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Protein Extraction from Tissue

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

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Abstract: *A picosecond IR laser (PIRL) can be used to blast proteins out of tissues through desorption by impulsive excitation (DIVE) of intramolecular vibrational states of water molecules in the cell in less than a millisecond. With PIRL-DIVE proteins covering a range of a few kDa up to several MDa are extracted in high quantities compared to conventional approaches. The chemical composition of extracted proteins remains unaltered and even enzymatic activities are maintained.*

The extraction of proteins from biological tissues is a critical, difficult, and time-consuming step in protein analysis. Proteins have to be released from their native environments, which requires a breakup of the outer cell membrane, intracellular membranes, and surrounding extracellular structures. This is usually achieved by the application of a homogenization technique, which varies depending on the nature of the tissue sample. In most cases, the first step includes cutting the tissue into small pieces with sharp blades or a meat grinder. Small pieces of soft tissue samples (brain, liver) can be disrupted by ultrasonic homogenizers.[1] Hard and filamentous tissues such as muscles, bones, and cartilage are disrupted by mechanical homogenizers.[2] The choice of an appropriate buffer system is important for an efficient protein extraction. The buffer usually guarantees a stable pH since deviation from the physiological pH may have denaturing effects on proteins. Buffer systems may require additives to stabilize proteins, protein–protein interactions, and enzymes. However, extraction buffers should not result in chemical reactions of the constituent proteins of the tissue.[3] After cell disruption, large particles like cell debris, large organelles, and insoluble compounds must be removed and proteins must be separated from other biomolecules and small organic molecules. This can be achieved by specific centrifugation and precipitation methods or a combination of both.[4]

Until now, there have been few methods for extracting a broad range of proteins with different chemical properties from intact tissue samples in a single experimental step. Most protocols are specific for the extraction of a distinct group of proteins from a defined tissue type.[5–7] Thus, it would be highly desirable to develop a sample preparation procedure that is capable of extracting proteins with a broad range of chemical properties in high yields, and which is soft and fast such that activities are maintained and the conversion of proteins by enzymatic or chemical reactions is minimized.

Romano and Levis showed that it is possible to ablate high-mass, single-stranded DNA intact with a UV laser using a chromophore and capture the ablated DNA for further analysis.[8] Some other groups use laser ablation sample transfer (LAST) prior to MALDI MS imaging (MALDI-MSI) or ESI-MS analysis.[9–14] However, in the above-mentioned studies proteins were not directly extracted from intact tissues by laser ablation. With MALDI-MSI it is possible to desorb and analyze proteins and peptides from intact tissue samples.[15,16] A detailed discussion is given in the Supporting Information.

Recently, a new concept in laser surgery has been developed by the Miller group based on a picosecond infrared laser (PIRL) specifically tuned to the strong OH vibration stretching band in water to drive ablation processes faster than nucleation growth or energy transfer to adjacent tissue. This method has been found to ablate entire proteins intact into the gas phase.[17] The key enabling feature is the elimination of unrestricted nucleation growth in the laser-driven phase transition, which otherwise leads to cavitation-induced shock waves and massive damage to surrounding tissues and disintegration of proteins.[18–20] The ablation process is made to happen faster than even acoustic transfer of energy to adjacent tissue and strong acoustic attenuation of the excited acoustics in the 100 GHz range also contributes to the ablation process.[18] All the absorbed energy is converted into translational degrees of freedom rather than being lost to surrounding tissue through thermal or acoustic transport. Most importantly, the ablation process occurs on timescales faster than even collisional exchange of the excited water molecules with the constituent proteins, which avoids thermal fragmentation in the ablation step. Here, it needs to be fully appreciated that liquid water couples vibrational energy directly to translational motions, the very motions leading to ablation. This transduction of the absorbed energy into translational motions occurs on the 100 fs timescale, nearly 100 times faster than any other material with respect to

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cascaded relaxation pathways that ultimately couple energy into translational motions.[21] The overall dynamics for ablation are limited by speed of sound propagation over the absorption length that dictates an impulse limit to the ablation process to be on the picosecond timescale.[17,18] For this reason, picosecond pulses are used instead of femtosecond pulses to avoid peak power conditions that lead to multiphoton ionization and fragmentation of constituent proteins. Effectively, this desorption by the impulsive excitation (DIVE) process drives the water molecules into the gas phase, along with constituent proteins and macromolecules, without thermal or shock-wave damage to the ambient tissue.[18] It is thus hypothesized that protein desorption is achieved without affecting their chemical composition.

To test the suitability of PIRL-based laser ablation for protein extraction, several experiments were performed. First, mouse muscle and liver tissue samples were ablated and the ablation products were analyzed by SDS-PAGE to investigate whether a broad range of proteins can be extracted by PIRL excitation and if these are suitable for immunoblotting. Furthermore, the integrity and enzymatic activity of ablated proteins were investigated by mass spectrometry. The experimental details are described in the Supporting Information.

To investigate whether PIRL is suitable for extracting proteins from solid tissue samples, mouse muscle tissue was irradiated over an area of 5 mm × 5 mm and 1.5 mm in depth and the ablation plume was captured with a cryo-trap (Figure S1). The condensate had a volume of 50 μL and a protein concentration of 19.33 $\mu g\,\mu L^{-1}$. Almost 1 mg of proteins was extracted from 37.5 mm^3 of solid muscle tissue in a single step. In contrast, only 175 μg of proteins was extracted from the same muscle tissue volume when a mortar and a pestle were used for homogenization. The entire extraction procedure took more than three days, whereas with PIRL excision, the yield of extracted proteins was almost six times higher and the whole extraction procedure required no more than five minutes. Condensate volumes of 7.5 μL were used for SDS-PAGE without any further sample preparation or fractionation step. The SDS-PAGE shows a large number of bands from a few kDa up to more than several hundred kDa (Figure 1). Further analysis was performed on selected bands by tryptic in-gel digest and tryptic peptides were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). MS analysis shows that the distinct band near the 250 kDa marker band contained different myosin proteins. The theoretical molecular weights of the myosin proteins of about 222 kDa coincided with the position of the band within the gel. The sequence coverage (35%) as well as the number of identified peptides (66), which were distributed over the entire protein sequence of myosin-4, suggest that this huge protein ($M_r = 222\,720$ Da) was extracted from the muscle tissue in high quantity. In the uppermost band, just under the loading pocket, the muscle-associated protein titin was identified. In total, 86 different peptides were identified from this 3.9 MDa protein. The identified peptides covered a region of 32212 amino acids corresponding to a molecular weight of 3.57 MDa. Because of this and the fact that titin was identified just beneath the loading pocket it is assumed that titin was desorbed intact in the same way as the various myosin proteins. A comparison between the SDS-PAGE patterns of the ablated and conventionally extracted protein samples (Figure S3) reveals that all distinct bands from the conventional extraction are also present in the ablated sample. This result indicates that PIRL and the conventional method extract a similar set of proteins. In addition the PIRL-ablated protein extract on the SDS-PAGE showed five bands that are not visible in the SDS-PAGE of the conventional protein extract.

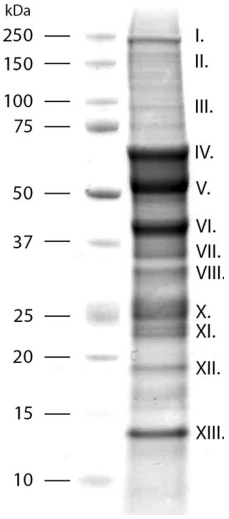

Figure 1. SDS-PAGE of proteins desorbed by PIRL. Left: Protein standard. Right: Mouse muscle proteins ($m = 145$ μg) extracted by PIRL. Bands are marked with Roman numerals. Bands detected in the gel electrophoresis of the ablated and conventionally extracted (Figure S3) tissue samples are labeled with the same numbers.

In addition, the extraction procedure was tested for compatibility with downstream western blot analysis. For this purpose mouse liver tissue was irradiated and the ablation plume was captured with a cryo-trap. Figure 2 shows liver tissue after this procedure. The ablated area of 5 mm × 5 mm is clearly visible. The condensate had a volume of 40 μL and a protein concentration of 20.1 $\mu g\,\mu L^{-1}$. As for the muscle tissue sample, high yields of proteins were extracted from a small tissue sample area in a single step. For immunoblotting, a 40 μg sample was loaded on a SDS-PAGE, and the proteins were transferred onto a blot membrane and incubated with the anti-CEACAM1-antiserum P1 antibody.[22] The western blot in Figure 3 shows a clear and abundant band slightly above the marker band for 100 kDa for the liver CEACAM1 protein, which is known to be highly glycosylated.[23,24] The theoretical molecular weight of the unmodified CEACAM1 is 53517.80 Da, and the detection of

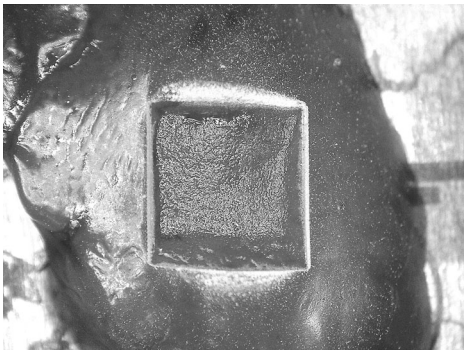

Figure 2. Mouse liver tissue after PIRL irradiation. A 5 mm × 5 mm area was irradiated for 5 min.

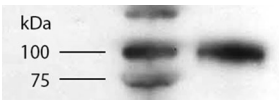

Figure 3. Detection of ablated liver CEACAM1 with anti-CEACAM1-antiserum P1. Left: Protein standard. Right: Ablated proteins from mouse liver ($m = 40$ µg). The thick band in the right lane runs slightly above 100 kDa and resembles the glycosylated liver CEACAM1.

CEACAM1 above 100 kDa suggests that labile posttranslational modifications like glycan chains survive the DIVE process.

These results show that the PIRL-DIVE-based extraction method is suitable for extracting proteins over a broad mass range including proteins in the MDa range without significant fragmentation. Because PIRL-extracted proteins were detectable by immunoblotting it can be assumed that the motifs of the proteins for binding the antibodies are not severely changed.

In order to address the question of whether the energy of PIRL affects the structure of the desorbed protein, we investigated whether the chemical composition of ablated proteins remains unaltered, since this is a prerequisite for an appropriate extraction method. For this purpose samples of PIRL-ablated and control RNase A without ablation were analyzed by MALDI-MS and LC-ESI-MS. The MALDI spectra (Figure 4) of the irradiated and the untreated sample

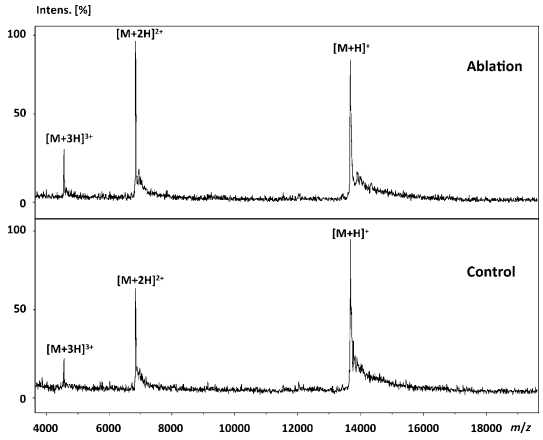

Figure 4. MALDI-TOF mass spectra of RNase A ablated by PIRL (top) and RNase A before irradiation with the PIRL (bottom, control). Matrix: 2,5-dihydroxyacetophenone (DHAP).

show signals of the singly, doubly, and triply protonated intact RNase A molecule. Both MALDI spectra are almost identical.

For a more detailed characterization of the protein composition both RNase A samples were analyzed by LC-MS on the intact protein level and on peptide level after tryptic digestion by LC-MS/MS. Comparison of the chromatograms and mass spectra of the intact protein as well as of the tryptic peptides revealed no signals in the ablated sample that were not present in the non-ablated sample, and vice versa (Figures S4 and S5 and Schemes S1 and S2).

In addition PIRL-ablated and non-ablated samples of alpha-S1 casein were analyzed by MALDI-MS (Figure S12). The two MALDI spectra are identical, showing signals for the singly and doubly protonated alpha-S1 casein carrying eight phosphate groups. This result further highlights the soft ablation process, since the protein was desorbed intact without loss of its labile posttranslational modifications.

Finally, it was investigated whether ablated proteins retain their enzymatic activity. Trypsin was dissolved in aqueous solution and ablated by PIRL. The condensed ablation plume was incubated with casein and analyzed by LC-ESI-MS. Non-incubated casein was analyzed by LC-ESI-MS as a control. The chromatogram of casein incubated with ablated trypsin shows a number of signals caused by tryptic peptides, while no signals for intact alpha-S1 casein and alpha-S2 casein were detectable (Figures S13 and S15, and Schemes S3 and S4). In contrast, no signals of tryptic peptides but abundant signals for intact alpha-S1 and alpha-S2 casein were observed in the LC-MS data of the untreated casein sample (Figures S13 and S14 and Scheme S5). This result shows that trypsin was still enzymatically active after being ablated by PIRL.

In addition, the condensed ablation plume of human blood plasma was investigated for the detection of angiotensin I (Ang 1–10) metabolizing proteolytic activities.[25] In the MALDI spectrum of the incubate collected after the ablated plasma proteins had been incubated with Ang 1–10 for six hours, signals of angiotensin II (Ang 1–8; m/z 1047.5) and Ang 6–9 (m/z 651.12) were detected, indicating the activity of at least two different plasma proteases (Figure 5). The presence of signals from Ang 4–10 (m/z 927.47) and Ang 1–7 (m/z 900.48) in the MALDI spectrum of the incubate after 24 h can be assigned to the presence of further angiotensin peptide metabolizing enzymes, thus giving additional evidence for the intactness of enzymes after PIRL ablation and

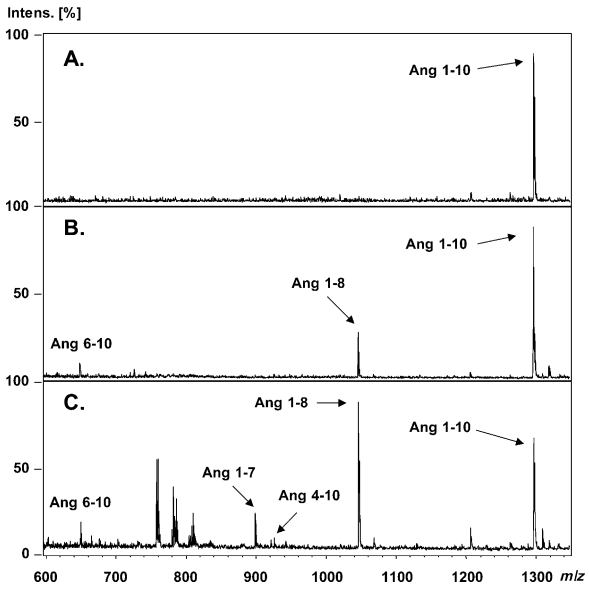

Figure 5. MALDI-TOF mass spectra of Ang 1–10 ($c = 10^{-5}$ M) incubated with ablated human plasma proteins. Reaction products were detected by MALDI-TOF MS after 0 h (A), 6 h (B), and 24 h (C). Signals of the generated angiotensin peptides are marked by arrows. Matrix: 2,5-dihydroxybenzoic acid (DHB).

the softness of the PIRL-DIVE process. As a control, the same amount of non-ablated human blood plasma was incubated with an equal amount of Ang 1–10. The MALDI spectrum of the incubate after 24 h shows very similar patterns of signals of angiotensin peptides after 24 h incubation time (Figure S16), indicating that almost all of the enzyme activity is maintained after PIRL irradiation.

In summary, we have demonstrated that PIRL-DIVE is a very effective extraction tool by which proteins are directly transferred from tissue into the gas phase rapidly, without altering the chemical composition of the proteins. Even enzymatic activities were found in protein fractions ablated by PIRL, underlining the softness of the ablation process. The qualitative and quantitative yield of proteins extractable by PIRL is very high. Thus the properties of PIRL protein extraction from tissues are suitable for many kinds of protein analytics—from proteomic studies in basic research to diagnostics targeting defined protein biomarkers.

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