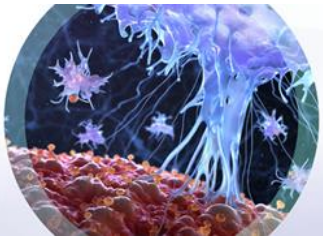

Top-down proteomics driven by high-sensitivity CID and EAD LC-MS/MS fragmentation

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This technical note describes a top-down proteomics workflow for the analysis of intact protein mixtures using multiple MS/MS fragmentation strategies on the ZenoTOF 8600 system. The superior sensitivity of the ZenoTOF 8600 system enables increases in protein sequence coverage by as much as 24% over previous-generation ZenoTOF systems. The ability to generate protein MS/MS data using either collision-induced dissociation (CID) or electron activated dissociation (EAD) pathways provides maximum versatility and complementarity for deep protein characterization.

Key features of top-down proteomics using CID and EAD MS/MS fragmentation on the ZenoTOF 8600 system

- **Streamlined protein characterization workflow:** Top-down proteomics eliminates the need for enzymatic digestion, thereby enabling direct characterization of post-translational modifications and enhanced sequence information in certain regions of proteins.
- **Superior sensitivity:** Protein sequence coverage is improved by as much as 24% relative with top-down proteomics workflows on the ZenoTOF 8600 system compared to the previous-generation ZenoTOF 7600+ system.
- **Powerful, versatile MS/MS strategies:** Fast, tunable EAD-based MS/MS offers complementary protein fragment information relative to CID-based MS/MS. Protein sequence coverage improves as much as 36% using EAD-MS/MS versus CID-MS/MS with the top-down proteomics workflow.

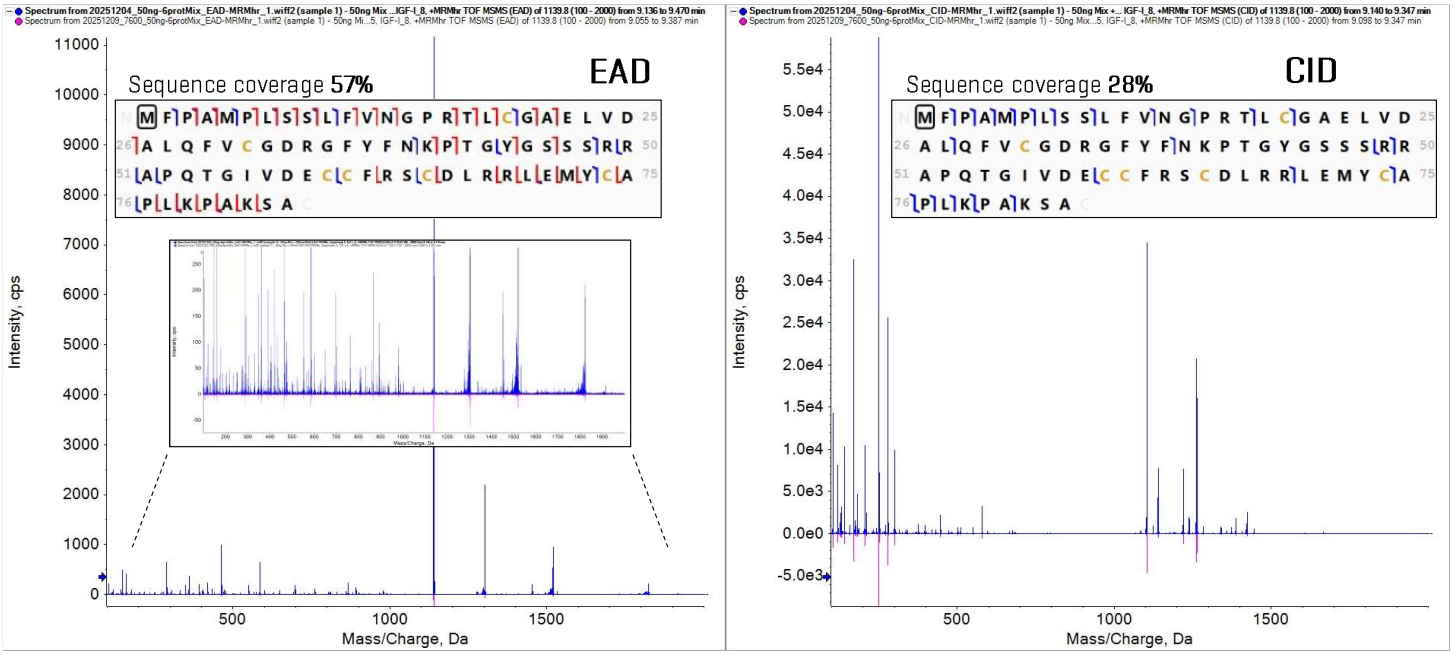

Figure 1. Top-down proteomics analysis using the ZenoTOF 8600 system. IGF-I protein (9.1 kDa; 50 ng on-column) was analyzed with the ZenoTOF 8600 system using either EAD-MS/MS (left) or CID-MS/MS (right). EAD-MS/MS predominantly generates c- and z-ions (sequence coverage map, red labels), while CID-MS/MS predominantly generates b- and y-ions (sequence coverage map, blue labels). The MS/MS spectra for the +8 charge-state precursor (1139.82 Da) show inverted overlays of the ZenoTOF 8600 system data (blue) versus the ZenoTOF 7600+ system (pink) for the same on-column loading, highlighting the superior sensitivity of the ZenoTOF 8600 system.

Introduction

Top-down proteomics is an advanced mass spectrometry-based approach that analyzes intact proteins rather than peptide fragments generated from enzymatic digestion. By preserving the full protein structure, this method enables comprehensive characterization of proteoforms, including post-translational modifications (PTMs), sequence variants, and truncations, which are often lost in bottom-up workflows. Top-down MS strategies provide deep insights into protein heterogeneity and functional regulation, making them essential for studying complex biological systems and advancing precision proteomics (1). Alternative MS/MS fragmentation techniques, namely electron activated dissociation (EAD), have shown to be highly effective for MS-based top-down proteomics by providing fragment ion information complementary to that obtained using traditional collision-induced dissociation (CID) fragmentation, thereby maximizing protein sequence coverage (2). The results here demonstrate that the increased sensitivity of the ZenoTOF 8600 system significantly improves protein sequence coverage relative to the ZenoTOF 7600+ system, and that higher sequence coverage was obtained using EAD-based MS/MS than with CID-based MS/MS. The sensitivity and versatility of the ZenoTOF 8600 system make it the ideal platform for top-down proteomics research.

Methods

Sample preparation: Pierce intact protein standard mix, comprising 6 intact protein standards ranging from 9.1 kDa to 68 kDa, was purchased from ThermoFisher. The mixture was diluted to final concentrations of 2 ng/µL, 10 ng/µL, 50 ng/µL, and 200 ng/µL in buffer containing water with 0.1% formic acid. Final mixture dilutions contained intact bovine serum albumin protein (Sigma) spiked to a final concentration of 20 fmol/µL to minimize non-specific protein binding.

Chromatography: Chromatographic separations were performed with a Waters M-Class UPLC system using a Waters nanoEase M/Z Protein BEH C4 column (50 mm x 0.3 mm, 300 Å, 1.7 µm particle size). The LC method used a 26-minute active separation gradient and 42-minute total run time at a flow rate of 10 µL/min (gradient profile shown in Table 1). The column was heated to 50°C for the analysis.

Table 1. LC gradient profile used in this work.

Time (Min)	Mobile phase A (%)	Mobile Phase B (%)
0	97	3
4	97	3
7	75	25
30	55	45
32	10	90
35	10	90
36	97	3
42	97	3

Mass spectrometry: Sample analysis was performed on both the ZenoTOF 7600+ and ZenoTOF 8600 systems, using the OptiFlow TurboV Ion Source and OptiFlow Pro Ion Source, respectively. The vertical microflow probe with the 1-10 µL/min electrode was used. Ion source parameters for both systems included a curtain gas setting of 40 psi, CAD gas setting of 7, gas 1 setting of 20, gas 2 setting of 60, interface temperature setting of 200°C, and an ionspray voltage setting of 4500 V. The MS method included both a TOF-MS scan and targeted MS/MS (i.e. MRM^{HR}) for the 3 most intense charge states of each intact protein, using either CID-MS/MS or EAD-MS/MS fragmentation. The MS parameters used are summarized in Table 2. The charge states selected for each protein for MRM^{HR} are summarized in Table 3. Acquisitions were performed in duplicate for each concentration and MS/MS fragmentation mode.

Table 2. EAD-MS/MS and CID-MS/MS parameters used.

Parameter	EAD-MS/MS	CID-MS/MS
TOF-MS mass range	500 – 2,000 Da	500 – 2,000 Da
TOF-MS accumulation time	100 msec	100 msec
Q1 resolution for MS/MS	Low	Low
MS/MS mass range	100 – 2,000 Da	100 – 2,000 Da
MS/MS accumulation time	125 msec	125 msec
Electron beam current	5,000 nA	N/A
Electron KE	7 eV	N/A
EAD reaction time	10 msec	N/A
Collision energy	12 V	See Table 3
Zeno pulsing	On	On

Table 3. List of proteins analyzed and their respective charge states.

Protein name	Average mass [Da]	Retention time [min]	Charge state	Precursor ion m/z [Da]	Collision energy for CID-MS/MS
Protein G	21,442.6	7.7	+26	825.68	37
			+27	795.14	
			+28	766.78	
IGF-I	9,111.5	10.2	+8	1139.82	40
			+9	1013.28	
			+10	912.05	
Protein AG	50,429.9	11.2	+54	935.43	30
			+55	918.44	
			+56	902.06	
Thioredoxin	11,865.5	16.0	+12	989.72	40
			+13	913.66	
			+14	848.47	
Carbonic anhydrase	28,981.3	23.4	+35	829.02	35
			+36	806.02	
			+37	784.23	
Klenow fragment	68,001.2	25.3	+87	782.62	27
			+88	773.74	
			+89	765.05	

Data processing: WIFF data files were converted to indexed centroided mzML format using the SCIEX MS Data Converter software. The resulting files were deconvoluted using FLASHDeconv software, and the deconvoluted files were subsequently processed using ProSight Lite v1.4 software to determine protein sequence coverage, as previously described [2]. To determine the sequence coverage for each protein, the lists of deconvoluted MS/MS fragment ion masses for each of the 3 charge states (limited to those near the experimentally observed retention times, summarized in Table 3) were combined and imported into ProSight Lite software. The fragment matching threshold was set to 10 ppm.

The top-down proteomics workflow using MRM^{HR}

The top-down proteomics workflow performed here utilized targeted MS/MS fragmentation of selected charge states from each protein in the analyzed mixture. Analysis of deconvoluted fragment ion masses generated from targeted MS/MS using available software tools (FLASHDeconv software and ProSight Lite software) [3,4] enabled the determination of protein sequence coverage. Different intact protein precursor charge states may yield different fragment ion profiles upon MS/MS fragmentation. By combining fragment ion lists from multiple charge states, the analysis efficiency is improved. In this case, the 3 most intense precursor charge states per protein were chosen for MS/MS (determined empirically, highlighted in Figure 2), thereby maximizing the sensitivity of the analysis.

As the ZenoTOF systems enable different MS/MS fragmentation modes, protein sequence coverage using either CID-MS/MS or EAD-MS/MS was directly compared. The sensitivities of both the ZenoTOF 8600 and ZenoTOF 7600+ systems were compared by analyzing a commercial protein mixture at on-column loadings ranging from 2 ng to 200 ng. The EAD-MS/MS parameters used were as previously described [2]. For CID-MS/MS, the optimal collision energies were determined empirically for each protein (data not shown).

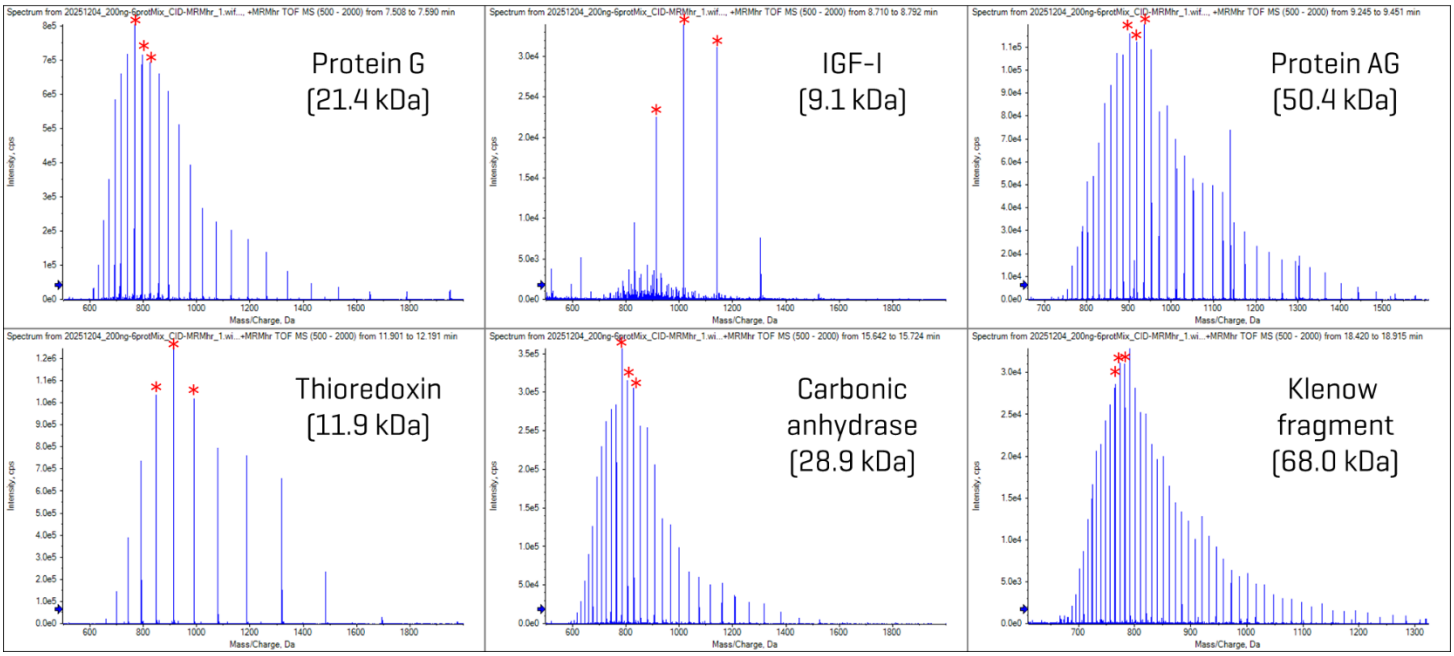

Figure 2. TOF-MS spectra for each protein in the commercial mixture, and the charge states selected for top-down MS/MS for each protein (red labels).

Superior protein sequence coverage using EAD-MS/MS with the ZenoTOF 8600 system

CID-MS/MS and EAD-MS/MS of peptides and proteins are known to yield distinct predominant fragment-ion series. CID-MS/MS primarily produces b- and y-ions, whereas EAD-MS/MS generates c- and z-ions (5). Consistent with these established dissociation characteristics, the data obtained in this study reflect clear differences between the two fragmentation modes. Figure 1 presents MS/MS spectra acquired for the +8 charge-state precursor of the IGF-I protein (50 ng on-column) using either EAD or CID. As is typical for intact-protein fragmentation, both methods predominantly produced ions mapping to the N- and C-terminal regions of the protein. However, the comparison underscores a substantial difference in sequence coverage: EAD-MS/MS achieved 57% coverage for this protein, whereas CID-MS/MS yielded 28%. In addition, the enhanced sensitivity of the ZenoTOF 8600 platform—relative to the ZenoTOF 7600+—is evident from the inverted spectral overlays shown in Figure 1, further highlighting the performance advantages observed in this work.

Figure 3 shows another example of the differences in fragmentation performance between EAD and CID MS/MS, as well as the comparative sensitivities of the ZenoTOF 8600 and ZenoTOF 7600+ platforms. In this figure, sequence coverage is shown for Thioredoxin protein at an on-column loading of 2 ng. Consistent with the distinct fragmentation chemistries of the two activation methods—EAD generating predominantly c- and z-type ions and CID yielding primarily b- and y-type ions—the EAD MS/MS workflow again produced superior sequence coverage relative to CID MS/MS. Moreover, the ZenoTOF 8600 system outperformed the ZenoTOF 7600+ system, emphasizing the advantage of enhanced sensitivity when analyzing low sample amounts.

Figures 4 and 5 summarize the protein sequence coverage for all proteins in the commercial mixture, across different on-column loadings, using EAD-MS/MS and CID-MS/MS, respectively. Consistently, across all proteins in the mixture, the performance difference between the two systems was most drastic at the lower on-column loadings, owing to the superior sensitivity of the ZenoTOF 8600 system. EAD-MS/MS also consistently provided higher sequence coverage than CID-MS/MS.

These results demonstrate the powerful capabilities of the ZenoTOF 8600 system for top-down proteomics, particularly when using EAD-MS/MS fragmentation. The fast MS/MS acquisition speed of the ZenoTOF 8600 system enables this workflow to be potentially applicable to the analysis of protein samples of varying complexity. Although EAD-MS/MS generally exhibited superior protein sequence coverage than CID-MS/MS, top-down MS/MS can be highly variable and dependent on the unique sequence and characteristics of each protein. CID-MS/MS may also yield important information about the protein, and a holistic approach to top-down protein characterization (combining the complementary information generated from multiple fragmentation and analysis strategies) is ideal.

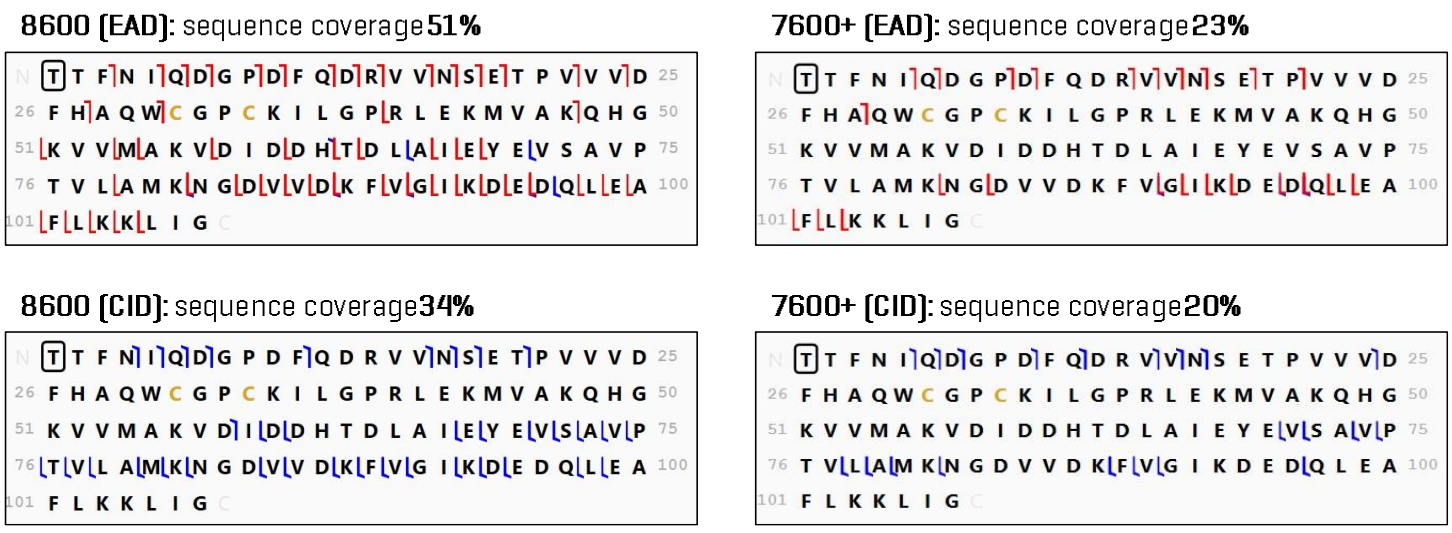

Figure 3. Superior protein sequence coverage obtained with top-down analysis on low sample loadings using EAD-MS/MS on the ZenoTOF 8600 system. Thioredoxin protein (11.9 kDa; 2 ng on-column) was analyzed on either the ZenoTOF 8600 system (left) or the ZenoTOF 7600+ system (right), using either EAD-MS/MS (top) or CID-MS/MS (bottom). EAD-MS/MS predominantly generates c- and z-ions (red labels), while CID-MS/MS predominantly generates b- and y-ions (blue labels).

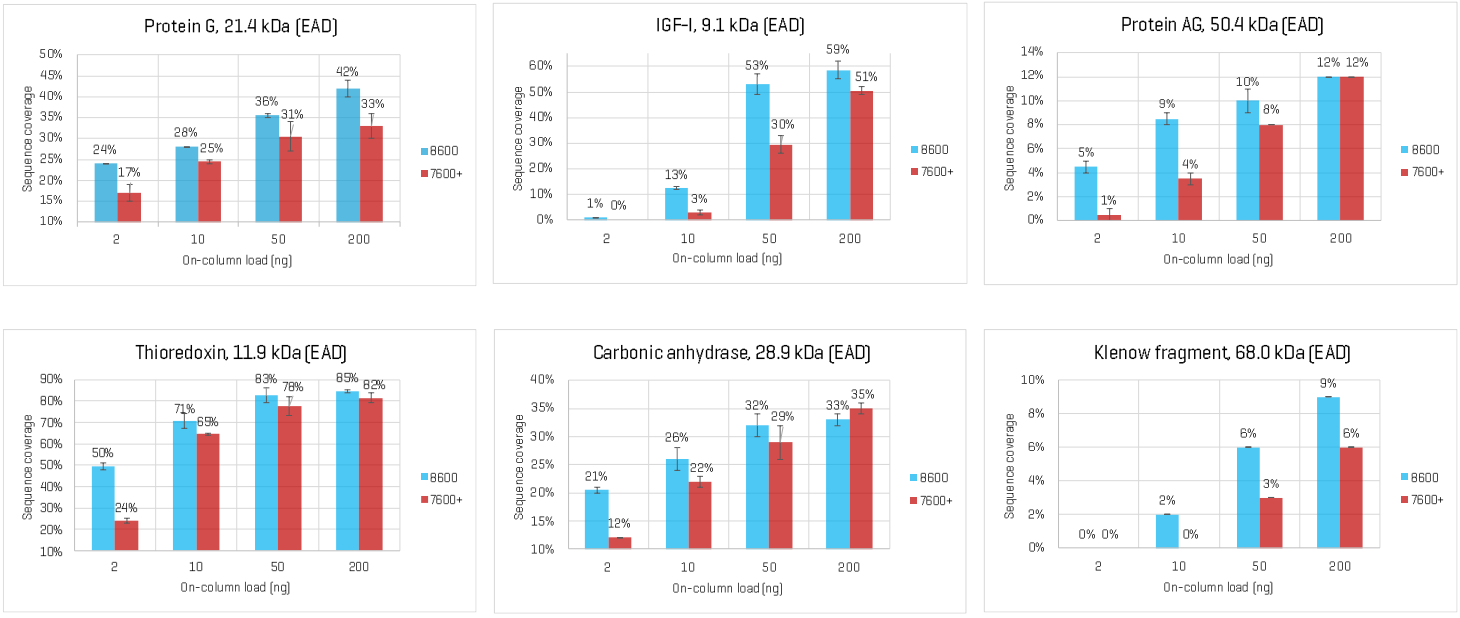

Figure 4. Summary of top-down proteomics analysis using EAD-MS/MS: comparisons of sequence coverage obtained per protein between the ZenoTOF 8600 and ZenoTOF 7600+ systems, at different on-column loadings. Bar graphs show the averages of duplicate measurements, with the variance highlighted by the error bars.

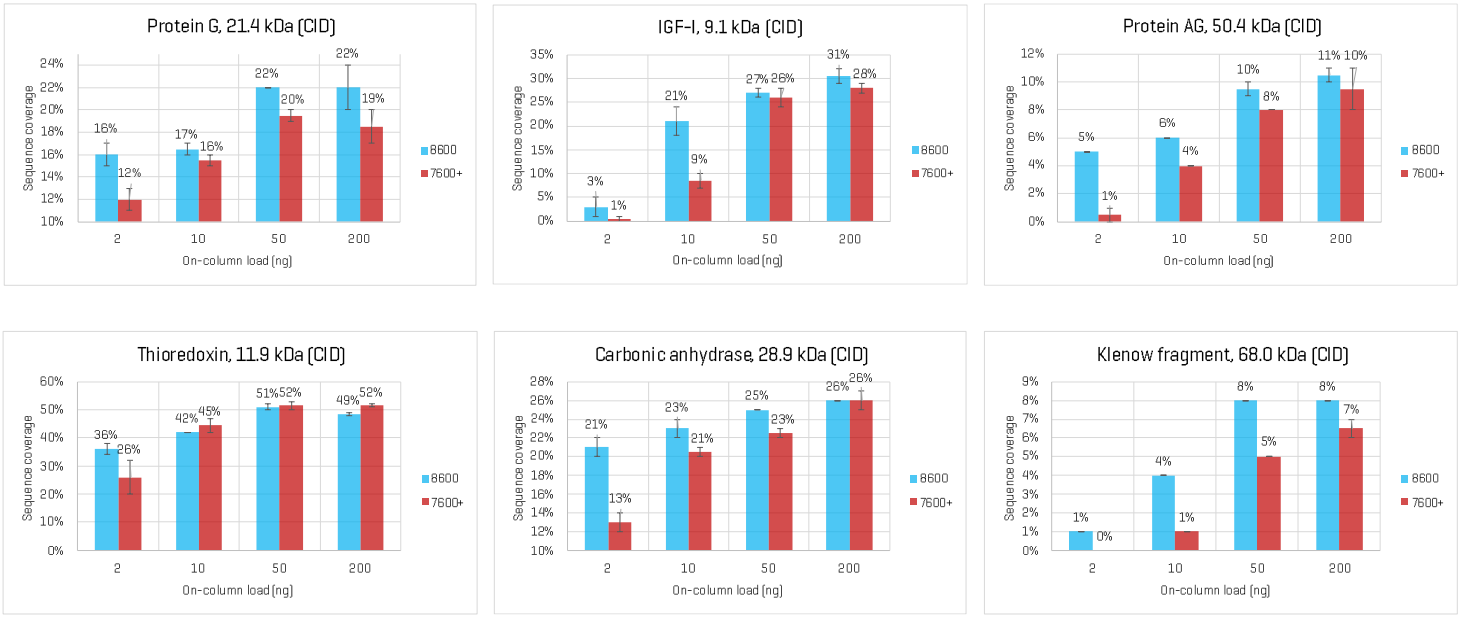

Figure 5. Summary of top-down proteomics analysis using CID-MS/MS: comparisons of sequence coverage obtained per protein between the ZenoTOF 8600 and ZenoTOF 7600+ systems, at different on-column loadings. Bar graphs show the averages of duplicate measurements, with the variance highlighted by the error bars.

Future points to consider

Although this study employed conventional LC separation methods, proteome-scale LC–MS analysis of intact proteins remains inherently challenging and demands extensive optimization of front-end separation, instrument settings, and data analysis workflows. Future improvements to top-down proteomics using the ZenoTOF 8600 system could explore alternative front-end approaches such as direct infusion or capillary electrophoresis (CESI), along with advanced software tailored for EAD data interpretation.

Conclusions

- A comprehensive workflow for top-down proteomics on the ZenoTOF 8600 system is presented, leveraging superior instrument sensitivity, EAD-MS/MS fragmentation, and freely available data processing tools.
- The sensitivity of the ZenoTOF 8600 system allowed for protein sequence coverage gains of up to 24% compared to the previous-generation ZenoTOF 7600+ system.
- For the top-down proteomics analysis of a commercial protein mixture, EAD-MS/MS fragmentation resulted in protein sequence coverage gains as much as 36% compared to traditional CID-MS/MS.
- The fast acquisition of EAD- and CID-based MS/MS on the ZenoTOF platforms enables top-down proteomics analysis of complex mixtures at LC timescales.

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