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Modification of citrulline residues with 2,3-butanedione facilitates their detection by liquid chromatography/mass spectrometry

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Citrullination is a post-translational modification (PTM) that results from the deimination of the amino acid arginine into citrulline by Peptidyl Arginine Deiminase enzymes and occurs in a wide range of proteins in health and disease. This modification causes a 1 Da mass shift, which can be used to identify citrullination sites in proteins by the use of mass spectrometry. However, other PTMs, such as deamidation from asparagine to aspartic acid or from glutamine to glutamic acid, can also cause a 1 Da mass shift, making correct interpretation of the data more difficult. We developed a chemical tagging strategy which, combined with an open source search application, allowed us to selectively pinpoint citrullinated peptides in a complex mixture after liquid chromatography/mass spectrometry (LC/MS) analysis. After incubation of a peptide mixture with 2,3 butanedione, citrulline residues were covalently modified which resulted in a 50 Da shift in singly charged mass. By comparison of the peptide mass fingerprint from a modified and an unmodified version of the same sample, our in-house search application was able to identify the citrullinated peptides in the mixture. This strategy was optimized on synthetic peptides and validated on a digest of *in vitro* citrullinated fibrinogen, where different proteolytic enzymes were used to augment the protein coverage. This new method results in easy detection of citrullinated residues, without the need for complex mass spectrometry equipment. Copyright © 2011 John Wiley & Sons, Ltd.

Citrulline is an amino acid that is not encoded in the DNA; instead, when present in a protein, it originates from a post-translational modification (PTM) (deimination) of arginine. This deimination, catalyzed by Peptidyl Arginine Deiminases (PADs), results in a neutral amino acid with a mass increase of 1 Da (Fig. 1).[1] It is clear that the loss of a positive charge can have severe effects on the tertiary structure of a protein, making citrullination an interesting modification to investigate, especially when examining immunogenicity in autoimmunity. After all, a difference in tertiary structure might bring new epitopes to the surface of a molecule, triggering an immune reaction. In addition, citrullination creates neo-epitopes that will activate the immune system.[2]

Citrulllination in the context of autoimmune disease is most often linked to rheumatoid arthritis, where the presence of citrullinated proteins and antibodies against these citrullinated proteins (ACPA) in the affected joints has been well documented.[3–5] In fact, the determination of ACPA-titres has proven to be very important for diagnosis of rheumatoid arthritis (RA), since they show a specificity of 90–98%.[6] The identification of the exact antigen that elicits this specific immune response, and most importantly the location of citrullinated residues within this antigen, would be of great interest in the research of the pathogenesis of RA.

A role for citrullination has also been demonstrated in multiple sclerosis,[7] indicating that this small modification and resulting conformation change might have an impact on antigen-TCR contact and the resulting antibody production in a variety of diseases.[8,9]

Physiologically, the deimination of arginine residues on cytoskeletal proteins causes loss of cell structure and plays a role in apoptosis.[10] When also considering histone citrullination as an important epigenetic regulatory mechanism,[11] it comes as no surprise that the interest in characterization of citrullinated proteins is on the rise. After all, the ability to localize the citrullinated residue in a protein will provide invaluable information about the changes in structure and function of this protein, and will therefore improve current understanding of the importance of this PTM and its role in several autoimmune diseases.

Although citrullination as a PTM plays a role in many physiological and pathological processes, relatively few methods exist to easily identify deiminated arginine residues in a protein. In most cases, citrullination is verified on Western blot, either with an antibody against citrulline itself or against an on-blot chemically modified version of the amino acid. However, using these detection methods, it is only possible to determine the citrullination state of an entire protein without being able to annotate the location of the converted arginine. The latter can be achieved by mass spectrometry (MS), but although a mass difference of 1 Da

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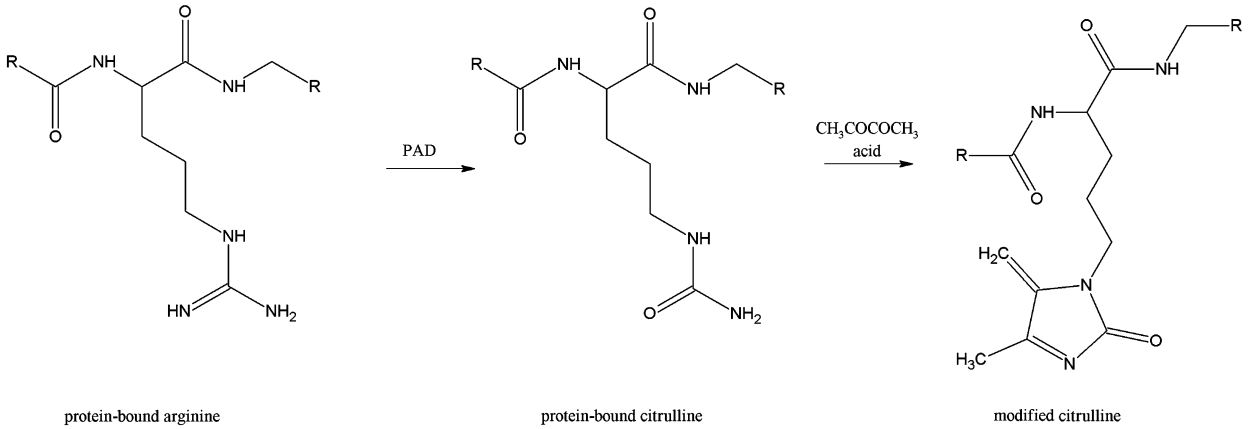

Figure 1. Modification of protein-bound arginine and citrulline. *In vivo* or *in vitro* deimination by PAD enzymes converts an arginine into a citrulline residue, which causes a mass shift of +1 Da. Afterwards, *in vitro* modification with 2,3-butanedione at low pH results in the formation of an imidazolone derivative, with a specific mass shift of +50 Da.

can be distinguished by high resolution mass spectrometry, there are other PTMs that also give rise to a 1 Da difference. Deamidation of Asn to Asp and of Gln to Glu or even the uptake by the peptide of a hydrogen atom can also account for this small difference. Moreover, a 1 Da mass difference can be especially difficult to detect on higher charged peptides. Therefore, another, more specific method of detecting citrulline residues by means of MS is needed.

A recent report described the neutral loss of isocyanic acid from citrulline and used this loss as a marker for citrullinated peptides.[12] This technique could be useful; however, the detection of a true neutral loss on a quadrupole time-of-flight (QTOF) instrument is not possible[13] and limits the researcher in instruments to a triple quadrupole mass spectrometer. Similarly, Stensland *et al.* used a combination of Collision-Induced Dissociation (CID) and Electron-Transfer Dissociation (ETD) for the fragmentation and identification of modified citrullinated peptides;[14] this technique, though very elegant, is not readily available to all research groups. Therefore, we developed a modification-based MS method for citrulline detection which could universally be performed on all electrospray ionization (ESI)-based mass spectrometers.

This approach involved 2,3-butanedione (BD), which is used in chemical modification of both arginine[15] and citrulline[16] (Fig. 1). In the case of arginine, this reaction is usually performed in basic environment in the presence of arylboronic acid; when performed in acidic conditions and followed by reaction with antipyrin, it is specific for citrulline-containing residues. The latter modification reaction was used in order to design a simple yet specific liquid chromatography/mass spectrometry (LC/MS) method for the detection of citrulline-containing peptides.

EXPERIMENTAL

Synthetic peptides

Two synthetic peptides derived from human vimentin, one without citrulline (vim = NH2-STSRSLYASSPG-COOH (MW 1213.9)) and one citrullinated (vim cit = NH2-STS Cit SLYASSPG-COOH (MW 1215.2)), were purchased from Thermo Scientific (Rockford, IL, USA).

Modification

Modification of citrullinated peptides (1 nmol unless otherwise specified) with 10 µL 50 mM 2,3-butanedione (BD) (Sigma-Aldrich, Germany) in 30 µL trifluoroacetic acid (TFA) (Sigma-Aldrich, Germany) was carried out at 37°C for 16 h. Other reaction conditions were tested and are specified throughout the text.

To mimic more complex protein samples, peptides were spiked into a synthetic cytochrome C digest (Dionex, Sunnyvale, CA, USA).

In vitro citrullination of human fibrinogen

In vitro citrullination was performed on human fibrinogen purified from plasma (Calbiochem, Darmstadt, Germany) in deimination buffer (0.1 M tris-HCl, pH 7.4, 100 mM $CaCl_2$, 5 mM dithiothreitol (DTT)), by adding 7 IU of PAD from rabbit skeletal muscle (Sigma-Aldrich, St. Louis, MO, USA) per mg of protein. After 2 h at 37°C, the reaction was stopped by adding EDTA to a final concentration of 50 mM.

In-solution digest of proteins

Proteins were digested using both LysC endoprotease (Sigma-Aldrich) and GluC endoproteinase (Sigma-Aldrich). Briefly, a protein was dissolved in 50 mM ammonium bicarbonate (Sigma-Aldrich) and 6 M urea (Sigma-Aldrich), after which it was first reduced with 20 mM DTT (Invitrogen, CA, USA) for 1 h at room temperature and subsequently alkylated with 200 mM iodoacetamide (MP, Solon, OH, USA) for 1 h at room temperature. Then, samples were diluted 1:10 with MilliQ ultrapure water and digested with GluC or LysC endoproteinase for 4 h at 37°C (1 part enzyme to 50 parts protein).

Fractionation on HPLC

Peptides originating from 10 to 20 µg of protein were separated on a PepMap 100 (C18) column (i.d. 75 µm, length 15 or 25 cm; Dionex, Sunnyvale, CA, USA) at 60°C, by use of a U3000 nano-LC system (Dionex) at a flow rate of 300 nL/min. Elution was performed with 0.1% formic acid (FA) in water (buffer A) and 80% acetonitrile/0.1% FA (v/v) in water (buffer B), increasing

from 4% to 65%B in 60 min, ending with 15 min of 100%B. Fractions were collected every 5 min (n = 15), subsequently dried in a SpeedVac and resuspended in 20 µL MilliQ ultrapure water. Half of each fraction was then modified as described above.

Mass spectrometric analysis

Samples were introduced into the mass spectrometer (QqTOF, Waters, Milford, MA, USA), through a nano-ESI source by infusion at a flow rate of 0.5 µL/min. LC/MS was used when more complex mixtures were analyzed and was performed using the U3000 system (Dionex, Sunnyvale, CA, USA), with a C18 column (i.d. 75 µm, length 15 or 25 cm; Dionex, Sunnyvale, CA, USA) at 25°C or 60°C. The same gradient was used as described above at a flow rate of 300 nL/min.

For tandem mass spectrometry (MS/MS), ions were fragmented by CID, with a collision energy of 10 eV for infused samples and with a custom collision energy profile for LC/MS/MS samples, ranging from 25 to 55 eV for doubly charged peptides between *m/z* 400 and 1200, and ranging from 11 to 26 eV for triply charged peptides between *m/z* 435 and 1000.

All solvents were MS-grade and purchased from Biosolve (Westford, MA, USA).

Peak picking and analysis

For MS-only samples, peak picking was performed using Mascot Distiller version 2.3.2 (Matrix Science, London, UK) for peaks with a maximum charge of 5. Correlation threshold was set to 0.6 and signal-to-noise ratio had to be at least 2.

Afterwards, peak lists from unmodified and modified samples were loaded into an in-house R application MsMod (available upon request). Basically, the program contains two data-filtering steps. The first step filters out peak data from the unmodified sample matching the modified data within a user-defined experimental mass tolerance (we used 0.1 Da). The second filter is based on the web-based tool PeptideMass,[17] a protein digest simulator of the protein(s) under investigation (in our case fibrinogen chain α, β and γ). The digestion parameters allow up to 1 and 3 miscleaved sites for LysC and GluC, respectively, and a user-defined mass range, in our case of 350–8192 Da. The theoretical isotopic profiles of the generated peptides[18] are compared with the filtered measured data. The filter parameters allow for a user-defined mass deviation, a fixed modification of carbamidomethylated cysteines, as well as a variable N-terminal carbamidomethylation, and a variable citrullination.

Manual confirmation of the data was performed using MassLynx software (Waters, Milford, MA, USA). MS/MS analysis was performed on a Mascot in-house server.

RESULTS AND DISCUSSION

Modification of citrullinated peptides with BD is specific and causes a mass shift of 50 Da

The reaction of citrullinated peptides with BD alone, as opposed to the widely used combination of BD and antipyrine, was evaluated on 1 nmol citrullinated synthetic peptide using previously described reaction conditions.[19] Different time points (30 min, 1 h, 2 h, 4 h, 8 h and 16 h) were tested and the reaction was verified by infusion MS at a concentration of 25 pmol/µL. The yield of the modification gradually improved when the reaction was allowed to take place for longer periods of time and, at 16 h, 96% of citrullinated peptides were modified (Fig. 2(A)). In all further experiments, incubation occurred overnight.

An attempt was made to optimize reaction conditions by adjusting the concentration of reagents. To obtain the lowest possible modification of arginine residues, a different volume (60 µL instead of 30 µL) of TFA was tested, resulting in a lower pH. Moreover, to minimize the background peaks in the spectrum originating from the modification reagent BD, a lower concentration (5 mM instead of 50 mM) was tested. To ascertain whether concentration of peptides influenced the quantity of reagents needed, different amounts of peptide (16 pmol, 0.8 pmol and 0.16 pmol) were modified. After analysing these different reaction conditions for traces of unreacted unmodified citrullinated peptides, the best results were obtained with 30 µl TFA and 10 µl 50 mM BD, as described in literature (data not shown).[19] Even at low concentration, full modification of the citrullinated peptide could be observed after overnight incubation.

After modification of 1 nmol of the synthetic citrullinated peptide (*m/z* 607.3 [2+]), new spectral peaks were observed at *m/z* 631.3 [2+] and 632.3 [2+] in the modified sample when compared to the unmodified sample (Fig. 2(B), *vim cit*, *m/z* 607.3 [2+], versus *vim cit mod*, *m/z* 632.3 [2+]). This shift of 50 Da (or in this case *m/z* 25 because of the doubly charged nature of the peptide) matched with the formation of an imidazolone derivative as proposed by Holm *et al.*[19] (Fig. 1).

The peptide showing the 50 Da mass shift was indeed the citrullinated chemically modified peptide as MS/MS analysis showed the 2,3-butanedione being covalently attached to the citrulline residue (Fig. 2(C)). Out of a total of 14 fragment ions, y-ions as well as b-ions, nine fragment ions were annotated in the MS/MS spectrum with an additional mass of 50 Da (denoted by * in Fig. 2(B)). Due to the non-tryptic nature of the peptide, y-ions were less frequently observed. The amino acid sequence of the peptide could still be established at a concentration of 25 pmol/µL.

The reaction was found to be specific for citrullinated peptides: no shift could be detected when non-citrullinated peptides were subjected to the same reaction (data not shown).

Modification of citrullinated peptides in mixtures

To determine the behaviour of peptides in more complex mixtures, both citrullinated and non-citrullinated synthetic peptides were spiked in a cytochrome C digest. The entire mixture was then modified as described in the Experimental section.

Different dilutions of synthetic peptides in the cytochrome C digest were tested (1:10, 1:20, 1:100); complete modification of the citrullinated peptides could still be observed at a 1:100 dilution (160 fmol peptide/16 pmol cytochrome C digest) (data not shown). In all modified samples, modification of the citrullinated synthetic peptide *vim cit* could be detected.

In a small portion of the runs (4/16) unmodified citrullinated peptides could still be found, with a reaction yield of >70% in all samples. This indicates an incomplete reaction when the modification is carried out on more complex samples, an observation also made by Stensland *et al.*[14]

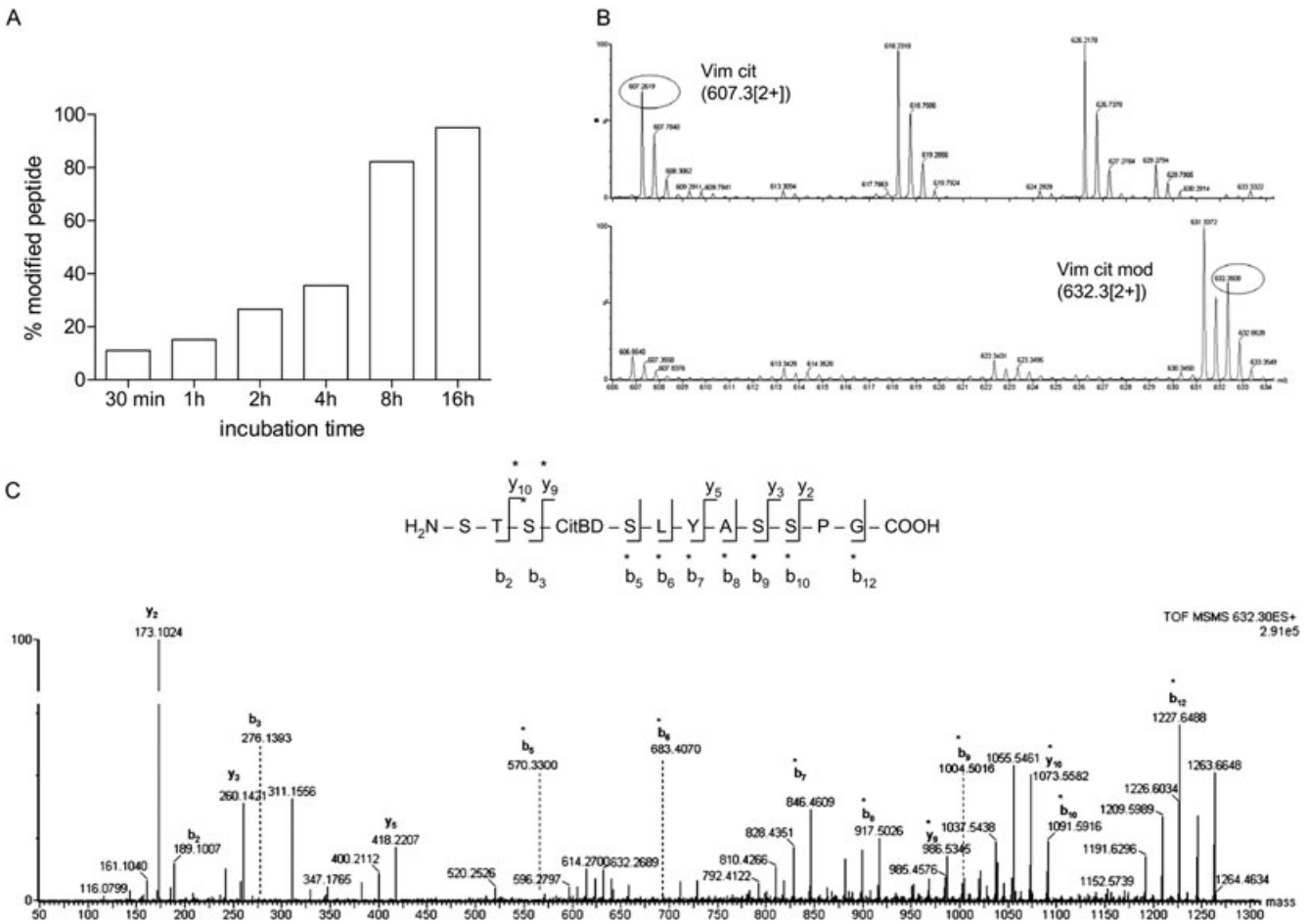

Figure 2. Modification of a citrullinated synthetic peptide (H2N-STSCitSLYASSPG-COOH) with 2,3 butanedione in 60% TFA. (A) Increasing incubation time results in higher amounts of modified peptides, with the highest (96%) after 16 h of incubation. (B) Specific modification of citrulline residues causes a 25 Da mass shift for doubly charged peptides, in this case from *m/z* 607.3 to 632.3. (C) MS/MS spectrum of the modified peptide. Due to the non-tryptic nature of the peptide, b-ions are most abundant in the spectrum. Seven out of 12 possible b-ions and 2 out of 12 possible y-ions have been found with a mass exceeding their theoretical mass by 50 Da (denoted by *), proving the covalent bond between BD and the citrulline residue.

In order to clarify whether non-citrullinated peptides from cytochrome C were affected by the reaction, we investigated two peptides, one without an arginine residue (KTGQAPGFSYTDANK, *m/z* 528.9 [3+]) and one with an arginine residue (GEREDLIAYLKK, *m/z* 717.6 [2+]). These *m/z* values were found in all samples, both before modification and after, while no *m/z* value of 50 Da higher could be detected in the modified sample (data not shown). This indicated that no modification of random peptides took place, not even when an arginine residue was present in the peptide.

Detection of citrullinated peptides originating from an *in vitro* citrullinated protein

As mentioned above, we noted that the reaction did not run to completion when applied to a more complex sample. We found that it was necessary to first fractionate more complex samples, such as whole protein digests, prior to modification. When utilizing our method for the elucidation of citrullinated sites in *in vitro* citrullinated human fibrinogen, 10 µg (approximately 100 pmol) of a LysC digest was first fractionated by HPLC (n = 2). Afterwards, the 15 fractions were split into two in order to obtain a modified and unmodified version of the same fraction. This fractionation was followed by consecutive LC/MS analysis of first the modified and next the unmodified sample, to minimize inter-run variability.

To facilitate comparison of the modified with the unmodified sample, an in-house application was developed in R to process the data.

Peak lists generated in Mascot Distiller for both the unmodified and the modified sample were compared to each other based on mass and charge, with a tolerance of 0.1 *m/z*, and peaks that were the same in both files were discarded. The resulting peak list was then compared to a theoretical digest of the citrullinated form of all three fibrinogen chains. Based on the list resulting from this semi-automated comparison, manual analysis was performed to check if the modified form of the peptide was present in the modified sample. Manual analysis was necessary because of the low ion counts of the modified peptides, which prevented accurate peak picking of some of these peptides.

This process of detection is illustrated in Fig. 3 for the peptide ESSHHPGIAEFPS**R**GK. In its citrullinated form, this

peptide should have a calculated mass of *m/z* 608.4 [3+], which could be observed in the unmodified sample (Fig. 3(A)). This, taken together with a loss of signal in the modified sample, resulted in detection by the search algorithm. Afterwards, manual analysis was performed to investigate modification.

In the modified sample, a peptide with *m/z* 625.2 [3+] could be found (Fig. 3(B)). The difference in *m/z* between both peptides mentioned was 16.6. Since the peptides were triply charged, this corresponded to a 50 Da mass shift indicative for a modified citrullinated residue. As can be seen in Fig. 3(C), a residual amount of unmodified peptide could be observed in the modified sample. However, the ion count was 85% lower, indicating that at least part of the total peptide amount was modified and preventing the peaks being efficiently picked by Mascot Distiller. This large loss in signal was the primary reason for detection by our search application MSMod.

No trace of the *m/z* signal for 625.2 [3+] could be found in the unmodified sample (Fig. 3(D)), indicating that this peptide was indeed a modification product, rather than a peptide resulting from the digest.

This approach was validated where possible by peptide sequence analysis. To this end, any peaks from the unmodified fraction not found in the modified fraction were compiled into an include list, after which the unmodified samples were run again, this time including MS/MS analysis. In total, six peptides originating from a LysC digest of citrullinated human fibrinogen could be positively identified as citrullinated with this method (see Table 1).

To ensure better coverage of the entire protein, the experiment was repeated using GluC as endoproteinase (n = 2). Using this protease, proteolysis resulted in shorter peptides with more easily detectable arginines/citrullines. Nine more peptides could be identified (Table 1) after comparison with the theoretical digest.

After both LysC and GluC digestion, 15 peptides, encompassing 17 citrullinated residues, were identified using the search algorithm due to loss of signal after modification (Table 1). For nine of the 15 peptides identified, the modified form could be found in the modified sample (italicized in Table 1); for the other six peptides, signal-to-noise was too low (S/N <2 in the MS spectrum) for reliable manual peak picking.

Of the 15 peptides in total discovered by modification and subsequent automatic comparison, eight could also be identified by MS/MS followed by Mascot analysis (shown in bold in Table 1). The other six could not be retrieved by a conventional LC/MS/MS approach, indicating that our method was more sensitive in picking up low-abundance citrullinated peptides in a complex mixture.

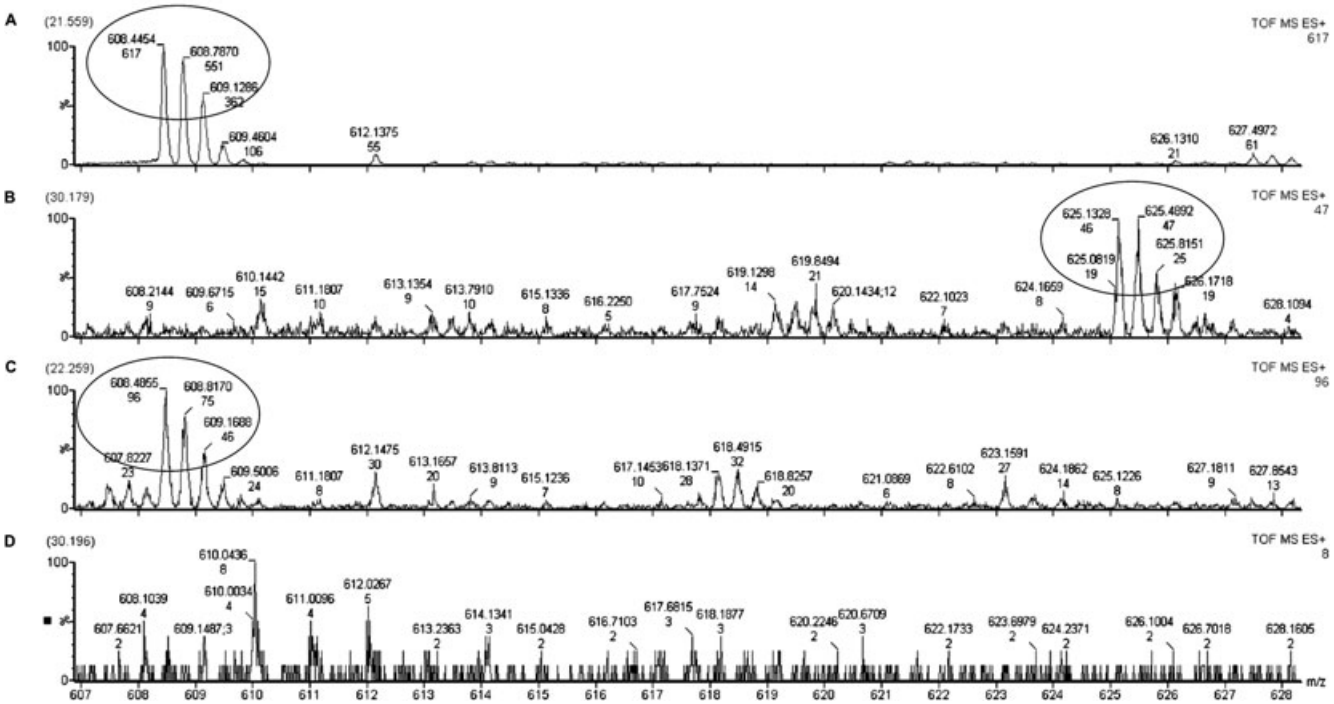

Figure 3. Detection of a citrullinated peptide in a LysC digest of *in vitro* citrullinated fibrinogen (single scan spectra are shown at the time of highest ion count). (A) MS spectrum of the unmodified sample at retention time (Rt) 21.5 min: Peptide ESSHHPGIAEFPSRGK should have a calculated mass of 608.1 [3+]. However, in the unmodified sample, a shift of +0.33 Da can be observed on the triply charged peptide, which corresponds to a 1 Da mass shift from arginine to citrulline. (B) MS spectrum of the modified sample at Rt 30.2 min: 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 16.6 Da. Since the peptides are triply charged, this corresponds to a 50 Da mass shift, also indicative for a modified citrullinated residue. The observed shift in Rt amounts to 11 min (from 22.3 min in the unmodified sample to 33.9 min in the modified sample). (C) MS spectrum of the modified sample at Rt 22.3 min: Modification is not quantitative, as can be seen here by the presence of a residual amount of unmodified peptide in the modified sample. (D) MS spectrum of the unmodified sample at Rt 30.2 min: No trace of the *m/z* 625.2 [3+] can be found in the unmodified sample, demonstrating that this peptide is indeed a product of modification, rather than a peptide resulting from the digest.

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Table 1. Peptides identified as being citrullinated in *in vitro* citrullinated fibrinogen. *m/z* (cit) was found in the unmodified sample by the search algorithm. Bold R denotes a citrullinated arginine residue, underlined amino acids denote carbamidomethylation. Masses are italicized when found by manual analysis and displayed in bold when confirmed by MS/MS

Peptide	Strand	Protease	*m/z* (cit)	*m/z* (mod)
S**R**GSESGIFTNTKE	A	GluC	*757.4* (2+)	*782.5* (2+)
TFPGFFSPMLGEFVSETES**R**GSESGIFTNTK	A	LysC	***1129.9* (3+)**	1146.6 (3+)
V̲SGNVSPGT**RR**E	A	GluC	***659.3* (2+)**	709.3 (2+)
G̲GGV**R**GPRVVE	A	GluC	*542.8* (2+)	*567.8* (2+)
G̲GGV**R**GPRVVE			*571.3* (2+)	*596.3* (2+)
K̲LVTSKGDKEL**R**TGKE	A	GluC	*895.5* (2+)	*920.5* (2+)
SGSF**R**PDSPGSGNA**R**PNNPDWGTFEE	A	GluC	***927,575* (3+)**	960.9 (3+)
ESSSSHHPGIAEFPS**R**GK	A	LysC	***608.5* (3+)**	*625.1* (3+)
QFTSSTSYN**R**GDSTFESK	A	LysC	***1022* (2+)**	1047 (2+)
FPS**R**GKSSSYSKQFTSSTSYN**R**GDSTFE	A	GluC	***1047.4* (3+)**	1080.6
DLLPSRD**R**QHLPLIK (*)	A	LysC	*601.4* (3+)	*618.1* (3+)
VTSGSTTTT**RR**SCSKTVTK	A	LysC	*687.2* (3+)	*720.5* (3+)
LRTGKEKVTSGSTTTT**RR**SCSKTVTK TVIGPDGHKE	A	GluC	***782.2* (3+)**	815.7 (3+)
K**R**EEAPSL**R**PAPPPISGGGYRA**R**PAK	B	LysC	***921.2* (3+)**	*971.2* (3+)
YC**R**TPCTVSC̲NIPVVSGKEC̲EE	B	GluC	*882.5* (3+)	*899.2* (3+)
AS̲ILTH̲DSSI**R**YLQE	C	GluC	*867.5* (2+)	*892.5* (2+)

(*)This peptide was citrullinated on one residue, although we could not determine on which one due to the close proximity of both arginine residues.

When looking at other methods for elucidating citrullination sites, such as enzymatic labelling[20] or accurate mass determination,[21] attention can be drawn to two clear advantages of our method. Five of the citrullinated arginines we identified in the α-strand of fibrinogen (QFTSSTSYN**R**GDSTFESK, GGGV**R**GPRVVE, ESSSSHHPGIAEFPS**R**GK and V̲SGNVSP GT**RR**E) have already been reported as being citrullinated *in vitro* by Kubota *et al.*[20] In that paper, the authors identified citrullination sites in fibrinogen α by incorporation of O^{18} by using trypsin, GluC and chymotrypsin. Using only two digestive enzymes, LysC and GluC, we were able to elucidate seven more citrullination sites in fibrinogen α (S**R**GSES GIFTNTKE, SGSF**R**PDSPGSGNA**R**PNNPDWGTFEE, KLVT SKGDKEL**R**TGKE, VTSGSTTTT**RR**SCSKTVTK and DLLPS RD**R**QHLPLIK) which were not covered in the analysis of Kubota *et al.* Additionally, this enzymatic labelling approach can only be used after *in vitro* labelling, while our method could be extrapolated to *in vivo* citrullinated samples for future research.

The most widely used approach to identifying citrullination sites to date is the detection of a 1 Da mass shift, most often in combination with MS/MS analysis. However, one of the identified peptides in this analysis illustrates the advantage of a specific modification of citrullinated residues. Peptide QFTSSTSYN**R**GDSTFESK has an *m/z* of 1022 [2+] and was correctly identified after MS/MS analysis with a 1 Da modification. However, since asparagine (N) and arginine (R) are in such close proximity, the Mascot search algorithm could not successfully distinguish between a deamidated asparagine and a citrullinated arginine (scored 144 and 139, respectively, from the same spectrum). The extra modification of the citrulline residue with BD, and the resulting loss of signal in the modified sample, dismissed the possibility that the 1 Da modification resided anywhere else but on the arginine residue.

CONCLUDING REMARKS

Different methods already exist for the detection of protein-bound citrulline. In most cases, these are based on the 1 Da mass difference when compared to the same peptide with arginine instead of citrulline. This analysis is often combined with a tryptic miscleavage to confirm the findings, since trypsin cannot cleave after a citrulline residue.[22] However, this method may incorrectly interpret the 1 Da mass shift, which will then be annotated as a deamidation of glutamine or asparagine. Other methods, based on the detection of a neutral loss[12] or chemical modification followed by a combination of CID and ETD peptide fragmentation,[14] have already been successfully used in the detection of protein-bound citrulline. Similarly, analysis of citrullinated peptides from *in vitro* citrullinated fibrinogen has been performed based on the observation of a 1 Da mass increase, combined with the use of both dissociation techniques.[23] However, this detection can only be performed by a selected group of mass spectrometers, respectively a triple quadrupole or an ion trap mass analyzer, thereby hampering widespread usage.

We set out to develop a simple, specific LC/MS method for the detection of peptide-bound citrulline based on the modification of citrulline with 2,3-butanedione in acidic environment that can be carried out on any mass spectrometer.

This modification resulted in a specific 50 Da mass shift, and could be carried out on peptide amounts as low as 160 fmol. In mixtures, the reaction was not complete, but still sufficient to allow detection of citrullinated peptides in a complex sample, as demonstrated by the identification of

15 citrullinated peptides from *in vitro* citrullinated fibrinogen. The method also avoids the incorrect interpretation of a 1 Da mass shift caused by deamidation of glutamine or asparagine, which can be especially problematic if these residues are situated in close proximity to the citrulline residue.

To aid data processing a search algorithm, MSMod, was developed, which facilitated the comparison of modified and unmodified samples and might also be used for the detection and evaluation of other post-translational modifications in different samples.

In conclusion, we developed a fast and simple method to identify and analyze citrullinated peptides in a mixture by selectively tagging the citrullinated residue with BD, followed by LC/MS analysis. In the future, this specific tagging strategy will be used in the analysis of *in vivo* citrullinated samples, in order to map the citrullinated epitopes in different autoantigens.

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