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Protocols for the analytical characterization of therapeutic monoclonal antibodies. II – Enzymatic and chemical sample preparation

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ABSTRACT

The analytical characterization of therapeutic monoclonal antibodies and related proteins usually incorporates various sample preparation methodologies. Indeed, quantitative and qualitative information can be enhanced by simplifying the sample, thanks to the removal of sources of heterogeneity (e.g. *N*-glycans) and/or by decreasing the molecular size of the tested protein by enzymatic or chemical fragmentation. These approaches make the sample more suitable for chromatographic and mass spectrometric analysis. Structural elucidation and quality control (QC) analysis of biopharmaceutics are usually performed at intact, subunit and peptide levels. In this paper, general sample preparation approaches used to attain peptide, subunit and glycan level analysis are overviewed. Protocols are described to perform tryptic proteolysis, IdeS and papain digestion, reduction as well as deglycosylation by PNGase F and EndoS2 enzymes. Both historical and modern sample preparation methods were compared and evaluated using rituximab and trastuzumab, two reference therapeutic mAb products approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA). The described protocols may help analysts to develop sample preparation methods in the field of therapeutic protein analysis.

1. Introduction

Due to their size and complexity, proper characterization of antibody-based pharmaceuticals requires their analysis at the peptide, subunit and intact levels. To overcome the limited information achievable at the intact protein level, cleavage of the amino acid chains and analysis of the resulting species are often necessary. Besides chemical methods, various enzymes are commercialized for generating peptides and larger subunits. Thanks to recent developments in enzyme technology, sample preparation now requires shorter time and shows improved reproducibility for various antibody subclasses and related products. Decreasing the molecular size of the tested protein, either by enzymatic digestion or chemical treatment, generates protein fragments with molecular properties more adapted to modern liquid chromatography and mass spectrometry. Peptides of less than 5 kDa and mAb fragments of 25–100 kDa possess indeed more favorable diffusion and adsorption properties than intact mAbs of 150 kDa, which enables the efficient liquid chromatographic separation of their variants. From the mass spectrometric (MS) point of view, subunit analysis increases sequence coverage from 30–50% to 50–70% compared to full length protein sequencing, and helps to identify modifications with minor mass shifts (e.g. deamidation) using state-of-the art high resolution MS instruments. In practice, the mass limit of an intact protein that can be analyzed using top-down approach is around 50 kDa. For 100% sequence coverage, peptide mapping is the method of choice, while subunit analysis helps to locate modifications and generates more reasonable amount of information within an acceptable analysis time. It is also worth to note that smaller protein fragments benefit better sensitivity in MS.

In this paper, recent trends in sample preparation for chromatographic and mass spectrometric characterization of protein biopharmaceuticals are overviewed and discussed. The aim is to help analysts developing reliable, state-of-the art methods for structural evaluation and quality assurance purposes. Benefits and drawbacks of enzymatic and chemical sample treatments will be critically discussed at the subunit, peptide and glycan levels.

2. Sample preparation protocols

2.1. Introduction to bottom-up proteolysis

Peptide level analysis (or "bottom-up" approach) involves the

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generation of peptides with molecular weights of 500–5000 Da, which are then subsequently separated by high performance liquid chromatography (HPLC) and sequenced by tandem MS. This technique is of great importance in protein structural elucidation and for batch quality purposes [1–3]. The selection and development of the proper sample preparation method at the bottom-up level is however still not straightforward and usually requires careful optimization to achieve appropriate sequence coverage, and also avoid miscleavages, over-digested, incompletely digested samples or any other artefacts generated during sample preparation. In addition, digestion of proteins may result in loss of information, such as connectivity of post-translational modifications (PTMs, relationship between such modifications located on different peptides derived from the same protein molecule), missed detection of sequence parts due to inadequate size, or unfavorable ionization of certain peptides [4]. Recent developments in digestion techniques, such as non-enzymatic digestion by chemicals or electrochemical oxidation as well as accelerated digestion have been recently reviewed by Switzar et al. [4]. The most widely used approach for protein digestion is the application of proteases. It is worth mentioning, that separation of the target analyte from the protease used for digestion may be necessary, since it might suppress ionization and complicate the MS analysis. As shown in Table 1, many proteases are available, each possessing their own specificity, optimum conditions and efficiency [5,6]. Trypsin is the gold standard in bottom-up proteomics, and has been modified to an autolysis-resistant protease for generating peptides possessing an average length of ~14 amino acids. Trypsin is easily available and cleaves the protein at the carboxyl sides of arginine (Arg) and lysine (Lys), except when followed by proline (Pro). In practice, variation in its specificity can be observed when obtained from different providers [7]. In-solution and in-gel tryptic digestion protocols are widely available, which may require optimization according to the sample type. Tryptic sample treatment generally involves the denaturation of the protein with chaotropic agents (e.g. urea, guanidine salts) or commercially available, MS friendly cleavable surfactants. Disulfide bridges are then reduced by dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) and alkylated by iodoacetamide or iodoacetic acid [8]. Alkylation may not be necessary if short digestion time is applied. Non-reduced peptide mapping applies the same sample preparation without reduction step. Non-reducing conditions help to identify the position and presence of disulfide bonds [9]. After reagent removal, tryptic digestion is usually performed under slightly basic pH conditions at 37 °C. Depending on the sample and the procedure, digestion time may take up to several hours or even a day and has to be quenched by acidification of the solution (e.g. by addition of formic acid or trifluoroacetic acid). Sample preparation is tedious and time-consuming, but can be completely automated. Other proteases, such as Lys-C, Asp-N and Glu-C may be used to improve sequence coverage [9,10], while other less specific enzymes such as chymotrypsin and pepsin are generally avoided, since they create complex peptide mixtures which are difficult to interpret. Peptide level analysis by RPLC- or HILIC-MS is a well-established technique, widely used for the structural and quality characterization of protein samples. However, it has to be kept in mind that it may be challenging to find optimal sample preparation conditions. Indeed, mild conditions (e.g. 37 °C) and short digestion time (e.g. 60 min) may result in incomplete digestion, while elevated temperature (e.g. 50–60 °C) and long digestion time (several hours to overnight) may produce artefacts, thus overestimating amino acid oxidation, truncation, deamidation, etc. Each step (from optimizing sample preparation conditions to data processing) should be critically evaluated to avoid misleading results [11,12].

The following trypsin digestion protocol can be used as a starting point for further optimization. The applicability of this protocol is illustrated by the digestion of two FDA/EMA approved IgG1 mAbs (rituximab and trastuzumab). Experimental conditions may vary depending on the sample and the trypsin activity. Further optimization of the sample preparation may be necessary in some cases.

2.1.1. Chemicals and reagents

Trypsin (Trypsin Gold, Mass Spectrometry Grade, art. V5280) was purchased from Promega (Dübendorf, Switzerland). Ammonium bicarbonate (art. 09830), iodoacetamide (IAA, art. I1149), DL-dithiothreitol (DTT, art. 43815), acetic acid (art. A6283) and trifluoroacetic acid (TFA, art. 302031) were purchased from Sigma-Aldrich (Buchs, Switzerland). Acetonitrile (art. A/0638/17) was purchased from Fisher Scientific (Reinach, Switzerland). Rapigest® SF surfactant (art. 186001861) was purchased from Waters (Baden-Dättwil, Switzerland). FDA approved, commercial mAb samples were kindly provided by Centre d'Immunologie Pierre Fabre (Saint-Julien-en-Genevois, France).

2.1.2. Laboratory device

Samples were homogenized using a vortex mixer Genie 2 (art. SI-0236, Scientific Industries, New York, USA) and thermostated using an Eppendorf Thermomixer comfort device (Vaudaux-Eppendorf AG, Schoenenbuch, Switzerland). Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). The pH was measured using a SevenMulti pH Meter S40 (Mettler Toledo, Greifensee, Switzerland). 1.5 mL HPLC vials with 150 µL conical glass inserts (31 × 5 mm, tip: 15 mm) were purchased from BGB Analytik Vertrieb GmbH (art. 110500, Rheinfelden, Germany). 0.5 mL Eppendrof Protein LoBind tubes (art. Z666491) were purchased from Sigma-Aldrich (Buchs, Switzerland).

2.1.3. Chromatographic system

Trypsin digested samples were analyzed using a Waters Acquity UPLC I-Class® system equipped with a binary solvent delivery pump, an autosampler (possessing flow-through needle (FTN) injection port with a 15 µL needle) and UV detector. A Waters Acquity CSH® C18 chromatographic column (130 Å, 1.7 µm, 2.1 mm x 150 mm, art. 186005298) was used for the analysis. Other UHPLC systems and C18 material dedicated for peptide mapping can obviously be used for this purpose.

Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. After 3 min initial isocratic segment at 2% B, a linear gradient from 2 to 60% B in 30 min was run. Flow rate was set to 0.3 mL/min, column temperature was 50 °C. Injection volume was set to 5 µL. Data were acquired at 214 nm with 5 Hz sampling rate and 0.4 s time constant.

2.1.4. Preparation of the reagents and samples

Samples and reagents should be stored according to providers' instructions. If no information is available, storage conditions must be validated. Before use, allow samples and reagents to reach room temperature. Vortex each sample gently. If required, dilute the mAb product to 1 mg/mL with the digestion buffer. Samples should be analyzed within 24 h when stored at 4 °C. Prepared samples can be stored for 1 month at −20 °C and for 6 months at −80 °C. 100 µg lyophilized trypsin was reconstituted in 200 µL 50 mM acetic acid (0.5 µg/µL). This enzyme solution was aliquoted to 3 µL fractions in 0.5 mL Eppendorf tubes. The aliquots can be stored up to 1 month at −20 °C and up to 12 months at −80 °C. Digestion buffer was 50 mM NH_4HCO_3 buffer at pH 7.8. 1 mg Rapigest® surfactant was reconstituted in 1 mL digestion buffer (0.1%, the solution can be stored for 1 week at 4 °C). Dissolve DTT and IAA in digestion buffer at 220 mM and 660 mM, respectively. DTT and IAA solutions have to be prepared freshly before starting the experiments. IAA solution has to be kept protected from light. TFA was diluted to 25% with water.

Perform digestion in 0.5 mL LoBind Eppendorf tubes. After each step (e.g. dilution, addition of reagents) the sample has to be vortexed. Dilute 50 µg mAb to 10 µL with the digestion buffer. Add 10 µL of 0.1% Rapigest®, then incubate the sample at 80 °C for 20 min. After denaturation, allow the sample to cool down to room temperature. Add 1 µL 220 mM DTT to the sample and perform reduction at 37 °C for 60 min. After reduction, add 1 µL 660 mM IAA and incubate the sample in the