Development of a protein 3-nitrotyrosine identification method using affinity labeling and solid phase capture

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Introduction:

3-Nitrotyrosine is a post-translational modification produced by the reaction of reactive nitrogen species (predominantly peroxynitrite) with tyrosine, present under physiological conditions, and in increased amounts in many diseases. Thus, 3-nitrotyrosine modification in proteins is a potential biomarker for oxidative stress. Many proteins exhibiting this modification (*e.g.* prostacyclin synthase and manganese superoxide dismutase) display a change in their enzymatic activity. In chemically modified proteins often times the same tyrosines are modified. These results suggest that there may be some specificity in modification. Of the currently available methods, only mass spectrometry is able to locate these sites specifically. However, only a relatively small portion of the population for a specific protein may be modified, which makes detection difficult. Even for abundant, almost fully modified proteins a nitrotyrosine or target protein specific affinity-based purification is necessary.

One method of affinity capture involves the reduction of nitrotyrosine with sodium dithionite to aminotyrosine followed by reaction with an amine specific biotin label (Sulfo-NHS-S-S-Biotin) at reduced pH (1). The pK_a of aminotyrosine is 4.7 (compared to the pK_a of other protein amines being — 7 to 11). With the labeling reaction performed at a reduced pH (pH 5 for example) non-tyrosine amines are much less reactive. Once the labeled protein is digested, the labeled peptides can then be captured with an avidin/streptavidin column and eluted by reduction of the label's disulfide. In our testing, this solution phase labeling method proved to have limited sensitivity. Solid-phase capture has been shown to have a higher recovery than solution-phase labeling followed by affinity capture (2) and has the potential to eliminate several purification steps, potentially increasing the sensitivity of the method.

Methods and Results

Several matrices and linker strategies were attempted, but a modified version of a commercially available thiol capture resin proved to yield the best results. Activated thiol sepharose from Amersham was reacted with 3-mercaptopropionic acid yielding a mixed disulfide bond at the end of the glutathione linker. The acid was activated immediately before incubation with dithionite reduced nitrotyrosine-containing peptide or protein (to limit hydrolysis of the active ester) using *N*-hydroxysulfosuccinimide (Sulfo-NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) yielding a primary amine reactive succinate ester (**I**).

The chemistry of the resins tested was first evaluated by using a synthetic tryptic BSA peptide with an incorporated nitrotyrosine. The HPLC purified peptide was reduced with sodium dithionite and desalted using a C18 SepPak from Waters, dried down, re-suspended in sodium acetate buffer (pH 5), and incubated with the activated resin (**I**). After elution, the tagged peptide was analyzed using a Bruker Reflex IV MALDI–TOF MS (Figure 1). Previous MALDI-QSTAR MS/MS data with reduced nitrated angiotensin II demonstrated that labeling was specific for the tyrosine amine and not the peptide amino terminus. To test the biological applicability of the resin, tetranitromethane treated bovine serum albumin (BSA) was used as a model protein. After thiol reduction and alkylation, nitrotyrosine reduction, and desalting with a Microcon® Centrifugal Filter Unit (Millipore), the protein was incubated with the amine reactive resin (**I**) at reduced pH. Non-captured protein was washed away and the remaining protein digested with trypsin on resin. After extensive washing the retained peptides were eluted with DTT and analyzed using MALDI–TOF MS (Figure 2).

Found masses from eluted peptides were searched against a single-protein database using ProteinLynx Global Server 2.2 (Waters Corp.) with custom post-translational modification databases. Results for the spectrum in Figure 2 are listed in Table 1. The masses in red (*) correspond to sites of nitrotyrosine modification that are found in the literature (3,4) and were confirmed by MALDI–TOF MS of the nitrated BSA. These results show that the chemistry for the capture of nitrotyrosine containing peptides is feasible, but whether the sensitivity of the method is adequate to detect endogenous levels of nitrotyrosine in a biological system is still in question. A ten-fold increase in sensitivity was already been observed for solid phase capture compared to solution phase labeling and capture. Further optimization is needed, but this method is a significant step toward the goal of characterizing specific nitrotyrosine modifications in complex protein mixtures in a proteomic manner.

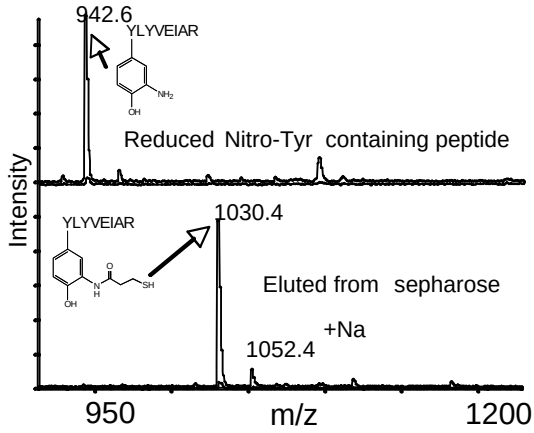
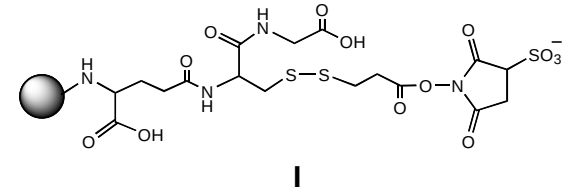


Figure 1: MALDI-TOF MS of reduced and eluted peptide



I

Table 1: Identified masses from MS in Figure 2

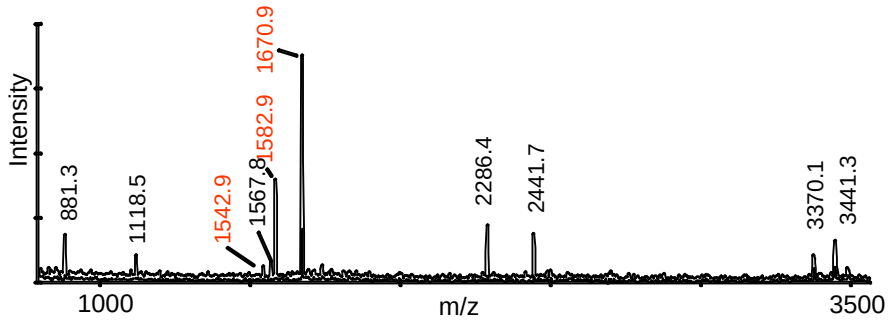


Figure 2: Captured and eluted tryptic digest peptides from nitrated BSA. Nitrated tryptic peptides shown in red

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