Specific modification of protein-bound citrulline residues facilitates their detection

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Although auto-antibodies against citrullinated proteins are well-known for diagnosis of rheumatoid arthritis, the exact position of citrulline in these proteins, important to evaluate the conformation of the epitopes, remains elusive.

The purpose of this study was to specifically modify citrullinated peptides to make them discernable from non-citrullinated peptides and to identify them by liquid chromatography coupled to mass spectrometry (LC-MS).

**Methods**

Citrullinated and non-citrullinated synthetic peptides were modified at 37°C with 2,3-butanedione (BD) in TFA and were analysed by nanoLC-MS and by infusion tandem MS.

Next, these peptides were spiked in a Cytochrome C-digest, after which the complete mixture was modified, followed by LC-MS analysis.

Peptides were also purified from mixtures by immunoprecipitation (IP) with anti-citrulline antibody bound to protein A-agarose beads and subsequently modified and identified.

**Results**

Modification with BD was specific for citrullinated peptides and produced a mass shift of 50Da. Complete modification was established after 16h with 50mM BD/30 µl TFA. Based on MSMS-spectra, the butanedione-adduct was proved to be covalently bound to the citrulline residue of the peptide and the amino acid sequence of the modified peptide could still be established at a concentration of 25pmol/µl.

When synthetic citrullinated peptides were spiked into a cytochrome C-digest, complete modification of the citrullinated peptide remained possible in a 1/100 dilution (160fmol citrullinated peptide/16pmol cytochrome C-digest), even though the reaction did result in significant reduction in ionization capacity (p<0.01).

Modified citrullinated peptides also showed a significant shift in retention time (p<0.0001). Non-citrullinated peptides of the cytochrome C-digest were not affected.

In order to obtain comparatively higher ion counts of the modified citrullinated peptides, an IP experiment was conducted. 2 nmol of citrullinated peptide could be purified from a mixture with its non-citrullinated counterpart by IP with an anti-citrulline antibody. Afterwards, modification of the purified citrullinated peptides resulted in the identification of the modified peptide by the specific additional mass shift and RT shift. Moreover a gain in ion count was observed; the proportion of citrullinated peptide as opposed to non-citrullinated peptide detected increased five-fold after IP (p< 0.05).

**Conclusion**

Specific modification of citrullinated proteins with 2,3-butanedione causes a 50Da-mass shift and a significantly longer retention of the modified citrullinated peptides on nanoLC-MS. Non-citrullinated peptides remained unaffected in mass and retention time. Also, peptides could be selectively purified from mixtures by immunoprecipitation. This opens possibilities for the identification of citrullinated peptides in complex mixtures and to identify the in vivo citrullinated status of proteins in the inflamed joint.