Supporting Information for:

Enzymatic synthesis of isotopically labeled hydrogen peroxide for mass spectrometry-based applications

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Materials and methods

Determination of the purity and concentration of $H_2{}^{18}O_2$

To determine the purity of $H_2{}^{18}O_2$, 10 µg of a synthetic peptide (MASLIKKLAVDR) was incubated with diluted $H_2{}^{18}O_2$ at 37 °C for 30 minutes and compared to an unoxidized control. Samples were frozen and lyophilized then desalted through homemade C18 columns and eluted in 50/50% acetonitrile (ACN)/H_2O in 0.1% formic acid (FA). Samples were analyzed by direct injection mass spectrometry as described below. Raw MS data was analyzed using XCailbur (Thermo). Relative levels of unmodified, ^{16}O- and ^{18}O-oxidized peptides were determined by analyzing the intact mass spectra as described previously.[1]

To determine the concentration of $H_2{}^{18}O_2$, 10 µg of a synthetic peptide (MASLIKKLAVDR) was incubated with differing concentrations of $H_2{}^{16}O_2$ (Fisher Bioreagents) or a 2x or 5x dilution of the generated $H_2{}^{18}O_2$ solution for 10 minutes at 37 °C. Oxidation was quenched with 400 mM sodium sulfite and samples were frozen and lyophilized. Samples were desalted through homemade C18 columns and eluted in 50/50% ACN/H_2O in 0.1% FA and analyzed by direct injection mass spectrometry as described below. Fractional oxidation was determined by least squares fitting the ^{16}O-oxidation standard curve to a single exponential equation with the software KaleidaGraph and using the resulting fitted equation to determine the concentrations of the ^{18}O-oxidized peptides.

Generation of peptide mixtures with different oxidation levels

To generate fully oxidized peptides, 25 µg (372 µM) of MASLIKKLAVDR at a concentration of 0.5 mg/ml was oxidized with 160 mM H_2O_2 for 30 minutes at 37 °C. The sample was frozen and lyophilized to remove the hydrogen peroxide, then desalted in a homemade C18 column and eluted in 50% ACN/H_2O in 0.1% trifluoroacetic acid (TFA). To generate fully unoxidized peptides, 25 µg of the peptide was incubated with 50 mM dithiothreitol (Sigma), 1.25 µM of Methionine Sulfoxide Reductase A and 12.3 µM of Methionine Sulfoxide Reductase B that were recombinantly expressed and purified from *E.coli* [2,3] in 50 mM Tris buffer for 45 minutes at 37 °C. Samples were lyophilized then desalted to remove enzymes and salts. After desalting, fully oxidized and fully reduced peptides were mixed to generate specific fractional oxidations. These pre-oxidized mixtures were then frozen and lyophilized before reconstitution in 1.25x diluted $H_2{}^{18}O_2$. The peptides were oxidized for 30 minutes at 37 °C, then frozen and lyophilized before desalting with homemade C18 columns into 50/50% ACN/H_2O in 0.1% FA. The sample was diluted to 15 µg/mL before analysis by mass spectrometry.

Mass Spectrometry

Peptides were diluted in 50/50% ACN/H_2O in 0.1% FA to 15µg/mL. 40ul of this peptide was directly injected into a Q Exactive Plus Mass Spectrometer (Thermo Fisher) using a Dionex Ultimate 3000 HPLC (Thermo Fisher) with a flow rate of 100µL/min. The solvent was a 50/50% mixture of 0.1% FA in H_2O and 0.1% FA in ACN that was injected for 3 minutes. A HESI source set in positive mode ionized the peptides at resolution of 70,000

at m/z 200 with a 240 ms maximum injection time, AGC target of 1e6, and an overall range of 300-2000 m/z. MS1 files were generated from raw files using MSConvert[4] and fractional oxidations were measured as previously described.[1] Under some conditions (e.g. oxidation ladder experiments shown in figure 3 where fractional ^{16}O oxidation was high prior to blocking with ^{18}O), a population of the peptide (up to 33% in the fully oxidized sample) was oxidized to form methionine sulfones in addition to methionine sulfoxides. In these cases, it was noted that ratios of $^{16}O/^{18}O$-oxidized relative to $^{18}O/^{18}O$-oxidized sulfone-containing peptides and ratios of ^{16}O-oxidized relative to ^{18}O-oxidized sulfoxide-containing peptides were similar in magnitude and reflected the expected initial ^{16}O oxidation levels. In such cases, fractional oxidation levels were determined by measuring the average of the two ratios normalized by their relative intensities.

Abbreviations

ACN- acetonitrile

FA- formic acid

TFA- trifluoroacetic acid

HESI – heated electrospray ionization

AGC – automatic gain control

References

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