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Reversed-phase liquid chromatography/mass spectrometry analysis of reduced monoclonal antibodies in pharmaceutics

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Abstract

A reversed-phase LC/MS method was developed for reduced antibodies that provides efficient separation of light chain and two variants of heavy chain containing N-terminal glutamine and pyroglutamic acid. The best separation was achieved on Zorbax CN and Varian Pursuit DiPhenyl columns eluted with increasing percentage of *n*-propanol and acetonitrile in 0.1% trifluoroacetic acid. Although glutamine was genetically coded for the N-terminal residue of heavy chain of a monoclonal antibody used in this study, we found that most of it (70%) was converted to pyroglutamate during production. The conversion process continued in vitro and was monitored by the method. Deconvoluted electrospray ionization mass spectrum of the heavy chain revealed the glycosylation profile of a single N-linked sugar including a-, mono-, and di-galactosylated biantennary glycans and a 5-mannose sugar form.
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1. Introduction

Human immunoglobulin gamma (IgG) antibodies are glycoproteins containing two identical copies of light chains (LC) and heavy chains (HC) connected by external disulfide bonds (Fig. 1). The light chains can be either kappa or lambda type and the heavy chain is gamma. The first complete sequence of human IgG antibody was uncovered by Edelman et al. [1]. Human monoclonal antibodies possess several conserved regions [2], which are identical to the Edelman sequence. N-termini of the chains are relatively conserved. N-terminal residue of immunoglobulin gamma heavy chain is typically either glutamine or glutamic acid or aspartic acid [2,3]. N-terminal residues for kappa light chain are typically either glutamic acid or aspartic acid, while for lambda light chain they are serine, proline or glutamine [2,3]. The most typical post-translational modification of monoclonal antibodies is conversion of the N-terminal glutamine (Q) to pyroglutamate (pE).

The role of N-terminal cyclization in protein biosynthesis and in biological function is not clear [4]. It is also not clear which enzymes, if any, may be involved in this cyclization process. As suggested by previous studies of several proteins including antibodies [5,6], N-terminal pyroglutamate is most likely formed either late in protein translation by cyclization of N-terminal glutamine or as a post-translational event just prior to cellular secretion of protein from the cell. In a recent study [7], a set of 270 secreted recombinant human proteins was analyzed by Edman analysis to examine the frequency of amino acid at the N-terminus. Q was found at the first position in 10.7% and was identified as the most preferred residue at this position. Because N-terminal Q is often cyclized to pE, the authors suggested that this modification may serve to protect the secreted proteins from degradation by extracellular aminopeptidase [7]. It was reported, that out of more than 200 human and murine immunoglobulin heavy and light chains subjected to amino terminal sequence analysis [2], Q residues are largely converted to pE residues with a few exceptions [8]. There have been also reports about glutamic to pyroglutamic acid conversion on N-terminus of immunoglobulin light chain [6] and in several N-terminal glutamic acid-containing peptides [9,10]. This conversion seems to be less often and, if happens, it takes place with slower rate than with Q to pE. Due to the unstable nature of N-terminal glutamine, it can be converted to pyroglutamic acid during fermentation process and storage in pharmaceutics

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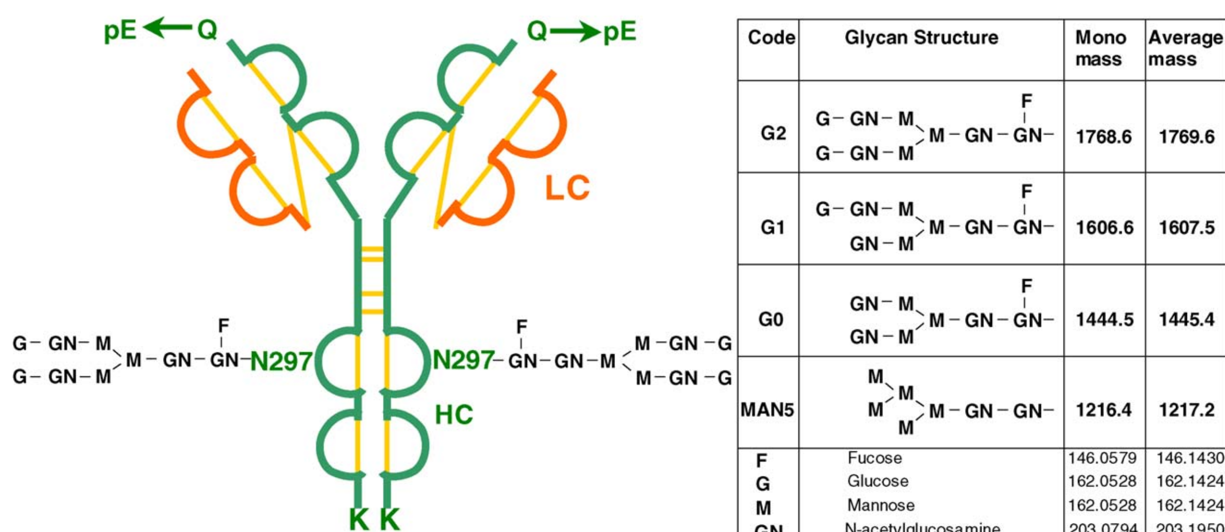

Fig. 1. Structure of recombinant monoclonal IgG antibody used in this study. Glycosylation profile includes two biantennary glycans with variable number of galactose residues (G). Most common isoforms are G0, G1 and G2. Conversion of N-terminal glutamine (Q) to pyroglutamate (pE) of heavy chain (HC) is also shown. Heavy chain C-terminal lysine (K) is often missing in antibodies due to the enzymatic activity. Disulfide bonds are shown in yellow, heavy chains in green and light chains (LC) in pink.

with exposure to elevated temperatures and near neutral pH [9,11–13]. N-terminal glutamine may convert to pyroglutamic acid during peptide mapping, which typically is conducted at pH 7.5 and 37 °C, the conditions which facilitate formation of pE on the N-termini of unfolded peptides [14,15]. Once formed, N-terminal pE may impede to protein sequence determination, since reactions, which require a free amino terminus, are not possible. However, several enzymes were described [13], which were utilized to remove pE in many blocked proteins [4,16].

The formation of pE from Q eliminates N-terminal amine and generates an acidic variant, because the remaining amide of the ring is neutral [4]. This modification is associated with the release of ammonia and mass decrease by 17 u (Fig. 2), similar to deamidation reaction. Previously, cation-exchange (CEX) chromatography [17] and isoelectric focusing (IEF) capillary electrophoresis (CE) [18,19] have been used to separate the antibody fragments (after papain digestion) containing none, one or two pyroglutamate residues, QQ, QpE, and pEpE. The problem is that these two separation techniques do not provide enough specificity to distinguish between the isoforms due to pE formation and other acidic charged variants such as deamidation, C-terminal lysine variants of the heavy chain, addition of sialic acid and partial refolding leading to solvent exposure of new negatively charged residues. Although N-terminal cyclization may not influence efficacy and immunogenicity, it is important to monitor and differentiate it from other modifications listed above. Unfortunately, CEX and IEF CE are not easily hyphenated with mass spectrometry for identification of these variants, but reversed-phase (RP) chromatography is readily attachable. On-line mass spectrometry has the potential to distinguish pyroglutamate (−17 u) from other “charged modifications” such as deamidation (+1 u), C-terminal lysine (+128 u), extra sialic acid (+291 u) and partial unfolding (0 u).

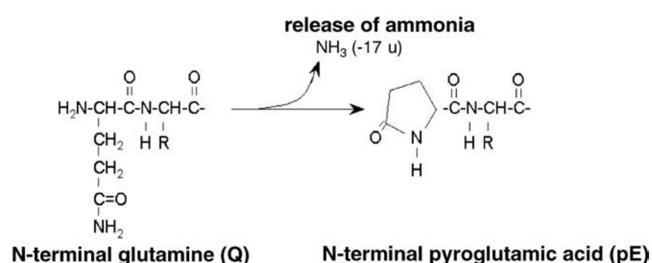

Fig. 2. Mechanism of formation of pyroglutamate (pE) from N-terminal glutamine (Q).

Perfusion chromatography using Poros column had been most often used for separation and purification of intact antibodies [20–22] and their reduced and alkylated chains [15]. Although an effective preparative technique, perfusion chromatography suffers from the following two limitations, i.e. poor separation of variants and unfriendliness to mass spectrometry, because of the use of the high flow rates required for separation, for example, 0.5–3 ml/min in ref. [21]. We recently introduced a method for RPLC/MS analysis of intact antibodies using column temperatures greater then 70 °C and stationary phases with long alkyl chains (C8 and C18) [23]. The method was further optimized by employing propyl alcohol and other solvents with high eluotropic strength [24]. The RPLC/MS method for the intact antibodies was used in this study as a starting point for method development for reduced antibodies.

This study was designed to develop an RPLC/MS method for use with reduced antibodies to clearly separate light and heavy chains, Q and pE variants of heavy chain, and other possible modifications of antibody chains. *N*-Glycosylation in a conserved site of heavy chain at asparagine 297 is the other typical source of polymorphism for monoclonal IgG antibodies [25]. Electrospray ionization mass spectrometry (ESI-MS) has been used previously to establish the glycosylation profile of an antibody after reduction/alkylation or limited proteolysis using direct infusion of desalted sample [26]. In this article, we

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