidases, the Endo-S from *Streptococcus pyogenes* and the Endo-S2 from the *Streptococcus pyogenes* of serotype M49 have been found to be highly active and specific for Fc deglycosylation of intact IgG antibodies¹⁷⁻¹⁹. We have previously demonstrated that Endo-S possesses transglycosylation activity and that glycosynthase mutants, such as EndoS-D233Q, can be generated which lack the hydrolysis activity on antibody glycoforms, but are able to use the highly active glycan oxazoline for transglycosylation to give homogeneous glycoforms²⁰. More recently, we have extended this glycosynthase approach to the Endo-S2 and have generated new glycosynthases that could use all major types of N-glycan oxazolines for transglycosylation²¹. Several groups including ours have used the glycosynthase approach for the synthesis of various homogeneous glycoforms of antibodies for structural and functional studies^{8,22-27}. Despite these successes, one drawback of this glycan remodeling method is that the wild-type enzyme (Endo-S or Endo-S2) used for the deglycosylation needs to be separated from the reaction mixture after the deglycosylation step in order to avoid hydrolyzing the final product, and the second step requires a glycosynthase mutant for transglycosylation, followed by another purification step.

Here, we report our findings that the wild-type Endo-S showed promiscuous activity on both the hybrid and high-mannose type glycan oxazolines for transglycosylation with marginal hydrolytic activity on the product glycoforms. The unique substrate specificity of Endo-S, coupled with its highly efficient hydrolysis on the complex-type N-glycans, permitted the development of an efficient, one-pot glycosylation remodeling method for therapeutic

antibodies such as rituximab as a model system. The one-pot enzymatic approach did not require removal of the enzyme and purification of the intermediate after deglycosylation step, and the Endo-S treatment was able to do both deglycosylation and transglycosylation. We also demonstrated that this one-pot strategy enabled chemoenzymatic synthesis of an azido-tagged N-glycoform of rituximab which could be further modified through orthogonal chemical ligation for various applications.

2. Results and Discussion

2.1. Comparative analysis of the Endo-S-catalyzed hydrolysis of different rituximab glycoforms and synthetic N-glycan oxazolines

Previously, Collin and co-workers have reported that Endo-S and Endo-S2 hydrolyze Fc-glycans of therapeutic antibodies with different substrate specificity. While Endo-S2 can release all three major types of N-glycans from the Fc domain of several therapeutic antibodies including cetuximab, Endo-S is only efficient for releasing bi-antennary complex type N-glycans from the antibodies¹⁹. To further confirm the substrate selectivity of the wild-type Endo-S, we performed side-by-side comparative analysis of Endo-S-catalyzed hydrolysis reactions for the selected homogeneous glycoforms of rituximab, as well as various synthetic N-glycan oxazolines (Figure 1).

We first tested three different homogeneous glycoforms of rituximab, namely, the sialylated complex type (**1**), the high-mannose (Man9GlcNAc2) type (**2**), and the hybrid type (**3**) (Figure 1). These homogeneous glycoforms (**1–3**) were synthesized following our recently published method, using the Endo-S2 glycosynthase mutant (EndoS2-D184M) as a key enzyme for transglycosylation²¹. The enzymatic reaction was monitored by LC-ESI-MS analysis. We found that Endo-S could hydrolyze the bi-antennary complex type glycoform of rituximab (**1**) fast, resulting in over 90% hydrolysis within 2 h under the reaction condition. However, only marginal hydrolysis (less than 5%) of the high-mannose and hybrid glycoforms (**2** and **3**) was observed under the same reaction condition (Figure 2A). A prolonged (overnight) incubation resulted in about 10% hydrolysis of the high mannose glycoform (**2**) and approximate 15% of the hybrid glycoform (**3**), respectively (data not shown). These data confirm that the high-mannose and hybrid type Fc glycoforms of antibodies are largely resistant to Endo-S-catalyzed hydrolysis although residual hydrolytic activity was detected, and the bi-antennary complex type glycoform is an excellent substrate for the wild-type Endo-S.

Next, we compared the Endo-S WT-catalyzed hydrolysis of various synthetic N-glycan oxazolines. Synthetic glycan oxazolines, the mimics of the oxazolinium intermediate during the endoglycosidase-catalyzed reaction in a substrate-assisted mechanism, are the key donor substrates for the endoglycosidase-catalyzed transglycosylation used for glycoprotein remodeling^{15,16,28}. Thus, it is important to know whether the glycan oxazolines will be hydrolyzed and how fast they will be hydrolyzed by the enzyme of interest. Various glycan oxazolines were incubated with Endo-S WT in PBS buffer, and the hydrolysis products (free glycans) were monitored and quantitated by HPAEC-PAD (the high performance anion exchange chromatography coupled with pulsed amperometric electrochemical detection). As shown in Figure 2B, the complex type glycan oxazoline (**4**)²⁹ was rapidly hydrolyzed by

Endo-S WT. In contrast, the high-mannose type N-glycans oxazolines, including the Man9 oxazoline (M9-Oxa **5**)³⁰ and the Man5 glycan oxazoline (Man5-Oxa **7**)³¹, were shown to be rather resistant to Endo-S hydrolysis. The hybrid type oxazoline (Hyb-Oxa **6**)²¹ showed moderate hydrolysis by Endo-S WT. Surprisingly, the Man3 oxazoline (M3-Oxa **8**)³², corresponding to the shared N-glycan core, was hydrolyzed even faster than the bi-antennary complex type N-glycan oxazoline (SCT-Oxa **4**) by Endo-S (Figure 2B). However, chemical modification at the two outer mannose moieties led to reduction of the enzymatic hydrolysis, as demonstrated by the azide-Man3 glycan oxazoline (AzideM3-Oxa **9**)³² (Figure 2B). These results clearly indicate that wild-type Endo-S possesses distinct enzymatic activities towards different glycan oxazolines, as well as different glycoforms of antibodies.

2.2. Endo-S-catalyzed transglycosylation of deglycosylated rituximab with different N-glycan oxazolines

The distinct substrate specificity of Endo-S towards different antibody glycoforms and N-glycan oxazolines prompted us to examine the wild-type Endo-S-catalyzed transglycosylation with the three major Fc N-glycans oxazolines, complex (SCT-Oxa **4**), Man9 (HM-Oxa **5**), and Hybrid (Hyb--Oxa **6**), to the deglycosylated rituximab (Fuc α 1,6GlcNAc-rituximab, **10**) (Scheme 1). The acceptor Fuc α 1,6GlcNAc-rituximab (**10**) was prepared by deglycosylation of rituximab with Endo-S, following our previously published procedure²⁰. We found that the wild-type Endo-S showed a significant transglycosylation activity with the complex type N-glycan oxazoline (**4**) to form the transglycosylation product (**1**) (Figure 3). However, the product was quickly hydrolyzed by the enzyme, as the resultant complex type glycoform turned out to be an excellent substrate for Endo-S-catalyzed hydrolysis. This result was consistent with our previous observation²⁰. We also observed that, under the same condition, wild-type Endo-S was also able to transfer the high-mannose glycan oxazoline (**5**) and hybrid glycan oxazoline (**6**), but at a much lower reaction rate than that with the complex type glycan oxazoline (**4**) (Figure 3).

Nevertheless, since the transglycosylation products (**2** and **3**) were largely resistant to Endo-S-catalyzed hydrolysis, the transglycosylation products could thus be accumulated. Previously we have generated novel Endo-S mutants, including EndoS-D233Q, which are glycosynthases that can use glycan oxazolines for transglycosylation but lack the activity to hydrolyze the product²⁰. The EndoS-D233Q mutant was efficient for transferring complex type N-glycan oxazolines to the deglycosylated antibody, however, it was not clear how the glycosynthase mutant would act on the high-mannose and hybrid type N-glycan oxazolines. Thus we tested EndoS-D233Q with oxazolines **5** and **6** for transglycosylation. Surprisingly, we found that the Endo-S mutant, EndoS-D233Q, was unable to act on either the high-mannose glycan oxazoline (**5**) or the hybrid glycan oxazoline (**6**) for transglycosylation with the Fuc α 1,6GlcNAc-rituximab (**10**), while under the same condition the wild-type Endo-S could achieve over 85% transglycosylation to form the corresponding antibody glycoforms (Figure 3B and Figure 3C). One explanation is that, in comparison with the wild-type enzyme, the D233Q mutation could reduce both the hydrolysis and transglycosylation activities of the enzyme, thus, resulting in decreased efficiency toward the less favorable glycan oxazoline substrates, while it was still active enough towards the complex type N-glycan oxazoline (**4**), as shown in the previous publication²⁰. These new experimental data

suggest that the wild-type Endo-S is complementary to the mutant Endo-S in terms of their substrate specificities, and the Endo-S WT will be particularly useful for the synthesis of antibodies with high-mannose and hybrid glycoforms, which could not be achieved by the previously reported Endo-S mutants.

2.3. One-pot N-glycosylation remodeling of commercial rituximab via wild-type Endo-S

The potent transglycosylation activity of Endo-S WT with the hybrid and high-mannose type oxazolines, together with its efficient hydrolysis of the complex-type N-glycans from the commercial antibody rituximab, prompted us to examine the possibility of remodeling antibody glycosylation in a one-pot fashion (Scheme 2). Thus, a mixture of the commercial rituximab, glycan oxazoline (**5** or **6**), and wild-type Endo-S was incubated in a buffer at room temperature and the reaction was monitored by LC-ESI-MS analysis. As expected, the Endo-S-catalyzed deglycosylation of rituximab took place rapidly and was completed within 5 min under the reaction condition, even before any significant transglycosylation could occur (data not shown). Then the transglycosylation product (**2** or **3**) was steadily formed over the time without apparent hydrolysis, reaching about 80% within 2 h (Figure 4A and 4B). As the IgG monoclonal antibody (rituximab) is a dimer consisting of two heavy chains and two light chains in which only the Fc domain of the heavy chain is glycosylated, the transglycosylation yield was calculated by the following equation: $\text{yield (\%)} = \frac{\text{fully transglycosylated rituximab}}{\text{deglycosylated rituximab} + \text{partially transglycosylated rituximab} + \text{fully transglycosylated rituximab}} \times 100$. The relative intensity of the deglycosylated, partially transglycosylated, and fully transglycosylated rituximab species in the LC-MS analysis was estimated for the calculation. This offered a quick estimate of the transglycosylation yield but did not imply an accurate quantification.

It was also observed that the transglycosylation with the hybrid type glycan oxazoline (**6**) went faster than that of the high-mannose type glycan oxazoline (**5**) under the same condition. The LC-ESI-MS profiles of the intact antibodies after transglycosylation were shown in Figure 4C and 4D. The experimental data indicated that the free N-glycans in the medium released from the deglycosylation step did not interfere with the transglycosylation step, and the one pot approach was able to transform the heterogeneous rituximab into high-mannose or hybrid type glycoforms without the need of separating the deglycosylated intermediate or changing the enzyme. It should be pointed out that this transformation could be optimized to push the reaction to completion by adding more glycan oxazoline and/or enzyme with prolonged incubation time.

2.4. One-pot glycan remodeling of commercial Rituximab with the azido-tagged azide-Man3GlcNAc oxazoline by the wild-type Endo-S

In addition to the hybrid and high mannose type N-glycans, we also tested the ability of Endo-S to act on the azido-tagged azide-Man3GlcNAc oxazoline (AzideM3-Oxa, **9**) for Fc glycan remodeling of rituximab in a one-pot manner (Scheme 3). Following the same one-pot reaction protocol described above, we found that the conversion took place relatively slowly, but the transglycosylation product, the azide-tagged glycoform of rituximab (**11**) was formed steadily. Within 6 h, nearly 40% conversion was achieved (Figure 5). We also tested the Man3 glycan oxazoline (M3-Oxa **8**). Interestingly, only marginal amount of

transglycosylation product was detected by LC-MS analysis in the case of using the Man3 glycan oxazoline (**8**) (Figure 5A). This result is consistent with our previous comparative study on Man3 glycan oxazoline (**8**) with Endo-S D233Q mutant and the wild-type Endo-S, where only transition formation of the transglycosylation product was observed by SDS-PAGE analysis when the wild-type Endo-S was used, whereas high-yield conversion was achieved using the Endo-S-D233Q mutant²⁰.

Indeed, as shown in the oxazoline hydrolysis experiment described above (Figure 2), the Man3-glycan oxazoline (**8**) was also hydrolyzed very rapidly by the wild-type Endo-S. In addition, the transglycosylation product was also hydrolyzed quickly by the wild-type Endo-S. Thus it was not a surprise that only transitional transglycosylation product was detected during the reaction. It should be pointed out that Davis and co-workers previously reported the Endo-S-catalyzed transglycosylation with Man3 glycan oxazoline on the deglycosylated IgG antibody, but no detailed quantitative characterization of the transglycosylation product was reported except MS analysis of the released N-glycans³³. In the present case of the azide-Man3 glycan oxazoline (**9**), our enzymatic glycan oxazoline hydrolysis experiment showed that the chemical modification increased to some extent its resistance toward Endo-S-catalyzed hydrolysis (Figure 2), which could partially explain the moderate transglycosylation yield. It is expected that the one-pot enzymatic glycan remodeling method might be extended to other site-selective modified glycan oxazolines to synthesize selectively tagged glycoforms of various antibodies.

3. Conclusion

We have demonstrated that wild-type Endo-S possesses remarkably different hydrolysis and transglycosylation activity on different N-glycan oxazolines and glycoforms of antibodies, using therapeutic monoclonal antibody rituximab as a model system. The potent activity to deglycosylate the recombinant complex type glycoforms of antibodies, the promiscuous transglycosylation activity on the high-mannose and hybrid type N-glycan oxazolines, and yet the lack of hydrolysis activity on the “ground-state” high-mannose and hybrid type glycoforms has enabled an efficient one-pot enzymatic glycan remodeling of heterogeneous glycoforms of rituximab to produce homogenous high-mannose or hybrid type glycoforms, without the need of intermediate separation and enzyme switching. This one-pot enzymatic glycan remodeling method should be also applicable to other therapeutic antibodies for obtaining specific glycoforms of antibodies for various applications.

4. Experimental Procedures

4.1. Materials

Monoclonal antibody, rituximab, was purchased from Genentech Inc., (South San Francisco, CA). Complex-type (SCT) sialoglycan oxazoline (**4**) and high mannose-type (HM) oxazoline (**5**) were harvested and synthesized from egg yolk and soybean flour, respectively, following previously described methods^{29,30}. The hybrid-type (Hyb) oxazoline (**6**) was converted from Man5GlcNAc (**7**) via a multi-step enzymatic treatment using a series of previously described enzymes, including the β -1,2-GlcNAc transferase (GnT1), β -1,4-galactosyltransferase, and sialyltransferase²¹. The Man3 (M3-Oxa **8**) and Azide-tagged

Man3 (AizdeM3-Oxa 9) oxazolines were synthesized according to our previously publications³².

4.2. Expression and Purification of Recombinant Endo-S WT and Endo-SD233Q

The pGEX plasmid vector encoding the wild-type Endo-S was a gift from Dr. M. Collin (Lund University, Sweden). Site-directed mutagenesis was conducted as described in the previous publication to generate Endo-S D233Q mutant DNA. The plasmid DNA encoding both the wild-type Endo-S and the Endo-S D233Q mutant was transformed in *E. coli* BL21 (DE3) cells for overexpression. For purification of the enzyme, the transformed cells were grown in 1L 2YT media with 100 µg/mL Carbenicillin added. Cells were incubated at 37 °C and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the cell density reached an OD600 of 0.8–1.0. The induced cells were incubated at 20°C overnight and harvested by centrifugation. The cell lysates were collected after treating with bacterial cell lysis buffer (Gold Biotechnology, Inc.) following manufacturer's instructions. The GST-tagged proteins were purified using the GST resin batch method (Thermo). The purified proteins were diluted into PBS at pH 7.4 after buffer exchange using Amicon ultra filtration units (10 kDa, Millipore). The purity of Endo-S proteins was over 90% on SDS-PAGE and the final concentration was recorded on a NanoDrop 2000c at absorbance 280 nm.

4.3. Liquid Chromatography Mass Spectrometry (LC-ESI-MS) Analysis

Antibody N-glycoforms were studied using an Exactive Plus Orbitrap instrument equipped with a HPLC system and an ESI source. The capillary temperature and the spray voltage were set up according to previous publications. Approximately 25 µL of intact rituximab solution was injected and separated via a Waters XBridge™ BEH300 C4 column (3.5 µm, 2.1 × 50 mm) at a flow rate of 0.4 mL/min. The program includes a 9 min linear gradient of 5–90% MeCN containing 0.1 % formic acid. The starting material deglycosylated rituximab (m/z 144879) and different transglycosylation products were measured using selected ion monitoring (SIM). The signal intensities of the target ions were optimized by automatic tuning function and the same optimized conditions were used in all LC-MS quantifications. The MagTran Software (Amgen) was used to analyze the data from the exported chromatogram results. An average of scans was analyzed through deconvolution to give a spectrum list for each analyzed sample. Integration of the deconvoluted spectrum shows the relative abundance of the analyzed ions. Total peak intensities within 0.7 mass units surrounding the center of the target ion peak were used to determine the ratio of the starting material and the product species. This relative ratio can then be used to calculate the reaction yield of each hydrolysis and transglycosylation reaction with the concentration of the starting material known.

4.4. HPAEC-PAD analysis of glycans and oxazolines

High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) was conducted on a DIONEX chromatography system (DIONEX Corporation, Sunnyvale, CA) using an anion exchange column (CarboPac PA200, 3 × 250 mm). The sample was delivered with a mobile phase consisting of deionized water (eluent A), 0.2 M NaOH (eluent B), 0.1M M NaOH (eluent C) and 0.1M M NaOH with 0.25M

NaoAc (eluent D). The program for the complex and hybrid type glycans and oxazolines used a gradient as follows: 0 min, 0% eluent A, 0% eluent B, 100% eluent C and 0% eluent D; 5.0 min, 0% eluent A, 0% eluent B, 100% eluent C and 0% eluent D; and 25.0 min, 0% eluent A, 0% eluent B, 60% eluent C and 40% eluent D at a flow rate of 1.0 mL/min. The program for oligomannose type glycans and oxazolines (Man9, Man5, Man3 and Azide-Man3) were analyzed using a different program: 0 min, 0% eluent A, 0% eluent B, 100% eluent C and 0% eluent D; 5.0 min, 0% eluent A, 0% eluent B, 100% eluent C and 0% eluent D; and 25.0 min, 0% eluent A, 0% eluent B, 80% eluent C and 20% eluent D with a flow rate of 1.0 mL/min. The relative abundance of each glycan and oxazoline was calculated from the integrated spectrum.

4.5. Comparison of the hydrolytic activity of Endo-S WT towards different rituximab glycoforms

The rituximab glycoforms, SCT-RTX **1**, HM-RTX **2** and Hyb-RTX **3** were synthesized according to the previously published protocol²¹. In three parallel reactions, each of the three N-glycoforms (0.01 mg, 6.9 μ M) was incubated with Endo-S WT (5 μ g/mL) at 30 °C in 20 μ l of PBS buffer (pH 7.4) for a given amount of time. The reaction was quenched by adding 20 μ l of 0.1% formic acid and the sample was placed at -20 °C. The hydrolysis yield was measured by calculating the ratio of the hydrolyzed product versus the starting material using LC-ESI-MS as shown above.

4.6. Comparison of the hydrolytic activity of Endo-S WT towards various oxazolines

In parallel reactions, SCT-Oxa **4**, HM-Oxa **5**, Hyb-Oxa **6**, M5-Oxa **7**, M3-Oxa **8** and AzideM3-Oxa **9** oxazolines (1 mM) was incubated with Endo-S WT (50 μ g/ml) at 30 °C in 25 μ l of PBS buffer (pH 7.4). Aliquots of reaction mixture (1 μ l) was sampled at the same time intervals and diluted in 100 mM NaOH solution to quench the reaction. All reactions were monitored by HPAEC-PAD and the relative percentage of the hydrolyzed oxazoline (free glycan) was quantified from the integrated spectrum as detailed in previous sections.

4.7. Evaluation of the transglycosylation activity of Endo-S WT towards the deglycosylated rituximab using SCT-Oxa **4**, HM-Oxa **5** and Hyb-Oxa **6** as glycosyl donors

The ability of Endo-S WT to transfer three different N-glycan oxazolines SCT-Oxa **4**, HM-Oxa **5** and Hyb-Oxa **6** to the deglycosylated rituximab **10** in a conventional two-step transglycosylation reaction was compared under the same condition. First, the commercial rituximab underwent deglycosylation by incubation with a catalytic amount of Endo-S WT (10 μ g/ml) at 30 °C for a short period of time. Once complete deglycosylation was confirmed, the deglycosylated rituximab was subsequently purified through Protein-A chromatography for further transglycosylation. The following reaction was performed using the deglycosylated rituximab **10** (10 mg/mL) as the glycosyl acceptor and N-glycan oxazolines as glycosyl donors with a catalytic amount of Endo-S WT (50 μ g/ml) at 30 °C in 20 μ l of PBS buffer (pH 7.4). The molar ratio of the donor substrate to the glycosyl acceptor was 40:1.

4. 8. Evaluation of transglycosylation activity of Endo-S WT and Endo-S D233Q towards the deglycosylated rituximab using HM-Oxa 5 and Hyb-Oxa 6 as glycosyl donor

The transglycosylation efficiency of Endo-S WT and Endo-S D233Q towards the deglycosylated rituximab was compared separately for HM-Oxa **5** and Hyb-Oxa **6**. The deglycosylated rituximab was prepared as mentioned above. For reaction with either oxazoline, the deglycosylated rituximab **10** (10 mg/mL) was incubated with each enzyme (500 µg/ml) at 30 °C in 20 µl of PBS buffer (pH 7.4). The molar ratio of the donor substrate to the glycosyl acceptor was also 40:1. The transglycosylation products were analyzed and quantified as mentioned previously.

4. 9. Transglycosylation of commercial Rituximab with Hyb-Oxa and HM-Oxa by Endo-S WT in a one-pot manner

Commercial Rituximab (0.1 mg, 69 µM) was dissolved in PBS buffer (pH 7.4) and was mixed with 40 equivalents of hybrid and high mannose oxazolines HM-oxa **5** (2.8 mM) and Hyb-oxa **6** (2.8 mM), respectively. The reaction was initiated by adding a catalytic amount of Endo-S WT (500 µg/ml) and incubated in PBS at 30°C (pH 7.4). For product quantification, the concentration of the product glycoform carrying two N-glycans, 148205 for HM-RTX and 148219 for Hyb-RTX (deconvoluted mass), were counted twice as each antibody molecule carries two glycosylation sites. The N-glycoform carrying only one N-glycan for each oxazoline was only counted once as the product formed. The same quantification method using LC-ESI-MS was used for the similar experiments conducted in this paper.

4. 10. Transglycosylation of commercial Rituximab with M3-Oxa and AzideM3-Oxa by the Endo-S WT in a one-pot fashion

Commercial Rituximab (0.1 mg, 69 µM) was incubated in PBS at 30°C (pH 7.4) with 40 equivalents of oxazolines M3-Oxa **8** (2.8 mM) and AzideM3-Oxa **9** (2.8 mM), respectively. To initiate the reaction, a fixed amount of Endo-S WT (500 µg/ml) was added into the reaction mixture at t = 0 min. The reactions with M3-Oxa and AzideM3-Oxa were maintained for a prolonged period of time to allow the accumulation of the transglycosylation product. Additional 40 equivalents of oxazolines were added to each reaction at t = 120 min. Transglycosylation with AzideM3-Oxa **9** showed three species at 144879 (starting material), 145618 (product with a single glycan attached) and 146357 (product with two glycans attached). The reaction yield was quantified as described above.

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Highlights

- Endo-S processes various IgG glycoforms and glycan oxazolines with different efficiencies
- Endo-S could transfer hybrid and high-mannose type glycan oxazoline to an intact antibody without product hydrolysis
- Wild-type Endo-S shows complementary activity to Endo-S D233Q mutant in producing different IgG Fc N-glycoforms.
- A one-pot IgG Fc glycan remodeling for transforming heterogeneous antibody to homogeneous glycoforms was established

Figure 1. Structures of rituximab glycoforms and various N-glycan oxazolines used for the assessment of Endo-S activity.

Figure 2. Hydrolytic activity of Endo-S WT on different rituximab glycoforms and N-glycan oxazolines. A) Comparison of the hydrolytic activity of Endo-S WT towards the complex (1), high-mannose (2) and hybrid (3) type glycoforms of rituximab; B) Comparison of the hydrolytic activity of Endo-S WT towards various N-glycan oxazolines. The results shown here are representative of three independently conducted experiments.

Figure 3.

Endo-S and EndoS-D233Q mutant catalyzed transglycosylation with different N-glycan oxazolines. A) Comparison of the transglycosylation activity of Endo-S WT with complex type (SCT-Oxa 4), high-mannose type (HM-Oxa 5), and hybrid type (Hyb-Oxa 6) glycan oxazolines; B) Comparison of the transglycosylation activity of Endo-S WT and Endo-S D233Q mutant with the high mannose-type oxazoline (HM-Oxa 5); C) Comparison of the transglycosylation activity of Endo-S WT and Endo-S D233Q mutant with the hybrid-type oxazoline (Hyb-Oxa 6). The results shown here are representative of three independently conducted experiments.

Figure 4. Endo-S catalyzed one-pot glycan remodeling of rituximab with high-mannose and hybrid glycan oxazolines. A) Enzymatic remodeling with high-mannose N-glycan oxazoline (5); B) enzymatic remodeling with hybrid N-glycan oxazoline (6); C) LC-ESI-MS profile of the transglycosylation products from the enzymatic remodeling with high-mannose N-glycan oxazoline (5); D) LC-ESI-MS profile of the transglycosylation products from the enzymatic remodeling with the hybrid N-glycan oxazoline (6). The results shown here are representative of two independently conducted experiments.

Figure 5.

Endo-S catalyzed one-pot glycan remodeling of rituximab with Man3 glycan oxazoline and azide-Man3 glycan oxazoline. A) the time course of reactions with the Man3 glycan oxazoline (Man3-Oxa **8**) and the azide-Man3 glycan oxazoline (azideM3-Oxa **9**); B) the LC-ESI-MS profile of the transglycosylation products from the enzymatic remodeling with the Man3 glycan oxazoline (Man3-Oxa **8**). The results shown here are representative of two independently conducted experiments.

Scheme 1.

Transglycosylation activity of Endo-S with different types of N-glycan oxazolines.

Scheme 2.

One-pot enzymatic glycan remodeling of commercial rituximab by Endo-S with high-mannose and hybrid type N-glycan oxazolines.

Scheme 3.

One-pot enzymatic glycan remodeling of commercial rituximab by Endo-S with azide-modified Man3 glycan oxazoline.