An Affinity Labeling Method for the Identification of Nitrotyrosine Containing Proteins and Peptides

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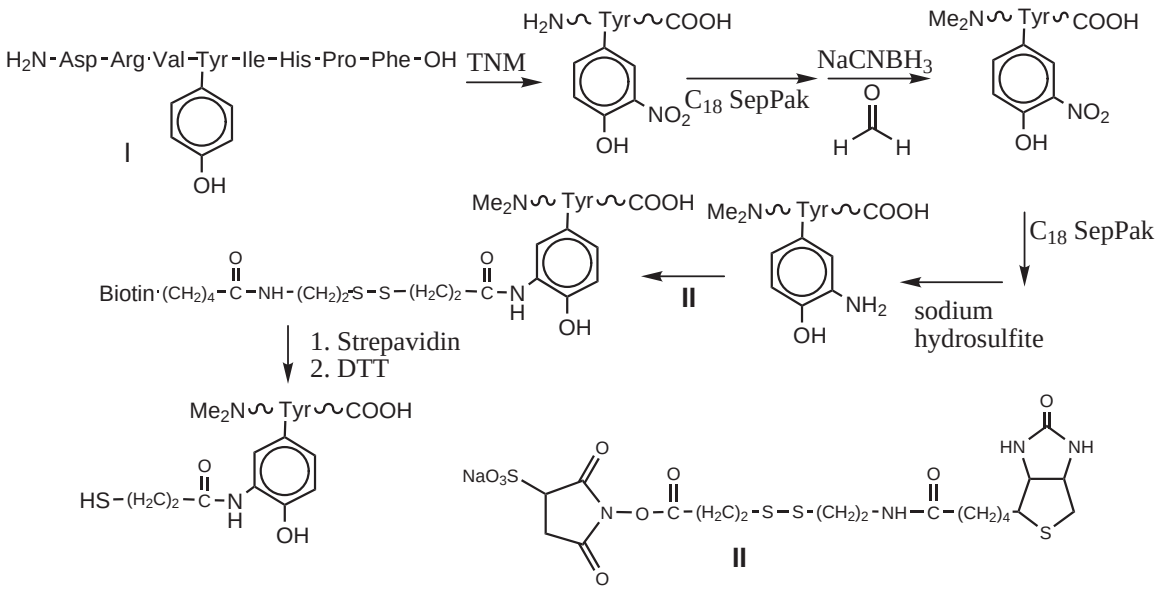
3-Nitrotyrosine is produced by the reaction of various redox reactive nitrogen species (predominately peroxynitrite) present under normal physiological conditions and in increased amounts during stress conditions. To that end, 3-nitrotyrosine is a potential marker for oxidative stress-induced modifications in proteins. Many proteins exhibiting this modification have displayed a change in their enzymatic activity. Here is presented a method to detect nitrotyrosine containing peptides in a proteomic manner via a functional group conversion and labeling with biotin.

As a proof of concept, angiotensin II (Ang II, **I**) nitrated with tetranitromethane (TNM), a reagent shown to specifically nitrate tyrosine (1), was used as a model compound (Scheme 1). Since this methodology uses an amine reactive labeling reagent, amines in the peptide or protein must be first converted to tertiary amines. Reductive methylation with sodium cyanoborohydride ($NaCNBH_3$) is an efficient and gentle reducing agent (compared with sodium borohydride) usable in aqueous solution (2). In the presence of sodium cyanoborohydride and formaldehyde the amino terminus of Ang II was converted to a tertiary amine (addition of two methyl groups). The methylation reagents must be removed before the next step to avoid undesirable reactions (SepPak C_{18} columns from Waters were used). With addition of sodium hydrosulfite (sodium dithionite), the reduction of nitrotyrosine was completed (3) and nitrotyrosine was converted to aminotyrosine. The newly formed aminotyrosine was labeled with an amine reactive, EZLink® Sulfo-NHS-S-S-Biotin (**II**) from Pierce. The labeling reagent contains a disulfide linker allowing for efficient removal from streptavidin bound to sepharose beads. The labeled peptide was eluted from the beads with dithiothreitol (DTT). Samples were prepared for mass spectrometric analysis with C_{18} ZipTips from Millipore.

Nitrotyrosine was confirmed by a strong absorbance of modified Ang II at 350 nm. MALDI-TOF analysis of methylated and nitrated Ang II shows the expected mass of 1119.34 Da as well as the expected photo-degradation products at 1109.37 Da and 1089.37 Da (Figure 1) (4). These additional masses reflect degradation during MALDI and were not observed in the ESI-QTOF spectrum. After reacting with the biotinylated linker, binding to streptavidin, and elution of the modified Ang II with DTT, the product (SH-Ang II) was confirmed by MALDI-TOF MS (Figure 2). All of the major intermediate products were also confirmed by ESI–QTOF MS/MS.

Studies continue using nitrotyrosine containing model proteins. Methods are being optimized to combine reaction steps, lowering the amount of potential loss during intermediate purification steps. The ultimate goal is to test the procedure with complex protein mixtures. At first this will be a cell lysate or tissue homogenate spiked with a previously tested nitrated model protein. The goal is development of a reagent that would be useful for the detection of proteins modified by nitrotyrosine.



Scheme 1: Synthetic plan for capture of nitrotyrosine containing peptides.

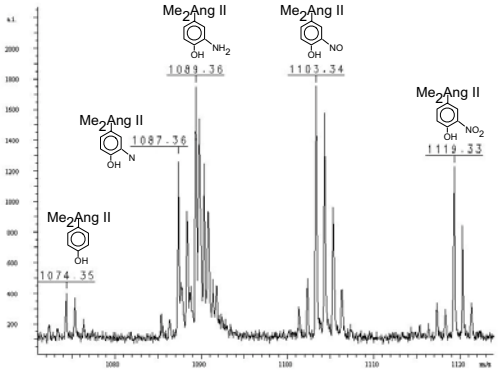


Figure 1: MALDI-TOF MS of nitrated Ang II

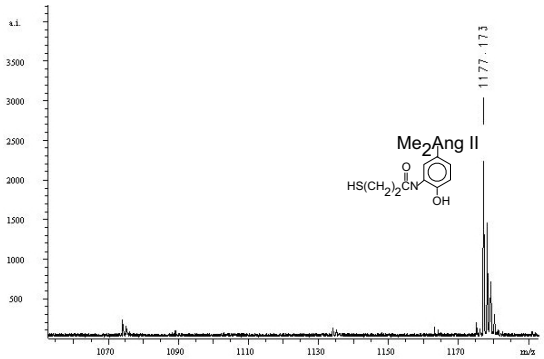


Figure 2: MALDI-TOF MS of biotinylated Ang II bound to streptavidin and after elution with DTT

Acknowledgements

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