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Note

Enzymatic Synthesis of Isotopically Labeled Hydrogen Peroxide for Mass Spectrometry-Based Applications

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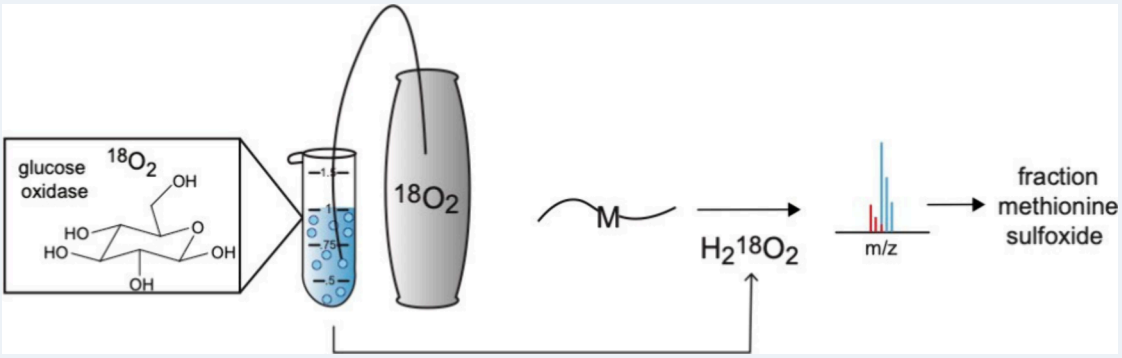

ABSTRACT: Methionine oxidation is involved in multiple biological processes including protein misfolding and enzyme regulation. However, it is often challenging to measure levels of methionine oxidation by mass spectrometry, in part due to the prevalence of artifactual oxidation that occurs during the sample preparation and ionization steps of typical proteomic workflows. Isotopically labeled hydrogen peroxide ($H_2{}^{18}O_2$) can be used to block unoxidized methionines and enables accurate measurement of *in vivo* levels of methionine oxidation. However, $H_2{}^{18}O_2$ is an expensive reagent that can be difficult to obtain from commercial sources. Here, we report a method for synthesizing $H_2{}^{18}O_2$ in-house. Glucose oxidase catalyzes the oxidation of β-D-glucose and produces hydrogen peroxide in the process. We took advantage of this reaction to enzymatically synthesize $H_2{}^{18}O_2$ from $^{18}O_2$ and assessed its concentration, purity, and utility in measuring methionine oxidation levels by mass spectrometry.

KEYWORDS: *Mass Spectrometry (MS), Glucose Oxidase (GOx), Methionine Oxidation*

INTRODUCTION

Side chains of methionines are susceptible to oxidation by reactive oxygen species (ROS) or monooxygenases.[1−6] This post-translational modification converts the nonpolar methionine residues (Met) to polar methionine sulfoxide residues (MetO).[1−4] MetO formation can induce protein misfolding and has been linked to a number of neurodegenerative disorders and pathological aging.[1−4,6] Additionally, regulated methionine oxidation can modulate diverse cellular processes and signaling pathways.[6] Due to its involvement in protein damage and functional regulation, global quantification of methionine oxidation can provide important insights into a number of diverse biological processes.

It is challenging to measure levels of *in vivo* methionine oxidation by mass spectrometry in part because unoxidized methionines can become spontaneously oxidized during typical proteomic workflows.[1−4] Selective blocking of unoxidized methionines can prevent this artifactual oxidation and allow for more accurate quantitation of methionine oxidation. An example of such an approach is methionine oxidation by blocking (MObB).[1−4] In MObB, unoxidized methionines within denatured proteins are fully oxidized with isotopically labeled hydrogen peroxide ($H_2{}^{18}O_2$) and blocked from spontaneous oxidation in subsequent steps of bottom-up proteomic workflows.[1,2] Relative levels of ^{16}O- and ^{18}O-modified peptides can then be measured and used to determine levels of endogenously oxidized methionines.[1,2] Furthermore, isotopically labeled hydrogen peroxide can be utilized to assess protein stability and protein−ligand binding using approaches such as stability of proteins from rates of oxidation (SPROX).[7] In addition to its utility in quantifying methionine oxidation, $H_2{}^{18}O_2$ has been employed in other mass spectrometric applications including quantitation of H_2O_2-producing reactions, analysis of the effects of H_2O_2 on

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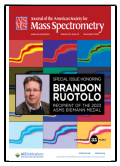

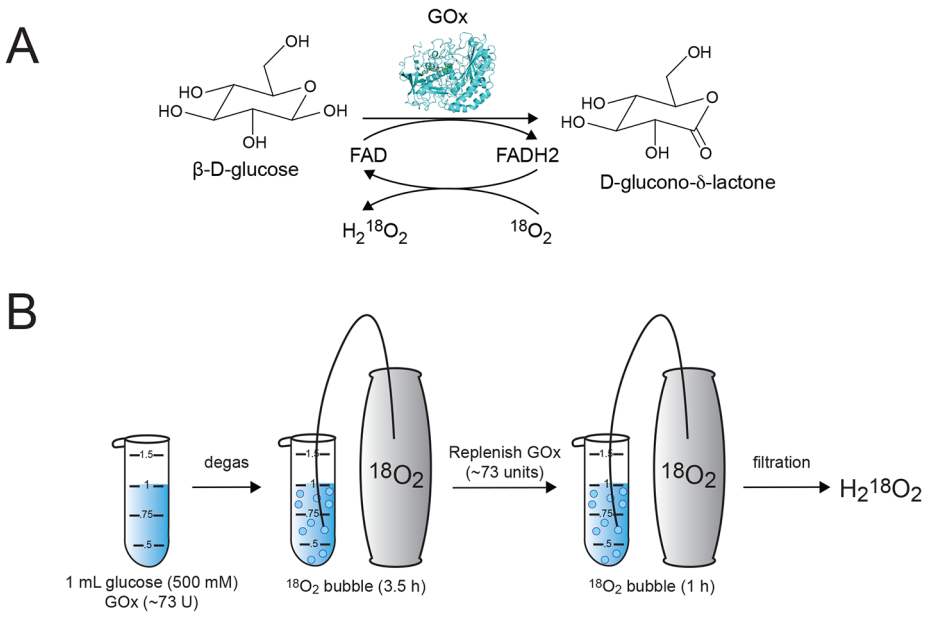

Figure 1. Reaction of glucose oxidase (PDB: 1GAL)[19] with β-D-glucose and $^{18}O_2$ generates $H_2{}^{18}O_2$. (A) Reaction mechanism of glucose oxidase. (B) Experimental protocol used for generation of $H_2{}^{18}O_2$. Details of the protocol are described in the Experimental Section.

metabolite synthesis, and quantitation of H_2O_2-induced oxidation of macromolecules.[8−10]

Despite its usefulness in diverse mass spectrometric applications, $H_2{}^{18}O_2$ is expensive and can be difficult to obtain from commercial sources. For example, the sale of $H_2{}^{18}O_2$ was entirely discontinued between 2021 and 2024, and currently there is only a single supplier for this reagent (Sigma). Traditionally, hydrogen peroxide is synthesized through the hydrogenation and subsequent oxidation of anthraquinone, an aromatic organic compound that acts as a catalyst in this reaction.[11,12] In the most popular synthetic methods, anthraquinone is hydrogenated by a trickle bed with a palladium catalyst. The hydrogenated anthraquinone is then oxidized by O_2 through a bubble column, restoring anthraquinone and producing hydrogen peroxide in the process.[11] The synthesized hydrogen peroxide is extracted using a sieve-plate extraction tower before it is purified via distillation. Alternative synthesis methods involving electrodes, biochemical approaches, or electrosynthesis reactions with different catalysts have also been reported.[12]

Enzymatic synthesis provides a more practical approach for in-house generation of $H_2{}^{18}O_2$ in typical biochemical laboratories. Glucose oxidase (GOx), an oxidoreductase that originates from insects and fungi, has been used in multiple industries including pharmaceuticals, food, textiles, and biofuels.[13−17] GOx catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone using a FAD cofactor and generates H_2O_2 by reduction of molecular oxygen (O_2).[5,13−15,17−19] Previously, GOx from *Aspergillus niger* has been used to generate ~11 mM H_2O_2 for use in textile bleaching studies.[16] In this study, we have optimized this enzymatic reaction and used $^{18}O_2$ as the substrate to generate ~200 mM $H_2{}^{18}O_2$ with high isotopic purity. We further demonstrated the efficacy of in-house generated $H_2{}^{18}O_2$ in conducting quantitative mass spectrometric analyses of methionine oxidation levels.

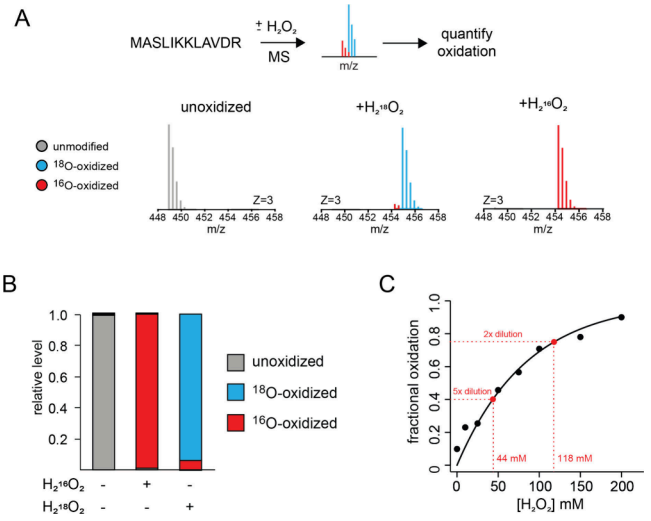

Figure 2. Synthesized $H_2{}^{18}O_2$ has high isotopic purity and concentration. (A, B) Synthetic peptide was oxidized with the in-house generated $H_2{}^{18}O_2$ (A) and shown to be 94% ^{18}O-labeled (B). (C) Concentration of in-house generated $H_2{}^{18}O_2$ was determined to be ~230 mM by comparing its efficiency in oxidizing a model peptide with known concentrations of the $H_2{}^{16}O_2$ ladder.

RESULTS AND DISCUSSION

Formation and Characterization of $H_2{}^{18}O_2$ Generated by GOx. The mechanism for production of hydrogen peroxide by glucose oxidase is illustrated in Figure 1A. We utilized this enzymatic reaction to synthesize $H_2{}^{18}O_2$ from $^{18}O_2$ as described in detail in the Experimental Section (Figure 1B). In brief, the enzymatic conversion of β-D-glucose to D-glucono-δ-lactone was carried out in the presence of a slow flow of $^{18}O_2$ into an initially degassed solution containing GOx. In initial experiments we observed that the evolution of H_2O_2 deactivates GOx over time. Thus, to maximize the yield of H_2O_2, the reaction was supplemented with additional GOx after an initial generation period of 3.5 h, allowing further

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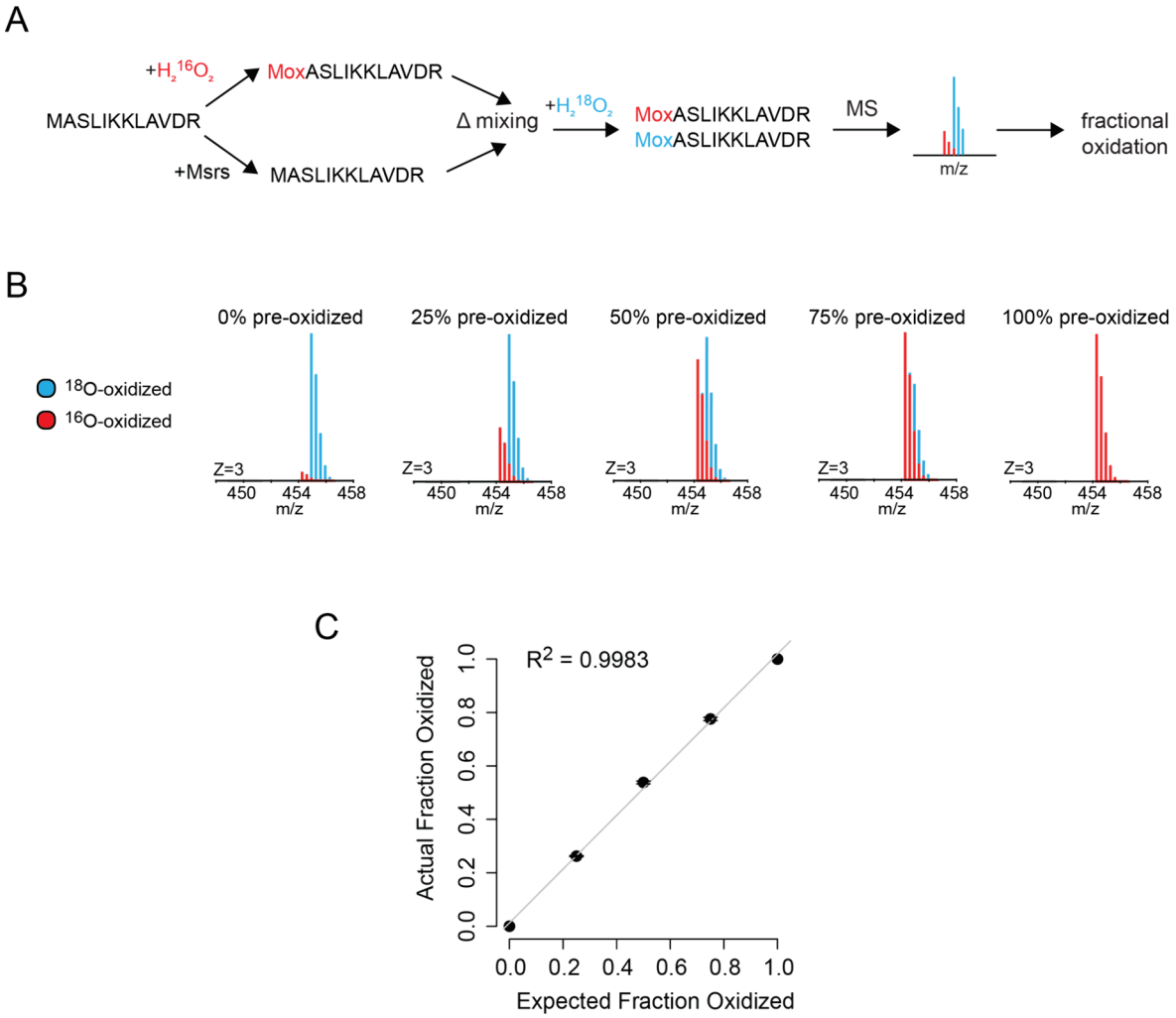

Figure 3. Synthesized $H_2{}^{18}O_2$ can be used to accurately measure levels of methionine oxidation by mass spectrometry. (A, B) Preoxidized peptide mixtures containing different levels of ^{16}O-methionines (0%, 25%, 50%, 75%, 100%) were fully oxidized with $H_2{}^{18}O_2$ (A) and analyzed by mass spectrometry (B). (C) Fractional oxidation with ^{16}O was measured and normalized to unoxidized and oxidized controls. The pairwise plot shows the correlation between measured and expected ^{16}O-oxidation levels for each mixture. The error bars indicate standard deviations of two replicate experiments.

generation of H_2O_2 for an additional hour. Following the reaction, GOx was removed from the mixture by filtration.

The purity and concentration of generated H_2O_2 was measured by mass spectrometry. An unoxidized synthetic peptide was fully oxidized with the generated $H_2{}^{18}O_2$ or commercially obtained $H_2{}^{16}O_2$ (Figure 2A). Relative levels of unmodified, ^{16}O-modified and ^{18}O-modified peptides were measured by mass spectrometry (Figure 2B). The peptide oxidized with in-house generated $H_2{}^{18}O_2$ contained ~94% ^{18}O-labeled methionines, indicative of the isotopic purity of the oxidant. In a second experiment, the peptide was partially oxidized with known concentrations of $H_2{}^{16}O_2$, and the resulting oxidation levels, as measured by mass spectrometry, were compared to peptides oxidized with various dilutions of in-house generated $H_2{}^{18}O_2$. This comparison indicated that the in-house generated $H_2{}^{18}O_2$ had a concentration of ~230 mM (Figure 2C).

Generated $H_2{}^{18}O_2$ Can Be Used to Accurately Measure Methionine Oxidation Levels. Next, we demonstrated that the generated $H_2{}^{18}O_2$ can be used as an effective blocking agent, enabling the accurate measurement of methionine oxidation levels. A synthetic peptide was fully reduced by methionine sulfoxide reductase A and methionine sulfoxide reductase B (Msrs) or fully oxidized with $H_2{}^{16}O_2$. Oxidized peptide was mixed with Msr-treated unoxidized peptide at variable ratios to generate mixtures with known predetermined methionine oxidation levels. These mixtures were then oxidized with diluted $H_2{}^{18}O_2$, resulting in ^{18}O-oxidation of the previously unoxidized methionines. Relative ^{16}O-oxidation levels of each peptide mixture were determined by measuring the fractional populations of ^{16}O- and ^{18}O-oxidized peptides. The pairwise comparison of expected versus measured ^{16}O-oxidation levels of peptide mixtures is shown in Figure 3. This analysis demonstrates that in-house generated $H_2{}^{18}O_2$ can be effectively used as an isotopically labeled blocking reagent for accurate measurement of methionine oxidation levels.

In-house synthesis also provides a more cost-effective approach for obtaining ^{18}O-labeled hydrogen peroxide (at the time of writing this manuscript, it reduced costs by approximately 50% in comparison to commercial sources). However, although the described method for in-house synthesis of hydrogen peroxide is straightforward and accessible, there are two important caveats that require special

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consideration. First, the isotopic purity of the $H_2{}^{18}O_2$ produced is dependent on the purity of the dissolved $^{18}O_2$ in the reaction buffer. Thus, to obtain isotopically pure $H_2{}^{18}O_2$, removal of $^{16}O_2$ by careful initial degassing, and subsequent use of highly pure $^{18}O_2$ as a substrate is required. Second, the $H_2{}^{18}O_2$ generated using the described protocol will also contain buffer components (in this case, sodium acetate) and glucose in oxidized and unoxidized forms. These impurities were inconsequential to the methionine blocking applications investigated in this study. However, if downstream applications require chemically pure $H_2{}^{18}O_2$, further purification of the generated product may be required.

CONCLUSIONS

This study describes a protocol that employs a widely available enzyme, glucose oxidase, for generation of concentrated and isotopically enriched ^{18}O-labeled hydrogen peroxide. The synthesized $H_2{}^{18}O_2$ can be used to block unoxidized methionines and facilitate the measurement of methionine oxidation levels in mass spectrometric workflows.

EXPERIMENTAL SECTION

To generate $H_2{}^{18}O_2$, 1.5 mL of $H_2{}^{18}O$ (Cambridge Isotope Laboratories, OLM-240-10G) was degassed in a 2 mL Eppendorf tube inside of a sealed vacuum flask connected to a vacuum. Glucose and sodium acetate were added to 1 mL of $H_2{}^{18}O$ to attain final concentrations of 500 mM and 50 mM, respectively. Activity units (72.8) of glucose oxidase (Sigma, G2133-10KU) was added to the solution, then $^{18}O_2$ (Sigma, 602892-1L) was slowly bubbled from a pipet tip attached to tubing connected to a 1 L gas tank for 4.5 h at 35 °C. Note that one activity unit is defined as 1.0 μmole of hydrogen peroxide per minute at 35 °C and pH 5.1. After 3.5 h, another 72.8 units of glucose oxidase was added to the tube and the slow bubble of $^{18}O_2$ continued for another hour. After incubation, the hydrogen peroxide was purified through centrifugation at 14,000g for 20 min at 4 °C in a 0.5 mL Amicon filter (3 kDa MWCO) to remove the glucose oxidase. The synthesized $H_2{}^{18}O_2$ was aliquoted and stored at −20 °C until use. Additional experimental information related to the determination of the purity and concentration of $H_2{}^{18}O_2$ and mass spectrometric analyses are provided in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.4c00326.

Experimental details, including determination of the purity and concentration of $H_2{}^{18}O_2$, generation of peptide mixtures with different oxidation levels, and mass spectrometry workflow (PDF)

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Author Contributions

The study concept was conceived by M.H., R.T., and S.G. The experiments were carried out by M.H., I.M., and R.T. Mass spectrometry was performed by K.W., K.S., and J.H. Data analysis was conducted by M.H., R.T., and S.G. The initial draft of the manuscript was written by M.H. and S.G.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ROS, reactive oxygen species; Msrs, methionine sulfoxide reductases; MObB, methionine oxidation by blocking; GOx, glucose oxidase; FAD, flavin adenine dinucleotide; H_2O_2, hydrogen peroxide; Met, methionine; MetO, methionine sulfoxide

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