

# Kinetics and Mechanism of the Reaction of Cysteine and Hydrogen Peroxide in Aqueous Solution

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**ABSTRACT:** The oxidation of thiol-containing small molecules, peptides, and proteins in the presence of peroxides is of increasing biological and pharmaceutical interest. Although such reactions have been widely studied there does not appear to be a consensus in the literature as to the reaction products formed under various conditions, the reaction stoichiometry, and the reaction mechanisms that may be involved. This study examines the reaction kinetics of cysteine (CSH) with hydrogen peroxide ( $H_2O_2$ ) in aqueous buffers (in the absence of metal ions) over a wide range of pH (pH 4–13) and at varying ratios of initial reactant concentrations to explore the range of conditions in which a two-step nucleophilic model describes the kinetics. The disappearance of CSH and  $H_2O_2$  and appearance of cystine (CSSC) versus time were monitored by reverse-phase high-performance liquid chromatography (HPLC). The effects of oxygen, metal ions ( $Cu^{2+}$ ), pH (4–13), ionic strength, buffer concentration, and temperature were evaluated. Data obtained at  $[H_2O_2]_0/[CSH]_0$  ratios from 0.01–2.3 demonstrate that the reaction of CSH with  $H_2O_2$  in the absence of metal ions is quantitatively consistent with a two-step nucleophilic reaction mechanism involving rate-determining nucleophilic attack of thiolate anion on the unionized  $H_2O_2$  to generate cysteine sulfenic acid (CSOH) as an intermediate. Second-order rate constants for both reaction steps were generated through model fitting. At  $[H_2O_2]_0/[CSH]_0 > 10$ , the % CSSC formed as a product of the reaction declines due to the increased importance of alternative competing pathways for consumption of CSOH. A thorough understanding of the mechanism in aqueous solution will provide valuable background information for current studies aimed at elucidating the influence of such factors on thiol oxidation in solid-state formulations.

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**Keywords:** cysteine; hydrogen peroxide; thiol oxidation; peptide stability; protein stability; nucleophilic substitution; disulfide formation; sulfenic acid; sulfinic acid; sulfonic acid

## INTRODUCTION

The oxidation of peptide and protein pharmaceutical products has become an increasingly important problem in drug development as more biotechnology derived products progress toward clinical studies and commercialization.<sup>1–3</sup> Pro-

teins containing one or more free cysteine residues (e.g., human serum albumin,<sup>4</sup> recombinant human  $\alpha$ 1-antitrypsin,<sup>5</sup> the superfamily of protein tyrosine phosphatases,<sup>6,7</sup> and a variety of others<sup>8</sup>), as well as thiol-containing peptides (e.g., glutathione<sup>9,10</sup> the angiotensin-converting enzyme inhibitor captopril<sup>11</sup> and the prostate-specific antigen peptides<sup>12</sup>) are particularly susceptible to thiol-oxidation through a variety of mechanisms. *In vitro*, these mechanisms may involve free-radical scavenging of various reactive oxygen species such as superoxide<sup>9,10</sup> or hydroxyl

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radical,<sup>13</sup> resulting in thiol loss and the consumption of oxygen. Alternatively, direct reaction of thiols via nucleophilic substitution with certain reactive oxygen species such as hydrogen peroxide may occur.<sup>4,5,7,14,15</sup>

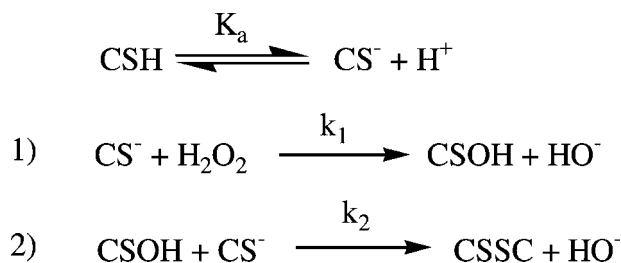
Because of their reactivity as scavengers of reactive oxygen species, thiol-containing compounds can be highly effective as antioxidants for stabilizing pharmaceuticals.<sup>13,16–20</sup> However, this propensity toward oxidation makes formulation of proteins and peptides containing free cysteine residues more difficult. In pharmaceutical formulations, thiol autoxidation is generally thought to involve a series of complex reaction processes catalyzed by traces of transitional metal ions (e.g., Cu<sup>II</sup> and Fe<sup>III</sup>).<sup>11,21–23</sup> For this reason, metal ion chelators such as EDTA have proven to be effective stabilizers of thiol containing peptides and proteins.<sup>24–26</sup> Considering, however, that peroxides are also found as impurities in some common formulation excipients (e.g., polyvinylpyrrolidone, polyethylene glycol, polysorbate 80, etc.),<sup>27–34</sup> and that H<sub>2</sub>O<sub>2</sub> is produced as a byproduct of metal-catalyzed oxidations,<sup>35–37</sup> an understanding of the mechanism of the thiol–H<sub>2</sub>O<sub>2</sub> reaction is a prerequisite for understanding the general case of thiol oxidation in formulations.

Our interest in stabilizing thiol-containing peptides stems from recent attempts to prepare lyophilized prototype formulations of several prostate-specific antigen (PSA) peptides under consideration by the National Cancer Institute for vaccine therapy for human prostate cancer,<sup>12,38</sup> including the cys ras peptide (KLVVVGAC-GVGKS),<sup>25</sup> PSA-3 (VISNDVCAQV),<sup>23,25</sup> and PSA-OP (FLTPKKLQCVDLHVISNDVCAQVH-PQKVTK).<sup>26</sup> Although EDTA was found to be highly effective in preventing metal-catalyzed disulfide formation in these lyophiles, the potential for the reaction of cysteine residues with trace peroxide impurities in excipients remains even in the presence of EDTA.

Several studies of the reaction between various low molecular weight thiols (e.g., cysteine, N-acetylcysteine, glutathione, and others) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or other peroxides (e.g., benzoyl peroxide, lipid hydroperoxides, etc.) have been reported, but there does not appear to be a consensus as to the reaction products formed under various conditions,<sup>10,14,15,39–42</sup> the mechanism(s) by which these products form,<sup>10,14,15,41–44</sup> or the rate constants for various reaction steps.

This study examines the reaction kinetics of cysteine with H<sub>2</sub>O<sub>2</sub> in aqueous buffers (in the

absence of metal ions) over a wide range of pH (pH 4–13) and at varying ratios of initial reactant concentrations to explore the range of conditions over which a two-step nucleophilic model as depicted in Scheme I accounts for the reaction kinetics and stoichiometry. In aqueous solution the first step in Scheme I is rate-determining, but it may be possible to estimate rate constants for each process, depending on the extent to which the two reaction steps differ.



Scheme I.

A thorough understanding of the mechanism of reaction between low molecular weight thiols and H<sub>2</sub>O<sub>2</sub> in aqueous solution should be useful in rationalizing the fate of thiol-containing proteins undergoing reaction with hydrogen peroxide, where the buildup of the sulfenic acid intermediate (RSOH) suggests that the second reaction step may become rate-limiting in some circumstances. This may reflect the increased importance of reactant mobility in the second reaction step. Similarly, the reaction of the RSOH intermediate with a thiol residue in a second molecule of a thiol-containing peptide or protein may also become rate limiting in amorphous (e.g., lyophilized) pharmaceutical formulations that contain peroxide impurities. Evidence that the disulfide may not be the sole reaction product under certain conditions led to an exploration of the possible factors influencing the fate of the sulfenic acid intermediate (RSOH) and the reaction products ultimately formed when RSOH reacts with H<sub>2</sub>O<sub>2</sub> or another molecule of RSOH.

## EXPERIMENTAL

### Reagents

Cysteine (>98% purity by TLC), cystine (Sigma-Ultra, >99% purity by TLC), cysteine sulfinic acid (water content 1 mol/mol), cysteic acid (monohydrate), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical

Co. (St. Louis, MO).  $\text{H}_2\text{O}_2$  (30% aqueous solution) and cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ). All reagents were analytical grade and used as supplied. Deionized ultra-filtered (DIUF<sup>®</sup>) water used to prepare CSH and  $\text{H}_2\text{O}_2$  solutions and HPLC grade acetonitrile were purchased from Fisher Scientific Co. (Pittsburgh, PA). Water used in mobile phase was deionized and further purified through a Milli-Q<sup>®</sup> UV Plus Ultrapure Water System, Millipore Ltd. (Billerica, MA).

### High-Performance Liquid Chromatography (HPLC)

The analyses of CSH, CSSC, and  $\text{H}_2\text{O}_2$  were carried out by reverse-phase high-performance liquid chromatography (HPLC). The system consisted of a Beckman 110A Solvent Delivery Module (Beckman Coulter, Fullerton, CA), a Waters<sup>™</sup> 717 plus Autosampler (Waters Corp., Milford, MA), and a Hewlett-Packard 1040M Series II HPLC detector (Hewlett-Packard Company, Palo Alto, CA) operating at 214 nm, which was connected to a PC for data acquisition and analysis using HP LC/MSD ChemStation software. A Supelcosil ABZ+Plus, 5- $\mu\text{m}$  ( $250 \times 4.6$  mm) (Supelco, Bellefonte, PA) analytical column and 5  $\mu\text{m}$  ( $2 \text{ cm} \times 2.1$  mm) guard column were employed. Elutions were performed isocratically using a mobile phase consisting of 50% (v/v) of an aqueous solution of 50 mM phosphoric acid (solution A) and 50% (v/v) of a solution containing 5 mM sodium 1-nonanesulphonate (99%, Lancaster Synthesis, Inc., Windham, NH), 50 mM phosphoric acid, and 5% (v/v) acetonitrile in water (solution B) at a flow rate 1.5 mL/min. Solution B was also used as the quench solution. The pH of the mobile phase was adjusted to 2.5 with NaOH. The retention times for  $\text{H}_2\text{O}_2$ , CSH, and CSSC were  $\sim 2$  min,  $\sim 4.8$  min, and  $\sim 6.5$  min, respectively. Cysteine sulfinic acid ( $\text{CSO}_2\text{H}$ ) and cysteic acid ( $\text{CSO}_3\text{H}$ ) eluted prior to  $\text{H}_2\text{O}_2$  at  $\sim 1.8$  min, but could not be adequately resolved for quantitation purposes under these conditions. Separations of  $\text{CSO}_2\text{H}$  from  $\text{CSO}_3\text{H}$  required two Supelcosil ABZ+Plus columns connected in series and a flow rate of 0.5 mL/min. Under these conditions, retention times for  $\text{CSO}_2\text{H}$  and  $\text{CSO}_3\text{H}$  were  $\sim 10.4$  and  $\sim 11$  min, respectively.

### General Procedure for Kinetic Studies

Fresh stock solutions of CSH were prepared by weighing  $\sim 20$  mg CSH into a 1.5-mL plastic microcentrifuge tube (VWR Scientific Products

Co., Buffalo Grove, IL) and dissolving in 1 mL phosphate buffer prepared from phosphoric acid solution (50 mM) adjusted to the desired pH with sodium hydroxide. Stock solutions of  $\text{H}_2\text{O}_2$  were prepared by combining 0.1 mL 30% aqueous  $\text{H}_2\text{O}_2$  solution and 0.9 mL of the same phosphate buffer (50 mM) in a 1.5-mL plastic microcentrifuge tube.

Reactions were initiated after first diluting the above stock solutions with the same buffer to a concentration twice that in the final reaction, equilibrating to the desired reaction temperature in a water bath set at  $25^\circ\text{C}$ . Equal volumes of each diluted solution were then combined by adding the diluted  $\text{H}_2\text{O}_2$  solution to the CSH solution and rapidly mixing in a 15 mL plastic centrifuge tube (Falcon<sup>®</sup>, Becton Dickinson Co., Franklin Lakes, NJ). Reaction solutions were stored at  $25^\circ\text{C}$  and aliquots (0.1 mL) were taken at predetermined time intervals and transferred into 1-mL HPLC vials. An aliquot of 0.1-mL solution B was added to quench the reaction by lowering the pH and the total volume was adjusted to 1 mL with the mobile phase. Samples were analyzed immediately by HPLC.

Kinetic studies to obtain a preliminary assessment of reaction order, metal ion effects, and the influence of oxygen utilized the initial rate method whereby the formation of cystine was monitored at several time points in the very early stages of the reaction (first minute) at varying initial concentrations of CSH and  $\text{H}_2\text{O}_2$ . More comprehensive studies for use in fitting mathematical models to the data (see Data Analyses), generating pH-rate profiles, and examining the effects of buffer concentration and ionic strength involved the determination of complete reactant (i.e., CSH and  $\text{H}_2\text{O}_2$ ) and product concentration versus time profiles.

### Metal Ion and Oxygen Effects

Solutions containing equal (5 mM) concentrations of CSH and  $\text{H}_2\text{O}_2$  and varying in EDTA concentration (0, 25, and 50 mM) were prepared by combining stock solutions in pH 6.0 (50 mM) sodium phosphate buffer either with or without addition of 50  $\mu\text{M}$  cupric sulfate ( $\text{CuSO}_4$ ). Buffers employed in the absence of added cupric ion were prepared using either Milli-Q treated deionized water or DIUF<sup>®</sup> water (see Reagents).

The influence of oxygen was explored using initial rate studies both in air-equilibrated samples and samples prepared and stored under a nitrogen atmosphere (in a glove box). Sample

temperatures were controlled by placing samples in a water jacketed container at 25°C. An OM-4 oxygen meter (Microelectrodes, Inc., Bedford, NH) was used to verify the removal of oxygen from diluted stock solutions that were bubbled with nitrogen prior to being combined to start a given reaction. The oxygen meter was calibrated by adjusting the reading to 0% in buffer solutions treated by bubbling with a N<sub>2</sub> stream for more than 30 min (only 20 min was necessary to achieve a constant reading) and to 100% in oxygen saturated buffer solutions. Experiments were at pH 6.0 and at a fixed starting concentration of CSH (25 mM) while H<sub>2</sub>O<sub>2</sub> starting concentrations were varied (i.e., 5, 10, 25, and 50 mM) or a fixed starting concentration of H<sub>2</sub>O<sub>2</sub> (10 mM) while CSH starting concentrations were varied (i.e., 2, 5, 10, and 25 mM).

#### Buffer, Ionic Strength, pH, and Temperature Effects

Phosphate buffers (pH 7) were prepared at different buffer concentrations (10, 30, and 50 mM) and at a constant ionic strength of 0.5 M (adjusted with sodium chloride (NaCl)). The kinetics of reaction in solutions containing 4 mM CSH and 4 mM H<sub>2</sub>O<sub>2</sub> were monitored as a function of buffer concentration and second-order reaction rate constants generated through model fitting were compared.

The effect of ionic strength was explored by comparing the kinetics of reaction between 4 mM CSH and 4 mM H<sub>2</sub>O<sub>2</sub> in 50 mM sodium phosphate buffers at pH 5.0, 7.0, 10.0, and 13.0 either in the absence of added NaCl or in buffers adjusted to an ionic strength of 0.5 M with NaCl. Second-order rate constants generated through model fitting of the experimental data were then compared.

The influence of pH on the reaction between 4 mM CSH and 4 mM H<sub>2</sub>O<sub>2</sub> was monitored in buffers ranging from pH 4–13 at 25°C. Buffers were prepared with 50 mM phosphoric acid solution and sodium hydroxide (NaOH). Ionic strength was not adjusted. The observed reaction rate constants ( $k_{\text{obs}}$ ) obtained through the model fitting of the experimental data were employed to construct the pH-rate profile.

Reactions between CSH and H<sub>2</sub>O<sub>2</sub> were conducted at different temperatures (0, 25, and 50°C) in pH 6.0 buffer solution using an ice bath for 0°C, a water bath at 25°C and an oven at 50°C. Rate constants ( $k_1$ ) generated from model fitting (see Data Analysis) were then fit to the Arrhenius

equation to obtain an estimate for the energy of activation.

#### Effect of [CSH]<sub>0</sub>/[H<sub>2</sub>O<sub>2</sub>]<sub>0</sub> Ratio on Kinetics and Reaction Products

To further explore the range of applicability of the simple nucleophilic mechanism depicted in Scheme I, additional experiments were carried out in which the ratio of starting concentrations of CSH and H<sub>2</sub>O<sub>2</sub> ([CSH]<sub>0</sub>/[H<sub>2</sub>O<sub>2</sub>]<sub>0</sub>) was varied from 100 to 0.001. A ratio of [CSH]<sub>0</sub>:[H<sub>2</sub>O<sub>2</sub>]<sub>0</sub> = 100 using 50 mM CSH and 500 μM H<sub>2</sub>O<sub>2</sub> at pH 6.0 was employed to assess the effect of a large excess of CSH on the rate of disappearance of H<sub>2</sub>O<sub>2</sub>. The effects of [CSH]<sub>0</sub>:[H<sub>2</sub>O<sub>2</sub>]<sub>0</sub> ratios ≪ 1.0 (i.e., 1 to 0.001) were evaluated at pH 5.0, 6.0, and 7.0 to explore the possible generation of new reaction products in the presence of excess H<sub>2</sub>O<sub>2</sub>. The concentration of [CSH]<sub>0</sub> in these experiments was 3 mM at [CSH]<sub>0</sub>:[H<sub>2</sub>O<sub>2</sub>]<sub>0</sub> ratios of 1 and 0.5 and 1 mM at ratios between 0.2 and 0.001. Solution aliquots were taken after the reactions were complete (72 h at pH 7.0, 96 h at pH 6.0, and 120 h at pH 5.0) and analyzed by HPLC for the CSSC product formed during the reaction. Analyses of some of the samples at later time points verified that the CSSC concentrations did not change after completion of the reaction.

#### DATA ANALYSIS

Concentration versus time curves for the disappearance of CSH and H<sub>2</sub>O<sub>2</sub> and appearance of CSSC were fit to a mathematical model derived from Scheme I using nonlinear least-squares regression analysis (SCIENTIST<sup>®</sup>, Micromath Inc., Salt Lake City, UT) to obtain estimates for the two second-order rate constants,  $k_1$  and  $k_2$ . The following differential equations can be generated from Scheme I:

$$-\frac{d[\text{CS}^-]}{dt} = k_1[\text{CS}^-][\text{H}_2\text{O}_2] + k_2[\text{CS}^-][\text{CSOH}] \quad (1)$$

$$-\frac{d[\text{HOOH}]}{dt} = k_1[\text{CS}^-][\text{H}_2\text{O}_2] \quad (2)$$

$$\frac{d[\text{CSOH}]}{dt} = k_1[\text{CS}^-][\text{H}_2\text{O}_2] - k_2[\text{CS}^-][\text{CSOH}] \quad (3)$$

$$\frac{d[\text{CSSC}]}{dt} = k_2[\text{CS}^-][\text{CSOH}] \quad (4)$$

where  $[CS^-]$ ,  $[H_2O_2]$ ,  $[CSOH]$ , and  $[CSSC]$  are the concentrations of cysteine thiolate anion, hydrogen peroxide, the cysteine sulfenic acid intermediate, and cystine, respectively, at time  $t$ .

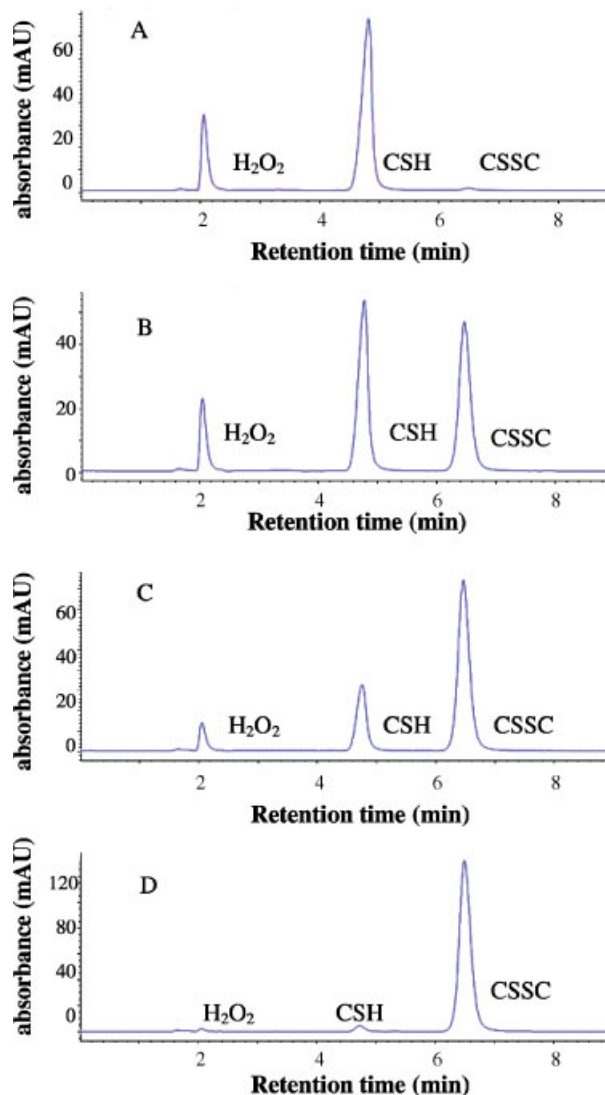
## RESULTS AND DISCUSSION

### Simultaneous analysis of CSH, $H_2O_2$ , and CSSC by HPLC

The reaction between CSH and  $H_2O_2$  has been previously studied by several other groups.<sup>10,14,43,45</sup> Normally in these studies, the concentrations of thiol and  $H_2O_2$  were measured by colorimetric methods and CSSC concentration was not analyzed. Although HPLC methods to separate CSH and CSSC<sup>46</sup> and to separately determine  $H_2O_2$  concentration in various media<sup>34,47</sup> have been reported, we are not aware of attempts to analyze all three reaction components in a single HPLC method. Figure 1 displays typical HPLC chromatograms obtained during the reaction of CSH with  $H_2O_2$  leading to the formation of CSSC. Response factors were verified to be linear for all three compounds over concentration ranges of 0.025–2.5 mM for CSH, 0.1–10 mM for  $H_2O_2$ , and 3–400  $\mu$ M for CSSC (solubility limited the range for CSSC) with intraday precision of <1% for all analytes. Interday precision in response factors was 1.6, 1.3, and 6.6% for CSH,  $H_2O_2$ , and CSSC, respectively. Detection limits at 214 nm estimated from three times the standard deviation for the lowest concentrations analyzed were 1.4  $\mu$ M (0.14 nmol), 6.5  $\mu$ M (.65 nmol), and 0.3  $\mu$ M (0.03 nmol), respectively, for CSH,  $H_2O_2$ , and CSSC. These detection limits indicate that HPLC detection at 214 nm is not as sensitive for  $H_2O_2$  as methods using electrochemical detection<sup>34</sup> nor as sensitive for CSH and CSSC as HPLC with coulometric detection.<sup>46</sup> However, Vignaud et al.<sup>46</sup> have observed that UV detection is more stable and easier to handle.

### Elimination of Metal Ions and Oxygen as Variables in the Kinetic Studies

Because autoxidation of CSH (i.e., the direct reaction between CSH and molecular oxygen) is a spin-forbidden process, this reaction was not expected to contribute to the kinetics in this study.<sup>48</sup> However, the presence of trace of transition metal ions will significantly accelerate thiol autoxidation. Transition metal ions such as  $Cu^{2+}$  and  $Fe^{3+}$  not only catalyze autoxidation, but also



**Figure 1.** HPLC chromatograms (214 nm) at various times during the reaction of CSH with  $H_2O_2$ . Conditions:  $[CSH]_0 = 4$  mM,  $[H_2O_2]_0 = 2$  mM in pH 6.0 (50 mM) phosphate buffer at 25°C. Times: (A) 15 s; (B) 30 min; (C) 90 min; (D) 900 min. [Color figure can be seen in the online version of this article, available on the website, [www.interscience.wiley.com](http://www.interscience.wiley.com).]

act as direct oxidants in thiol oxidation.<sup>22,36,37,49–52</sup> Given the above, it is clear that transition metal ions had to be avoided to minimize the contribution of alternate routes of thiol oxidation.

The ability of the metal ion chelator EDTA to eliminate the effect of metal ions on the reaction between CSH and  $H_2O_2$  was investigated in pH 6.0 solutions containing equal (5 mM) concentrations of CSH and  $H_2O_2$  and varying in EDTA concentration (0, 25, and 50 mM) either with or without the addition of 50  $\mu$ M cupric sulfate ( $CuSO_4$ ). Initial rates of formation of CSSC, shown in Figure 2,

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