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Detection and Identification of Protein Citrullination in Complex Biological Systems

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Abstract

Protein citrullination is a post-translational modification of arginine that is catalyzed by the Protein Arginine Deiminase (PAD) family of enzymes. Aberrantly increased citrullination is associated with a host of inflammatory diseases and cancer and PAD inhibitors have shown remarkable efficacy in a range of diseases including rheumatoid arthritis, lupus, atherosclerosis, and ulcerative colitis. In rheumatoid arthritis, citrullinated proteins serve as key antigens for rheumatoid arthritis-associated autoantibodies. These data suggest that citrullinated proteins may serve more generally as biomarkers of specific disease states, however, the identification of citrullinated residues remains challenging due to the small 1 Da mass change that occurs upon citrullination. Herein, we highlight the available techniques to identify citrullinated proteins/residues focusing on advanced MS techniques as well as chemical derivatization strategies that are currently being employed to identify citrullinated proteins as well as the specific residues modified within those proteins.

Citrullinated proteins are generated upon the deimination of peptidyl-arginine by the Protein Arginine Deiminase (PAD) family of enzymes (Figure 1). This posttranslational modification (PTM) occurs on a wide array of proteins with a variety of impacts on cell signaling [1], immune response [2–4] and gene regulation [5–8]. Dysregulated PAD activity, resulting in aberrant levels of citrullinated proteins, has been observed in a number of disorders including rheumatoid arthritis (RA) [9–14], type 1 diabetes [15], lupus [16], ulcerative colitis [17, 18*], multiple sclerosis [19–22], Parkinson's disease [23, 24],

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Alzheimer's disease [25] and cancer [6, 11, 26, 27]. More recently, protein citrullination has been demonstrated to be critical for the formation of neutrophil extracellular traps (NETs), a mixture of DNA and associated proteins that is released from the neutrophil in response to extracellular stimulation (i.e., infection, inflammation) [28]. In RA, the most well studied of these examples, aberrant protein citrullination appears to be a key driver of disease, as citrullinated protein levels are elevated in RA synovium, and antibodies that bind citrullinated proteins represent a key diagnostic for RA [9, 14].

RA is an autoimmune disease in which inflammation of the joints leads to erosion of the bone and severe pain. Analysis of the synovial fluid around the joints in RA patients compared to healthy individuals has alluded to anti-citrullinated protein antibodies (ACPAs) as a specific factor in RA pathogenicity [29, 30]. Specific antigens to these ACPAs include the citrullinated forms of filaggrin [31], fibrinogen [32], fibronectin [33] and vimentin [34] (Table 1). One of the most important ACPAs is the family of anti-cyclic citrullinated peptide antibodies (anti-CCPs). Given the high specificity of anti-CCPs and their presence in patients with both early and advanced stages of disease, the anti-CCP test is now commonly used to diagnose RA, and thereby differentiate between other inflammatory diseases, and as a prognostic marker of disease severity [35].

Given that abnormal citrullination is a hallmark of inflammatory disease and potentially cancer, the development of methods to detect protein citrullination in complex biological samples is imperative. We hypothesize that citrullinated proteins will ultimately serve as powerful biomarkers for a wide range of diseases. Herein, we discuss current methods to detect protein-bound citrulline.

COLDER Assay

One of the earliest methods to detect protein citrullination is the so-called COLDER assay (COlor DEvelopment Reagent). This assay involves the chemical derivatization of the urea group that is uniquely present in citrulline. This reaction involves the acid-catalyzed modification of the urea with diacetyl monooxime in the presence of thiosemicarbazide and ammonium iron (III) sulfate, as well as phosphoric and sulfuric acid [36]. While the COLDER assay is commonly used to measure *in vitro* PAD activity, this assay has poor sensitivity as demonstrated by the very high limit of detection (~60 nmol), thus making it impractical for samples with low protein concentrations. Additionally, the chemical species formed during this chemical derivatization step has been difficult to identify, making this reaction impractical for MS-based identification of citrullinated proteins.

Antibody-Based Detection of Protein Citrullination

Antibody systems are more sensitive for samples with low protein concentrations. The first citrulline-specific antibody was described in 1992 by Senshu *et al.* [37] Their antibody does not recognize protein-citrulline but instead recognizes a chemically modified form of citrulline similar to the species formed during the COLDER assay. The antibody was produced using recombinant histones that were deiminated and subsequently derivatized with diacetyl monooxime and antipyrine under acidic conditions. This antibody was shown to recognize rat pituitary proteins that had been deiminated *in vitro* and was later used as the

basis for a commercially available kit for the detection of protein citrulline. In 2011, Moelants *et al.* generated an antibody to a 2,3-butanedione-modified citrulline which was used in a sandwich ELISA format to detect as low as 1 ng of citrullinated cytokines, with high specificity [38]. This assay method was also used to quantify the citrullination response of granulocytes and PBMCs to lipopolysaccharide (LPS) [38].

Nicholas and colleagues also developed an antibody to a decacitrullinated peptide. This antibody, denoted F95, was used to stain human brain samples to show anatomical localization of citrullinated proteins [39, 40]. F95 was later shown to have a limited range of efficacy. For example, in lung samples, F95 was only able to detect a small portion of citrullinated proteins [41]. Shortly after the development of F95, a set of citrulline-reactive antibodies was developed from lymphocytes of RA patients [42]. These antibodies were selected by their ability to recognize citrullinated peptides in ELISA and Western blots with RA patient sera. A variety of antibodies to specific targets of deimination have also been produced. These include citrullinated histones as well as a few other key targets of the PADs. These antibodies are listed in Table 2.

Mass-Spectrometric Analysis of Citrullinated Samples

Detection of citrulline by mass spectrometry (MS) is challenging due to the small change in mass that occurs when an arginine residue is deiminated; upon citrullination, the observed parent mass of a peptide is increased by 0.98 Da. This small change, combined with the low abundance of citrulline in the body, is easily confused with a ^{13}C isotope or a deamidation event. Another common difficulty is the change in protease cleavage induced by the deimination of an arginine – by neutralizing the positive charge of an arginine residue, proteases that selectively cleave after positively charged residues (e.g., trypsin) will no longer cleave after the citrulline formed from the deimination reaction.

As such, direct detection of citrullinated peptides from biological samples is feasible but laborious and requires high mass accuracy. In a study comparing synovial fluid in RA patients compared to patients with other inflammatory diseases, citrullinated peptides derived from fibrinogen were detected only after careful analysis of retention time compared to their arginine-containing counterparts [12]. In a similar study also using synovial fluid from RA patients, three novel citrullinated proteins were identified, including the sites of modification [43] (Table 1). This direct MS method is useful for monitoring small amounts of protein in a sample for potential diagnostic purposes in RA. However, direct detection by this method is not ideal to identify new citrullinated proteins or for global citrulline detection in a complex biological sample. In 2013, an improvement upon these methods was made by Jin *et al.* who were focused on identifying the sites of citrullination in brain samples. In this optimized MS method, collision-induced dissociation (CID) fragmentation of citrullinated peptides results in the neutral loss of isocyanic acid, which triggers higher-energy collision dissociation (HCD) fragmentation to obtain diagnostic peptide fragments. This CID-triggered HCD fragmentation allows for both confirmation of citrullination through the neutral loss of isocyanic acid and peptide sequencing through backbone fragmentation. [44*]. Using this method, researchers identified three novel substrates for deimination along with the modified residues. In a very recent paper, Fert-Bober and

colleagues used SWATH-MS, a data-independent acquisition method, to explore citrullinated proteins in human heart tissue [45**]. Using this advanced MS technique, low abundance peptides are detected more easily and sites of modification can more often be determined without enrichment techniques. In this study, 304 citrullination sites were identified on 145 proteins [45**].

Chemical Derivatization of Citrulline

To aid the MS-based detection of citrullinated proteins, Holm *et al.* showed that it was possible to selectively derivatize the urea group in citrulline with 2,3-butanedione alone [46, 47] or in combination with antipyrine [46] (Figure 2). These modifications result in a gain of either 50 or 238 daltons, respectively, enabling the differentiation of citrullinated peptides from their arginine counterparts using MS. Additionally, these methods were also used to manufacture antibodies specifically for modified citrulline residues [38].

Phenylglyoxal-based Probes of Protein Citrullination

Due to the low abundance of citrullinated proteins, the enrichment of citrullinated proteins of interest from non-citrullinated and abundant background proteins, will greatly improve the detection of citrullinated species from complex proteomes. To this end, chemical probes for citrulline have been developed using similar chemistry to the derivatization with 2,3-butanedione. Phenylglyoxals can be used in acidic environments to cyclize specifically with the urea group present in citrulline (Figure 2). Using this concept, Bicker *et al.* used a rhodamine-tagged phenylglyoxal probe (RhPG) (Figure 3) to label citrullinated proteins in complex mixtures [18*]. RhPG has an advantage over other detection methods due to its low limit of detection (~20 fmol citrullinated PAD4) allowing for the monitoring of kinetics of protein citrullination. Due to the high throughput of the assay format, many samples can also be run and compared simultaneously. Using this method, researchers used RhPG to show that a PAD inhibitor reduces citrullination in a mouse model of ulcerative colitis and that the levels of specific citrullinated proteins correlate with disease severity [18*]. These data provide a mechanism to monitor the pharmacodynamics of PAD inhibitors and to identify potential citrullinated proteins that might serve as disease biomarkers.

To marry this concept with MS, Choi and colleagues used 4-bromophenyl glyoxal (Figure 3) to selectively label citrullinated peptides. The bromine signature was then exploited in MALDI-MS analysis to unambiguously differentiate labeled peptides from others [48]. To further improve upon MS analysis of complex protein and peptide mixtures, Tuttunen and coworkers conjugated a biotin tag to phenylglyoxal to generate biotin-PG (BPG) (Figure 3) for streptavidin pull down [49, 50]. Using this covalent probe, the authors were able to identify more than 150 unique citrullination sites in proteins present in the synovial fluid of RA patients. In a methodological comparison, the spectral counts for citrulline-containing peptides from synovial fluid was increased more than 30-fold post-enrichment with BPG compared to direct analysis [49]. In this approach, BPG modification was performed on protease digested samples, and the resulting citrullinated peptides were enriched for MS analysis. One potential caveat with this workflow is that citrullinated peptides that are not amenable to MS analysis due to low or high masses, or the presence of other PTMs, will

result in a lack of identification of the parent protein as a citrullinated species. In a later publication, Lewallen *et al.* synthesized a similar biotin-tagged phenylglyoxal probe (Figure 3) to monitor cellular PAD activity [51**]. In this method, intact citrullinated proteins were enriched and trypsin digested for MS analysis. This enables identification of the parent citrullinated protein, but does not allow for identification of the exact site of citrullination. Using this probe, novel citrullinated proteins were observed in cell lysates including RNA splicing proteins, suggesting a role for the PADs in RNA splicing [51**]. With this tool at hand, the citrullinome can be explored in more detail in biological samples than previously possible.

Previous methods for enrichment had proposed the use of a “citrulline-reactive bead” that directly incorporated the phenylglyoxal moiety onto PL-DMA resin with a base-labile linker (Figure 3) [52**]. This method was only verified with single peptides and a tryptic digest of BSA and not in a more complex mixture or with larger proteins containing citrulline.

Concluding Remarks

Citrullination has proved to be an important biomarker for a variety of diseases in which PAD activity is dysregulated. Methods that allow for the detection of citrullinated proteins with high sensitivity and specificity, could serve to identify novel disease-relevant citrullination events, as well as provide diagnostics for early detection of a wide array of diseases. Recent advances in chemical derivatization and MS analysis of citrullinated species have afforded insight into the ubiquity of protein citrullination and its relevance to a variety of disease states. Specifically, in the field of RA, dysregulated citrullination is a well-validated marker of disease progression and severity. In addition to RA, other autoimmune diseases and numerous cancers are characterized by dysregulated protein citrullination. These other disease areas will benefit from advances in methods to detect protein citrullination, including the development of more quantitative and high-throughput methods to detect both known and uncharacterized sites of disease-relevant citrullination.

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Highlights

Protein citrullination is an inflammation dependent process that is aberrantly upregulated in inflammatory disease and cancer.

Citrullinated proteins serve as key antigens for rheumatoid arthritis-associated autoantibodies, suggesting that citrullinated proteins may serve more generally as biomarkers of specific disease states.

The identification of citrullinated residues remains challenging due to the small 1 Da mass change that occurs upon citrullination.

Improved MS techniques and chemical derivatization strategies are markedly improving our ability to identify citrullinated proteins as well as the specific residues modified within those proteins.

Figure 1.
PAD-Catalyzed Deimination of Peptidyl-Arginine to Form Citrulline.

Figure 2.
Chemical Derivitization of Citrulline.

Figure 3.
Chemical Probes for Citrulline.

Table 1

Select Citrullinated Proteins Identified from Biological Samples by MS

Substrate	Sample/Tissue	Reference
Fibrinogen α -chain	RA Synovial Fluid	12
PPRC1	RA Synovial Fluid	12
Fibrinogen β -chain	RA Synovial Fluid	49
α -Enolase	RA Synovial Fluid	49
Vimentin	RA Synovial Fluid	49
Importin-9	RA Synovial Fluid	49
Importin-5	RA Synovial Fluid	49
Rab21	RA Synovial Fluid	49
HSPA1A	RA Synovial Fluid	49
14-3-3	RA Synovial Fluid	49
Apo E	RA Synovial Fluid	43
MNDA	RA Synovial Fluid	43
β -Actin	RA Synovial Fluid	43
Cytochrome C	Cardiac Tissue	45
Carbonic anhydrase 3	Cardiac Tissue	45
Adenylate Kinase 4	Cardiac Tissue	45
Troponin I	Cardiac Tissue	45
Myelin Basic Protein	Brain Protein Extract	44
GFAP	Brain Protein Extract	44
NRGN	Brain Protein Extract	44
SNRNP200	Cell Lysate	51
U2AF2	Cell Lysate	51
SRSF7	Cell Lysate	51
HNRNPAB	Cell Lysate	51
CPSF6	Cell Lysate	51

Table 2

Commercially Available Antibodies for Specific Sites of Citrullination

Antigen	Site of Citrullination	Vendor	Catalog No.
Histone H3	R2	Abcam,	ab174992
Histone H3	R2, R8, R17	Abcam,	ab5103
Histone H3	R26	Abcam,	ab19847
Histone H4	R3	Abcam,	ab81797
HIF-1alpha	R11	Novus Biologicals,	nb100-105c