Intact Protein Multi-Attribute Method (MAM) that Includes the Identification and Quantification of Protein Clipping Events

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Overview

- Proteolytic enzymes or chemical hydrolysis can cause clipping events in biopharmaceuticals.
- Clipping needs to be monitored as **CQA** to develop processes yielding low levels of clipped side products.
- Identification of clipping variants is a first step to optimize the process minimizing side products.
- Specific workflows in the BioPharma Compass (**BPC**) software to detect and identify such protein clipping variants, which we applied to two antibody models:
- **NISTmAb IdeS digest:** detection of production process related clipping variants.
- **Panitumumab (IgG2κ) SpeB digest:** elucidation of the unknown proteolytic activity of SpeB in IgG2. (*Note: in IgG1 SpeB cleaves specifically near the hinge region*)

Methods

Sample Handling

The mAbs were digested using IdeS or SpeB (both Genovis), denatured, reduced and separated by reversed phase chromatography. Intact subunit monoisotopic molecular weight (**MW**) and Middle-Down Sequencing (**MDS**) spectra were acquired on a maXis II ETD ultra-high resolution QTOF and a rapifleX MALDI-TOF/TOF (all Bruker), respectively. Middle-Down sequencing spectra were acquired by MALDI-ISD and ESI-MS after capillary- and analytical flow LC, respectively.

Data Analysis

Based on the LC and HC sequences, all possible clipping variants were computed using BioPharma Compass 2021 (Bruker) in an automatic workflow. Experimental data based on intact masses or MDS were automatically matched to the corresponding N- and C-termini of the clipping products.

Results

LC-ESI-MS based screening for clipping variants in NISTmAb IdeS digest

The dataset was screened for clipping variants using the NISTmAb subunit sequences with variable modifications (**Fig.1**). Major clipping sites were found at Fd: D88|P89 and Fc: D34|P35; a minor site at Fc/2: C85|K86.

The MW based screening allowed to detect internal cleavage products directly, while the MDS screening requires one terminus to be included to reduce analysis time.

LC-MALDI-ISD based MDS screening of cleavage sites of SpeB in panitumumab

All MALDI-ISD spectra were screened against all possible truncation products that include either the N- or the C-terminus.

The major SpeB cleavage site HC:E224|C225 was identified amid the 4 cysteine crosslinks of the hinge region, thus establishing the Fc/2 and Fd termini (**Fig.3**) of the primary SpeB cleavage products.

These were used in a 2nd round search to find multiple unspecific cleavage sites in all subunits and, again, P|D as major chemical hydrolysis site (**Fig.2**).

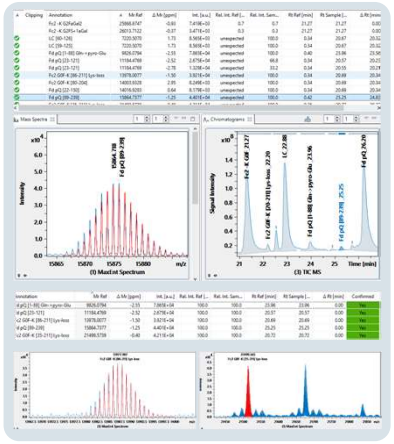

Fig. 1 LC-ESI-QTOF-MS analysis of NISTmAb clipping products

Top Screening for clipping variants in BPC is a workflow option. The result is shown here with clipping variant candidates check-marked.

The total ion chromatogram displays possible clipping variants, the deconvoluted mass spectrum was calculated from the selected chromatographic peak and the assignment was confirmed by the matching calculated isotope pattern (red).

Center List of clipping variant candidates that were accepted in the software for further investigation.

Bottom Candidates were validated using the match with the theoretical isotope pattern and the link to known modification profiles (here: G0F/G1F/G2F).

Also MDS (**see Fig.2**) or tryptic digest analyses are very suitable to validate clipping sites.

Fig. 2 MALDI-ISD analysis of panitumumab clipping variants

Top MALDI-ISD spectrum matching panitumumab clipping product LC-[75-214], 90% of the sequence were confirmed and both termini safely assigned.

Center LC-MS Survey view of the LC-MALDI-MS dataset in which the unspecific cleavage products from the panitumumab digestion with SpeB are highlighted. Colour code indicates which fragments originated from the same subunit.

Bottom Panitumumab HC and LC sequences with all identified cleavage sites from a single LC run.

Fig. 3 Main cleavage site of endoprotease SpeB in human IgG2 panitumumab

MALDI-ISD spectrum from fraction in which Panitumumab Fd fragment Mr 23968.6 was detected after SpeB cleavage. The cleavage site was obseved at E224|C225. The MALDI-ISD spectrum of the corresponding Fc/2 fragment confirmed the cleavage site (data not shown).

Conclusions

- Protein clipping variants were identified by intact mass as well as by Middle-Down-Sequencing.
- Detection of clipping events is the basis to reduce clipping variant-related side activities in biopharmaceuticals.
- The clipping analysis workflows in BioPharma Compass were also used to identify unspecific cleavage products of SpeB digestion in IgG2 – in contrast, IgG1 hinge is cleaved very specifically.

BioPharma