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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

Table 1
Properties of selected protease enzymes for peptide level applications.

Protease	Trypsin	Lys-C	Arg-C	Asp-N	Glu-C
cleavage sites	C-terminal of Lys, Arg. Does not cleave if Lys and Arg are followed by Pro. Asp and Glu at C-terminal side of Lys and Arg inhibit cleavage	C-terminal of Lys. Does not cleave if Lys is followed by Pro. Asp and Glu at C −terminal side of Lys inhibit cleavage.	C-terminal of Arg. Also cleaves at Lys with lower efficiency.	N-terminal of Asp	C-terminal of Glu. Low level cleavages might occur at Asp residues.
enzyme/protein	1:20–1:100	1:20–1:50	1:20–1:350	1:20–1:200	1:20–1:200
working pH	7–9	8–9	7.6–7.9	4–9	4–9
cleavage buffers	50–100 mM TRIS-HCl or NH_4HCO_3.	50–100 mM TRIS-HCl or NH_4HCO_3.	50 mM TRIS-HCl or NH_4HCO_3, 5 mM $CaCl_2$, 2 mM EDTA, > 2 mM DTT	50 mM TRIS-HCl	50–100 mM TRIS-HCl or NH_4HCO_3.
incubation time	several hours-overnight	several hours-overnight	several hours-overnight	several hours-overnight	several hours-overnight
working temperature	37 °C	37 °C	37 °C	37 °C	37 °C
remarks	Most widely used protease in peptide mapping. TPCK treatment inactivates chymotrypsin contamination and chemical modification (methylation) minimizes autoproteolysis. Resistant to mild denaturing conditions (1–2 M urea or guanidine and 0.1% SDS). Incomplete digestion or overdigestion should be avoided by optimizing the method for a particular protein. Trypsin/Lys-C mix is used to improve general protein digestion. It uses the same conditions as trypsin alone.	Tolerates high denaturing conditions (8 M urea). Used to digest proteolytically resistant proteins. Also used as a trypsin alternative if larger peptides are preferable for the analysis. If urea is used in protein sample preparation, avoid high temperature. High temperature induces protein carbamylation in the presence of urea.	Used in analysis of histone modifications. Requires DTT, cysteine or other reducing agent and $CaCl_2$ for activity	Can be used as a trypsin alternative to achieve better distribution of cleavage sites. Activity is retained in the presence of urea (up to 3.5 M), guanidine (1 M), acetonitrile (up to 60%), EDTA (up to 2 mM); DTT or ß-mercaptoethanol.	Can be used as a trypsin alternative to achieve better distribution of cleavage sites. Activity and cleavage specificity is affected by buffer conditions: in NH_4HCO_3 and other non-phosphate buffers cleaves at C-term of Glu. Glu-C cleaves at C-term of Glu and Asp in phosphate buffer.

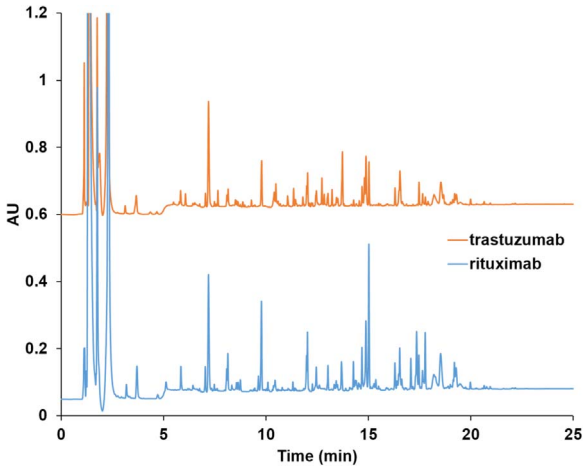

Fig. 1. Peptide level analysis: reversed phase separation of rituximab and trastuzumab peptides following overnight tryptic digestion. Column: Waters Acquity CSH® C18 (130 Å, 1.7 µm, 2.1 mm x 150 mm), mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile. Gradient: 3 min at 2% B, then 2–60% B in 30 min. Flow: 0.3 mL/min, T: 50 °C, injection volume: 5 µL. Data were acquired at 214 nm (5 Hz).

dark at room temperature for 30 min. After alkylation, add the sample to the previously aliquoted 3 µL 0.5 µg/µL trypsin solution. Enzyme/protein ratio is 1:33. Incubate and mix the resulting mixture at 37 °C for overnight (16 h) digestion. Quench reaction by adding 1 µL 25% TFA and incubating the sample at 37 °C for 30 min. The resulting cloudy (Rapigest® precipitates) solution should be centrifuged and the supernatant can be transferred to the HPLC vial. The final sample volume is 25 µL from which around 20 µL can be transferred to the insert for the analysis.

2.1.5. Results of the tryptic digestion

Tryptic peptides of rituximab and trastuzumab were separated by reversed phase liquid chromatography (Fig. 1.). Reagents and hydrophilic peptides eluted close or within to the dead time. Note that small unretained peptides may not be detected when digestion is efficiently performed at the expected cleavage sites. However, they might be present when less specific enzymes are used and miscleavages occur. When using the protocol for performing peptide mapping of mAb, between 90 and 99% sequence coverage can be expected, depending on the sample [9,13,14]. Note that besides having an established sample preparation protocol, the settings employed for data treatment (e.g. existence of post-translational modifications, sequence variations, etc.) as well as the instrument conditions (e.g. sensitivity, resolution, etc.) also play a crucial role to achieve reasonable sequence information and reliable data quality. For full sequence coverage, multiple enzyme digestions (or the use of less specific enzymes) is generally recommended, while for QC purposes the most specific and robust methods should be used.

2.2. Introduction to middle-up proteolysis

Limited proteolysis (middle-up level of analysis) is an alternative approach to bottom-up or intact mAbs analysis, which consists in using digestion with specific proteases for subunit analysis of mAbs and related products. The moderate size fragments of 25–100 kDa possess better chromatographic properties than intact proteins [15], enabling the separation of protein variants. Even minor mass shifts of the subunits related to PTMs can be routinely assessed using high resolution MS instruments. Recent developments in limited proteolysis lead to a balanced approach between peptide mapping and intact protein analysis, thus combining the advantages of relatively simple sample preparation, analysis and data interpretation. The most frequently used enzymes for this approach include papain, pepsin, KGP (lys-gingipain protease from *Porphyromonas gingivalis*), SpeB (recombinant *streptococcal pyogenic* exotoxin B), IdeZ (immunoglobulin-degrading enzyme from *Streptococcus equi ssp. Zooepidemicus*) and IdeS (from *Streptococcus pyogenes*). Pepsin and papain have traditionally been used to generate antibody fragments but their limited specificity, long digestion time and need for extensive digestion method optimization were hardly compatible with general routine analytical workflows [16,17]. Nowadays, IdeS is the most frequently used enzyme in limited proteolysis. The use of IdeS digestion for the analysis of mAbs was first described by Chevreux et al. [18]. IdeS is a bacterial cysteine protease, which specifically cleaves human IgGs under their hinge region (Fig. 2) resulting in Fc/2 (single chain of fragment crystallizable, sFc) and F(ab')2 (full fragment antigen-binding) fragments. This enzyme can be considered today as a gold standard for mAbs analysis. Sjörgen et al. reviewed the most relevant applications of IdeS, including antibody glycosylation evaluation, identity assessment of originator, biosimilar and biobetter

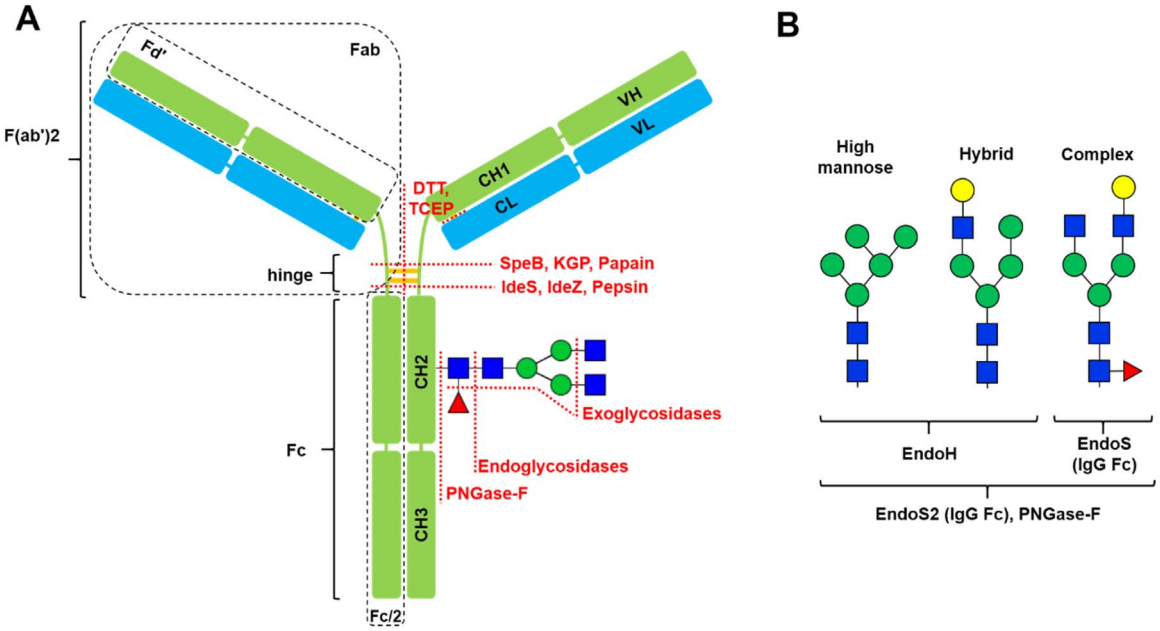

Fig. 2. (A) Cleavage sites of selected enzymes and reducing agents, (B) specificity of selected glycosidases.

Table 2
Properties of selected protease enzymes for middle level applications.

Protease	IdeS	IdeZ	SpeB	KGP	Pepsin	Papain
Specificity	Human IgG1 (moderate efficiency for IgG2a and IgG3)	IgG2a (further optimization may be required)	IgGs under reducing conditions	Human IgG1 under mild reducing conditions (further optimization may be required)	IgG and IgM (extensive optimization is required)	IgG (extensive optimization and preactivation may be required)
Cleavage site	under hinge domain	under hinge domain	over hinge domain	over hinge domain	under hinge domain	over hinge domain
Cleavage buffers	10–20 mM phosphate/TRIS/MES/HEPES/bicarbonate/acetate	10–20 mM phosphate/TRIS/MES/HEPES/bicarbonate/acetate	phosphate, TRIS, acetate	100 mM TRIS	100–200 mM acetate	100–200 mM phosphate 50–100 mM TRIS
Additives	–	–	reducing agents: DTT/TCEP: 1–5 mM cysteine/cysteamine/mercaptoethanol: 50–100 mM	reducing agent: 2 mM cysteine	stop reagent: 2 M TRIS-base	preactivation may be required: 2–3 mM cysteine stop reagent: 0.03 M IAA
c IgG (mg/mL)	0.5–10	0.5–10	0.5–10	0.5–5	1–5	1–5
enzyme/protein	1 U/μg IgG*	1 U/μg IgG*	1 U/μg IgG*	1 U/μg IgG*	1:20–1:100	1:20–1:100
working pH	6–8	6–8	6.5–8	8	4–4.5	5–8
incubation time	30 min	120 min	60 min	60–120 min	1–48 h	6–12 h
working temperature	37 °C	37 °C	37 °C	37 °C	37 °C	37 °C
risks	not known	not known	reduction of IgG thiols	not known	under/over digestion	under/over digestion

* may depend on unit definition.

products as well as identification of many common PTMs [19]. Various authors highlighted IdeS advantages, including simple sample preparation, straightforward data interpretation, limited method artefacts and more easily accessible knowledge to any kind of modifications. Indeed, sample preparation can be performed within an hour with high reproducibility and specificity, making this approach suitable for high-throughput analysis. General digestion protocol suggested by the vendor is usually appropriate. IdeZ is more specific to mouse IgG2a and IgG3, KGP cleaves human IgG1 above its hinge region, while SpeB is non-specific to antibody subclasses and cleaves IgGs above the hinge domain. Again, like in the case of peptide mapping enzymes, each protease used in subunit analysis has its own specificity, optimum conditions and efficiency described in Table 2. The following protocols are suggested for papain and IdeS digestion of IgG1 mAbs. These conditions may serve as a starting point for method development. Further optimization of the sample preparation may be necessary depending on the enzyme source and on the sample itself.

2.2.1. Chemicals and reagents

IdeS (FabRICATOR®, art. A0-FR1-050) was purchased from Genovis AB (Lund, Sweden), papain (art. P4762), TRIS-HCl (art. T3253), L-cysteine (art. 30089), and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, art. E5134) was purchased from Sigma-Aldrich (Buchs, Switzerland).

2.2.2. Laboratory device

The same laboratory device was used, as specified in Section 2.1.2.

2.2.3. Chromatographic system

Middle-up level experiments were performed using a Waters Acquity UPLC® system equipped with a binary solvent delivery pump, an autosampler (loop volume was 5 μL) and fluorescence detector (FL). Samples were analyzed using a Waters Acquity UPLC® Peptide BEH C18 chromatographic column (300 Å, 1.7 μm, 2.1 mm × 150 mm, art. 186003687). Data acquisition and instrument control were performed by Empower Pro 3 Software (Waters). Data processing and visualization were achieved with Excel (Microsoft). Other UHPLC systems and comparable wide pore C18 material 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. A generic linear gradient from 30 to 38% B in 12 min was performed. Flow rate was set to 0.4 mL/min, column temperature was 80 °C. Injection volume was set to 3 μL in partial loop mode. Data were acquired using 280 nm excitation and 360 nm emission wavelengths with 5 Hz sampling rate and 0.4 s time constant. Detection can be performed also in UV at 214 and/or 280 nm.

2.2.4. Preparation of the reagents and samples

Samples and reagents should be stored according to instructions from providers. 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 month at −80 °C.

2.2.4.1. IdeS digestion. Reconstitute 5000 U lyophilized IdeS enzyme in 75 μL water (67 U/μL). This enzyme solution can be aliquoted to 2 μL fractions in Eppendorf tubes. The aliquots can then be stored up to 6 months at −20 °C. Digestion solvent is 10 mM TRIS buffer at pH 7.5. Dilute protein sample to 1 mg/mL with the digestion buffer and add it to the IdeS enzyme aliquot. Final sample volume should be 100 μL. Incubate and mix the resulting solution at 37 °C for 30 min. Samples can then be transferred to HPLC vial inserts. For minimizing sample transfer steps, vial inserts can be used for digestion, or injection can be performed directly from Eppendorf tubes. Note that in this case the

inserts should fit to the thermomixer, or the injector of the LC system should be able to inject from these tubes.

2.2.4.2. Papain digestion. Digestion buffer for papain is 50 mM TRIS buffer at pH 7.5. Prepare papain solution at 250 µg/mL in digestion buffer. The final papain/protein ratio is 1:20. Prepare 10 mM L-cysteine and 50 mM EDTA solutions in digestion buffer. L-cysteine and papain solutions should be freshly prepared before performing the experiments. Dilute 25 µg mAb to 15 µL with the digestion buffer using 0.5 mL Eppendorf tubes. Add 3 µL 10 mM L-cysteine, 2 µL 50 mM EDTA and 5 µL 250 µg/mL papain to the mAb solution. The final sample volume is 25 µL, and the solution should be mixed and incubated at 37 °C for 3–4 h. Depending on the papain source and the protein, even longer incubation time may be necessary for complete digestion. Prepared samples can either be transferred into conical glass inserts or injected directly from Eppendorf tubes.

2.2.5. Results of middle-up level papain and IdeS proteolysis

Rituximab and trastuzumab samples were prepared according to the above described protocols and analyzed by RPLC, as described in Section 2.2.3. The resulting chromatograms of the digested samples are shown in Fig. 3. The IdeS digestion of both mAbs was completed within 15–30 min, while papain required several hours for digestion. Papain digestion of trastuzumab was still incomplete even after 4 h of incubation. Finally, the cost of papain remains significantly lower than the one of IdeS digestion.

2.3. Introduction to N-deglycosylation

Glycosylation is probably the most common and relevant PTM of therapeutic proteins. Carbohydrate structures are attached either to Asp (*N*-glycosylation) or Ser/Thr (*O*-glycosylation) residues. Glycosylation pattern of therapeutic proteins affects their stability, bioactivity and pharmacokinetics and is considered as a critical quality attribute (CQA) that must be monitored during manufacturing and in the finished product [20]. The recent work of Largy et al. described the comprehensive characterization of both *O*- and *N*-glycosylation of therapeutic glycoproteins using LC/MS methods. An optimized sample preparation workflow was presented suitable for detailed analysis in regulated environment [21]. Zhang et al. reviewed the most relevant analytical approaches in glycan analysis of therapeutic proteins from monosaccharide to intact protein level [22]. Here, we focus only on new trends in sample preparation. Historical methods for glycan analysis are slow and laborious and require excessive optimization. Those multistep methods generally involve the denaturation and alkylation of the protein, chemical or enzymatic release of the glycans from peptides or proteins, the purification of the glycans, the labelling with fluorophore and/or mass spectrometric entities and finally, removal of the excess labelling reagent from sample before analysis. Since the details and acceptance criteria for glycan analysis are not defined by the authorities [23,24], there is no standard protocol for the analysis of mAbs glycosylation. Generally, simultaneous use of orthogonal methods is required for correct structural elucidation and quantitation [25]. In spite of the availability of various innovative approaches, released glycan analysis remained the reference method up to now. *N*-glycans are usually released by specific enzymes, while chemical treatment cleaves both *N*- and *O*-glycans [26,27]. When performing glycan release it should be kept in mind that chemical treatment (hydrazinolysis or β-elimination in alkali/reducing conditions) might degrade the protein, while enzymatic cleavage may be more specific to certain glycan structures,

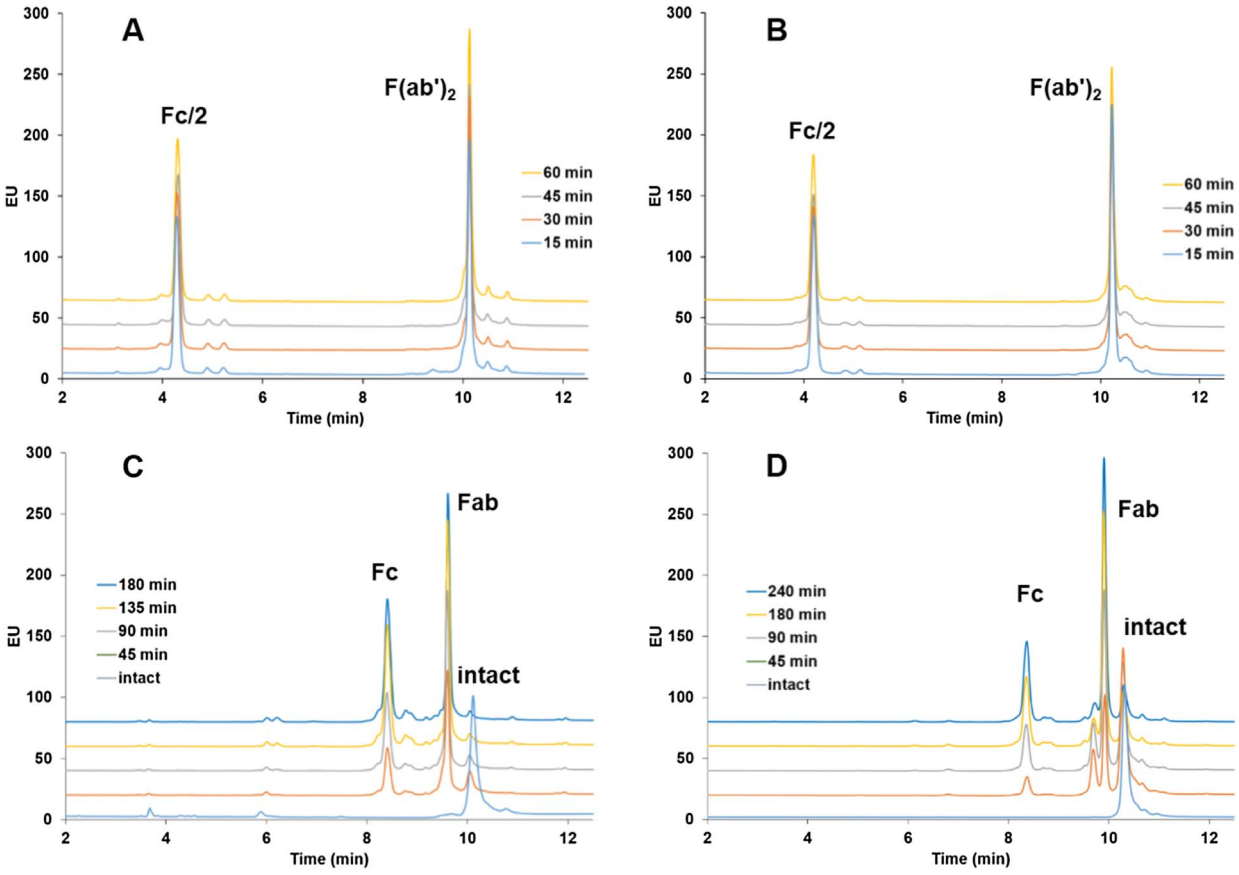

Fig. 3. Middle-up approach: enzymatically fragmented mAbs using various reaction times. (A) IdeS digested rituximab, (B) IdeS digested trastuzumab, (C) papain digested rituximab and (D) papain digested trastuzumab. Column: Waters Acquity UPLC® Peptide BEH C18 (300 Å, 1.7 µm, 2.1 mm × 150 mm), mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile. Gradient: 30–38% B in 12 min. Flow: 0.4 mL/min, T: 80 °C, injection volume: 3 µL, detection: fluorescence at λ_{ex} 280 and λ_{em} 360 nm (5 Hz).

Table 3
Properties of selected enzymes for deglycosylation.

Glycosydase	PNGase F	EndoH	EndoS	EndoS2
Specificity	various glycoproteins and glycopeptides: high mannose, complex and hybrid oligosaccharides	various glycoproteins: high mannose and limited number of hybrid oligosaccharides	Complex glycans on IgG Fc domains	Complex, hybrid and high mannose glycans on IgG Fc domains
cleavage sites	between the Asn and innermost *N*-Acetylglucosamine (GlcNAc) residues	cleaves between the *N*-acetylglucosamine residues of the chitobiose core of N-linked glycans (cleaves after the first GlcNAc)	cleaves between the *N*-acetylglucosamine residues of the chitobiose core of N-linked glycans (cleaves after the first GlcNAc)	cleaves between the *N*-acetylglucosamine residues of the chitobiose core of N-linked glycans (cleaves after the first GlcNAc)
enzyme/protein	1:250–1:500	200–500 U/µg IgG*	1 U/µg IgG*	1 U/µg IgG*
working pH	6–10	5–6	7.4	7.4
cleavage buffers	50 mM NH_4HCO_3 or phosphate (0.5% SDS, 50 mM DTT may be required for denaturation)	50 mM NH_4HCO_3 or phosphate (0.5% SDS, 50 mM DTT may be required for denaturation)	10 mM phosphate, 150 mM NaCl	10 mM phosphate, 150 mM NaCl
incubation time	several hours-overnight	several hours-overnight	30 min	30 min
working temperature	37 °C	37 °C	37 °C	37 °C
remarks	Rapid® PNGase-F requries 10 min incubation time. Detergents may affect enzyme activity.	EndoH does not cleave complex glycans	no denaturation of the substrate is required	no denaturation of the substrate is required

* may depend on unit definition

showing biased picture of the real glycosylation pattern. Both methods may be affected by detergents used for denaturation, and deglycosylated proteins may precipitate due to their limited solubility in aqueous buffers. Fig. 2 shows the cleavage sites and specificities of glycosydases, while their optimum conditions are presented in Table 3. The reference glycosydase is the recombinant PNGase F (from *Elizabethkingia miricola*), which cleaves high mannose, complex and hybrid oligosaccharides between the asparagine (Asn) and innermost N-acetylglucosamine residues of glycopeptides and glycoproteins. Additionally, endoglycosydases can be used for glycan release, which cleave within the chitobiose core of glycans. Among other endoglycosidases, EndoH (*Streptomyces plicatus*), EndoS and EndoS2 (*Streptococcus pyogenes*) are the most widely used alternatives to PNGase F. EndoH is specific to high mannose and hybrid glycans, while EndoS is specific to complex ones [25,26]. EndoS2 shows specificity to all of these groups [28–31]. Sample preparation time may be shortened by using Rapid PNGase F or EndoS and EndoS2 glycosydases, requiring less than one hour for the deglycoslylation. Additionally, EndoS and EndoS2 cleave under physiological conditions without the need for denaturation with surfactants and DTT. Glycan release may also be applied for simplifying MS data of complex protein samples by removing glycan variation. Generally, if analysis of glycosylation is not the objective of the study, it is valuable to include this step into sample preparation for a number of reasons: i) deglycosylation considerably simplifies mass spectra ii) peaks of glycovariants will coelute in a single peak, thus increasing signal to noise ratio (S/N) and sensitivity iii) deglycosylation reduces the probability of peak overlapping in the mass spectra obtained by low resolution instruments [32]. As an example, mass spectra of antibody drug conjugates [33,34] and polyclonal IgGs [35] was found much easier to interpret after deglycosylation. Released glycans can be further fragmented for in-depth structural analysis and structural validation using tandem mass spectrometry and exoglycosidase digestion [32,36–38].

The following protocols describe suggested sample preparation for deglycosylation using PNGase F, Rapid® PNGase F and EndoS2 enzymes. Glycosylated and deglycosylated species can be separated in HILIC conditions, while they usually coelute in RPLC. For this reason, deglycosylation was followed by hydrophilic interaction liquid chromatography. These conditions may serve as starting point for method development.

2.3.1. Chemicals and reagents

PNGase F (art. P0704S) and Rapid® PNGase F (art. P0710S) enzymes were purchased from New England Biolabs Inc. (Ipswich, MA, USA). Reaction buffers, denaturation buffer and NP40 detergent for PNGaseF and Rapid® PNGase F deglycosylation were provided together with the enzymes. EndoS2 enzyme (GlycINATOR®, art. A0-GL1-020) was purchased from Genovis AB (Lund, Sweden). NaH_2PO_4 (art. S8282) and NaCl (art S7653) were purchased from Sigma–Aldrich (Buchs, Switzerland). FDA approved mAb samples were kindly provided by Centre d'Immunologie Pierre Fabre (Saint-Julien en Genevois, France).

2.3.2. Laboratory device

The same laboratory device was used as specified in Section 2.1.2.

2.3.3. Chromatographic system

The same chromatographic system was used as specified in Section 2.1.3. with a Waters Acquity UPLC® Glycoprotein Amide chromatographic column (300 Å, 1.7 µm, 2.1 mm × 150 mm, art. 186007963). Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. A linear gradient from 85 to 72% B in 0.2 min, then from 72 to 64% B in 10 min was performed for EndoS degylcosylated mAbs (protein level) and a gradient from 95 to 75% B in 0.2 min, then from 75 to 67% B in 10 min was run for PNGaseF and Rapid® PNGase F deglycosylated mAbs (subunit level). Flow rate was set to 0.45 mL/min, column temperature was 60 °C. Injection volume was 0.5 µL. Data were acquired using 280 nm excitation and 360 nm emission wavelengths

with 5 Hz sampling rate and 0.4 s time constant.

2.3.4. Preparation of the samples and reagents

Sample handling and storage conditions are similar to Section 2.2.4.

2.3.4.1. PNGase F deglycosylation. Deglycosylate samples in LoBind Eppendorf tubes. Combine 20 µg mAb, 1 µL "10× Glycoprotein Denaturing Buffer" and water to have 10 µL total reaction volume. Vortex and heat the mixture at 100 °C for 10 min. After denaturation, add 2 µL "10× G7 Reaction Buffer", 2 µL 10% NP40, 5 µL water and 1 µL PNGase F to the sample. Then, incubate the sample at 37 °C for 1 h according to the provider protocol. After deglycosylation, the samples can be transferred to HPLC vial inserts.

2.3.4.2. Rapid® PNGase F deglycosylation. Deglycosylate samples in LoBind Eppendorf tubes. Dilute 20 µg mAb to have 16 µL sample, then add 4 µL "5× Rapid PNGase F Buffer" and 1 µL Rapid PNGase F enzyme. Vortex and incubate the mixture at 50 °C for 15 min. After deglycosylation, the samples can be transferred to HPLC vial inserts.

2.3.4.3. EndoS2 deglycosylation. Reconstitute 2000 U lyophilized EndoS2 enzyme in 50 µL water (40 U/µL). This enzyme solution can be aliquoted to 1 µL fractions in Eppendorf tubes. The aliquots can be stored for up to 1 month at 4 °C. Digestion solvent is 10 mM sodium phosphate buffer with 150 mM NaCl at pH 7.4. Dilute protein sample to 1 mg/mL with the digestion buffer and add diluted sample to the EndoS2 enzyme aliquot. Final sample volume is 40 µL. The resulting solution should be thermostated and mixed at 37 °C for 30 min. After deglycosylation, the samples can be transferred to HPLC vial inserts.

2.3.5. Results of deglycosylation

Rituximab and trastuzumab samples were prepared according to the above described protocols and analyzed by HILIC, as described in section 2.3.3. The resulting HILIC chromatograms of the deglycosylated samples are shown in Fig. 4. As expected, deglycosylated species are less hydrophilic and therefore less retained than glycosylated ones [39,40]. Both rituximab and trastuzumab have been deglycosylated within 30 min, regardless of the glycosidase. Here we used reducing conditions for PNGase F and Rapid® PNGase F glycan release, which resulted in the dissociation of heavy chain (HC) and light chain (LC) fragments. Note, that PNGase F can be used with non-denaturating conditions, too. In this case overnight incubation at lower temperature may be necessary according to the protocol of the provider. EndoS2 preserved the native-like structure of the deglycosylated mAbs.

2.4. Introduction to reduction

Chromatographic and MS profiles of therapeutic proteins can be simplified by enzymatic treatment and/or reduction. Reduction of the disulfide bridges between heavy chains and light chains of mAbs leads to fragments possessing molecular weights of 25 and 50 kDa. Measuring the molecular weight of HC and LC provides rapid and cost effective sequence information, which facilitates the identification of PTMs and sequence variations. This approach now has become a routine technique for the analysis of mAbs. The most widely used chemicals for reduction are DTT and TCEP [9]. Both provide effective, cheap and fast solutions for the reduction of mAbs and related proteins. 1–10 mg/mL protein sample should be treated with around 50–100 mM DTT or TCEP. Lower reductant concentration may result in incomplete reduction. Using elevated temperatures of 40–80 °C and/or addition of guanidine-HCl, urea or Tris-HCl in few moles affects the protein structure, making it more accessible for reduction. DTT is an efficient reducing agent for inter-chain disulfides, while TCEP in combination with a denaturant is able to rapidly reduce both inter- and intra-chain disulfides. It is worth mentioning that certain conditions, e.g. extended reduction (high temperature and/or long reaction time) may create unwanted species and therefore additional peaks can appear on the chromatogram [9,41]. The addition of chaotropic agents can result in adducts observed in mass spectra [42]. DTT is more frequently applied than TCEP, even TCEP is more stable and odorless. DTT reduction can be quenched by acidification of the sample, while TCEP generally works in a wider pH-range (e.g. pH 2–8). Alkylation of the resulting sulfhydryl residues can minimize reformation of disulfide bonds, however it is usually not necessary if samples are analyzed within a limited time (e.g. few hours)

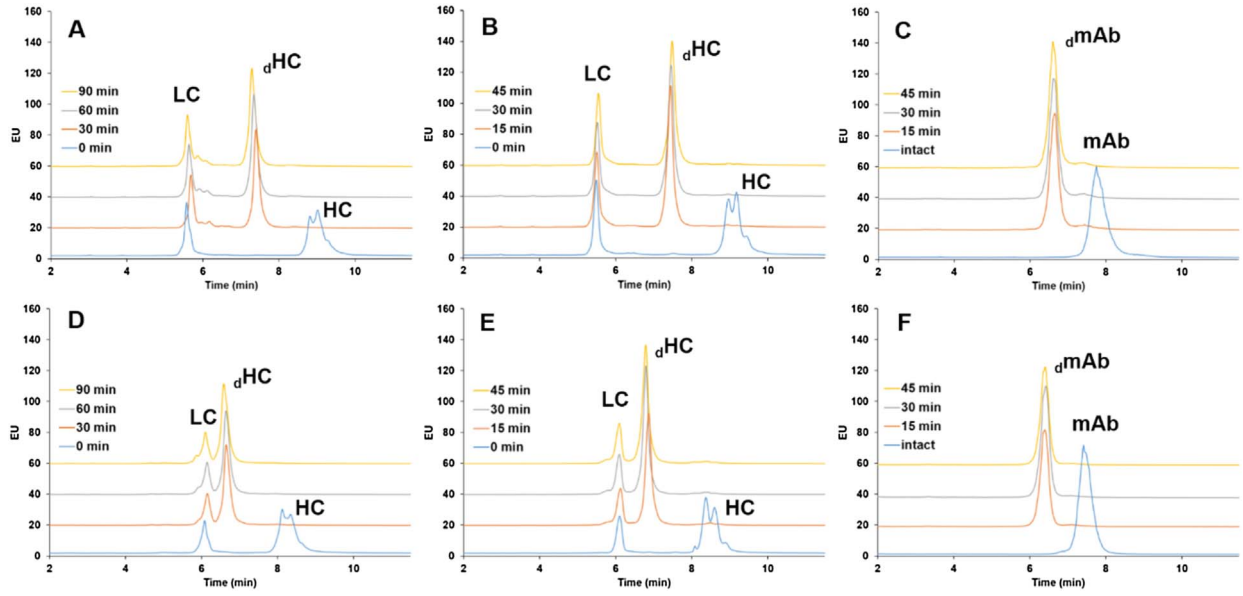

Fig. 4. HILIC chromatograms of deglycosylated mAbs using various reaction times. Rituximab (A–C) and Trastuzumab (D–E) deglycosylated by PNGase F (A, D), Rapid® PNGase F (B, E) and EndoS2 (C, F). Column: Waters Acquity UPLC Glycoprotein BEH Amide (300 Å, 1.7 µm, 2.1 mm × 150 mm), mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile. Gradient: 85–72% B in 0.2 min, then 72–64% B in 10 min for EndoS degylcosylated mAbs (protein level) and 95–75% B in 0.2 min, then 75–67% B in 10 min for PNGaseF and Rapid® PNGase F deglycosylated mAbs (subunit level). Flow: 0.45 mL/min, T: 60 °C. Injection volume 0.5 µL (flow through needle), detection: fluorescence at λ_{ex} 280 and λ_{em} 360 nm (5 Hz). $_{d}$HC and $_{d}$mAb denotes deglycosylated heavy chain and deglycosylated mAb, respectively.

after the reduction [38], or the reduction has not been quenched and excess amount of DTT/TCEP is present in the sample. The following protocols describe the DTT reduction of inter-chain disulfides and the TCEP/guanidine-HCl reduction of both inter- and intra-chain disulfides of two IgG1 mAbs.

2.4.1. Chemicals and reagents

DTT (art. 43815), TCEP-HCl (art. C4706) and guanidine-HCl (art. G4505) were purchased from Sigma-Aldrich (Buchs, Switzerland).

2.4.2. Laboratory device

The same laboratory device has been used as described in Section 2.1.2.

2.4.3. Chromatographic system

The same chromatographic system has been used as described in Section 2.2.3.

2.4.4. Preparation of the reagents and samples

Sample handling and storage conditions are similar to Section 2.2.4.

2.4.4.1. Inter-chain disulfide reduction by DTT. Dilute protein sample to 1 mg/mL with water. Final sample volume is 100 µL. Dissolve DTT in water at 1 M concentration. DTT solution should be freshly prepared before performing the experiments. Add 10 µL of 1 M DTT solution to 90 µL 1 mg/mL mAb solution. Final DTT concentration is 100 mM. Vortex and incubate the sample at 45 °C for 30 min. The prepared samples should then be analyzed within 24 h when kept at 4 °C.

2.4.4.2. Inter- and intra-chain disulfide reduction by TCEP/guanidine-HCl. Dilute protein sample to 1 mg/mL by water. Place 38 ± 0.5 mg solid guanidine-HCl into the Eppendorf tube. Dissolve TCEP in water at 1 M concentration. TCEP solution should be freshly prepared before performing the experiments. Add 90 µL of 1 mg/mL protein sample and 10 µL of 1 M TCEP to the solid guanidine-HCl. The final sample volume is around 120 µL, final guanidine-HCl concentration is around 4 M and final TCEP concentration is around 100 mM. Vortex samples until complete dissolution of the solid material and incubate at 45 °C for 30 min. The prepared samples should then be analyzed within 24 h when kept at 4 °C.

2.4.5. Results of DTT and TCEP/guanidine-HCl reduction

Rituximab and trastuzumab samples were prepared according to the above described protocols and analyzed by RPLC, as described in Section 2.2.3. The resulting chromatograms of the digested samples are shown in Fig. 5. DTT completely reduced inter-chain disulfide bridges of both mAbs within 15–30 min. Longer reaction time may result in partial reduction of intra-chain disulfides. TCEP/guanidine-HCl completely reduced both inter- and intra-chain disulfide bridges within 30 min. Shorter reaction time may lead to incomplete reduction of intra-chain disulfides. Based on our experience, DTT combined with guanidine-HCl and TCEP alone are less efficient in intra-chain disulfide reduction, leading to highly heterogeneous sample containing partially reduced intra-chain disulfides.

2.5. Combination of limited proteolysis and reduction

As previously discussed, sub-unit analysis is nowadays widely used in mAbs analysis, combining the possibility of site specific determination of various modifications and the ease of sample preparation. Combined sample preparation, (e.g. the use of multiple enzymes and/or enzymatic digestion, followed by disulfide bond reduction) can further increase the data quality and selectivity of the method. Here, we wanted to highlight the possibility to perform combined sample preparation with limited proteolysis and disulfide bond reduction.

IdeS digestion experiments and/or reduction are probably the two most widely used middle-up level approaches [43–47]. Prior to disulfide bond reduction, digestion with IdeS can be performed in a one-pot manner within a reasonable preparation time. The whole analysis time including sample preparation and LC–MS analysis can be reduced down to 1–2 h [2,48] making the method appropriate for high throughput analysis. This approach results in 2 LC, 2 Fc/2 (or sFc) and 2 Fd' (a portion of the heavy chain which is included in the Fab fragment) fragments of ~25 kDa, which are more easily analyzed by LC-ESI/MS [9,49,50]. Top down sequence analysis of such sub-units performed by gas phase fragmentation approaches allows obtaining comparable sequence coverage as the one achieved by traditional peptide mapping. However, fragmentation is often incomplete for sub-units, therefore traditionally used bottom-up methods may be required to increase sequence coverage and localize PTMs [9].

Fig. 6 shows chromatograms of rituximab and trastuzumab prepared using various middle-up level fragmentation methods. The above described sample preparation protocols were used. Digestion was followed by reduction, the digestion temperature was increased from 37 °C to 45 °C. This minor modification simplified the sample preparation, since the whole process could be performed in a single pot, at constant temperature. The slight increase of temperature did not modify the chromatographic profiles. In combined sample preparation experiments, 1 M DTT solution was added to the digested sample in a volume maintaining 100 mM final DTT concentration. Note that the localization of the disulfide containing hinge region is different after papain and IdeS digestion. For this reason, the Fc/2 and Fd fragments are not identical in the digested and reduced samples.

3. Conclusion

The present paper was aimed to share basic information on sample preparation for the characterization of therapeutic monoclonal antibodies and related products. Recent and most relevant proteolytic, chemical and combined treatment approaches were described. Generic protocols were provided for peptide mapping after tryptic digestion, protein fragmentation using papain and IdeS, deglycosylation using PNGase F and EndoS2, and finally, chemical reduction using DTT and TCEP/guanidine-HCl for disulfide cleavage. Applicability of the protocols was illustrated by analyzing two commercially available mAbs, namely rituximab and trastuzumab. At bottom-up level, expected sequence coverage for overnight tryptic digestion is around 90–99%, depending on the sequence, on the source of trypsin and the reaction conditions. At middle-up level, both rituximab and trastuzumab were fully fragmented to F(ab')2 and Fc/2 subunits by IdeS within 15–30 min. As expected, papain showed some differences in efficacy of cleaving the mAbs to Fc (fragment crystallizable) and Fab (fragment antigen-binding) sub-units, and the complete reaction may require 3–4 h or even more. DTT efficiently reduced the inter-chain dislufides, while TCEP with guanidine-HCl reduced both the inter- and intra-chain disulfide bridges within 30 min resulting in heavy chain and light chain fragments. Finally, rapid deglycosylation was performed by PNGase F, Rapid PNGase F and EndoS2 within 15–30 min for both mAbs. The possibility to combine sample preparation was illustrated by successive limited proteolysis and reduction in a single pot manner. Papain and IdeS digested samples were reduced by DTT to convert intact proteins into LC, Fc/2* and Fd, as well as LC, Fc/2 and Fd* fragments, respectively. With keeping in mind that the risk of producing sample preparation artefacts is always present and has to be carefully evaluated [15,51]. These protocols may be used as described, or can serve as a starting point for further sample preparation optimization, allowing the structural elucidation and/or quality assurance purposes.

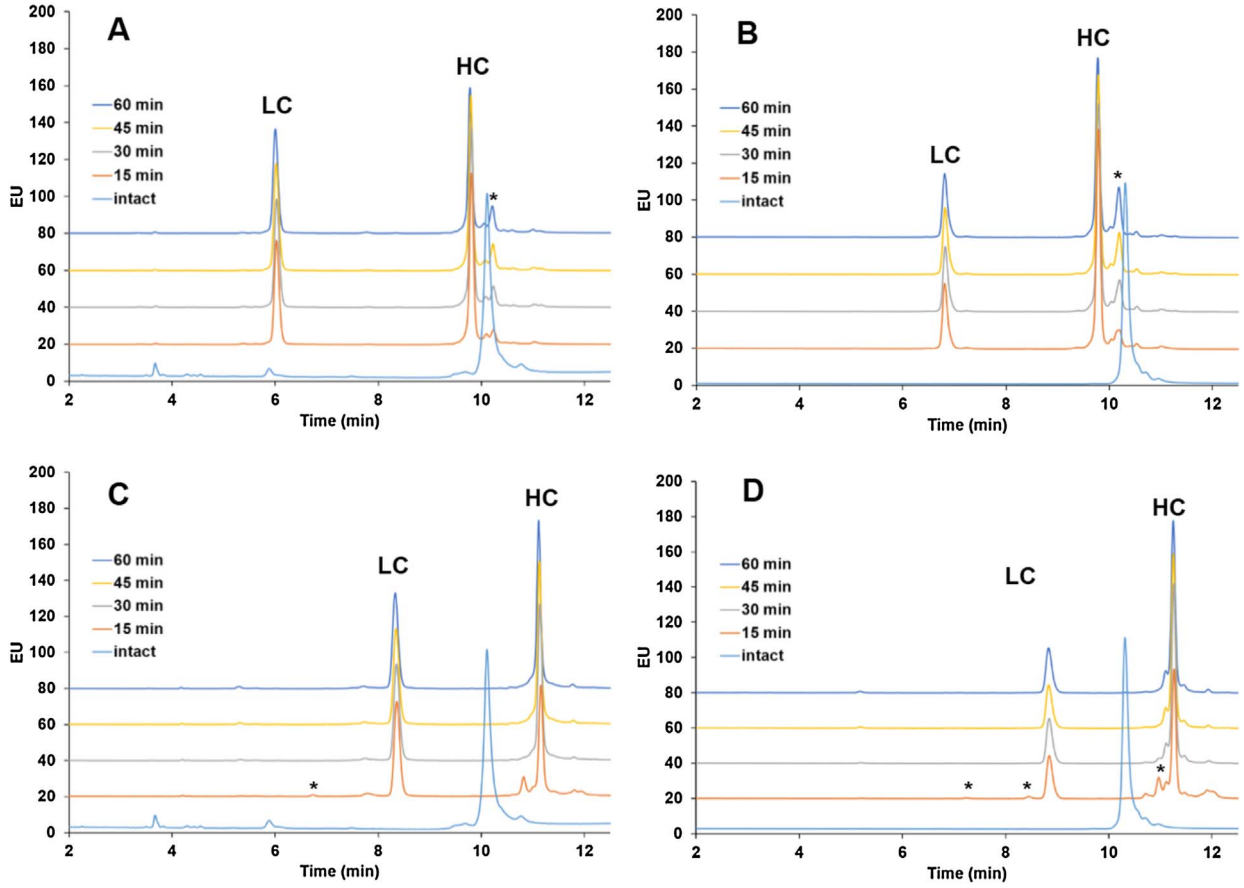

Fig. 5. RPLC chromatograms of chemically fragmented (reduced) mAbs. Rituximab (A and C) and Trastuzumab (B and D) reduced by DTT (A, B) and TCEP/guanidine-HCl (C, D) using various reaction times. Column: Waters Acquity UPLC® Peptide BEH C18 (300 Å, 1.7 µm, 2.1 mm × 150 mm), mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile. Gradient: 30–38% B in 12 min. Flow: 0.4 mL/min, T: 80 °C, injection volume: 3 µL, detection: fluorescence at λ_{ex} 280 and λ_{em} 360 nm (5 Hz).
*partially reduced intra-chain disulfide variants.

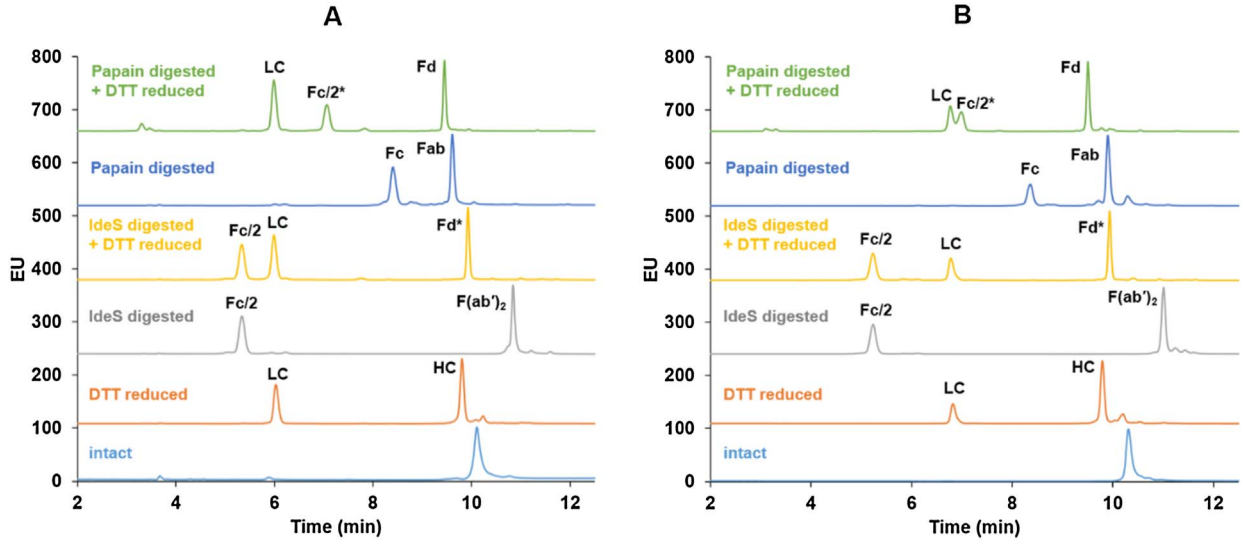

Fig. 6. RPLC chromatograms of reduced, digested, and reduced + digested (A) rituximab, (B) trastuzumab. Column: Waters Acquity UPLC® Peptide BEH C18 (300 Å, 1.7 µm, 2.1 mm × 150 mm), mobile phase A: 0.1% TFA in water, mobile phase B: 0.1% TFA in acetonitrile. Gradient: 30–38% B in 12 min. Flow: 0.4 mL/min, T: 80 °C, injection volume: 3 µL, detection: fluorescence at λ_{ex} 280 and λ_{em} 360 nm (5 Hz).
*subunit + hinge disulfide containing sequence.

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