Identification and Quantification of Oxidation Sites on Monoclonal Antibodies Using Capillary Electrophoresis and Quadrupole Time-of-Flight Mass Spectrometry

Application Note

Biotherapeutics & Biosimilars

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Abstract

This Application Note describes the identification and quantification of oxidation sites on a monoclonal antibody (mAb) using an Agilent 7100 Capillary Electrophoresis (CE) system coupled to an Agilent Accurate-Mass 6520 Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometry (MS). Forced oxidation of mAb experiments with t-butyl hydroperoxide (t-BHP) was performed to stimulate potential oxidative modifications. The oxidized and control samples were digested with trypsin to generate peptide fingerprint maps, and analyzed with CE/MS to identify oxidation sites and quantify the degree of oxidation. Peptides containing amino acid residues including methionine and tryptophan are sensitive to oxidation, and were easily identified using this CE/MS approach. Quantitative analysis demonstrated varying levels of mAb oxidation, and methionine residue in DTLMISR peptide was found to be more prone to oxidation.

Introduction

Protein oxidation is a covalent modification of amino acids. Amino acids such as methionine (Met) cysteine (Cys), tryptophan (Trp), and histidine (His) are known to be sensitive towards oxidation. As with other therapeutic proteins, mAb's can undergo oxidation at various stages such as production, formulation, and storage. Therefore, it is very important to analyze potential oxidation changes during such processes. Forced oxidation studies using either hydrogen peroxide or t-butyl hydroperoxide (t-BHP) have been performed in order to investigate the possible sites that are susceptible to oxidation and the effect of oxidation on activity[1]. Oxidation of methionine residue in monoclonal antibody (mAb) is one of the major concerns as it can lead to a product with altered binding.

Agilent Technologies

Capillary electrophoresis (CE) shows great potential for biopharmaceuticals analysis. There is growing interest in exploring CE coupled to mass spectrometry (MS) to achieve higher sensitivity and specific compound identification with accurate mass measurements. CE, as an electrophoretic separation method, provides an orthogonal tool nicely complementing chromatographic LC separations, enhancing confidence in analysis of complex samples. Quadrupole time-of-flight (Q-TOF) MS provides high mass accuracy and resolution at high acquisition rates. Previously, CE/MS has been successfully employed for characterization of mAb.

This Application Note demonstrates the technical capability of CE/Q-TOF/MS for accurate identification of monoclonal antibody oxidation sites. The monoclonal antibody was subjected to forced oxidation with t-BHP to simulate potential oxidative modifications. To investigate the modification sites and quantify the degree of oxidation, either t-BHP treated or control samples were digested with trypsin to generate peptide fingerprint maps. The resulting peptide mixture was analyzed with CE/Q-TOF/MS to identify modification sites and quantify the degree of oxidation.

Experimental

Materials

Immunoglobulin G (IgG) was a proprietary therapeutic protein. 2,2,2-trifluoroethanol (TFE), DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, acetic acid, and solvents were purchased from Sigma Aldrich. High quality sequence grade trypsin was obtained from Agilent Technologies, Inc.

CE materials: water (5062-8578), polyvinyl alcohol (PVA) coated capillary (G1600-67219), sample vials (5183-4623) were obtained from Agilent Technologies, Inc.

Oxidation of mAb using t-BHP

The monoclonal antibody samples were incubated with 3 % t-BHP in Tris-HCl, pH 7.5, overnight at ambient temperature. Untreated samples were used as controls. After overnight reaction, the treated samples were vacuum dried and subjected to trypsin digestion.

Trypsin digestion

Before the digestion of the mAb with trypsin, the disulfide bonds were reduced (DTT) and alkylated (IAA) under denaturing conditions (TFE). This pretreatment ensured complete monoclonal antibody denaturation, solubilization, and efficient access of protease to the target substrate[2]. After reduction and the alkylation step, the pH of the solution was adjusted to pH 7–8 and trypsin digestion (20:1, protein to protease w/w) was performed overnight, incubating at 37 °C. The samples were either immediately analyzed by CE/MS or stored at –80 °C until use.

CE/MS instrumentation

The CE/Q-TOF/MS analysis was performed using the Agilent 7100 CE system with a CE/MS adapter kit (G1603A) coupled to the Agilent 6520 Accurate-Mass Q-TOF LC/MS equipped with an electrospray source and an orthogonal triple tube sheath liquid interface (G1607B)[3]. This sheath-liquid CE/MS interface with a low flow rate (4 µL/min with a 1:100 flow splitter) allows for high efficiency separation of CE, and provides stable flow and optimal spray conditions essential for electrospray ionization. Q-TOF parameters were optimized automatically through the MS tuning programs, and the MS system was calibrated using an ESI tuning mixture.

The spectra were recorded in positive ion and centroid modes. The data obtained from MS and MS/MS were analyzed using features contained in the following software packages: Agilent MassHunter Qualitative Analysis and Agilent MassHunter BioConfirm. The CE/MS raw data were processed using the Molecular Feature Extractor (MFE) algorithm and matched with a theoretical peptide digest list with predicted oxidation modifications. Table 1 shows the CE/MS parameters.

Table 1. CE/MS parameters.

CE conditions	
CE	Agilent 7100 CE
Sample	mAb digest
Injection	10 sec at 50 mbar
Capillary	PVA coated capillary (85 cm* 50 µm id)
Buffer	20 mM acetic acid
Voltage	27 kV (0.3 minutes ramp)
Temperature	20 °C
Preconditioning	15 minutes flush with buffer at 1 bar
MS conditions	
MS	Agilent 6520 Q-TOF
Ionization mode	ESI
Acquisition mode	MS (mass range 100–3,200 *m/z*)
Sheath liquid	0.5 % acetic acid in 50% methanol, 4 µL/minute
Drying gas flow	5 L/min
Nebulizer	10 psi
Drying gas temperature	175 °C
Fragmentor	175 V
Vcap	3,500 V

Results and Discussion

The tryptic digest of the mAb was subjected to CE/MS analysis. Figures 1A and 1B show the base peak electropherogram (BPE) of untreated (control) and 3 % t-BHP treated samples respectively. The peptide masses obtained for both mAb samples from the MFE analysis were matched with the theoretical digest, with a preferred modification of oxidation included for the theoretical peptide digest generated using the BioConfirm sequence editor (define and match sequences). It was clear that the oxidized peptide was much more abundant in the sample treated with 3 % t-BHP. Figures 1C and 1D show the extracted compound electropherograms for oxidized peptides. In the untreated sample, some degree of oxidation was detected, which corresponds to oxidized peptide NFDYWGQGTLVTVSSASTK (Trp oxidation). When forced oxidation was carried out with 3 % t-BHP, there was enhanced oxidation of the mAb, as expected. The oxidized peptide sequences are illustrated in Figure 1. This indicates that t-BHP induces mAb oxidation under current experimental conditions.

Figure 2 shows the MassHunter Bioconfirm results for both control and oxidized peptides. The peptides containing methionine (Met) and tryptophan (Trp) are readily oxidized, and this resulted in increased molecular weights of approximately 16 or approximately 32 Da, depending on the degree of oxidation.

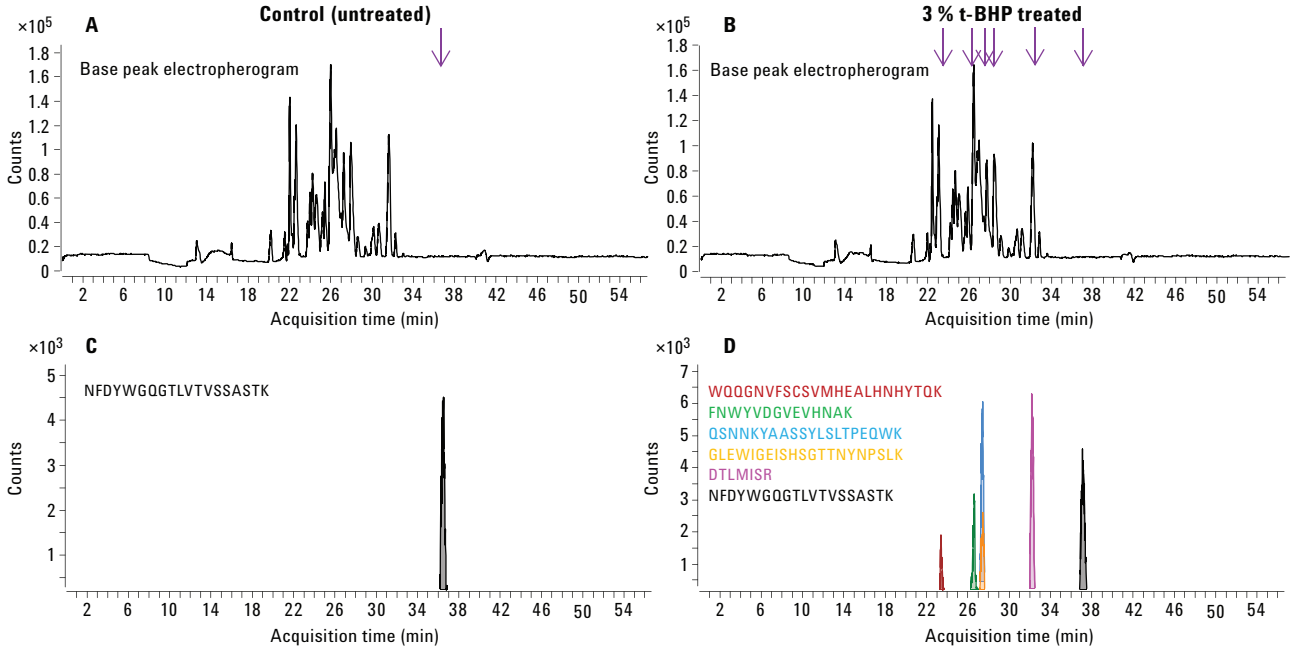

Figure 1. CE/Q-TOF/MS of monoclonal antibody tryptic digest peptide mapping. (A) and (B) Base peak electropherogram of untreated and 3 % t-BHP treated tryptic peptides. (C) and (D) Extracted compound electropherogram of mAb oxidised without 3 % t-BHP. The arrows correspond to the oxidized peptide region.

Show/Hid	Cpd	ID Source	RT	m/z	Mass	Avg Mass	Tgt Seq Mass	Diff (Bio, ppm)	Vol	Height	Sequence	Pred Mods	Polar
☑	27	BioConfirm	23.291	561.0648	2800.283	2802.1502	2800.2598	8.28	1079896	15048	WQQGNVFSCSVMHEALHNHYTQK		Positive
☑	28	BioConfirm	23.318	567.4622	2832.2718	2833.8778	2832.2497	7.81	19708	674	WQQGNVFSCSVMHEALHNHYTQK	2*Oxidation(+15.994915)B42...	Positive
☑	180	BioConfirm	27.28	587.5339	2346.0954	2347.5745	2346.1128	-7.41	91285	1168	QSNNKYAASSYLSLTPEQWK	1*Dioxidation(+31.98983)A191	Positive
☑	288	BioConfirm	31.647	687.673	2059.9931	2061.3112	2059.9851	3.87	1031964	11306	NFDYWGQGTLVTVSSASTK		Positive
☑	376	BioConfirm	36.963	698.3415	2091.9993	2093.2097	2091.9749	11.64	93882	868	NFDYWGQGTLVTVSSASTK	1*Dioxidation(+31.98983)B103	Positive
☑	179	BioConfirm	27.28	768.3856	2302.1359	2302.9123	2302.123	5.6	2151705	48444	GLEWIGEISHSGTTNYNPSLK		Positive
☑	183	BioConfirm	27.303	773.7159	2318.1263	2319.1231	2318.1179	3.62	39277	838	GLEWIGEISHSGTTNYNPSLK	1*Oxidation(+15.994915)B48	Positive
☑	131	BioConfirm	26.286	559.9461	1676.8152	1677.3666	1676.7947	12.21	2786072	95371	FNWYVDGVEVHNAK		Positive
☑	142	BioConfirm	26.48	565.275	1692.8019	1693.7082	1692.7896	7.28	41955	1071	FNWYVDGVEVHNAK	1*Oxidation(+15.994915)B273	Positive
☑	307	BioConfirm	32.008	418.2263	834.4374	834.9555	834.4269	12.56	2434448	97947	DTLMISR		Positive
☑	310	BioConfirm	32.081	426.2227	850.4303	850.9291	850.4219	9.98	96553	3860	DTLMISR	1*Oxidation(+15.994915)B248	Positive

Figure 2. Agilent MassHunter Bioconfirm results.

To confirm the oxidation modification, CE/MS/MS experiments were performed to map the specific oxidation sites on peptides. The MS/MS data was searched against the mAb sequence with preferred modification of oxidation using Agilent MassHunter BioConfirm software. Figure 3 shows an example of the analysis, with the representative MS and MS/MS spectrum for DTLMISR peptide in both modified and unmodified form. The comparison of y4 and y5 ions mass showed an increase in mass of approximately 16 Da in modified peptide. As both spectra show other y and b ions at the same mass, the oxidation must occur on the Met position.

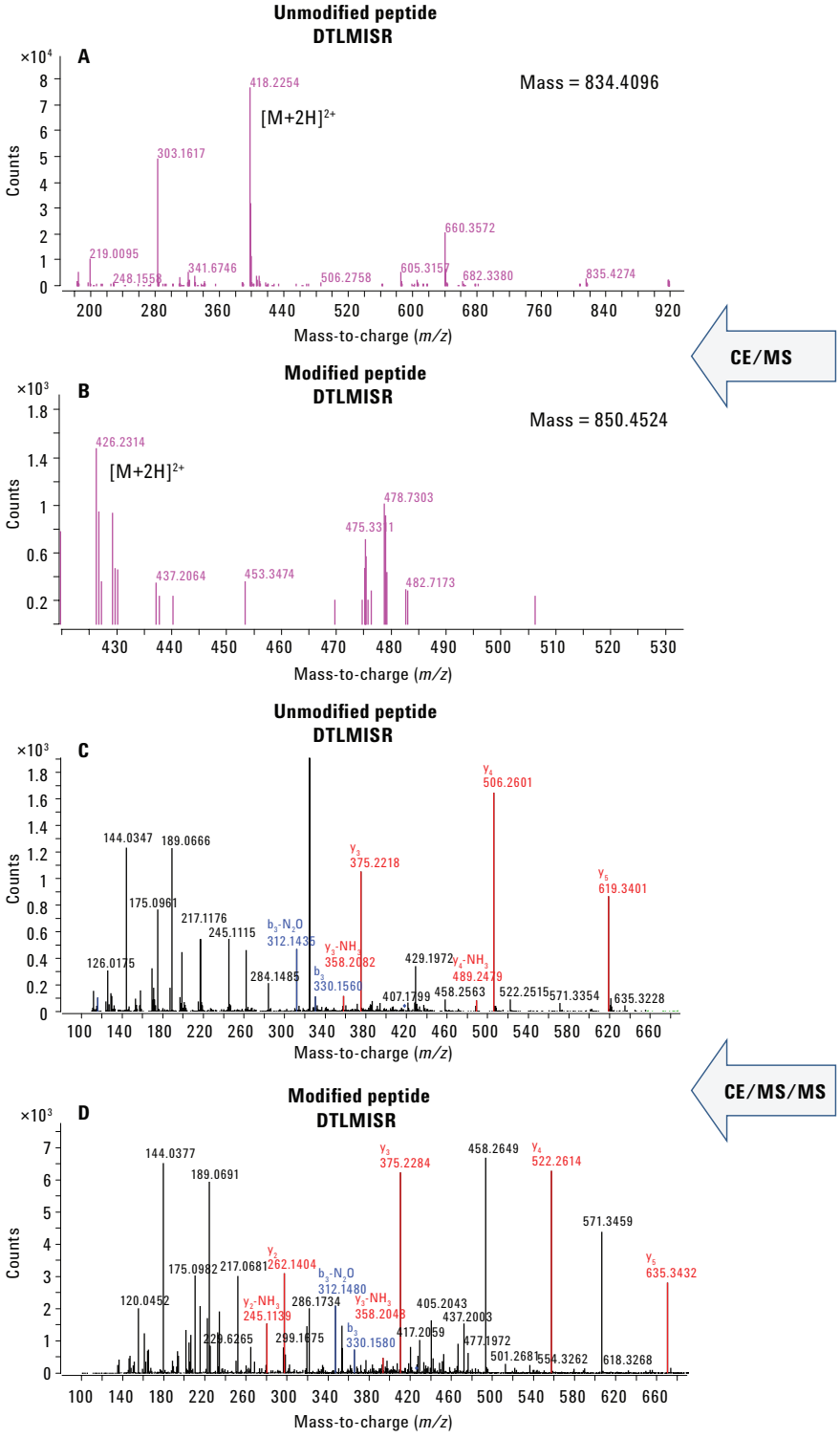

Figure 3. CE/Q-TOF/MS and MS/MS spectrum of DTLMISR peptide.
(A) and (B): Mass spectrum of unmodified and modified DTLMISR peptide.
(C) and (D): Unmodified and modified CE/MS/MS spectra of DTLMISR peptides and their assignments, y4 and y5 ions shows a difference of approximately 16 Da between the unmodified and modified peptide.

Quantitative analysis of the mAb oxidation was performed, and the relative percentage oxidation was calculated by dividing the height of modified peptide by the sum of modified and nonmodified peptide height[4]. Figure 4 shows the peptide oxidation levels versus the percent t-BHP plot. The degree of oxidation was found to be higher for the commonly used DLTMISR peptide sequence of mAb as compared to other oxidized peptides. This shows the different susceptibility of oxidation sites of mAb peptides and that Met residue in DTLMISR peptide is more prone to oxidation. The result found in this study was in good agreement with previous published studies on mAbs[5,6].

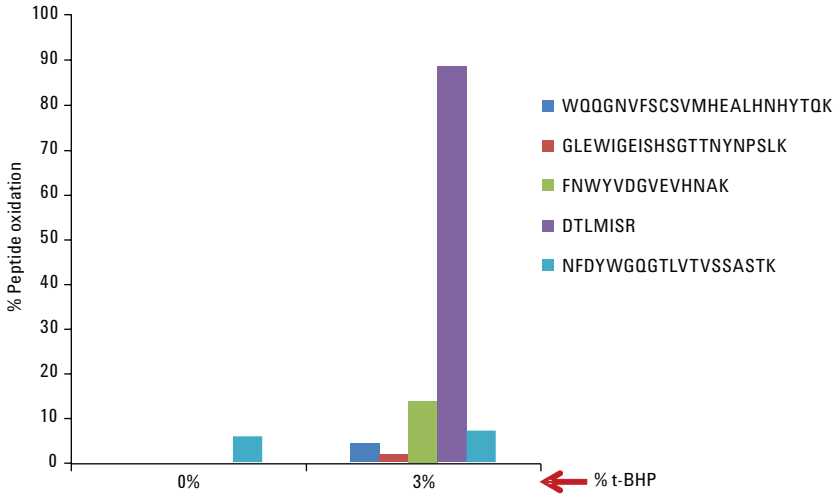

Figure 4. Quantitative assessment of monocional antibody peptide oxidation levels incubated with or withouit t-BHP.

Conclusion

This Application Note provides an Agilent CE/MS based solution for identification and quantification of mAb oxidation sites. Use of the CE/MS technique to achieve high-quality results could be extremely beneficial in early stage antibody characterization studies, widely used in biopharmaceutical product development.

References

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