ABRF-PRG-08 Study Anonymous Participant: 27960

Methods used

LC-MALDI prep: CAP-HPLC (Agilent 1200), monolithic PS-DVB column I.D. 200 µm (Dionex), 2µl/min flow rate, 45 min separation gradient (A: 0,1% TFA; B: ACN + 0,1% TFA; 20-40%B), fraction collection onto MTP AnchorChip targets with PROTEINEER fc (all Bruker Daltonics), 30 sec. collection time per fraction
MALDI-TOF/TOF: ultraflex III (Bruker Daltonics) with 200 Hz smartbeam laser, linear, reflector mode, LID (PSD), CID and reISD fragmentation options.
Software: BioTools 3.1 (Bruker) for all detailed analysis, Top-Down analysis and sequence tag generation, MS-BLAST (EMBL) for Sequence tag BLAST queries, Mascot (Matrix Science) for Bottom-Up protein ID.

Top-Down

1. Samples A and B were MW analyzed by MALDI-TOF MS, using sinapinic acid on MTP AnchorChip 2000/384 (Bruker). 3 molecular species were detected: 12519 Da, 24587 Da (both: B only) and 33170 Da (samples A and B).
2. After reduction and alkylation, Sample A was spotted on a stainless steel target using Sinapinic acid as matrix and analyzed by reflector mode In Source Decay (reISD) . Protein RAGE_HUMAN was identified by automated Sequence Tag determination in the BioTools 3.1 software (Bruker) combined with an MS-BLAST search (EMBL). With the known N-terminal sequence GSHM and the Top-Down analysis features in BioTools, the N-terminus was determined by elimination of a pre-peptide sequence and the N-terminal addition of GSHM. Using the intact protein mass, the C-terminus was predicted and confirmed by c-terminal ISD fragment ions y and z. A match with the intact MW with < 100 ppm was achieved and Glu-C identified as endoproteinase well suitable for the Bottom-Up approach.
3. Sample B was analyzed by direct LC-MALDI on MTP AnchorChip 2000/384 targets with sinapinic acid sheath flow. ReISD Top-Down sequencing spectra were obtained from the 3 chromatographically separated species. All provided identical c-ion patterns (identical N-termini!) but different y/z ion patterns (C-terminal heterogeneity!). Different extend of C-terminal truncation provided a) matching intact MWs derived from the sequence in Sample A, and b) matching y/z-ions: 33kDa: modified RAGE_HUMAN (1-249), 25 kDa: (1-225), 12.5 kDa: (1-114).

Basically, the analysis was completed at this point, as the proper MWs and N-terminal/C-terminal matches of the Top-Down analysis provided sufficient evidence to establish the complete sequences of 3 different C-terminal truncation forms of one protein. Sample A contained one form of the protein and sample B the same protein plus 2 C-terminally truncated forms.
The only possible sequence variations that could not be excluded by the existing data were those leaving the terminal sequences and the protein MWs largely unchanged. With that restriction, even at that point 100 % sequence coverage was achieved for the 3 protein forms.

Bottom-Up

4. Both samples were Glu-C digested and analyzed by LC-MALDI on Prespotted AnchorChip targets (PACs, Bruker). The LC-MS/MS data were matched against the proposed sequences without any enzyme restriction.
5. 79 peptides covered 100 % of the sequence of the 33 kDa species in sample A.
6. 100 % of the 12.5 kDa species sequence in sample B were matched to 35 peptides
7. The C-terminal sequence of the 24 kDa was not directly detected by this Bottom-Up analysis although its mass was known from the protein sequence obtained from the Top-Down work.

The Bottom-Up analysis confirmed the Top-Down findings and, together, provided 100 % sequence coverage for all 3 detected protein forms on the basis of peptide MS/MS spectra matching to "No enzyme" sequence specificity. If only peptides were considered that perfectly followed the nominal Glu-C specificity, sequence coverage would be significantly lower for the Bottom-Up approach.