

## Supporting Information

### Highly Efficient Ionization of Phosphopeptides at Low pH by Desorption Electrospray Ionization Mass Spectrometry

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#### Experimental section

##### *Chemicals*

*L*-Tyrosine (MW: 181.2 Da), *O*-phospho-*L*-tyrosine (MW: 261.2 Da), bradykinin (sequence: RPPGFSPFR; MW: 1060.2 Da), angiotensin II (DRVYIHPF, MW 1046.2 Da),  $\alpha$ -casein (MW: ~ 23557.9 Da),  $\beta$ -casein (MW: ~ 23734.1 Da), ovalbumin (MW: ~ 42881 Da) and trypsin, (MW: ~23800 Da) were purchased from Sigma-Aldrich (St. Louis, MO). Sulfated hirudin (DFEEIPEE-Y(SO<sub>3</sub>H)-LQ, MW 1491.5 Da) and endoproteinase Glu-C (sequencing grade) were acquired from Protea Biosciences (Morgantown, WV) and Roche (Indianapolis, IN), respectively. Acetic acid and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ) and GFS Chemicals (Columbus, OH), respectively. The deionized water used for sample preparation was obtained using a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA).

##### *Protein digestion*

Casein proteins were digested using the following protocol.<sup>1</sup> 3.3 mg proteins were dissolved in

330  $\mu\text{L}$  of 25 mM ammonium bicarbonate. For the trypsin digestion, the proteins were denatured by heating in 95  $^{\circ}\text{C}$  water for 5 min and then digested in 25 mM ammonium bicarbonate by trypsin at a molar ratio of 1:50 (enzyme/protein) for 15 h at 37  $^{\circ}\text{C}$ . For the Glu-C digestion, the proteins were digested in 25 mM ammonium bicarbonate by Glu-C at a molar ratio of 1:100 (enzyme/protein) for 15 h at room temperature.

Ovalbumin was digested using the following protocol.<sup>2</sup> 6.2 mg protein in 330  $\mu\text{L}$  of 25 mM ammonium bicarbonate was added with DTT at a molar ratio of 10:1 (DTT/protein) for disulfide bond reduction, and then denatured by heating in 95  $^{\circ}\text{C}$  water bath for 5 min. After denaturation, the solution was cooled down to room temperature, added with iodoacetamide at a molar ratio of 20:1 (iodoacetamide/protein) and incubated in dark for 15 minutes at room temperature. Then, the solution was added with trypsin at a molar ratio of 1:50 (enzyme/protein) for digestion at 37  $^{\circ}\text{C}$  for 16 h.

#### *Sample preparation*

After digestion, for ESSI-MS analysis, 100  $\mu\text{L}$  of sample digestion was mixed with 900  $\mu\text{L}$  of MeOH/H<sub>2</sub>O/HOAc (50:50:1 by volume). For DESI-MS analysis, 100  $\mu\text{L}$  of sample digestion was mixed with 900  $\mu\text{L}$  of MeOH/H<sub>2</sub>O (50:50 by volume) and then the solution pH was adjusted to 2, unless specified otherwise.

#### *DESI-MS Apparatus*

A home-built apparatus (shown in Figure 2S) of liquid sample DESI<sup>3,4</sup> was used throughout the experiments. A Thermo Finnigan LCQ DECA ion trap mass spectrometer (Thermo Scientific, San Jose, CA) was mainly employed for ion detection. The sample flowing out of the fused silica transfer capillary (i.d., 0.1 mm) underwent interactions with the charged microdroplets generated from DESI spray for ionization. The capillary outlet was placed about 1 mm downstream from the DESI spray probe tip and kept in line with the sprayer tip and the mass spectrometer's inlet. The spray solvent for DESI was methanol/water (1:1 by volume) containing 1% acetic acid and was injected at a flow rate of 10  $\mu\text{L}/\text{min}$

with a high voltage of 5 kV applied to the sprayed solvent. The flow rate of the sample solutions injected for DESI ionization was 2-5  $\mu\text{L}/\text{min}$ .

#### *ECD MS/MS analysis*

ECD MS/MS experiments were conducted using a Bruker 12 Tesla Solarix Fourier-transform ion cyclotron mass spectrometer (FTICR MS, Bruker Daltonik GmbH, Bremen, Germany). The cathode heater was conditioned at 1.6 A and the ECD lens was set to 10 V, a pulse length of 100 ms, and a bias voltage of 1.2 V. Each spectrum was an average of 100-120 scans of broadband 1 M time-domain transient scans.

### $\alpha$ -S1-casein sequence

1 RPKHPIKHQGLPQEVLENLLRFFVAPFPEVFGKEKVNELSKDIG<sup>46</sup>SE<sup>48</sup>STEDQAMEDIKQMEAE<sup>64</sup>SI<sup>66</sup>S<sup>67</sup>S<sup>68</sup>SEE 70  
71 IVPN<sup>75</sup>SVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPN<sup>113</sup>SAEERLHSMKEGIHAQQKEPMIGVNVQ 140  
141 ELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW 199

### $\alpha$ -S2-casein sequence

1 KNTMEHV<sup>8</sup>S<sup>9</sup>S<sup>10</sup>SEESII<sup>16</sup>SQETYKQEKMAINPSKENLCSTFCKEVVRNANEEYSIG<sup>56</sup>S<sup>57</sup>S<sup>58</sup>SEE<sup>61</sup>SAEVATEEVK 70  
71 ITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIVLPWDQVKRNAVPITPTLNREQL<sup>129</sup>ST<sup>131</sup>SEENSKKTVD 140  
141 ME<sup>143</sup>STEVFTKTKLTHEEKNRLNFKKISQRYQKFALPQYLKTVYQHQAAMKPWIQPKTKVIPYVRYL 207

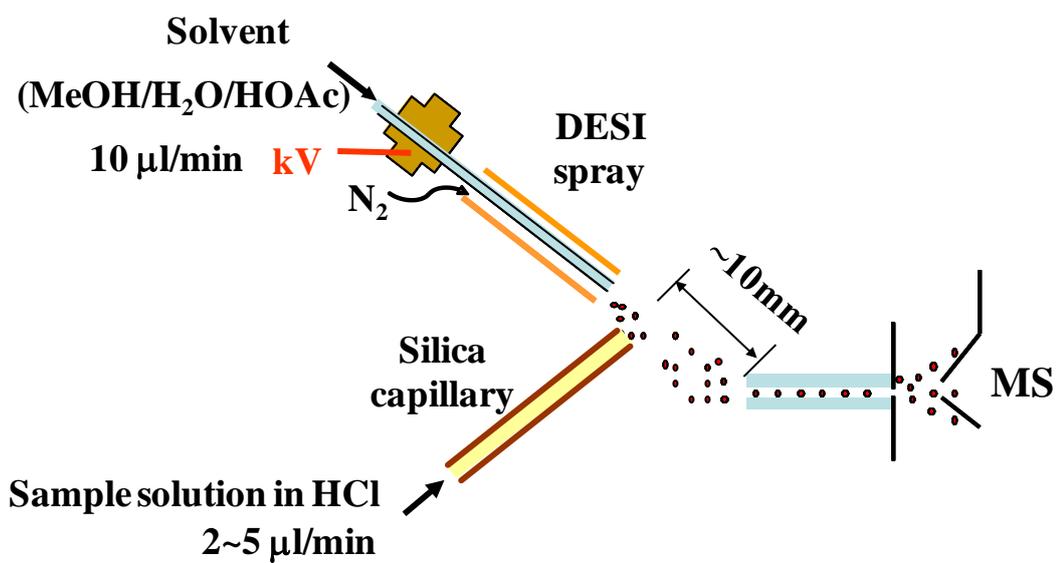
### $\beta$ -casein sequence

1 RELEELNVPGEIVE<sup>15</sup>SL<sup>17</sup>S<sup>18</sup>S<sup>19</sup>SEESITRINKKIEKFQ<sup>35</sup>SEEQQTEDELQDKIHPPAQTQSLVYPPFGPIPNL 70  
71 PQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFKYPVEPFTEQSLLTLDVENLHLPLPLL 140  
141 QSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLPVGRPFPIIV 209

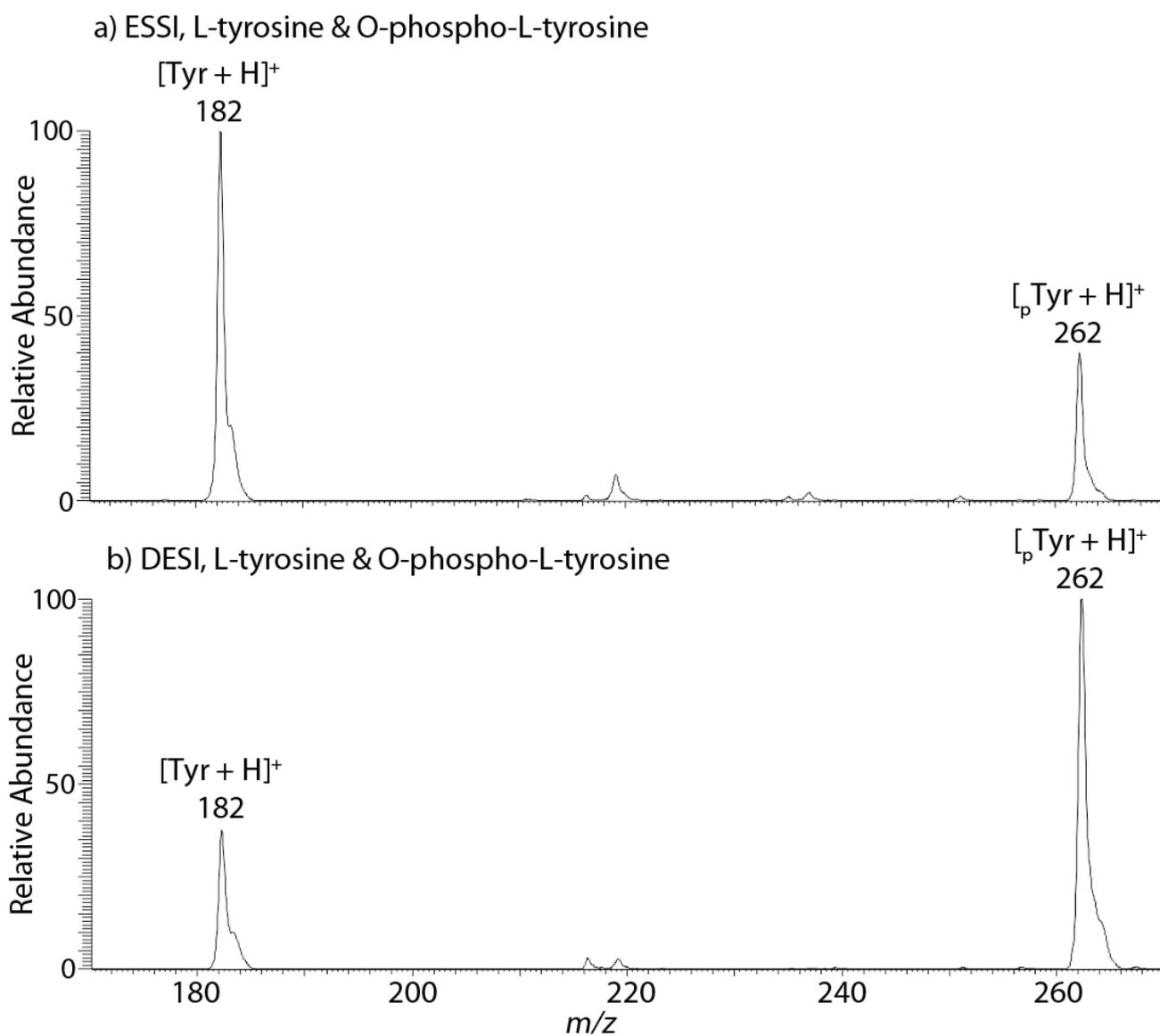
### ovalbumin

1 GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRFDKLPFGFD<sup>68</sup>SI<sup>68</sup>E 70  
71 AQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAERYPILPEYLQCVKELYRGGLEPINFQTAADQ 140  
141 ARELINSWVESQTNGIIRNVLPSSVDSQTAMVLVNAIVFKGLWEKAFKDEDTQAMPFRVTEQESKPVQM 210  
211 MYQIGLFRVASMASEKMKILELPPFASGTMSMLVLLPDEVSGLEQLESIIINFEKLTWTSSNVMEERKIKV 280  
281 YLPRMKMEEKYNLTSVLMAMGITDVFSSANLSGISSAESLKISQAVHAAHAEINEAGREVVG<sup>344</sup>SAEAGVD 350  
351 AASVSEEFRADHPFLFCIKHIATNAVLFFGRCVSP 385

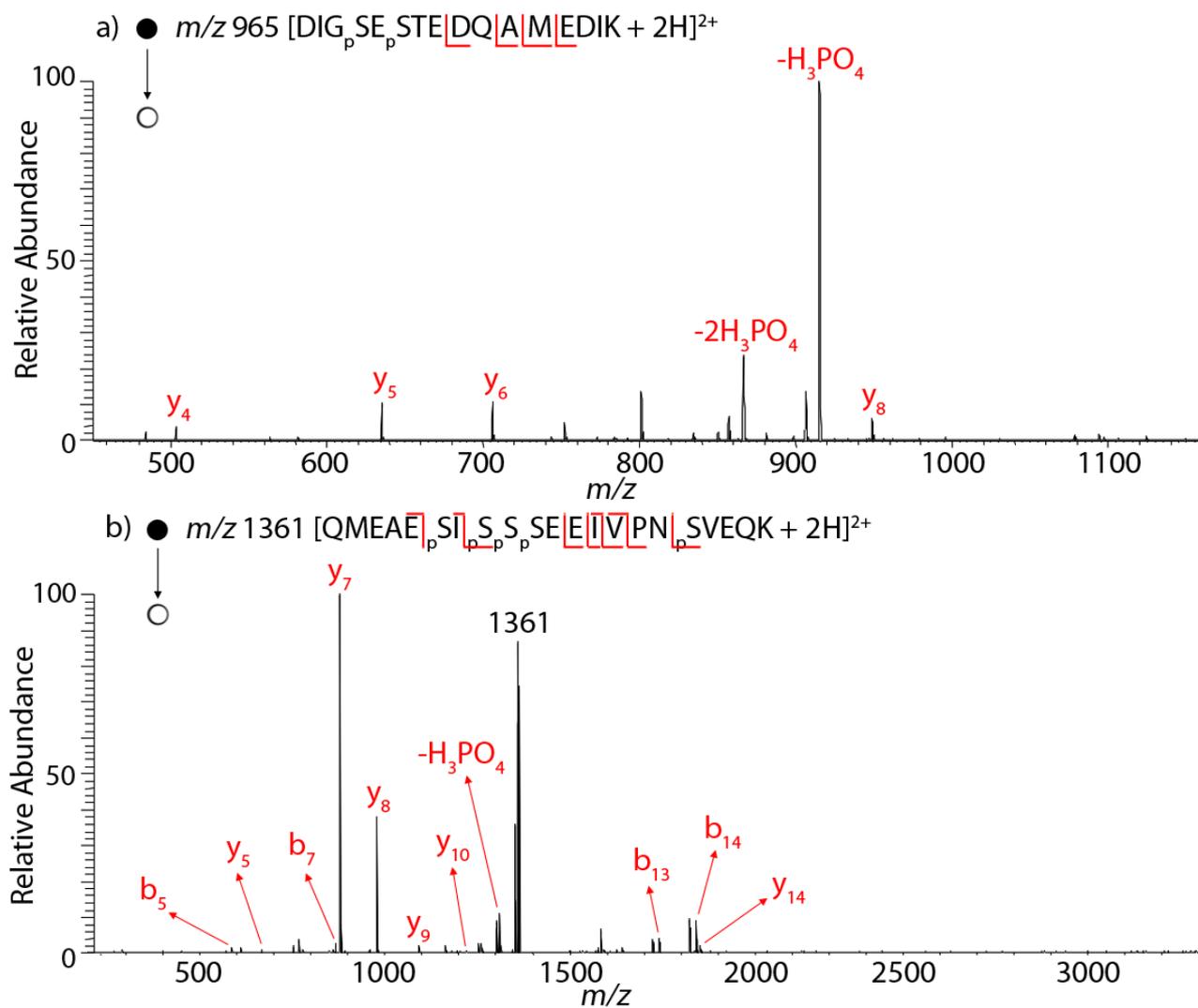
**Figure 1S.** Sequences of  $\alpha$ -casein (including  $\alpha$ -S1-casein and  $\alpha$ -S2-casein),  $\beta$ -casein and ovalbumin proteins.



**Figure 2S.** Scheme showing the apparatus of DESI-MS



**Figure 3S.** a) ESSI-MS spectrum of a mixture of *L*-tyrosine and *O*-phospho-*L*-tyrosine (molar ratio=1:3) at pH 3.3 and b) DESI-MS spectrum of a mixture of *L*-tyrosine and *O*-phospho-*L*-tyrosine (molar ratio=1:3) at pH 2.0. The subscript “p” indicates the presence of a phosphate group.

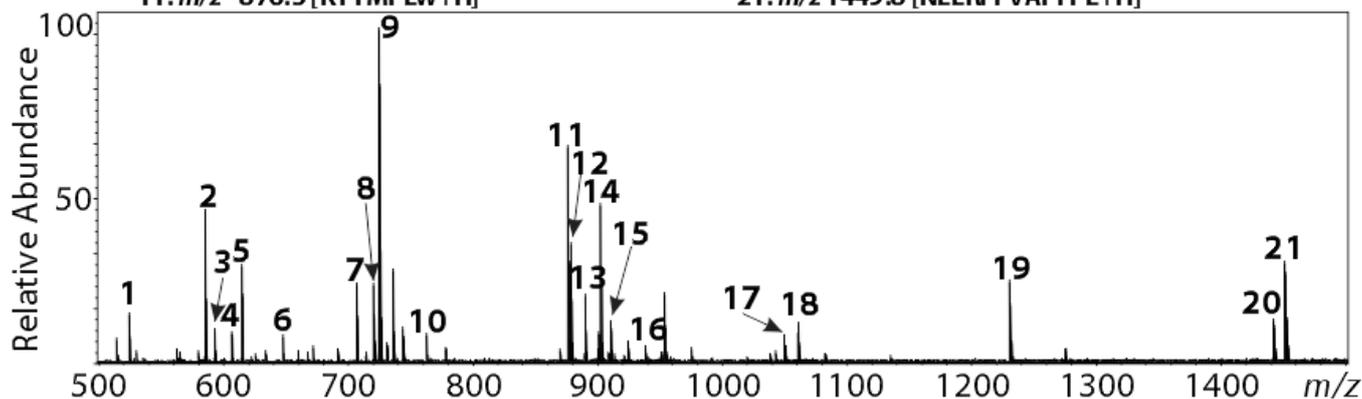


**Figure 4S.** CID MS/MS spectra of a) [DIG<sub>p</sub>SE<sub>p</sub>STEDQAMEDIK+2H]<sup>2+</sup> ( $m/z$  965) and b) [QMEAE<sub>p</sub>SI<sub>p</sub>SpSpSEEIVPN<sub>p</sub>SVEQK+2H]<sup>2+</sup> ( $m/z$  1361)

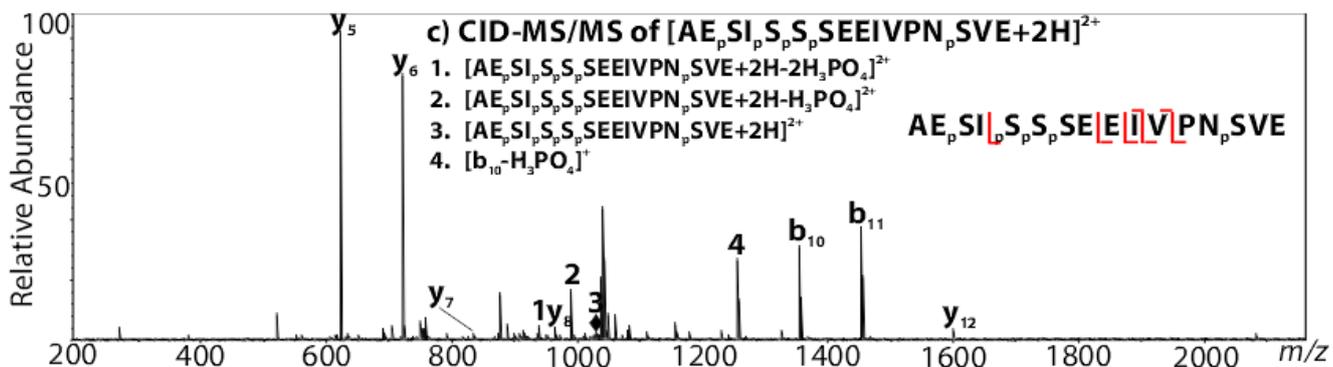
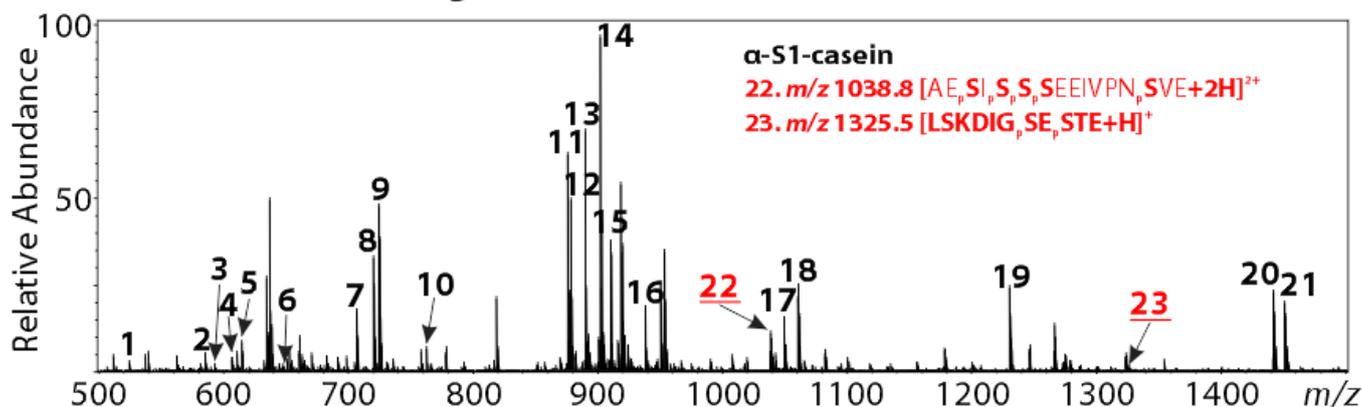
**a) ESSI-MS of Glu-C digested  $\alpha$ -casein**

$\alpha$ -S1-casein

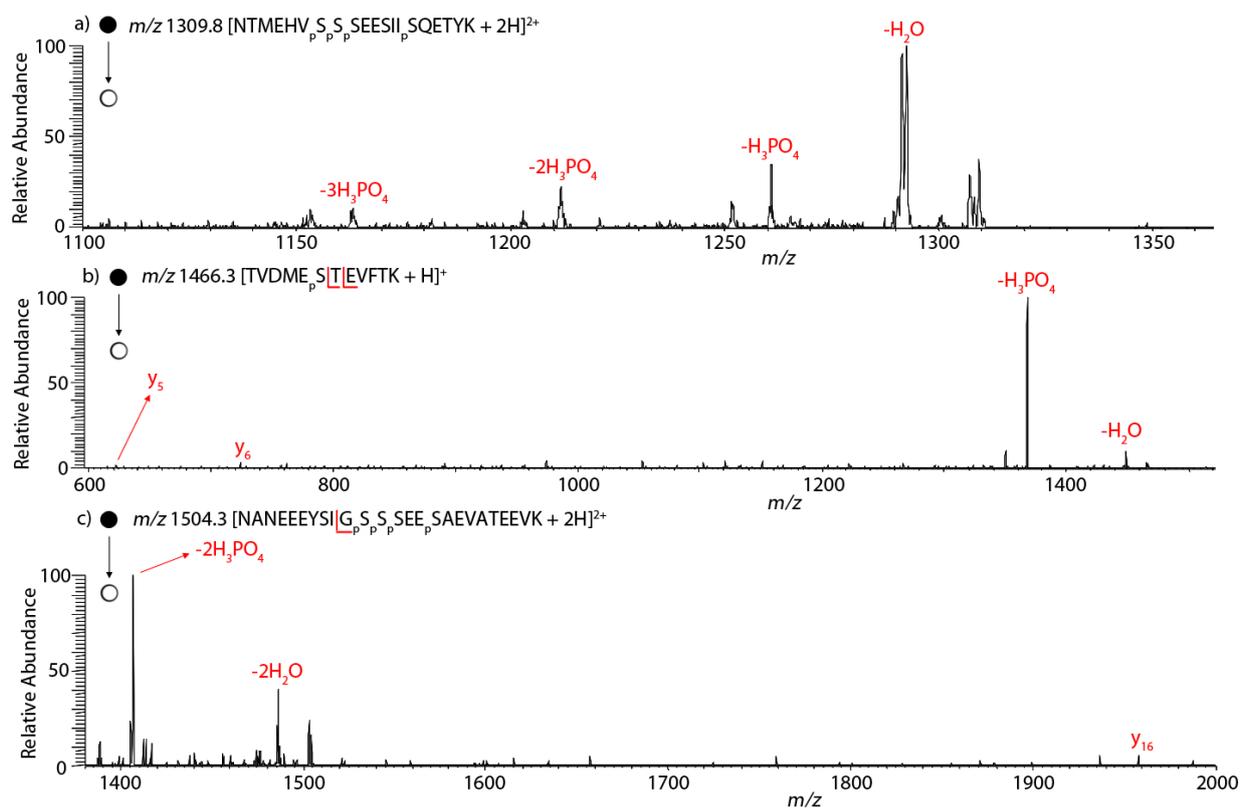
- |   |  |
|---|--|
| 1. $m/z$ 525.3 [VFGKEKVNE+2H] <sup>2+</sup>                     | 12. $m/z$ 878.5 [QLLRLKKYKVPQLE+2H] <sup>2+</sup>                |
| 2. $m/z$ 586.0 [QLLRLKKYKVPQLE+3H] <sup>3+</sup>                | 13. $m/z$ 889.9 [GIHAQQKEPMIGVNQE+2H] <sup>2+</sup>              |
| 3. $m/z$ 593.6 [GIHAQQKEPMIGVNQE+3H] <sup>3+</sup>              | 14. $m/z$ 902.4 [LAYFYPE+H] <sup>+</sup>                         |
| 4. $m/z$ 607.3 [IVPN <sub>p</sub> SAEERLHSMKE+3H] <sup>3+</sup> | 15. $m/z$ 910.4 [IVPN <sub>p</sub> SAEERLHSMKE+2H] <sup>2+</sup> |
| 5. $m/z$ 615.3 [LFRQFYQLD+2H] <sup>2+</sup>                     | 910.5 [QKHIQKE+H] <sup>+</sup>                                   |
| 6. $m/z$ 648.3 [IKQME+H] <sup>+</sup>                           | 16. $m/z$ 938.4 [IVPN <sub>p</sub> SAEE+H] <sup>+</sup>          |
| 7. $m/z$ 707.4 [RPKHPIKHQGLPQEVLE+3H] <sup>3+</sup>             | 17. $m/z$ 1049.6 [VFGKEKVNE+H] <sup>+</sup>                      |
| 8. $m/z$ 720.9 [DVPSERYLGYLE+2H] <sup>2+</sup>                  | 18. $m/z$ 1060.6 [RPKHPIKHQGLPQEVLE+2H] <sup>2+</sup>            |
| 9. $m/z$ 725.4 [NLLRFFVAPFPE+2H] <sup>2+</sup>                  | 19. $m/z$ 1229.6 [LFRQFYQLD+H] <sup>+</sup>                      |
| 10. $m/z$ 763.4 [DIKQME+H] <sup>+</sup>                         | 20. $m/z$ 1440.7 [DVPSERYLGYLE+H] <sup>+</sup>                   |
| 11. $m/z$ 876.5 [KTTMPLW+H] <sup>+</sup>                        | 21. $m/z$ 1449.8 [NLLRFFVAPFPE+H] <sup>+</sup>                   |



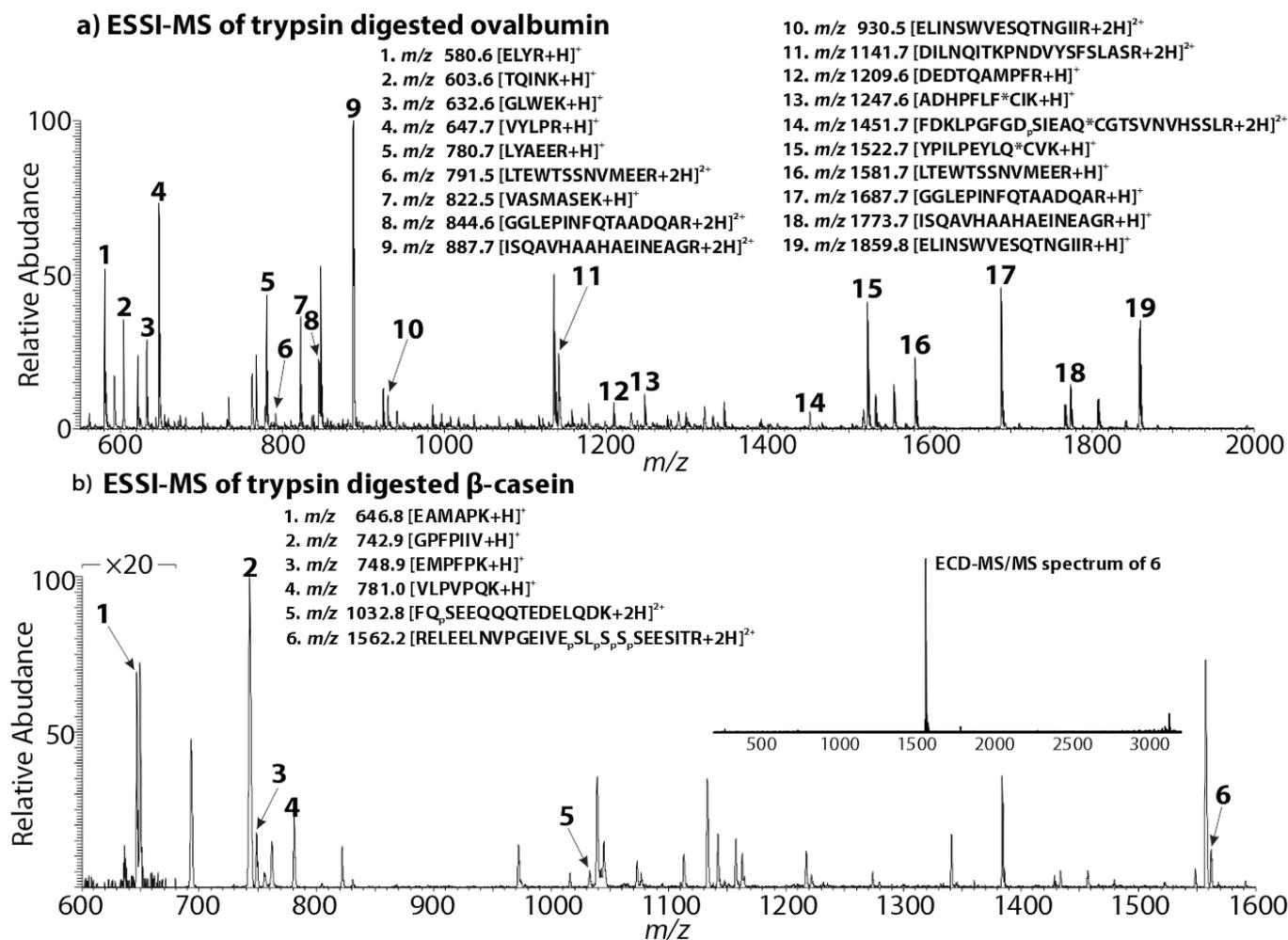
**b) DESI-MS of Glu-C digested  $\alpha$ -casein**



**Figure 5S.** a) ESSI-MS spectrum of  $\alpha$ -casein Glu-C digest at pH 3.7 and b) DESI-MS spectrum of  $\alpha$ -casein Glu-C digest at pH 2.0. Note the generation of  $[\text{AE}_p\text{SI}_p\text{S}_p\text{S}_p\text{SEEIVPN}_p\text{SVE}+2\text{H}]^{2+}$  ( $m/z$  1038.8) ion in b), which is missing in a). c) CID-MS/MS spectrum of the  $[\text{AE}_p\text{SI}_p\text{S}_p\text{S}_p\text{SEEIVPN}_p\text{SVE}+2\text{H}]^{2+}$  ( $m/z$  1038.8) ion.



**Figure 6S.** CID MS/MS spectra of a) [NTMEHV<sub>p</sub>S<sub>p</sub>S<sub>p</sub>SEESI<sub>p</sub>SQETYK+2H]<sup>2+</sup> ( $m/z$  1310), b) [TVDME<sub>p</sub>STEVFTK+H]<sup>+</sup> ( $m/z$  1466) and c) [NANEEEYSIG<sub>p</sub>S<sub>p</sub>S<sub>p</sub>SEE<sub>p</sub>SAEVATEEVK+2H]<sup>2+</sup> ( $m/z$  1504)

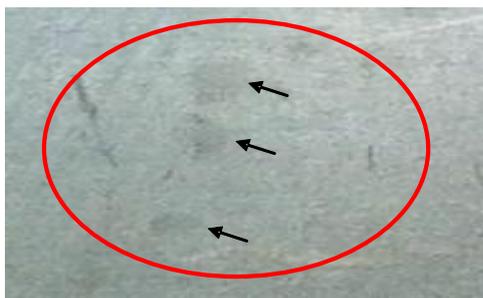


**Figure 7S.** ESSI-MS spectra of a) ovalbumin tryptic digest at pH 3.7 and b) β-casein tryptic digest at pH 3.8, \*C indicates the protected cysteine residue. Inset of b) shows ECD-MS/MS spectrum of doubly charged [RELEELNVPGEIVE<sub>p</sub>SL<sub>p</sub>S<sub>p</sub>S<sub>p</sub>SEESITR+2H]<sup>2+</sup> ( $m/z$  1562), which does not provide fragment ions from backbone cleavages.

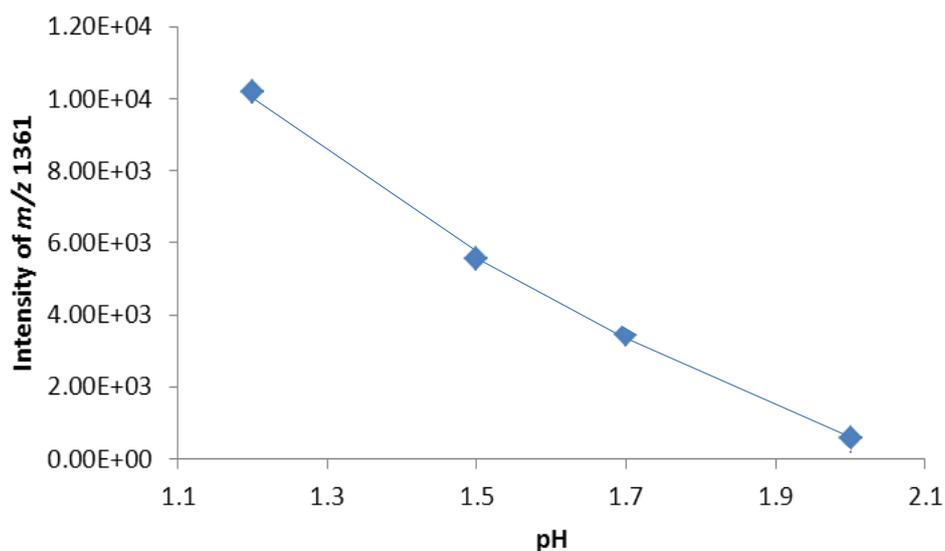
a) DESI



b) ESSi



**Figure 8S.** Images of a stainless steel metal surface after receiving sprays from a) DESI and b) ESSi for 30 min. The arrows in b indicate the corrosion marks.



**Figure 9S.** Chart showing the increasing intensity for the doubly charged phosphopeptide QMEAE<sub>p</sub>SI<sub>p</sub>S<sub>p</sub>S<sub>p</sub>SEEIVPN<sub>p</sub>SVEQK ( $m/z$  1361) with decreasing pH.

## References

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- (2) Mirza, M. R.; Rainer, M.; Güzel, Y.; Choudhary, I. M.; Bonn, G. K. *Anal. Bioanal. Chem.* **2012**, *404*, 853-862.
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