# Supporting Information

# Fast Protein Analysis Enabled by High-Temperature Hydrolysis

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## Experimental

**Chemicals and Materials.** Recombinant ubiquitin (MW: 8.56 kDa) was obtained from UB-biotech (Jilin, China). Bovine serum albumin (BSA, MW: 66.7 kDa) was purchased from Sigma-Aldrich (MO, USA), 11AA-phosphorylated-peptide (VLSPE-pY-LWDDR, MW: 1472 Da) from Guoping Pharmaceutical (Anhui, China), and horseradish peroxidase (HRP, MW: 44.3 kDa) from Acmec Biochemical (Shanghai, China). Recombinant antigens of *Mycobacterium tuberculosis*, including ESAT-6 (MW: 11.11 kDa) and CFP-10 (MW: 11.73 kDa) were purchase from Kitgen Bio-tech (Hangzhou, China). Formic acid (A.R.) was obtained from Fisher Scientific (NH, USA). Other chemicals and reagents were purchased from Sigma-Aldrich (MO, USA). The capillaries with pulled tips for nanoESI were prepared from glass capillaries (o.d. 1.5 mm, i.d. 0.86 mm, Sutter Instrument, CA, USA) using a P-1000 capillary puller (Sutter Instrument, CA, USA)

**Fabrication of the high-temperature microreactor.** The microreactor was homebuilt as shown in Figure S1a). A PFA tubing (o.d. 1/16 inch, i.d. 0.125 inch) of 10 cm was inserted into a stainless-steel tube (o.d. 2.5 mm, i.d. 2 mm), which was wrapped with an alloy heating wire (Ni80Cr20, o.d. 0.25 mm, 10 Ω) and coated with silicon grease for thermo insulation. A high temperature of 150 °C could be reached in 20 s at 12 V. The inlet and outlet of the PFA tubing were connected with spiral gated valves for fast sealing and opening. The heating process was controlled by a single-chip microcomputer and a thermistor was installed on the stainless-steel tube for temperature feedback. A relatively stable temperature control (±2 °C) was achieved with the control program.

**Protein hydrolysis and nanoESI-MS analysis.** Protein samples were prepared in purified

water. For analysis, the stock solutions were diluted by water and then mixed with formic acid or acetic acid. The sample solution was injected into the microreactor, sealed by the valves, heated up to 150 °C, and kept for 2-5 min for fast hydrolysis. After protein hydrolysis, the solution was transferred into a glass capillary with a pulled tip for direct analysis by nanoESI-MS. MS experiments were performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (MaXis Impact, Bruker Daltonics, Bremen, Germany) with a nanoESI source. Mass spectra were converted to MGF format by Bruker Compass DataAnalysis Software (Bruker Daltonics, Bremen, Germany) and subjected to MASCOT searches (Matrix Science Ltd., London, U.K.). Peptide mass tolerance was set as 0.05 Da.

A miniature mass spectrometer (57 cm (length) × 24 cm (width) × 32 cm (height)) was also used for protein and peptide analysis. It was a modified Mini β (PURSPEC Technologies, Beijing, China) instrument, equipped with a discontinuous atmospheric pressure interface (DAPI) for introduction of ions, and two linear ion traps. The mini MS system was coupled with nanoESI , by which voltages of 1500-1800 V were used for analysis of proteins and peptides.

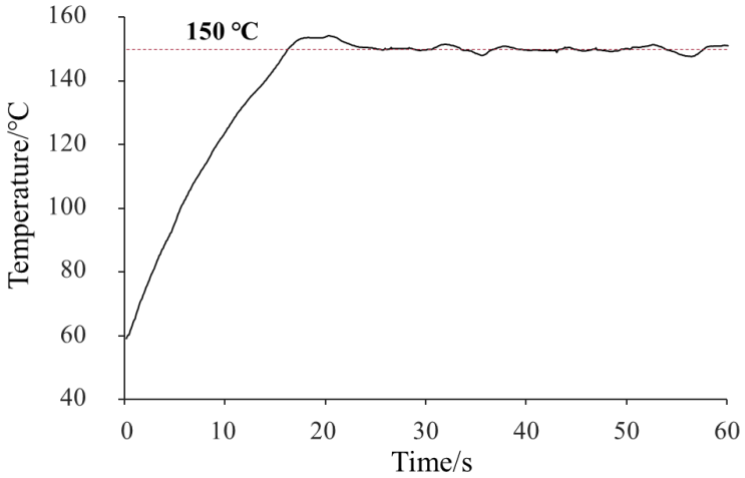



**Fig. S1.** Change of temperatures detected by the feedback thermistor of the microreactor by setting at 150 °C. It shows that the required temperature reaches within 20 s after heating, and the temperature keeps relatively stable afterwards.

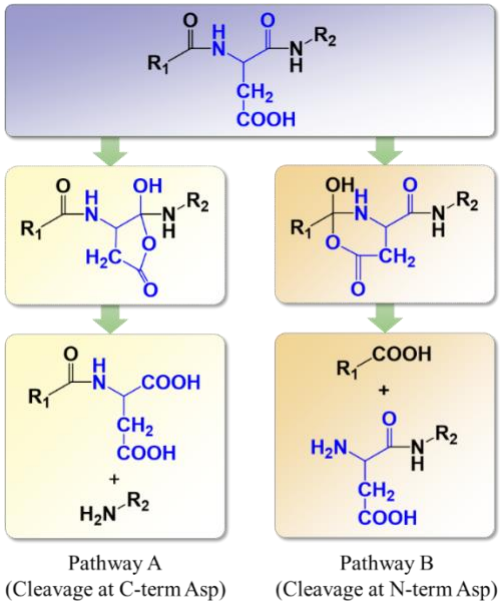



**Fig. S2.** Mechanism of the selective hydrolysis cleavage at X-Asp and Asp-X sites by high-temperature microreaction. Under acidic condition, an anhydride or a cyclic imide can be formed between the β-carboxyl group and the amide group of the peptide/protein at either terminal. Finally, Asp-X or X-Asp can be cleaved through elimination of one water molecule.[1, 2]

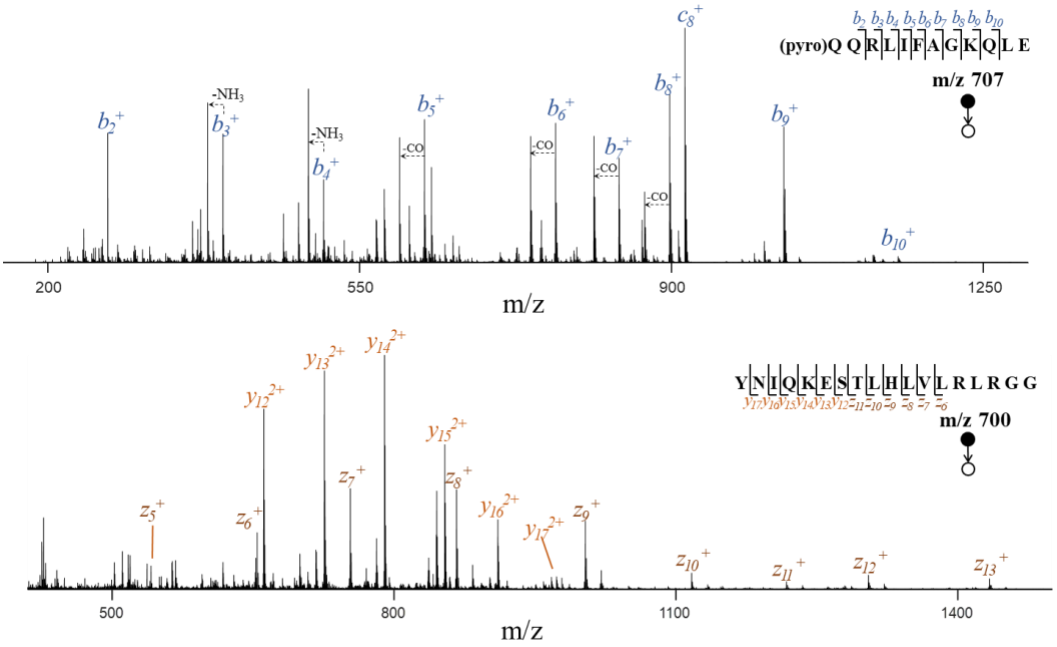



**Fig. S3.** MS/MS spectra of characteristic peptides hydrolyzed from ubiquitin. The analysis was performed by nanoESI-MS/MS in positive ion mode.

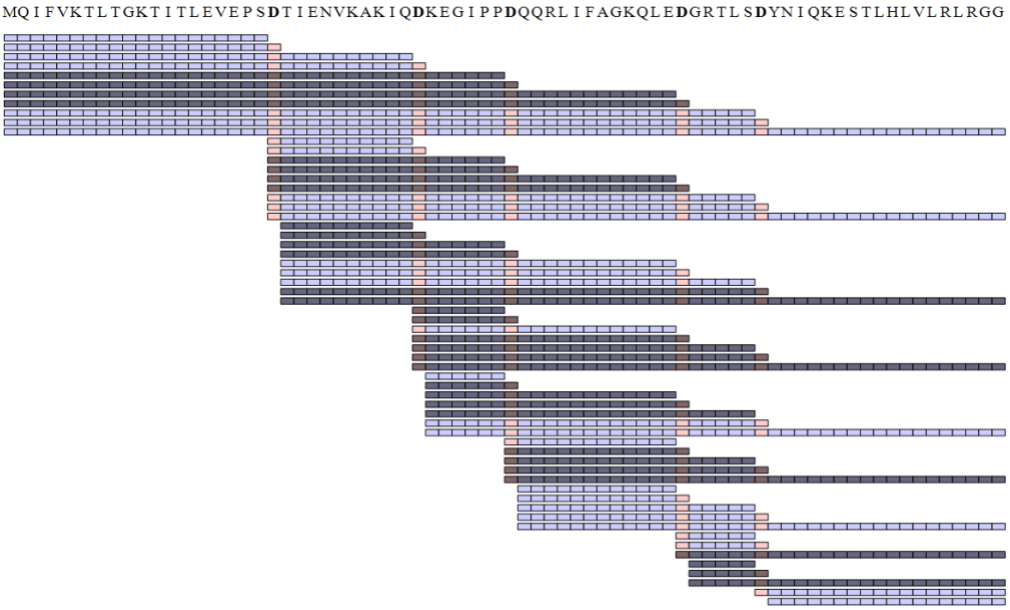



**Fig. S4.** Theoretical peptide distribution of ubiquitin through breakdown at the aspartyl site. Peptides detected after hydrolysis are labeled in dark grey.

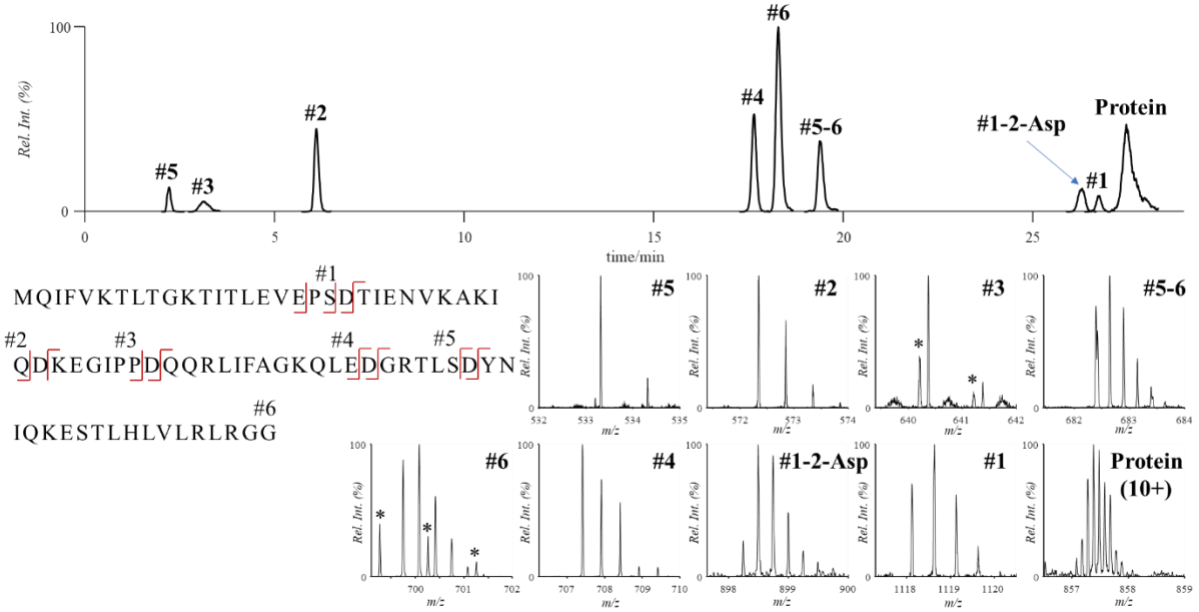



**Fig. S5.** LC-MS chromatogram and related MS spectra of peptides hydrolyzed from ubiquitin. The analysis was performed by an RPLC-ESI-Q-TOF system in positive ion mode. Impurities were marked with *.

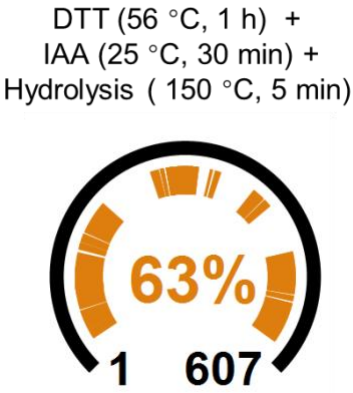



**Fig. S6.** Recovery of peptides of BSA treated by separated procedures: reduction by incubation with DTT for 1 h, incubation with IAA for 30 min and then high-temperature hydrolysis for 5 min.

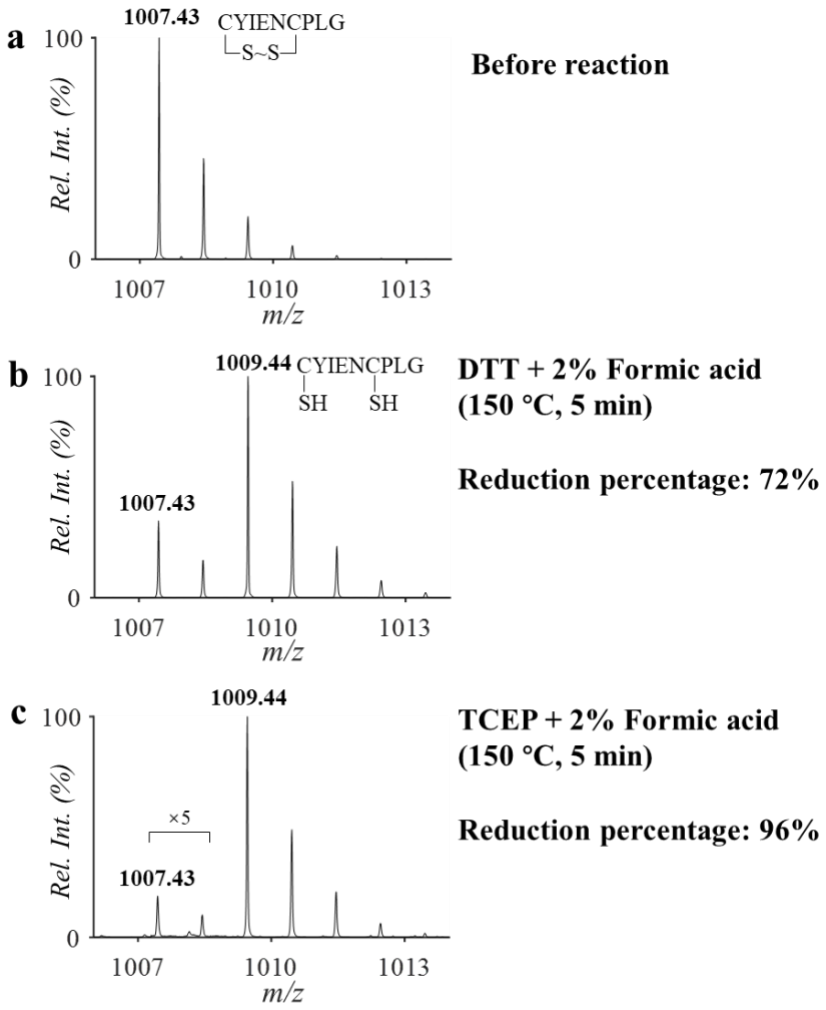



**Fig. S7.** Mass spectra of oxytocin (a) before reaction, (b) after treatment with DTT (12 mM) for 5 min (150 °C), (c) after treatment of TCEP (12 mM) for 5 min (150 °C). Oxytocin was prepared in water at 20 μM, with 2% formic acid (v/v). nanoESI-MS in positive ion mode was used for analysis. The reduction product was observed at m/z 1009.44, the reduction yield was calculated by the ratio of the reduced intensity of *m/z* 1007.43 to its intensity before reaction.

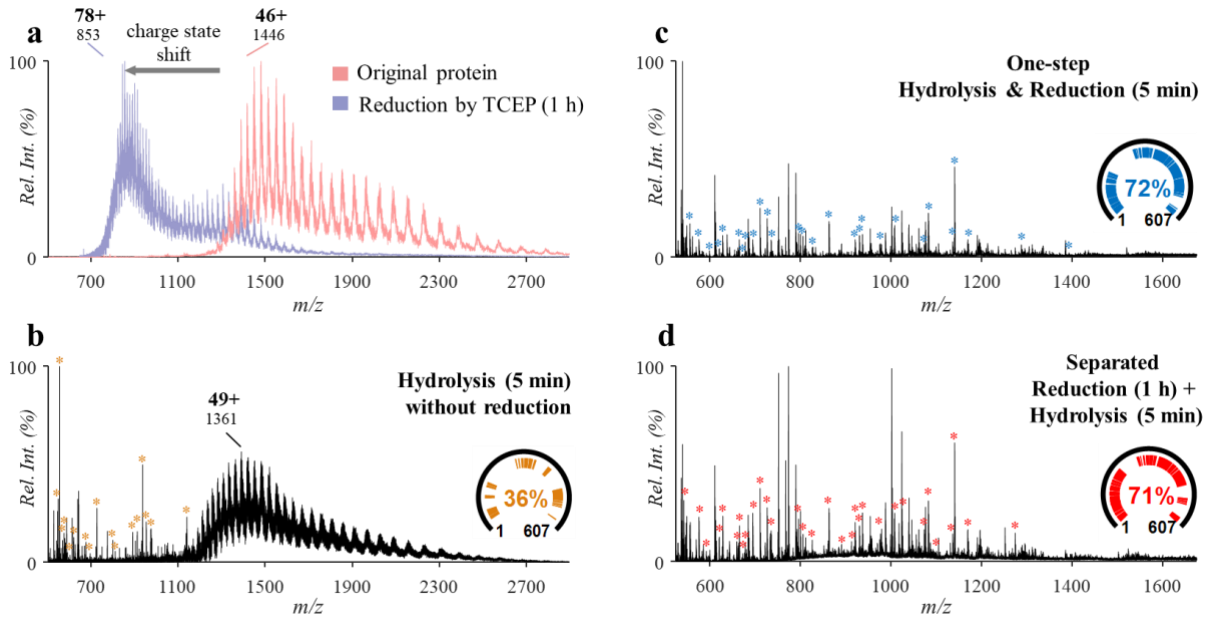



**Fig. S8.** (a) Mass spectra of BSA before (red) and after (blue) reduction by with TCEP (12 mM) for 1 h. (b) Mass spectrum of BSA after high-temperature hydrolysis without reduction. (c) Mass spectrum of BSA treated by separated procedures: reduction by incubation with TCEP for 1 h and then high-temperature hydrolysis for 5 min. (d) Mass spectrum of BSA by one-step high-temperature hydrolysis and reduction for 5 min. Peptides identified by MASCOT were marked with *.

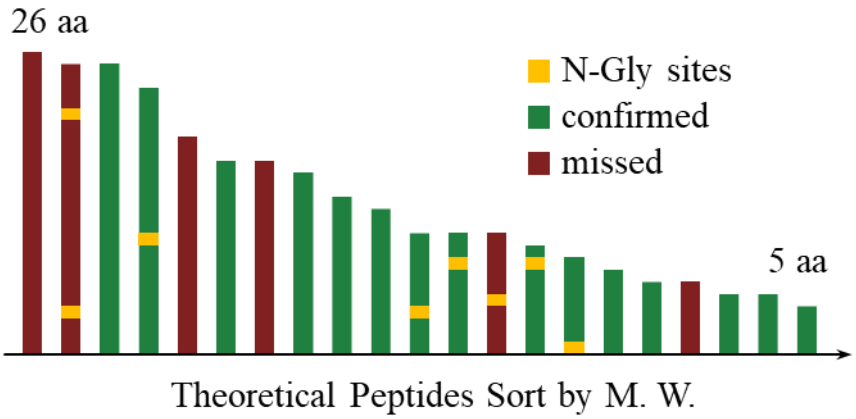
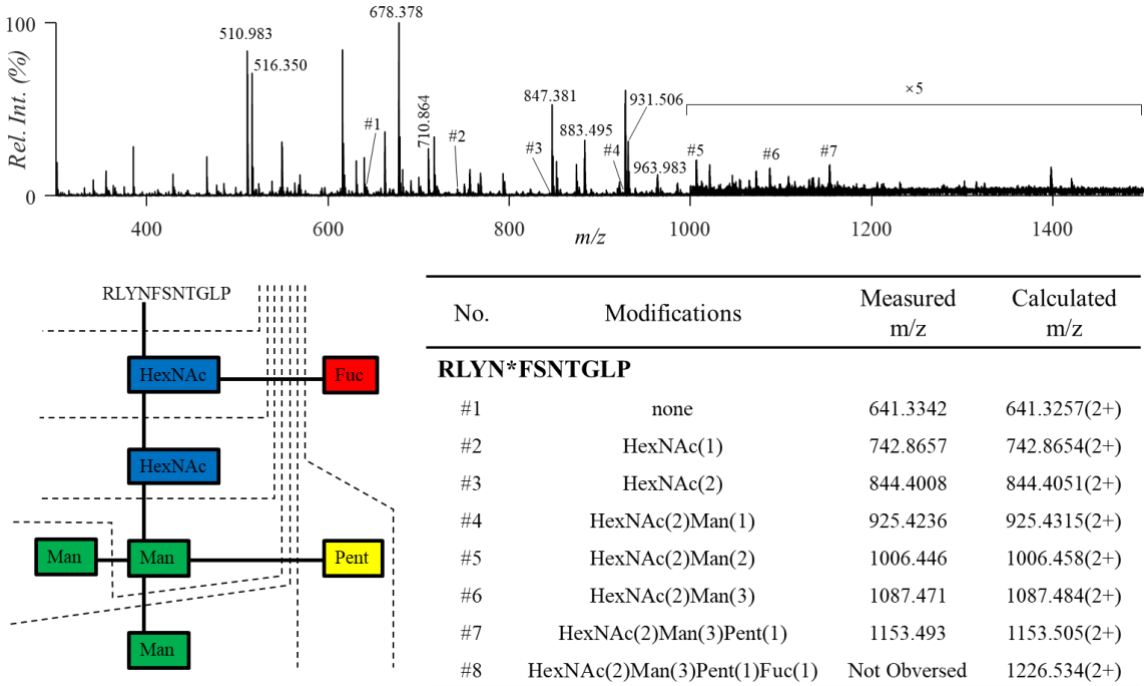



**Fig. S9.** Amino acid sequence coverage of HRP analyzed by hydrolysis and nanoESI-MS.

Peptides detected after hydrolysis are listed in Table S4.




| No. | Modifications | Measured m/z | Calculated m/z |
|---|---|---|---|
| **RLYN*FSNTGLP** | | | |
| #1 | none | 641.3342 | 641.3257(2+) |
| #2 | HexNAc(1) | 742.8657 | 742.8654(2+) |
| #3 | HexNAc(2) | 844.4008 | 844.4051(2+) |
| #4 | HexNAc(2)Man(1) | 925.4236 | 925.4315(2+) |
| #5 | HexNAc(2)Man(2) | 1006.446 | 1006.458(2+) |
| #6 | HexNAc(2)Man(3) | 1087.471 | 1087.484(2+) |
| #7 | HexNAc(2)Man(3)Pent(1) | 1153.493 | 1153.505(2+) |
| #8 | HexNAc(2)Man(3)Pent(1)Fuc(1) | Not Obversed | 1226.534(2+) |

**Fig. S10.** Mass spectra and possible cleavage pathways for characterization of glycopeptide peaks from HRP.

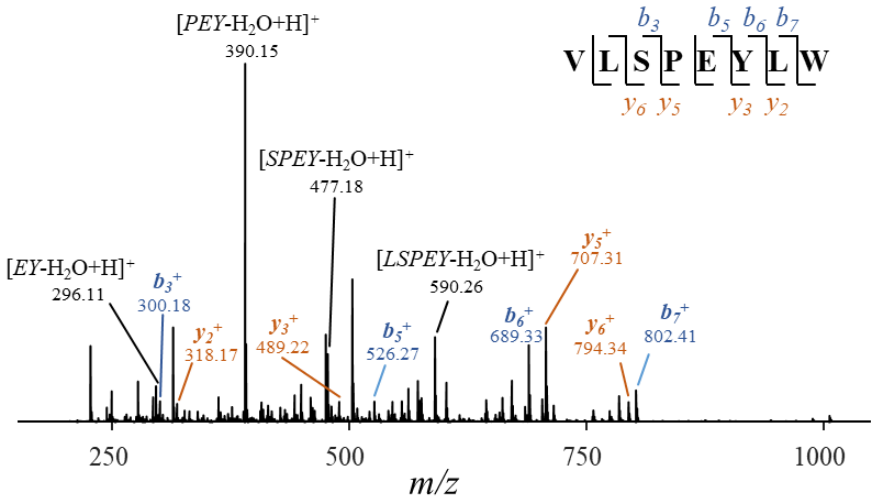



**Fig. S11.** MS/MS spectrum of peptide residue (VLSPEYLW) after hydrolysis of the phosphopeptide.

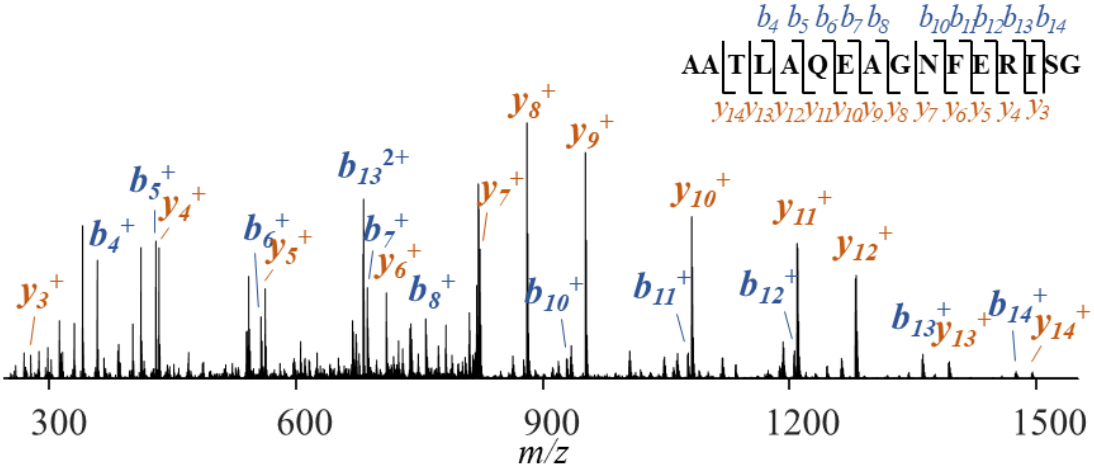



**Fig. S12.** MS/MS spectrum of peptide #1 (AATLAQEAGNFERISG) hydrolyzed from CFP-10.

**Table S1.** Comparison of the trypsin-based enzymatic digestion method with the high-temperature hydrolysis method.

| | **Trypsin-based enzymatic digestion*** | **High-temperature hydrolysis** |
|---|---|---|
| Time | ~ 12 h | 2-5 min |
| Salt | 100 mM ammonium bicarbonate | None |
| Enzyme | Trypsin | None |
| Reagent | Urea | Formic acid |
| Temperature | ~ 37 ℃ | 120 ℃ - 180 ℃ |
| Post-treatment | Desalting and purification | None |
| Analytical methods | LC-MS<br>MALDI-MS | nanoESI-MS<br>LC-MS<br>Miniature MS |

* Information from *Nature Biotechnology* 2001, 19, 242–247

**Table S2.** MASCOT searching results of 2-min BSA high-temperature hydrolysis without DTT.

| Start | End | Observed | Mr(expt) | Mr(calc) | Delta | M | Peptide |
|---|---|---|---|---|---|---|---|
| 25 | 37 | 1583.8004 | 1582.7931 | 1582.7852 | 0.0079 | 2 | R.DTHKSEIAHRFKD.L |
| 26 | 36 | 1353.7428 | 1352.7355 | 1352.7313 | 0.0042 | 0 | D.THKSEIAHRFK.D |
| 26 | 37 | 1468.7858 | 1467.7785 | 1467.7582 | 0.0203 | 1 | D.THKSEIAHRFKD.L |
| 38 | 60 | 2666.3467 | 2665.3394 | 2665.3489 | -0.0094 | 0 | D.LGEEHFKGLVLIAFSQYLQQCPF.D |
| | | | | | | | + Dehydro (C) |
| 38 | 61 | 2782.3494 | 2781.3421 | 2781.3836 | -0.0415 | 1 | D.LGEEHFKGLVLIAFSQYLQQCPFD.E |
| 97 | 109 | 1450.737 | 1449.7297 | 1449.7286 | 0.0011 | 0 | D.ELCKVASLRETYG.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 135 | 142 | 925.5381 | 924.5308 | 924.528 | 0.0028 | 2 | P.DLPKLKPD.P |
| 136 | 141 | 695.4837 | 694.4764 | 694.4741 | 0.0023 | 0 | D.LPKLKP.D |
| 136 | 142 | 810.5108 | 809.5035 | 809.5011 | 0.0025 | 1 | D.LPKLKPD.P |
| 143 | 152 | 1137.5379 | 1136.5306 | 1136.5172 | 0.0134 | 2 | D.PNTLCDEFKA.D |
| 149 | 152 | 476.2519 | 475.2446 | 475.2431 | 0.0016 | 0 | D.EFKA.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 149 | 153 | 591.2799 | 590.2726 | 590.27 | 0.0026 | 1 | D.EFKAD.E |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 273 | 277 | 547.2675 | 546.2602 | 546.2598 | 0.0005 | 0 | D.LLECA.D |
| | | | | | | | + Dehydro (C) |
| 273 | 277 | 548.2758 | 547.2685 | 547.2676 | 0.001 | 0 | D.LLECA.D |
| 283 | 288 | 710.3927 | 709.3854 | 709.3833 | 0.0022 | 0 | D.LAKYIC.D |
| 293 | 302 | 1109.5376 | 1108.5303 | 1108.5257 | 0.0047 | 0 | D.TISSKLKECC.D |
| | | | | | | | + 2 Dehydro (C) |
| 304 | 318 | 1723.9457 | 1722.9384 | 1722.9338 | 0.0046 | 0 | D.KPLLEKSHCIAEVEK.D |
| 320 | 330 | 1135.6415 | 1134.6342 | 1134.6284 | 0.0058 | 0 | D.AIPENLPPLTA.D |
| 320 | 334 | 1597.8044 | 1596.7971 | 1596.8035 | -0.0064 | 2 | D.AIPENLPPLTADFAE.D |
| 338 | 346 | 1082.5367 | 1081.5294 | 1081.5226 | 0.0068 | 0 | D.VCKNYQEAK.D |
| 389 | 404 | 1857.9759 | 1856.9686 | 1856.9607 | 0.0079 | 2 | D.PHACYSTVFDKLKHLV.D |
| 399 | 404 | 737.4991 | 736.4918 | 736.4959 | -0.0041 | 0 | D.KLKHLV.D |
| 399 | 405 | 852.529 | 851.5217 | 851.5229 | -0.0011 | 1 | D.KLKHLVD.E |
| 475 | 516 | 4757.4735 | 4756.4662 | 4756.4689 | -0.0027 | 0 | D.YLSLILNRLCVLHEKTPVSEKVTK CCTESLVNRRPCFSALTP.D |
| | | | | | | | + 4 Dehydro (C) |
| 475 | 517 | 4872.4878 | 4871.4805 | 4871.4959 | | 1 | D.YLSLILNRLCVLHEKTPVSEKVTK |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | -0.0153 | | CCTESLVNRRPCFSALTPD.E |
| | | | | | | | + 4 Dehydro (C) |
| 518 | 525 | 936.4826 | 935.4753 | 935.4753 | 0.0001 | 0 | D.ETYVPKAF.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 527 | 534 | 974.5112 | 973.5039 | 973.5022 | 0.0018 | 0 | D.EKLFTFHA.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 536 | 540 | 545.2888 | 544.2815 | 544.2805 | 0.001 | 0 | D.ICTLP.D |
| | | | | | | | + Dehydro (C) |
| 536 | 540 | 546.2987 | 545.2914 | 545.2883 | 0.0031 | 0 | D.ICTLP.D |
| 580 | 585 | 610.1839 | 609.1766 | 609.2251 | -0.0484 | 1 | D.KCCAAD.D |

**Table S3.** MASCOT searching results of 5-min "one-step" BSA high-temperature hydrolysis mixed with DTT.

| Start | End | Observed | Mr(expt) | Mr(calc) | Delta | M | Peptide |
|---|---|---|---|---|---|---|---|
| 26 | 36 | 1353.7084 | 1352.7011 | 1352.7313 | -0.0302 | 0 | D.THKSEIAHRFK.D |
| 26 | 37 | 1468.7671 | 1467.7598 | 1467.7582 | 0.0016 | 1 | D.THKSEIAHRFKD.L |
| 62 | 79 | 2013.0566 | 2012.0493 | 2012.0401 | 0.0092 | 0 | D.EHVKLVNELTEFAKTCVA.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 81 | 95 | 1597.7598 | 1596.7525 | 1596.7355 | 0.017 | 0 | D.ESHAGCEKSLHTLFG.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 97 | 109 | 1450.7464 | 1449.7391 | 1449.7286 | 0.0105 | 0 | D.ELCKVASLRETYG.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 111 | 130 | 2394.9527 | 2393.9454 | 2393.9861 | -0.0407 | 2 | D.MADCCEKQEPERNECFLSHK.D |
| | | | | | | | + 2 Dehydro (C) |
| 114 | 130 | 2077.9108 | 2076.9035 | 2076.8815 | 0.022 | 0 | D.CCEKQEPERNECFLSHK.D |
| | | | | | | | + 2 Dehydro (C) |
| 136 | 141 | 695.4846 | 694.4773 | 694.4741 | 0.0032 | 0 | D.LPKLKP.D |
| 143 | 147 | 546.2301 | 545.2228 | 545.2394 | -0.0165 | 0 | D.PNTLC.D |
| | | | | | | | + Dehydro (C) |
| 143 | 152 | 1137.5051 | 1136.4978 | 1136.5172 | -0.0194 | 2 | D.PNTLCDEFKA.D |
| 143 | 152 | 1137.5421 | 1136.5348 | 1136.5172 | 0.0176 | 2 | D.PNTLCDEFKA.D |
| 148 | 153 | 724.3238 | 723.3165 | 723.3075 | 0.009 | 2 | C.DEFKAD.E |
| 154 | 195 | 5208.5402 | 5207.5329 | 5207.489 | 0.0439 | 0 | D.EKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAE.D |
| | | | | | | | + 2 Dehydro (C); Glu->pyro-Glu (N-term E) |
| 154 | 196 | 5341.5553 | 5340.548 | 5340.5266 | 0.0215 | 1 | D.EKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAED.K |
| | | | | | | | + 2 Dehydro (C) |
| 261 | 271 | 1252.5897 | 1251.5824 | 1251.5853 | -0.0028 | 0 | D.LTKVHKECCHG.D |
| | | | | | | | + 2 Dehydro (C) |
| 261 | 277 | 1897.9039 | 1896.8966 | 1896.877 | 0.0196 | 2 | D.LTKVHKECCHGDLLECA.D |
| | | | | | | | + Dehydro (C) |
| 273 | 277 | 547.2653 | 546.258 | 546.2598 | -0.0017 | 0 | D.LLECA.D |
| | | | | | | | + Dehydro (C) |
| 273 | 279 | 778.3427 | 777.3354 | 777.3215 | 0.014 | 2 | D.LLECADD.R |
| 279 | 282 | 476.2251 | 475.2178 | 475.2027 | 0.0152 | 2 | D.DRAD.L |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 283 | 288 | 710.3935 | 709.3862 | 709.3833 | 0.003 | 0 | D.LAKYIC.D |
| 293 | 318 | 2930.4849 | 2929.4776 | 2929.4837 | -0.006 | 2 | D.TISSKLKECCDKPLLEKSHCIAEVEK.D |
| | | | | | | | + Dehydro (C) |
| 303 | 319 | 1953.994 | 1952.9867 | 1952.9877 | -0.001 | 2 | C.DKPLLEKSHCIAEVEKD.A |
| 304 | 318 | 1722.9467 | 1721.9394 | 1721.926 | 0.0134 | 0 | D.KPLLEKSHCIAEVEK.D |
| | | | | | | | + Dehydro (C) |
| 320 | 330 | 1135.6436 | 1134.6363 | 1134.6284 | 0.0079 | 0 | D.AIPENLPPLTA.D |
| 332 | 335 | 481.1964 | 480.1891 | 480.1856 | 0.0035 | 1 | D.FAED.K |
| 332 | 336 | 609.2925 | 608.2852 | 608.2806 | 0.0046 | 2 | D.FAEDK.D |
| 336 | 346 | 1325.6074 | 1324.6001 | 1324.6445 | -0.0444 | 2 | D.KDVCKNYQEAK.D |
| 338 | 346 | 1081.5482 | 1080.5409 | 1080.5148 | 0.0261 | 0 | D.VCKNYQEAK.D |
| | | | | | | | + Dehydro (C) |
| 338 | 347 | 1197.5725 | 1196.5652 | 1196.5495 | 0.0157 | 1 | D.VCKNYQEAKD.A |
| 338 | 386 | 5730.7902 | 5729.7829 | 5729.787 | -0.0041 | 2 | D.VCKNYQEAKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAK.D |
| | | | | | | | + 3 Dehydro (C) |
| 348 | 386 | 4553.3119 | 4552.3046 | 4552.2559 | 0.0488 | 0 | D.AFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAK.D |
| | | | | | | | + 2 Dehydro (C) |
| 348 | 387 | 4668.3299 | 4667.3226 | 4667.2828 | 0.0398 | 1 | D.AFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKD.D |
| | | | | | | | + 2 Dehydro (C) |
| 389 | 397 | 1024.419 | 1023.4117 | 1023.4484 | -0.0367 | 0 | D.PHACYSTVF.D |
| 389 | 404 | 1857.9717 | 1856.9644 | 1856.9607 | 0.0037 | 2 | D.PHACYSTVFDKLKHLV.D |
| 399 | 404 | 737.5068 | 736.4995 | 736.4959 | 0.0036 | 0 | D.KLKHLV.D |
| 406 | 415 | 1167.5848 | 1166.5775 | 1166.5628 | 0.0147 | 0 | D.EPQNLIKQNC.D |
| | | | | | | | + Dehydro (C); Glu->pyro-Glu (N-term E) |
| 406 | 416 | 1300.6406 | 1299.6333 | 1299.6003 | 0.033 | 1 | D.EPQNLIKQNCD.Q |
| | | | | | | | + Dehydro (C) |
| 417 | 473 | 6428.292 | 6427.2847 | 6427.2464 | 0.0384 | 0 | D.QFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTE.D |
| | | | | | | | + Dehydro (C); Gln->pyro-Glu (N-term Q) |
| 417 | 474 | 6544.2892 | 6543.2819 | 6543.2811 | 0.0008 | 1 | D.QFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTED.Y |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | | + Gln->pyro-Glu (N-term Q) |
| 475 | 516 | 4759.4943 | 4758.487 | 4758.4845 | 0.0025 | 0 | D.YLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTP.D |
| | | | | | | | + 2 Dehydro (C) |
| 475 | 517 | 4873.5564 | 4872.5491 | 4872.5037 | 0.0454 | 1 | D.YLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPD.E |
| | | | | | | | + 3 Dehydro (C) |
| 475 | 525 | 5811.0175 | 5810.0102 | 5809.9946 | 0.0157 | 2 | D.YLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAF.D |
| | | | | | | | + Dehydro (C) |
| 527 | 534 | 992.5367 | 991.5294 | 991.5127 | 0.0167 | 0 | D.EKLFTFHA.D |
| 580 | 584 | 493.1999 | 492.1926 | 492.1825 | 0.0101 | 0 | D.KCCAA.D |
| | | | | | | | + 2 Dehydro (C) |
| 580 | 585 | 610.1861 | 609.1788 | 609.2251 | -0.0462 | 1 | D.KCCAAD.D |
| 587 | 607 | 2161.1609 | 2160.1536 | 2160.1375 | 0.0162 | 0 | D.KEACFAVEGPKLVVSTQTALA.- |
| | | | | | | | + Dehydro (C) |

**Table S4.** MASCOT searching results of 2-min BSA high-temperature hydrolysis reduction after incubation with DTT for 1 h.

| Start | End | Observed | Mr(expt) | Mr(calc) | Delta | M | Peptide |
|---|---|---|---|---|---|---|---|
| 81 | 95 | 1596.7644 | 1595.7571 | 1595.7277 | 0.0295 | 0 | D.ESHAGCEKSLHTLFG.D |
| | | | | | | | + Dehydro (C); Glu->pyro-Glu (N-term E) |
| 81 | 96 | 1711.7738 | 1710.7665 | 1710.7546 | 0.0119 | 1 | D.ESHAGCEKSLHTLFGD.E |
| | | | | | | | + Dehydro (C); Glu->pyro-Glu (N-term E) |
| 97 | 109 | 1450.744 | 1449.7367 | 1449.7286 | 0.0081 | 0 | D.ELCKVASLRETYG.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 97 | 109 | 1467.7617 | 1466.7544 | 1466.7313 | 0.0231 | 0 | D.ELCKVASLRETYG.D |
| | | | | | | | + Dehydro (C) |
| 97 | 110 | 1564.7475 | 1563.7402 | 1563.7477 | -0.0075 | 1 | D.ELCKVASLRETYGD.M |
| | | | | | | | + Dehydro (C); Glu->pyro-Glu (N-term E) |
| 97 | 112 | 1767.8435 | 1766.8362 | 1766.8331 | 0.0031 | 2 | D.ELCKVASLRETYGDMA.D |
| | | | | | | | + Glu->pyro-Glu (N-term E) |
| 133 | 141 | 994.5536 | 993.5463 | 993.5859 | -0.0395 | 2 | D.SPDLPKLKP.D |
| 143 | 147 | 546.204 | 545.1967 | 545.2394 | -0.0426 | 0 | D.PNTLC.D |
| | | | | | | | + Dehydro (C) |
| 143 | 152 | 1137.54 | 1136.5327 | 1136.5172 | 0.0155 | 2 | D.PNTLCDEFKA.D |
| 154 | 195 | 5209.5264 | 5208.5191 | 5208.4969 | 0.0223 | 0 | D.EKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAE.D |
| | | | | | | | + Dehydro (C); Glu->pyro-Glu (N-term E) |
| 279 | 282 | 476.1695 | 475.1622 | 475.2027 | -0.0404 | 2 | D.DRAD.L |
| 280 | 288 | 1051.5376 | 1050.5303 | 1050.5406 | -0.0103 | 2 | D.RADLAKYIC.D |
| | | | | | | | + Dehydro (C) |
| 303 | 319 | 1952.9894 | 1951.9821 | 1951.9799 | 0.0022 | 2 | C.DKPLLEKSHCIAEVEKD.A |
| | | | | | | | + Dehydro (C) |
| 304 | 318 | 1722.9434 | 1721.9361 | 1721.926 | 0.0101 | 0 | D.KPLLEKSHCIAEVEK.D |
| | | | | | | | + Dehydro (C) |
| 320 | 330 | 1135.6433 | 1134.636 | 1134.6284 | 0.0076 | 0 | D.AIPENLPPLTA.D |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 332 | 335 | 481.2038 | 480.1965 | 480.1856 | 0.0109 | 1 | D.FAED.K |
| 348 | 386 | 4553.3045 | 4552.2972 | 4552.2559 | 0.0414 | 0 | D.AFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAK.D |
| | | | | | | | + 2 Dehydro (C) |
| 348 | 387 | 4668.3334 | 4667.3261 | 4667.2828 | 0.0433 | 1 | D.AFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKD.D |
| | | | | | | | + 2 Dehydro (C) |
| 389 | 404 | 1857.9675 | 1856.9602 | 1856.9607 | -0.0005 | 2 | D.PHACYSTVFDKLKHLV.D |
| 406 | 415 | 1185.5854 | 1184.5781 | 1184.5734 | 0.0048 | 0 | D.EPQNLIKQNC.D |
| | | | | | | | + Dehydro (C) |
| 406 | 416 | 1300.6401 | 1299.6328 | 1299.6003 | 0.0325 | 1 | D.EPQNLIKQNCD.Q |
| | | | | | | | + Dehydro (C) |
| 417 | 473 | 6429.3023 | 6428.295 | 6428.2542 | 0.0409 | 0 | D.QFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTE.D |
| | | | | | | | + Gln->pyro-Glu (N-term Q) |
| 475 | 516 | 4759.4982 | 4758.4909 | 4758.4845 | 0.0064 | 0 | D.YLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTP.D |
| | | | | | | | + 2 Dehydro (C) |
| 475 | 517 | 4875.5507 | 4874.5434 | 4874.5193 | 0.0241 | 1 | D.YLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPD.E |
| | | | | | | | + Dehydro (C) |
| 518 | 534 | 2042.9821 | 2041.9748 | 2042.0149 | -0.0401 | 2 | D.ETYVPKAFDEKLFTFHA.D |
| 536 | 540 | 545.2702 | 544.2629 | 544.2805 | -0.0176 | 0 | D.ICTLP.D |
| | | | | | | | + Dehydro (C) |
| 536 | 541 | 660.3017 | 659.2944 | 659.3074 | -0.013 | 1 | D.ICTLPD.T |
| | | | | | | | + Dehydro (C) |
| 542 | 584 | 4886.5631 | 4885.5558 | 4885.5908 | -0.0349 | 2 | D.TEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAA.D |
| | | | | | | | + 2 Dehydro (C) |
| 580 | 585 | 610.1853 | 609.178 | 609.2251 | -0.047 | 1 | D.KCCAAD.D |
| 587 | 607 | 2162.1598 | 2161.1525 | 2161.1453 | 0.0072 | 0 | |

**Table S5.** Observed hydrolyzed peptides of HRP which sequence according to UniProtKB - P00433. N* denotes glycosylated Asn.

| No. | Calculated. M. W. | Observed Ions | Position | Sequence |
|---|---|---|---|---|
| 1 | 2646.51 | 883.49(3+), 662.88(4+) | 100-124 | LLTIAAQQSVTLAGGPSWRVPLGRR |
| 2* | 2590.29 | 932.11(3+), 1107.84(3+), 1161.85(3+), 1205.86(3+) | 259-281 | TIPLVRSFAN*STQTFFNAFVEAM |
| 3 | 1924.99 | 963.98(2+) | 231-246 | NKYYVNLEEQKGLIQS |
| 4 | 1854.04 | 928.51(2+) | 133-149 | LANANLPAPFFTLPQLK |
| 5 | 1529.89 | 510.98(3+), 765.94(2+) | 30-42 | PRIAASILRLHFH |
| 6 | 1419.73 | 710.86(2+) | 67-80 | AFGNANSARGFPVI |
| 7* | 1280.65 | 641.33(2+), 742.87(2+), 844.41(2+), 925.43(2+), 1006.46(2+), 1087.48(2+), 1153.51(2+), | 183-193 | RLYNFSN*TGLP |
| 8* | 1235.64 | 1064.96(2+), 1130.98(2+) | 151-161 | SFRNVGLN*RSS |
| 9* | 1092.51 | 802.37(2+), 903.91(2+), | 248-257 | QELFSSPN*AT |
| 10* | 1082.54 | 907.40(2+), 988.42(2+), 1054.44(2+) | 57-65 | N*TTSFRTEK |
| 11 | 930.51 | 931.50(1+) | 21-28 | TIVNELRS |
| 12 | 846.50 | 847.49(1+) | 223-229 | LRTPTIF |
| 13 | 677.37 | 678.38(1+) | 126-131 | SLQAFL· |
| 14 | 639.21 | 640.22(1+) | 44-49 | CFVNGC (with an internal disulfide bond) \|_____\| |
| 15 | 515.33 | 516.35(1+) | 51-55 | ASILL |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