Methods in Citrullination and Analysis of Recombinant Human Histones

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KEY FINDING

Recombinant histone H3 can be citrullinated by PAD4 at 4°C with high yields at *in vivo*-modified sites and additional sites.

Introduction

Most natively expressed proteins are modified by one or more processes that are collectively called post-translational modifications (PTMs). PTMs result in chemical and physical changes to proteins that drive essential regulatory functions. PTMs can affect thermodynamic stability, folding, activity, and interactions with other proteins and/or molecules like DNA. Citrullination is a PTM where the positively charged amino acid arginine is converted to the neutral amino acid citrulline by replacing the primary ketimine in arginine with a ketone (**Figure 1**).

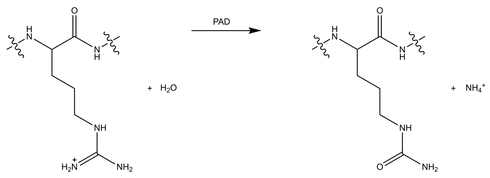
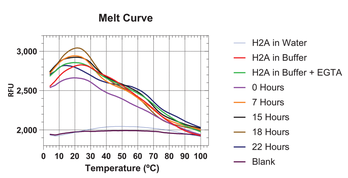


Figure 1. Reaction scheme of arginine citrullination by PAD.

This reaction is catalyzed by a family of calcium-dependent enzymes called peptidylarginine deiminases (PADs), and histones are one of many endogenous targets for citrullination. Histones are proteins that package and order DNA into structural units called nucleosomes, in which 146 base pairs wrap twice around a histone octamer comprised of two histone dimers (H2A/H2B) and one histone tetramer (H3/H4). PAD4 citrullinates histone tails, leading to the decondensation of nucleosomes and making the DNA more exposed for cleavage and fragmentation. In this way, hypercitrullinated histones play a role in the inflammatory response as a component of neutrophil extracellular traps (NETs), a complex fibrous network of protein and DNA fragments released by neutrophils to trap and eradicate extracellular pathogens like bacteria, fungi, viruses, and parasites. Failure to clear NET components following inflammation can result in the production of autoantibodies and anti-citrullinated protein antibodies (ACPAs), which are associated with several human autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, Alzheimer's disease, and multiple sclerosis. Citrullinated histones are a key component of the pathological progression of these diseases. This study seeks to investigate how purified, recombinant histones can be successfully citrullinated and how effectively the citrullination reaction can be manipulated to form specific citrullinated products to be used as tools in autoimmune disease research.

Citrullination of Recombinant Histones H2A and H2B

Initial attempts to citrullinate histones H2A and H2B (human, recombinant; Cayman Item Nos. 11080 and 11081, respectively) utilized our general citrullination protocol wherein proteins are citrullinated at 37°C for 30 minutes with PAD4 (Cayman Item No. 10500). These attempts resulted in substantial loss of product due to precipitation, which seems to be a general problem with citrullinating histones. Thermal shift assay (TSA) was used to assess thermal stability of histones H2A and H2B. TSA monitors thermally induced protein denaturation using SYPRO™ Orange dye, which fluoresces when bound to hydrophobic regions. A well-folded protein has few hydrophobic amino acids exposed and therefore displays low fluorescence, but increasing temperature will drive protein unfolding, exposing hydrophobic regions and increasing fluorescence. TSA showed that histones H2A and H2B had very low T_m values of ~10°C in the citrullination buffer (**Figure 2**).

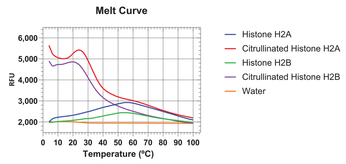


Observed Melting Temperature (°C)

Sample	T_{m1}	T_{m2}	T_{m3}
H2A in Water	23	24	24
H2A in Buffer	17	9	8.5
H2A in Buffer + EGTA	6.5	8	8.5
0 Hours	8.5	16	17
7 Hours	6.5	9	9.5
15 Hours	8.5	8	9
18 Hours	8.5	9.5	9.5
22 Hours	8.5	8	8.5

Figure 2. Histone H2A was purified and then citrullinated at 4°C for 22 hours, taking samples out at different time points and stopping the reaction using 20 mM EGTA. The citrullination buffer destabilizes the protein, as shown in the shift of the unfolding peaks to lower temperatures. Soluble aggregates also form in citrullination buffer, indicated by high fluorescence at 4°C. Experiments were performed in triplicates (T_{m1}, T_{m2}, and T_{m3}).

This result explained the poor yields at 37°C since the protein is already thermally denatured. We postulate that upon citrullination, the surface charge of the unfolded protein is altered significantly, resulting in protein destabilization and precipitation. To maintain histone stability, we modified the method to perform the citrullination at 4°C. With this method, the PAD4 activity decreased 15-fold relative to its activity at 37°C, however optimized conditions resulted in up to a 600% increase in yield. The citrullination of histones H2A and H2B (Cayman Item Nos. 30132 and 30133) resulted in a decrease in T_m (**Figure 3**), implying that the change in surface charge decreased protein stability at higher temperatures.



Observed Melting Temperature (°C)

Sample	T_{m1}	T_{m2}	T_{m3}
H2A	35.1	37.2	35.7
Citrullinated H2A	19.5	20.1	19.5
H2B	41.4	42.0	39.0
Citrullinated H2B	15.9	16.2	15.9

Figure 3. Histones H2A and H2B were purified separately and then citrullinated at 4°C for 18 hours with a final formulation in Milli-Q water. The unmodified histones show a broad peak at 35-42°C, while the citrullinated histones have a much lower T_m. The citrullinated samples also show evidence of aggregates, with high fluorescence being observed at 4°C. Experiments were performed in triplicates (T_{m1}, T_{m2}, and T_{m3}).

Citrullination of Recombinant Histone H3

We then turned our focus to histone H3 (Cayman Item No. 10263) with two objectives: (1) determine if the protein could also be citrullinated at 4°C and (2) accomplish an in-depth analysis of the citrullinated species generated over the reaction time to determine if the citrullination reaction could be tuned to drive citrullination at specific arginine residues. Histone H3 (0.2 mg per sample) was citrullinated with 8 units* PAD4 at 4°C in 100 mM Tris, pH 7.4, with 10 mM calcium chloride and 5 mM DTT for 15 minutes up to 24 hours. The reaction was halted at each time point by adding EGTA to a final concentration of 20 mM to chelate the Ca^{2+} necessary for enzymatic activity. A "0 hour" control sample was also made, where the reaction contained 20 mM EGTA prior to the addition of PAD4. All time point samples were divided into aliquots, flash-frozen in liquid nitrogen, and stored at -80°C prior to analysis. Previous studies determined that unmodified histone H3 and citrullinated histone H3 were stable after multiple freeze/thaw cycles.

Each sample was analyzed by five methods: (1) SDS-PAGE, (2) citrulline-specific chemiluminescence blot using Cayman's Citrulline-specific Probe-biotin (Cayman Item No. 17450), (3) Western blot using Cayman's Histone H3 (Citrullinated R2 + R8 + R17) Monoclonal Antibody (mAb) (Cayman Item No. 17939), (4) two-dimensional gel electrophoresis, and (5) PTM profiling of 2D gel spots by mass spectrometry. SDS-PAGE and citrulline-specific chemiluminescence blotting showed an ensemble of citrullinated histone H3. Western blotting with the R2 + R8 + R17 mAb provided specific information about citrullination in one region of the protein. Two-dimensional gel electrophoresis and PTM profiling by mass spectrometry provided data about citrullination in the various populations of histone H3 with different extents of modification.

* One unit is defined as the amount of enzyme required to produce 1 nmol NH_4^+ per minute at 37°C in 50 mM HEPES, pH 7.7, containing 10 mM calcium chloride, 5 mM DTT, and 2 mM N-benzoyl-L-arginine ethyl ester (BAEE).

Characterization of Citrullinated Histones

SDS-PAGE:

Citrullinated proteins migrate to a lower molecular weight (MW) on SDS-PAGE due to the protein's decrease in positive charge resulting in less SDS bound to it. For histone H3, this shift is very subtle, but it is more obvious for other histones. The time course of histone H3 citrullination shows a very small shift at ~1 hour with no obvious degradation of the protein when analyzed by SDS-PAGE (**Figure 4**).

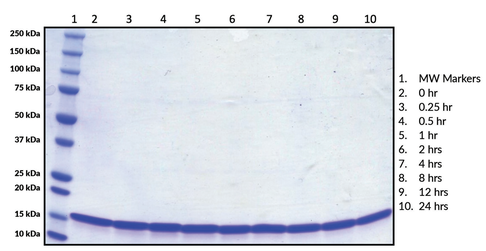


Figure 4. Time course (0-24 hours) of PAD4 citrullinated H3 analyzed by SDS-PAGE. 4 µg of each samples was loaded on gel.

Citrulline-specific Probe:

A citrulline-specific biotin probe, which contains a phenylglyoxal group and a biotin moiety, was used for the detection of histone H3 citrullination by chemiluminescence on a blot. Under acidic conditions, the phenylglyoxal group reacts specifically with citrulline and visualization occurs after incubation with streptavidin-HRP and a chemiluminescent substrate (**Figures 5A, B**). The blot shows a small amount of chemiluminescence is observed at 15 minutes (**Figure 6**), indicating that citrullination has occurred even after a very short time at 4°C. This is a shorter citrullination onset time than observed by SDS-PAGE, suggesting that the citrulline-specific chemiluminescence method is more sensitive for detection of citrullination.

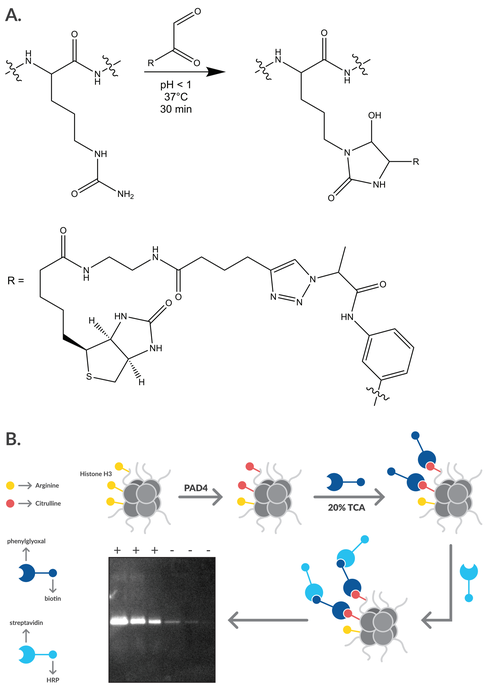


Figure 5. A. The reaction of citrulline-specific biotin probe with citrulline side chain. **B.** Detection scheme using citrulline-specific biotin probe.

Western Blot:

Western blot analysis with an anti-citrullinated histone H3 (R2 + R8 + R17) monoclonal antibody showed citrullination was first detected at 2 hours (**Figure 7**), much later than the onset of citrullination observed when using the citrulline-specific probe (**Figure 6**). These data indicate that the cluster of arginines at positions R2, R8, and R17 are modified at later time points than other sites, suggesting that the R2/R8/R17 cluster is less accessible to citrullination by PAD4 than other sites. These different onset times may also reflect that multiple populations of modified histone H3 are present with varied citrullination patterns.

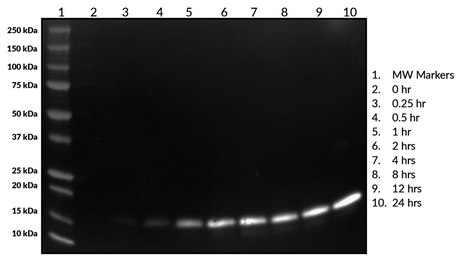


Figure 6. PAD4 citrullinated H3 over 24 hours blotted and probed with the citrulline-specific probe. 50 ng of each sample was loaded on gel.

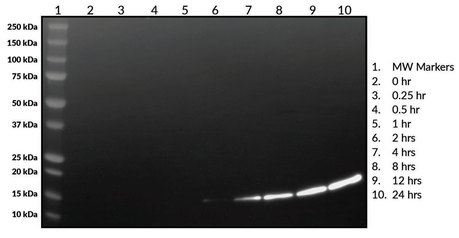


Figure 7. Western blot of PAD4 citrullinated H3 over 24 hours probed with anti-citrullinated histone H3 (R2 + R8 + R17) mAb. 100 ng of each sample was loaded on gel.

2D Gel Electrophoresis:

2D gel analysis of citrullinated histone H3 showed several spots (5-9 per time point) without much change in apparent MW, reflecting multiple populations of citrullinated histone H3 and indicating different extents of citrullination (**Figure 8**). Upon citrullination, a basic arginine residue is changed to a neutral citrulline residue, such that the pI of the protein would shift to be more acidic. As expected, the spots collectively migrated to more acidic pIs with longer reaction times as more arginines were converted to citrullines.

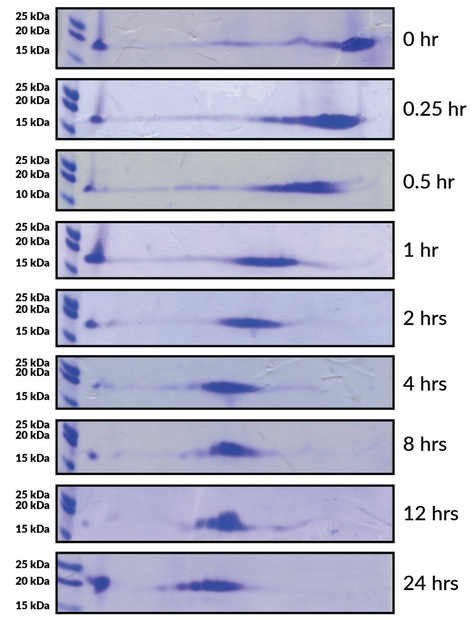


Figure 8. 2D gel electrophoresis of PAD4 citrullinated H3. 50 µg of each sample was loaded on gel.

Mass Spectrometry:

PTM profiling by mass spectrometry was performed (MS BioWorks, Ann Arbor, MI). This analysis included the identification and localization of citrulline and homocitrulline on histone H3 from excised 2D gel spots. Data from individual spots showed that the citrullination patterns for histones with different pIs were rather similar, but these spots also displayed different carbamylation patterns of lysine residues, presumably caused by urea used in the recombinant protein preparation. No citrullination of histone H3 was observed at the 0 time point but was observed in all subsequent time points, suggesting that the entire population of histone H3 has some level of citrullination within 15 minutes. The mass spectrometry data also show that at early times, arginines near the DNA-protein interface are modified. At later times, additional citrullination sites are identified at the DNA-protein interface, buried in the histone complex, and on the unstructured C-terminal tail (**Figure 9B**).

Native citrullination of arginines 2, 8, 17, and 26 in histone H3 have been previously reported. Mass spectrometry of 2D gel spots initially detected citrullination of R8, R17, and R26 at 2 hours, 15 minutes, and 1 hour, respectively. R2 was not observed by mass spectrometry. In addition, Western blots of citrullinated histone H3 detected R2/R8/R17 citrullination at 2 hours. These data support that after 2 hours, we can citrullinate the same arginine residues as identified in studies of native citrullinated histone H3. However, at 2 hours, citrullination also occurs at R40, R49, R64, and R69 as shown by mass spectrometry. Since the purified histone H3 used in these studies was overexpressed in *E. coli* as a single construct and was depleted of DNA through refolding in urea, histone H3 should not be packaged into a nucleosomal complex (**Figure 9A**). It is possible that the additional citrullination sites identified in this study are due to PAD4 having greater accessibility to arginines outside of the nucleosomal complex.

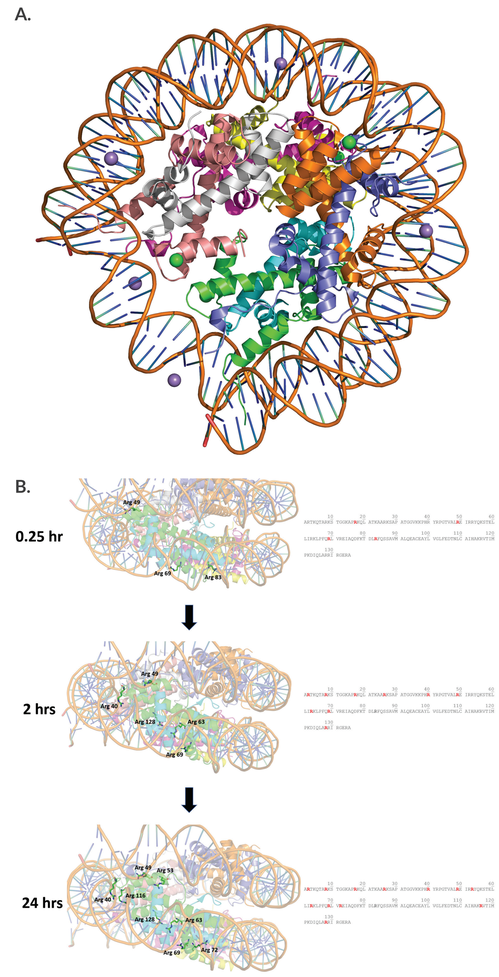


Figure 9. A. Top-down view of the crystal structure of the human nucleosome (PDB ID 3WKJ*): histone H2A type 1-B/E (pink and blue), histone H2B type 1-A (orange and yellow), histone H3 (green and salmon), histone H4 (grey and cyan), DNA (orange), Mn^{2+} (purple spheres), Cl^- (green spheres). **B.** Side view of the crystal structure of the human nucleosome (PDB ID 3WKJ*) with the progression of histone H3 citrullination over time. Subunit colors are the same as shown in Figure 9.A. Citrullination sites highlighted for 0.25, 2, and 24 hours.

* Urahama, T., Horikoshi, N., Osakabe, A., *et al. Acta Crystallogr. F Struct. Biol. Commun.* **70(Pt 4)**, 444-449 (2014).

Conclusions

- Lowering the reaction temperature of the PAD4 citrullination of H2A and H2B to 4°C resulted in a significant increase in yield of citrullinated histone.
- PAD4 citrullination of histone H3 at 4°C was detected by the citrulline-specific probe in as little as 15 minutes.
- R2/R8/R17 are citrullinated after 2 hours, indicating that these sites are not readily accessible for citrullination, and that there might be multiple populations of citrullinated histone H3.
- At all time points, multiple populations of citrullinated histone H3 were observed by 2D gel electrophoresis.
- Mass spectrometry data showed that with increasing time more arginines are converted to citrullines.
- Mass spectrometry showed that 2D gel spots from the same time point display highly similar citrullination patterns, suggesting that we can make a largely homogeneous population of citrullinated histone H3.
- Citrullinated arginines reported to be present in native histone H3 were also observed in our recombinant citrullinated histone H3 preparations. However, additional citrullination sites were also observed, likely due to PAD4 having greater accessibility to these regions when histone H3 is not in the nucleosomal complex.