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Introduction of a glycosylation site in the constant region decreases the aggregation of adalimumab Fab

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ABSTRACT

The production of therapeutic monoclonal antibodies is costly; therefore, antigen-binding fragments (Fabs) can be used instead. However, their tendency toward aggregation can reduce the half-life in the plasma and the therapeutic effectiveness. To examine the effect of glycosylation on the properties of the Fab of a therapeutic antibody, an N-glycosylation site was introduced at position 178 of the H-chain constant region of adalimumab Fab through site-directed mutagenesis of L178 N (H:L178 N Fab), and then H:L178 N Fab was expressed in *Pichia pastoris*. SDS-PAGE analysis with treatment of N-glycosidase F or periodic acid–Schiff reagent showed that H:L178 N Fab contained a relatively low glycan level. Moreover, the H:L178 N mutation did not decrease the binding activity and thermal stability of Fab, and H:L178 N Fab was more resistant to protease digestion than wild-type Fab. The aggregation of Fab induced by pH-shift stress was measured by monitoring the optical density at 350 nm. Although the wild-type Fab showed a large increase in optical density with an increase of protein concentration, no such increase of turbidity during aggregation was found in H:L178 N Fab. These results demonstrated that glycosylation at position 178 of the H-chain constant region of adalimumab Fab can prevent protein aggregation, and therefore serve as a potentially effective platform for drug development.

1. Introduction

Monoclonal antibodies (mAbs) have achieved important therapeutic applications in the treatment of various human diseases. However, the application of therapeutic mAbs is limited owing to the high cost of producing the quantities required to realize the effective therapeutic doses. As an alternative, antigen-binding fragments (Fab) can be rapidly produced at a large scale in a microbial system, which show useful applications in the clinical diagnosis and therapy of various diseases [1,2]. For example, PEGylated Fab' fragments have been demonstrated to be practical for the treatment of Crohn's disease and rheumatoid arthritis, such as centolizumab pegol (Cimzia®) [3].

However, protein aggregation is a challenge associated with the use of such therapeutic protein fragments, which can potentially occur at various stages of the manufacturing processes such as during purification, transportation, and storage [4–8]. Administration of a biological drug with a tendency for protein aggregation could cause anaphylactic shock and induce anti-idiotypic antibody production [9–12]. In particular, since therapeutic mAbs are typically administered at high doses, the probability of aggregate formation is increased [13,14].

Immunoglobulin G (IgG) has two conserved glycosylation sites located in the CH_2 domains of the Fc region. N-glycan in the Fc domains covers the hydrophobic regions, which are more aggregation-prone, to consequently avoid protein aggregation [15,16]. Development of rational glycoengineering strategies can increase the solubility of a protein as well as reduce the aggregation propensity. Aggregation-prone residues on the surface of the Fab domain of a model IgG_1, the therapeutic antibody bevacizumab (Avastin®), were previously identified using the spatial aggregation propensity (SAP) tool [17]. In particular, L180 in the CH_1 domain of bevacizumab showed a high SAP value. Thus, introduction of a glycosylation mutation of L118 N in the CH_1 domain of bevacizumab could potentially mask the aggregation-prone residue of L180 due to the presence of a carbohydrate motif, since L118 was found to be located near L180 in the tertiary structure. Indeed, the L118 N glycosylated mutant showed reduced aggregation of bevacizumab

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compared to the wild type [17]. Although rational glycoengineering can be effective in reducing aggregation of an antibody, this strategy has not yet been investigated for a Fab molecule.

Recently, an expression system of a Fab fragment of IgG_1 developed as the therapeutic antibody adalimumab (Humira®) was constructed in *Pichia pastoris* [18]. In the present study, we focused on the L178 residue (corresponding to L180 for bevacizumab) in the H-chain constant region of adalimumab Fab and examined whether site-directed mutagenesis, in which Leu 178 is mutated to Asn, could result in addition of N-glycan at position 178 in the N-glycosylation sequence (Asn-X-Ser/Thr). In addition, we examined the effect of this glycosylation on the aggregation of adalimumab Fab.

2. Materials and methods

2.1. Construction of expression plasmids

The adalimumab Fab expression vector, which contains sequences encoding both the Fab-H chain and -L chain, was cloned according to a previously reported method [18]. Site-directed mutagenesis of the adalimumab gene was performed by polymerase chain reaction, as described previously [19]. The introduced mutations were confirmed by DNA sequence analysis. Each gene was fused directly in-frame with the α-factor secretion signal of the expression vector pPICZαA (Invitrogen, USA) and pPICZαA−ΔPmeI, and a nucleotide sequence encoding the Kex2 cleavage site was placed upstream of the Fab gene. An extra Ser residue was added to the N-terminus of each Fab-H chain and -L chain to achieve more efficient signal sequence processing [20].

2.2. Expression and purification of adalimumab Fab

The adalimumab Fab was expressed in *Pichia pastoris* X-33 cells and purified as described previously [18]. In brief, the culture supernatant was saturated with 60% ammonium sulfate, and the precipitated protein was removed by centrifugation at 10,000×*g* for 15 min. The precipitated protein was resuspended in 50 mM acetate buffer, pH 4.0. After dialysis against 50 mM acetate buffer (pH 4.0), the dialysate was applied to a cation-exchange column (2.2 × 12.0 cm; SP-Toyopearl 650 M, Tosoh, Japan) equilibrated with 50 mM acetate buffer at pH 4.0. The column was eluted with 50 mM acetate buffer (pH 4.0) containing 1.0 M NaCl, the elution fractions were dialyzed against 50 mM acetate buffer (pH 5.0), and the dialysate was applied to a Blue Sepharose 6 affinity column (1.5 × 6.0 cm; GE Healthcare, USA) equilibrated with 50 mM acetate buffer, pH 5.0. The column was then eluted with equilibration buffer containing 0.4 M NaCl, and the Fab fraction was finally isolated by cation-exchange chromatography on a resource S column (column volume: 1 mL) connected to an AKTA purifier system (GE Healthcare, USA) previously equilibrated with 50 mM sodium acetate buffer, pH 5.0. The proteins were eluted by a linear NaCl gradient (0−0.1 M) with flow rate of 1 mL/min for 40 min.

2.3. Periodic acid-Schiff (PAS) staining for glycoconjugates

PAS reagent staining was performed according to a previously reported method [21]. In brief, SDS−PAGE gel was immersed in a 12.5% trichloroacetic acid solution for 15 min, washed with distilled water, and then transferred to 1% periodic acid solution containing 3% acetate acid for 1 h. The gel was washed thoroughly with distilled water to remove the periodic acid, followed by the addition of Schiff reagent, and incubated in the dark for 1 h. Finally, the gel was removed by immersion in 0.5% sodium metabisulfite solution, followed by distilled water.

2.4. Enzyme-linked immunosorbent assay (ELISA) of adalimumab Fab

To measure the binding of adalimumab Fab, 96-well ELISA plates were coated with human tumor necrosis factor-alpha (TNFα; 0.2 μM) in 0.05 M sodium carbonate buffer, pH 9.6, overnight at 4 °C. The plates were washed three times with TBS-Tween wash solution (0.02% Tween 20, 0.15 M NaCl, 0.02% NaN_3) and blocked with blocking buffer (TBS-Tween containing 2% non-fat dry milk) at room temperature for 1 h, and then washed again three times with the wash solution. The plates were incubated with dilutions of purified adalimumab Fab in blocking buffer at room temperature for 1 h, washed three times with wash solution, and incubated with 1:2000-diluted horseradish peroxidase-conjugated sheep-anti-human IgG (Fab) (PAR-2424-00, Funakoshi, Japan) at room temperature for 1 h. The plate was washed four times with wash solution and incubated with ELISA POD Substrate TMB Solution (Nacalai Tesque, Japan) at room temperature. The reaction was stopped by adding an equal volume of 1 M H_2SO_4 and the absorbance was measured at 450 nm using a Photometer Multiskan FC microplate (Thermo Scientific, USA).

2.5. Differential scanning calorimetry (DSC) measurements

DSC measurements and data analyses were carried out using a Nano DSC system (TA-instrument, USA). Thermograms of the wild-type or H:L178 N Fab were obtained between 60°C and 90 °C at a scan rate of 1.0 °C/min. The sample solutions were prepared by dissolution in 50 mM KH_2PO_4 buffer (pH 6.5) and the protein concentration was 0.2 mg/mL. The calorimetric data were analyzed using Nanoanalyzer software (TA-instrument, USA) to obtain the melting temperature (T_m).

2.6. Proteolytic stability assay

The wild-type or H:L178 N Fab (0.1 mg/mL) was incubated in 40 μL of 20 mM Tris-HCl buffer (pH 7.8) at 50 °C with 0.4 μg of proteinase K for 12 h or 24 h. At each stage of the reaction, 10% aqueous acetic acid was added to the protein solutions in order to stop protease reactions. The digested protein solution was loaded onto a 12% SDS-PAGE gel and stained with Simply Blue (Invitrogen, USA). Each protein band was quantified using the freeware ImageJ.

2.7. Protein aggregation experiments

The wild-type or H:L178N Fab (2 mg/mL each) were incubated in 10 mM glycine-HCl buffer pH 2.0 at 4 °C overnight. The acid-treated Fab was diluted to a protein concentration of 0.01, 0.02, and 0.04 mg/mL in 50 mM NaH_2PO_4 buffer (pH 7.4) with stirring, and pre-incubated at 40 °C for the induction of aggregation. The turbidity during aggregation was measured with a fluorescence spectrophotometer F-2500 (Hitachi, Japan) at an excitation wavelength of 350 nm and emission wavelength of 350 nm. After the induction of aggregation at 40 °C, the extent of monomer in the protein solution was measured by size-exclusion high-performance liquid chromatography (SEC-HPLC) with a TSK G3000SW column (Tosoh, Japan), and the peak area of the monomer was evaluated against that of untreated Fab at the same protein concentration. The proteins were eluted with 50 mM KH_2PO_4 buffer (pH 6.5) containing 0.2 M NaCl at a flow rate of 0.5 mL/min.

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