Published in final edited form as:

Carbohydr Res. 2018 March 22; 458-459: 77–84. doi:10.1016/j.carres.2018.02.007.

Site-specific immobilization of endoglycosidases for streamlined chemoenzymatic glycan remodeling of antibodies

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Abstract

Chemoenzymatic glycan remodeling of antibodies using an endoglycosidase and its mutant is emerging as an attractive approach for producing homogeneous antibody glycoforms. We report in this paper a site-specific covalent immobilization of the endoglycosidases (Endo-S2 and its glycosynthase mutant D184M) using a recombinant microbial transglutaminase (MTG) and evaluation of the immobilized enzymes in deglycosylation and glycosylation of a therapeutic antibody. The site-specific covalent immobilization was achieved by introduction of a Q-tag at the C-terminus of the recombinant enzymes followed by conjugation of the enzymes to a primary amine-containing solid support through MTG-catalyzed transglutamination. Using rituximab as a model system, we found that the Endo-S2 wild-type and D184M glycosynthase mutant immobilized by this approach were efficient in the two step antibody glycan remodeling to generate homogeneous antibody glycoforms. Notably using the covalently immobilized enzymes can efficiently avoid the need of intermediate purification and eliminate the residual contamination of wild type enzyme for product hydrolysis, thus streamlining the chemoenzymatic Fc glycan remodeling of antibodies.

Graphical Abstract

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Keywords

Enzyme immobilization; Endoglycosidase; Transglycosylation; Antibody glycosylation; Transglutaminase; Glycan remodeling

1. Introduction

Antibodies of the IgG class are essential components of the immune system. IgG antibodies mediate immune responses through both the Fab domain that recognizes antigens and the Fc domain that interacts with Fc receptors. All IgG Fc domains contain a conserved N-glycosylation site to which a biantennary complex-type N-glycan is attached, but significant structural diversity arises from terminal and core modifications, e.g. variation in terminal galactosylation and sialylation, addition of bisecting N-acetylglucosamine and core fucose. The fine structure of the Fc N-glycan has profound impact on the effector function of IgG antibodies. For example, many therapeutic monoclonal IgG antibodies exert their effector functions through antibody-dependent cellular cytotoxicity (ADCC) which is mediated by Fc-Fc γ III receptor interaction. It is well-established that removal of core fucose significantly increases the binding affinity of Fc to human Fc γ III receptor thereby augments ADCC activity both *in vitro* and *in vivo* [1–4]. On the other hand, it has been suggested that a minor sialylated Fc glycoform of intravenous immunoglobulin (IVIG) is mainly responsible for the anti-inflammatory activity in several animal model experiments [5–8]. Thus, developing methods for efficient antibody glycoengineering are of great interest.

Various glycoengineering strategies, including *in vivo* genetic engineering of the antibody expression cell line [9–11] and *in vitro* glycan remodeling using glycosyltransferases [12–14], have been attempted to control antibody glycosylation. The endoglycosidase-catalyzed glycan remodeling is emerging as a promising approach to construct antibodies with homogeneous, well-defined glycan structures at the Fc domain [15, 16]. This chemoenzymatic approach involves two key steps: a deglycosylation step catalyzed by wild-type endoglycosidase to remove heterogeneous N-glycans, and the subsequent reglycosylation by an enzymatic transfer of a homogeneous N-glycan *en bloc* from a synthetic N-glycan oxazoline using an endoglycosidase or its glycosynthase mutant [17–22]. In 2012, we reported an efficient chemoenzymatic glycan remodeling of an intact therapeutic monoclonal antibody and IVIG by the discovery of glycosynthase mutants (D233A and D233Q) of Endo-S, an endoglycosidase from *Streptococcus pyogenes* together with the wild type enzyme for deglycosylation. In 2016, we reported new glycosynthases such as mutant D184M derived from Endo-S2, another endoglycosidase from *S. pyogenes* serotype M49, which showed more relaxed substrate specificity capable of acting on high-mannose, hybrid, and complex-type Fc N-glycans and much higher efficiency in catalysis [22]. These developments have enabled the synthesis of various homogeneous glycoforms of antibodies for structural and functional studies [4, 23–27]. Despite these successes, the chemoenzymatic glycan remodeling approach requires the isolation of the deglycosylated antibody intermediate from the wild type enzyme used in the deglycosylation step, as even a residual contamination of the wild type enzyme could hydrolyze the final product generated in the second step. In addition, a simplified protocol with enhanced overall efficiency

including the repeat use of the enzymes would be desirable for future basic and industrial applications. For these purposes, we sought to immobilize the enzymes and evaluate their efficiency. In this paper, we describe a site-specific covalent immobilization of wild type Endo-S2 and its glycosynthase mutant D184M on an agarose resin through site-specific transglutamination by a recombinant microbial transglutaminase (MTG). We found that agarose-immobilized enzymes were efficient to perform the two-step Fc glycan remodeling of antibodies without the need of isolating the deglycosylated antibody intermediate and the covalent immobilization could effectively eliminate the contamination of the antibody products with any residual enzymes. In addition, a one-pot streamlined protocol could be implemented for glycan remodeling using the immobilized enzymes.

2. Results and Discussion

2.1. Design of the enzyme immobilization protocol

Various enzyme immobilization methods have been developed. These include irreversible immobilization such as covalent binding, entrapment, and aggregation, and reversible immobilization such as adsorption, ionic binding, affinity binding, and metal binding [28]. We sought to explore a site-specific covalent enzyme immobilization for two considerations. First a covalent conjugation between the enzyme and a support will avoid any possible leaking of the enzymes into the media in comparison with non-covalent immobilization and, second, site-directed conjugation could minimize the destruction of the enzyme activities in comparison with random conjugation methods. Thus we selected the site-directed covalent immobilization of the enzymes using a microbial transglutaminase (MTG)-catalyzed transglutamination as the method of choice. MTG catalyzes an acyl transfer reaction between a γ -carboxyamide group of a glutamine residue of a peptide and a primary amine, resulting in the formation of a new γ -glutaminy l covalent link [29, 30]. It shows selectivity to peptide sequences flanking the glutamine residue. Several short peptide sequences (Q-tags) have been identified that serve as preferred substrates for MTG [31, 32]. On the other hand, MTG displays broad acyl-acceptor substrate specificity [29], enabling the use of a variety of scaffolds with primary amine groups as solid supports. We have developed several applications of this versatile enzyme including functionalization of many support structures, such as the polysaccharide chitosan [33], proteins spider silk [34], and gelatin [35]. This enables not only immobilization of the enzyme, but also its localization among many different structures, surfaces, and delivery vehicles [34, 36, 37].

The overall design of the immobilization was depicted in Figure 1. Briefly, a Q-tag sequence (WALQRPH) that was shown to be an efficient substrate for MTG [32] is fused to the C-terminus of Endo-S2 with a flexible linker (Fig. 1A). On the other hand, the agarose resin surface is functionalized with primary amine by inserting a water soluble PEG linker to facilitate enzymatic transglutamination. Introducing a PEG spacer is also expected to make the beads more hydrophilic and may increase the flexibility of the immobilized enzyme.

2.2. Site-specific covalent immobilization of the wild-type endoglycosidase S2 and its glycosynthase mutant D184M

cDNA sequences encoding Endo-S2 (43–842) WT and D184M were amplified by PCR and cloned into pET28a vector. The resulting plasmids encode Endo-S2 proteins with the C-terminal addition of a Q-tag followed by 6xHis-tag. The Q-tagged Endo-S2 WT and D184M were readily expressed in *Escherichia coli* and was purified using immobilized metal ion affinity chromatography to obtain the soluble enzyme with a yield of more than 20 mg/L. The Q-tagged Endo-S2 showed comparable activity with our previous Endo-S2 construct [22]. Microbial pro-transglutaminase from *Streptomyces mobaraensis* was expressed in *E. coli* and purified following a previously reported procedure [30]. MTG was activated by cleaving off the pro-sequence and used for catalysis of the transglutamination as shown in Fig. 1B. Briefly, MTG was mixed with Endo-S2 Q-tag proteins and agarose resin modified with PEG linker terminated with a primary amine group at room temperature for 1 hour in PBS buffer. After washing off the MTG and unimmobilized Endo-S2, the amount of immobilized Endo-S2 Q-tag proteins were determined by a modified Bradford dye-binding assay [38]. The concentration of immobilized Endo-S2 WT and D184M was determined to be about 1 mg/ml (50% v/v resin).

2.3. Assessment of enzymatic activity of immobilized Endo-S2

The Endo-S2 enzymes immobilized using the method described above were tested for their catalytic deglycosylation and reglycosylation activities (Scheme 1). We found that the immobilized WT Endo-S2 could efficiently deglycosylate a number of antibodies, including rituximab and Herceptin. To facilitate time-course monitoring of the reaction progress by LC-MS, a synthesized homogeneous S2G2F-rituximab (disialylated bi-antennary complex type, core-fucosylated) was used as the substrate. The preparation of this glycoform has been described in our previous studies [20, 22]. S2G2F-rituximab was efficiently and completely deglycosylated (Fig. 2A). A preliminary study indicated that the immobilized Endo-S2 was stable and could be repeatedly used for antibody deglycosylation for several runs without significant loss of activity, although we did not quantitatively measure the activity of the immobilized enzymes after each repeated use.

To test the transglycosylation activity of immobilized Endo-S2 glycosynthase mutant D184M, deglycosylated Herceptin and the sialylated bi-antennary complex type N-glycan oxazoline (SCT-oxazoline) were used as substrates. We observed efficient transglycosylation and near complete synthesis of S2G2F-Herceptin (Fig. 2B). Addition of more glycan oxazoline can further push the reaction towards completion.

2.3. Immobilization prevented the co-purification of endoglycosidase activity with the antibody during Protein A purification

The homogeneous IgG glycoforms synthesized by the chemoenzymatic approach developed by our group occasionally showed slow Fc glycan hydrolysis during extended storage at 4 °C. LC-MS analysis showed that N-glycans were hydrolyzed between the N,N'-diacetylchitobiose, suggesting that the antibody may be contaminated by endoglycosidase activity. We reasoned that due to the high and specific hydrolytic activity of the wild-type endoglycosidase S and S2 on IgG N-glycan, residual contamination of the enzyme in the

antibody glycoform products might cause the unwanted cleavage. We further reasoned that using covalently immobilized wild-type endoglycosidase for deglycosylation could prevent this problem. We attempted to validate our assumption following the research design described below. First, commercial rituximab was deglycosylated with either free or immobilized Endo-S2 WT followed by Protein A purification. To test whether the deglycosylated rituximab purified from the two different preparations above contain residual endoglycosidase hydrolytic activity, they were incubated with commercial Herceptin (trastuzumab) 1:1, w/w) that are mixtures of the G0F, G1F, and G2F glycoforms, where G0, G1, and G2 indicate the numbers of terminal galactose moieties, and F indicates core fucosylation (Fig. 3A). After overnight incubation at 37 °C, the incubation samples were checked by LC-MS. N-glycan hydrolysis was observed in Herceptin that was co-incubated with deglycosylated rituximab prepared with free Endo-S2 WT, as shown by the emergence of deglycosylated Herceptin (Fig. 3B). On the contrary, no hydrolysis activity was observed in Herceptin co-incubated with deglycosylated rituximab that was prepared with immobilized Endo-S2 (Fig. 3C). The peak representing deglycosylated rituximab in each sample was detected to show its presence (Fig. 3 D and E). The result presented here demonstrated that residual endoglycosidase N-glycan hydrolytic activity was present even after antibody purification on protein A column, and covalent immobilization of the endoglycosidase on the agarose resin could efficiently eliminate such residual hydrolytic activity.

2.4. Streamlined IgG Fc glycan remodeling using the immobilized enzymes

The chemoenzymatic approach for IgG glycoengineering using soluble endoglycosidases requires two purification steps, one after the deglycosylation and the other after the transglycosylation step, to remove the endoglycosidases from the resulting IgG antibody glycoform [20]. Because enzyme immobilization enables easy separation of IgG glycoengineered products, we designed a streamlined IgG glycoengineering procedure using immobilized Endo-S2 WT and D184M mutant without intermediate purification steps (Scheme 2). Commercial Herceptin was first incubated with immobilized Endo-S2 WT agarose resin in a column and deglycosylation was readily achieved after a short period of incubation time. Since the released free N-glycans do not affect the second step transglycosylation, the deglycosylated Herceptin was directly eluted from the first column and added together with the complex-type (CT) N-glycan oxazoline to a second column containing the immobilized Endo-S2 D184M agarose resin. Incubation of the column at ambient temperature for 1–2 hours to give the glycoengineered rituximab (Scheme 2). The efficiency of the process was monitored and confirmed by LC-MS analysis of the intermediate and final product (Fig. 4). The commercial rituximab contains three major Fc glycoforms (G0F, G1F, and G2F) (Fig. 4A). The deglycosylation of rituximab with the immobilized Endo-S2 gave a single Fc deglycosylated form (Fig. 4B). Finally, glycosylation of the deglycosylated rituximab with the immobilized Endo-S2 D184M mutant and the N-glycan oxazoline afforded a pure glycoform (G2F) of rituximab (Fig. 4C). The IgG glycoengineered products synthesized by this streamlined glycan remodeling approach can be directly stored in the buffer of choice after buffer exchange.

3. Conclusion

An efficient site-oriented immobilization of endoglycosidase Endo-S2 and its glycosynthase mutant D184M on an agarose resin is described. The immobilized enzymes retained their catalytic activity and were demonstrated to catalyze efficient *in vitro* Fc glycan remodeling of monoclonal IgG antibody to homogeneous glycoforms. The use of covalently immobilized enzymes avoids the need of intermediate separation, thus streamlining the glycan remodeling procedures. In addition, covalent immobilization of the endoglycosidases efficiently eliminates the contamination of residual enzymatic activity in the final product that might hydrolyze the product over time. This work represents the first example of using covalently immobilized endoglycosidases for *in vitro* glycan remodeling of IgG antibodies for producing homogeneous antibody glycoforms. The site-oriented covalent immobilization protocol should be also applicable for other endoglycosidases and mutants for glycosylation remodeling of various glycoproteins.

4. Experimental

4.1. General procedures and methods

Monoclonal antibodies rituximab and trastuzumab (Herceptin) were products from Genentech Inc., (South San Francisco, CA). Asialo and sialoglycan complex type oxazoline was synthesized following the previously reported procedure [39]. Silica gel (200–425 mesh) for flash chromatography was purchased from Sigma–Aldrich. ^1H NMR spectra was recorded on a 400 MHz spectrometer (Bruker, Tokyo, Japan) with CDCl_3 as the solvent.

4.2. Synthesis of Fmoc-NH-PEG-NH₂

A solution of Fmoc-Cl (1.14 g, 4.42 mmol) in THF (20 mL) was slowly added dropwise into a solution of diethylene glycol bis(3-aminopropyl) ether (DGBE) (TCI Amercia) (1.0 g, 4.42 mmol) in *i*-PrOH (50 mL) at -10°C . After adding, the reaction was allowed to warm up to room temperature and stirred overnight. The reaction was monitored by TLC. The crude product was purified by flash silica gel chromatography to give the product as a white solid (2.18 g, 95%). ^1H NMR (CDCl_3 , 400 MHz): δ 7.75 (d, $J = 7.4$ Hz, 2 H), 7.68 (d, $J = 7.6$ Hz, 2 H), 7.37 (t, $J = 16$ Hz, 2 H), 7.29 (t, $J = 14$ Hz, 2 H), 3.68 (m, 3 H), 3.60 (m, 12 H), 3.19 (t, $J = 6.0$ Hz, 4 H), 2.05 (m, 4 H). ESI-MS: calc. for Fmoc-NH-PEG-NH₂, $M = 442.25$; found (m/z), 443.31 [$M + \text{H}$]⁺.

4.3. Expression and Purification of microbial transglutaminase (MTG)

MTG was expressed following a previous published approach [30] with modifications. DNA sequence encoding *Streptomyces mobaraensis* (DSM 40847) MTG with a pro-peptide was codon-optimized and inserted into pET-20b(+) vector between the NdeI and XhoI sites. The resulting plasmid encoding pro-MTG was transformed into BL21(DE3) cells and pro-MTG was expressed by auto-induction as described [30]. After harvest, cells were lysed by sonication and pro-MTG was activated by incubating the cell lysate with trypsin (1 mg/ml) at 1:10 ratio (v/v) at 37°C for 1 hour to remove the pro-peptide. The activated MTG was purified using a 1-ml HisTrap Ni-NTA column (GE Healthcare). The purified MTG was buffer exchanged in 1×PBS and protein concentration was determined by NanoDrop A280

method. The activity of MTG was confirmed by a modified colorimetric hydroxamate assay as previously described [40].

4.4. Cloning of endoglycosidase S2 and D184M mutant with Q-tag

Genes encoding Endo-S2 and its mutant D184M were amplified from pET22b-CPD plasmid derivatives [22] using forward primer 5'-

GATATACCATGGGCGGAAAGACAGATCAGCAGGTTGGTGC-3' and reverse primer 5'-

TATATCCTCGAGGCCGCTGCCGCTGCCGTGCGGGCGCTGCAGCGCCAGCCGCTG CCGCTGCCATCTTCAGCGTATTAGCGAC-3'. The PCR products were cloned into pET-28a(+) vector between NcoI and XhoI sites, resulting in plasmids encoding Endo-S2 proteins with a C-terminal Q-tag (WALQRPH) followed by a 6×His-tag. The resulting plasmids were transformed into BL21(DE3) cells. For overexpression, 1-liter 2×YT media was inoculated with 10 ml transformant start culture and was grown to OD₆₀₀ ~0.6 at 37°C. The culture was then cooled to 20 °C and protein expression was induced by adding 0.5 mM IPTG. After 16 h induction at 20 °C, cells were harvested and lysed by Bacterial cell lysis buffer (Gold Biotechnology, Inc.). Endo-S2 proteins were purified by Ni-NTA chromatography and stored in 1×PBS buffer. The activity of the Q-tagged Endo-S2 and D184M mutant were tested and were comparable to the previously reported constructs [22].

4.5. Site-specific immobilization of Endo-S2 WT and D184M through MTG-mediated transglutamination

The Q-tagged Endo-S2 WT and D184M were separately immobilized on agarose resin via a MTG-catalyzed transglutamination reaction. First, NHS-activated agarose dry resin (0.15 g) (Pierce) was incubated with Fmoc-DGBE (0.1M, synthesized as described above) for 6 hour at room temperature. After quenching remaining active sites, the Fmoc groups were removed by piperidine (20%), HOBt (0.1 M) in DMF. The agarose beads modified with DGBE group were incubated with Endo-S2 (0.5 mg/ml), MTG (0.1 mg/ml) in 1×PBS in a total volume of 500 µl at room temperature for 1 hour. The beads were washed thoroughly to remove excess enzymes and re-suspended in 1×PBS. The concentration of immobilized protein were measured by a previously described "465 Bradford assay" [38], which measures the decrease in the absorbance of the solution at 465 nm due to absorption of the dye by the protein bound to the solid support.

4.6. Liquid Chromatography Mass Spectrometry (LC-ESI-MS)

The LC-MS analysis was performed on an Exactive Plus Orbitrap (Thermo Scientific). Intact IgG antibody was analyzed by injecting on the Waters XBridge™ BEH300 C4 column (3.5 µm, 2.1 × 50 mm) with a linear gradient of 5–90% MeCN containing 0.1% formic acid within 9 min at a flow rate of 0.4 ml/min. For analysis of the light chain and heavy chain, the IgG antibody were treated with 50 mM tris(2-carboxyethyl)-phosphine and heated at 37 °C for 15 min, then analyzed with an Agilent Poroshell 300SB-C8 column (5µm, 75 × 1 mm). Analysis was performed at 60 °C with a linear gradient of 25–35% MeCN containing 0.1% formic acid within 6 min at a flow rate of 0.40 ml/min. Raw data were deconvoluted using MagTran (Amgen).

4.7. Testing the activity of the immobilized enzymes

To test N-glycan hydrolysis activity of immobilized wild-type Endo-S2, S2G2F-IgG (1 mg/ml) was incubated with immobilized Endo-S2 WT (0.1 mg/ml) in Tris (0.1 M, pH 7.4). Reaction samples were collected at 5, 15, 30 and 60 minute and analyzed by LC-MS. To test the transglycosylation activity of immobilized Endo-S2 D184M, deglycosylated Herceptin (trastuzumab, 2.5 mg/ml) and 40 equivalents of SCT oxazoline were incubated with immobilized enzyme (0.25 mg/ml) in Tris (0.1 M, pH 7.4). Reaction samples were collected at 5, 15, 30 and 60 minute and analyzed by LC-MS.

4.8. Synthesis of Herceptin G2F glycoform without purification steps using site-specific immobilized Endo-S2 enzymes

Commercial Herceptin (1 mg) was applied to a spin column (Pierce #69705) containing immobilized Endo-S2 WT (20 µg) in a total volume of 0.2 ml (Tris, pH 7.4) and incubated for 1 hour. The deglycosylated Herceptin was passed through the spin filter and separated from the agarose resins by centrifugation. Near full recovery of the antibody sample was achieved. The flow through (deglycosylated Herceptin, 0.2 ml, 5 mg/ml) was applied to another spin column containing immobilized Endo-S2 D184M (100 µg) in a total volume of 0.4 ml (Tris, pH 7.4). Transglycosylation reaction was initiated by addition of 40 equivalents of the asialylated bi-antennary complex type N-glycan oxazoline (CT oxazoline). After the reaction has proceeded for 30 minutes, another 20 equivalents of CT oxazoline was added and the reaction was allowed to proceed for another 15 minutes. The final product (0.9 mg G2F-Herceptin) was separated from agarose resin by centrifugation and analyzed by LC-MS.

4.9. Identification of the endoglycosidase activity in purified IgG product and testing whether immobilization prevent this activity

Commercial rituximab (1 mg) was deglycosylated either with free (1 µg) or immobilized (10 µg) Endo-S2 WT. The reactions were monitored by LC-MS and deglycosylated antibodies were purified by Protein A chromatography. To check the potential contamination of enzymes in the deglycosylated antibody, the differentially prepared deglycosylated rituximab were incubated with commercial Herceptin (both antibodies were at 1 mg/ml) in Tris buffer (0.1 M, pH 7.4) at 37 °C overnight to detect residual hydrolytic activity on the commercial Herceptin. Samples were taken after overnight incubation and were analyzed by LC-MS.

Acknowledgments

We thank other members of the Wang lab for technical assistance and helpful discussions. This work was supported by the US National Institutes of Health (NIH grant R01 GM096973) and DTRA (HDTRA1-13-1-0037).

References

1. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG. J Biol Chem. 2002; 277:26733–26740. [PubMed: 11986321]
2. Nimmerjahn F, Ravetch JV. Science. 2005; 310:1510–1512. [PubMed: 16322460]

3. Ferrara C, Grau S, Jager C, Sondermann P, Brunker P, Waldhauer I, Hennig M, Ruf A, Rufer AC, Stihle M, Umana P, Benz J. *Proc Natl Acad Sci USA*. 2011; 108:12669–12674. [PubMed: 21768335]
4. Li T, DiLillo DJ, Bournazos S, Giddens JP, Ravetch JV, Wang LX. *Proc Natl Acad Sci USA*. 2017; 114:3485–3490. [PubMed: 28289219]
5. Kaneko Y, Nimmerjahn F, Ravetch JV. *Science*. 2006; 313:670–673. [PubMed: 16888140]
6. Anthony RM, Nimmerjahn F, Ashline DJ, Reinhold VN, Paulson JC, Ravetch JV. *Science*. 2008; 320:373–376. [PubMed: 18420934]
7. Schwab I, Nimmerjahn F. *Clin Exp Immunol*. 2014; 178(Suppl 1):97–99. [PubMed: 25546778]
8. Washburn N, Schwab I, Ortiz D, Bhatnagar N, Lansing JC, Medeiros A, Tyler S, Mekala D, Cochran E, Sarvaiya H, Garofalo K, Meccariello R, Meador JW 3rd, Rutitzky L, Schultes BC, Ling L, Avery W, Nimmerjahn F, Manning AM, Kaundinya GV, Bosques CJ. *Proc Natl Acad Sci USA*. 2015; 112:E1297–1306. [PubMed: 25733881]
9. Umana P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. *Nat Biotechnol*. 1999; 17:176–180. [PubMed: 10052355]
10. Yamane-Ohnuki N, Kinoshita S, Inoue-Urakubo M, Kusunoki M, Iida S, Nakano R, Wakitani M, Niwa R, Sakurada M, Uchida K, Shitara K, Satoh M. *Biotechnol Bioeng*. 2004; 87:614–622. [PubMed: 15352059]
11. Stanley P, Sundaram S, Tang J, Shi S. *Glycobiology*. 2005; 15:43–53. [PubMed: 15329358]
12. Raju TS, Briggs JB, Chamow SM, Winkler ME, Jones AJ. *Biochemistry*. 2001; 40:8868–8876. [PubMed: 11467948]
13. Warnock D, Bai X, Autote K, Gonzales J, Kinealy K, Yan B, Qian J, Stevenson T, Zopf D, Bayer RJ. *Biotechnol Bioeng*. 2005; 92:831–842. [PubMed: 16187338]
14. Thomann M, Schlothauer T, Dashivets T, Malik S, Avenal C, Bulau P, Ruger P, Reusch D. *PLoS One*. 2015; 10:e0134949. [PubMed: 26266936]
15. Wang LX, Amin MN. *Chem Biol*. 2014; 21:51–66. [PubMed: 24439206]
16. Fairbanks AJ. *Chem Soc Rev*. 2017; 46:5128–5146. [PubMed: 28681051]
17. Wei Y, Li C, Huang W, Li B, Strome S, Wang LX. *Biochemistry*. 2008; 47:10294–10304. [PubMed: 18771295]
18. Zou G, Ochiai H, Huang W, Yang Q, Li C, Wang LX. *J Am Chem Soc*. 2011; 133:18975–18991. [PubMed: 22004528]
19. Fan SQ, Huang W, Wang LX. *J Biol Chem*. 2012; 287:11272–11281. [PubMed: 22318728]
20. Huang W, Giddens J, Fan SQ, Toonstra C, Wang LX. *J Am Chem Soc*. 2012; 134:12308–12318. [PubMed: 22747414]
21. Giddens JP, Lomino JV, Amin MN, Wang LX. *J Biol Chem*. 2016; 291:9356–9370. [PubMed: 26966183]
22. Li T, Tong X, Yang Q, Giddens JP, Wang LX. *J Biol Chem*. 2016; 291:16508–16518. [PubMed: 27288408]
23. Kuroguchi M, Mori M, Osumi K, Tojino M, Sugawara S, Takashima S, Hirose Y, Tsukimura W, Mizuno M, Amano J, Matsuda A, Tomita M, Takayanagi A, Shoda S, Shirai T. *PLoS One*. 2015; 10:e0132848. [PubMed: 26200113]
24. Lin CW, Tsai MH, Li ST, Tsai TI, Chu KC, Liu YC, Lai MY, Wu CY, Tseng YC, Shivatare SS, Wang CH, Chao P, Wang SY, Shih HW, Zeng YF, You TH, Liao JY, Tu YC, Lin YS, Chuang HY, Chen CL, Tsai CS, Huang CC, Lin NH, Ma C, Wong CH. *Proc Natl Acad Sci USA*. 2015; 112:10611–10616. [PubMed: 26253764]
25. Quast I, Keller CW, Maurer MA, Giddens JP, Tackenberg B, Wang LX, Munz C, Nimmerjahn F, Dalakas MC, Lunemann JD. *J Clin Invest*. 2015
26. Tang F, Yang Y, Tang Y, Tang S, Yang L, Sun B, Jiang B, Dong J, Liu H, Huang M, Geng M, Huang W. *Org Biomol Chem*. 2016
27. Guo W, Tang F, Qin K, Zhou M, Le Z, Huang W. *Carbohydr Res*. 2017; 446–447:32–39.
28. Karav S, Cohen JL, Barile D, de Moura Bell JM. *Biotechnol Prog*. 2016
29. Gundersen MT, Keillor JW, Pelletier JN. *Appl Microbiol Biotechnol*. 2014; 98:219–230. [PubMed: 23615739]

30. Marx CK, Hertel TC, Pietzsch M. *Enzyme Microb Technol.* 2007; 40:1543–1550.
31. Sugimura Y, Yokoyama K, Nio N, Maki M, Hitomi K. *Arch Biochem Biophys.* 2008; 477:379–383. [PubMed: 18616926]
32. Oteng-Pabi SK, Pardin C, Stoica M, Keillor JW. *Chem Commun (Camb).* 2014; 50:6604–6606. [PubMed: 24824734]
33. Liu Y, Wu HC, Chhuan M, Terrell JL, Tsao CY, Bentley WE, Payne GF. *ACS Biomater Sci Eng.* 2015; 1:320–328. [PubMed: 26501127]
34. Wu HC, Quan DN, Tsao CY, Liu Y, Terrell JL, Luo X, Yang JC, Payne GF, Bentley WE. *Biotechnol Bioeng.* 2017; 114:83–95. [PubMed: 27478042]
35. Yung CW, Wu LQ, Tullman JA, Payne GF, Bentley WE, Barbari TA. *J Biomed Mater Res A.* 2007; 83:1039–1046. [PubMed: 17584898]
36. Bhokisham N, Pakhchanian H, Quan D, Tschirhart T, Tsao CY, Payne GF, Bentley WE. *Metab Eng.* 2016; 38:1–9. [PubMed: 27237361]
37. Wu LQ, Bentley WE, Payne GF. *Int J Artif Organs.* 2011; 34:215–224. [PubMed: 21374563]
38. Bonde M, Pontoppidan H, Pepper DS. *Anal Biochem.* 1992; 200:195–198. [PubMed: 1595895]
39. Huang W, Yang Q, Umekawa M, Yamamoto K, Wang LX. *ChemBioChem.* 2010; 11:1350–1355. [PubMed: 20486148]
40. Folk JE, Cole PW. *Biochim Biophys Acta (BBA) - Enzymol Biol Oxidation.* 1966; 122:244–264.

Highlights

- Site-specific covalent immobilization of Endo-S2 and its glycosynthase mutant
- Immobilized enzymes catalyze efficient chemoenzymatic IgG Fc glycan remodeling
- Immobilization prevents residual contamination of endoglycosidase in products
- A one-pot glycoengineering approach was implemented using the immobilized enzymes

(A) Endo-S2 'Q-tag' protein design

(B) Site-specific covalent immobilization design

Figure 1. Schematic presentation of the strategy for site-specific covalent immobilization of Endo-S2

A) Design of Endo-S2 with a Q-tag for immobilization; B) Site-specific immobilization of Q-tagged Endo-S2 by MTG-catalyzed transglutamination to surface-modified agarose resins. DGBE, diethylene glycol bis(3-aminopropyl) ether.

Figure 2. Testing the activity of immobilized enzymes after site-specific covalent immobilization on agarose beads

A) The hydrolysis activity of immobilized Endo-S2 WT was tested by hydrolysis of N-glycans from homogeneous rituximab glycoform S2G2F; B) The transglycosylation activity of immobilized Endo-S2 D184M was tested by transglycosylation of deglycosylated Herceptin with SCT-oxazoline.

Figure 3. LC-ESI-MS analysis of full antibody for the detection of residual endoglycosidase N-glycan hydrolytic activity co-purified with glycoengineering products

In the LC-MS analysis, the intact whole antibodies that contain two N-glycans are represented by the nature of the glycans (G0F, G1F or G2F) separated by /, such as G0F/G0F, G0F/G1F and so on. When one of the N-glycan is hydrolyzed on the whole antibody, the missing N-glycan is represented by –, and the antibody glycoforms are labeled as G0F/– or G1F/– or G2F/–. A), Herceptin incubated in PBS as control; B), Herceptin incubated with deglycosylated rituximab that was prepared with free Endo-S2 WT; C), Herceptin incubated with deglycosylated rituximab that was prepared with immobilized Endo-S2 WT; D), Rituximab deglycosylated with free Endo-S2; E), Rituximab deglycosylated with immobilized Endo-S2.

Figure 4. LC-ESI-MS analysis of the heavy chain and light chain of Herceptin starting material and product glycoforms in the one-pot glycoengineering approach

A) Heavy chain and light chain of the commercial Herceptin starting material; B) Heavy chain and light chain of Fuca1,6GlcNAc-Herceptin after the deglycosylation step catalyzed by immobilized Endo-S2 WT; C) Heavy chain and light chain of G2F-Herceptin after the transglycosylation step catalyzed by immobilized Endo-S2 D184M.

Scheme 1.

Scheme 2.