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M. Lj Rankovic, Francis Canon, L. Nahon, Alexandre A. Giuliani, A. R. Milosavljevic. Photoinduced fragmentation of gas-phase protonated leucine-enkephalin peptide in the VUV range. 29. International Conference on Photonic, Electronic, and Atomic Collisions, Universidad Autónoma de Madrid. ESP., Jul 2015, Tolède, Spain. pp.6, 10.1088/1742-6596/635/1/012034 . hal-02740171

**HAL Id: hal-02740171**

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Submitted on 2 Jun 2020

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2015 J. Phys.: Conf. Ser. 635 012034

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## Photoinduced fragmentation of gas-phase protonated leucine-enkephalin peptide in the VUV range

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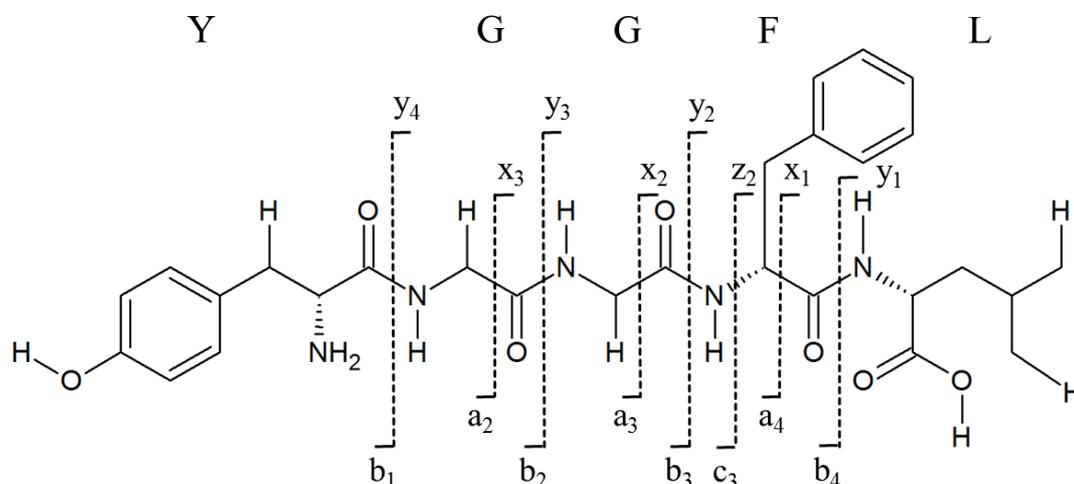
**Abstract.** In this article we report new results for action spectroscopy of protonated peptide Leucine enkephalin (YGGFL). By coupling a linear ion trap mass spectrometer with a vacuum ultraviolet (VUV) synchrotron radiation beamline, we investigate photofragmentation pattern of this peptide, through the analysis of tandem mass spectra recorded over a range of VUV photon energies, below and above the ionization energy. The obtained fragmentation patterns are discussed and compared to previous results.

### 1. Introduction

Development of electrospray ionization (ESI) [1], along with the advances in mass spectrometry techniques in recent years, has allowed manipulation of large bio-molecular ionic species in the gas phase. Therefore, fundamental properties of peptides, proteins, and nucleic acids such as ionization energies, bond energies and electronic energy levels could be investigated through action spectroscopy methods. In the present work ESI technique is used to produce intact biomolecular ionic species in the gas phase, from liquid solutions of these molecules. Tandem mass spectrometry by using VUV as an activation method was employed to investigate targets of interest.

Leucine enkephalin (Leu-enk) peptide is formed from five amino acids joined through peptide bonds, with the sequence tyrosine-glycine-glycine-phenylalanine-leucine (or YGGFL, in one letter coding). Leu-enk is an ideal candidate to study because it is small enough to be easily manageable with mass spectrometry techniques and to allow clear analysis of fragmentation products, while still being big enough to represent peptides. Usually the site of protonation is at N-terminus of the first amino acid - tyrosine, although other protonation sites were reported in the literature, as a consequence of proton mobility (see [2] and references therein). A standard nomenclature of the backbone fragments is based on where the positive charge (proton) stays upon bond scission and which particular bond is cleaved. If the proton stays at C-terminal, fragments a, b, c are formed, while fragments x, y, z originate from the N-terminal. Numbers in the subscripts of the fragment letters indicate the number of amino acid residues left in the particular fragment. Cleavage of the peptide C-N bonds is the origin of b and y fragment ions. Figure 1 displays a schematic structure of the Leu-enk peptide and denotation of some backbone fragments relevant for the present work.





**Figure 1.** Schematic structure of Leucine enkephalin peptide with denoted fragments (dashed lines) and constituent amino acids (in one letter code).

Leu-enk has been probed with a vast number of different techniques, covering fragmentation pattern information and fragment yields. The reported results include collision induced dissociation (CID) [3], surface induced dissociation (SID) [4], blackbody infrared radiative dissociation (BIRD) [5] and laser-induced dissociation (LID) [6]. Each of these methods produces different conditions which favor certain decomposition pathways governed by certain fragmentation mechanisms. An extensive study of fragmentation schemes has been reported for protonated Leu-enk in the experiment involving multiple-resonance CID, by V. Rakov *et al.* in [3]. One of the reasons Leu-enk is used as a standard peptide is because it is very useful for testing and tuning new experimental setups, since it has been found that abundance ratios of some fragment ions can indicate an amount of internal energy deposited in the precursor ions [7]. Therefore, experimental parameters of a setup are adjusted in such a way that various ratios of Leu-enk's fragment intensities are kept constant. On the other hand, ratios of  $a_4/b_4$  and  $b_3/y_2$  can indicate at least qualitatively the degree of excitation of the precursor ions.

Amino acids and peptides strongly absorb VUV light [8]. Therefore an investigation of VUV interaction with peptides is of great interest. The only VUV light source with high enough brilliance and flexibility to continuously change the photon energy over a wide range is the synchrotron radiation source. A comprehensive study of VUV-induced fragmentation of protonated Leu-enk was reported by S. Bari *et al* in [9]. In the present work, we extend this investigation by means of mass resolution, the number of assigned ionic fragments and the photon energy range. The obtained results are also compared with existing data.

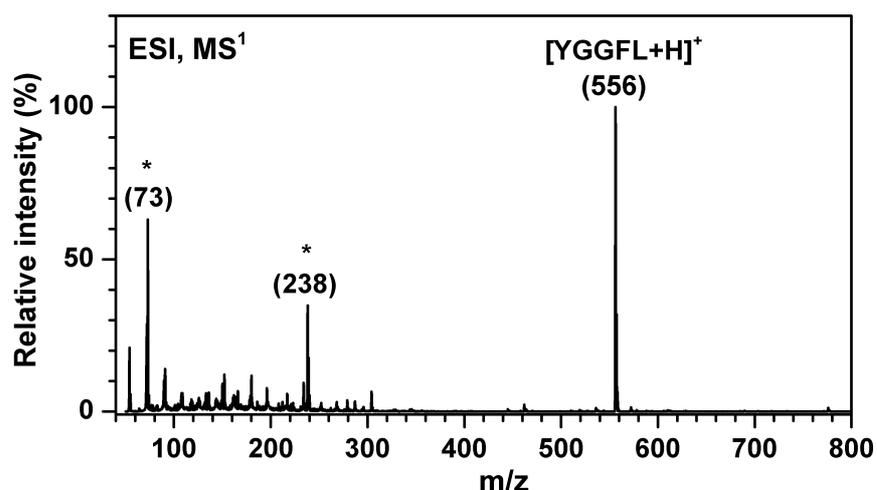
## 2. Experiment

Mass spectra in this article were obtained using the experimental setup located at the synchrotron SOLEIL near Paris, France. A commercial mass spectrometer Thermo Phiningan LTQ XL (LTQ) equipped with ESI was connected to the synchrotron VUV beamline DESIRS [10], with custom made turbo differential vacuum manifold [11-13]. Leu-enk ions produced by ESI were isolated in a linear quadrupole ion trap and subjected to VUV photons. After well-defined time of irradiation (500 ms in the present experiment) at particular photon energy, tandem mass spectra ( $MS^2$ ) were recorded.

The LTQ mass spectrometer was connected to the synchrotron beamline from the back side of the LTQ. A dedicated vacuum manifold has been made to accommodate the pressure difference between

the LTQ and the beamline. One side of the vacuum manifold was fixed to the beamline where the pressure is in the order of  $10^{-8}$  mbar, while the other side was connected via flexible bellow to the back plate window of the mass spectrometer. The pressure of the Helium buffer gas inside the ion trap of LTQ is of the order of  $10^{-3}$  mbar while the pressure in the spectrometer is of the order of  $10^{-5}$  mbar. During the operation and photon irradiation, the pressure inside the vacuum manifold was in the order of  $10^{-6}$  mbar. A home-made rotating mechanical shutter driven by an electric motor was built and positioned in front of the photon beam inside the vacuum manifold. Cooling of the electric motor in the vacuum was established through heat conduction through a massive copper heat sink (holder) tightly surrounding the motor [14]. Alignment of the photon beam with respect to the ion trap's axis was achieved by using a custom supporting frame, mounted under the LTQ. It has several degrees of freedom, both translational and rotational, which allow for a fine alignment of the ion trap position with respect to the incident photon beam. An optimal alignment provides the highest overlap between cylindrical ion trapping region and the photon beam and is essential for the experiment.

Leu-enk was provided from Sigma-Aldrich as a powder and it was diluted with water/acetonitrile 75:25% v/v solution to the final concentration of  $10 \mu\text{M}$ . The ESI source is positioned on the front side of LTQ. The ions formed by ESI source from the solution are guided by a system of ion lenses and stored in the ion trap. Parameters of ESI were optimized to obtain highest possible abundance of protonated Leu-enk cations  $[\text{YGGFL}+\text{H}]^+$ . A typically recorded mass spectrum ( $\text{MS}^1$ ) produced by ESI is displayed in figure 2.

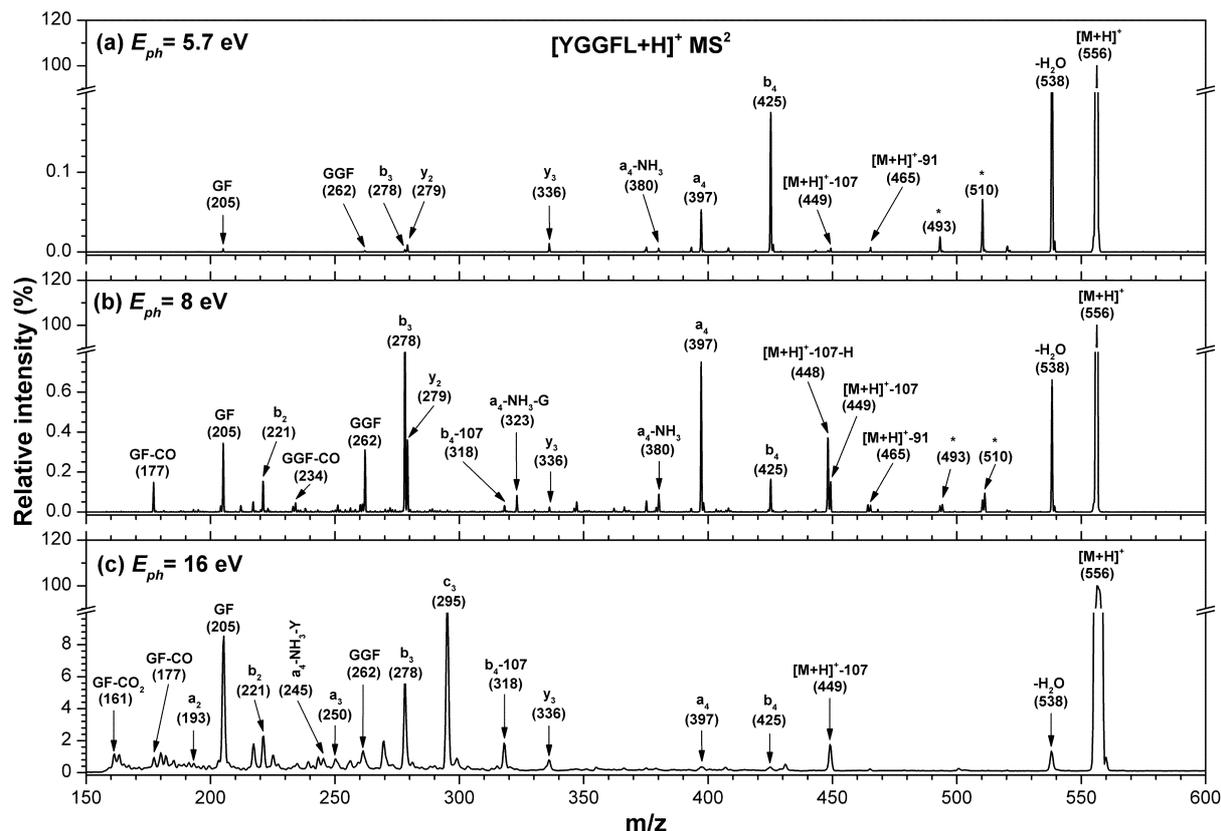


**Figure 2.** The mass spectrum of electro-sprayed Leu-enk ions from water/acetonitrile 75/25% solution with  $10 \mu\text{M}$  concentration of peptide molecules. The peak in the spectrum at  $m/z$  556 corresponds to the protonated Leu-enk cation  $[\text{YGGFL}+\text{H}]^+$ , while two peaks denoted with a star originate from pollutions.

The precursor ions of interest, in this case  $[\text{YGGFL}+\text{H}]^+$ , were selected and isolated in the ion trap, by means of ejecting all other ions. When enough precursor ions are accumulated in the ion trap or when a time limit for ion accumulation is reached, the mechanical shutter opens and the monochromatic VUV photon beam of defined energy irradiates the precursor ions. Synchrotron beamline DESIRS [10] is equipped with a gas filter cutting off higher order harmonics, which can create an additional signal originating from higher photon energies. If filled with Krypton, the gas filter cuts off all photon energies above 14 eV. Additionally, a  $\text{MgF}_2$  glass filter is inserted as the part of the vacuum manifold assembly, to cut off the higher harmonics over 10.6 eV. The photon beam produced by the beamline undulator is monochromatized by using a normal incidence monochromator, resulting in final energy resolution of around 10 meV in the present case.

### 3. Results and discussion

J. Sztáray *et al.* in [15] performed a review of the studies about Leu-enk energetics and reaction pathways, so we will focus here only on the discussion of the most prominent fragments prevailing under our experimental conditions. Figure 3 displays the tandem mass spectra obtained for protonated Leu-enk precursor ions  $[\text{YGGFL}+\text{H}]^+$  after activation with synchrotron VUV photons at three different energies. The mass spectra have been normalized to show the precursor ions  $[\text{YGGFL}+\text{H}]^+$  at  $m/z$  556 with 100 % relative intensity.



**Figure 3.** Tandem mass spectrum of protonated Leucine enkephalin precursor ions after irradiation with photons of a) 5.7 eV b) 8 eV and c) 16 eV.

Fragmentation patterns in our spectra are in good agreement with the ones reported in [9], which is expected considering the similar experimental conditions: VUV synchrotron photon activation of trapped ions. The lack of x and z and low abundant y-sequence ions in our spectra confirm that N-terminal ions are favoured. The ionization energy (IE) of protonated Leu-enk was determined by DFT calculations to be 8.87 eV [16]. The radical cation is not observed in our spectra and it is likely that it is not stable and fragments readily after its formation, as it has been proposed by Bari *et al.* [9]. For sub-ionisation energies absorption of photons by precursor ions leaves them in an excited electronic state. This energy may be redistributed internally via intramolecular vibrational processes, causing the weak peptide C-N bonds to break first, forming the backbone ions b and y. According to the fragmentation scheme proposed in [3], the major reaction pathway is following:  $\text{YGFFL}-b_4-a_4-b_3-b_2-a_2-\text{Y}$ . All these ions are present in our mass spectra, except Y (136), due to the mass cut off at  $m/z$  150. Backbone ion  $b_4$  ( $m/z$  425) needs the lowest photon energy to form and it shows up as the strongest fragment at 5.7 eV in our mass spectrum. This ion is formed directly from dissociation of the precursor ions. The fragment at  $m/z$  538 corresponds to the loss of a water molecule from the

precursor ions. Its intensity is highest among all fragments in the lowest energy region but falls quickly with the increase of the photon energy. Note that this fragment has not been discussed in the previous work by Bari et al. [9]. Following figure 1, after CO loss,  $b_4$  forms into the fragment ion  $a_4$  ( $m/z$  397). Ion  $a_4$  dissociates with the neutral loss of  $NH_3$  into fragment at  $m/z$  380. After Glycine residue loss (-57) near ionization energy, this ion is observed at  $a_4-NH_3-G$  ( $m/z$  323). It is also reported and discussed in detail by a group of Glish, as a rearrangement fragment FYG ( $m/z$  323) [17]. The intensity of the ion  $a_4$  exceeds the  $b_4$  intensity and peaks at around 7 eV, where it is the most prominent fragment in the mass spectrum. This energy corresponds to the peak of the absorption band coming from the  $\pi-\pi^*$  peptide transition. Fragment  $b_3$  ( $m/z$  278) is the next in line to show up as a dominant fragment with further increase of the photon energy. Ion  $b_3$  is formed from  $a_4$  ion while further dissociation of  $b_3$  forms  $b_2$  ( $m/z$  221). C-terminal ion  $y_2$  ( $m/z$  279) has a higher intensity than  $b_3$  (278) in the low energy region. With the increase of photon energy, the internal energy of the precursor ions rises, resulting in the drop of the intensity of  $y_2$  ions compared to the intensity of  $b_3$  ions, similarly as for  $b_4$  and  $a_4$  ions, respectively. The peak designated at  $m/z$  449 corresponds to the tyrosine side chain loss of the precursor ions while the loss of the phenylalanine side chain is responsible for a small peak at  $m/z$  465. H loss from 449 leads to a fragment designated at  $m/z$  448. Loss of tyrosine side chain is also noticed from backbone fragment  $b_4$ , so fragment  $b_4-107$  (318) is observed. As the photon activation energy goes over the IE of Leu-enk (8.87 eV) more reaction channels become open. New reaction channels above the IE lead to internal fragments GGF ( $m/z$  262) and GF ( $m/z$  205). These fragments are also present in the sub ionization energies, but with very small abundances. Suffering the CO loss, these ions form GGF-CO ( $m/z$  234) and GF-CO ( $m/z$  177). In the second channel with another  $CO_2$  loss from GF, fragments at  $m/z$  161 are created. Above the IE internal fragments dominate over the backbone by more than a factor of 10. At 16 eV, the most prominent fragments are  $c_3$  ( $m/z$  295) and GF ( $m/z$  205). Mass-to-charge ratio of doubly ionized precursor ions  $[Leu-enk+H]^{2+}$  is the same as for  $b_3$  ion at  $m/z$  278. According to [9], it is possible that small abundance of doubly ionized precursor ions contributes to the intensity of the  $m/z$  278 peak. Internal C-terminal fragment  $a_3$  ( $m/z$  250) is formed upon CO loss from  $b_3$  ion and along with ion  $a_2$  ( $m/z$  193) shows up at energies above 14 eV. Both backbone and internal fragment ions peak at around 20 eV with a very broad peak, which is also reported in [9]. At 24 eV being the final energy point in our scans, all fragments are in a decline with GF (205),  $b_2$  (221),  $b_3$  (278) and  $c_3$  (295) dominating the spectra at around 1% of the precursor ion intensity.

#### 4. Conclusions

A linear quadrupole ion trap mass spectrometer was coupled to a synchrotron beamline to study the VUV photo-induced dissociation of gas-phase protonated Leu-enk cations. The present experiment extends previous studies by means of high spectral purity of the photon beam, increased sensitivity and mass resolution (in the case of VUV/ion trap results) and increased energy range. Presented mass spectra are in good agreement with the existing fragmentation data in the literature. The fragmentation of the Leu-enk peptide shows a clear and interesting energy dependence that can be related to electronic excitation processes, which will be investigated in more details in future papers.

#### 5. Acknowledgments

This work was supported by the French ANR (Project ANR-08-BLAN-0065), the "Pavle Savic" bilateral scientific project between Serbia and France (N27482TE) and the COST Action MP1002 (Nano-IBCT). Milosavljević A R and Ranković M Lj acknowledge support by the Ministry of education, science and technological development of Republic of Serbia under the Project 171020. We are grateful to the SOLEIL synchrotron general staff for providing the beamtime under the Projects 20110324 and 20130388.

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