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Supporting Information

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Ultrafast Extraction of Proteins from Tissues Using Desorption by Impulsive Vibrational Excitation**

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Supporting Information

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Experimental section

Chemicals

Water, methanol (MeOH) and acetonitrile (ACN; all HPLC-grade) were obtained from Merck (Darmstadt, Germany). Sequence-grade trypsin and resuspension buffer was purchased from Promega (Mannheim, Germany). Roche complete proteases inhibitor was obtained from Hoffmann-La Roche (Penzberg, Germany). Any other chemicals and proteins were obtained from Sigma-Aldrich (Munich, Germany).

PIRL ablation

The PIRL is a commercially available model PIRL-HP2-1064 OPA-3000 from Attodyne Inc. (Toronto, Canada), with the wavelength of 3 μm, the repetition rate of 1 kHz and the pulse width of 300 ps. A home-built optical system delivers and focuses the PIRL beam onto the sample surface. The optical power at the sample surface was approximately 450 mW. The optical energy density at the sample surface was 3.39 J/cm^2 and the average optical power density was 3.39×10^3 W/cm^2. The PIRL beam was scanned at the speed of 130 mm/s during the ablation process. Aliquots of RNase A solution, trypsin solution and human plasma were transferred onto caps of reaction vials. The PIRL ablation was performed on the surface of the sample solutions in a square pattern of 4 mm x 4 mm until the aliquot was gone. The mouse muscle and liver tissues were ablated by the PIRL in a square pattern of 5 mm x 5 mm for 5 min each.

Ablation of tissue samples

Liver biopsies and mouse muscle samples were taken from male 16 wk old C57BL/6 mouse after asphyxia and cervical dislocation.[1] Mouse was kept in an SPF-certified germ-free facility and fed ad libitum. All animal experiments were approved by the local animal care committee (Behörde Soziales, Familie, Gesundheit und Verbraucherschutz, Hamburg). The tissue samples were mounted on an ablation block and an area of 5 mm x 5 mm was ablated

with the PIRL for 5 min. The ablation plume was captured with a cryo-trap using liquid N_2 (Figure S1).

Gel electrophoresis of ablated tissue samples

The protein concentrations of the ablated mouse liver and muscle tissue samples were determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). For SDS-PAGE 7.5 µL of the muscle sample (c= 19.33 µg/µL) and 2 µL of the liver sample (c= 20.10 µg/µL) were dissolved in 5 µL 4x sample buffer, 1 µL 20x reducing agent and filled up to 20 µL with HPLC-grade water. The samples were incubated at 95°C for 5 min and loaded onto a 10% Criterion™ XT Bis-Tris gel (Bio-Rad, Munich, Germany). Proteins were separated at a constant voltage of 120 V for 45 min. The gel was stained over night in accordance to Dyballa and Metzger[2] and destained with 40% MeOH.

Western Blotting

Western transfers and detection of the CEACAM1 protein with P1 rabbit polyclonal anti-CEACAM1 antiserum in Western Blotting have been performed as described.[3]

Conventional protein extraction from mouse muscle tissue and gel electrophoresis

A small piece of tissue (5 mm x 5 mm and 1.5 mm in depth) was cut out of a mouse muscle. The extracted tissue was frozen using liquid nitrogen, lyophilised and subsequently homogenized with a mortar and a pestle. The homogenate was incubated for 30 min in 500 µL lysis buffer (25 mM Tris, 150 mM NaCl, 10% glycerol, 1% triton, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM glycerol phosphate, Roche complete proteases inhibitor) on ice. Afterwards the lysate was centrifuged for 20 min at 14680 rpm at 4°C. The supernatant was collected and the protein concentration was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The sample volume was reduced using a speed-vac (Thermo Scientific, Waltham, MA, USA) and 14 µL (c= 10.35 µg/µL) were used for SDS-PAGE. Sample preparation and gel electrophoresis were performed as described above in the section “Gel electrophoresis of ablated tissue samples”.

Ablation of RNase A molecules

The ablation plume from RNase A samples was captured with a home-built collection device (Figure S2). The system consisted of a vacuum pump and tubing system equipped with a plastic housing in which a C8 reversed-phase extraction membrane disc (3M Empore^{TM} SPE, Sigma-Aldrich, Munich, Germany) was placed. The extraction discs were conditioned with 300 µL pure ACN and equilibrated with 0.1% formic acid (FA) using a vacuum pump. 300 µL of the RNase A solution (c= 5.9 µg/µL, dissolved in 0.1% FA) were transferred into the sampling device and ablated by PIRL. Due to the vacuum pump the ablation plume was drawn over the extraction disc and the RNase A molecules were trapped on the reversed-phase membrane. In addition, RNase A solution (300 µL) was transferred onto the extraction disc and vacuumed over the membrane. The non-ablated RNase A molecules acted as a control sample for analyzing protein integrity.
The ablated and non-ablated RNase A reversed-phase membranes were put into P-200 tip (Eppendorf, Hamburg, Germany) and washed with 200 µL 0.1% trifluoroacetic acid (TFA). Elution was achieved with 200 µL 70% ACN, 0.1% TFA (dissolved in HPLC-H_2O) and the samples were subsequently vaporized with a speed-vac (Thermo Scientific, Waltham, MA, USA). For further mass spectrometric analysis and tryptic in solution digestion, the dried samples were dissolved in 20 µL 0.1% FA.

Ablation of alpha-casein molecules

The ablation plume from alpha casein (c= 1.5 µg/µL, dissolved in 0.1% FA) was captured as described above in the section “Ablation of RNase A molecules”. The ablated and non-ablated alpha casein samples were eluted from the reversed-phase membrane using 200 µL 70% ACN, 0.1% TFA (dissolved in HPLC-H2O) and the samples were subsequently evaporated to dryness. For further MALDI-TOF MS analysis the samples were dissolved in 20 µL 0.1% FA.

Trypsin ablation and alpha-casein digestion

Trypsin (non-sequencing grade, Sigma-Aldrich, Munich, Germany) was dissolved in 100 mM NH_4HCO_3 (c= 4.55 µg/µL). Trypsin solution (300 µL) was transferred to the sampling device and irradiated with PIRL. The ablation plume was captured with a cryo-trap using liquid N_2. For testing the enzyme activity, 1 µL of the condensate was dissolved in 79 µL trypsin resuspension buffer. For tryptic digestion, alpha-casein (2.9 mg) was dissolved in 100 µL 6 M urea. For reduction, 2.3 µL 100 mM dithiotreitol (dissolved in 100 mM NH_4HCO_3) was added and incubated for 10 min at 56°C. Afterwards 2.3 µL 300 mM iodacetamid (dissolved in 100 mM NH_4HCO_3) were added and incubated for 30 min in the dark. Then 100 mM NH_4HCO_3 (850 µL) was added. Finally the 80 µL trypsin digest solution (see above) was added and the sample was digested over night at 37°C. The digest was acidified with FA and evaporated. The sample was dissolved in 70 µL 0.1% TFA and desalted with a home-made Oligo R3 (Life Technologies, Darmstadt, Germany) micro-column as described in an earlier publication.[4] After desalting the sample was evaporated and dissolved in 20 µL 0.1% FA for liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis. As a control sample intact alpha-casein was dissolved in 0.1% FA (c= 1 µg/µL) and analyzed without further trypsin incubation by LC-MS/MS.

Ablation of human plasma serum and angiotensin I incubation

For the ablation of human blood plasma a volunteer was recruited. In accordance to the ethics committee of the medical association Hamburg (Ethikkommission der Ärztekammer Hamburg, Germany) the participant provided signed informed consent.
Human venous citrate blood (ratio 1:9, blood to sodium citrate 3,13% (Eifelfango)) was obtained by catheterization from the cubital vein of a healthy male volunteer (Age: 53 years, blood pressure: normal, <120/80 mmHg).
Human plasma serum (300 µL) was transferred to the sampling device and irradiated with PIRL without any further treatment. The ablation plume was trapped with a cryo-trap using liquid N_2.The protein concentration of the ablated human plasma serum was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) and 4.752 µg of the ablated plasma serum was incubated with angiotensin I (Ang-I, c= 10^{-5} M,

dissolved in MS-H_2O) at 37°C. At defined incubation times (0 h, 6 h and 24 h) aliquots with a volume of 0.5 µL were taken for MALDI-TOF MS analysis.

Angiotensin I incubation with human plasma serum

The protein concentration of human plasma serum was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). 4.75 µg of plasma protein were incubated with angiotensin I (Ang-I, c= 10^{-5} M, dissolved in MS-H_2O) at 37 °C. Aliquots with a volume of 0.5 µL were taken after 0 h, 6 h and 24 h for MALDI-TOF MS analysis.

Tryptic Digestion

In-gel digestion was performed in accordance to Shevchenko et al.[5] Briefly, gel shrinking and swelling was achieved with pure ACN and 100 mM NH_4HCO_3, respectively. For in-gel reduction and alkylation 10 mM dithiothreitol (dissolved in 100 mM NH_4HCO_3) and 55 mM iodacetamide (dissolved in 100 mM NH_4HCO_3) were used. Gel pieces were covered with a trypsin solution (13 ng/µL sequencing-grade trypsin, dissolved in 10 mM NH_4HCO_3 containing 10% ACN) and digestion was carried out over night at 37°C. Peptides were extracted with 5% FA, 50% ACN and evaporated. For further LC-MS/MS analysis, samples were dissolved in 20 µL 0.1% FA.

In-solution digestion was performed in a centrifuge filter (Merck Millipore, Darmstadt, Germany) with a 10 kDa cut-off.[6] The sample was transferred to the centrifuge filter and centrifuged for 20 min at 14680 rpm. Then 500 µL 6 M urea was added and the sample was centrifuged for 20 min at 14680 rpm. This step was repeated twice. For reduction and alkylation, 1.3 µL 100 mM dithiothreitol (dissolved in 100 mM NH_4HCO_3) and 1.3 µL 300 mM iodacetamid (dissolved in 100 mM NH_4HCO_3), respectively, were added and the sample was centrifuged for 20 min at 14680 rpm. Following this, 425 µL 100 mM NH_4HCO_3 and 1 µL trypsin solution (0.25 µg/µL sequencing-grade trypsin, dissolved in trypsin resuspension buffer) were added and the sample was incubated over night at 37°C. After digestion was finished the samples were centrifuged for 20 min at 14680 rpm and the retentate was acidified with 0.9 µL FA. Afterwards the samples were evaporated and dissolved in 70 µL 0.1% TFA for desalting using a home-made Oligo R3 micro-column as described in an earlier

publication.[4] After desalting the sample was evaporated and dissolved in 20 µL 0.1% FA for further LC-MS/MS analysis.

MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed on a Reflex IV (Bruker, Bremen, Germany) equipped with a N_2-laser in positive ion-mode. Intact proteins were measured in linear mode using 2,5-dihydroxyacetophenon (DHAP) as a matrix. For matrix preparation, DHAP (7.6 mg) was dissolved in 375 µL ethanol. After vortexing and sonification, diammonium citrate (125 µL) was added and 2 µL from the dissolved sample were mixed in a 0.5 mL tube with 2 µL 2% TFA and 2 µL DHAP-solution. Due to friction on the tube surface, crystallisation seeds were created using a small pipette tip and 1 µL of the solution was loaded on a MALDI anchor chip target.
MALDI-TOF MS analysis of angiotensin I incubated with ablated plasma proteins were carried out in reflector mode. Samples were prepared in accordance to the dried-droplet method using 0.5 µL 2,5-dihiydroxbenzoic acid (DHB) matrix solution (20 mg/mL DHB, 30:70 [v/v] ACN:0.1% TFA, dissolved in HPLC-H_2O) and 0.5 µL of sample solution.

Analysis by LC-MS/MS

LC-MS measurements were performed by injecting the samples on a nano-ultra pressure liquid chromatography system (nano-UPLC; nanoACQUITY, Waters, Manchester, UK) coupled via electrospray-ionization (ESI) to a quadrupole time-of-flight (QTOF) mass spectrometer (QTOF Premier, Micromass/Waters, Manchester, UK). The samples were loaded (5 µL/min) on a trapping column (nanoAcquity UPLC PST trap column, C18, 180 µm × 20 mm, 5 µm, 100 Å, Waters, Manchester, UK; buffer A: 0.1% FA in HPLC-H_2O; buffer B: 0.1% FA in ACN) with 2% buffer B. After sample loading the trapping column was washed for 5 min with 2% buffer B (5 µL/min) and the peptides were eluted (200 nL/min) onto the separation column (nanoAcquity UPLC BEH column, C18, 75 µm × 150 mm, 100 Å Waters, Manchester, UK; 200 nL/min, gradient: 2−50% B in 30 min in case of the short gradient or in 90 min in case of the long gradient). The spray was generated from a fused-silica emitter (I.D. 10 µm, New Objective, Woburn, USA) at a capillary voltage of 1520 V, a source temperature of 100 °C and

a cone voltage of 40 V in positive ion mode. For MS/MS measurements, data were recorded in the data dependant acquisition mode (DDA). MS survey scans were performed over an m/z range from 400-1500 with a scan-time of 0.6 s and an interscan delay of 0.05 s. The two most abundant signals were used for fragmentation. MS/MS spectra were obtained from 100-1500 m/z with a scan-time of 0.95 sec and a collision ramp from 22-30 eV. An online exclusion was used to prevent multiple fragmentation events (exclusion time: 20 sec, exclusion window: +/- 2 m/z).

For intact protein measurements, only full scan MS spectra were recorded over an m/z range from 500-3000. For calibration, a lockspray spectrum was recorded every 10 seconds (1 pMol/µL [Glu1] Fibrinopeptide B (Sigma, Munich, Germany)) over an m/z range from 100-1500 using a collision energy of 22 eV.

Data Analysis

Manual validation and data interpretation of the LC-MS/MS measurements were performed using MassLynx 4.1 (Waters, Manchester, UK). The raw spectra were smoothed using a savitzky golay algorithm (smooth window channel: 3, number of smooths: 2). Extracted ion chromatograms were generated using the width (full width at half maximum, FWHM) of the monoisotopic peak or the whole isotopic envelope if no isotopic resolution was achieved. Mass spectra were deconvoluted with MaxEnt1 using the following parameters: the resolution was set to 0.2 Da/channel and the uniform Gaussian width at half height to 0.6 Da. Iteration was performed until convergence was reached.

For peptide identification, the raw spectra were smoothed (savitzky golay, smooth window channel: 3, number of smooths: 2), centered (min peak width at half height: 4, centroid to: 80%) and lock mass corrected using Protein Lynx Global Server version 2.5.2 (Waters, Manchester, UK). The resulted peak lists were exported as a mzML file and searched against a mammalian decoy database in case of the RNase A and casein samples or against a mouse decoy database in case of the ablated mouse muscle tissue samples using mascot (SwissProt, www.matrixscience.com) and the OpenMS Proteomic Pipeline (TOPP)[7] using two different search engines (open mass spectrometry search algorithm (OMSSA),[8] XTANDEM[9]). The searches were performed using the following parameters: precursor mass tolerance was set to 35 ppm and fragment mass tolerance was set to 0.2 Da. Furthermore, two missed

cleavages were allowed and a carbamidomethylation on cysteine residues as well as an oxidation of methionine and a phosphorylation on serine and threonine residues were considered as variable modifications. Peptides were regarded as correctly identified if the ion score was equal or lower than p= 0.05.

Supplementary results

LC-MS/MS analysis of the ablated and non-ablated intact RNase A

The base peak chromatograms of the ablated and non-ablated sample (Figure S6) show peaks in the retention time interval from 41 min to 64 min that are caused by impurities and not by intact RNase A protein species. A manual inspection of the MS raw data in this retention time window revealed no differences between the two samples. The broad peak in the retention time interval from 65 min to 71 min in both chromatograms is caused by different protein species of the RNase A, as this was the only protein that was identified in the LC-MS/MS analysis of the tryptic digests of the ablated and non-ablated sample. Extracted ion chromatograms (EIC, Figure S7) and deconvoluted spectra (Figure S 9-11) were generated for three different RNase A species. The EICs in Figure S7 a-b are evoked by a protein species of the RNase A with an average mass of 13694.2 Da for the ablated and 13695 Da for the non-ablated sample (Figure S8, Figure S9). Compared to the theoretical average mass this results in a mass deviation of 285 ppm and 343 ppm, respectively, indicating that the EICs in Figure S7 a-b are caused by the unmodified RNase A species. The MS raw spectra (Figure S8) as well as the deconvoluted spectra (Figure S9) were almost identical for both the ablated and the non-ablated RNase A sample. The other EICs in Figure S7 show the elution of two distinct RNase A protein species in both chromatograms with a molecular weight of 13779.6 Da (Figure S7 c-d, Figure S10) and 12036 Da (Figure S7 e-f, Figure S11). If a thermal degradation or changes in the chemical structure would have been occurred during the DIVE process, additional signals only present in the ablated sample should be detectable, which is not the case.

Supplementary discussion

Desorption of proteins from tissue sections by irradiating the tissue, covered with a MALDI matrix, with a laser is established and applied for mass spectrometry imaging (MSI) since the beginning of the last decade.[10] MALDI-MSI is a powerful tool linking signal intensities of ions defined by their m/z value with their spatial distribution. Spatial resolution of 10 µm have already been achieved.[11] Desorption of proteins from tissues by PIRL is a little bit comparable with desorbing proteins from tissues by MALDI, since in the case of PIRL induced desorption the water molecules can be interpreted as MALDI matrix molecules which enable the desorption. However, in the case of MALDI-MSI the desorbed proteins are directly driven to the MALDI-MS detector. The proteins desorbed by the MALDI process can not be trapped and subjected to a tryptic digestion, SDS-PAGE or other methods of protein analysis, as this is the case and shown in this study with extraction of proteins by PIRL. Furthermore the resolution regarding the number of different proteins of the current MALDI-MSI technology for proteins is much lower than the resolution of the PIRL extraction of proteins. In typical MALDI-MSI spectra the number of protein signals with m/z values larger than 30000 Da usually is in the range of less than a dozen,[12, 13] which is a very small fraction of the number of cellular proteins, which were reported by *Wilhelm et. al.* recently. They have detected a core proteome of approximately 12000 proteins.[14] In this study the SDS-PAGE of a protein mixture (Figure 1) extracted by PIRL already comprises more than 20 bands. Typically the number of identifiable proteins per stained SDS-PAGE band is in the range of more than 10. In case of PIRL extracted SDS-PAGE more than 500 proteins were identified.

Figures and Schemes

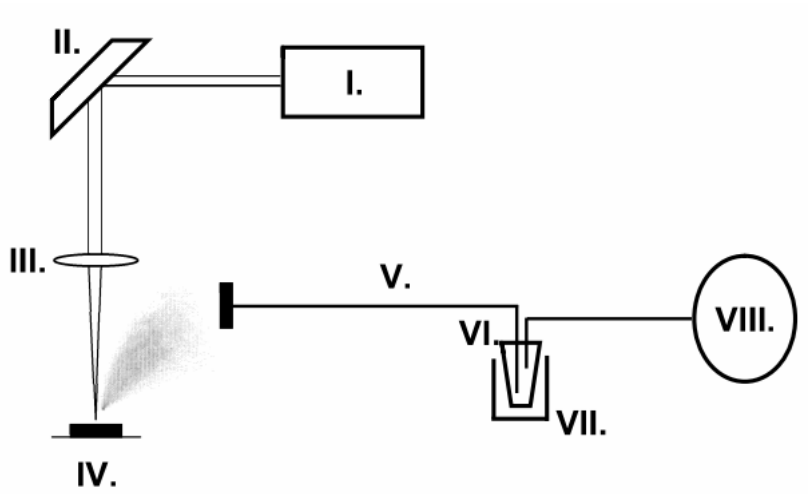


Figure S1: Schematic diagram of the cryo-trap used to capture PIRL extracted proteins. I. PIRL II. Scanning mirror III. Focusing lens IV. Sample holder V. Tubing with funnel VI. Wash bottle VII. Cryo box filled with liquid nitrogen VIII. Vacuum pump

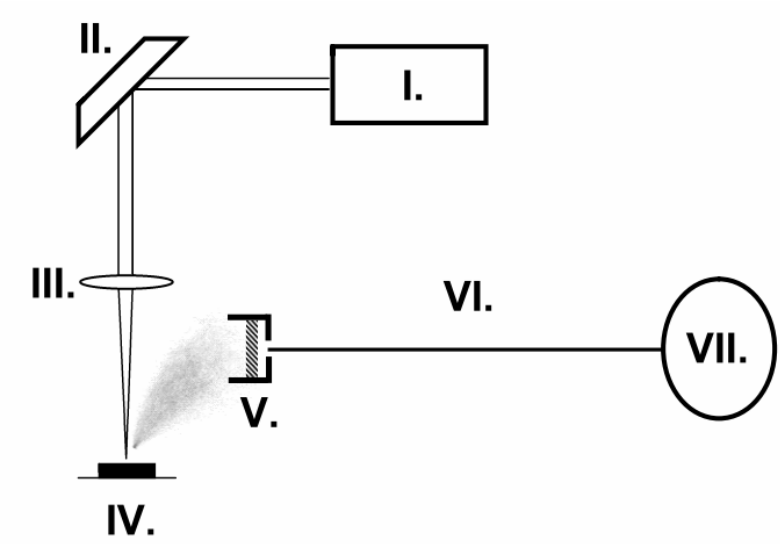


Figure S2: Schematic diagram of the membrane trapping device used to capture PIRL extracted proteins. I. PIRL II. Scanning mirror III. Focusing lens IV. Sample holder V. Plastic housing filled with a C8 reversed-phase extraction membrane disc (cross-hatched area) VI. Tubing VII. Vacuum pump

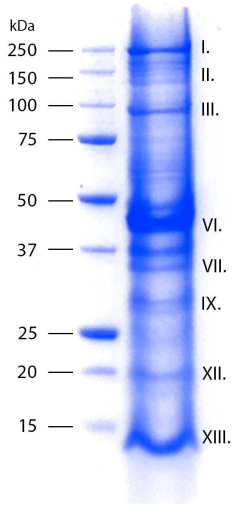


Figure S3: SDS-PAGE of the conventional mouse muscle tissue protein extract. Left: Protein Standard Right: Mouse muscle proteins extracted using mortal and pestle. In total 145 µg were loaded onto the electrophoresis gel. Bands are marked with Roman numeral. Bands detected in the gel electrophoresis of the conventional extracted and ablated (Figure 1) tissue are represented with the same numbers.

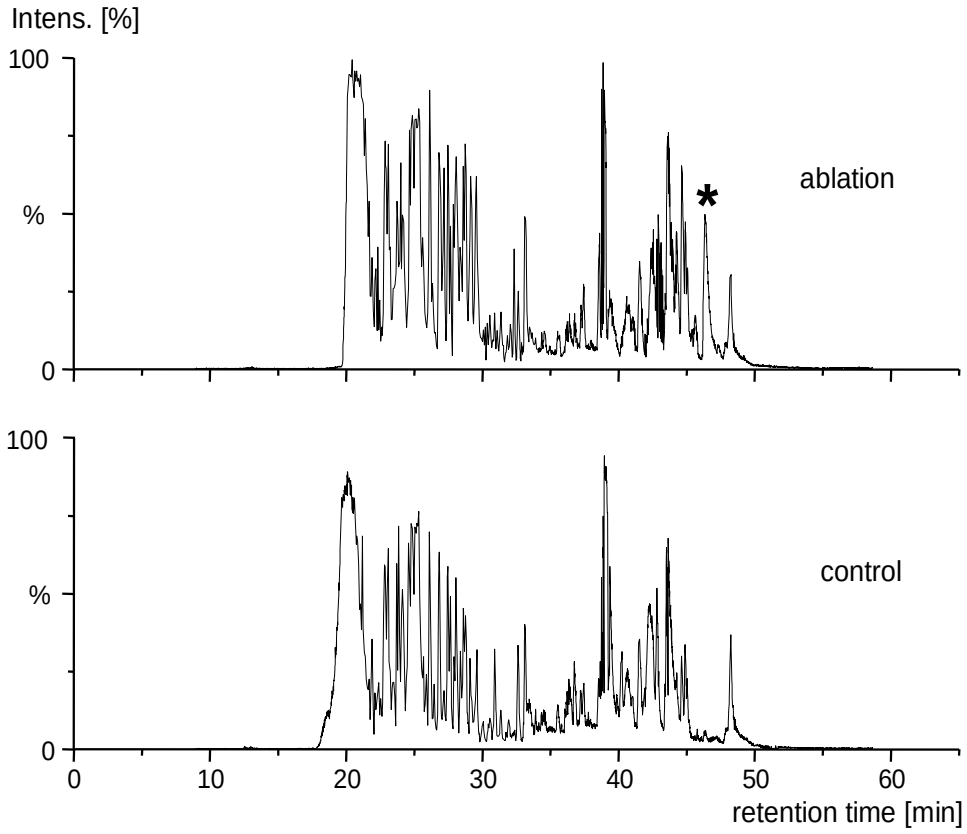


Figure S4: Basepeak-chromatogram of the LC-MS/MS analysis of the tryptic peptides of the ablated (above) and non-ablated (below; control) RNase A. Ordinate: Intensity [%]. Abscissa: retention time [min]. *By comparing the chromatograms of both samples one abundant signal is observed in the ablated sample at a retention time of 46.35 min, which seemed to be not present in the control sample. A manual inspection of the MS spectra in the corresponding retention time window showed that the peak is not caused by a peptide but by a singly charged contaminant with m/z 437.36 (Figure S5). Furthermore a comparison between both samples showed that this signal is also detected as the most abundant one in the control sample. Both spectra are almost identical and show a series of singly charged contaminants that differ by 74 m/z next to the signal m/z 437.36.

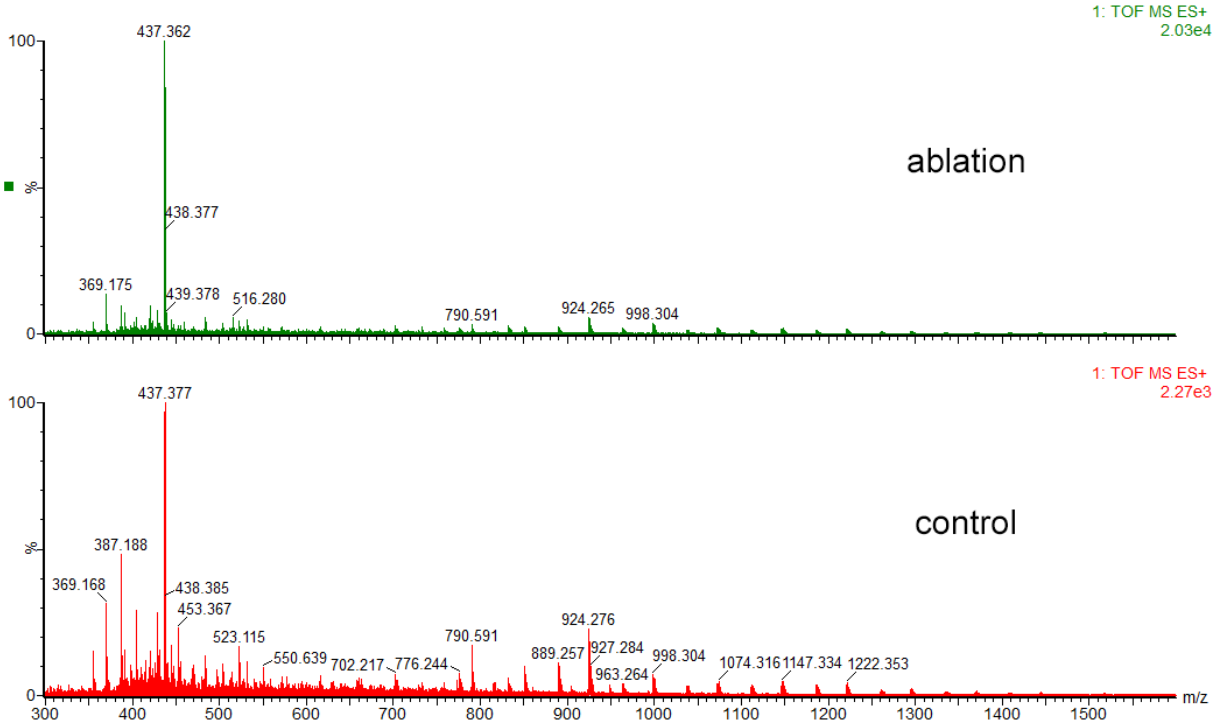


Figure S5: Summed spectra (RT 46.2-46.7 min) of the LC-MS/MS analysis of the tryptic peptides of the ablated and non-ablated RNase A. Ablation: ablated RNase A sample. Control: non-ablated RNase A sample. Ordinate: Intensity [%]. Abscissa: mass-to-charge ratio [m/z]

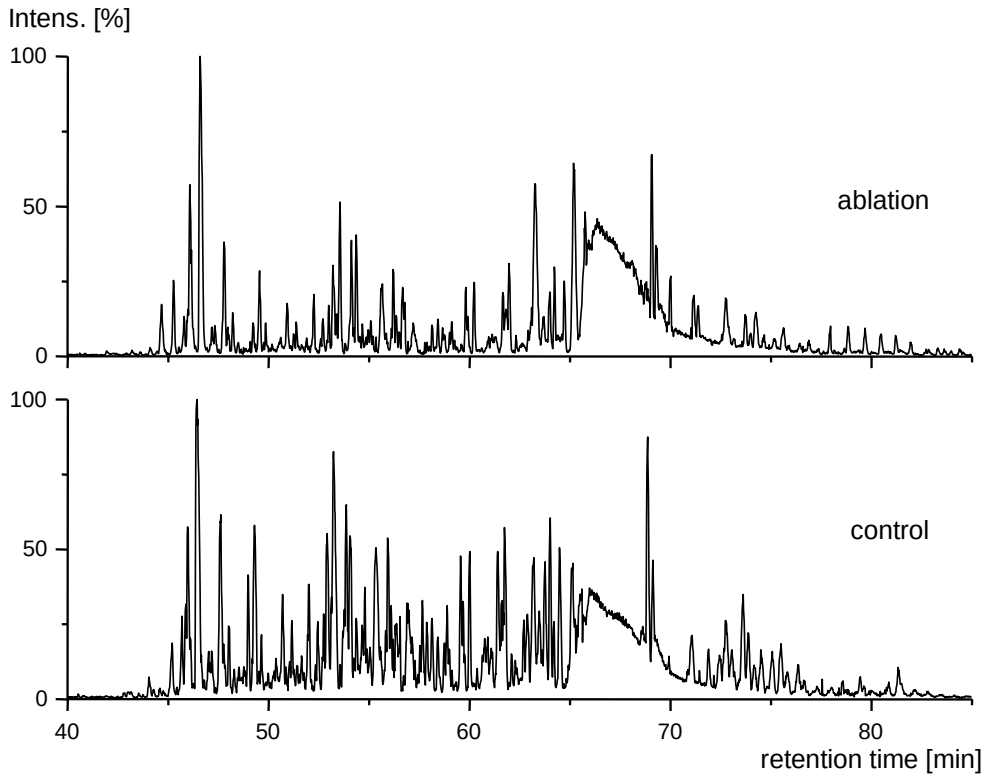


Figure S6: Basepeak-chromatogram of the LC-MS analysis of ablated intact RNase A (above) and non-ablated intact RNase A (below; control). Ordinate: Intensity [%]. Abscissa: retention time [min].

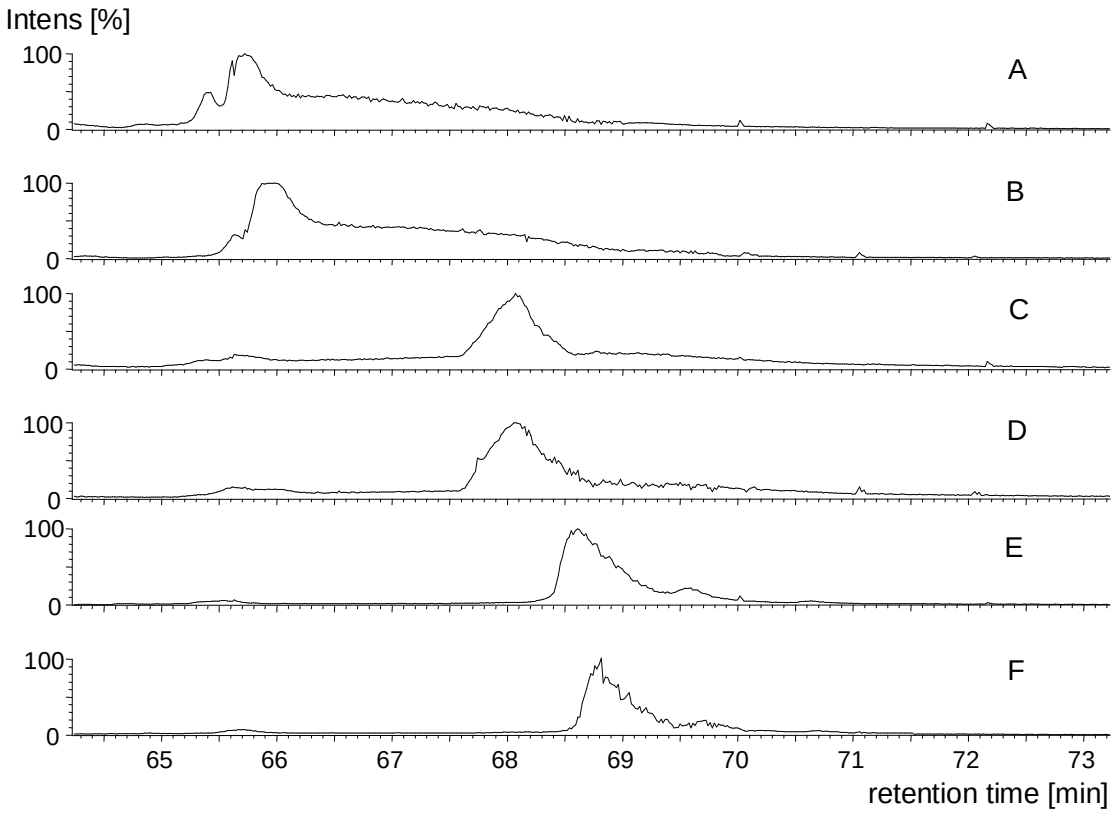


Figure S7: Extracted ion chromatograms (EIC) of the LC-MS analysis of three different RNase A species of the non-ablated (A,C,E) and ablated (B.D,F) RNase A [Figure S6]. A: EIC of the unmodified RNase A species with a molecular weight (m_r) of 13695 Da. B: EIC of the unmodified RNase A species, m_r= 13694.2 Da. C and D: EIC of the RNase A species with m_r= 13779.6 Da. E and F: EIC of the RNase A species with m_r= 12036 Da.

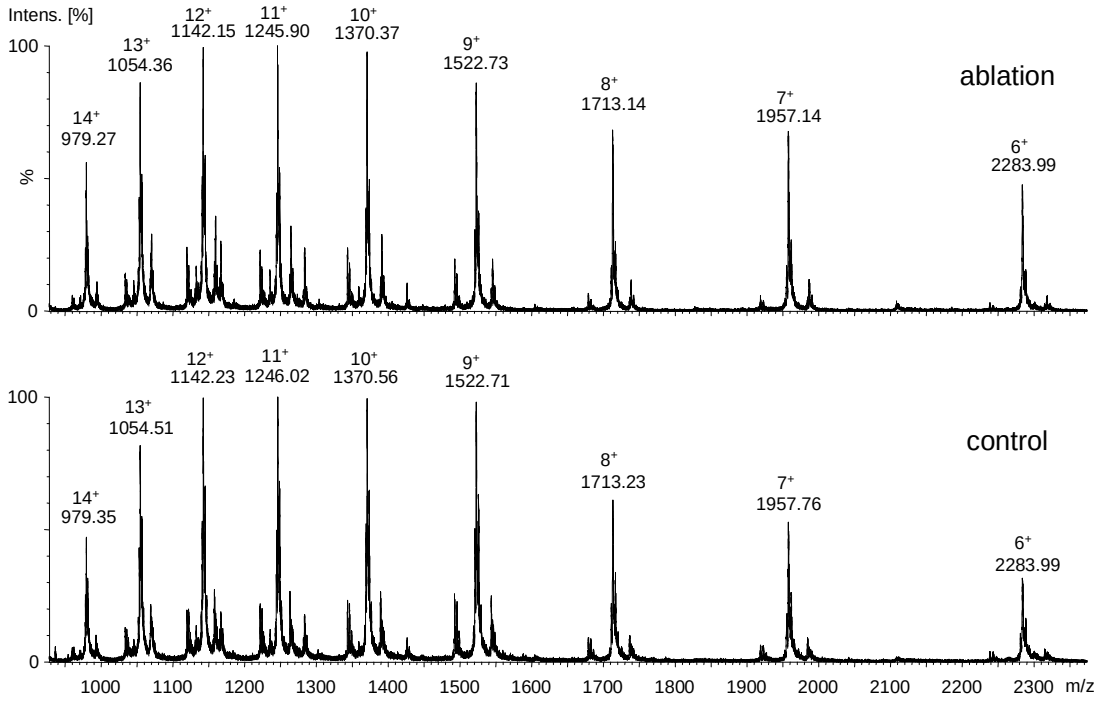


Figure S8: LC-MS spectra of the intact RNase A species. Ablation: ablated RNase A sample [Figure S7 B]. Control: non-ablated RNase A sample [Figure S7 A]. Ordinate: Intensity [%]. Abscissa: mass-to-charge ratio [m/z]

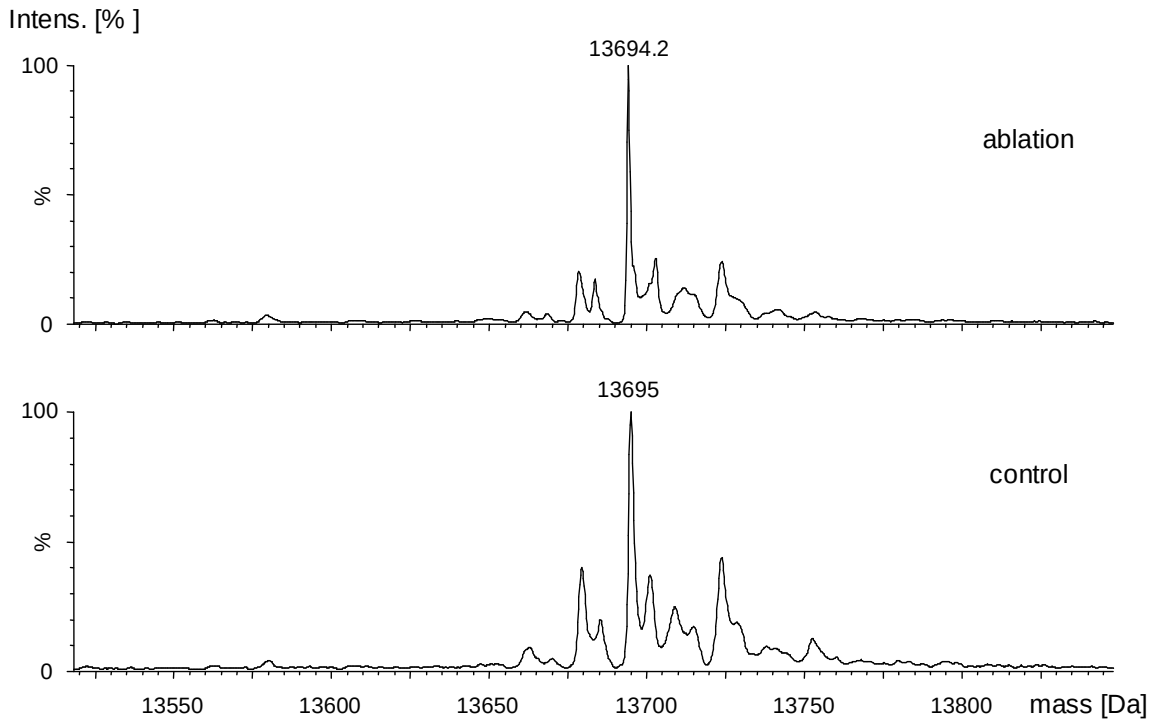


Figure S9: Deconvoluted spectra of the unmodified RNase A species measured with LC-MS. Ablation: deconvoluted spectra of the ablated RNase A sample [Figure S7 B]. Control: deconvoluted spectra of the non-ablated RNase A sample [Figure S7 A]. Ordinate: intensity [%]. Abscissa: mass [Da]

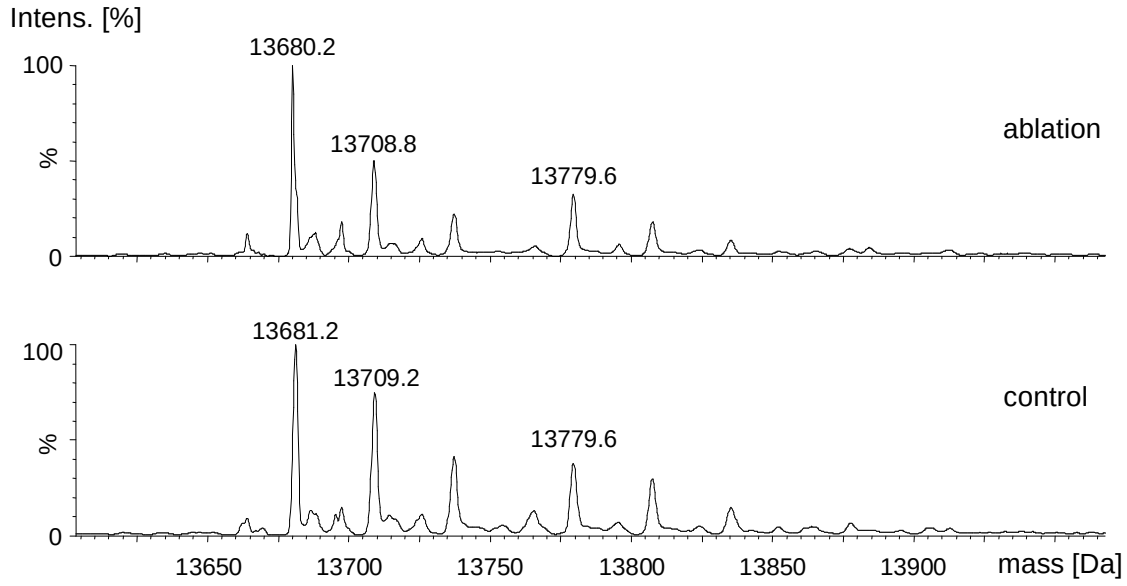


Figure S10: Deconvoluted spectra of the RNase A species with M_r= 13779 Da measured with LC-MS. Ablation: deconvoluted spectra of the ablated RNase A sample [Figure S7 D]. Control: deconvoluted spectra of the non-ablated RNase A sample [Figure S7 C]. Ordinate: intensity [%]. Abscissa: mass [Da]

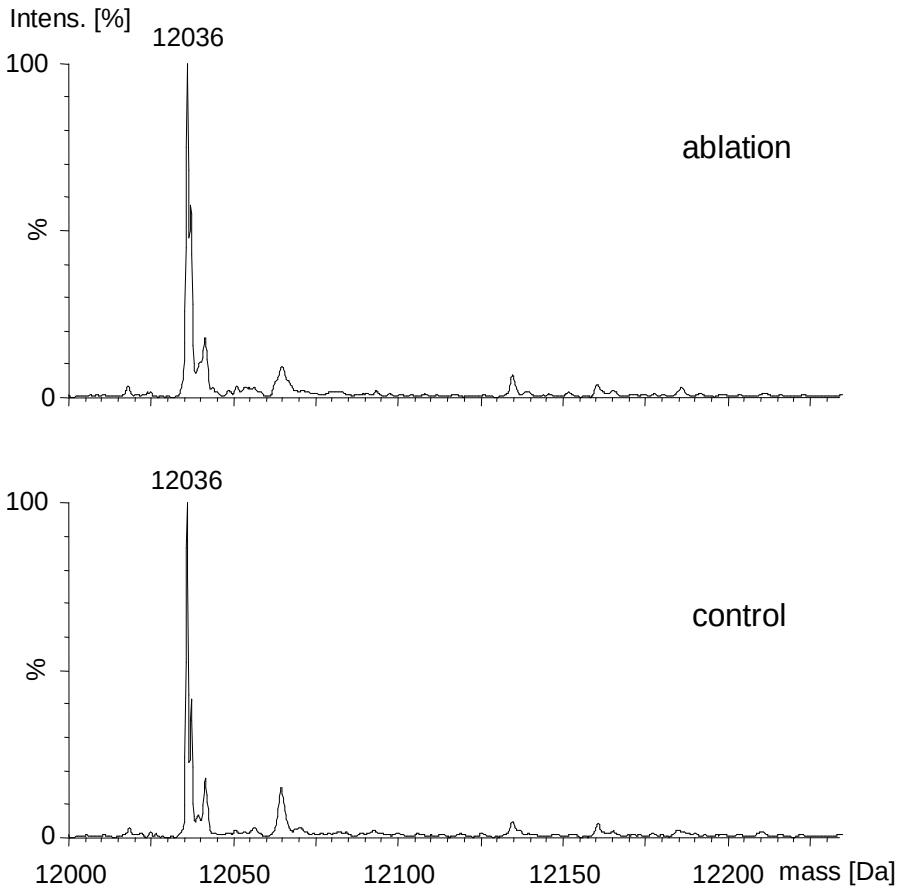


Figure S11: Deconvoluted spectra of the RNase A species with M_r= 12036 Da measured with LC-MS. Ablation: deconvoluted spectra of the ablated RNase A sample [Figure S7 F]. Control: deconvoluted spectra of the non-ablated RNase A sample [Figure S7 E]. Ordinate: intensity [%]. Abscissa: mass [Da]

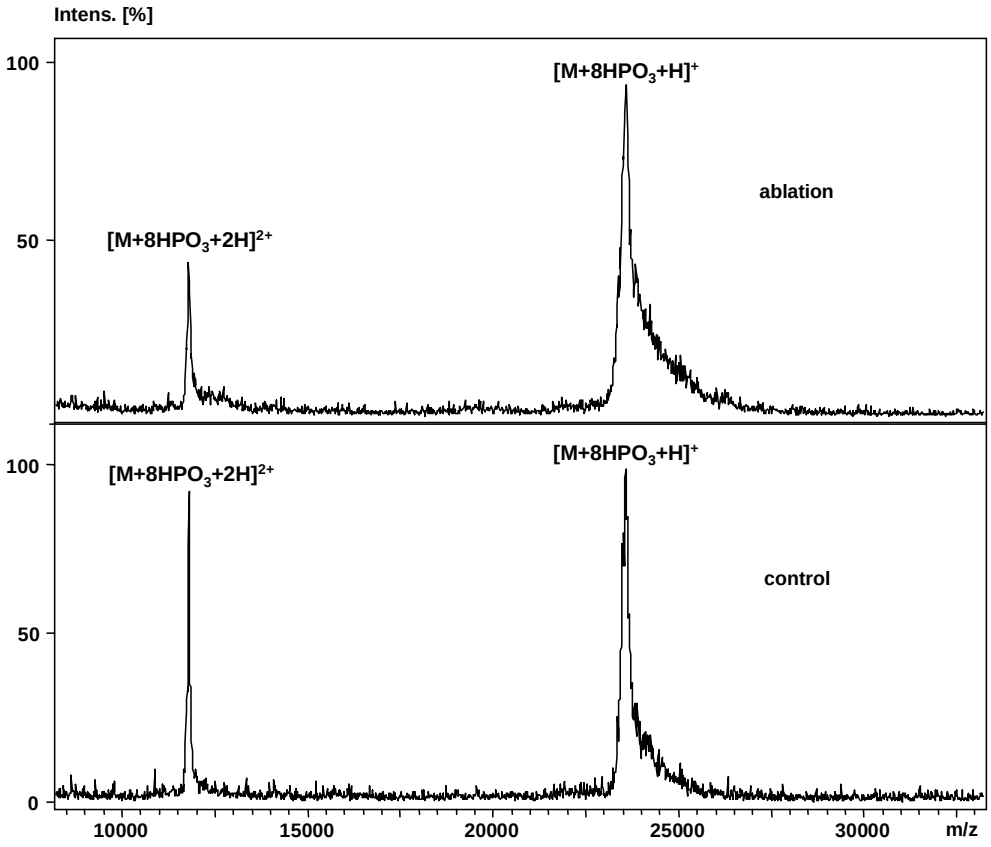


Figure S12: MALDI-TOF-MS spectra of alpha-S1 casein ablated by the PIRL (panel above) and before irradiation (panel below, control). Matrix: 2,5-dihydroxyacetophenon (DHAP). Ordinate: Intensity [%]. Abscissa: mass-to-charge ration [m/z]

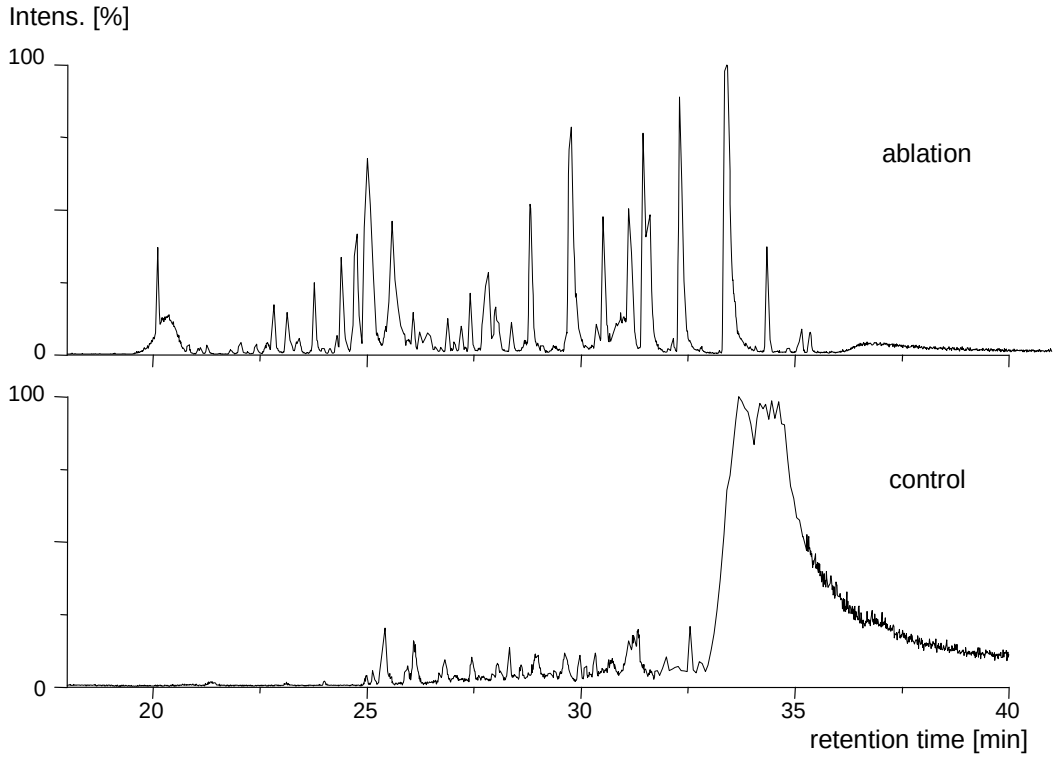


Figure S13: Base peak-chromatogram (BPC) of the LC-MS/MS analysis of alpha-casein incubated in the absence (control) and in the presence of ablated trypsin. Ordinate: Intensity [%]. Abscissa: retention time [min].

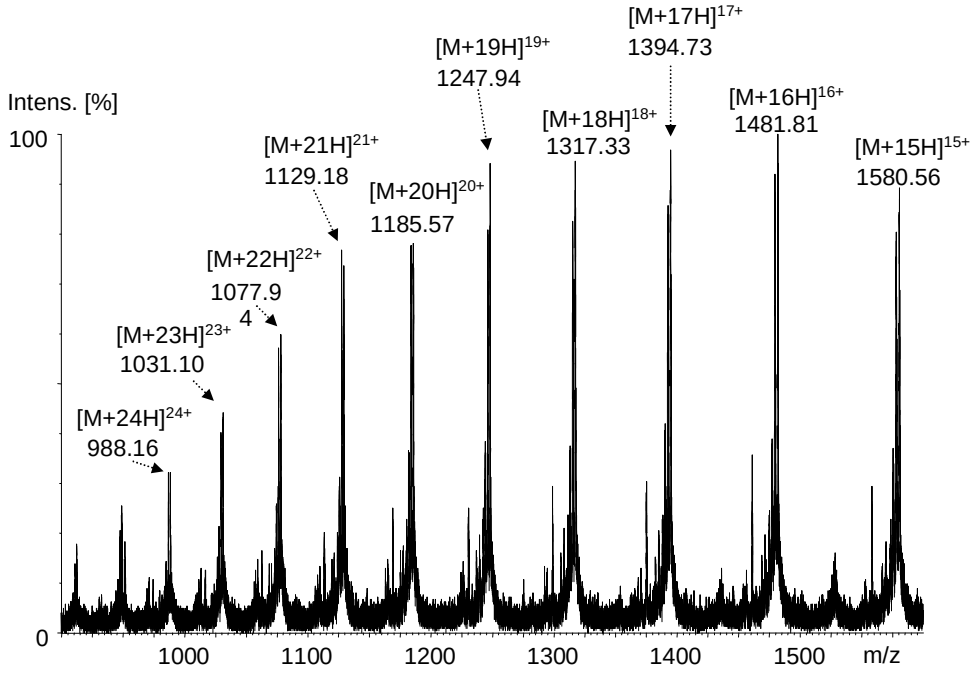


Figure S14: LC-MS spectrum of alpha-casein incubated in the absence of ablated trypsin. The spectra shows multiply charged signals of the intact alpha-S1-casein species phosphorylated with nine phosphate groups (M_r= 23691.4 Da). Ordinate: Intensity [%]. Abscissa: mass-to-charge ratio [m/z].

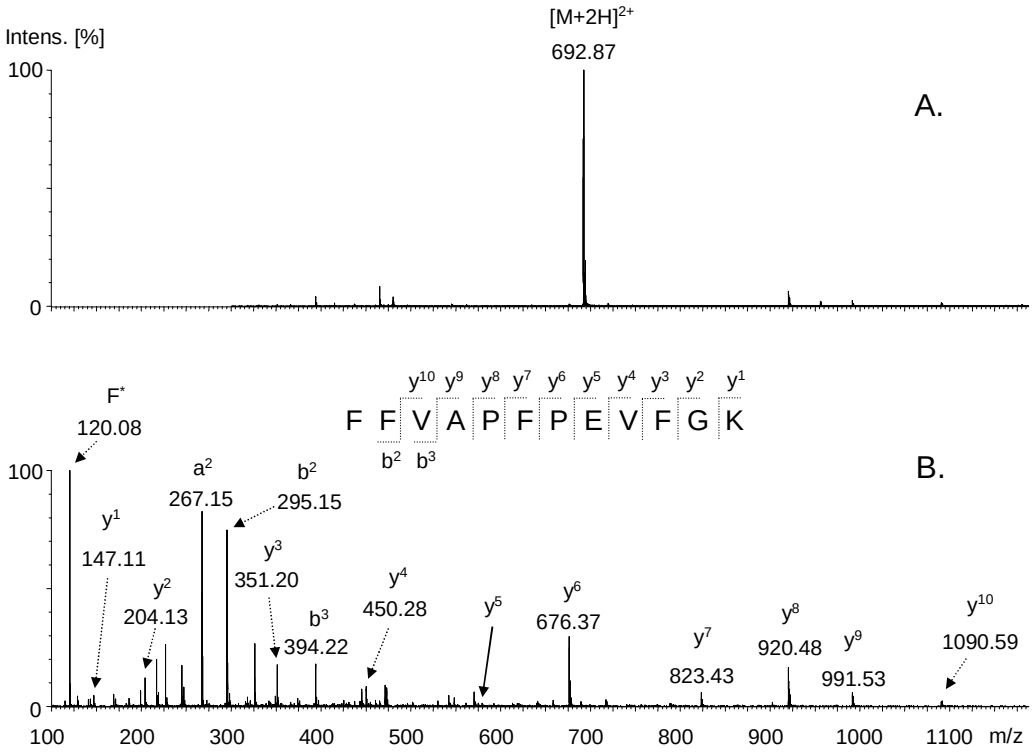


Figure S15: LC-MS and LC-MS/MS spectra of a tryptic alpha-S1-casein peptide from the LC-MS/MS analysis of alpha-casein that was incubated with the ablated trypsin. A: Full-Scan MS spectrum showing the doubly charged precursor m/z 692.87. B: MS/MS spectra of the tryptic peptide FFVAPFPEVFGK with annotated y- and b-ions. F*: Phenylalanine immonium-ion. Ordinate: Intensity [%].Abscissa: mass-to-charge ratio [m/z]

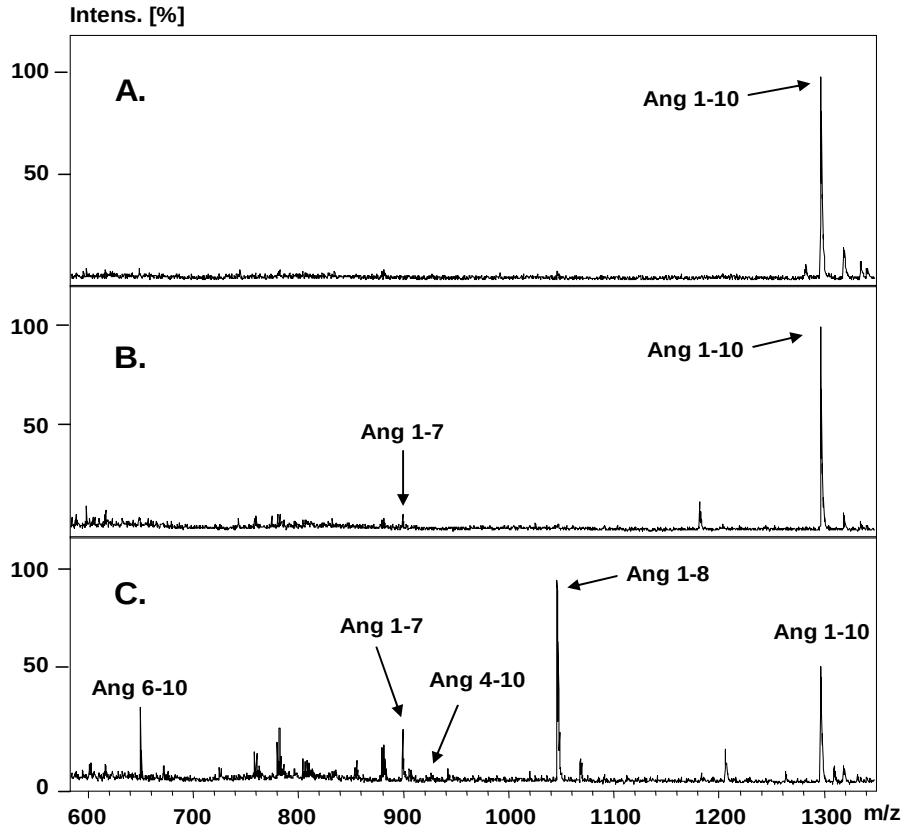


Figure S16: MALDI-TOF MS spectra of Ang 1-10 (C= 10^{-5} M) incubated with human plasma proteins, not ablated with PIRL (control experiment). The same plasma protein concentration was used as in the experiment with PIRL ablated plasma proteins (Figure 5). Reaction products were detected by MALDI-TOF MS after 0 h (A), 6 h (B) and 24 h (C). The generated angiotensin peptides are marked by arrows. Matrix: 2,5-dihydroxybenzoic acid (DHB). Ordinate: Intensity [a.u.] Abscissa: mass-to-charge ratio [m/z]

Scheme S1: RNase A identified by Mascot in the LC-MS/MS analysis of the ablated RNase A sample.

amino acid sequence (identified amino acids are highlighted in red):

KETAAAKFER QHMDSSTSAA SSSNYCNQMM KSRNLTKDRC KPVNTFVHES

LADVQAVCSQ KNVACK**NGQT NCYQSYSTMS ITDCRETGSS KYPNCAYK**TT

QANK**HIIVAC EGNPYVPVHF DASV**

Sequence coverage: 66%

Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
1 – 10	575.8146	1149.6146	1149.6142	0.41	2	63	8e-06	1		-.KETAAAKFER.Q
11 – 31	788.9742	2363.9008	2363.9239	-9.77	0	58	1.9e-06	1	U	R.QHMDSSTSAASSSNYCNQMMK.S + Carbamidomethyl (C)
11 – 31	794.3018	2379.8836	2379.9188	-14.8	0	36	0.0003	1	U	R.QHMDSSTSAASSSNYCNQMMK.S + Carbamidomethyl (C); Oxidation (M)
11 – 31	799.6397	2395.8973	2395.9137	-6.86	0	25	0.0058	1	U	R.QHMDSSTSAASSSNYCNQMMK.S + Carbamidomethyl (C); 2 Oxidation (M)
11 – 31	804.9698	2411.8876	2411.9086	-8.73	0	48	2.2e-05	1	U	R.QHMDSSTSAASSSNYCNQMMK.S + Carbamidomethyl (C); 3 Oxidation (M)
11 – 31	1206.9636	2411.9126	2411.9086	1.67	0	28	0.0028	1	U	R.QHMDSSTSAASSSNYCNQMMK.S + Carbamidomethyl (C); 3 Oxidation (M)
67 – 85	1143.4634	2284.9122	2284.9147	-1.08	0	96	6.8e-10	1		K.NGQTNCYQSYSTMSITDCR.E + 2 Carbamidomethyl (C)
67 – 85	1151.4568	2300.8990	2300.9096	-4.60	0	116	5.4e-12	1		K.NGQTNCYQSYSTMSITDCR.E + 2 Carbamidomethyl (C); Oxidation (M)
86 – 98	752.8371	1503.6596	1503.6664	-4.47	1	41	0.00067	1		R.ETGSSKYPNCAYK.T + Carbamidomethyl (C)
105 – 124	1112.5271	2223.0396	2223.0783	-17.4	0	31	0.011	1		K.HIIVACEGNPYVPVHFDASV.- + Carbamidomethyl (C)
105 – 124	742.0284	2223.0634	2223.0783	-6.71	0	37	0.0042	1		K.HIIVACEGNPYVPVHFDASV.- + Carbamidomethyl (C)

Scheme S2: RNase A identified by Mascot in the LC-MS/MS analysis of the non-ablated RNase A sample.

amino acid sequence (identified amino acids are highlighted in red):

KETAAAKFER **QHMDSSTSAA SSSNYCNQMM K**SRNLTKDRC KPVNTFVHES

LADVQAVCSQ KNVACK**NGQT NCYQSYSTMS ITDCRETGSS KYPNCAYK**TT

QANK**HIIVAC EGNPYVPVHF DASV**

Sequence coverage: 58%

Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
11 – 31	788.9752	2363.9038	2363.9239	-8.50	0	37	0.00022	1	U	R.QHMDSSTSAASSSNYCNQMMK.S + Carbamidomethyl (C)
11 – 31	794.3065	2379.8977	2379.9188	-8.87	0	64	5.2e-07	1	U	R.QHMDSSTSAASSSNYCNQMMK.S + Carbamidomethyl (C); Oxidation (M)
67 – 85	1143.4692	2284.9238	2284.9147	4.00	0	109	3.5e-11	1	U	K.NGQTNCYQSYSTMSITDCR.E + 2 Carbamidomethyl (C)
67 – 85	1151.4645	2300.9144	2300.9096	2.09	0	127	4.7e-13	1	U	K.NGQTNCYQSYSTMSITDCR.E + 2 Carbamidomethyl (C); Oxidation (M)
86 – 98	752.8425	1503.6704	1503.6664	2.71	1	66	1.9e-06	1		R.ETGSSKYPNCAYK.T + Carbamidomethyl (C)
105 – 124	1112.5518	2223.0890	2223.0783	4.84	0	34	0.0071	1	U	K.HIIVACEGNPYVPVHFDASV.- + Carbamidomethyl (C)

Scheme S3: Alpha-S1-casein identified by Mascot in the LC-MS/MS analysis of the ablated trypsin after being incubated with alpha-casein

amino acid sequence (identified amino acids are highlighted in red):

MKLLILTCLV AVALARPK**HP IKHQGLPQEV LNENLLRFFV APFPEVFGKE KVNELSK**DIG SESTEDQAME DIKQMEAESI SSSEEIVPNS VEQK**HIQKED VPSERYLGYL EQLLRLKKYK VPQLEIVPNS AEERLHSMKE GIHAQQKEPM IGVNQELAYF YPELFR**QFYQ LDAYPSGAWY YVPLGTQYTD APSFSDIPNP IGSENSEKTT MPLW

Sequence coverage: 51%

Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
19 – 37	745.7476	2234.2210	2234.2283	-3.30	1	45	0.00029	1	U	K.HPIKHQGLPQEVLNENLLR.F
23 – 37	587.3208	1758.9406	1758.9377	1.66	0	50	0.00016	1	U	K.HQGLPQEVLNENLLR.F
23 – 37	880.4801	1758.9456	1758.9377	4.55	0	70	1.5e-06	1	U	K.HQGLPQEVLNENLLR.F
38 – 49	692.8679	1383.7212	1383.7227	-1.07	0	40	0.0015	1	U	R.FFVAPFPEVFGK.E
38 – 49	692.8700	1383.7254	1383.7227	1.96	0	29	0.02	1	U	R.FFVAPFPEVFGK.E
38 – 49	692.8704	1383.7262	1383.7227	2.54	0	30	0.017	1	U	R.FFVAPFPEVFGK.E
38 – 49	692.8709	1383.7272	1383.7227	3.26	0	38	0.0027	1	U	R.FFVAPFPEVFGK.E
38 – 51	821.4395	1640.8644	1640.8603	2.54	1	64	8.1e-06	1	U	R.FFVAPFPEVFGKEK.V
38 – 57	771.4107	2311.2103	2311.2252	-6.48	2	52	0.00013	1	U	R.FFVAPFPEVFGKEKVNELSK.D
95 – 105	669.3451	1336.6756	1336.6735	1.60	1	61	1.9e-05	1	U	K.HIQKEDVPSER.Y
95 – 115	647.3314	2585.2965	2585.3601	-24.6	2	33	0.015	1	U	K.HIQKEDVPSERYLGYLEQLLR.L
106 – 115	634.3563	1266.6980	1266.6972	0.68	0	38	0.002	1	U	R.YLGYLEQLLR.L
106 – 115	634.3572	1266.6998	1266.6972	2.10	0	30	0.012	1	U	R.YLGYLEQLLR.L
106 – 115	634.3601	1266.7056	1266.6972	6.68	0	37	0.0023	1	U	R.YLGYLEQLLR.L
106 – 117	754.9515	1507.8884	1507.8762	8.12	1	72	2.5e-07	1	U	R.YLGYLEQLLRLK.K
106 – 118	818.9952	1635.9758	1635.9712	2.86	2	47	4.7e-05	1	U	R.YLGYLEQLLRLKK.Y
119 – 134	976.4771	1950.9396	1950.9452	-2.83	1	56	8.1e-05	1	U	K.YKVPQLEIVPNSAEER.L + Phospho (ST)
119 – 134	651.3245	1950.9517	1950.9452	3.34	1	56	7.4e-05	1	U	K.YKVPQLEIVPNSAEER.L + Phospho (ST)
135 – 166	951.7273	3802.8801	3802.8963	-4.26	2	37	0.0063	1	U	R.LHSMKEGIHAQQKEPMIGVNQELAYFYPELFR.Q
140 – 166	802.6516	3206.5773	3206.5859	-2.67	1	53	0.00018	1	U	K.EGIHAQQKEPMIGVNQELAYFYPELFR.Q
140 – 166	1069.8735	3206.5987	3206.5859	4.00	1	57	6e-05	1	U	K.EGIHAQQKEPMIGVNQELAYFYPELFR.Q

Scheme S4: Alpha-S2-casein identified by Mascot in the LC-MS/MS analysis of the ablated trypsin after being incubated with alpha-casein

amino acid sequence (identified amino acids are highlighted in red):

MKFFIFTCLL AVALAKNTME HVSSSEESII SQETYKQEK**N MAINPSKENL CSTFCKEVVR** NANEEEYSIG SSSEESAEVA TEEVKITVDD KHYQK**ALNEI NQFYQKFPQY LQYLYQGPIV LNPWDQVK**RN AVPITPTLNR EQLSTSEENS KK**TVDMESTE VFTKKTK**LTE EEKNRLNFLK KISQR**YQKFA LPQYLKTVYQ HQKAMKPWIQ PKTKVIPYVR** YL

Sequence coverage: 46%

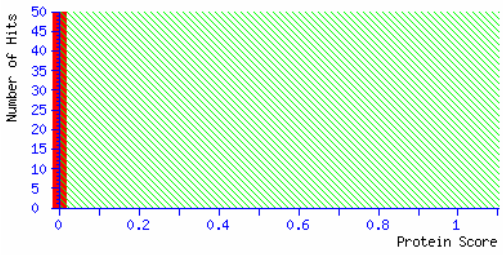
Start – End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
40 – 56	671.9817	2012.9233	2012.9118	5.71	1	67	6.1e-06	1	U	K.NMAINPSKENLCSTFCK.E + 2 Carbamidomethyl (C)
40 – 60	625.0430	2496.1429	2496.1923	-19.8	2	97	8.5e-09	1	U	K.NMAINPSKENLCSTFCKEVVR.N + 2 Carbamidomethyl (C)
40 – 60	833.0671	2496.1795	2496.1923	-5.14	2	58	6e-05	1	U	K.NMAINPSKENLCSTFCKEVVR.N + 2 Carbamidomethyl (C)
40 – 60	645.0385	2576.1249	2576.1586	-13.1	2	46	0.00098	1	U	K.NMAINPSKENLCSTFCKEVVR.N + 2 Carbamidomethyl (C); Phospho (ST)
96 – 128	1353.3645	4057.0717	4057.0778	-1.50	1	46	0.00058	1	U	K.ALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVK.R
153 – 164	733.8121	1465.6096	1465.6048	3.32	0	71	1.3e-06	1	U	K.TVDMESTEVFTK.K + Phospho (ST)
153 – 165	797.8616	1593.7086	1593.6997	5.59	1	49	0.00028	1	U	K.TVDMESTEVFTKK.T + Phospho (ST)
153 – 167	608.6171	1822.8295	1822.8424	-7.08	2	35	0.0084	1	U	K.TVDMESTEVFTKKTK.L + Phospho (ST)
186 – 196	699.8945	1397.7744	1397.7707	2.69	1	40	0.0011	1	U	R.YQKFALPQYLK.T
189 – 203	932.5058	1862.9970	1863.0043	-3.89	1	41	0.0014	1	U	K.FALPQYLKTVYQHQK.A
204 – 214	664.3825	1326.7504	1326.7482	1.71	1	42	0.00071	1	U	K.AMKPWIQPKTK.V
213 – 220	488.3017	974.5888	974.5913	-2.51	1	39	0.00065	1	U	K.TKVIPYVR.Y

Scheme S5: Mascot results of the LC-MS/MS analysis of alpha-casein without trypsin incubation:

	SwissProt	Decoy	False discovery rate
Peptide matches above identity threshold	0	0	
Peptide matches above homology or identity threshold	0	0	

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 26 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



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