

Methods for the Detection of Peptidylarginine Deiminase (PAD) Activity and Protein Citrullination*

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The post-translational conversion of peptidylarginine to peptidylcitrulline, a process also known as citrullination, is catalyzed by the enzyme family of peptidylarginine deiminases (PADs) and has been demonstrated to be involved in many physiological processes, including the regulation of gene expression. In addition, citrullination has been shown to be associated with several diseases, such as cancer, multiple sclerosis, rheumatoid arthritis, and Alzheimer's disease. To get more insight into the role of PAD enzymes and citrullination in both health and disease, experimental strategies to study PAD activity and to characterize citrullinated proteins in complex biological samples are crucial. Here, we describe the chemical, proteomic and antibody-based procedures that are currently available and discuss their applicability for the analysis of complex samples. The methods that have been developed can be used to provide more insight in the substrate specificity of PAD enzymes. Because the evidence that PADs play a pathophysiological role in the diseases mentioned above is increasing, they become attractive targets for therapeutic interventions. More knowledge of PAD specificity and the availability of reliable, high-throughput assays for PAD activity will facilitate the development of highly specific PAD inhibitors. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.R113.033746, 388–396, 2014.

Peptidylarginine deiminases (PADs)¹ are Ca²⁺-dependent enzymes that catalyze the post-translational conversion of

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¹ The abbreviations used are: PAD, peptidylarginine deiminase; ACPA, anti-citrullinated protein/peptide antibodies; BAEE, N_α-benzoyl-L-arginine ethyl ester; CID, collision-induced dissociation; DAMO, diacetyl monoxime; ETD, electron transfer dissociation; HCD, higher-energy collisional dissociation; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; (N)ET, (neutrophil) extracellular trap; RA, rheumatoid arthritis.

peptidylarginine to peptidylcitrulline (Fig. 1; (1)). The guanidine group of the arginine side chain is converted to an ureido group, a process also known as deimination or citrullination. This conversion results in a mass increase of ~1 Da and the concomitant loss of charge under physiological conditions can affect the structure and function of the deiminated protein. In humans, five different PAD isotypes exist, PAD1–4 and PAD6, which have ~50% sequence similarity (2). PAD enzymes are distributed over a wide range of cells and tissues and each isotype has a tissue-specific expression pattern (3–6). They have been reported to be involved in hair growth, myelin formation, the regulation of gene expression and many other processes (reviewed in (7)). Citrullination of histones by PAD4 has also been shown to be involved in (neutrophil) extracellular trap (NET/ET) formation. (N)ETs are large extracellular structures of decondensed chromatin (8) and PAD4 may aid chromatin decondensation through histone citrullination (9–11).

For their enzymatic activity PAD enzymes need relatively high amounts of calcium. Because the cytosolic and nucleoplasmic calcium concentrations are relatively low, PADs are inactive under normal conditions. PADs become activated in dying cells, when calcium concentrations increase because of the influx of calcium ions from the extracellular environment and to the release from intracellular calcium stores. Calcium binding alters the conformation of these enzymes, which results in their activation and the subsequent citrullination of intracellular target proteins. When cells become necrotic and cellular contents are released into the extracellular space, extracellular proteins can also be citrullinated.

In addition to its involvement in many physiological processes, citrullination has been demonstrated to be associated with several diseases, including cancer, neurodegenerative diseases, and autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (RA). Patients suffering from chronic multiple sclerosis have been shown to have two- to threefold higher levels of citrullinated myelin basic protein in their brains compared with healthy subjects (12, 13). The majority of RA patients produce autoantibodies against proteins containing citrulline (14). These autoantibodies are now known as anticitrullinated protein or peptide antibodies (ACPA) and their production is strongly associated with genetic and environmental factors. ACPA production can result

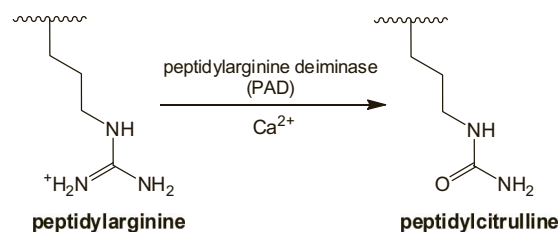


FIG. 1. **Citrullination.** The conversion of peptidylarginine to peptidylcitrulline, also known as citrullination or deimination, is catalyzed by peptidylarginine deiminase (PAD) in a Ca^{2+} -dependent manner.

in the formation of immune complexes, the up-regulation of proinflammatory cytokines and ultimately in chronic inflammation of the joints.

To better understand the involvement of citrullinated proteins in these diseases, it is important to get more insight into the conversion of peptidylarginine into peptidylcitrulline and the activity of PADs in complex biological samples, such as patient material. More information about the substrate specificity of these enzymes would allow us to develop inhibitors for a specific PAD isotype, instead of pan-PAD inhibitors. Several methods that can be used for the detection of PAD activity and the assessment of the capacity of PAD inhibitors are described here. To study PADs and citrullination, it is not only important to have methods for the evaluation of PAD activity, but procedures to detect citrullinated proteins in complex biological samples are required as well. Therefore, we will also describe the methods that are currently used for the detection and identification of citrullinated proteins and discuss their applicability for analyzing citrullination in such samples.

Methods for the Detection of PAD Activity—Detection methods for PAD activity and citrullination are related, because substrate conversion is frequently used as a read-out for PAD activity. Below we will describe a number of methods that are focused on the detection of PAD activity (summarized in Table I), but most of these use the formation of citrulline (or a citrulline derivative) as a measure for the extent of PAD activity.

Colorimetric Assay—In 1939, Fearon introduced a colorimetric assay for the detection of citrulline with diacetyl monoxime (DAMO) (15) and this method has subsequently been optimized by several researchers (16–21). The method is based on the specific reaction of DAMO with a ureido group under highly acidic conditions, followed by the detection of the resulting pink or orange product by absorbance measurements at 530 nm. The detection limit of citrulline was shown to be around 0.1 μmol , but later the assay was adapted to a 96-well microtiter plate format requiring less material, which increased the sensitivity to 0.2 nmol (20). The reaction product was later shown to be highly reactive with nucleophiles and the citrulline conversion is now generally not only performed with DAMO, but also antipyrine is added, resulting in a product of increased stability and a yellow color. The reaction

mechanism of citrulline conversion by DAMO and antipyrine was characterized in 2006 by Holm and colleagues (22).

Thirty years ago, this method was adapted to detect citrullination in a peptide-like molecule, N_{α} -benzoyl-L-arginine ethyl ester (BAEE). For the detection of PAD activity the test sample is incubated with BAEE in the presence of sufficient amounts of calcium to allow the PAD enzyme to citrullinate this substrate. After snap-freezing, the reaction product is incubated at 95 °C with a so-called color solution, containing strong acids, DAMO and antipyrine. The conversion of BAEE by PAD, DAMO, and antipyrine results in a yellow product with an absorption maximum of 464 nm. In parallel, a standard curve for citrulline is taken along, making it possible to calculate the extent of citrullination. This colorimetric assay to determine PAD activity is fairly easy to perform, but major disadvantages are the relatively low sensitivity and reproducibility (23) (and our own experience). Another disadvantage is that biological samples for obvious reasons should be devoid of free citrulline, urea or other ureido-containing compounds. This makes this method less suitable for the determination of PAD activity in complex biological samples. Nevertheless, the assay can be used for determining the activity of purified enzymes, and is therefore appropriate to get a first clue on the inhibitory capacity of PAD inhibitors.

High-performance Liquid Chromatography (HPLC) Fluorometric Method—A much more sensitive assay for determining PAD activity was developed in 2000 (24). In this HPLC-based method a fluorescent substrate, N -dansyl-glycyl-arginine, is converted to N -dansyl-glycyl-citrulline by PAD. After incubation with the enzyme, the reaction product is analyzed on a HPLC-system equipped with a C18 reverse phase column and the fluorescence intensity of the different peaks is measured with a fluorometer. In this way, as little as 0.2 pmol of N -dansyl-glycyl-citrulline could be detected, which makes this a highly sensitive assay for determining PAD activity. Other groups have, however, not used this assay frequently, which may at least in part be because of the requirement for specific equipment and the fact that this rather laborious method is not suitable for analyzing multiple samples in a short period of time.

Continuous Spectrophotometric Assay of NADH Oxidation—In 2005, a continuous spectrophotometric assay for the determination of PAD4 activity was described. This method is based on the formation of the side product ammonia during the conversion of arginine to citrulline (25). The released ammonia molecule is coupled to α -ketoglutarate by glutamate dehydrogenase in the presence of NADH resulting in the formation of glutamate and NAD^+ . The latter reaction can be monitored in real-time by UV/VIS spectrometry, because the conversion of NADH to NAD^+ changes the absorbance of 340 nm. This assay is very easy to perform and would be suitable for kinetic studies, as all handlings can be performed in one cuvette and the absorbance is continuously monitored. In contrast to the HPLC fluorometric assay described above,

TABLE I
Available assays for the detection of PAD activity

Assay	Sensitivity	Suitable for complex samples?	Comments
Colorimetric assay (15–21)	Low	No	Quick assay for determining purified PAD activity.
HPLC fluorometric assay (24)	High	Yes	Laborious assay. Unsuitable for the simultaneous analysis of multiple samples.
Spectrophotometric assay of NADH oxidation (25)	Low	No	Appropriate assay for the continuous monitoring of purified PAD activity.
ABAP assay (26)	High	Yes	Easy-to-perform assay for determining PAD activity in biological samples.
Fluorescence quenching assay (28)	High	Yes	Allows the simultaneous monitoring of different enzymatic activities.
Trypsin assay (29)	?	?	Has only been tested with purified PAD enzyme.

this assay is unable to detect small quantities of PAD activity in cultured cells. As the endogenous amounts of NAD(P)/NAD(P)H, ammonia and dehydrogenases can severely interfere with the reaction, this assay is less suitable for the determination of PAD activity in complex biological samples. For continuously monitoring the activity of purified PAD, however, this is an appropriate assay.

Antibody-based Assay for Peptidylarginine Deiminase Activity (ABAP)—The ABAP assay for determining PAD activity was developed in 2007 (26) and is based on the detection of citrullinated peptides by an antibody that reacts in a citrulline-dependent manner with these peptides. Arginine-containing peptides, e.g. a peptide corresponding to a citrullinated filaggrin epitope recognized by RA autoantibodies, are immobilized on a 96-well microtiter plate and incubated with PAD containing samples. The conversion of peptidylarginine to peptidylcitrulline can subsequently be detected with an antibody specifically reactive with the citrullinated peptide, for example, the single chain variable fragment RA3 (27) or antibodies from ACPA-positive RA patient sera (26). Finally, the detection and visualization of the bound antibodies are performed by classical ELISA procedures. The ABAP assay was shown to be relatively sensitive, as the detection threshold of citrulline was around 5 pmol, which is in between that of the colorimetric assay in microtiter plates and that of the HPLC fluorometric method. Furthermore, the presence of endogenous ureido-containing compounds does not influence the assay, which makes it very suitable for determining PAD activity in complex biological samples.

Fluorescence-based Detection of PAD Activity—Recently, a PAD activity assay based on fluorescence quenching was developed (28). This method is characterized by a fluorescently labeled arginine-containing substrate peptide and the repression of its fluorescence by a quencher dye. The positively charged arginine residue forms a noncovalent complex with the negatively charged quencher dye molecule, resulting in quenching of the fluorescence of the substrate. On conversion of the arginine residue to a neutral citrulline residue by a PAD enzyme, the quencher dye will not or much less effi-

ciently bind to the peptide, resulting in elevated fluorescence levels. A major advantage of this assay is that the activities of different enzymes can be monitored simultaneously in one sample by using fluorophores that can be detected at different wavelengths. It has been demonstrated that this assay also allows the detection of PAD activity in cell lines, indicating that also this assay is relatively sensitive and can be used for complex biological samples. However, the exact sensitivity of the assay was not investigated yet; a standard amount of substrate peptide of about 1 nmol was used in 96-well microtiter plates.

A related assay was recently developed by Wildeman and Pires (29) based on the fact that the conversion of an arginine to a citrulline interferes with the ability of trypsin to hydrolyze the amide bond flanking the arginine at the C terminus. A masked fluorophore (7-amino-4-methylcoumarin) was introduced at the C-terminal side of an arginine and a carboxy-benzyl group was fused to the N-terminal side of the arginine to mimic a protein-based substrate. The incubation of this molecule with trypsin resulted in hydrolysis of the amide bond and the generation of a fluorescent signal on release of the fluorophore. When the labeled arginine-containing substrate was incubated with PAD4 before trypsin digestion, no increase in fluorescence was observed, indicating that trypsin was indeed unable to hydrolyze the amide bond on the conversion of arginine to citrulline. This assay has, so far, only been tested with purified recombinant PAD enzyme. Therefore, it remains to be investigated whether this method is suitable and sensitive enough to detect PAD activity in complex biological samples.

Methods for the Detection of Citrullinated Proteins on Western Blots—

Antimodified Citrulline and Anticitrulline Antibodies—Antibodies that have been widely used over the last two decades are the so-called anti-modified citrulline antibodies (30). These antibodies bind to citrullinated residues that have been modified with the same reagents as used in the colorimetric assay for the detection of PAD activity, DAMO and antipyrine. The generation of these antibodies was accomplished by the

in vitro citrullination of histones by PAD isolated from rabbit muscle, followed by the chemical modification with DAMO and antipyrine in a highly acidic environment and the subsequent immunization of rabbits with the reaction products. This resulted in an antibody recognizing the modified citrulline independently of the amino acids flanking the citrulline residues. The antibodies are therefore highly specific and can detect amounts of peptidylcitrulline as low as 3–10 fmol, which means that this is a very sensitive method for citrullinated protein detection. Despite several attempts to generate new sera containing these antibodies, these antibodies are not available anymore. It is currently unclear why the immunization of rabbits with the procedure previously published by Senshu and coworkers fails to result in the production of antimodified citrulline antibodies with properties similar to the antibodies originally produced by these researchers.

A similar detection method was recently developed by Moelants and colleagues (31), who generated antibodies against a peptide containing a citrulline residue that was chemically modified by 2,3-butanedione and antipyrine under acidic conditions. The specificity of the antibodies was determined by ELISA using three different peptides containing modified citrulline and they showed that the antibodies could be successfully used for the detection of citrullinated chemokines.

Another approach to detect citrullinated proteins on a Western blot would be the use of antibodies against nonmodified citrulline. Indeed, both polyclonal and monoclonal antibodies against citrulline have been developed and are commercially available. A widely used monoclonal antibody is the F95 antibody that was raised against a decacitrullinated peptide linked to the activated carrier protein keyhole limpet hemocyanin (32). However, in bronchoalveolar lavage cells of smokers a larger percentage of stained cells was found with the antimodified citrulline approach described above than with the F95 antibody (33). Whether this difference is caused by a restricted recognition pattern of the F95 antibody remains unclear (34, 35).

Furthermore, two different polyclonal antibodies against citrulline, which have been developed by the coupling of citrulline to keyhole limpet hemocyanin via glutaraldehyde, are commercially available from Abcam. Although the available antibodies display a wide reactivity against citrullinated proteins, some bias for citrulline in a certain amino acid context exists (23), resulting in an incomplete profile of citrullinated proteins present in the sample of interest.

Phenylglyoxal-rhodamine Probe—Another method that was recently described to be applicable for the detection of citrullinated proteins is based on the chemical reaction of the ureido group of citrulline with phenylglyoxal (36, 37). Phenylglyoxal has been reported to react specifically with arginine residues under neutral or basic conditions and with citrulline residues under highly acidic conditions. This property was used to develop a chemical procedure to detect citrullinated

proteins. Phenylglyoxal was fused to rhodamine and the resulting probe was added to citrullinated proteins in a highly acidic solution. This led to the attachment of rhodamine to the citrullinated residues of proteins, which were visualized by fluorescence imaging after separation of the proteins by SDS-PAGE. Bicker and colleagues found that this probe also reacted with cysteines in an acidic environment, but the resulting bond appeared to be hydrolyzed at neutral pH and a neutralization step was already included before gel loading. Detection of *in vitro* citrullinated proteins using a similar approach also appeared to be successful in our lab (unpublished results). However, in our hands, this approach appeared to be unsuitable for the detection of citrullinated proteins in complex samples because of strong background reactivities.

Methods for the Identification of Citrullinated Residues in Proteins—To get more insight into the proteins that are citrullinated in biological samples mass spectrometry (MS) is a commonly used method. MS also allows the mapping of the exact citrullination sites, and this information might be important for the development of biomarker detection assays and therapeutic strategies. Citrullinated proteins can be characterized directly by tandem MS, based on the 0.98 Da mass difference between citrulline and arginine, or after chemical modification of the citrullinated residues to increase the mass difference.

Direct MS Analysis of Citrullinated Proteins—The mass spectrometric analysis of citrullinated proteins is the only method that can identify the exact citrullination sites, but it requires precautions to obtain reliable data. Because citrullination leads to an additional mass of ~1 Da and the commonly applied shotgun type liquid chromatography tandem MS (LC-MS/MS) approaches rely on automated database screening with the obtained spectra, such analyses are susceptible to misidentification of ^{13}C isotopes or modifications that lead to a similar mass increase (in particular the commonly occurring deamidation of glutamine or asparagine residues). Misidentification of ^{13}C isotopes can be circumvented by searching the database that has a small enough parent mass tolerance (ideally <5 ppm), because ^{13}C leads to a slightly larger mass increase (1.0036 Da) than the conversion of arginine into citrulline (0.9802 Da) (38). Deamidation, however, cannot be discriminated from citrullination based on mass difference alone and proper characterization of the exact citrullination site in the MS/MS spectrum, or additional information, is therefore required for peptides containing both potential deamidation and citrullination sites (38).

When searching MS/MS data against a database for citrullinated peptides, it is imperative that always also deamidation is included to prevent false positives corresponding to deamidated peptides. In addition, it should ideally be checked if either the (potential) citrullination site or the (potential) deamidation site is covered by the fragment ions in the MS/MS spectrum. If that is the case, the peptide can safely be identified as containing either modification. However, because

most scoring algorithms (including the commonly used Mascot software) take into account the number of matching product ions, the correctly modified peptide will often be given a higher identification score compared with the other potential modification state (38). Even so, other information can provide additional proof for a peptide being citrullinated rather than deamidated. For example, a shift in retention time of several minutes during liquid chromatography in a C_{18} -column is often observed for citrullinated peptides compared with the corresponding unmodified peptides. For deamidation events this shift will be much smaller, because under the acidic conditions used in LC-MS/MS, citrullination leads to loss of positive charge in the peptide, whereas deamidation does not (38). Also, for peptides with a parent charge higher than two often a reduction of the charge is seen for the citrullinated peptide (and not for the deamidated peptide), for the same reason (38). Additional information can also come from the cleavage state of the identified peptide, as trypsin will no longer specifically cleave at citrullinated arginine residues and lead to a “missed cleavage.” It should be noted that C-terminal citrulline residues can be observed in datasets (39), but these will be because of either post-cleavage citrullination or to the low level of nonspecific cleavage that is often observed for trypsin (40).

Using such straightforward methods, Van Beers and co-workers recently characterized citrullinated proteins present in the synovial fluid of RA patients (41). Proteins from synovial fluid samples were separated by SDS-PAGE and the resulting gels were cut into discrete molecular weight regions. Proteins were in-gel digested with trypsin and the resulting peptides were extracted from the gels and analyzed by LC-MS/MS to determine the identity of the proteins and to map the sites of citrullination. This approach allowed the identification of 53 citrullinated proteins occurring in the synovial fluid of RA patients.

A related procedure was applied by other researchers, who first separated the samples by two-dimensional isoelectric focusing/SDS-PAGE (42, 43). One of two identical gels was blotted and stained with the anti-modified citrulline antibodies to determine the positions of citrullinated proteins and the second gel was used to excise areas containing these citrullinated proteins. These gel slices were subsequently subjected to trypsin digestion and LC-MS/MS analysis.

In more complex samples like tissues, however, such strategies are not always feasible, as the abundance of the citrullinated peptides is simply too low to provide high quality MS/MS spectra and the corresponding non-citrullinated peptides will not always be present. Hermansson and colleagues (44) isolated citrullinated fibrinogen from RA synovial tissue and identified citrullinated fibrinogen peptides based on the accurate mass and retention time of the peptides, which were known from the analysis of *in vitro* citrullinated fibrinogen. Peptides that contained glutamines and asparagines were disregarded during the analysis of synovial fibrinogen, be-

cause the researchers could not distinguish peptides containing deamidated glutamines and asparagines from citrullinated arginines and because the MS/MS data on citrullinated peptides from *in vivo* samples were frequently missing or of poor quality. Another possibility is to monitor the occurrence of neutral loss of isocyanic acid from citrullinated peptides in collision-induced dissociation (CID) spectra (45). As the elimination of isocyanic acid is only present in the CID spectra of citrullinated peptides and not in the spectra of deamidated peptides, this can be used to differentiate between peptides that are either citrullinated or deamidated. CID preferentially cleaves bonds with the lowest bond energies, which might result in the preferential loss of post-translational modifications, thereby hampering the identification and localization of the modification site. Hence, Creese and colleagues (46) combined CID with electron transfer dissociation (ETD), as ETD cleaves randomly along the peptide backbone, while often leaving side chains and modifications intact. If neutral loss of isocyanic acid was observed in the CID spectrum, ETD of the parent ion was performed, which improved the identification and localization of the modification site.

As an alternative approach, Jin and colleagues (47) used CID in combination with higher-energy collisional dissociation (HCD), because HCD spectra allow the detection of lower m/z ions as compared with CID spectra. In the Jin study, several *in vitro* citrullination sites of glial fibrillary acidic protein and myelin basic protein, as well as *in vivo* citrullination sites of these proteins, were identified in brain extracts. In addition, it was shown that CID-triggered HCD fragmentation and high-resolution MS with reverse phase HPLC separation can differentiate between citrullinated peptides and peptides with glutamine or asparagine deamidation with great confidence, because of the low m/z ions in the HCD spectrum.

Finally, for experiments where *in vitro* citrullination of proteins by PAD enzymes can be employed, these issues can be partially mitigated by doing the citrullination in $H_2^{18}O$ water. This leads to stable isotope labeling of the citrulline residue with ^{18}O (48), increasing the mass difference on citrullination to 3 Da and no other common naturally occurring modifications resulting in such a mass increase are known.

Modification of Citrulline Residues Followed by MS—As mentioned above, citrullinated peptides often have very low abundance in complex samples. Therefore, they can be difficult to detect in the context of all other non-citrullinated peptides. Hence, several strategies based on chemical modification of citrulline residues have been developed to increase the chance of detection or to enrich the citrullinated peptide before mass spectrometric analysis (Fig. 2). Holm and colleagues (22) used 2,3-butanedione and antipyrine to modify peptidylcitrulline and subsequently subjected the modified peptides to matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) MS. The observed mass increase of 238 Da and the UV-VIS absorption at 464 nm, resulting from the modification, were used to detect citrullinated pep-

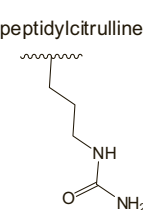
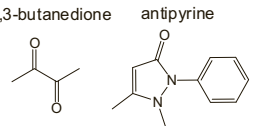
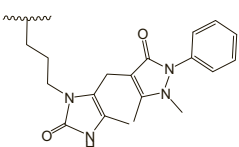
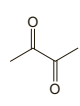
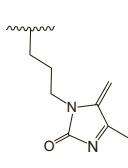
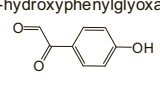
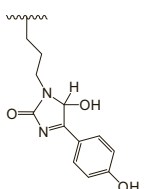
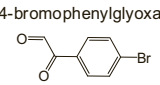
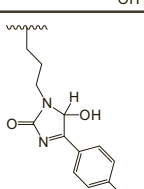
	Compounds used for modification	Peptidylcitrulline-reaction product	Mass increase
peptidylcitrulline 	2,3-butanedione antipyrine 		238 Da
	2,3-butanedione 		50 Da
	4-hydroxyphenylglyoxal 		132 Da
	4-bromophenylglyoxal 		194 Da

FIG. 2. **Compounds used for the modification of citrulline residues before MS.** The compounds that are shown are used for the chemical modification of citrulline residues under highly acidic conditions. The resulting reaction products are presented together with the mass increase resulting from the chemical modification.

tides. However, this approach did not allow the identification of the site of citrullination. They optimized the procedure further and subjected the chemically modified citrullinated peptides to LC-MS/MS with alternating CID and ETD (36). In this way, citrullinated peptides were detected in the CID spectrum, which showed a dominant peak with an m/z value of 201.1, corresponding to a specific modification-derived fragment ion, and the exact site of citrullination could be determined from the ETD spectrum. De Ceuleneer and colleagues (49) modified citrullinated residues with 2,3-butanedione only and compared modified and unmodified samples to identify the citrullinated peptides in the mixture by LC-MS analysis. Several proteolytic enzymes were used to increase the protein coverage, thereby allowing the identification of the citrullination sites. This method was shown to detect 160 fmol of citrullinated peptide and was suggested to be suitable for the detection of citrullinated peptides in complex samples. Although this approach is less dependent on the availability of tandem mass spectrometers, the localization of citrullination sites by this method in many cases, e.g. for sequences with multiple arginines, will not be possible without tandem MS/MS. In addition, it should be noted that any chemical modifi-

cation step before the analysis will have, to some extent, an effect on the accuracy by which peptides can be identified and quantified (50).

Tuttoren and coworkers (51) developed a method for the enrichment of citrulline-containing proteins/peptides that is based on the reaction of phenylglyoxal with citrulline residues under acidic conditions. Sarcosine dimethylacrylamide resin was functionalized with 4-hydroxyphenylglyoxal via a cleavable linker (cleaved at high pH) and allowed to react with a peptide mixture in an acidic environment to immobilize the citrullinated peptides present in the mixture. After the removal of unbound peptides, bound peptides were cleaved off at high pH after which they were analyzed by MALDI-TOF/TOF MS. A similar approach was performed by Choi and colleagues (52), who modified citrulline residues with 4-bromophenylglyoxal. Both the increase in mass and the unique isotopic distribution of Br as observed by MALDI-TOF MS were used for the determination of citrullination sites. However, for the identification of citrullinated proteins in complex samples the sensitivity of these methods might be too low (51). Therefore, Tuttoren and colleagues (53) tried to improve their approach by the specific biotinylation of citrullinated peptides followed

by their selective enrichment. Biotin-labeled 4-glyoxalbenzoic acid specifically reacted with citrulline residues under acidic conditions and streptavidin beads were used to enrich for these modified biotinylated peptides. Subsequently, the peptides were analyzed by MALDI-TOF/TOF MS. Compared with the enrichment strategy in which they used beads that were functionalized with 4-hydroxyphenylglyoxal via a cleavable linker (51), as described above, the enrichment of biotinylated citrullinated peptides was demonstrated to lead to a 20-fold higher sensitivity.

PAD Substrate Specificity—The increased evidence for the pathophysiological role of PAD enzymes in several diseases makes them attractive therapeutic targets. It is therefore of high importance to gain more insight into the structure and substrate specificity of these enzymes. Most PAD isoforms are exclusively expressed in the cytoplasm, except for PAD4, which contains a nuclear localization signal and is indeed found to reside at least in part in the nucleus. It would therefore be reasonable to assume that the substrate specificity differs between the PAD isotypes. It has indeed been described that incubation of HL-60 cell lysates with either recombinant h(uman) PAD2, hPAD3 or hPAD4 results in different citrullination patterns (54). In addition, Nakayama-Hamada and coworkers (55) reported that the residues in human fibrinogen citrullinated by hPAD2 and hPAD4 do not fully overlap. In total, 21 arginine residues were citrullinated *in vitro* by both enzymes, whereas 9 were only citrullinated by hPAD2 and 1 only by hPAD4. These data were confirmed by Van Beers and colleagues (56), who found that hPAD2 and hPAD4 citrullinated 39 and 28 arginine residues of human fibrinogen *in vitro*, respectively, with an overlap of 25 residues. hPAD2 thus appears to be less restrictive in the selection of peptidylarginine substrates than hPAD4, and, depending on the amino acid context, some peptidylarginines are only converted to peptidylcitrulline by one of these PAD isoforms.

To explore the substrate specificity of hPAD2 and hPAD4 in a more global way, we recently mapped citrullination sites for these enzymes in heterogeneous protein samples and by using a library of synthetic peptides (manuscript submitted for publication). COS-1 and HEK293 cells were transfected with hPAD2 or hPAD4 and 48 h after transfection cell lysates were incubated with calcium to allow citrullination of cellular proteins. Citrullinated proteins were isolated, subjected to LC-MS/MS and peptide sequences were analyzed. In both cell lines significantly more citrullination sites were found for hPAD2 (90 and 102 in COS-1 and HEK293 cells, respectively) than for hPAD4 (57 and 38 in COS-1 and HEK293 cells, respectively). In addition, lysates of nontransfected HEK293 cells supplemented with recombinant hPAD2 or hPAD4 expressed in bacteria yielded 121 citrullination sites for hPAD2 and 81 for hPAD4. Subsequently, the frequency of amino acids flanking the citrulline was evaluated, which confirmed that hPAD4 displays a higher degree of substrate specificity than hPAD2 (Fig. 3). Amino acid substitution experiments with syn-

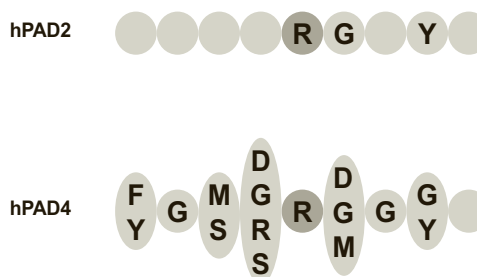


FIG. 3. Substrate specificity of hPAD2 and hPAD4. hPAD2 or hPAD4 was ectopically expressed in COS-1 or HEK293 cells and citrullination was allowed to occur in cell lysates. Alternatively, bacterially expressed hPAD2 or hPAD4 was added to HEK293 cell lysates, followed by a citrullination reaction. Citrullinated proteins were digested with trypsin and subjected to tandem mass spectrometry. Residues that were found at least twice more frequently than expected based on their natural occurrence in at least two out of three data sets are shown at the positions flanking the citrullinated arginine (dark gray circle).

thetic peptides also showed that for hPAD2 many substitutions close to the converted arginine are allowed without reducing the efficiency of citrullination, whereas for hPAD4 this appeared to be more critical. These data are further supported by the results of Stensland and coworkers (57), who reported that residues flanking the targeted arginine are important for citrullination by hPAD4. They also reported that methylation of a lysine flanking the targeted arginine influenced citrullination.

Insight into the structure of PAD substrates and into the specificity of different PAD isoforms is important for the design of highly selective PAD inhibitors, which may find applications for therapies in the longer term. The availability of reliable assays to determine PAD activity and citrullination are crucial for the development of PAD inhibitors and the assessment of their inhibitory capacity.

Conclusions and Future Perspectives—PAD-mediated citrullination is not only interesting from a physiological point of view, but also from a pathophysiological perspective. Increasing evidence indicates that the substrate specificity of PAD isotypes at least partially differs and a more detailed analysis of these differences will be needed to obtain a comprehensive inventory of PAD substrates and the amino acids that are crucial for their recognition. Various methods exist to determine PAD activity and the applicability of these methods depends on the specific research question and the source and sample complexity of PAD. The required sensitivity, the available quantities and number of samples to be analyzed, and the convenience of performing the assay are important factors that influence the choice of the most optimal assay.

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REFERENCES

- Vossenaar, E. R., Zendman, A. J., van Venrooij, W. J., and Pruijn, G. J. (2003) PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays* **25**, 1106–1118
- Arita, K., Hashimoto, H., Shimizu, T., Nakashima, K., Yamada, M., and Sato, M. (2004) Structural basis for Ca(2+)-induced activation of human PAD4. *Nat. Struct. Mol. Biol.* **11**, 777–783
- Rus’d, A. A., Ikejiri, Y., Ono, H., Yonekawa, T., Shiraiwa, M., Kawada, A., and Takahara, H. (1999) Molecular cloning of cDNAs of mouse peptidylarginine deiminase type I, type III and type IV, and the expression pattern of type I in mouse. *Eur. J. Biochem.* **259**, 660–669
- Vossenaar, E. R., Radstake, T. R., van der Heijden, A., van Mansum, M. A., Dieteren, C., de Rooij, D. J., Barrera, P., Zendman, A. J., and van Venrooij, W. J. (2004) Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. *Ann. Rheum. Dis.* **63**, 373–381
- Foulquier, C., Sebbag, M., Clavel, C., Chapuy-Regaud, S., Al Badine, R., Mechin, M. C., Vincent, C., Nachat, R., Yamada, M., Takahara, H., Simon, M., Guerrin, M., and Serre, G. (2007) Peptidyl arginine deiminase type 2 (PAD-2) and PAD-4 but not PAD-1, PAD-3, and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation. *Arthritis Rheum.* **56**, 3541–3553
- van Beers, J. J., Zendman, A. J., Rajmakers, R., Stammen-Vogelzangs, J., and Pruijn, G. J. (2013) Peptidylarginine deiminase expression and activity in PAD2 knock-out and PAD4-low mice. *Biochimie* **95**, 299–308
- Wang, S., and Wang, Y. (2013) Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. *Biochim. Biophys. Acta* **1829**, 1126–1135
- Kaplan, M. J. (2011) Neutrophils in the pathogenesis and manifestations of SLE. *Nat. Rev. Rheumatol.* **7**, 691–699
- Li, P., Li, M., Lindberg, M. R., Kennett, M. J., Xiong, N., and Wang, Y. (2010) PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J. Exp. Med.* **207**, 1853–1862
- Neeli, I., Khan, S. N., and Radic, M. (2008) Histone deimination as a response to inflammatory stimuli in neutrophils. *J. Immunol.* **180**, 1895–1902
- Wang, Y., Li, M., Stadler, S., Correll, S., Li, P., Wang, D., Hayama, R., Leonelli, L., Han, H., Grigoryev, S. A., Allis, C. D., and Coonrod, S. A. (2009) Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J. Cell Biol.* **184**, 205–213
- Moscarello, M. A., Wood, D. D., Ackerley, C., and Boulias, C. (1994) Myelin in multiple sclerosis is developmentally immature. *J. Clin. Invest.* **94**, 146–154
- Moscarello, M. A., Pritzker, L., Mastronardi, F. G., and Wood, D. D. (2002) Peptidylarginine deiminase: a candidate factor in demyelinating disease. *J. Neurochem.* **81**, 335–343
- van Venrooij, W. J., van Beers, J. J., and Pruijn, G. J. (2011) Anti-CCP antibodies: the past, the present and the future. *Nat. Rev. Rheumatol.* **7**, 391–398
- Fearon, W. R. (1939) The carbamid diacetyl reaction: a test for citrulline. *Biochem. J.* **33**, 902–907
- Boyde, T. R., and Rahmatullah, M. (1980) Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. *Anal. Biochem.* **107**, 424–431
- Zarabian, B., Koushesh, F., and Vassef, A. (1987) Modified methods for measuring citrulline and carbamoyl-beta-alanine with reduced light sensitivity and sucrose interference. *Anal. Biochem.* **166**, 113–119
- Archibald, R. (1944) Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. *J. Biol. Chem.* **156**, 121–142
- Guthohrein, G., and Knappe, J. (1968) Modified determination of citrulline. *Anal. Biochem.* **26**, 188–191
- Knipp, M., and Vasak, M. (2000) A colorimetric 96-well microtiter plate assay for the determination of enzymatically formed citrulline. *Anal. Biochem.* **286**, 257–264
- Hunninghake, D., and Grisolia, S. (1966) A sensitive and convenient micro-method for estimation of urea, citrulline, and carbamyl derivatives. *Anal. Biochem.* **16**, 200–205
- Holm, A., Rise, F., Sessler, N., Sollid, L. M., Undheim, K., and Fleckenstein, B. (2006) Specific modification of peptide-bound citrulline residues. *Anal. Biochem.* **352**, 68–76
- Slade, D. J., Subramanian, V., Fuhrmann, J., and Thompson, P. R. (2013) Chemical and biological methods to detect posttranslational modifications of arginine. *Biopolymers* doi: 10.1002/bip. 22256
- Chikuma, T., Yamada, M., Tsuda, A., Yamamoto, M., Nakashima, K., Yajima, R., and Kato, T. (2000) A highly sensitive high-performance liquid chromatography–fluorometric method for the assay of peptidylarginine deiminase activity. *Anal. Biochem.* **285**, 230–234
- Liao, Y. F., Hsieh, H. C., Liu, G. Y., and Hung, H. C. (2005) A continuous spectrophotometric assay method for peptidylarginine deiminase type 4 activity. *Anal. Biochem.* **347**, 176–181
- Zendman, A. J., Rajmakers, R., Nijenhuis, S., Vossenaar, E. R., Tillaart, M., Chirivi, R. G., Raats, J. M., van Venrooij, W. J., Drijfhout, J. W., and Pruijn, G. J. (2007) ABAP: antibody-based assay for peptidylarginine deiminase activity. *Anal. Biochem.* **369**, 232–240
- Raats, J. M., Wijnen, E. M., Pruijn, G. J., van den Hoogen, F. H., and van Venrooij, W. J. (2003) Recombinant human monoclonal autoantibodies specific for citrulline-containing peptides from phage display libraries derived from patients with rheumatoid arthritis. *J. Rheumatol.* **30**, 1696–1711
- Wang, Q., Priestman, M. A., and Lawrence, D. S. (2013) Monitoring of protein arginine deiminase activity by using fluorescence quenching: multicolor visualization of citrullination. *Angew. Chem. Int. Ed. Engl.* **52**, 2323–2325
- Wildeman, E., and Pires, M. M. (2013) Facile fluorescence-based detection of PAD4-mediated citrullination. *ChemBiochem* **14**, 963–967
- Senshu, T., Sato, T., Inoue, T., Akiyama, K., and Asaga, H. (1992) Detection of citrulline residues in deiminated proteins on polyvinylidene difluoride membrane. *Anal. Biochem.* **203**, 94–100
- Moelants, E. A., Van Damme, J., and Proost, P. (2011) Detection and quantification of citrullinated chemokines. *PLoS One* **6**, e28976
- Nicholas, A. P., and Whitaker, J. N. (2002) Preparation of a monoclonal antibody to citrullinated epitopes: its characterization and some applications to immunohistochemistry in human brain. *Glia* **37**, 328–336
- Makrygiannakis, D., Hermansson, M., Ulfgren, A. K., Nicholas, A. P., Zendman, A. J., Eklund, A., Grunewald, J., Skold, C. M., Klareskog, L., and Catrina, A. I. (2008) Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Ann. Rheum. Dis.* **67**, 1488–1492
- De Rycke, L., Nicholas, A. P., Cantaert, T., Kruijthof, E., Echols, J. D., Vandekerckhove, B., Veys, E. M., De Keyser, F., and Baeten, D. (2005) Synovial intracellular citrullinated proteins colocalizing with peptidyl arginine deiminase as pathophysiologically relevant antigenic determinants of rheumatoid arthritis-specific humoral autoimmunity. *Arthritis Rheum.* **52**, 2323–2330
- Makrygiannakis, D., Revu, S., Engstrom, M., af Klint, E., Nicholas, A. P., Pruijn, G. J., and Catrina, A. I. (2012) Local administration of glucocorticoids decreases synovial citrullination in rheumatoid arthritis. *Arthritis Res. Ther.* **14**, R20
- Stensland, M., Holm, A., Kiehne, A., and Fleckenstein, B. (2009) Targeted analysis of protein citrullination using chemical modification and tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **23**, 2754–2762
- Bicker, K. L., Subramanian, V., Chumanevich, A. A., Hofseth, L. J., and Thompson, P. R. (2012) Seeing citrulline: development of a phenylglyoxal-based probe to visualize protein citrullination. *J. Am. Chem. Soc.* **134**, 17015–17018
- Rajmakers, R., van Beers, J. J., El-Azzouy, M., Visser, N. F., Bozic, B., Pruijn, G. J., and Heck, A. J. (2012) Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. *Arthritis Res. Ther.* **14**, R114
- Zhao, X., Okeke, N. L., Sharpe, O., Batliwalla, F. M., Lee, A. T., Ho, P. P., Tomooka, B. H., Gregersen, P. K., and Robinson, W. H. (2008) Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. *Arthritis Res. Ther.* **10**, R94
- Vandermarliere, E., Mueller, M., and Martens, L. (2013) Getting intimate with trypsin, the leading protease in proteomics. *Mass Spectrom. Rev.* **32**, 453–465
- van Beers, J. J., Schwarte, C. M., Stammen-Vogelzangs, J., Oosterink, E.,

- Bozic, B., and Pruijn, G. J. (2013) The rheumatoid arthritis synovial fluid citrullinome reveals novel citrullinated epitopes in apolipoprotein E, myeloid nuclear differentiation antigen, and beta-actin. *Arthritis Rheum.* **65**, 69–80
42. Chang, X., Zhao, Y., Wang, Y., Chen, Y., and Yan, X. (2013) Screening citrullinated proteins in synovial tissues of rheumatoid arthritis using 2-dimensional western blotting. *J. Rheumatol.* **40**, 219–227
43. Kinloch, A., Lundberg, K., Wait, R., Wegner, N., Lim, N. H., Zendman, A. J., Saxne, T., Malmstrom, V., and Venables, P. J. (2008) Synovial fluid is a site of citrullination of autoantigens in inflammatory arthritis. *Arthritis Rheum.* **58**, 2287–2295
44. Hermansson, M., Artemenko, K., Ossipova, E., Eriksson, H., Lengqvist, J., Makrygiannakis, D., Catrina, A. I., Nicholas, A. P., Klareskog, L., Savitski, M., Zubarev, R. A., and Jakobsson, P. J. (2010) MS analysis of rheumatoid arthritic synovial tissue identifies specific citrullination sites on fibrinogen. *Proteomics Clin. Appl.* **4**, 511–518
45. Hao, G., Wang, D., Gu, J., Shen, Q., Gross, S. S., and Wang, Y. (2009) Neutral loss of isocyanic acid in peptide CID spectra: a novel diagnostic marker for mass spectrometric identification of protein citrullination. *J. Am. Soc. Mass Spectrom.* **20**, 723–727
46. Creese, A. J., Grant, M. J., Chapple, I. L. C., and Cooper, H. J. (2011) On-line liquid chromatography neutral loss-triggered electron transfer dissociation mass spectrometry for the targeted analysis of citrullinated peptides. *Anal. Methods* **3**, 259–266
47. Jin, Z., Fu, Z., Yang, J., Troncosco, J., Everett, A. D., and Van Eyk, J. E. (2013) Identification and Characterization of Citrulline-modified Brain Proteins by Combining HCD and CID Fragmentation. *Proteomics* **13**, 2682–2691
48. Kubota, K., Yoneyama-Takazawa, T., and Ichikawa, K. (2005) Determination of sites citrullinated by peptidylarginine deiminase using ¹⁸O stable isotope labeling and mass spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 683–688
49. De Ceuleneer, M., De Wit, V., Van Steendam, K., Van Nieuwerburgh, F., Tilleman, K., and Deforce, D. (2011) Modification of citrulline residues with 2,3-butanedione facilitates their detection by liquid chromatography/mass spectrometry. *Rapid Commun. Mass Spectrom.* **25**, 1536–1542
50. Rajmakers, R., Berkers, C. R., de Jong, A., Ovaa, H., Heck, A. J., and Mohammed, S. (2008) Automated online sequential isotope labeling for protein quantitation applied to proteasome tissue-specific diversity. *Mol. Cell. Proteomics* **7**, 1755–1762
51. Tuttunen, A. E., Holm, A., Jorgensen, M., Stadtmuller, P., Rise, F., and Fleckenstein, B. (2010) A technique for the specific enrichment of citrulline-containing peptides. *Anal. Biochem.* **403**, 43–51
52. Choi, M., Song, J. S., Kim, H. J., Cha, S., and Lee, E. Y. (2013) Matrix-assisted laser desorption ionization-time of flight mass spectrometry identification of peptide citrullination site using Br signature. *Anal. Biochem.* **437**, 62–67
53. Tuttunen, A. E., Holm, A., and Fleckenstein, B. (2013) Specific biotinylation and sensitive enrichment of citrullinated peptides. *Anal. Bioanal. Chem.* **405**, 9321–9331
54. Darrah, E., Rosen, A., Giles, J. T., and Andrade, F. (2012) Peptidylarginine deiminase 2, 3 and 4 have distinct specificities against cellular substrates: novel insights into autoantigen selection in rheumatoid arthritis. *Ann. Rheum. Dis.* **71**, 92–98
55. Nakayama-Hamada, M., Suzuki, A., Kubota, K., Takazawa, T., Ohsaka, M., Kawaida, R., Ono, M., Kasuya, A., Furukawa, H., Yamada, R., and Yamamoto, K. (2005) Comparison of enzymatic properties between hPADI2 and hPADI4. *Biochem. Biophys. Res. Commun.* **327**, 192–200
56. van Beers, J. J., Rajmakers, R., Alexander, L. E., Stammen-Vogelzangs, J., Lokate, A. M., Heck, A. J., Schasfoort, R. B., and Pruijn, G. J. (2010) Mapping of citrullinated fibrinogen B-cell epitopes in rheumatoid arthritis by imaging surface plasmon resonance. *Arthritis Res. Ther.* **12**, R219
57. Stensland, M. E., Pollmann, S., Molberg, O., Sollid, L. M., and Fleckenstein, B. (2009) Primary sequence, together with other factors, influence peptide deimination by peptidylarginine deiminase-4. *Biol. Chem.* **390**, 99–107