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Chemoenzymatic glycan remodeling of natural and recombinant glycoproteins

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Abstract

N-glycosylation plays important roles in modulating the biological functions of glycoproteins, such as protein folding, stability, and immunogenicity. However, acquiring homogeneous glycoforms of glycoproteins has been a challenging task for functional studies and therapeutic applications. In this chapter, we describe an efficient chemoenzymatic glycan remodeling protocol for making homogeneous glycoproteins that involves enzymatic deglycosylation and subsequent reglycosylation procedures. Two therapeutic glycoproteins, Herceptin (trastuzumab, a therapeutic monoclonal antibody) and erythropoietin (EPO, a glycoprotein hormone) were chosen as the model systems. The detailed protocol includes the deglycosylation of the Herceptin or EPO with a wild type endo- β -N-acetylglucosaminidase, to remove the heterogeneous N-glycans, leading to the GlcNAc-protein or Fuca1,6GlcNAc-protein intermediate. Then desired homogeneous N-glycans are attached to the acceptor by using an activated sugar oxazoline as the donor substrate and a specific glycosynthase (mutant of endoglycosidase) as the catalyst to reconstitute a homogeneous glycoform. Using this approach, Herceptin was remodeled to an afucosylated complex glycoform and a Man₉GlcNAc₂ glycoform, with the former showing significantly enhanced antibody-dependent cellular cytotoxicity. EPO was engineered to carry azide-tagged Man₃GlcNAc₂ glycans that could be further modified via click chemistry to introduce other functional groups.

Keywords

glycoprotein; antibody; Herceptin; erythropoietin; glycosynthase; oxazoline; chemoenzymatic synthesis

1. Introduction

Glycoproteins account for approximately 50% of total proteins in nature. N-glycosylation is critical for the folding, secretion, solubility, and stability of glycoproteins. It also modulates the biological activities of glycoproteins, as related to cellular functions, and in vivo therapeutic efficacy when used as therapeutics (Dalziel, Crispin, Scanlan, Zitzmann, & Dwek, 2014; Dwek, Butters, Platt, & Zitzmann, 2002; Haltiwanger & Lowe, 2004; Helenius & Aebi, 2001). One example is that afucosylated antibodies exhibit enhanced binding to Fc γ IIIa receptor, which translates into a 50–100 fold increase in antibody-dependent cellular

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cytotoxicity (ADCC) (Arnold, Wormald, Sim, Rudd, & Dwek, 2007; Jefferis, 2009). Aberrant N-glycosylation is involved in a number of diseases, such as cancer and inflammation (Dube & Bertozzi, 2005; Taniguchi & Kizuka, 2015). Natural glycoproteins often carry heterogeneous N-glycans, due to the complexity of N-glycosylation processing in the biosynthesis. Preparation of homogenous glycoproteins still poses a great challenge for the functional study of glycoproteins. To address this issue, our group and others have established an efficient chemoenzymatic approach to glycan remodeling of N-glycoproteins (Parsons et al., 2016; Wang & Amin, 2014). The approach exploits a glycosynthase to transfer a sugar oxazoline that mimics the transition state of glycan hydrolysis of the GlcNAc residue of a peptide or protein acceptor. Glycosynthases used in this approach are either mutants of endo- β -N-acetylglucosaminidase (ENGase), which lack the hydrolase activity and retain the transferase activity (Huang, Giddens, Fan, Toonstra, & Wang, 2012), or a wild type ENGase that can transfer some specific glycan oxazoline while lacking the activity to hydrolyze the final product (Ochiai, Huang, & Wang, 2008; Wei et al., 2008). This approach generally involves two steps: first deglycosylation of a glycoprotein with a wild type ENGase (with or without a fucosidase) to generate the GlcNAc- or Fuc α 1,6-GlcNAc-protein acceptor, then transfer of a desired glycan from the corresponding glycan oxazoline by a glycosynthase to reconstitute a homogeneous glycoform of the glycoprotein with desired functions and properties.

In this chapter, we describe detailed protocols for glycan remodeling of two important therapeutic glycoproteins: Herceptin (trastuzumab) and erythropoietin (EPO). Herceptin is a therapeutic monoclonal antibody widely used for the treatment of breast cancer. It binds to the HER2 receptor of breast cancer cell and induces ADCC as one of its mechanisms to combat tumor (Hudis, 2007). A typical IgG type antibody is composed of two heavy chains and two light chains that form three distinct domains, including two identical Fab domains and a Fc domain. The Fc domain, a homodimer of the heavy chain, carries a conserved N-glycan at the N297 glycosylation site, which is usually a biantennary, core-fucosylated complex type N-glycan (Fig. 1). This essential glycan is critical for the folding and secretion of IgG. It also modulates the binding of IgGs with different Fc receptors and affects IgG effector functions (Arnold et al., 2007) (Jefferis, 2009). As mentioned earlier, the most dramatic effect is the influence on interaction with Fc γ IIIa receptor. EPO is a biologically important protein, which stimulates the proliferation of red blood cells. It is a widely used therapeutic for the treatment of anemia after chemotherapy. It is also used illegally as a doping agent to improve an athlete's aerobic capacity and endurance. EPO contains three N-glycosylation sites at Asn-24, Asn-38, and Asn-83 and one O-glycosylation site at Ser-126. Most N-glycans of EPO, either from natural sources, or from recombinant expression in CHO cell lines, are mainly core-fucosylated bi- tri- and tetra-antennary complex-type glycans (Harazono, Hashii, Kuribayashi, Nakazawa, & Kawasaki, 2013). The sialylation state of the N-glycans of EPO is crucial to its serum half-life (Takeuchi & Kobata, 1991).

In glycan remodeling, Herceptin was remodeled into two different glycoforms, an afucosylated biantennary complex glycoform (Sial₂Gal₂GlcNAc₂Man₃GlcNAc₂, S2G2) and a fucosylated Man₉GlcNAc₂ (M9F) glycoform (Fig. 1). It was first deglycosylated with wild-type Endo-S2 (ENGase from *Streptococcus pyogenes*) plus AlfC (an α 1,6-fucosidase from *Lactobacillus casei*), to generate the GlcNAc-Herceptin acceptor. This acceptor was then

used as substrate for transfer with S2G2 glycan oxazoline to obtain the Herceptin-S2G2 glycoform with EndoS2-D184M glycosynthase. This afucosylated glycoform has been demonstrated to have enhanced ADCC activity (Li et al., 2017; Lin et al., 2015). The Herceptin-M9F glycoform was remodeled by EndoS2 treatment of Herceptin, then transfer of the M9 oxazoline to the Fuca1,6GlcNAc-Herceptin acceptor. In contrast, EPO was remodeled to carry a homogenous azide-tagged Man₃GlcNAc₂ glycan, which could be further engineered to carry polysialylated glycan or other functional groups. As shown in Fig. 2, EPO with a high-mannose type glycan was first treated with Endo-H (ENGase from *Streptomyces picatus*) to generate the GlcNAc-EPO acceptor, which contains three GlcNAc acceptor sites. Later, it was treated with Endo-A (ENGase from *Arthrobacter protophormiae*) glycosynthase, which did not hydrolyze the final transfer product. The transfer reaction and purity of proteins was monitored with LC-ESI-MS analysis of intact protein. The final product of Herceptin was also analyzed with LC-ESI-MS under reducing conditions.

2. Materials

All buffers and solutions are prepared with Millipore water (ddH₂O). Most chemicals are from Sigma-Aldrich in reagent grade (> 95%) or higher. His-tagged EPO used in this protocol contains a mutation (S126V) on the O-glycosylation site (Yang & Wang, 2016), and is expressed from HEK293T cells in presence of 5 µg/ml of kifunensine, a mannosidase inhibitor (Yang, Li, Wei, Huang, & Wang, 2010). Endo-S2 and the corresponding mutant EndoS2-D184M were expressed and purified from *E. coli* following published protocols (Li, Tong, Yang, Giddens, & Wang, 2016). Endo-A was prepared according to (Umekawa et al., 2008). AlfC, α1,6-fucosidase from *Lactobacillus casei*, was prepared in following procedures described in (Rodriguez-Diaz, Monedero, & Yebra, 2011). The biantennary sialylated complex glycan oxazoline (S2G2-Oxa) was synthesized according to (Noguchi, Tanaka, Gyakushi, Kobayashi, & Shoda, 2009; Seko et al., 1997). Man₉GlcNAc oxazoline was made according to (Wang, Ni, Singh, & Li, 2004). Azido tagged Man₃GlcNAc₂ oxazoline (M₃N₃-Oxa) was prepared following (Ochiai et al., 2008).

2.1 Glycoproteins, enzymes, and substrates

- Monoclonal antibody Herceptin: Genentech Inc. (San Francisco, CA)
- GlcNAc-Herceptin
- Fuca1,6-GlcNAc-Herceptin
- EPO (His-tagged) with high-mannose type glycan (Yang & Wang, 2016)
- GlcNAc-EPO
- Endo Hf: New England Biolabs (Ipswich, MA)
- Endo-A (Umekawa et al., 2008)
- Endo-S2 (Li et al., 2016)
- EndoS2-D184M (Li et al., 2016)
- AlfC (α1,6-Fucosidase) (Rodriguez-Diaz et al., 2011)

- PNGase F: New England Biolabs (Ipswich, MA)
- Sial₂Gal₂GlcNAc₂Man₃GlcNAc oxazoline (S2G2-Oxa) (Seko et al., 1997)
- Man₉GlcNAc₂ oxazoline (M9-Oxa) (Wang et al., 2004)
- Azide-tagged Man₃GlcNAc oxazoline (M3_{N3}-Oxa) (Ochiai et al., 2008)

2.2 Buffers, columns, and reagents

- HiTrap Protein A column (GE Healthcare, Little Chalfont, UK)
- HisTrap Ni-NTA column (GE Healthcare, Little Chalfont, UK)
- Poroshell 300SB-C8 column 5 μ m 1 $\times$ 75 mm (Agilent, Santa Clara, CA)
- XBridge BEH300 C4 column 3.5 μ m 2.1 $\times$ 50 mm (Waters, Milford, MA)
- Amicon Ultra Centrifugal Filters Ultracel 30,000 NMWL (EMD Millipore, Billerica, MA)
- LC-MS buffer A: ddH₂O with 0.1% formic acid
- LC-MS buffer B: acetonitrile with 0.1% formic acid
- Protein A wash buffer: Phosphate-buffered saline (PBS), pH 7.4
- Protein A elution buffer: 100 mM Glycine-HCl, pH 2.7
- Protein A neutralization buffer: 1.5 M Tris-HCl, pH 8.8
- Ni-NTA binding buffer: PBS containing 0.5 M NaCl and 5 mM imidazole, pH 7.4
- Ni-NTA wash buffer: PBS containing 0.5 M NaCl and 20 mM imidazole, pH 7.4
- Ni-NTA elution buffer: PBS containing 0.5 M NaCl and 200 mM imidazole, pH 7.4
- Ni-NTA stripping buffer: PBS containing 0.5 M NaCl and 50 mM EDTA
- Tris(2-carboxyethyl)phosphine HCl (TCEP)

2.3 Equipment and software

- AKTA FPLC with UPC-900 and P-920 (GE Healthcare, Little Chalfont, UK).
- Syringe pump NE-300 (New Era Pump System, Farmingdale, NY)
- Side-A-Lyzer mini dialysis cassette (Thermo Scientific, Waltham, MA)
- NanoDrop 2000c (Thermo Scientific, Waltham, MA)
- MyCycler Thermal Cycler (Bio-Rad, Hercules, CA)
- ExactivePlus Orbitrap mass spectrometer system (Thermo Scientific, Waltham, MA)
- Ultimate 3000 LC system with autosampler (Thermo Scientific, Waltham, MA)

- Unicorn manager (GE Healthcare, Little Chalfont, UK)
- Xcalibur for raw MS data analysis (Thermo Scientific, Waltham, MA)
- Chromeleon 7 chromatography management system (Thermo Scientific, Waltham, MA)
- Magtran for raw data deconvolution (Agilent, Santa Clara, CA)

3. Methods

3.1 Glycan-remodeling of Herceptin with complex type glycan

3.1.1. LC-ESI-MS analysis of intact protein (Note 1)

1. Sample preparation: Add 0.5 μ l of Herceptin (1 μ g/ μ l) of in 20 μ l of ddH₂O with 0.1% formic acid.
2. LC-MS operation: Inject 20 μ l of the sample into the LC-ESI-MS system. Run the LC program (buffer A with 5 – 90% buffer B over 9 min) at flow rate of 0.4 ml/min on XBridge C4 column. Mass spectrometer scans in the m/z range of 1500–5000.
3. Data analysis: Use XCaliber to analyze the LC chromatography. The peak of Herceptin appears at approximately 3.5 min under these conditions. Load the raw data in Magtran software to deconvolute the measured molecular weight.

3.1.2 Deglycosylation of Herceptin

1. Dissolve 2 mg of Herceptin power in 0.2 ml of PBS (10 mg/ml). Add 2 μ l of EndoS2-WT (20 μ g) and 7 μ l of AlfC (100 μ g) into the Herceptin solution, incubate at 37°C for 16 hr. Monitor the reaction with LC-ESI-MS (Section 3.1.1) at 4 and 16 hr. After overnight incubation, the resulting Herceptin-GlcNAc acceptor is as shown in Fig. 3B (intact protein) and in Fig. 4B (heavy chain) (Note 2,3).
2. Purify the Herceptin-GlcNAc acceptor with Protein A affinity chromatography.

3.1.3. Protein A affinity purification of modified Herceptin

1. Precondition the column: Wash the column with 3 ml of protein A elution buffer and equilibrate the column with 10 ml pf protein A binding buffer at flow rate of 1 ml/min. (Note 4)

¹IgG can be analyzed under both non-reducing and reducing conditions. Analysis of the intact protein is more convenient and suitable for reaction monitoring. Analysis of IgG under reducing conditions also provides detailed information on changes in the heavy or light chains.

²AlfC cannot remove fucose from IgGs that carry full length glycan, presumably due to steric hindrance. Only when IgG is treated with EndoS2, to convert to Fuca1,6GlcNAc-IgG, can fucose be removed by AlfC (unpublished data).

³EndoS2 treatment of Herceptin is rapid and the reaction is complete within 1 hr. Removal of fucose from Fuca1,6GlcNAc-Herceptin by Alf takes longer and generally requires overnight incubation.

⁴This step could be carried out either on an FPLC system or with a syringe pump.

2. Dilute the deglycosylation reaction mixture with protein A binding buffer to final volume of 5 ml, load onto the Protein A column at a flow rate of 0.5 ml/min using a syringe pump.
3. Wash the protein A column with 10 ml of protein A binding buffer at flow rate of 1 ml/min using FPLC system.
4. Elute the bound Herceptin with 5 ml of protein A elution buffer at 1 ml/min and monitor the elution at absorbance of OD₂₈₀. Collect the eluate in one-ml fractions, neutralize immediately with 150 µl neutralization buffer for each fraction. Combine all fractions containing Herceptin.
5. Buffer exchange of Herceptin-GlcNAc: Add combined elution fractions to a 4 ml Amicon centrifugal filter, spin down at 4200 rpm for 25 min. Wash the concentrate with 5 ml of PBS for three times, and finally adjust the volume of sample with PBS to a concentration of 10 mg/ml.

3.1.4. Transfer of a sialylated glycan from glycan oxazoline (S2G2-Oxa) to Herceptin-GlcNAc

1. Add 5.3 µl of S2G2-Oxa (530 µg, 40 molar equivalents, 20 equivalents for each glycosylation site) to 100 µl Herceptin-GlcNAc solution (1 mg). Check the mixture with pH paper to ensure pH of the reaction is in neutral range (Note 5).
2. Add 10 µg of EndoS2-D184M (final concentration 0.1 µg/ µl) and incubate at 30°C for 30 min. Monitor the reaction with LC-MS analysis of the intact protein (Fig. 3C).
3. Add an additional 10 µg of EndoS2-D184M (final concentration 0.2 µg/ µl) and another 40 molar equivalents of S2G2-Oxa to reaction mixture, incubate at 30°C for another 30 min. Monitor with LC-MS analysis of intact protein to confirm completion of transfer (Fig. 3D). LC-MS analysis of heavy chain of protein under reducing conditions is shown in Fig. 4D (Note 6).
4. Purification of final transglycosylation product Herceptin-S2G2: Follow the procedures in Section 3.1.3, concentrate it to 10 mg/ml.
5. PNGase F treatment of Herceptin-S2G2: Mix 0.5 µl of PNGase F (250 units, NEB definition), 1 µl of Herceptin-S2G2 (10 µg), and 8.5 µl of PBS. Incubate at 37°C for six hours. LC-MS analysis of heavy chain under reducing conditions is shown in Fig. 4E (Note 7).

⁵Oxazolines are not stable at acidic or neutral pH for storage, therefore must always be dissolved in 20 mM NaOH as the stock. Small volumes of oxazoline solutions are easily neutralized using the PBS buffer employed for protein dissolution. If the pH of the mixture is not neutral (pH >8), a small volume of 0.2 M of phosphate buffer (pH 7) should be added to adjust it to neutral before adding the glycosynthase.

⁶The efficiency of the EndoS2-D184M-catalyzed reaction may vary slightly between runs. In some cases, a single reaction is sufficient to achieve the full transfer, in other cases additional enzyme and oxazoline are required to drive the reaction to completion. This may be due to batch variability in the activities of EndoS2-D184M, or due to storage time and/or conditions. The quality of the oxazoline also influences the transglycosylation efficiency.

⁷Occasionally non-specific transfer of glycan oxazoline to a position other than GlcNAc may occur (Parsons et al., 2016) (Huang, Yang, Umekawa, Yamamoto, & Wang, 2010). To exclude this undesired possibility, the final product is examined after treatment with PNGase F. As illustrated in Fig. 4D specific transfer is indicated by the absence of additional peak. If non-specific transfer occurs,

3.1.5. LC-ESI-MS analysis of heavy and light chain of starting material and transfer product under reducing conditions

1. Sample preparation: Add 0.5 μ l of Herceptin (0.5 μ g), 2 μ l of 0.5 M TECEP into 18 μ l of ddH₂O with 0.1% formic acid, incubate at room temperature for 20 min.
2. LC-MS operation: inject 20 μ l of the mixture into the LC-ESI-MS system. Run the LC program (buffer A with 25–35% buffer B over 6 min), at 60°C with a flow rate of 0.4 ml/min on Poroshell C8 column to separate the heavy and light chains of Herceptin. Mass spectrometer scans in the m/z range of 400–3000.
3. Data analysis: The peak of light chain eluted at approximately 2.5 min and the heavy chain eluted at 3.5 min under these conditions. Load the raw data of each peak in Magtran software to deconvolute the measured molecular weight (Fig. 4).

3.2. Glycan-remodeling of Herceptin with high-mannose glycan

3.2.1. Deglycosylation of Herceptin

1. Dissolve 2 mg of Herceptin powder in 0.2 ml of PBS (10 mg/ml). Add 2 μ l of EndoS2-WT (20 μ g) into the Herceptin solution, incubate at 37°C for 1 hr. Monitor the reaction with LC-ESI-MS of analysis of the intact protein (see subheading 3.1.1). The reaction is complete within 1 hr. The result of the LC-MS analysis of heavy chain under reducing conditions is as shown in Fig. 5A.
2. Purify the Fuca1,6GlcNAc-Herceptin (Herceptin-GnF) acceptor with Protein A affinity chromatography (see Section 3.1.3).

3.2.2. Transfer of M9-Oxa to Herceptin-GnF acceptor

1. Add 4.3 μ l of M9-Oxa (430 μ g, 40 molar equivalents) to 100 μ l Herceptin-GnF solution (1 mg). Check the mixture with pH paper to ensure that the pH of the reaction is in neutral range.
2. Add 10 μ g of EndoS2-D184M (final concentration 0.1 μ g/ μ l) and incubate at 30°C for 30 min. Monitor the reaction by LC-MS analysis of intact protein.
3. Add an additional 10 μ g of EndoS2-D184M (final concentration 0.2 μ g/ μ l) and another 40 molar equivalents of S2G2-Oxa to reaction mixture, incubate at 30°C for another 30 min. LC-MS analysis of the intact protein confirmed completion of the reaction. The result of LC-MS analysis of the heavy chain under reducing conditions is shown in Fig. 5B.
4. Purification of final transglycosylation product Herceptin-M9F: Follow the procedures in Section 3.1.3, and performing buffer exchange according to step 5 in Section 3.1.4, finally concentrating Herceptin-M9F to 10 mg/ml.

additional peaks, other than the product peak (addition of one or more the glycan moiety to the product peak), will appear either in the heavy or light chain.

5. PNGase F treatment of Herceptin-M9F: Mix 0.5 μ l of PNGase F (250 unit), 1 μ l of Herceptin-M9F, and 8.5 μ l of PBS. Incubate at 37°C for six hours. The result of the LC-MS analysis of the heavy chain under reducing conditions is as shown in Fig. 5C. (Note 7).

3.2.3. LC-ESI-MS analysis of starting material and transfer product in reducing condition

—Follow procedures in Section 3.1.5. Results are as shown in Fig. 5.

3.3 Glycan remodeling of EPO (Note 8) with azide-modified glycan

3.3.1 LC-ESI-MS analysis of EPO

1. Sample preparation: Add 0.8 μ g of EPO of in 20 μ l of ddH₂O with 0.1% formic acid.
2. LC-MS operation: Inject 20 μ l of the mixture into the LC-ESI-MS system. Run the LC program (buffer A with 5 – 90% buffer B over 6 min) at a flow rate of 0.4 ml/min. Mass spectrometer scan in the m/z range of 400–3000.
3. Data analysis: The peak of EPO appears at approximately 2.5 min under such conditions. Load the raw data in Magtran software to deconvolute the measured molecular weight.

3.3.2 Deglycosylation of EPO

1. Add 1 μ l of Endo Hf (500 units) to 200 μ l of EPO-HM (400 μ g, 2 μ g/ μ l, Note 9), incubate at 37°C for 30 min. Monitor the reaction with LC-MS (Fig. 6B, Note 9).

3.3.3. Purify the GlcNAc-EPO with Ni-NTA affinity chromatography

1. Preparation of Ni-NTA column: Strip the Ni cation from the column with the 5 ml stripping buffer at flow rate of 1 ml/min. Wash the column with 10 ml of ddH₂O at a flow rate of 1 ml/min, recharge the column with 0.1 M of NiSO₄. Finally equilibrate the column with 10 ml of Ni-NTA binding buffer.
2. Load the GlcNAc-EPO on the Ni-NTA column. Wash with 5 ml of binding buffer at a flow rate of 1 ml/min, then with 5 ml of wash buffer. Finally elute with 5 ml of elution buffer, collecting 0.5 ml fractions at the flow rate of 0.5 ml/ml. Pool the two or three fractions that contain the EPO elution peak.
3. Buffer exchange to PBS: Dialyze with a Side-A-Lyzer cassette against 30 ml of PBS at room temperature for 1 hr, then again against fresh 30 ml PBS at 4°C overnight (Note 10).

⁸The EPO used in present research contains a mutation in the S126 O-glycosylation site (serine to valine), as previous studies have indicated that O-glycosylation is not essential for either the stability or bioactivity of EPO (Delorme et al., 1992). Elimination of this O-glycan simplifies analysis of the deglycosylation and transglycosylation reactions of EPO.

⁹Do not use EPO in concentrations above 2 μ g/ μ l in the deglycosylation reaction. N-glycosylation is important for the solubility and stability of EPO. Deglycosylated EPO is unstable and rapidly precipitates at concentrations above 2 μ g/ μ l. Do not heat the deglycosylated EPO at 37°C for a prolonged time as this also causes EPO precipitation.

¹⁰Use a gentle method, such as dialysis, to do buffer exchange for deglycosylated EPO. Centrifugal methods involving high-speed centrifugation result in a dramatic loss of deglycosylated EPO mainly due to protein aggregation..

3.3.4. Transfer of M3_{N3}-Oxa to GlcNAc-EPO

1. Add 1.4 µl M3_{N3}-Oxa (140 µg, 90 molar equivalent, 30 equivalents for each site) to 200 µl of GlcNAc-EPO (40 µg, 0.2 µg/µl). Check the mixture with pH paper to ensure that the pH of the reaction is in neutral range.
2. Add 1.8 µl of Endo-A (20 µg, final concentration 0.1 µg/µl) to the mixture, incubate at 37°C for 30 min. Monitor the reaction with LC-MS. (Fig. 6C, Note 11)
3. (Optional) Add 1.4 µl M3_{N3}-Oxa (140 µg, 90 molar equivalents) to the reaction mixture and extend the incubation for another 30 min (Note 12).

4. Summary

In this chapter, we describe detailed procedures of chemoenzymatic glycan remodeling of two therapeutic glycoproteins, Herceptin and erythropoietin, to provide homogeneous glycoforms. This chemoenzymatic method, which involves a single step enzymatic deglycosylation and subsequent enzyme-catalyzed transfer of a pre-assembled glycan *en bloc* to the protein acceptor, should be generally applicable for glycan remodeling to produce homogeneous glycoprotein glycoforms for structural and functional studies and for biomedical applications.

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References

- Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annual Review of Immunology*. 2007; 25:21–50.
- Dalziel M, Crispin M, Scanlan CN, Zitzmann N, Dwek RA. Emerging principles for the therapeutic exploitation of glycosylation. *Science*. 2014; 343(6166):1235681. [PubMed: 24385630]
- Delorme E, Lorenzini T, Giffin J, Martin F, Jacobsen F, Boone T, Elliott S. Role of glycosylation on the secretion and biological activity of erythropoietin. *Biochemistry*. 1992; 31(41):9871–9876. [PubMed: 1390770]
- Dube DH, Bertozzi CR. Glycans in cancer and inflammation—potential for therapeutics and diagnostics. *Nature Reviews Drug Discovery*. 2005; 4(6):477–488. [PubMed: 15931257]
- Dwek RA, Butters TD, Platt FM, Zitzmann N. Targeting glycosylation as a therapeutic approach. *Nature Reviews Drug Discovery*. 2002; 1(1):65–75. [PubMed: 12119611]
- Haltiwanger RS, Lowe JB. Role of glycosylation in development. *Annual Review of Biochemistry*. 2004; 73:491–537.

¹¹For the three N-glycosylation sites (N24, N38, and N83) of EPO, we observed that two sites are more accessible to the glycosynthase, while the third site is more hindered. As a result, the transglycosylation mainly results in the transfer of two azide-tagged Man₃GlcNAc glycans. In a LC-MSⁿ analysis of EPO modified with S2G2 glycan, we have shown that the two sites that are more accessible are N38 and N83 (unpublished data).

¹²When the EPO acceptor is at a low concentration, such as 0.2 µg/µl, forcing conditions with the addition of more oxazoline still does not help result in additional glycan transfer to the third asparagine (N24) site.

- Harazono A, Hashii N, Kuribayashi R, Nakazawa S, Kawasaki N. Mass spectrometric glycoform profiling of the innovator and biosimilar erythropoietin and darbepoetin by LC/ESI-MS. *Journal of Pharmaceutical and Biomedical Analysis*. 2013; 83:65–74. [PubMed: 23708432]
- Helenius A, Aebi M. Intracellular functions of N-linked glycans. *Science*. 2001; 291(5512):2364–2369. [PubMed: 11269317]
- Huang W, Giddens J, Fan SQ, Toonstra C, Wang LX. Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. *Journal of the American Chemical Society*. 2012; 134(29):12308–12318. [PubMed: 22747414]
- Huang W, Yang Q, Umekawa M, Yamamoto K, Wang LX. Arthrobacter endo-beta-N-acetylglucosaminidase shows transglycosylation activity on complex-type N-glycan oxazolines: one-pot conversion of ribonuclease B to sialylated ribonuclease C. *Chembiochem*. 2010; 11(10):1350–1355. [PubMed: 20486148]
- Hudis CA. Trastuzumab—mechanism of action and use in clinical practice. *The New England Journal of Medicine*. 2007; 357(1):39–51. [PubMed: 17611206]
- Jefferis R. Glycosylation as a strategy to improve antibody-based therapeutics. *Nature Reviews Drug Discovery*. 2009; 8(3):226–234. [PubMed: 19247305]
- Li T, DiLillo DJ, Bournazos S, Giddens JP, Ravetch JV, Wang LX. Modulating IgG effector function by Fc glycan engineering. *Proceedings of the National Academy of Sciences of the United States of America*. 2017; 114(13):3485–3490. [PubMed: 28289219]
- Li T, Tong X, Yang Q, Giddens JP, Wang LX. Glycosynthase Mutants of Endoglycosidase S2 Show Potent Transglycosylation Activity and Remarkably Relaxed Substrate Specificity for Antibody Glycosylation Remodeling. *Journal of Biological Chemistry*. 2016; 291(32):16508–16518. [PubMed: 27288408]
- Lin CW, Tsai MH, Li ST, Tsai TI, Chu KC, Liu YC, ... Wong CH. A common glycan structure on immunoglobulin G for enhancement of effector functions. *Proceedings of the National Academy of Sciences of the United States of America*. 2015; 112(34):10611–10616. [PubMed: 26253764]
- Noguchi M, Tanaka T, Gyakushi H, Kobayashi A, Shoda S. Efficient synthesis of sugar oxazolines from unprotected N-acetyl-2-amino sugars by using chloroformamidine reagent in water. *Journal of Organic Chemistry*. 2009; 74(5):2210–2212. [PubMed: 19203234]
- Ochiai H, Huang W, Wang LX. Expeditious chemoenzymatic synthesis of homogeneous N-glycoproteins carrying defined oligosaccharide ligands. *Journal of the American Chemical Society*. 2008; 130(41):13790–13803. [PubMed: 18803385]
- Parsons TB, Struwe WB, Gault J, Yamamoto K, Taylor TA, Raj R, ... Davis BG. Optimal Synthetic Glycosylation of a Therapeutic Antibody. *Angew Chem Weinheim Bergstr Ger*. 2016; 128(7):2407–2413. [PubMed: 27546920]
- Rodriguez-Diaz J, Monedero V, Yebra MJ. Utilization of natural fucosylated oligosaccharides by three novel alpha-L-fucosidases from a probiotic *Lactobacillus casei* strain. *Applied and Environmental Microbiology*. 2011; 77(2):703–705. [PubMed: 21097595]
- Seko A, Koketsu M, Nishizono M, Enoki Y, Ibrahim HR, Juneja LR, ... Yamamoto T. Occurrence of a sialylglycopeptide and free sialylglycans in hen's egg yolk. *Biochimica et Biophysica Acta*. 1997; 1335(1–2):23–32. [PubMed: 9133639]
- Takeuchi M, Kobata A. Structures and functional roles of the sugar chains of human erythropoietins. *Glycobiology*. 1991; 1(4):337–346. [PubMed: 1820196]
- Taniguchi N, Kizuka Y. Glycans and cancer: role of N-glycans in cancer biomarker, progression and metastasis, and therapeutics. *Advanced Cancer Research*. 2015; 126:11–51.
- Umekawa M, Huang W, Li B, Fujita K, Ashida H, Wang LX, Yamamoto K. Mutants of *Mucor hiemalis* endo-beta-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities. *Journal of Biological Chemistry*. 2008; 283(8):4469–4479. [PubMed: 18096701]
- Wang LX, Amin MN. Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chemical Biology*. 2014; 21(1):51–66.
- Wang LX, Ni J, Singh S, Li H. Binding of high-mannose-type oligosaccharides and synthetic oligomannose clusters to human antibody 2G12: implications for HIV-1 vaccine design. *Chemical Biology*. 2004; 11(1):127–134.

- Wei Y, Li C, Huang W, Li B, Strome S, Wang LX. Glycoengineering of human IgG1-Fc through combined yeast expression and in vitro chemoenzymatic glycosylation. *Biochemistry*. 2008; 47(39):10294–10304. [PubMed: 18771295]
- Yang Q, Li C, Wei Y, Huang W, Wang LX. Expression, glycoform characterization, and antibody-binding of HIV-1 V3 glycopeptide domain fused with human IgG1-Fc. *Bioconjugate Chemistry*. 2010; 21(5):875–883. [PubMed: 20369886]
- Yang Q, Wang LX. Mammalian alpha-1,6-Fucosyltransferase (FUT8) Is the Sole Enzyme Responsible for the N-Acetylglucosaminyltransferase I-independent Core Fucosylation of High-mannose N-Glycans. *Journal of Biological Chemistry*. 2016; 291(21):11064–11071. [PubMed: 27008861]

Fig. 1.
Reaction scheme for glycan remodeling of Herceptin.

Fig. 2.
Reaction scheme for glycan remodeling of EPO.

Fig. 3.

LC-MS analysis of intact Herceptin to monitor transfer of S2G2 glycan to Herceptin-GlcNAc acceptor. A) Herceptin. B) GlcNAc-Herceptin (Herceptin-Gn). C) Intermediate product during transfer of S2G2 to Herceptin-Gn at 30 min. SM, starting material. D) Final Herceptin-S2G2 transglycosylation product.

Fig. 4. LC-MS analysis under reducing conditions for the heavy and light chains of different glycoforms of Herceptin in the glycan remodeling with S2G2 glycan. A) Heavy chain of Herceptin. B) Heavy chain of Herceptin-Gn. C) Heavy chain of Herceptin-S2G2. D) Heavy chain of Herceptin-S2G2 treated with PNGase F. E) Light chain of Herceptin. F) Light chain of Herceptin-S2G2.

Fig. 5.

LC-MS analysis under reducing conditions for the heavy and light chains of different glycoforms of Herceptin in the glycan remodeling with M9 glycan. A) Heavy chain of Fuca 1,6GlcNAc-Herceptin (Herceptin-GnF). B) Heavy chain of Herceptin-M9F. C) Heavy chain of Herceptin-M9F treated with PNGase F. D) Light chain of Herceptin-M9F.

Fig. 6.

Mass spectrometry analysis of glycan remodeling of EPO. A) MALDI-TOF analysis of glycan released from EPO with high-mannose type glycan. The glycan release and MALDI-TOF analysis was performed as described in (Yang & Wang, 2016). B) LC-MS analysis of GlcNAc-EPO (EPO-Gn). C) LC-MS analysis of EPO transferred with M3N3 glycan.