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Comparative studies on the substrate specificity and defucosylation activity of three α-L-fucosidases using synthetic fucosylated glycopeptides and glycoproteins as substrates

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Abstract

Core fucosylation is the attachment of an α-1,6-fucose moiety to the innermost *N*-acetyl glucosamine (GlcNAc) in *N*-glycans in mammalian systems. It plays a pivotal role in modulating the structural and biological functions of glycoproteins including therapeutic antibodies. Yet, few α-L-fucosidases appear to be capable of removing core fucose from intact glycoproteins. This paper describes a comparative study of the substrate specificity and relative activity of the human α-L-fucosidase (FucA1) and two bacterial α-L-fucosidases, the AlfC from *Lactobacillus casei* and the BfFuc from *Bacteroides fragilis*. This study was enabled by the synthesis of an array of structurally well-defined core-fucosylated substrates, including core-fucosylated *N*-glycopeptides and a few antibody glycoforms. It was found that AlfC and BfFuc could not remove core fucose from intact full-length *N*-glycopeptides or *N*-glycoproteins but could hydrolyze only the truncated Fucα1,6GlcNAc-peptide substrates. In contrast, the human α-L-fucosidase (FucA1) showed low activity on truncated Fucα1,6GlcNAc substrates but was able to remove core fucose from intact and full-length core-fucosylated *N*-glycopeptides and *N*-glycoproteins. In addition, it was found that FucA1 was the only α-L-fucosidase that showed low but apparent activity to remove core fucose from intact IgG antibodies. The ability of FucA1 to defucosylate intact monoclonal antibodies reveals an opportunity to evolve the human α-L-fucosidase for direct enzymatic defucosylation of therapeutic antibodies to improve their antibody-dependent cellular cytotoxicity.

Graphical Abstract

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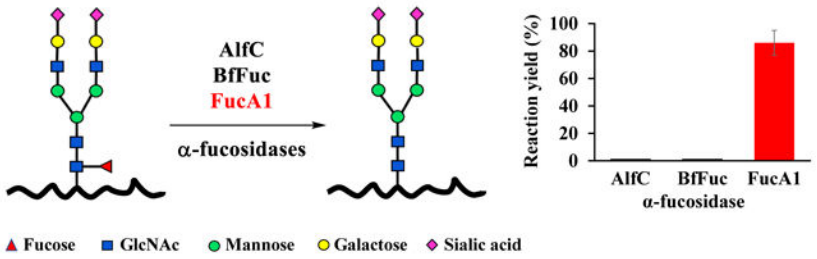
Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Keywords

Core fucosylation; monoclonal antibody; glycoproteins; fucosidase; glycopeptides; chemoenzymatic synthesis; *N*-glycans

1. Introduction

Protein *N*-glycosylation is a common post-translational modification of proteins that plays essential roles in many important biological recognition processes such as protein folding, trafficking, cell adhesion, and immune responses [1-2]. Core fucosylation, the attachment of an α-1,6-fucose moiety to the innermost *N*-acetyl glucosamine (GlcNAc) in *N*-glycans in mammalian systems, is one of the most frequent modifications on *N*-glycoproteins that can modulate the glycan conformations and regulates biological processes including cell adhesion, signal transduction, and development[3-5]. Further, the role of core fucose in tumor growth and progression has been demonstrated in several studies [6-9]. For example, gene knockout of FUT8, the α-1,6-fucosyltransferase that is responsible for core fucosylation in mammalian systems, in liver cancer cell lines and xenograft mouse models has shown reduced signaling through growth factor receptors and subsequent tumor suppression[10-11]. Additionally, core-fucosylated α-fetoprotein (AFP-L3) is upregulated in liver cancers and is an FDA approved biomarker in hepatocellular carcinoma [12]. In addition to regulating biological functions, core fucosylation also presents structural implications. Presence of α-1,6 core fucose in glycoproteins impacts the conformations of *N*-glycans and thus modulates their affinity for respective lectins [4, 13]. In the case of monoclonal antibodies, core fucosylation of IgG-Fc *N*-glycans at Asn-297 interferes with the favorable carbohydrate-carbohydrate interactions between the *N*-glycans from antibody Fc domain and Fc receptors, thus decreasing the affinity of antibody for FcγRIIIa receptor [14]. Compelling data have shown that removal of core fucosylation in IgG-Fc *N*-glycans can significantly enhance FcγRIIIa receptor binding (up to 50-fold), resulting in remarkably increased antibody-dependent cellular cytotoxicity (ADCC) and enhanced therapeutic efficacy [15-17].

Given the structural effects and biological importance of core fucosylation, it is crucial to develop tools that can modulate the fucose content of glycoproteins and antibodies for structure-function relationship studies as well as for therapeutic applications. Many α-L-fucosidases have been reported but only a few have been confirmed to be able to act on the α-1,6-glycosidic linkage to the innermost GlcNAc residue (the core fucose) [18-26]. We have previously shown that a nonspecific α-L-fucosidase from bovine kidney could remove core fucose from endoglycosidase S (Endo-S) treated IgG antibody but it has only low activity

and requires a long incubation time to achieve complete defucosylation [21]. Later, we have found that the α-L-fucosidases from *Bacteroides fragilis* (BfFuc) and *Lactobacillus casei* (AlfC) could much more efficiently remove the core fucose from Fc-deglycosylated antibodies than the bovine kidney enzyme, although none of the enzymes show detectable defucosylation activity on intact IgG antibodies [22-23]. Wong and co-workers have independently reported that the two α-L-fucosidases (BfFuc and AlfC) could not act on intact glycoproteins including antibodies either but hydrolyze the core fucose when the external *N*-glycans were removed [24]. On the other hand, FucA1, the human α-L-fucosidase, is responsible for the defucosylation of fucosylated substrates in lysosomes and its activity of defucosylation has been associated with tumor suppression [27-2930]. Furthermore, an *in vitro* study with FucA1 has demonstrated that FucA1 could remove core fucose from free complex-type *N*-glycans[31]. However, the substrate specificity and substrate structure-activity relationships of FucA1 and the above-mentioned bacterial α-L-fucosidases still remain to be further characterized. One challenge in probing the substrate specificity of these α-L-fucosidases is the availability of structurally well-defined core-fucosylated *N*-glycans, glycopeptides, and glycoproteins due to the difficulties in isolation and synthesis of core-fucosylated oligosaccharides and glycoconjugates [31-33]. We describe in this paper the chemoenzymatic synthesis of an array of well-defined core-fucosylated oligosaccharides, glycopeptides, and glycoproteins including antibodies (Figure 1). These fucosylated compounds were used for a comparative study on the substrate specificity and activities of the human α-L-fucosidase (FucA1) and two bacterial α-L-fucosidases (AlfC and BfFuc). Our results showed that the three α-L-fucosidases had different substrate specificity and activities. The two bacterial enzymes were found to hydrolyze the core fucose only when the core fucose was exposed by endoglycosidase treatment to remove the external sugar residues of the N-glycans. In contrast, the human α-L-fucosidase, FucA1, was the only enzyme capable of removing core fucose from some intact full-length glycopeptides and glycoproteins. FucA1 also demonstrated a low but detectable activity to remove core fucose from intact antibodies. Furthermore, the nature of *N*-glycosylation on antibodies appeared to influence the defucosylation activity of FucA1, which demonstrated higher hydrolytic activity on the high mannose glycoform than the complex-type glycoform of an antibody.

2. Results and discussion

2.1. Synthesis of the fucosylated glycopeptides (2, 3 and 4) and a fucosylated biantennary complex-type N-glycan (5)

A total of ten fucosylated substrates (1-10) (Figure 1) were applied to study the substrate specificity of the three enzymes. The synthesis of fucosylated glycopeptides (**2** and **3**) started with the preparation of the GlcNAc-peptide, CD52-GN (**11**), a 19-mer containing the CD52 antigen sequence and the sortase A signal sequence (LPKTGGS), followed by enzymatic core fucosylation and transglycosylation (Scheme 1). The inclusion of a sortase A signal sequence was for the purpose of further enzymatic site-specific conjugation for future applications [34-35]. Solid-phase peptide synthesis on an automated peptide synthesizer following our previously published procedure [36] gave CD52-GN (**11**) in 70% yield after HPLC purification. Core fucosylation was achieved by using the fucoligase AlfC E274A that we have previously reported, which was able to use simple synthetic α-fucosyl fluoride

as the donor substrate to attach an α1,6-linked fucose to a GlcNAc moiety in peptide [22]. The resulting core-fucosylated glycopeptide CD52-GN-F (**2**) was purified using preparative HPLC in 62% yield and characterized by LC-MS (Figure S2). Transfer of a biantennary complex-type *N*-glycan to **2** was achieved by using an EndoF3-D165A mutant, which was specific for glycosylation of fucosylated GlcNAc acceptor substrate [37], giving the full-length glycopeptide (**3**) (Scheme 1). The glycopeptide (**3**) was purified by preparative HPLC in 42% yield and characterized by LC-MS (Figure S3).

To further assess the hydrolysis of core fucose from a glycopeptide containing more than one *N*-glycosylation site, we also synthesized fucosylated glycopeptide **4**, a 24-mer cyclic glycopeptide derived from the V1V2 region of envelope glycoprotein gp120 of HIV-1 strain ZM109 [38]. First, the precursor GlcNAc-peptide, V1V2-GN2 (**12**), was synthesized using SPPS by modifying our previously described procedure [38]. The purified glycopeptide (62% yield) was fucosylated by AlfC E274A fucoligase to provide fucosylated glycopeptide **4**, which was purified by preparative HPLC in 70% yield and its identity was confirmed by LC-MS analysis (Figure S4). We also synthesized a fucosylated biantennary complex-type N-glycan (**5**) using the fucoligase AlfC E274A for direct core fucosylation (Scheme 2). But in this case, we found that the full-length *N*-glycan was a poor substrate, and a large amount of the enzyme was required to drive the reaction. The fucosylated product (**5**) was purified by size exclusion chromatography on a Sephadex G-15 column in 27% yield and its identity was confirmed by MALDI-TOF-MS analysis (Figure S5).

2.2. Preparation of the core-fucosylated glycoproteins (6 and 7) and antibodies (9 and 10)

To study the hydrolysis of core fucose in the context of intact glycoproteins, two types of glycoforms of the granulocyte macrophage colony stimulating factor (GM-CSF) were produced. GM-CSF-HM-F (**6**) was expressed in HEK293T FUT8$^+$ cells in the presence of kifunensine, an α1,2-mannosidase inhibitor[39]. The His-tagged protein was purified using immobilized metal affinity chromatography (IMAC). A smear of protein bands on SDS-PAGE indicated a heterogeneously glycosylated protein (Figure S6A). Glycan characterization was done by releasing *N*-glycan from the protein using PNGase F, followed by glycan enrichment and MALDI-TOF-MS analysis. A mixture of partially fucosylated high mannose (Man_{5-9}) glycoforms was seen in the released *N*-glycan (Figure S6B). On the other hand, the complex-type glycoform, the His-tagged GM-CSF-WT (**7**), was expressed using the above method in the absence of kifunensine. Purification and characterization of the glycoprotein were done as explained above (Figure S7A). The released *N*-glycan showed a mixture of glycoforms with tri- and tetra-antennary complex-type *N*-glycans as the predominant forms (Figure S7B).

For testing the activity of the α-L-fucosidases on intact antibodies, we chose Rituximab (**8**), a therapeutic monoclonal antibody as a model and prepared two variants of core-fucosylated glycoforms (**9,10**). First, Rituximab was treated with endoglycosidase EndoS2 to hydrolyze the Fc glycan, giving the truncated fucosylated glycoform, Fucα1,6GlcNAc-RTX (RTX-GN-F, **9**). Then, a high-mannose (Man_5) *N*-glycan was transferred to **9** using the EndoS2-D184M mutant [40] to afford the Man_5 glycoform (**10**) (Scheme 3). The identities of the antibody glycoforms (**8-10**) were confirmed by LC-MS analysis (Figure S8A, S9A, S10A).

2.3. Hydrolysis of p-nitrophenyl α-L-fucoside by α-L-fucosidases

Recombinant expression and purification of α-L-fucosidases was performed by following previously reported procedures. The human α-L-fucosidase, FucA1, was expressed as a fusion protein with an N-terminal green fluorescent protein (GFP) tag in HEK293T cells [31]. AlfC and BfFuc were expressed in *E. coli* BL21(DE3) strain [23]. The three His-tagged enzymes were purified by IMAC and analyzed using SDS-PAGE (Figure S1). Previous studies have characterized that the optimal pH of AlfC[18] and BfFuc [24] is around pH 7, while FucA1 has an acidic condition (pH 4.5) for the optimal enzymatic hydrolysis[30]. To compare the substrate specificity and hydrolytic efficiency of the enzymes, we performed reactions at the respective optimum pH of each enzyme. Reactions with AlfC and BfFuc were done in PBS at pH 7.4. Meanwhile, FucA1 reactions were set up in sodium acetate buffer at pH 4.5.

The purified enzymes were first tested for activity with *p*NP-Fuc (**1**) at 37°C. Reaction progress was monitored by measuring the UV absorbance (at 410 nm) of the *p*NP product formed. BfFuc and FucA1 showed efficient hydrolysis of **1** while AlfC was required at a 10-fold higher concentration to achieve comparable hydrolysis (Figure 2).

2.4. Hydrolysis of Fucα1,6GlcNAc-peptides

Next, we evaluated the hydrolysis of glycopeptides containing core-fucosylated GlcNAc. We used two glycopeptide substrates - the 19-mer CD52-GN-F (**2**) and the 24-mer V1V2-GN2-F2 (**4**) carrying one and two core fucose residues, respectively. The yields of defucosylation were estimated based on relative abundance of the cleaved product using mass spectrometric analysis. Additionally, a mixture of **4** and its hydrolysis product, V1V2-GN2 (**12**) were separated and quantified using RP-HPLC (Figure S4E) and the results were confirmed using mass spectrometry.

In case of glycopeptide **2**, AlfC and BfFuc hydrolyzed the substrate efficiently, while FucA1 showed much lower hydrolytic activity than the two bacterial α-L-fucosidases (Figure 3A). Similar trends were observed with glycopeptide **4** (Figure 3B). Thus, the two bacterial α-L-fucosidases (AlfC and BfFuc) are much more active on the truncated Fucα1,6GlcNAc-peptides than the human α-L-fucosidase (FucA1).

2.5. Hydrolysis of full-length fucosylated complex-type N-glycan and intact glycopeptide by α-L-fucosidases

Next, we turned our attention to test the substrate specificity of the three α-L-fucosidases on full-length fucosylated *N*-glycans and glycopeptides. Recent studies have demonstrated *in vitro* activity of FucA1 with synthetic aryl α-fucosides [30] and with core-fucosylated biantennary sialylated complex-type free *N*-glycan[31]. We first assessed the activity of FucA1 and the other two α-L-fucosidases with core-fucosylated biantennary complex-type *N*-glycan (**5**). As expected, FucA1 efficiently defucosylated *N*-glycan (**5**). However, AlfC and BfFuc did not show any fucosidase activity on the full-length core-fucosylated *N*-glycan (**5**) (Figure 4A). Then, we tested the activity of these enzymes on the core-fucosylated full-length *N*-glycopeptide (**3**). The glycopeptide (**3**) was incubated with the α-L fucosidases at 37°C. The reactions were monitored by LC-MS analysis. We found that FucA1 showed considerable hydrolysis of the glycopeptide at 1 mg/ml enzyme concentration and the

reaction could be pushed to completion by increasing the amount of enzyme and/or prolonging the incubation time (Figure 4B). In contrast, like the case of the full-length *N*-glycan (**5**), AlfC and BfFuc did not show hydrolytic activity on the intact full-length fucosylated glycopeptide (**3**) either (Figure 4B). Taken together, these results reveal that AlfC and BfFuc are much more active to defucosylate the truncated Fucα1,6GlcNAc-peptide substrates than FucA1, but the human enzyme (FucA1) is the only one that showed apparent activity to defucosylate intact full-length core-fucosylated *N*-glycopeptides.

2.6. Hydrolysis of fucosylated intact glycoproteins by the human α-L-fucosidase FucA1

The successful defucosylation of intact glycopeptide by FucA1 encouraged us to examine its activity with biologically relevant glycoproteins. Human granulocyte macrophage colony stimulating factor, GM-CSF is a cytokine involved in immune functions [41] and contains two conserved *N*-glycosylation sites. We expressed two types of glycoforms of GM-CSF using HEK293T cells – a) a fucosylated high mannose variant and b) the wild-type protein containing mainly complex-type *N*-glycans. First, we tested the hydrolysis of GM-CSF-HM-F (**6**) by the α-L-fucosidases. The extent of defucosylation was estimated by release of the total *N*-glycans and measurement of the released *N*-glycans using MALDI-TOF-MS analysis. We have previously found that the estimate by MALDI-TOF-MS analysis of the fucosylated and non-fucosylated high-mannose N-glycans is consistent with the more accurate HPLC quantification.[42] Gratifyingly, an overnight incubation of GM-CSF-HM-F with FucA1 showed a high amount of defucosylation (estimated as 70% of released total *N*-glycan) (Figure 5A, S6C). Prolonging the incubation to two overnights under the reaction conditions showed 85% of released total *N*-glycan to be defucosylated (data not shown).

Next, we assessed the defucosylation of GM-CSF-WT. The WT protein contains a mixture of bi- to tetra-antennary complex-type *N*-glycans, with fucosylated tetra- and tri-antennary glycans terminating in GlcNAc as the two most abundant glycoforms, respectively. MALDI-TOF-MS analysis of *N*-glycan released from FucA1-treated GM-CSF-WT showed defucosylation but the reaction progress was slow. We observed about 50% of total *N*-glycan released from the glycoprotein to be hydrolyzed in seven days (Figure 5B, S7C), while the protein control incubated without the enzyme did not show any loss of fucose. Upon comparing defucosylation of the two variants of GM-CSF by FucA1, hydrolysis of complex-type glycoprotein appears less efficient than that of the high-mannose type.

2.7. Defucosylation of intact IgG antibody by the three α-L-fucosidases

Next, we evaluated the removal of core fucose from intact antibodies. The conserved glycosylation site at N297 in antibody Fc is buried within the antibody dimer making the site difficult to access for modifications. Hence, discovery of an enzyme that can efficiently modulate core fucose of intact antibodies is very valuable. To study the hydrolytic capability of FucA1 with antibodies, we used commercial monoclonal antibody, Rituximab (**8**). About 10% hydrolysis of the antibody was observed in seven days at an equimolar ratio of antibody to enzyme (Figure 6A, S8B). The result was reproducible in multiple independent reactions. Although the defucosylation yield was low, to our knowledge, this was the first example of direct enzymatic defucosylation of intact antibodies by an α-L-fucosidase. In contrast, AlfC and BfFuc did not show any hydrolytic activity on the intact antibody.

Wong and co-workers as well as our group have previously reported that AlfC and BfFuc could efficiently remove the core fucose from the endoglycosidase-pretreated; truncated Fucα1,6GlcNAc-antibody intermediate for glycan remodeling to produce non-fucosylated antibody glycoforms [22-24, 40]. We thus compared the hydrolysis of RTX-GN-F (**9**) by the three α-L-fucosidases. In agreement with previous reports, AlfC and BfFuc were found to be efficient in removing core fucose from the deglycosylated antibody (Figure 6B, S9B). Interestingly, FucA1 showed only low hydrolytic activity on the Fucα1,6GlcNAc-antibody (**9**) under a similar condition and a large amount of enzyme was required to give hydrolytic activity comparable to the AlfC and BfFuc (Figure 6B).. We also observed that at a high antibody substrate concentration (>20 mg/ml), the AlfC showed higher apparent initial rate for the defucosylation of the Fucα1,6GlcNAc-antibody (**9**) than BfFuc, confirming our previous result [23]. Interestingly, when the substrate concentration is low (<5 mg/ml), the BfFuc showed a higher apparent rate of hydrolysis of **9** (data not shown), consistent with the observation from Wong and co-workers [24]. These results suggest that AlfC may have lower substrate affinity (higher K_M) for the antibody but higher turnover rate than the BfFuc enzyme, which should be clarified in future kinetic studies.

As an initial study to evaluate the effect of different *N*-glycans on the defucosylation of antibody by FucA1, we prepared a Man_5 glycoform of Rituximab, RTX-M5-F (**10**) and tested it with FucA1. We found that FucA1 had much higher activity on the fucosylated Man_5 glycoform (**10**) than the parent Rituximab (**8**), giving ca. 55% yield of defucosylation in a total of seven-day incubation (Figure 6C, S10B), while incubation of Rituximab (**8**) under similar condition gave ca. 10% defucosylation (Figure 6A). This result suggests that FucA1 can distinguish between different core-fucosylated *N*-glycans for defucosylation. In contrast, AlfC and BfFuc did not show hydrolytic activity on the core-fucosylated high-mannose antibody glycoform (**10**) (Figure 6C). The finding that FucA1 possesses low but apparent defucosylation activity on intact monoclonal antibodies is significant, as this discovery raises an exciting opportunity to enzymatically remove the core fucose directly from recombinant monoclonal antibodies to enhance their antibody-dependent cellular cytotoxicity and the overall therapeutic efficacy [15-17]. The defucosylation activity of FucA1 on intact antibodies may be further improved through directed evolution [43-44].

2.8. Surface plasmon resonance (SPR) analysis on the binding of FcγIIIa receptor with the antibodies treated by FucA1

To characterize the structural and functional integrity of the antibodies treated with FucA1, we assessed the receptor binding affinities of the partially defucosylated glycoforms obtained by FucA1 treatment. It is well established that the core fucose on the Fc N-glycans impacts the local conformations of Fc domain [45] and affects the antibody binding to FcγIIIa receptor (FcγRIIIa).[14-15] Defucosylation has been shown to significantly enhance the antibody's FcγRIIIa affinity (up to 50-fold) for the typical complex type Fc glycoform [46] but only moderately (2-3 fold) enhance the affinity for FcγRIIIa in the case of high-mannose type glycoform. [47-48] Thus, the affinity of the partially defucosylated antibody mixtures for the FcγRIIIa might serve as a quick estimate on the structural and functional integrity of the antibodies after treatment with the fucosidase, as Fc denaturation might occur during the long incubation to potentially contribute to the observed defucosylation. First, we used the

core-fucosylated rituximab and the partially defucosylated antibody generated by FucA1 treatment to measure the binding to recombinant FcγRIIIA VI58 receptor using surface plasmon resonance (SPR) analysis. Our data showed a marginal enhancement (1.3-fold) in the binding affinity of the FucA1-hydrolyzed antibody (Figure 7A,7B). The K_D value changed from 71 nM of the intact rituximab to 56 nM of the partially (5-10%) defucosylation of rituximab. The data suggest that the Fc domain maintained its active conformations without denaturation during the long fucosidase incubation and a small fraction of the antibody was defucosylated, as reflected by the slight increase in the FcγRIIIa affinity. Next, we analyzed the binding affinities of the Man_5 glycoforms of rituximab. We found that the mixture, which contains ca. 55% defucosylated glycoforms after FucA1 treatment, showed about a 1.5-fold enhancement in binding affinity to the FcγRIIIa (Figure 7C, 7D). The K_D value changed from 103 nM of the fucosylated Man_5 glycoform to 70 nM of the partially defucosylated Man_5 glycoform. To provide an accurate side-by-side comparison of the impact of defucosylation of the Man_5 glycoforms, we produced a non-fucosylated rituximab Man_5 standard through glycoengineering (Figure S11). Binding analysis of the rituximab Man_5 glycoform with FcγRIIIa V158 showed a K_D of 43 nM (Figure 7E), which translates to a 2.4-fold enhancement in binding affinity over the core-fucosylated glycovariant. The K_D value of rituximab Man_5 glycoform agrees with the previous reported values of 32 nM for the Man_5 glycoform and 27 nM for the Man_{8-9} glycoforms of IgG1[49]. Indeed, in comparison to the core-fucosylated complex-type Fc glycoforms of antibodies, the non-fucosylated oligomannose glycoform appears to possess only a 2-3 fold enhancement in affinity for FcγRIIIa, as demonstrated in several previous studies.[47-48] The moderate impact of core-fucosylation on FcγRIIIa affinity was in sharp contrast to the dramatic increase (up to 50-fold) in binding affinity upon defucosylation of core-fucosylated complex-type antibody Fc glycoforms. Our results are consistent with previous studies and the 1.5-fold enhancement in FcγRIIIa affinity of the partially defucosylated Man_5 glycoforms confirmed that about 50% defucosylation occurred in the sample and that the Fc domain maintained an active conformation without denaturation during the FucA1 treatment.

3. Conclusion

A comparative study of the substrate specificity and relative activity of the bacterial α-L-fucosidases from *Bacteroides fragilis* (BfFuc) and *Lactobacillus casei* (AlfC) and the human α-L-fucosidase (FucA1) is described. This study was enabled by the synthesis of an array of structurally well-defined core-fucosylated *N*-glycopeptides and glycoproteins including a few antibody glycoforms. The experimental data reveal that the two bacterial α-L-fucosidases hydrolyze only truncated Fucα1,6GlcNAc-peptide substrates and they are not active to remove the core fucose when it is present in intact full-length *N*-glycans or *N*-glycoproteins. In contrast, the human α-L-fucosidase (FucA1) shows low activity on truncated Fucα1,6GlcNAc-peptide substrates but FucA1 can remove the core fucose from intact full-length *N*-glycopeptides and glycoproteins. In addition, FucA1 appears to be the only α-L-fucosidase that shows low but apparent activity to remove core fucose from intact IgG antibodies. The ability of FucA1 to defucosylate intact antibodies points to a promising

way to directly remove the core fucose from intact therapeutic antibodies to improve their antibody-dependent cellular cytotoxicity for treatment of human diseases.

4. Experimental

4.1 Materials

p-Nitrophenyl α-L-fucoside was purchased from Sigma-Aldrich. Monoclonal antibody Rituximab (Genentech Inc., South San Francisco, CA) was purchased from Premium Health Services Inc. (Columbia, MD). Sialoglycan complex-type oxazoline (SCT-oxazoline) was synthesized using previously established procedure [40]. Man_5-oxazoline was synthesized using previously described method [38]. AlfC E274A, EndoF3 D165A, EndoS2 WT and EndoS2-D184M were overexpressed and purified using previously reported methods [2237, 40].

4.2 Methods

Analytical HPLC was performed with a Waters 626 HPLC system using a YMC column (5 μm, 4.6 x 250 mm). Preparative HPLC was performed with a Waters 600 HPLC system equipped with a SymmetryPrep™ C18 column (7 μm, 19 x 300 mm) using a flow rate of 12 ml/min or an XBridge™ Prep Shield RP18 column (5 μm, 10 x 250 mm) using a flow rate of 4 ml/min. Water containing 0.1% TFA and acetonitrile containing 0.1% TFA were used as Solvents A and B respectively for both analytical and preparative chromatography purposes. LC-MS analysis of saccharides and glycopeptides was performed using a Waters Alliance e2695 HPLC system connected to an SQ Detector 2. The C18 columns used for analysis include a long Thermo Scientific Hypersil column (3 μm, 4.6 x 250 mm) employing a flow rate of 1 ml/min and a short XBridge™ column (3.5 μm, 2.1 x 50 mm) using a flow rate of 0.4 ml/min. Mass spectrometric analysis of CT-F glycan was performed using Bruker Autoflex III MALDI-TOF (Bruker Daltonics) in reflectron positive mode using 2,5-dihydroxybenzoic acid (DHB) matrix. The matrix was prepared by dissolving 100 mg DHB and 20 μl of N, N-dimethylaniline (DMA) in 50% aqueous acetonitrile solution. LC-MS analysis of antibodies was carried out using Thermo Scientific Exactive™ Plus Orbitrap mass spectrometer on a Waters XBridge™ BEH300 C4 column (3.5 μm, 2.1 x 50 mm). The method involved a 9-minute linear gradient of 5 to 90% acetonitrile containing 0.1% formic acid at a flow rate of 0.4 ml/min. Deconvolution of raw data was done using MagTran (Amgen).

4.3 Expression and purification of AlfC, BfFuc and FucA1 α-L-fucosidases

α-L-fucosidases from *Lactobacillus casei* (AlfC) and *Bacteroides fragilis* (BfFuc) were overexpressed in *E. coli* BL21(DE3) competent cells and purified as previously reported [23]. DNA construct for expression of human α-L-fucosidase (FucA1), pGEn2-FucA1 containing the catalytic domain of the enzyme was purchased from Glycozyme, CCRC, UGA through DNASU plasmid repository. Overexpression of FucA1 was done in HEK293T cells following the reported procedures [31]. Briefly, a day prior to transfection, HEK293T cells were seeded at a density of 7 x 10^5/ml in serum-free FreeStyle™ F17 Expression Medium (Thermo Fisher Scientific) and cultured at 37°C, 8% CO_2, 150 rpm. The following day, transient transfection was performed with 1 μg DNA (pGEn2-FucA1 expression vector) and

2 μg polyethylenimine (PEI) per million cells. The expression culture was incubated at 37°C, 8% CO_2, 150 rpm for 3 days. At harvest, the broth was spun down at 1000 rpm for 10 min at 4°C and the supernatant was purified using a HisTrap™ column (GE Healthcare), following manufacturer's protocol. The eluted protein was buffer exchanged to PBS, pH-7.4 and characterized using SDS-PAGE. Long-term storage of enzyme aliquots was done at −80°C.

4.4 Synthesis of core-fucosylated substrates

4.4.1 Synthesis of CD52-GN-F (2)—Core-fucosylated CD52 antigen (**2**) was synthesized from CD52-GN containing a signal peptide sequence (**11**) following previously outlined procedure[36]. **11** (10 mg, 5 μmol) and α-fucosyl fluoride (1.7 mg, 10 μmol) were mixed with AlfC E274A (0.5 mg/ml) in 1 ml PBS, pH-7.4 and incubated at 37°C. Reaction progress was monitored by LC-MS. Complete transfer was achieved with further addition of α-fucosyl fluoride (3.7 mg, 21.8 μmol). The fucosylated product was purified on a C18 column using RP-HPLC using a linear gradient of 5 to 35% B over 30 min at a flow rate of 12 ml/min. t_R = 14.96 min. ESI-MS: calcd. for **2,** *M* = 2197.0 Da; found (*m/z*), 733.49 $[M + 3H]^{3+}$, 1099.60 $[M + 2H]^{2+}$.

4.4.2 Synthesis of CD52-SCT-F (3)—Core-fucosylated biantennary sialylated CD52 (**3**) was generated from **2** as reported before [37]. EndoF3-D165A (0.5 mg/ml) was added to a mixture of **2** (2 mg, 0.91 μmol) and SCT-oxazoline (4 mg, 2 μmol) in 120 μl PBS, pH-7.4. The reaction mixture was incubated at 30°C for 0.5 h. The final product was purified using RP-HPLC on a C18 column using a linear gradient of 5 to 25% B over 40 min at a flow rate of 4 ml/min. t_R = 10.01 min. ESI-MS: calcd. for **3**, *M* = 4199.1 Da; found (*m/z*), 1051.09 $[M + 4H]^{4+}$, 1400.93 $[M + 3H]^{3+}$.

4.4.3 Synthesis of V1V2-GN2-F2 (4)—Solid-phase peptide synthesis of V1V2-GN2 (**12**) was performed under microwave synthesis conditions using a CEM Liberty Blue microwave peptide synthesizer. Synthesis was based on Fmoc chemistry using Rink Amide resin (0.66 mmol/g) on a 0.1 mmol scale. Couplings were performed using 5 equiv of Fmoc-protected amino acids (or 3 equiv of glycosyl-amino acids), 5 equiv of DIC and 5 equiv of HOBt in DMF. Double couplings were performed at 45°C for 20 min (2×) for Fmoc-Cys(Trt)-OH, Fmoc-His(Trt)-OH and Fmoc-Arg(Pbf)-OH. The selectively protected glycosyl-amino acid building block Fmoc-Asn(Ac_3GlcNAc)-OH (1a) was introduced at pre-determined sites and the coupling was performed at 45°C for 40 min. All other amino acids were coupled at 90°C for 2 min. Fmoc deblocking was performed in 20% piperidine in DMF containing 0.1 M HOBt. The N-terminus was capped with an alkyne tag by treatment with 4-pentynoic acid following the same method as introduction of glycosyl-amino acid. After synthesis, the resin was washed with DMF (3×) and DCM (3×) and dried. Resin cleavage and global peptide deprotection were achieved using freshly prepared cocktail R (TFA/Thioanisole/Ethanedithiol/Anisole [90/5/3/2]) and shaking for 2 h. The peptide was separated from the resin by filtration and then precipitated onto cold (−20°C) diethyl ether. A solution of the crude peptide in water (1 mg/mL) with 2.5% hydrazine was shaken at RT for 3 h to cyclize the peptide and remove the acetyl protecting group simultaneously. The crude peptides were purified by RP-HPLC and the purity and identity were confirmed by

analytical HPLC and LC-MS analysis. Analytical RP-HPLC method: C18 column (YMC-Triart C18, 4.6 × 250 mm, 5 μm) at a flow rate of 1 mL/min using a linear gradient of 5-40% MeCN containing 0.1% TFA over 30 min. Compound **12** (1.43 mg, 0.44 μmol) and α-fucosyl fluoride (0.29 mg, 1.76 μmol) were incubated with AlfC E274A (0.5 mg/ml) in 0.5 ml PBS, pH-7.4 at 37°C. The reaction was monitored by LC-MS. The reaction was pushed to completion with further addition of α-fucosyl fluoride (0.45 mg, 2.64 μmol) while maintaining the pH of the mixture. After 1 h of incubation, the fucosylated peptide was purified using RP-HPLC on a C18 column using stepwise gradient of solvent B starting with 5 to 20% over 10 min followed by 20 to 40% over 30 min at a flow rate of 4 ml/min. t_R = 10.12 min. ESI-MS: calcd. for **4**, *M* = 3533.7 Da; found (*m/z*), 707.78 $[M + 5H]^{5+}$, 884.57 $[M + 4H]^{4+,}$ 1179.04 $[M + 3H]^{3+}$.

4.4.4 Synthesis of the fucosylated biantennary complex-type *N*-glycan (5)—

Sialylated biantennary complex-type glycan (SCT) was extracted from SGP present in egg yolk powder (Henningsen Foods) and treated with neuraminidase to generate asialo complex-type *N*-glycan, using previously reported methods [50]. Purified CT-glycan (**13**) was fucosylated by incubating with AlfC E274A (2 mg/ml) and α-fucosyl fluoride (10 mol equivalent of **13**) in PBS, pH-7.4 at 37°C for 1 h. The final product was purified using a Sephadex G-15 column. MALDI-TOF-MS: calcd. for **5**, *M* = 1787 Da; found (*m/z*), 1809.7 $[M + Na]^+$.

4.4.5 Expression and purification of GM-CSF (6,7)—

GM-CSF-HM-F (**6**) was expressed using HEK 293T FUT8$^+$ cells following a previously reported procedure [51]. Briefly, cells were seeded at a density of 7 x 10^5/ml in serum-free FreeStyle™ FI7 Expression Medium (Thermo Fisher Scientific) and cultured at 37°C, 8% CO_2, 150 rpm. The overnight grown culture was transiently transfected with 1 μg DNA (pcDNA3.1-GM-CSF plasmid) and 3 μg polyethylenimine (PEI) per million cells. 4 μM of kifunensine was supplemented post-transfection to restrict protein glycosylation to high mannose glycoform. The expression culture was incubated at 37°C, 8% CO_2, 150 rpm for 3 days. At harvest, the broth was spun down at 1000 rpm for 10 min at 4°C and the supernatant was purified using a HisTrap™ column (GE Healthcare), following manufacturer's protocol. The eluted protein was buffer exchanged to PBS, pH-7.4 and characterized using SDS-PAGE and mass spectrometric analysis. Expression and purification of GM-CSF-WT (**7**) was performed as above using HEK293T cells without supplementation of kifunensine.

4.4.6 Chemoenzymatic remodeling of Rituximab—

Commercial Rituximab (**8**, 4 mg/ml) was deglycosylated by incubating with immobilized EndoS2 WT (0.1 mg/ml) in PBS, pH-7.4 at room temperature for 3 h and purified on a Protein A column to obtain RTX-GN-F, **9** as reported previously [23]. The samples were analyzed by MALDI-TOF-MS. **9** (10 mg/ml) was incubated with EndoS2 D184M (0.2 mg/ml) and Man_5-oxazoline (80 mol equivalent of **9**) in PBS, pH-7.4 at 30°C to generate RTX-M5-F (**10)**. Reaction progress was monitored using LC-MS and the final product was purified using Protein A chromatography. ESI-MS analysis of the Fc monomer released by IdeS treatment of the RTX-M5-F showed a species of 25116 Da (after deconvolution) which agrees with the calculated molecular mass of the Fc monomer carrying a Man_5-F glycan. To prepare the RTX-M5 standard, **9** (10

mg/ml) was first treated with AlfC (0.1 mg/ml) in PBS, pH-7.4 at 37°C to produce RTX-GN and purified using Protein A chromatography. Purified RTX-GN was then incubated with EndoS2 D184M (0.2 mg/ml) and Man_5-oxazoline (80 mol equivalent) in PBS, pH-7.4 at 30°C. Purification and analysis were done as described above. ESI-MS analysis of the Fc monomer released by IdeS treatment of the RTX-M5 showed a species of 24971 Da (after deconvolution).

4.5 Testing hydrolytic activity of α-L-fucosidases with fucosylated saccharide

The hydrolytic activity of the α-L-fucosidases with simple saccharides was tested by incubating **1** (5mM) and the respective enzyme (0.0005 mg/ml) in 100 μl PBS, pH-7.4 or sodium acetate, pH 4.5 at 37°C. Samples were collected through the course of the reaction and quenched with 0.1 M sodium hydroxide solution. Reaction products were monitored by measuring the absorbance at 410 nm. The above reaction was also conducted with 10-fold higher concentration of AlfC (0.005 mg/ml) while maintaining other reaction conditions constant.

4.6 Testing hydrolytic activity of α-L-fucosidases with Fucα1,6GlcNAc-peptides

To test the hydrolysis of core α-1,6 fucose linked to GlcNAc peptides, **2** (5 mg/ml) was incubated with each of the enzymes (0.01 mg/ml) in 10 μl PBS, pH-7.4 or NaOAc, pH-4.5 at 37°C. Intermediate reaction samples were collected and analyzed by LC-MS. ESI-MS: calcd. for hydrolysis product, M = 2051.4 Da; found (m/z), 684.85 $[M + 3H]^{3+}$, 1026.58 $[M + 2H]^{2+}$. The activity of FucA1 was tested at ten-fold higher concentration (1 mg/ml) by incubating with **2** (5 mg/ml) under similar reaction conditions. Similarly, **4** (4 mg/ml) was mixed with respective enzymes (0.01 mg/ml) in 10 μl PBS, pH-7.4 or NaOAc, pH-4.5 at 37°C. Samples were collected at 10, 20, 40 and 60 min and analyzed using LC-MS. ESI-MS: calcd. for hydrolysis product, M = 3241.7 Da; found (m/z), 649.38 $[M + 5H]^{5+}$, 811.43 $[M+4H]^{4+,}$ 1081.58 $[M+3H]^{3+}$.

4.7 Testing hydrolytic activity of α-L-fucosidases with complex-type *N*-glycan and intact glycopeptide

To test the hydrolysis of complex-type glycoform by the α-L-fucosidases, **5** (5 mg/ml) was incubated with respective enzymes (1 mg/ml) in 8 μl PBS, pH-7.4 at 37°C. Reaction samples were analyzed by MALDI-TOF-MS. MALDI-TOF-MS: calcd. for hydrolysis product **5**, M = 1641 Da; found (m/z), 1666.3 $[M + Na]^{+}$. Hydrolysis of intact glycopeptide was tested by mixing **3** (3 mg/ml) and FucA1 (3 mg/ml) in 10 μl of 100 mM NaOAc, pH-4.5 at 37°C for 21 h. Reactions with AlfC (2.9 mg/ml) and BfFuc (1.3 mg/ml) were performed in 8 μl PBS, pH-7.4 at 37°C. Sample analysis was performed by LC-MS. ESI-MS: calcd. for hydrolysis product, M = 4053.1 Da; found (m/z), 1014.36 $[M + 4H]^{4+}$, 1352.29 $[M + 3H]^{3+}$.

4.8 Testing hydrolytic activity of α-L-fucosidases with glycoproteins

The hydrolytic activity of the α-L-fucosidases with intact glycoproteins was tested by incubating **6** (1.5 mg/ml) and FucA1 (1.5 mg/ml) in 70 mM NaOAc, pH-4.5 in 10 μl total volume at 37°C for 45h. Reactions with AlfC and BfFuc were performed in 10 μl PBS, pH-7.4 at 37°C. Sample analysis was done by *N*-glycan release and MALDI-TOF-MS.

Hydrolysis of complex-type intact glycoprotein was tested by incubating **7** (0.55 mg/ml) and the respective enzymes (0.6 mg/ml) in 180 μl of 40 mM NaOAc, pH-4.5 or PBS, pH-7.4 at 37°C for 7 days.

4.9 Testing hydrolytic activity of α-L-fucosidases with antibodies

The hydrolytic activity of the α-L-fucosidases with intact antibody was tested by incubating **8** (2.8 mg/ml) and the respective enzymes (2.8 mg/ml) in 18 μl of 70 mM NaOAc, pH-4.5 or PBS, pH-7.4 at 37°C. Samples were treated with IdeS protease (0.02 mg/ml) in PBS at 37°C for 15 min to generate Fc monomers and analyzed by LC-MS. To test the hydrolysis of RTX-GN-F, **9** (22 mg/ml) was incubated with respective enzymes (0.15 mg/ml) in 10 μl PBS, pH-7.4 or NaOAc, pH-4.5 at 37°C. Samples were collected through the course of the reaction and analyzed by LC-MS post-IdeS treatment. The hydrolytic activity of the α-L-fucosidases with antibody containing fucosylated Man_5 glycan was tested by incubating **10** (1.6 mg/ml) and the respective enzymes (1.6 mg/ml) in 18 μl of 100 mM NaOAc, pH-4.5 or PBS, pH-7.4 at 37°C. Samples were analyzed by LC-MS post-IdeS treatment.

4.10 *N*-glycan release and MALDI-TOF-MS analysis

N-glycans were released from glycoproteins using Peptide: *N*-glycosidase F (PNGase F) under denaturing condition following the protocol recommended by New England Biolabs (NEB). The cleaved glycans were purified using HyperSep™ Hypercarb™ SPE cartridges (Thermo Scientific). The eluted samples were lyophilized and analyzed by MALDI-TOF-MS in reflectron positive mode using 2,5-dihydroxybenzoic acid (DHB) matrix. Glycan assignment was done using GlycoWorkbench.

4.11 SPR binding analysis

The experiment was carried out by capturing each antibody glycoform onto the protein A chip and flowing serial dilutions of FcγRIIIA V158 as the analyte. After each cycle, the surface was regenerated by injecting a glycine HCl buffer (10 mM, pH 2.0). The antibodies were captured at 200 RU. The receptor in 2-fold serial dilutions (from 500 nM to 0.976 nM) was injected at 30 μL/min for 180 s, followed by a 300 s dissociation. The experimental data were fit to a 1:1 Langmuir binding model using the BIA Evaluation software (GE Healthcare) to obtain the steady state kinetic data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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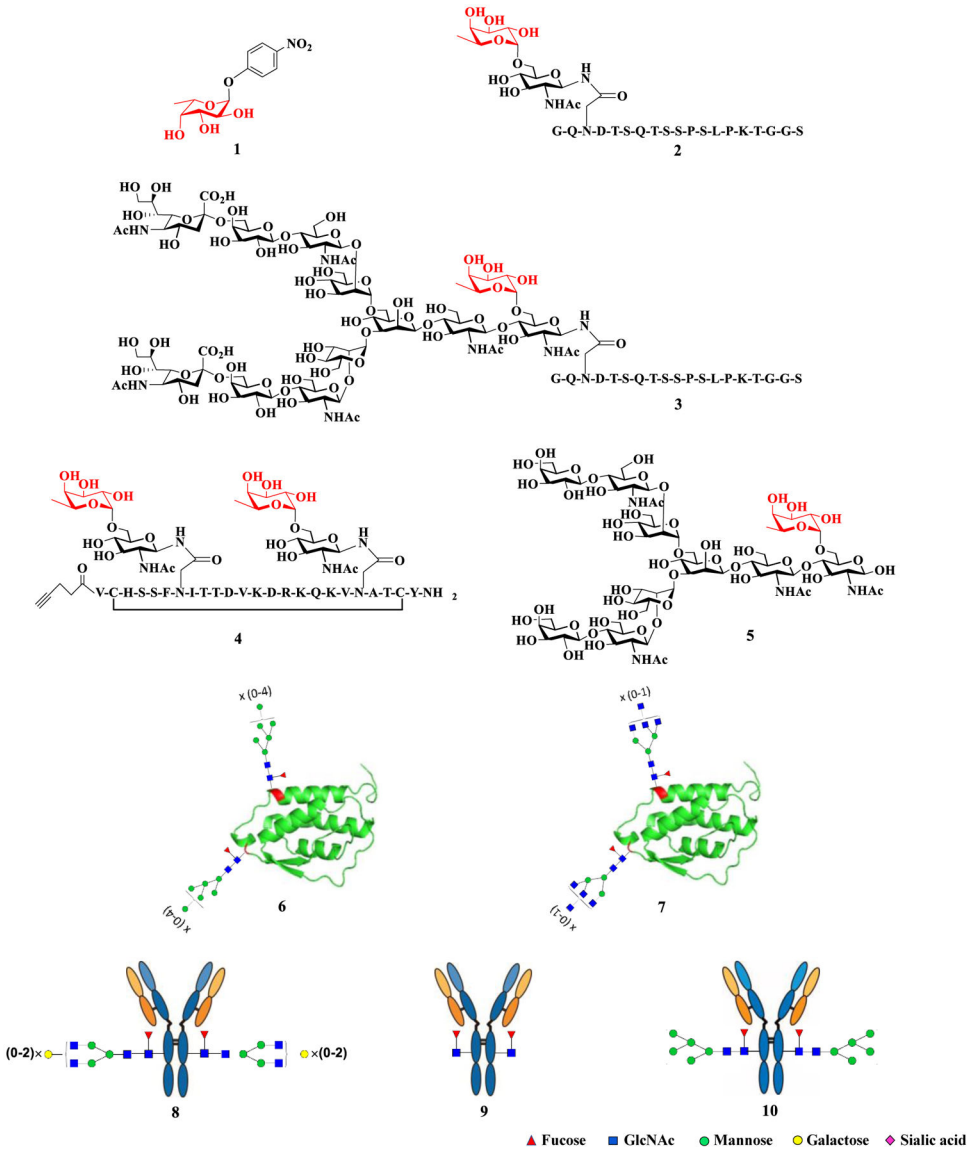


Figure 1: Substrates used for testing hydrolytic activity of α-fucosidases.

p-nitrophenyl α-L-fucoside (**1**); CD52-GN-F: a 19-mer glycopeptide containing CD52 antigen, sortase A signal sequence and truncated *N*-glycan (**2**); CD52-SCT-F: CD52 glycopeptide containing core-fucosylated biantennary sialylated complex type glycan (**3**); V1V2-GN2-F2: a 24-mer cyclic glycopeptide derived from the V1V2 region of HIV-1 gp120 glycoprotein (**4**); CT-F: core-fucosylated complex-type free *N*-glycan (**5**); GM-CSF-HM-F: granulocyte macrophage colony stimulating factor containing fucosylated high mannose glycan (**6**); GM-CSF-WT: GM-CSF wild type containing a mixture of complex-type *N*-glycans (**7**); Rituximab: IgG1 antibody (**8**); RTX-GN-F: Deglycosylated Rituximab containing Fucα1,6GlcNAc (**9**); RTX-M5-F: Rituximab containing core-fucosylated mannose-5 glycan (**10**)

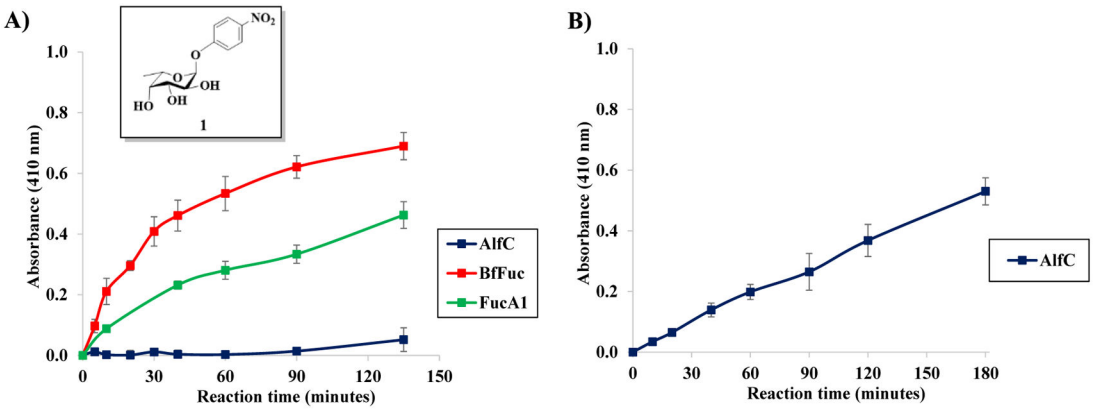


Figure 2: Hydrolysis of fucosylated saccharide by α-fucosidases.

Time course profiles of hydrolysis of (A) 5 mM *p*-nitrophenyl L-fucoside (**1**) by 0.5 μg/ml enzyme (n = 3). (B) 5 mM *p*-nitrophenyl L-fucoside (**1**) by 5 μg/ml AlfC (n = 3).

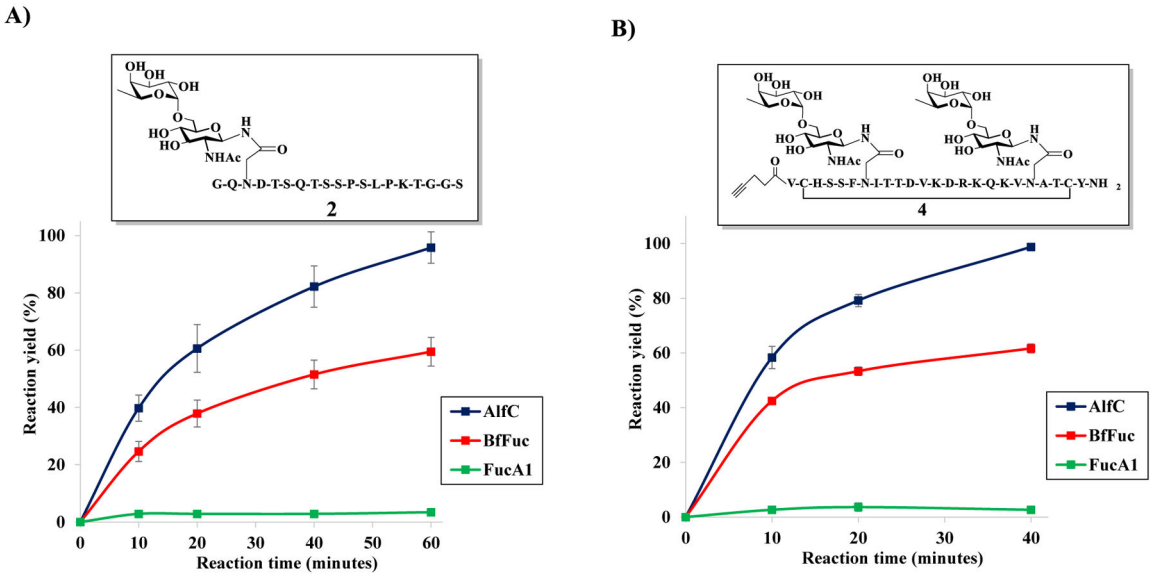


Figure 3: Hydrolysis of Fucα1,6GlcNAc-peptides by α-fucosidases.

Time course profiles of hydrolysis of (A) CD52-GN-F (**2**) at 0.01 mg/ml enzyme concentration (n = 3) and (B) V1V2-GN2-F2 (**4**) at 0.01 mg/ml enzyme concentration (n = 3).

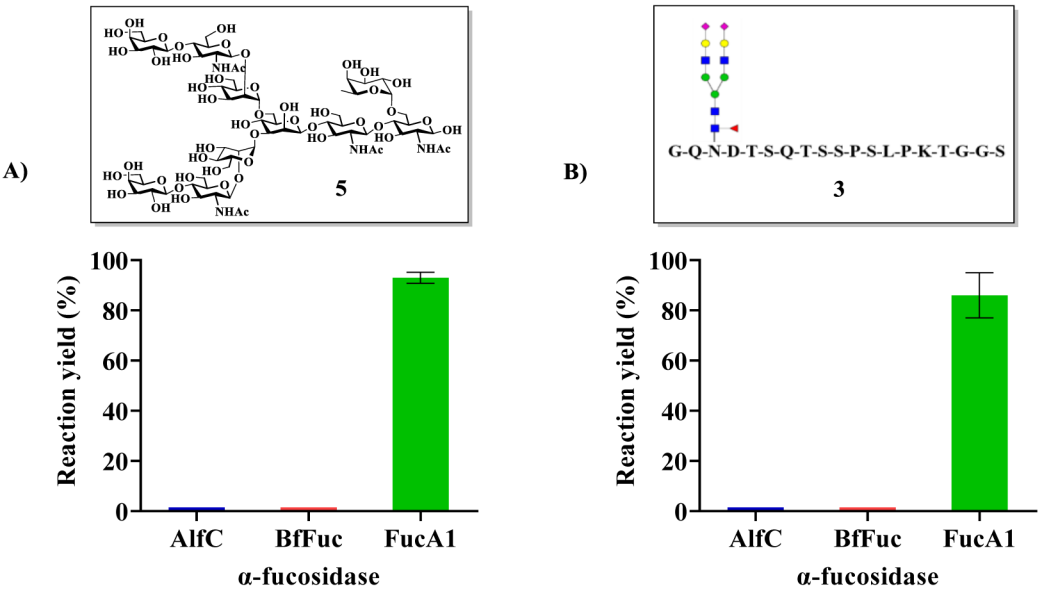


Figure 4: Hydrolysis of full-length fucosylated complex-type *N*-glycan and intact glycopeptide by α-fucosidases.

(A) Hydrolysis of complex-type free *N*-glycan **(5)** by 1 mg/ml enzyme in 7 h (n = 3) (B) Hydrolysis of intact glycopeptide CD52SS-SCT-F **(3)** by 3 mg/ml enzyme in 21 h (n = 2).

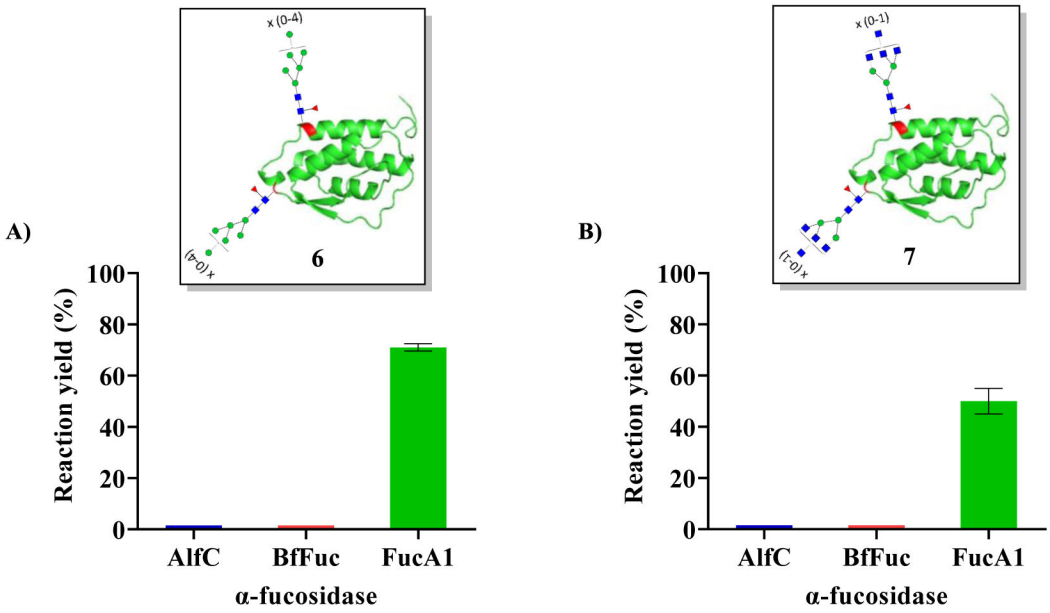


Figure 5: Hydrolysis of fucosylated intact glycoproteins by α-fucosidases.

(A) Hydrolysis of GM-CSF-HM-F (**6**) by 1.5 mg/ml enzyme in 21 h (n = 3) (B) Hydrolysis of GM-CSF-WT (**7**) by 0.6 mg/ml enzyme in 7 days (n = 2)

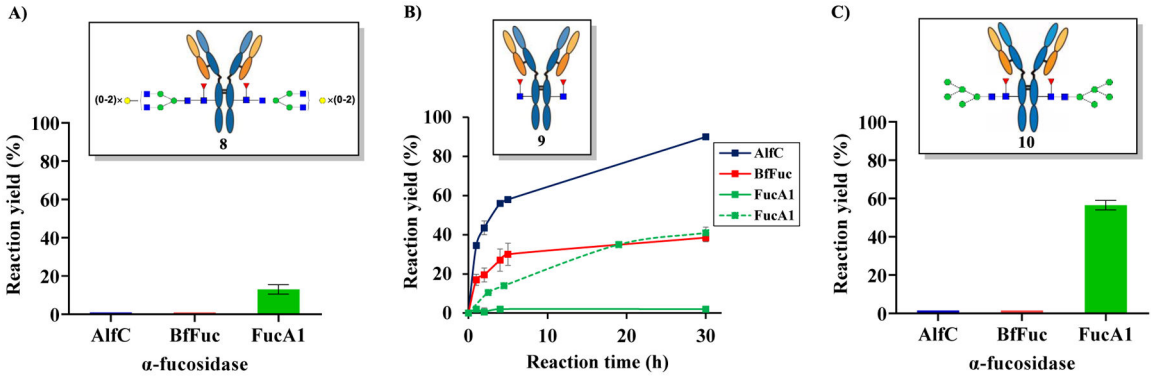


Figure 6: Hydrolysis of fucosylated intact antibody by α-fucosidases.

(A) Hydrolysis of Rituximab (**8**) by 2.8 mg/ml enzyme in 7 days (n = 4). (B) Time-course profile of hydrolysis of RTX-GN-F (**9**) by 0.15 mg/ml enzyme (n = 2). Green dotted line shows activity of FucA1 at 1 mg/ml enzyme concentration (n = 2). (C) Hydrolysis of RTX-M5-F (**10**) by 1.6 mg/ml enzyme in 7 days (n = 3).

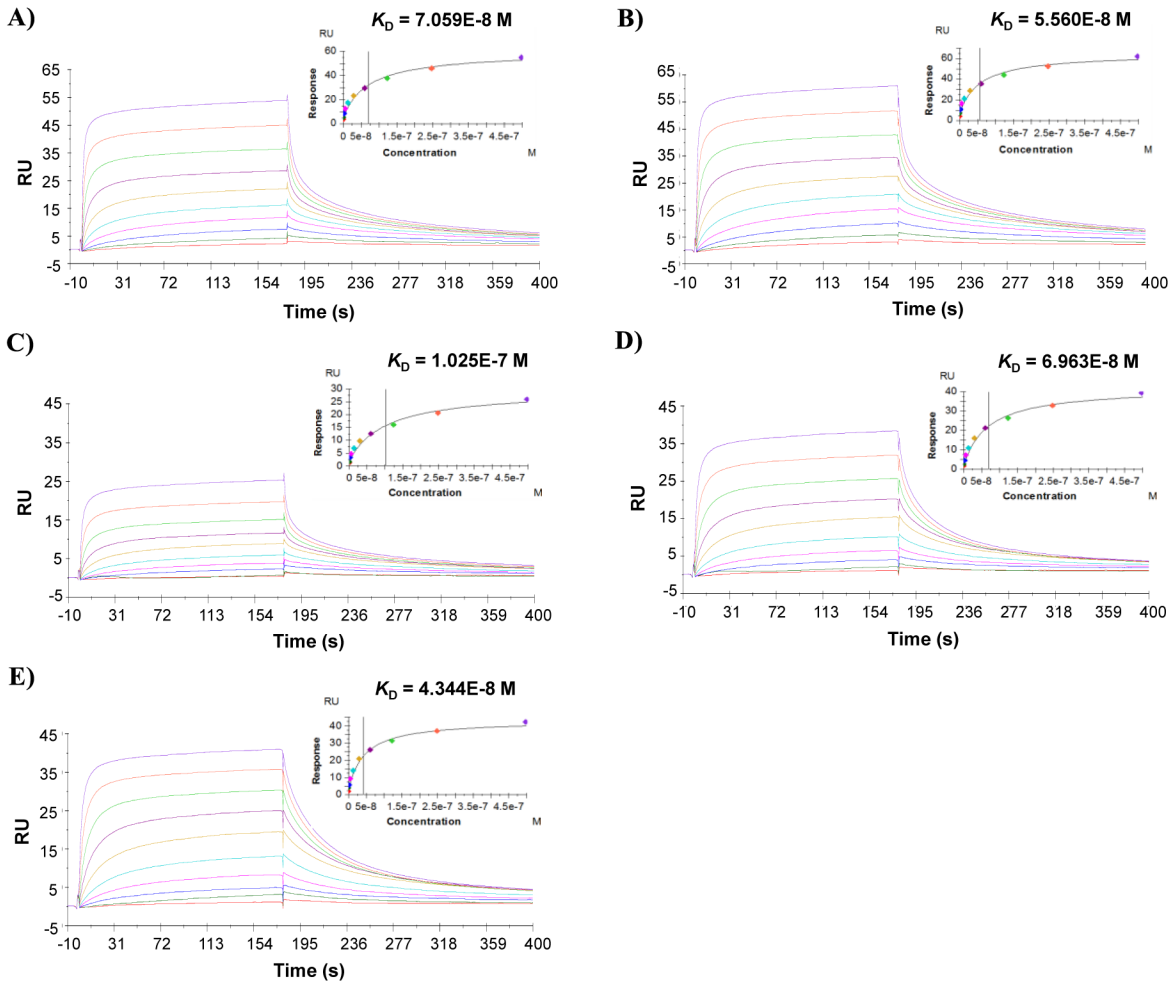
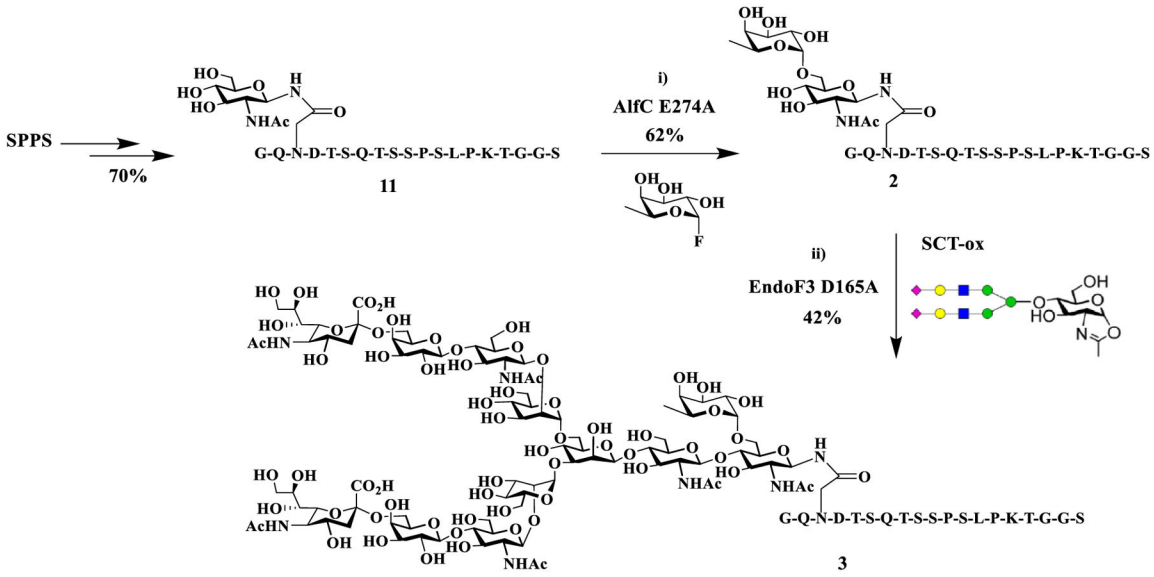


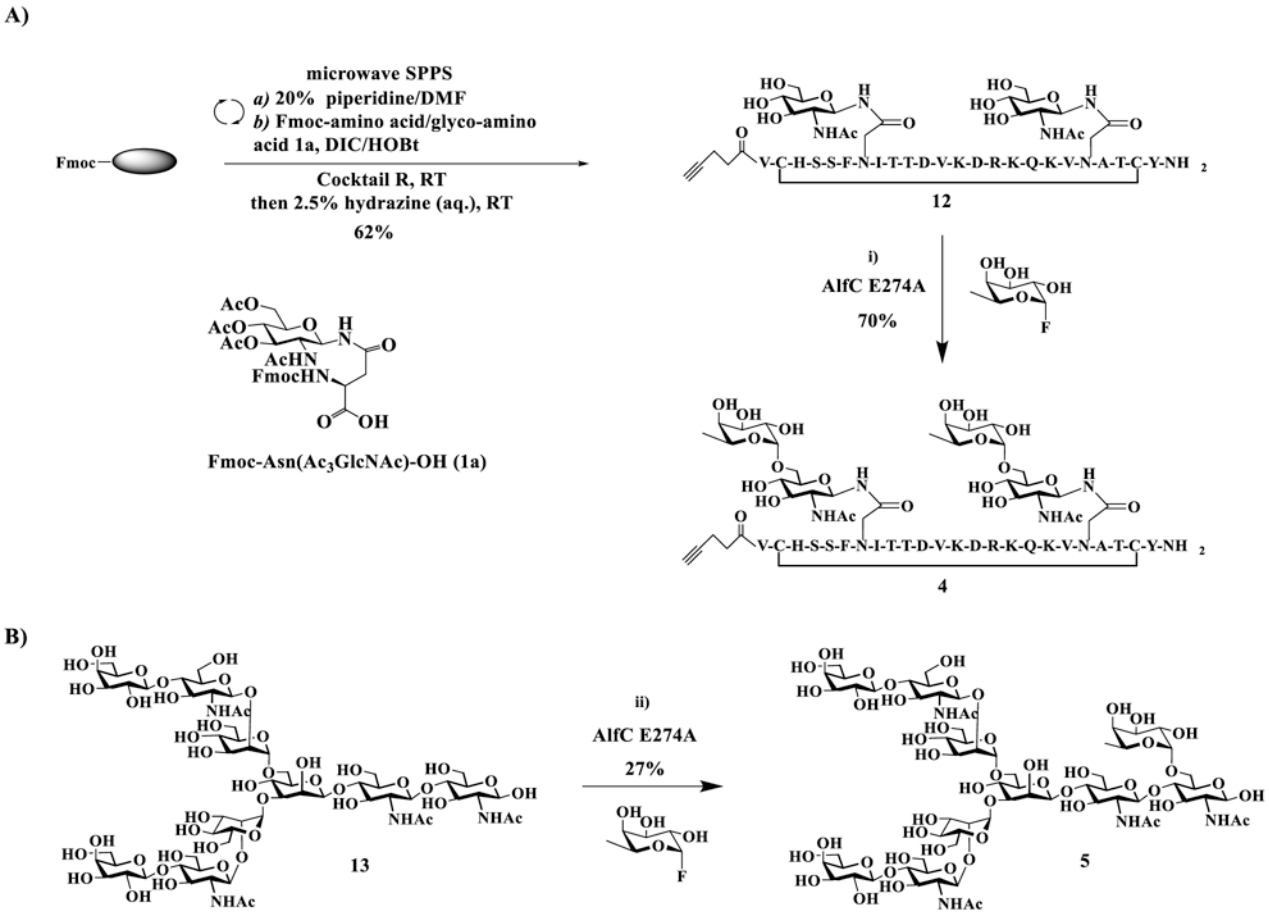
Figure 7: SPR sensorgrams showing binding profiles of antibody glycoforms to FcγRIIIA receptor.

(A) Intact Rituximab (B) Rituximab defucosylated by FucA1 (10% yield) (C) RTX-M5-F (D) RTX-M5-F defucosylated by FucA1 (55% yield) (E) RTX-M5 standard



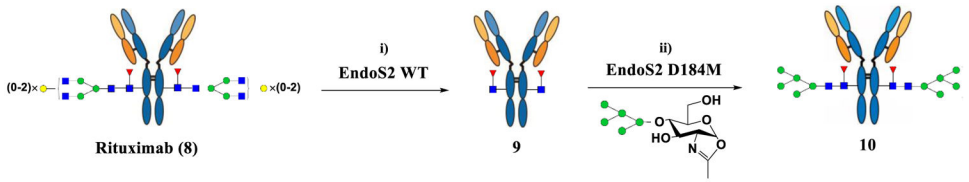
Scheme 1: Chemoenzymatic synthesis of core fucosylated glycopeptides

Reagents and conditions: (i) AlfC E274A (0.0014 mol equiv of **11**), α-fucosyl fluoride (6.36 mol equiv of **11**), PBS (pH-7.4), 37°C, 2 h, 62% (ii) Endo F3 D165A (0.0009 mol equiv of **2**), SCT-ox (2 mol equiv of **2**), PBS (pH-7.4), 30°C, 0.5 h, 42%



Scheme 2: Chemoenzymatic synthesis of core fucosylated glycopeptide 4 and *N*-glycan 5

Reagents and conditions: (i) AlfC E274A (0.008 mol equiv of **12**), α-fucosyl fluoride (10 mol equiv of **12**), PBS (pH-7.4), 37°C, 70% (ii) AlfC E274A (2 mg/ml), α-fucosyl fluoride (10 mol equiv of **13**), PBS (pH-7.4), 37°C, 3 h, 27%



Scheme 3: Chemoenzymatic synthesis of defined core-fucosylated glycoforms of antibody (9, 10)

Reagents and conditions: (i) Commercial Rituximab (4 mg/ml), immobilized EndoS2 WT (0.1 mg/ml), PBS (pH-7.4), RT, 3 h; (iii) **9** (10 mg/ml), EndoS2 D184M (0.2 mg/ml), Man-5 oxazoline (80 mol equiv of **9**), PBS (pH-7.4), 30°C