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Detection and identification of protein citrullination in complex biological systems

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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.

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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], 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].

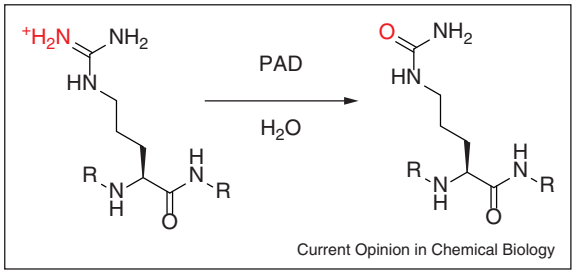
RA is an autoimmune disease in which inflammation of the joints leads to erosion of the bone and severe pain. Analysis of the synovial fluid around the joints in RA patients compared to healthy individuals has alluded to anti-citrullinated protein antibodies (ACPAs) as a specific factor in RA pathogenicity [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 progonostic marker of disease severity [35].

Given that abnormal cirullination 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

Figure 1



PAD-catalyzed deimination of peptidyl-arginine to form citrulline.

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.

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]

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