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# Characterization of asparagine 330 deamidation in an Fc-fragment of IgG1 using cation exchange chromatography and peptide mapping

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ABSTRACT

Deamidation is one of the most common degradation pathways for proteins and frequently occurs at "hot spots" with Asn-Gly, Asn-Ser or Asn-Thr sequences. Occasionally, deamidation may occur at other motifs if the local protein structure can participate or assist in the formation of the succinimide intermediate. Here we report the use of a chymotryptic peptide mapping method to identify and characterize a deamidated form of an IgG1 which was observed as an acidic peak in the cation exchange chromatography (CEX). The antibody was formulated in sodium acetate buffer, pH 5.3 and this deamidated form was observed mainly under thermal stress conditions. It was found that the IgG1 molecule with deamidation in the Fc region at asparagine residue 330 (in a Val-Ser-Asn-Lys motif) is the predominant form in this CEX peak, and was missed by tryptic mapping because the peptides are hydrophilic and elute near the void volume. In addition, a domain-based CEX method using papain digestion was developed to monitor the Asn 330 deamidation. These methods revealed that the Fc deamidation occurs mainly at Asn 330 in the VSNK motif at pH 5.3, whereas at pH 7.5, deamidation occurs predominantly at Asn 389 and Asn 394 in the NGQPENNYK motif.



## 1. Introduction

Deamidation is a common degradation pathway for therapeutic proteins and may cause changes in protein structure, function and immunogenicity [1,2]. Deamidation can occur during manufacturing, transit, normal sample storage, as well as under various stress conditions [3–5]. Typically, deamidation has been associated with "hot spots" such as Asn-Gly (NG), Asn-Ser (NS), Asn-Thr (NT) and Asn-Asn (NN) sites within the protein sequence. These motifs are theorized to have faster deamidation rates due to the flexibility in the residue adjacent to the C-terminal of the asparagine [6]. Protein structure is also thought to play a role, since rates of deamidation are known to be higher in regions with greater solvent accessibility and structural flexibility [7–9]. For IgG1 monoclonal antibodies (MAbs), deamidation has been observed in both Fab and Fc regions. Fab deamidation could potentially reduce biological activity and thus has been at the focus of most analytical scrutiny [10–12]. However, the amino acid sequence in the Fc region is highly conserved for MAbs and certain Fc deamidation sites have also been well-documented. Among these are two hot spots in the NGQPENNYK sequence as well as other NG-containing sites. For example, Chelius reported three deamidation sites within the Fc region – NGQPENNYK and DWLNGK [13]. In general, deamidation can occur after therapeutic antibodies have been injected into serum and these modifications have been observed in endogenous IgG as well [14,15].

The major methodology for identification of deamidation is reversed phase chromatography (RP-HPLC) tryptic peptide mapping coupled with mass spectrometer detection. Tryptic peptide mapping techniques are powerful for deamidation analysis but have two major limitations. First, the protein samples are typically incubated at above neutral pH buffers at 37 °C for several hours or overnight, which could generate deamidation artifacts during digestion. Recently, several groups have reported improved peptide mapping procedures to reduce levels of deamidation caused by digestion conditions or to differentiate sample preparation-related deamidation artifacts [16–18]. Second, tryptic mapping alone may miss the potential peptide degradants because a few small, hydrophilic peptides elute near the void volume [19]. Missing these hydrophilic peptides is considered a low risk because they are not associated with so-called "hot spots" for deamidation. One exception is the Val-Ser-Asn-Lys (VSNK) peptide in the Fc region,

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where the asparagine (referred to as N330) in this tetrapeptide could be a critical residue due to its proximity to effector function binding sites. To our knowledge, N330 deamidation has been reported only once and has not been well studied [20]. For N330 deamidation, the tryptic mapping method is not effective since the VSNK and VSDK peptides coelute and identity is based on isotopic shifts in the stressed samples studied by Liu et al. [20]. From that study, it is unclear whether N330 degrades into either aspartic acid or isoaspartic acid. An orthogonal peptide mapping approach is needed to fully characterize N330 deamidation.

Cation exchange chromatography (CEX) is an effective separation technique for analyzing charge variants. This, in turn, also makes CEX a potentially powerful characterization tool for deamidation [21]. Deamidation introduces a negative charge to the protein and drastically changes interactions with the stationary phase, causing retention times to shift. In addition, conformational changes from deamidation could also affect retention of the species [22]. Prior work has been done using CEX to study Fab deamidation. The resolution and selectivity of CEX separation for deamidation species is impressive considering the subtlety of the modification [10]. Compared with LC–MS peptide mapping approach, CEX method is relatively easy and may avoid the artifacts in peptide mapping. One limitation of the CEX method for studying deamidation in full length MAbs is that MAbs are multi-domain tetramers containing two heavy chains and two light chains. Thus the separation can be complicated with different combinations of Fab and Fc deamidation. To reduce such complexity, a CEX method can be used in conjunction with a method to separate Fab and Fc domains through papain or limited Lys-C digestion [23]. Such an approach, a domain based CEX would enable the analysis and quantitation of Fab and Fc deamidation separately. Alternatively, capillary electrophoresis has been used for characterization of monoclonal antibodies and for peptide mapping proteins [24–26].

The aim of this paper is to characterize a previously unknown ion exchange chromatography (IEC) degradant peak for an IgG1 (MAb A thereafter). It was determined that the observed peak is related to N330 deamidation. Furthermore, a domain based CEX method to characterize this Fc degradation was developed. Our results indicate that N330 deamidation is a major and common Fc degradation pathway for thermally-stressed IgG1s stored in a pH 5.3 sodium acetate buffer.

## 2. Materials and methods

### 2.1. Materials

Acetonitrile (ACN) and propan-2-ol were purchased from Burdick & Jackson (Muskegon, MI, USA). 2-(N-morpholino)ethanesulfonic acid (MES), N-2-aminoethanesulfonic acid (ACES), Tris, sodium acetate, trehalose and antipain were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, USA). Related peptides were synthesized in Genentech. MAb A and other MAbs were produced at Genentech using a CHO cell culture process and purified through the typical downstream multistep chromatography process to a high degree of purity intended for therapeutic applications. MAbs with 5–30 mg/mL concentration (formulated at 20 mM sodium acetate pH 5.3 or 20 mM Tris, pH 7.5) were thermally stressed at 40 °C for 4 weeks.

### 2.2. Low pH reduction and chymotrypsin and trypsin digest conditions

Samples were denatured and reduced with 50 mM tris(2-carboxyethyl)phosphine (TCEP) in a denaturing buffer (8 M guanidine hydrochloride, 300 mM sodium acetate, pH 5.0) at 60 °C for 10 min. The reduced samples were then buffer exchanged into a digest buffer (20 mM MOPS, 1 mM TCEP, pH 7.0) using NAP-5 columns GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and digested with chymotrypsin (Roche Applied Science, Mannheim, Germany) at a 1:100 (m/m) chymotrypsin:IgG ratio or digested with trypsin (Promega, Madison, WI, USA) at1:40 (m/m) trypsin: IgG ratio at 37 °C for 3 h.

### 2.3. Low pH reduction, alkylation and Asp-N digest conditions

Samples were incubated at 37 °C in a denaturing and reducing buffer (6 M guanidine hydrochloride, 250 mM Tris and 10 mM DTT, pH 7.5). After one hour, iodoacetic acid was added and the mixture was further incubated at 37 °C for 15 min. The Alkylation was then quenched with DTT. Samples were finally buffer exchanged into digest buffer (25 mM Tris, pH 7.5) using NAP-5 columns and digested with Asp-N (Roche Applied Science, Mannheim, Germany) at a 1:100 (m/m) Asp-N:IgG ratio at 37 °C for 3 h.

### 2.4. LC–MS/MS peptide mapping analysis

Digested peptides were separated using a Phenomenex Jupiter C18 column (5-μm particle size, 300-Å pore size, 1.0 mm × 250 mm). Elution was achieved using a two-segment linear gradient from 100% buffer A (0.1% TFA in water, % v/v) at 0–3 min to 10% buffer B (0.09% TFA in 90% acetonitrile, % v/v) at 23 min and from 10% B to 40% buffer B at 160 min. The flow rate and column temperature were maintained at 70 μL/min and 55 °C, respectively. Online mass spectrometry data were acquired using an LTQ Orbitrap system (Thermo Fisher Scientific, San Jose, CA).

### 2.5. Cation exchange chromatography (CEX) and fraction collection for MAb A

CEX separation and fraction collection were carried out on an Agilent 1200 HPLC system with UV monitoring at 280 nm. Dionex ProPac WCX-10 columns (9.0 mm × 250 mm with temperature controlled at 40 °C) were used for semi-preparative collection. Mobile phase A consisted of 20 mM ACES, pH 7.2 and mobile phase B consisted of 20 mM ACES pH 7.2 with 0.25 M NaCl. Protein was eluted with a linear gradient from 15% to 25% B in 30 min at a flow rate of 2.5 mL/min. The samples were fraction collected, concentrated, and buffer exchanged into 20 mM sodium acetate at pH 5.3 and stored at −20 °C.

### 2.6. Papain digestion and domain CEX

MAb A samples were first diluted to 5 mg/mL in acetate buffer. Then 1.5 mg of antibody (300 μL), 150 μL cysteine (10 mM), 150 μL of papain (0.1 mg/mL concentration), 150 μL of 1.0 M Tris, 40 mM EDTA at pH 7.4 and 750 μL D.I. water were mixed for a final reaction volume of 1.5 mL. The reaction mixture was subsequently vortexed and incubated at 37 °C for 2 h and the reaction was quenched with antipain at a 1:40 ratio of antipain to papain. As the final step, the digested samples were buffer exchanged into 20 mM MES pH 6.0 prior to running IEC.

The domain CEX method was carried out on an Agilent Technologies, Inc. 1100 series HPLC with 20 mM MES (pH 6.0) as the mobile phase A and 250 mM NaCl in mobile phase A as the mobile phase B. 100 μg of the papain-digested sample was loaded onto a Dionex ProPac WCX-10 (4 mm × 250 mm) column controlled at 40 °C. The flow rate was 0.8 mL/min and gradient was started from 5% B to 45% B in 40 min after an initial 3-min hold. Dionex ProPac WCX-10 columns were used for semi-preparative collection (9.0 mm × 250 mm with temperature controlled at 40 °C) and