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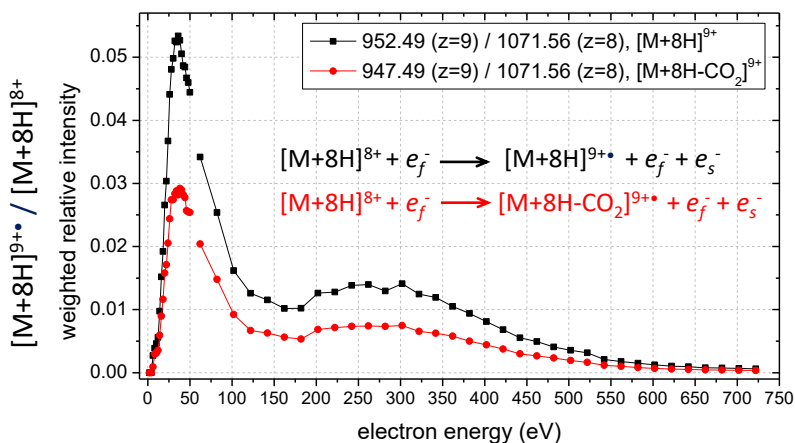
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## 1. D4.1 – CED gun prototype installed and tested

Coulombic explosion dissociation (CED) is accomplished when molecular ions undergo multiple ionization by intense pico- and femto-second laser light irradiation. The concept has been demonstrated experimentally for small molecules and atomic clusters. The idea explored within the TopSpec project is to detach multiple electrons from polyprotonated intact mAbs using a high-energy high-intensity electron beam. The experiments discussed in delivery D2.3 indicate that multiple electron detachment is possible and polyprotonated proteins with many electron holes and enriched charge states can be generated. A number of lower  $m/z$  fragments are also generated at the lower electron energies examined, typical to an EID process. In experiments with intact mAbs, the ionized species are not isotopically resolved and precise determination of the hydrogen atoms shifts that would allow an estimation of the number of radical sites on the protein to be made is not possible.

A more systematic investigation to explore these effects was performed with ubiquitin ions. **Figure 1** shows the effect of electron energy on the signal intensity ratio of the ionized species  $[M+8H]^{9+}$  relative to the precursor ions  $[M+8H]^{8+}$ ,  $[M+8H]^{9+}/[M+8H]^{8+}$ . The ratio is normalized to the number of electrons available for ionization since at higher electron energies the transmission of electrons through the trapping region is considerably enhanced. The normalization process shows two maxima that should correspond to the ionization cross section curve. The first maximum is relatively sharp and located around 35 eV. In the Omnitrap platform this electron energy is used almost exclusively for generating high quality EID spectra. The second maximum observed at  $\sim 300$  eV is located on a broad shoulder extending to the higher energies. The efficiency of ionization drops considerably as the energy of the electrons is increased further. Experiments with different charge states of ubiquitin produced very similar curves. **Appendix I** shows an example of the relative intensity curves and also the electron current measured experimentally and used for calculating the “weighted relative intensity” parameter presented in **Figure 1**.



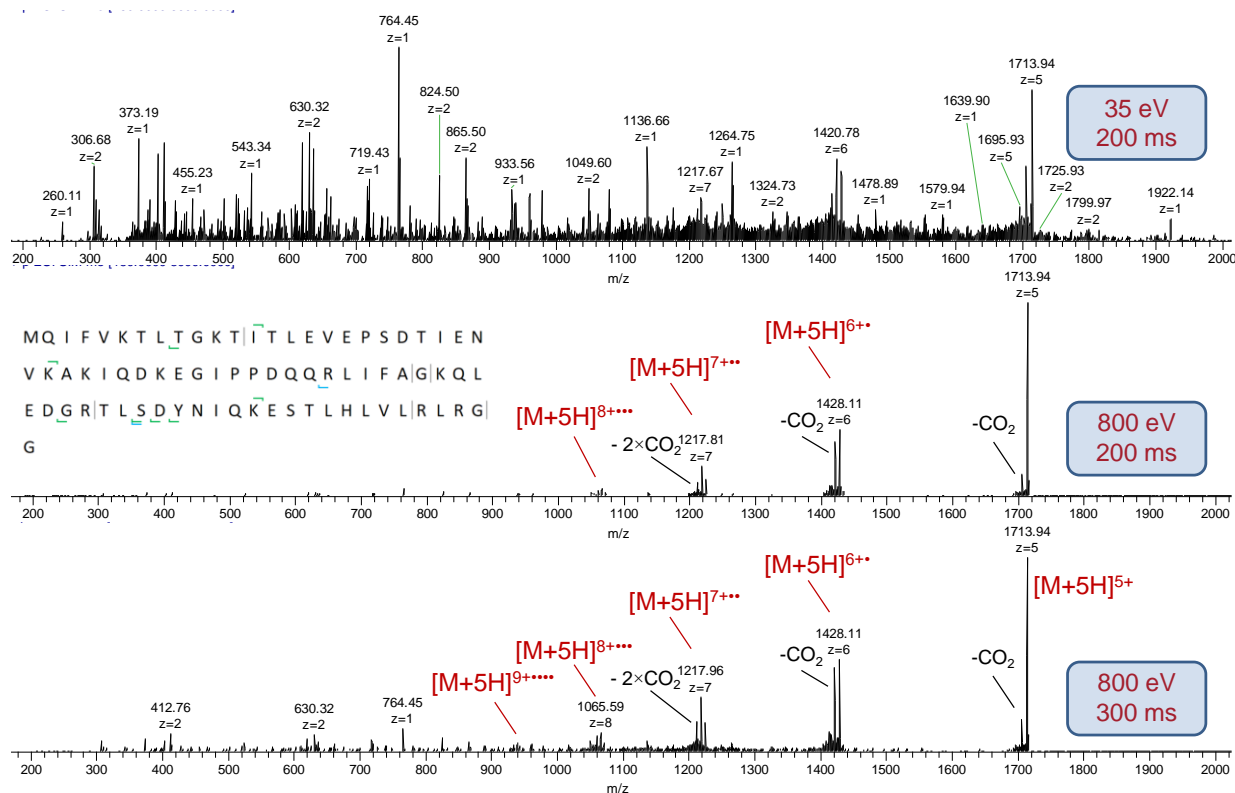
**Figure 1.** Ionization efficiency of ubiquitin  $[M+8H]^{8+}$  ions measured experimentally as a function of electron energy

Characteristic mass spectra produced at different electron energies and irradiation times are presented in **Figures 2 (a), (b)** and **(c)** for ubiquitin  $[M+5H]^{5+}$  ions. **Figure 2 (a)** shows the standard EID experiment performed with 35 eV electrons. A very rich fragmentation spectrum is produced

and complete sequence coverage is obtained with all types of primary fragment ions generated throughout the backbone within 200 ms irradiation time.

A very different mass spectrum is produced when electron energy is raised to 800 eV. At the higher energies electron ionization is enhanced, multiply ionized radical ions are formed with high abundance, while the generation of fragment ions at 200 ms irradiation time remains rather low. A conclusion that can be drawn by comparing the mass spectra in **Figures 2 (a)** and **(b)** indicate that higher energy electrons enhance the formation of the radical ion produced by ionization while simultaneously reduce the efficiency of fragment ion formation. This can be explained by the fragmentation mechanism proposed in EID where low energy electrons detached from the ions during ionization are re-captured by positive ions in a process similar to ECD, ultimately leading to fragmentation. The kinetic energy of the detached electrons increases as the energy of the incoming electron beam is raised, and consequently the re-capturing process becomes less efficient giving rise to higher charge state ionized precursor ions.

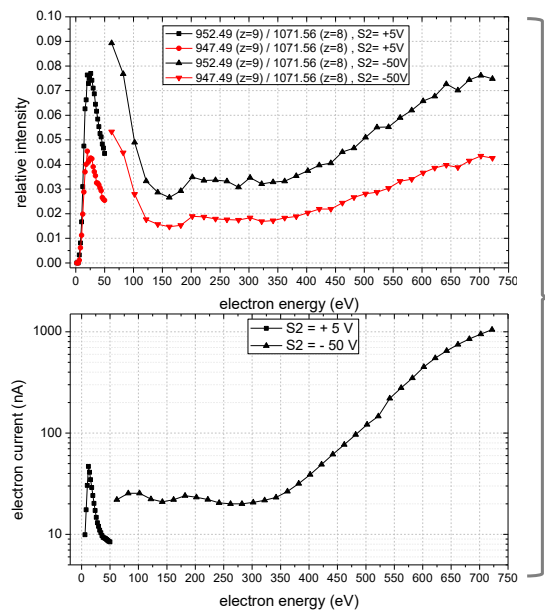
Also shown in **Figure 2 (c)** is the enhancement in ionization as well as in fragment ion formation by extending irradiation time to 300 ms. EID fragment ions are generated with higher efficiency compared to the shorter interaction times examined in **Figure 2 (b)**. Higher charge state ions are also observed where at least four electrons are detached from the  $[M+5H]^{5+}$  precursor to form the tetraradical cation  $[M+5H]^{9+}$ . The ionization process appears to be accompanied by the loss of one or more  $CO_2$  groups, whereby the effect is enhanced as the number of electrons detached from the protein increases. At least two  $CO_2$  losses are observed for the higher charge states generated during ionization starting from the  $[M+5H]^{7+}$  ions and beyond. New cleavages generated at 800 eV and not present in the EID mass spectrum produced by 35 eV electrons are also shown in the sequence map of ubiquitin in **Figure 2**. The enhanced activity in terms of cleavage frequency suggests that the second  $CO_2$  loss may originate from the side chain of D58. Additional losses are observed on basic residues (K, R).



**Figure 2.** (a) EID mass spectrum produced by 35 eV electrons and a 200 ms irradiation time, (b) Electron ionization of ubiquitin using 800 eV electrons obtained with minimal fragmentation at 200 ms irradiation time and (c) EID mass spectrum using 800 eV electrons and irradiation time extended to 300 ms where ionization efficiency and fragment ion formation are simultaneously enhanced.

These experiments suggest that electron ionization with energies far greater compared to the 800 eV investigated experimentally will promote direct ionization further, while fragment ion formation may remain a low efficiency mechanism. An important feature to consider is the increasing number of CO<sub>2</sub> losses observed as the number of electrons detached from the proteins is increased and the spectral complexity this effect will impose on fragment identification, assuming the coulombic explosion effect will eventually occur. New electronics are currently being developed to increase electron energy to 5 KeV, however, it is emphasized that in experiments with several micro-ampere of electron current transmitted through segment Q5 the performance of the Omnitrap platform is no longer stable.

## Appendix I



weighted relative intensity = relative intensity × correction factor

