Specific modification of citrullinated peptides facilitates their detection

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- Introduction

Citrullinated proteins are modified proteins in which an arginine is enzymatically converted to a citrulline. Antibodies against citrullinated proteins (anti-CPP) are highly specific for Rheumatoid Arthritis (RA) and can be detected very early in the course of the disease, even before clinical onset. Since it is becoming clear that citrullinated epitopes instead of the mere presence of citrulline may be relevant for the induction of ACPA, it is of great importance to characterize the exact position of this citrulline in the citrullinated proteins.
The purpose of this study was to specifically modify citrullinated peptides in such a way that makes them discernible from non-citrullinated peptides and to identify them by liquid chromatography coupled to mass spectrometry (LC-MS).

- Method

Citrullinated and non-citrullinated synthetic peptides were modified at 37°C with 2,3-butanedione (BD) in TFA and were analyzed by nanoLC-MS and by infusion tandem mass spectrometry using a QqTOF instrument (Waters).
Next, these peptides were spiked in a Cytochrome C-digest after which the complete mixture was modified, followed by LC-MS analysis.
Recombinant human fibrinogen was citrullinated *in vitro* by Peptidyl Arginine Deiminase 4 (PAD4) from rabbit skeletal muscle. After tryptic digest, peptides were fractionated on RP-LC and fractions were divided in two, one to be modified and one as reference sample. After modification, peptides were analysed by LC-MS.

- Results

Modification with BD was specific for synthetic citrullinated peptides and produced a mass shift of 50Da (m/z = 25 Da for doubly charged ions). Complete modification was established after 16h with 50mM BD/30 µl TFA. Based on MSMS-spectra, the butanedione-adduct was shown 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).
Besides the additional mass, modified citrullinated peptides also showed a significant shift in retention time ($p<0.0001$). Peptides of the cytochrome C-digest that were not citrullinated were not affected by this shift in retention time or by the additional mass characteristic.
To test for the presence of citrullinated peptides in a whole protein, we digested 10µg of *in vitro* citrullinated recombinant human fibrinogen and modified the peptides after fractionation. Results are shown in figure 1; a citrullinated peptide could be identified and its presence was confirmed by modification.

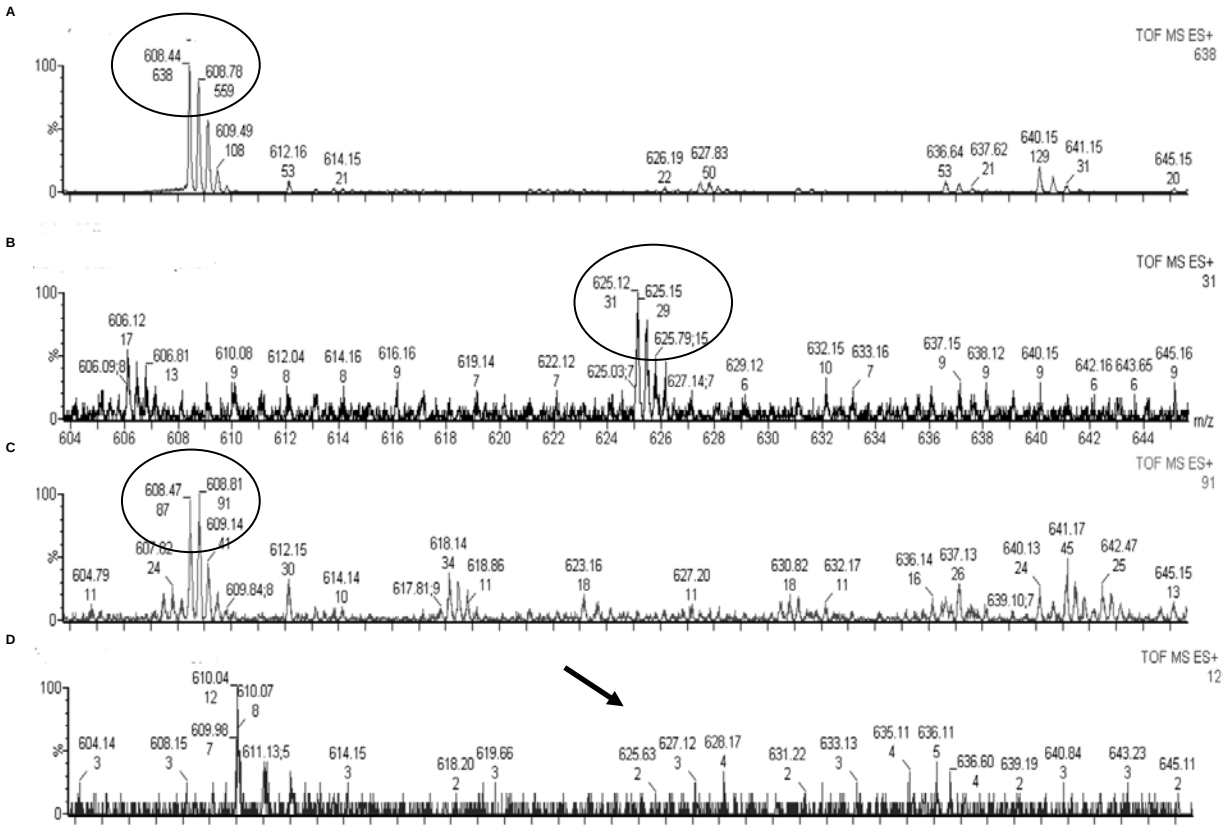

Figure 1A: Peptide ESSHHPGIAEFPSRGK should have a calculated mass of 608.1[3+]. However, in the unmodified sample, a shift of +0.33Da can be observed, indicative for the 1Da mass shift from arginine to citrulline; Figure 1B: In the modified sample, a peptide with m/z 625.2 [3+] can be found. The difference in m/z between both peptides mentioned is 16.6Da. Since the peptides are triply charged, this corresponds to a 50Da mass shift, also indicative for a modified citrullinated residue. The observed shift in Rt amounts to 11mins (from 22.3 mins in the non-modified sample to 33.9mins in the modified sample); Figure 1C: Modification is not quantitative, as can be seen here by the presence of a residual amount of unmodified peptide in the modified sample; Figure 1D: No trace of the m/z 625.2 [3+] can be found in the unmodified sample, proving that this peptide is indeed a product of modification rather than a peptide resulting from the digest.

- Conclusions

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.
When applied to an *in vitro* citrullinated protein, our method could identify a citrullinated peptide in the mixture of tryptic peptides. Therefore, it should be possible to find citrullinated peptides in *in vivo* citrullinated proteins, found in the joint of patients with Rheumatoid Arthritis. This might give valuable insights into the immunology of this autoimmune disorder.