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Liquid chromatography–fluorescence and liquid chromatography–mass spectrometry detection of tryptophan degradation products of a recombinant monoclonal antibody

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ABSTRACT

Light exposure is one of several conditions used to study the degradation pathways of recombinant monoclonal antibodies. Tryptophan is of particular interest among the 20 amino acids because it is the most photosensitive. Tryptophan degradation forms several products, including an even stronger photosensitizer and several reactive oxygen species. The current study reports a specific peptide mapping procedure to monitor tryptophan degradation. Instead of monitoring peptides using UV 214 nm, fluorescence detection with an excitation wavelength of 295 nm and an emission wavelength of 350 nm was used to enable specific detection of tryptophan-containing peptides. Peaks that decreased in area over time are likely to contain susceptible tryptophan residues. This observation can allow further liquid chromatography–mass spectrometry (LC–MS) analysis to focus only on those peaks to confirm tryptophan degradation products. After confirmation of tryptophan degradation, susceptibility of tryptophan residues can be compared based on the peak area decrease.

Recombinant monoclonal antibodies exhibit extensive heterogeneity, which is reflected in variants differing in molecular weight, charge, and hydrophobicity and is attributed to various post-translational modifications [1–6]. Recombinant monoclonal antibody therapeutics are required to be thoroughly characterized to determine modifications and their impact on the stability, efficacy, and safety of the products. In addition, various stress conditions are used to degrade recombinant monoclonal antibodies in order to understand their major degradation pathways. This information is useful in designing appropriate formulation and storage conditions. Identification of posttranslational modifications and degradation products has been an integral part of the development of recombinant monoclonal antibody therapeutics.

One degradation hotspot of recombinant monoclonal antibodies is the light sensitive tryptophan residue. Tryptophan is the principal amino acid that absorbs ultraviolet (UV) light [7,8]. The major tryptophan degradation product, *N*-formylkynurenine, is an even stronger photosensitizer at wavelengths greater than 320 nm [9,10]. Several reactive oxygen species, including superoxide, hydrogen peroxide, and free radicals, are formed during the degradation process that can further oxidize other substrates [9–12]. Various oxidation products of tryptophan have been identified during the degradation process and by reaction with the oxygen species [11,13,14]. Susceptibility of tryptophan residues and the various degradation products is highly dependent on the neighboring amino acids based in the primary sequence [15], solvent accessibility, and microenvironment of the residue [16–18].

Oxidation of tryptophan residues of recombinant monoclonal antibodies has been reported in several studies [16,18–20]. Degradation of a solvent-exposed tryptophan caused by light exposure in the heavy chain complementarity-determining region (CDR) resulted in a significant decrease of its binding affinity [20]. Color change of a recombinant monoclonal antibody after exposure to light and heat has been attributed to the degradation of tryptophan and the formation of various oxidation products [13,19]. Exposure of recombinant monoclonal antibodies to intense light also leads to fragmentation, covalent cross-linking, aggregation, generation of acidic species, and loss of potency [19].

Abbreviations: UV, ultraviolet; DTT, dithiothreitol; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; MS/MS, tandem mass spectrometry.

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In the current study, a specific peptide mapping procedure was established to detect various tryptophan degradation products. It is challenging to detect posttranslational modifications and degradation products of antibodies because of the presence of many peptides after trypsin digestion. Degradation products from various amino acids are usually present at very low levels and coelute with other peptides. The targeted peptide mapping procedure focuses on tryptophan-containing peptides only using excitation and emission wavelengths that are specific for tryptophan residues.

Materials and methods

Materials

The recombinant monoclonal IgG antibody was expressed in a Chinese hamster ovary (CHO) cell line and purified at Alexion (Cheshire, CT, USA). Acetonitrile, dithiothreitol (DTT), formic acid (30%), iodoacetic acid, and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). Trypsin was purchased from Worthington (Lakewood, NJ, USA).

Light exposure

The recombinant monoclonal antibody was diluted to 5 mg/ml using phosphate-buffered saline (PBS). The diluted sample was filled into 3-ml clear glass vials and then incubated in an ES2000 benchtop photostability chamber (Environmental Specialties, Raleigh, NC, USA) with white light (9.92 klux) and UV light (15.57 W/m^2) at 22 °C for 1, 2, and 3 weeks. The same antibody without light exposure stored at the same temperature was used as a control.

Trypsin digestion

All samples were denatured and reduced using 10 mM DTT and 6 M guanidine hydrochloride in 20 mM Tris (pH 7.8) at 37 °C for 30 min. The reduced samples were alkylated using 25 mM iodoacetic acid at 37 °C for 30 min at a slightly basic pH of approximately 7–8 based on measurement using pH paper. The samples were buffer exchanged into 20 mM Tris (pH 7.8) using NAP-5 columns (GE Healthcare, Pittsburgh, PA, USA). The samples were then digested using trypsin at a 1:10 trypsin/antibody ratio (w/w) at 37 °C for 4 h. The digestion was stopped by the addition of formic acid to lower the sample pH.

Reversed-phase HPLC with fluorescence or UV detection

Tryptic peptides were analyzed using a Vydac C18 column (150 × 4.6 mm) and a Waters high-performance liquid chromatography (HPLC) system (Waters, Bedford, MA, USA) with fluorescence detection. Mobile phase A contained 0.1% TFA in water. Mobile phase B contained 0.1% TFA in acetonitrile. The elution gradient was optimized to separate tryptophan-containing peptides. Tryptic peptides were injected using 98% mobile phase A and 2% mobile phase B. After 5 min, the percentage of mobile phase B was set to increase to 6% over 20 min, to 15% over 3 min, and then to 35% within 150 min. The column was washed using 95% mobile phase B and then equilibrated using 2% mobile phase B. The flow rate was set at 1 ml/min, and the column temperature was set at 60 °C throughout the run. Peptides were monitored using an excitation wavelength of 295 nm and an emission wavelength of 350 nm. The major fluorescence peaks were collected and analyzed by liquid chromatography–mass spectrometry (LC–MS) to determine their identities.

For comparison purposes, the same set of samples was also analyzed using the same HPLC conditions except that the gradient was optimized for separation of all peptides and elution was monitored using UV 214 nm. The samples were injected at 2% mobile phase B. After 5 min, the percentage of mobile phase was increased to 35% within 130 min and then to 95% within 10 min. The column was then washed and equilibrated.

LC–MS analysis

Tryptic peptides were analyzed using a Maxis 4G mass spectrometer (Bruker, Billerica, MA, USA) and an ultra-performance liquid chromatography (UPLC) system (Waters) with a Proto 200 C18 column (1.0 × 250 mm, Higgins Analytical). The samples were loaded at 98% mobile phase A (0.1% TFA in water) and 2% mobile phase B (0.1% TFA in acetonitrile). After 2 min, mobile phase B was increased to 35% within 118 min and then to 60% within 15 min. The column was then washed and equilibrated. The column was heated at 60 °C, and the flow rate was set at 50 μl/min. The mass spectrometer was tuned and calibrated following the manufacturer's procedure and was run at the positive scan mode with m/z in the range of 150–3000. Capillary voltage was set at 4500 V. Dry gas was set at 10 L/min. Dry temperature was set at 220 °C, and the nebulizer was set at 2.0 bar.

Results and discussion

Principle of the method

Peptide mapping with UV and MS detection has been used to analyze protein modifications and degradation. Trypsin digestion of a typical antibody results in approximately 60 peptides; therefore, it is challenging to detect posttranslational modifications and degradation products because of overlapping peaks. In the current study, the intrinsic fluorescence property of the tryptophan residue was used to establish a method to specifically monitor tryptophan degradation. Instead of detecting all peptides using UV absorption at 214 nm, fluorescence detection using an excitation wavelength of 295 nm and an emission wavelength of 350 nm was used to specifically detect only tryptophan-containing peptides. Because various tryptophan degradation products have excitation and emission wavelengths that are different from the tryptophan residue, a decrease in peak areas over time indicates potential degradation of the tryptophan residue contained in the peptide. This information can thus help, following LC-MS analysis, to focus on those susceptible residues only. It is worthwhile to mention that degradation of other amino acids in the tryptophan-containing peptides can also lead to a decrease of the original fluorescence peak areas as a result of shifting the retention times of the peptides containing the degradation products of other amino acids.

Degradation of tryptophan residues of a recombinant monoclonal antibody

The fluorescence chromatograms of the recombinant monoclonal antibody digested with trypsin after various lengths of exposure to lights are shown in Fig. 1. As a comparison, UV chromatograms of the same samples are shown in Fig. 1 of the online supplementary material. In contrast to the many peaks observed by UV 214 nm, only nine major peaks are detected in the fluorescence chromatograms. Each peak from the time 0 sample was collected and analyzed by LC–MS to determine the identity. The data are summarized in Table 1. Mass spectra corresponding to each peak are shown in Fig. 2 of the supplementary material. The monoclonal antibody contains three tryptophan residues on each