Leveraging ion-ion and ion-photon reactions to improve the sequencing of proteins carrying multiple disulfide bonds: the human albumin case study

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ABSTRACT

Gas-phase sequencing of large intact proteins (>30 kDa) is an inherently challenging process, complicated by extensive overlap of multiply charged product ion peaks which are often characterized by low signal-to-noise ratio. Disulfide bonds exacerbate this issue, since their presence implies that two covalent bonds (the S-S bond and the backbone bond) must be cleaved in order to liberate sequence-informative fragments. While electron-based ion activation techniques such as electron transfer dissociation (ETD) are known to cleave disulfide bonds, in practice regular ETD struggles to produce extensive sequencing of large proteins with multiple disulfide bonds. Here we evaluate the increase in sequence coverage obtained by combining activated ion ETD (AI-ETD) and proton transfer charge reduction (PTCR) in the analysis of recombinant human albumin.

INTRODUCTION

Human serum albumin (HSA) is the most abundant protein found in serum, it regulates oncotic pressure of the plasma and is responsible for many of the fluidics in the body[1]. HSA is a single chain composed of 585 amino acids for a molecular weight of 66 kDa. 35 of the 585 amino acids are cysteines, which form a total of 17 disulfide bridges[1]. HSA serves as a critical biomarker in many diseases such as cancer, cardiovascular diseases, glycemic diseases[2]. Therefore, it has many applications in genetics, biochemistry, and medicine and has been extensively studied. Mass spectrometry (MS) is a powerful analytical tool that can characterize significant biologics like albumin. However, the presence of the 17 disulfide bridges limits the capability of generating detectable sequence fragments. Traditional bottom-up MS circumvents this issue through chemical reduction of the S-S bonds followed by enzymatic digestion. Although it eliminates problems connected with disulfide bonds, the chemical reduction step can introduce unnecessary artifacts and may result in the loss of cysteine-linked post-translational modifications (PTMs)[3].

Top-down MS (TD MS) is a valuable alternative method since it fragments the intact protein, preventing the aforementioned problems. Still, TD MS suffers from the challenges of sequencing large intact proteins (>30 kDa): the extensive overlap of multiply charge product ion peaks with low signal-to-noise ratio (SNR) prevents a simple interpretation of fragmentation spectra of whole proteins[4]. This scenario is further complicated by the presence of the disulfide bonds, that require both the backbone bond and the S-S bond to be cleaved to liberate fragment ions[5]. Although electron-based ion activation techniques such as electron transfer dissociation (ETD) can effectively cleave disulfide bonds[6], in practice regular ETD still struggles to produce extensive sequencing of large intact proteins with multiple disulfide bonds. Hybrid ion fragmentation methods such as higher energy collisional activation following ETD reaction (ETHcD) and infrared photoactivation concurrent with ETD reaction (activated ion-ETD or AI-ETD) were shown to increase sequence coverage of intact proteins[7]. The MS^2 spectra generated from these dissociation techniques are often highly complex and congested due to fragment ion signal overlap. Proton transfer charge reduction (PTCR) can be employed to reduce such complexity[8]. In PTCR, multiply charged cations are deprotonated by reacting with a perfluorinated reagent anion without the production of additional fragment ions[9]. Here, we employ various ion dissociation techniques in combination with PTCR in the analysis of recombinant human albumin and analyze the increase in sequence coverage for AI-ETD MS^2 - PTCR MS^3 experiments.

MATERIALS AND METHODS

Sample Preparation: Recombinant human albumin was purchased from Albumin Bioscience and desalted via buffer exchange using Zeba spin columns (Thermo Scientific).

MS Instrumentation and Methods: Mass spectrometry measurements were performed on a modified Thermo Scientific™ Orbitrap Eclipse™ Tribrid™ mass spectrometer equipped with a 10.6 μm CO_2 laser. Sample was directly infused using a NanoFlex electrospray source. Ion fragmentation was carried out in intact protein mode (2 mTorr N2 in IRM) using: higher-energy collisional dissociation (HCD), infrared multiphoton dissociation (IRMPD), ETD, ETD with HCD post-activation (ETHcD), AI-ETD and AI-ETD with HCD post activation (AI-ETHcD). Spectral simplification was carried out using 7-30 ms PTCR, by isolating all fragmentation products with a single, wide isolation window. MS^2 and corresponding PTCR MS^3 spectra were recorded in full profile mode at 240,000 resolving power (at *m/z* 200) by averaging 300 or 600 mass spectra.

Data Analysis: Fragmentation maps were obtained and manually validated using TDValidator (Proteinaceous, Inc), which includes an isotope fitting algorithm that matches the experimental ion isotopic *m/z* peak clusters from the original .raw spectrum against theoretical ion isotopic *m/z* peak clusters generated using fragment ions' molecular formulae. The SNR threshold for peak picking was set to 10. The fragment tolerance was set to 10 ppm, while the inter-isotopic tolerance for a single isotopic cluster was set to 3 ppm. The maximum charge state for fragment ions was set to +20. Automatically assigned fragments were manually validated. Plots were generated using GraphPad Prism 9 (GraphPad Software).

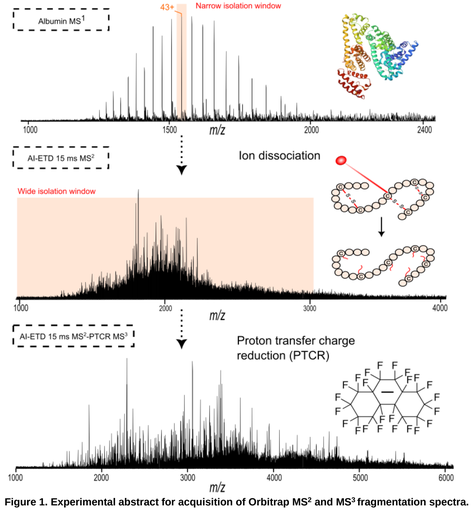


Figure 1. Experimental abstract for acquisition of Orbitrap MS^2 and MS^3 fragmentation spectra.

Results

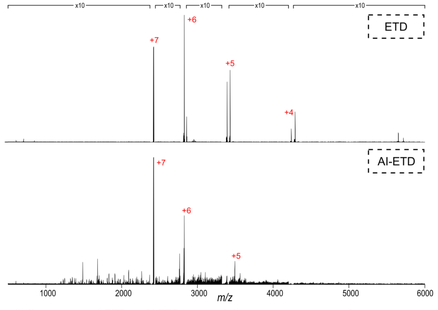


Figure 2. Comparison of ETD vs AI-ETD fragmentation of apomyoglobin. A single charge state of myoglobin (+20) was isolated for 17 ms PTCR. An additional re-isolation of the +7 charge state was conducted in the LTQ. 6 ms ETD or AI-ETD was then performed on the +7 precursor ion resulting in the two mass spectra shown here. An additional amplification by x10 of the regions annotated was performed in QualBrowser to display fragments present in AI-ETD but not in regular ETD.

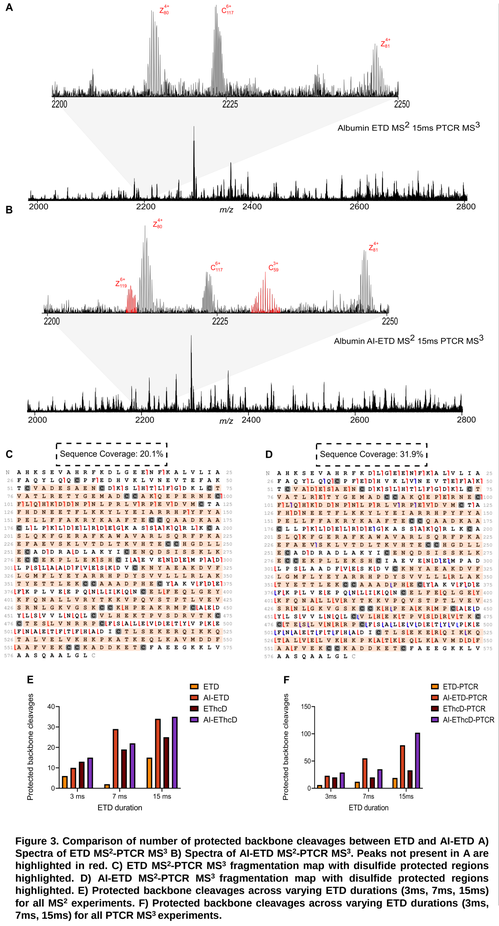
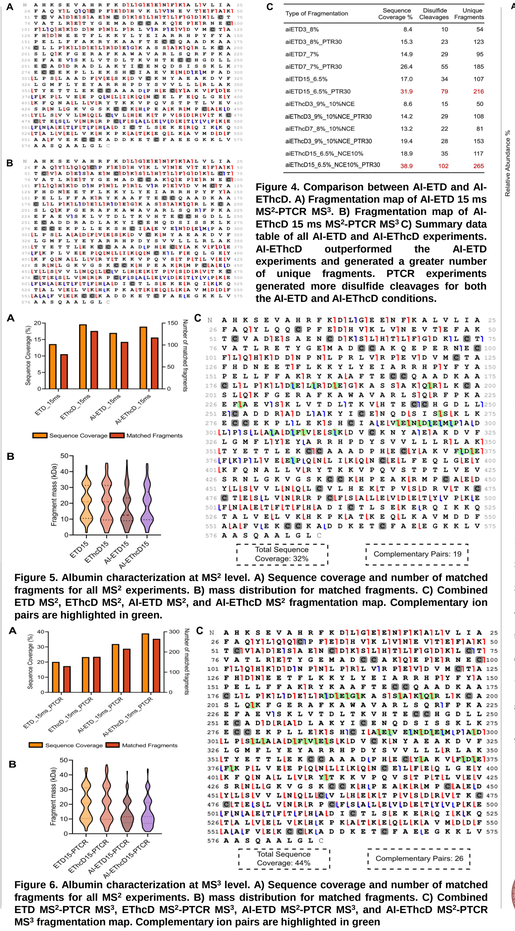


Figure 3. Comparison of number of protected backbone cleavages between ETD and AI-ETD A) Spectra of ETD MS^2-PTCR MS^3 B) Spectra of AI-ETD MS^2-PTCR MS^3. Peaks not present in A are highlighted in red. C) ETD MS^2-PTCR MS^3 fragmentation map with disulfide protected regions highlighted. D) AI-ETD MS^2-PTCR MS^3 fragmentation map with disulfide protected regions highlighted. E) Protected backbone cleavages across varying ETD durations (3ms, 7ms, 15ms) for all MS^2 experiments. F) Protected backbone cleavages across varying ETD durations (3ms, 7ms, 15ms) for all PTCR MS^3 experiments.



Type of Fragmentation	Sequence Coverage %	Disulfide Cleavages	Unique Fragments
aiETD3_8%	8.4	10	54
aiETD3_8%_PTR30	15.3	23	123
aiETD7_7%	14.9	29	95
aiETD7_7%_PTR30	26.4	55	185
aiETD15_6.5%	17.0	34	107
aiETD15_6.5%_PTR30	31.9	79	216
aiEThcD3_9%_10%NCE	8.6	15	50
aiEThcD3_9%_10%NCE_PTR30	14.2	29	108
aiEThcD7_8%_10%NCE	13.2	22	81
aiEThcD3_9%_10%NCE_PTR30	19.4	28	153
aiEThcD15_6.5%_NCE10%	18.9	35	117
aiEThcD15_6.5%_NCE10%_PTR30	38.9	102	265

Figure 4. Comparison between AI-ETD and AI-EThcD. A) Fragmentation map of AI-ETD 15 ms MS^2-PTCR MS^3. B) Fragmentation map of AI-EThcD 15 ms MS^2-PTCR MS^3 C) Summary data table of all AI-ETD and AI-EThcD experiments. AI-EThcD outperformed the AI-ETD experiments and generated a greater number of unique fragments. PTCR experiments generated more disulfide cleavages for both the AI-ETD and AI-EThcD conditions.

Figure 6. Albumin characterization at MS^3 level. A) Sequence coverage and number of matched fragments for all MS^2 experiments. B) mass distribution for matched fragments. C) Combined ETD MS^2-PTCR MS^3, EThcD MS^2-PTCR MS^3, AI-ETD MS^2-PTCR MS^3, and AI-EThcD MS^2-PTCR MS^3 fragmentation map. Complementary ion pairs are highlighted in green

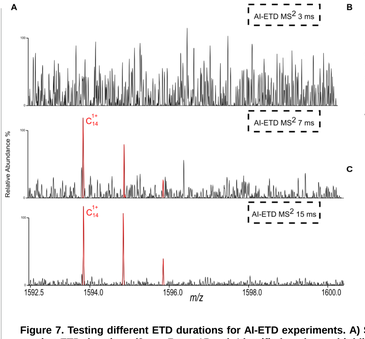


Figure 7. Testing different ETD durations for AI-ETD experiments. A) Spectral representation of varying ETD durations (3 ms, 7 ms, 15 ms). Identified peaks are highlighted in red. B) Sequence coverage and number of matched fragments for AI-EThcD MS^2 and PTCR MS^3 experiments C) mass distribution for match fragments for AI-EThcD MS^2 and PTCR MS^3 experiments.

CONCLUSIONS

Our results demonstrate that AI-ETD and the related AI-EThcD can drastically improve the sequence coverage of disulfide-protected regions of human serum albumin. We report that longer ETD durations, as demonstrated with 7 ms and 15 ms AI-ETD and AI-EThcD, provided both better sequencing of disulfide protected regions and overall coverage. Global coverage was also significantly increased when AI-ETD and AI-EThcD experiments were summed. Additionally, the inclusion of PTCR facilitated the identification of product ions not previously matched in the corresponding MS^2 experiments.

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