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Analysis of isoaspartate in a recombinant monoclonal antibody and its charge isoforms

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Abstract

Monoclonal antibody (mAb) therapy applications have been growing rapidly in recent years. Like other proteins, therapeutic mAbs can under go various enzymatic and non-enzymatic reactions that can affect their structural integrity and stability. Among the degradation reactions, isoaspartate (isoAsp) formation is one of the major sources of charge heterogeneity of mAbs. This paper reports the detection and quantification of isoAsp in a recombinant mAb and its charge isoforms resolved by cation exchange high performance liquid chromatography. The assay utilizes the enzyme protein isoaspartyl methyltransferase in conjunction with strong cation exchange separation and UV detection (at 260 nm) of *S*-adenosyl-L-homocysteine, which is produced stoichiometrically in the enzymatic reaction. The mAb is found to contain an average 0.2 mol of isoAsp per mol of protein, however, various charge isoforms were found to contain different levels of isoAsp. The most acidic isoforms contain approximately 0.7 mol of isoAsp per mol of protein, and no isoAsp is detected in the most basic isoform. It appears that the majority of isoAsp in the mAb is formed as a result of asparagine deamidation.

Keywords: Monoclonal antibody; Charge heterogeneity; Isoaspartate; Deamidation; SCX-HPLC

1. Introduction

Recombinant monoclonal antibodies (mAbs), similar to other proteins, are subject to a variety of degradation reactions that can occur during manufacturing, formulation and storage. These reactions, enzymatic or non-enzymatic, can affect the size or charge heterogeneity of mAbs, and may or may not modify their antigen binding affinity. Therefore, like other protein pharmaceuticals, therapeutic mAbs also require extensive and stringent characterization of purity, structural integrity and stability [1]. As one of the important

Abbreviations: mAb, monoclonal antibody; isoAsp, iosaspartate; CEX, cation exchange; HPLC, high performance liquid chromatography; SAH, *S*-adenosyl-L-homocysteine; SCX, strong cation exchange; PIMT, protein isoaspartyl methyltransferase; SAM, *S*-adenosyl-L-methionine; Asn, asparagine; isoAsp-DSIP, isoAsp-δ-sleep-inducing peptide; IEF, isoelectric focusing; Asp, aspartate.

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pathways for protein degradation, isoaspartate (isoAsp) formation is a major source of protein microheterogeneity and, subsequently, may have significant impact on protein structure and/or function [2–7]. Previous studies have shown that isoAsp formation resulting from asparagine (Asn) deamidation or aspartate (Asp) isomerization (Fig. 1) is the leading cause of charge heterogeneity of mAbs [8–13]. This paper reports the detection and quantification of isoAsp in a recombinant IgG4 mAb and its charge isoforms resolved by cation exchange-high performance liquid chromatography (CEX-HPLC). The assay is based on methylation of isoAsp by the enzyme protein isoaspartyl methyltransferase (PIMT) using *S*-adenosyl-L-methionine (SAM) as the substrate. *S*-adenosyl-L-homocysteine (SAH), produced stoichiometrically in the enzymatic reaction, is separated from other reaction components by strong cation exchange (SCX) HPLC and quantified by UV detection at 260 nm [14]. Our work suggests that the charge heterogeneity of the mAb is primarily due to Asn deamidation and isoAsp formation. The method described here could be applied to the analysis of other mAb therapeutics.

2. Experimental

2.1. Materials

The mAb analyzed in this study was purified at small scale at Wyeth BioPharma, Genetics Institute Campus in Andover, MA, during development of the manufacturing process. The mAb was prepared at a concentration of 188 μM, in 20 mM sodium succinate, pH 6.0. SAH and SAM were obtained from Sigma (St. Louis, MO). IsoAsp-δ-sleep-inducing peptide (isoAsp-DSIP; WAGGβDASGE) was purchased from Bachem California, Inc. (Torrance, CA). ISOQUANT protein deamidation detection kit containing solution of purified PIMT was purchased from Promega Corporation (Madison, WI).

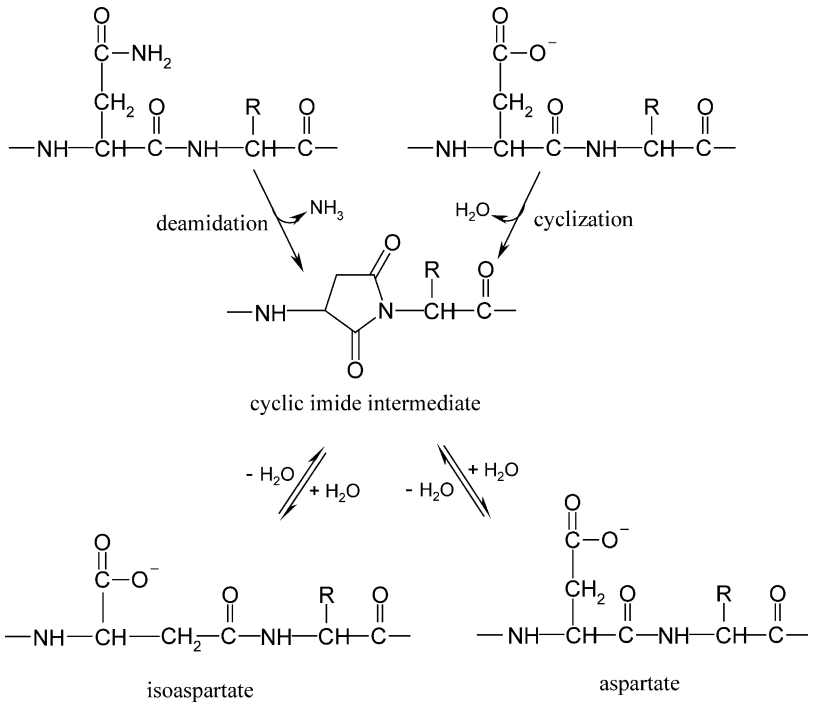

Fig. 1. Scheme of Asn deamidation and Asp isomerization reactions leading to formation of isoAsp.

2.2. HPLC equipment

All HPLC analyses were performed with a Waters Alliance system with a Model 2487 dual wavelength absorbance detector and a refrigerated autosampler with temperature controlled at 4 °C.

2.3. CEX-HPLC of intact mAb

A Dionex ProPac WCX-10 weak cation exchange column (4 × 250 mm, Dionex) was employed for CEX-HPLC fractionating of the intact mAb. A solution of 10 mM HEPES, pH 8.0, was used for mobile phase A, and 10 mM HEPES, 200 mM NaCl, pH 8.0, was used as mobile phase B. The column was equilibrated at 100% mobile phase A at a flow rate of 1.0 ml min^{-1}. After the sample was injected, the column was washed with 100% A for 5 min followed by a gradient of 0–50% mobile phase B over 45 min, 50–100% B over 5 min, and 100–0% B over 0.1 min. The column was then equilibrated for additional 20 min in 100% mobile phase A before the next injection. The detection was at 280 nm.

Separated mAb peak fractions were collected manually and pooled from several 1.5 mg injections. The pooled fractions were then dialyzed to 20 mM sodium succinate, pH 6.0. Protein concentration of the mAb isoforms was determined by measuring absorbance at 280 nm using a molar extinction coefficient of 2.25×10^5 M^{-1} cm^{-1}.

2.4. Isoelectric focusing

The isoelectric focusing (IEF) analysis of the intact mAb and its charge isoforms collected from CEX-HPLC was carried out using a pH 3–10 Novex IEF gel (Invitrogen). Ten μl of protein solution with the concentration of approximately 1 mg ml^{-1} was mixed with 10 μl of the sample buffer (50% (v/v) glycerol, pH 3–10, Invitrogen) and 2 μl of 0.05% (w/v) Bromphenol blue (BioRad). Cathode and anode buffers were from Invitrogen. The IEF gel was focused at 100 V for 1 h, 200 V for 1 h and finally 500 V for 30 min. After electrophoresis, the gel was fixed using the fixing solution containing 0.14 M sulphosalicylic acid and 0.7 M tricholoacetic acid, washed with water, stained with 0.1% (w/v) Serva Violet 17, and destained in 10% (v/v) acetic acid and 15% (v/v) methanol.

2.5. SAH and isoAsp-DSIP standards

SAH stock solutions were made by dissolving SAH solid with purified water. The concentration of SAH stock solutions was determined by measuring absorbance at 260 nm and using a molar extinction coefficient of 15 400 M^{-1} cm^{-1}. The concentration of isoAsp-DSIP solutions used for PIMT enzymatic reaction was determined at 280 nm using a molar extinction coefficient of 5540 M^{-1} cm^{-1}.

2.6. PIMT enzymatic reaction

Samples subjected to PIMT enzymatic reaction included a 24.6 μM isoAsp-DSIP solution and the intact mAb and its charge isoforms. Methylation reactions were carried out by mixing 1 vol of the PIMT solution, 1 vol of SAM, and 1 vol of 5 × reaction buffer provided in the ISOQUANT kit with 2 vol of purified water and 1 vol of test protein (peptide) solution. A blank was prepared using purified water instead of test solution. The mixture was incubated at 30 °C for 40 min. The reaction was stopped by adding 1 vol of stop solution supplied with the ISOQUANT kit. A single lot of PIMT was used throughout the study.

2.7. SCX-HPLC analysis of isoAsp

Two SCX columns were used for analysis of isoAsp: a Partisil 10 SCX column (HiChrom, 85-Å, 10-μm particle size, 4.6 × 250 mm) and a PL-SCX column (Polymer Laboratories, 1000-Å, 8-μm particle size, 2.1 × 150 mm). The detection was at 260 nm. Mobile phase A was 15% (v/v) acetonitrile in 0.01 M ammonium formate, pH 2.6. Mobile phase B was 15% (v/v) acetonitrile in 1.0 M ammonium formate, pH 4.3. The samples analyzed included SAH standards, and the complete PIMT reaction mixtures incubated as described above. The injection volume was 20 μl and each sample was analyzed in triplicate.

For the Partisil 10 SCX column, the flow rate was 1.5 ml min^{-1}. The column was equilibrated at 100% mobile phase A. After the sample was injected, the column was washed with 100% A for 5 min followed by a step gradient of 0–30% mobile phase B over 5 min, 30–100% B over 1 min, and 100–0% B over 4 min. The column was then equilibrated for additional 5 min in 100% A before the next injection. For the PL-SCX column, the flow rate was 0.5 ml min^{-1}. The column was equilibrated with 100% A. Upon sample injection, the column was washed with 100% A for 2 min, followed by a gradient of 0–20% B over 6 min, 20–100% B in 0.5 min, washed with 100% B for 3.5 min, 100–0% B in 0.5 min, and then equilibrated for another 2.5 min in 100% A before the next injection.

The SAH peak in the methylation reaction of each sample was then integrated. The SAH peak area corresponding to SAH produced in the blank reaction was subtracted from those of SAH produced in isoAsp-DSIP or the mAb sample containing reactions when calculating the recovery. The isoAsp content was calculated based on the area of SAH peak in the sample and the SAH standard curve.

3. Results

3.1. Separation of mAb charge isoforms by CEX-HPLC

CEX-HPLC profile of the purified mAb preparation is shown in Fig. 2. The major portion of the mAb eluted as a single peak that appeared to be the most basic isoform (peak 5 in Fig. 2). A marked charge heterogeneity was observed in the acidic region of the chromatogram, with three distinct peaks eluting adjacent to the main peak (peaks 2–4 in Fig. 2) and a more complex mixture of acidic species observed at the beginning of the chromatogram (peak 1 in Fig. 2). These five isoform fractions were collected for the IEF and isoAsp analyses. Fraction 1 was collected as one peak due to insufficient resolution between various forms and low protein amount. Protein concentration of the purified mAb isoforms was determined by measuring absorbance at 280 nm. Isoform 1 pool had a concentration of approximately 15.4 μM, isoform 2–14.6 μM, isoform 3–7.5 μM, isoform 4–3.6 μM and isoform 5–15.6 μM.

3.2. IEF Analysis of the mAb and purified charge isoforms

Charge heterogeneity of the mAb was also evident based on the IEF analysis. Two major bands with pI values of 8.5 and 8.4, respectively, and 1–2 minor bands were observed in the intact mAb (Fig. 3, lanes 2–4). The most basic band (pI = 8.5), as expected, corresponded to the most basic isoform resolved by CEX-HPLC (Fig. 3, lane 9). Though a slightly more acidic minor band was present in isoform 2 and a basic minor band was seen in isoform 4, the major bands in isoforms 2, 3, and 4 had essentially the same pI (Fig. 3, lane 6–8). The comigration of these isoforms during the IEF analysis may indicate that net charge alone does not determine the order of isoform elution in CEX-HPLC. The pool of most acidic mAb isoforms (CEX-HPLC fraction 1), contained predominantly the minor acidic bands, with pI lower than 8.4 (Fig. 3, lane 5).

3.3. IsoAsp analysis

It is well known that deamidation of Asn to Asp or isoAsp can increase the negative charge of a protein. Isomerization of Asp to isoAsp, on the other hand, even though not affecting the net charge of a protein, can potentially change local protein structure, and subsequently, surface charge distributions. Therefore, detection and quantification of isoAsp resulting from Asn deamidation or Asp isomerization may help to explain mAb charge heterogeneity. In this study, the isoAsp content in a mAb and its charge isoforms was determined by a non-radioactive assay developed recently [14]. This assay is based on methylation of isoAsp by the enzyme PIMT. SAH, which is produced in the enzymatic reaction in stoichiometric amounts to isoAsp content, is separated from other reaction components by SCX-HPLC and quantified by UV detection at 260 nm. Large excess of SAM is required for the assay to over-

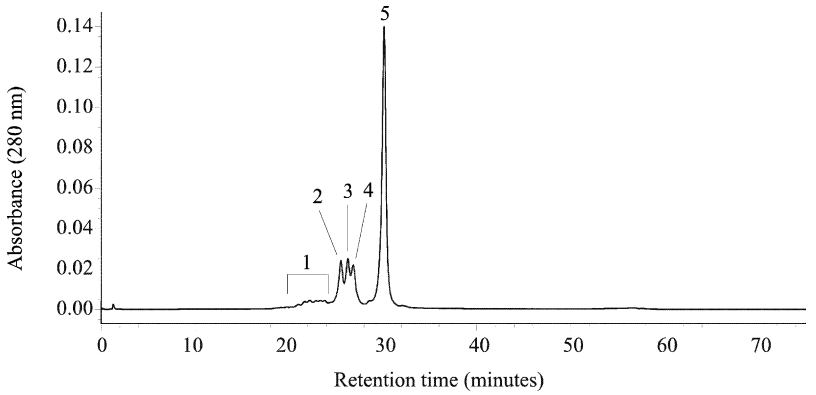

Fig. 2. CEX-HPLC profile of the mAb. Five fractions were collected for the IEF and isoAsp analysis, and were designated isoforms 1–5. Chromatographic conditions are described in Experimental. The injection amount was 100 μg.

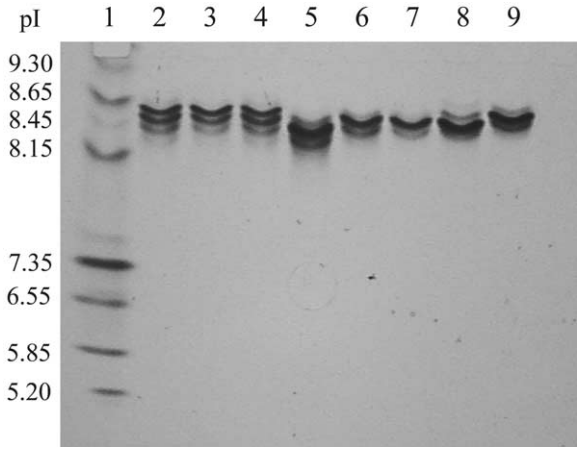

Fig. 3. IEF profile of the mAb and its charge isoforms. Electrophoretic conditions are described in the Experimental. The loading was approximately 10 μg for each lane. The pI values for standards are indicated. Lane 1, IEF pI markers; lanes 2–4, the mAb; lane 5, isoform 1 resolved by CEX-HPLC; lane 6; isoform 2; lane 7, isoform 3; lane 8, isoform 4; lane 9,

come the inhibitory effects of SAH. Two different SCX columns were used to assess the effects of HPLC matrix pore size on the analysis of isoAsp in the mAb.

Analysis of the SAH standard curves indicated that the method is linear over entire SAH concentration range studies using either of the two columns (Fig. 4). The accuracy of the method was determined by using isoAsp-DSIP control peptide that contains 1 mol of isoAsp per mol of peptide. Typical chromatograms of the methylation reaction of isoAsp-DSIP and a water blank are shown in Fig. 5 ((A), Partisil 10 SCX column; (B), PL-SCX column). In the blank reaction, a small but reproducible amount of SAH was generated, which might be due to automethylation of PIMT. The SAH peak area in the blank was subtracted from that of SAH produced in isoAsp-DSIP or mAb sample containing reactions when calculating the recovery. SAH produced in the enzymatic reaction eluted at 9.40 ± 0.02 min from the Partisil 10 SCX column and at 6.97 ± 0.02 min from the PL-SCX column, as a symmetric peak with baseline separation from other reaction components. In a reaction containing 82 pmol of isoAsp-DSIP, 79 pmol (97%) of SAH was detected when using the Partisil 10 SCX column, and for the PL-SCX column, the recovery was 95%.

Chromatographic profiles of enzymatic reaction of the mAb and its most basic and acidic isoforms are shown in Fig. 6 ((A), Partisil 10 SCX column; (B), PL-SCX column). The results obtained for all isoforms are summarized in Fig. 7. IsoAsp content obtained by two different SCX columns was similar for all isoforms analyzed (Fig. 7). The intact mAb was found to contain approximately 0.2 mol of isoAsp per mol of protein. Various levels of isoAsp were found in the charge isoforms resolved by CEX-HPLC. Not surprisingly, isoform 1, the most acidic in the CEX-HPLC and IEF analyses, contained an average of 0.72 mol of isoAsp per mol of protein, significantly higher than other isoforms. Isoforms 2 and 3 contained

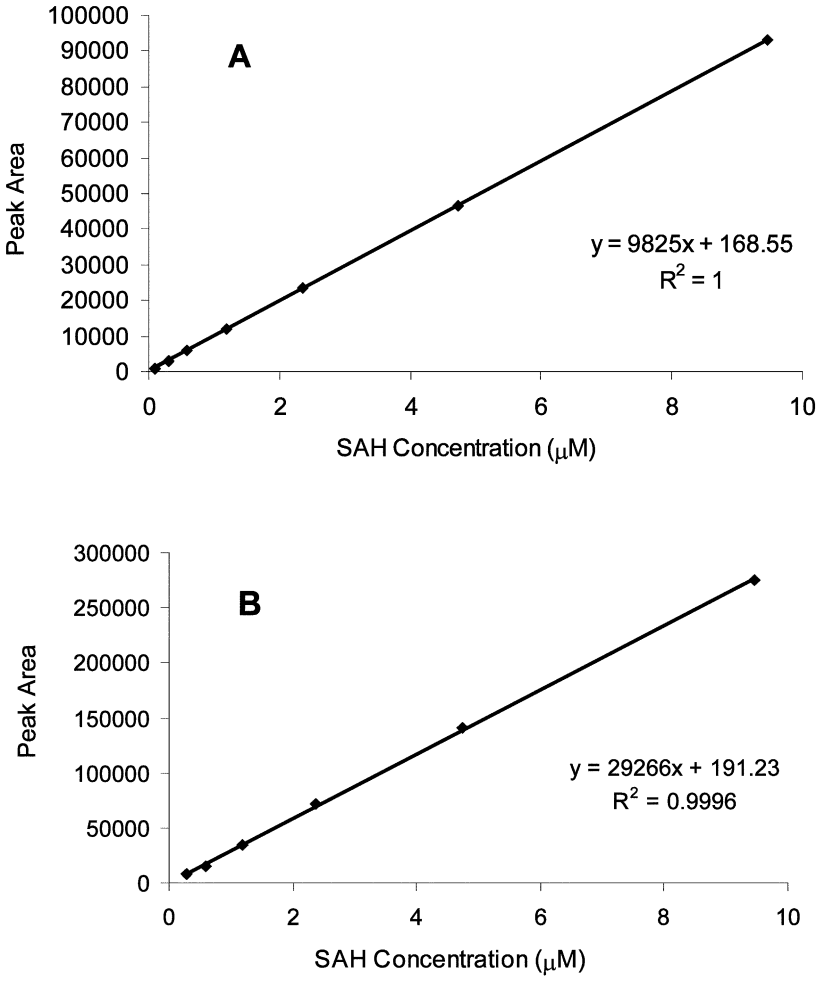

Fig. 4. Linear fit of SAH standards analyzed by SCX-HPLC. (A), Partisil 10 SCX column, 0.098–9.46 μM range; (B) PL-SCX column, 0.296–9.46 μM range.

approximately 0.07 and 0.15 mol of isoAsp per mol of protein, respectively, where as in isoforms 4 and 5, only a trace amount of isoAsp caused by automethylation of PIMT was detected.

4. Discussions

Charge heterogeneity has been observed frequently in mAbs [8–13]. Three major factors contribute to the charge heterogeneity of recombinant IgG mAbs: incomplete removal of C-terminal heavy chain lysine, sialic acid content and Asn deamidation. In the mAb preparation analyzed in this study, no C-terminal lysine and very little amount of sialic acid was detected (data not shown). In addition, the isoforms resolved by CEX-HPLC were homogeneous in size and no difference in secondary or tertiary structure was found by circular dichroism, fluorescence spectroscopy and analytical ultracentrifugation (data not shown). However, isoforms 1–4 were found less active in antigen binding than isoform 5 (data not shown). To determine a potential cause of charge

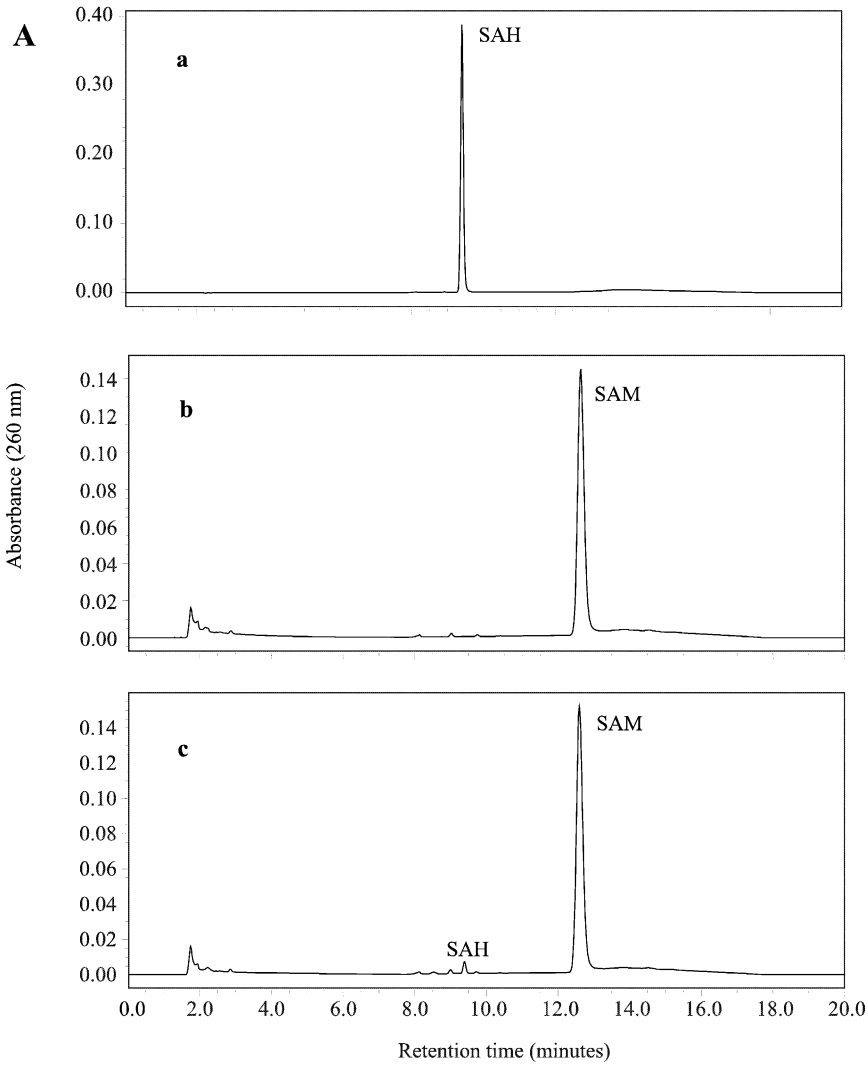

Fig. 5. SCX-HPLC profiles of isoAsp-DSIP PIMT enzymatic reactions analyzed using (A) the Partisil 10 SCX column and (B) the PL-SCX column. The injection volumes were 20 μl. (a), 113.5 μM SAH; (b), blank reaction; (c), methylation reaction of a 24.6-μM isoAsp-DSIP solution.

heterogeneity of this mAb, the content of one of the deamidation product, isoAsp was analyzed in the intact protein and its charge isoforms by a convenient and accurate HPLC-based method [14].

Both Asn deamidation and Asp isomerization can lead to isoAsp formation. Under neutral and alkaline conditions, Asn deamidation and Asp isomerization proceeds through a cyclic imide intermediate. Hydrolysis of this cyclic imide generates a mixture of by-products usually consisting of approximately 75% L-isoAsp and 25% L-Asp [15,16]. IsoAsp formation often occurs at the sequences of Asn-Gly, Asn-Ser and Asp-Gly, especially in regions with flexible conformations [17–20]. Protein turnover and aging may be triggered by Asn deamidation and Asp isomerization [21,22]. However, for protein drug develop-

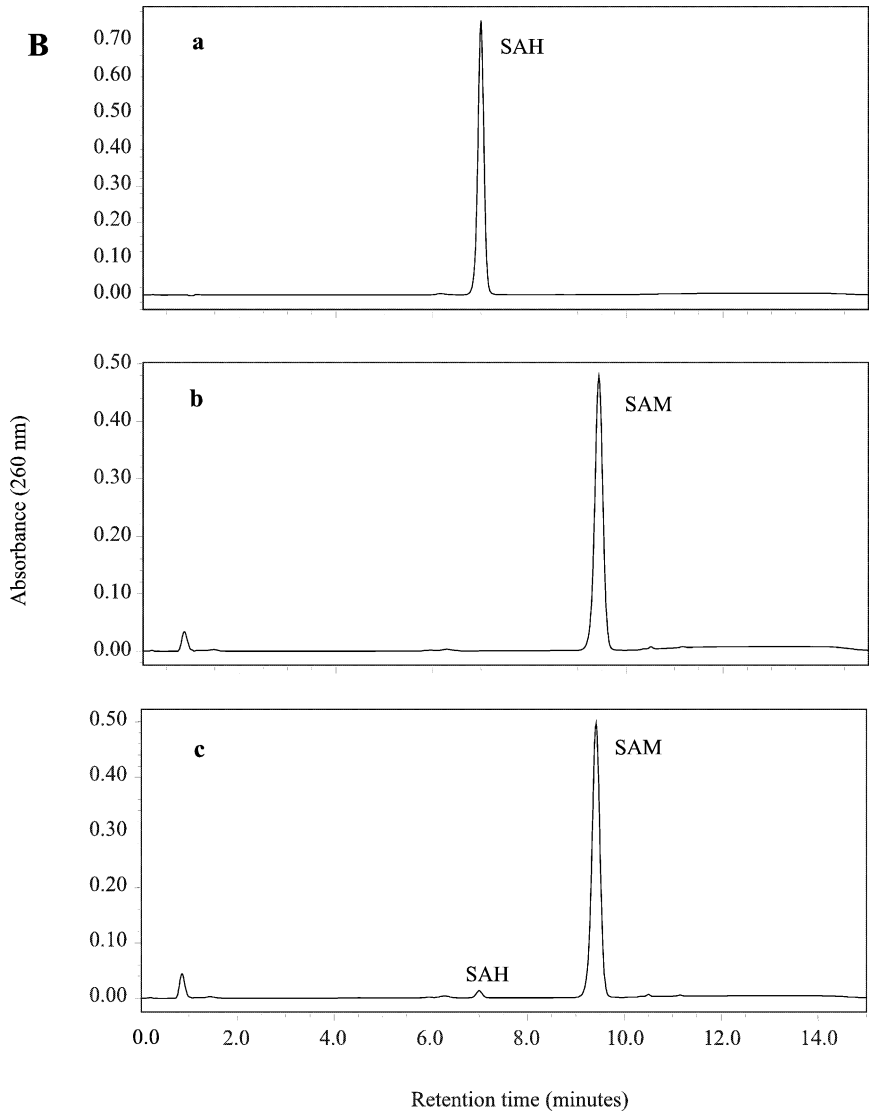

Fig. 5 (*Continued*)

ment, the major concern is the impact of Asn deamidation and Asp isomerization on protein structure and function [2–7], resulting in the decreased efficacy and shelf life of degraded proteins [5], and increased immunogenicity [23]. For recombinant mAbs, like other therapeutic proteins, isoAsp formation can occur during manufacturing, formulation and storage. Therefore, it is important to monitor isoAsp levels during biopharmaceutical drug development, which could increase product quality, provide understanding of structure/function relationships early in the development process and minimize regulatory concerns. Given the importance of isoAsp formation in protein therapeutics, methods for detection and quantification of isoAsp are of great interest to biopharmaceutical industry [2–4,6]. IsoAsp levels in proteins have been measured

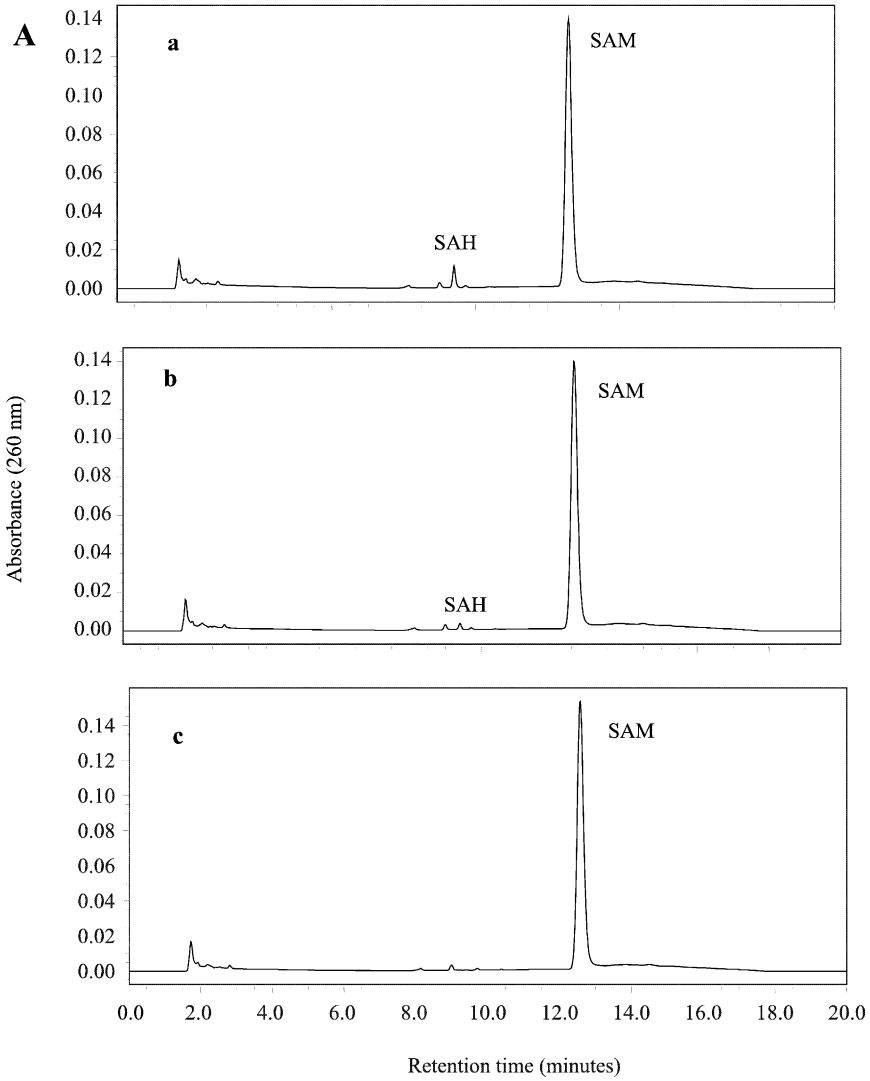

Fig. 6. SCX-HPLC profiles of the mAb and its charge isoforms' PIMT enzymatic reactions analyzed using (A) the Partisil 10 SCX column and (B) the PL-SCX column. (a), methylation reaction of the unfractionated mAb; (b), methylation reaction of isoform 1; (c), methylation reaction of isoform 5.

using PIMT and tritium scintillation counting [24]. Recently, alternative procedures that obviate the need for radioactive materials have been described to have similar accuracy and sensitivity as the radio-isotopic assay [14,25,26]. These methods are based on reversed phase- and SCX-HPLC and take advantage of the quantitative UV detection of SAH, produced in stoichiometric amounts to isoAsp content by PIMT in the isoAsp methylation reaction.

Asn deamidation has been detected in several therapeutic mAbs, including murine mAb OKT3 [8], a humanized anti-CD18 [9], a murine mAb used as a surrogate antigen for LeY [12], and humanized IgG mAb HER2 [13]. Asp isomerization has also been found in humanized mAb E25

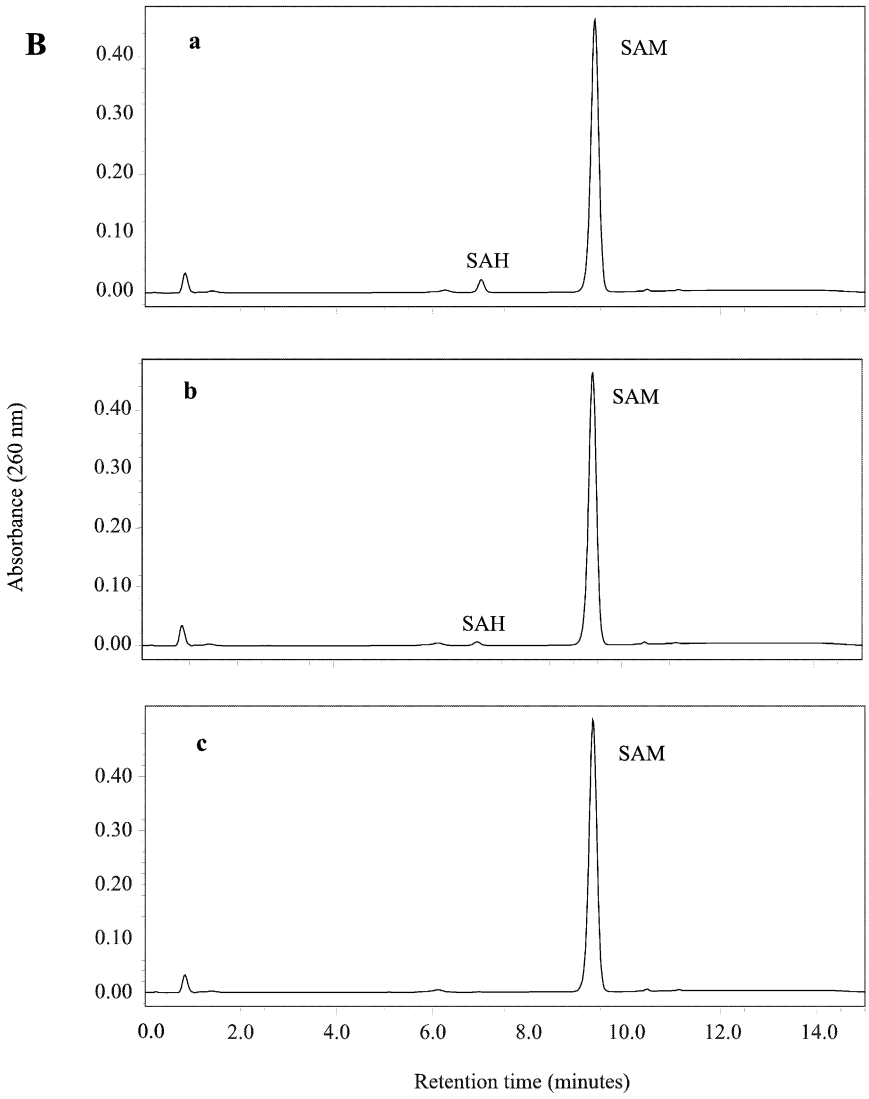

Fig. 6 (*Continued*)

[10] and HER2 [13]. IsoAsp formation in the complementarity-determining regions (CDRs) of mAbs, resulting from Asn deamidation [13] or Asp isomerization [10,13], was of particular importance as it reduced their antigen binding affinities. In the present study, isoAsp contents in a recombinant mAb and its charge isoforms resolved by CEX-HPLC were analyzed with a non-isotopic SCX-HPLC method [14]. The accuracy of the method was demonstrated by quantitative recovery of SAH produced in the methylation reaction of isoAsp-DSIP control peptide. The intact mAb was found to contain approximately 0.2 mol of isoAsp per mol of protein, and different levels of isoAsp were detected in its charge isoforms. The mAb analyzed contains a number of Asn-Gly, Asn-Ser and Asp-Gly sequences, and some of them are located in the CDRs. Preliminary results from tryptic peptide mapping of the mAb and its charge isoforms indicate no Asp isomerization in

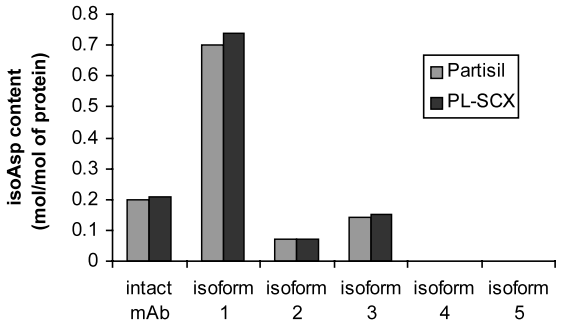

Fig. 7. IsoAsp content of the unfractionated mAb and its charge isoforms.

the mAb, but various levels of Asn deamidation in isoforms 1 through 4 and no deamidation in isoform 5 (data not shown). It is worth pointing out that the isoAsp analysis is performed on the protein in its native conformation. Hence, the method may potentially underestimate isoAsp content. However, deamidation and isoAsp formation typically occur in the exposed and flexible sequences of a protein [17–20], therefore, should be quantitatively detectable by the method used. We envision that this method, by reducing concerns and costs associated with the use and disposal of radioactive materials, will be applicable to other protein and mAb pharmaceuticals for isoAsp formation monitoring.

5. Conclusions

Charge heterogeneity of a recombinant mAb was assessed by CEX-HPLC, IEF and isoAsp measurement. The charge isoforms were found to contain different levels of isoAsp. It appears that deamidation is the major source of charge heterogeneity of this antibody.

The PIMT enzymatic reaction coupled with SCX-HPLC method described in this study allows detect and quantify isoAsp formation in recombinant protein pharmaceuticals, including mAb therapeutics, and will have applications in protein drug development.

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