Sulfitolysis of Disulfide Bonds in Proteins Using a Solid-State Copper Carbonate Catalyst

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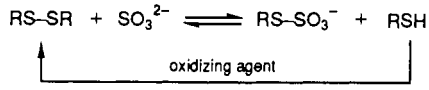
A novel method involving copper carbonate impregnated on silica beads as a solid-state catalyst to S-sulfonate the cysteine and cystine residues in proteins is described. The rate constant of the sulfonation reaction in the presence of the solid-state catalyst was severalfold higher than that of the reaction catalyzed by copper in the solution state. The activation energy of the reaction was about 8.5 kcal/mol, which was about 2-fold smaller than that for the solution-phase catalysis. The advantages of this method over that of the other chemical modification methods to improve the functional properties of food proteins are discussed.

Chemical modification of reactive groups in proteins is a strategy commonly used to induce physical and chemical changes in the molecule to achieve various purposes (Feeney et al., 1980; Glazer, 1977). In the case of food proteins, chemical modification approaches have been used to improve the functional properties of novel proteins (Feeney, 1977; Kinsella and Shetty, 1979). The most commonly used method for the modification of proteins is the acylation reaction, which involves covalent modification of the nucleophilic groups with acid anhydrides such as acetic and succinic anhydrides. The various nucleophilic groups that readily react with acid anhydrides include the ϵ-amino group of lysine, the imidazole group of histidine, the sulfhydryl group of cysteine, the hydroxyl group of threonine and serine, and the phenolic group of tyrosine (Kinsella and Shetty, 1979). Although acylation improves several functional properties of food proteins (Meyer and Williams, 1977; Franzen and Kinsella, 1976a,b; Beuchat, 1977; Thompson and Reyes, 1980), it has detrimental effect on the nutritional quality and safety of the protein intended for food applications. For example, succinylation of proteins decreases their digestibility by proteases and impairs the biological availability of lysine and other essential amino acids (Siu and Thompson, 1982a,b; Groninger and Miller, 1979).

In order to overcome these undesirable effects of acylation on protein quality, development of alternative chemical modification methods is warranted. One of the promising approaches is the sulfitolysis of disulfide bonds in proteins with sulfite. The method is based on a reduction–oxidation reaction system, which can be depicted as



where RS–SR represents the disulfide bonds in proteins. Addition of sulfite to the protein initially cleaves the disulfide bonds exposed to the solution, resulting in the formation of one *S*-SO_3^- derivative and one free SH group for each disulfide bond cleaved. In the presence of an oxidizing agent, such as copper, the free SH groups are oxidized back to disulfide, which is again cleaved by the sulfite present in the system. The reaction cycle repeats itself until all the disulfide bonds and the sulfhydryl groups in the protein are converted to *S*-SO_3^- derivatives. The advantages of this method over other chemical modification methods are that (1) conformation of proteins (hence, the hydrophobicity/hydrophilicity character of the protein surface) can be altered to a greater extent with cleavage of a fewer number of disulfide bonds, (2) introduction of SO_3^- groups would increase the negative charge of the protein and thus might improve its solubility characteristics, and (3) the reaction is very specific to cysteine and cystine residues in the protein and hence the bioavailability of other essential amino acids would not be impaired.

The sulfitolysis reaction with several modifications has previously been reported by other researchers (Swan, 1957; Pechere et al., 1958; Bailey and Cole, 1959; Chan, 1968; Kella and Kinsella, 1985). Of all the catalysts studied, the cupric ion has been shown to be very specific for oxidation of thiol groups. However, when copper is used in the soluble form as cupric sulfate, the rate of sulfitolysis of disulfide bonds in proteins is very slow and the reaction usually takes several hours to reach completion (Kella and Kinsella, 1985). Furthermore, stepwise reduction and modification of disulfide bonds, which is useful in elucidating the structure–function relationship of proteins, tends to be difficult when copper is used in the solution state. To overcome these difficulties, in this paper we describe a sulfitolysis method employing a solid-state copper catalyst. Bovine serum albumin, which contains one free sulfhydryl group and 17 disulfide bonds, has been selected as a model protein for this study. It is shown that in the presence of a solid copper catalyst the rate of sulfitolysis is several-fold greater than when copper is used in the soluble form. Furthermore, the method provides better control of the extent of cleavage of disulfide bonds in proteins.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA, fraction V) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co (St. Louis, MO). 2-Nitro-5-thiosulfobenzoate (NTSB) was synthesized from DTNB according to the method of Thannhauser et al. (1984). Copper carbonate–silica gel catalyst (6% Cu, 4–10 mesh) was from Alfa Products. All other chemicals used were of reagent grade.

Methods. *Determination of Copper Content of Silica Beads.* The copper carbonate on silica beads was washed several times with distilled water to remove loosely attached particles, then dried overnight in a convection oven, and stored in a dry place. To obtain a standard curve for copper estimation, increasing amounts of $CuCO_3$ were dissolved in 5 N HCl; the absorbance of these solutions at 690 nm was measured. To determine the copper content of the washed silica beads, a sample of 1.5 g of $CuCO_3$ was treated with 5 N HCl to dissolve the copper; the sample was then filtered and rinsed, the filtrate

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was made up to a known volume, and the absorbance at 690 nm was measured. Estimation of copper content from the standard curve gave a value of 63.6 mg of copper/g of silica bead.

Sulfitolysis. Sulfitolysis of the disulfide bonds in BSA using $CuCO_3$ impregnated on silica beads as the solid-phase catalyst was studied as follows. An Erlenmeyer flask with 20 mL of BSA solution was placed in a temperature-controlled water bath shaker and equilibrated for 20 min to attain the required temperature. Sodium sulfite was added to a final concentration of 0.1 M, and the pH was adjusted to 6.5 with HCl. The sulfitolysis reaction was initiated by adding 1.5 g of $CuCO_3$–silica beads catalyst. The flask was shaken at 110 oscillations/min. Under these conditions it was assumed that the solution was saturated with oxygen during the reaction. Aliquots (0.15 mL) of protein solution were withdrawn at various time intervals and centrifuged immediately in an Eppendorf microcentrifuge to separate loose $CuCO_3$ particles in the solution. This facilitated complete stoppage of the oxidative sulfitolysis reaction. In order to determine whether there was any residual catalytic activity of copper in the centrifuged solution, the disulfide content was determined over a period of time. There was no decrease in disulfide content, indicating that removal of $CuCO_3$ particles from the solution completely stopped the oxidative sulfitolysis of disulfide bonds. The concentration of the unsulfonated disulfide bonds remaining in the protein as a function of reaction time was determined by the NTSB method (Thannhauser et al., 1984).

To determine the extent of adsorption of protein onto the surface of the $CuCO_3$–silica bead particles during the course of the reaction, the change in the concentration of the protein in the reaction solution was monitored as a function of time. It was found that after 1 h of reaction time the decrease in protein concentration was about 1.7–2.7% of the original concentration. This small change in the bulk concentration of the protein did not significantly affect the calculation of the rate constants.

The dependence of the reaction as a function of temperature in the range 15–35 °C and the pH in the range 6.0–9.0 was studied. For pH between 6.0 and 7.5, BSA solutions were prepared in 0.1 M phosphate buffer, and for pH between 8.0 and 9.0, 0.1 M borate buffer was used. The effect of sulfite concentration on the sulfitolysis reaction was studied in the range 0.025–0.2 M sodium sulfite. The effect of protein concentration was studied in the range 0.16–3.0% (w/v). In all cases, the experimental conditions and procedures were the same unless otherwise specified. The kinetic data presented here are averages of at least triplicate measurements.

Sulfhydryl and Disulfide Estimation. Protein concentration was determined by the micro-biuret assay described by Itzhaki and Gill (1964). Sulfhydryl and disulfide bond concentrations were determined by 2-nitro-5-thiosulfobenzoate (NTSB) method as described by Thannhauser et al. (1984) and as modified by Damodaran (1985). To determine the amount of free sulfhydryl groups in the protein during the sulfitolysis reaction, an aliquot of the protein solution withdrawn from the reaction was mixed with an excess amount of NTSB containing no denaturant. The color that developed was measured at 412 nm, and the number of moles of sulfhydryl groups per mole of protein was calculated. Since no denaturant was present in this assay, the amount of sulfhydryl groups calculated by this procedure would represent only the available free SH groups. To determine the amount of disulfide bonds present in the protein during the sulfitolysis reaction, an aliquot of the protein solution was mixed with an excess amount of NTSB containing GuSCN. The final concentration of GuSCN in the mixture was about 2.0 M. Under these conditions, denaturation and exposure of the buried disulfide bonds to the solvent would result in the cleavage of the disulfide bonds by the sodium sulfite present in the solvent. For every disulfide bond in the protein cleaved, one free SH groups will be liberated. These liberated SH groups would then react with the NTSB. Thus, the SH determined by this procedure would represent the sum of the free SH and the SH liberated from the disulfide bonds. The amount of disulfide bonds could then be determined by subtracting the free SH from the total SH.

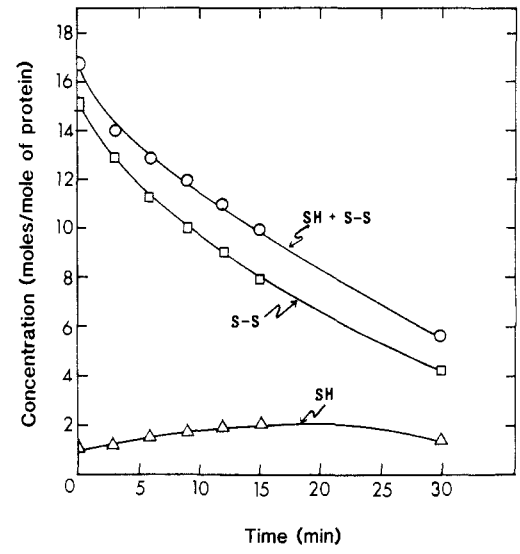

Figure 1. Time course of changes in the SH and SH + S–S content of BSA during sulfitolysis. Conditions: 1% BSA, 0.1 M sodium sulfite, pH 7.0, and 25 °C.

RESULTS

The time course of changes in the sulfhydryl and disulfide content of bovine serum albumin during the sulfitolysis reaction is shown in Figure 1. In the native stage, BSA contains one free sulfhydryl group and 17 disulfide bonds (Brown, 1977). During the sulfitolysis reaction, while the sum of free SH and the disulfide bonds decreased with time, the free SH content increased from 1 to 2 during the first 15 min of reaction. This indicates that although the ordinate for the curve A in Figure 1 is expressed as SH + S–S content, it represents basically the rate of disappearance of disulfide bonds as a result of the sulfitolysis reaction. Two important inferences can be made from this observation: First, addition of 0.1 M sodium sulfite alone did not cause any cleavage of disulfide bonds in BSA, indicating that most of these disulfide bonds were buried in the interior of the protein and were unavailable for cleavage by sulfite. Second, reoxidation of the free sulfhydryl groups to S–S bond caused structural changes in the protein, which in turn systematically exposed increasing numbers of disulfide bonds for sulfitolysis.

The effect of sulfite concentration on the copper carbonate catalyzed sulfitolysis of disulfide bonds of BSA is shown in Figure 2A. In all these experiments, the protein concentration was 1% and the copper content was 4.5 mg/mL (i.e., 75 mg of $CuCO_3$–silica beads/mL). In the absence of copper carbonate no appreciable decrease in disulfide bonds with time was observed at all sulfite concentrations studied. However, in the presence of the catalyst, both the rate and the extent of cleavage and sulfonation of disulfide bonds increased with sulfite concentration and reached a maximum at around 0.12 M sulfite; further increases in sulfite concentration did not influence the rate and extent of sulfitolysis. At 0.15 M sulfite concentration, about four disulfide bonds per mole of BSA remained unsulfonated after 30 min of reaction time; under these experimental conditions the reaction did not reach completion even after several hours.

Analysis of the results according to first-order kinetic plots indicated that the copper carbonate catalyzed sulfitolysis of disulfide bonds followed a pseudo-first-order kinetics (Figure 2B). However, the pseudo-first-order rate constant increased with sulfite concentration and reached a constant value at about 0.12 M sulfite (Figure 2B, inset),

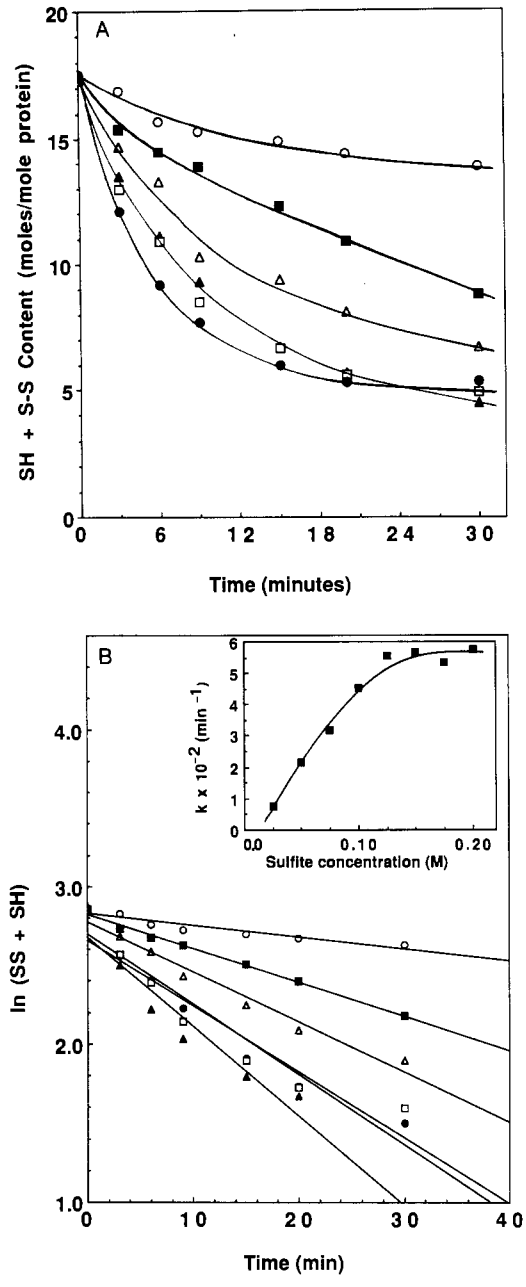

Figure 2. (A) Effect of sulfite concentration on the sulfitolysis of 1% BSA at pH 6.5 and 25 °C. (B) Pseudo-first-order plots for the sulfitolysis of 1% BSA at various sulfite concentrations. Key: ○, 0.025 M; ■, 0.05 M; △, 0.075 M; ▲, 0.1 M; □, 0.125 M; ●, 0.15 M.

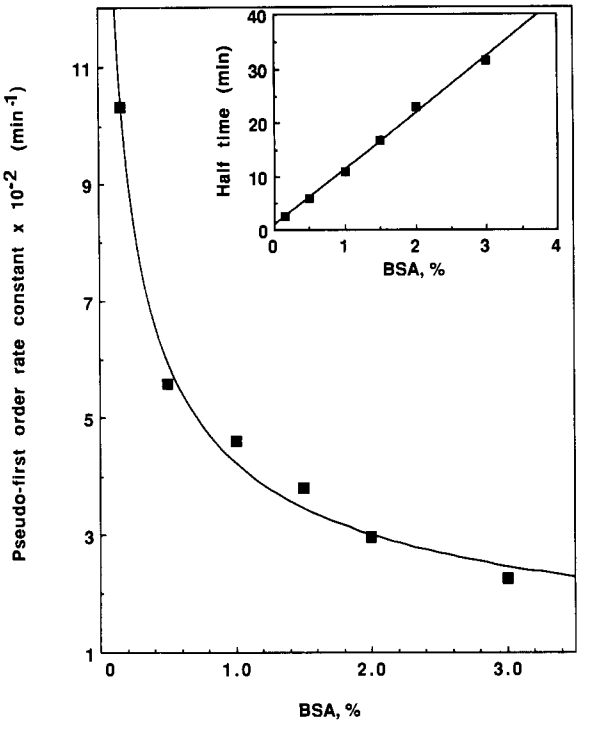

Figure 3. Effect of BSA concentration on the pseudo-first-order rate constant of sulfitolysis at 0.1 M sodium sulfite, pH 6.5, and 25 °C.

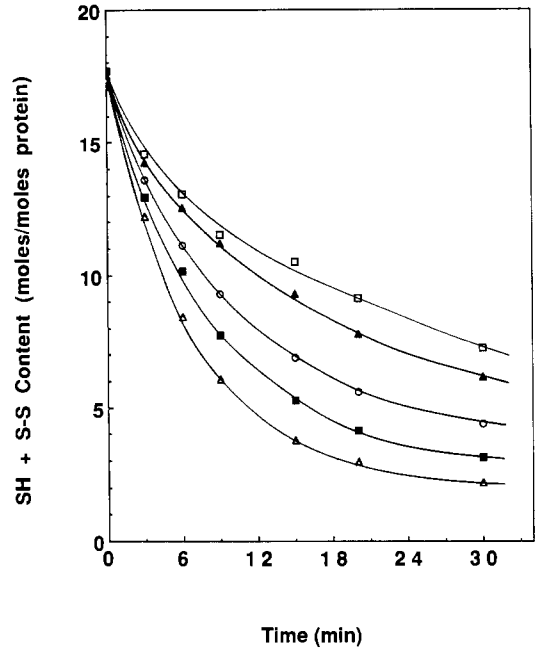

Figure 4. Effect of temperature on the sulfitolysis of BSA at pH 6.5 and 0.1 M sodium sulfite. Protein concentration was 1%. Key: □, 15 °C; ▲, 20 °C; ●, 25 °C; △, 30 °C; ■, 35 °C.

indicating that at 1% protein concentration the sulfitolysis reaction was truly pseudo-first-order only above 0.12 M sulfite.

The effect of protein concentration on the sulfitolysis of disulfide bonds in the presence of 0.1 M sulfite and 4.5 mg/mL copper catalyst is shown in Figure 3. The $t_{1/2}$, that is, the time required to sulfonate 50% of the disulfide bonds, increased almost linearly with protein concentration (Figure 3, inset). The pseudo-first-order rate constant decreased with an increase of protein concentration; a dramatic decrease in the rate constant occurred between 0.16 and 1.0% protein concentration followed by only slight decrease up to 3% protein concentration. Previously it has been reported that when copper in the soluble form (as copper sulfate) was used as the catalyst, the protein concentration had no effect on the pseudo-first-order rate constant (Kella and Kinsella, 1985). However, the protein concentration range used in that study was between 0.016 and 0.16%. This suggests that although the plots of logarithm of disulfide bonds versus time apparently exhibit a linear relationship in the concentration range 0.16–3.0% BSA, indicating first-order kinetics, the true order of the reaction in this concentration range might be greater than 1.

The rate of copper carbonate catalyzed sulfitolysis increased with temperature between 15 and 35 °C (Figure 4). At 15 °C, about eight disulfide bonds remained unsulfonated after 30-min reaction time, whereas at 35 °C only about two disulfide bonds remained unsulfonated after 30-min reaction time. In the temperature range studied, the pseudo-first-order rate constant

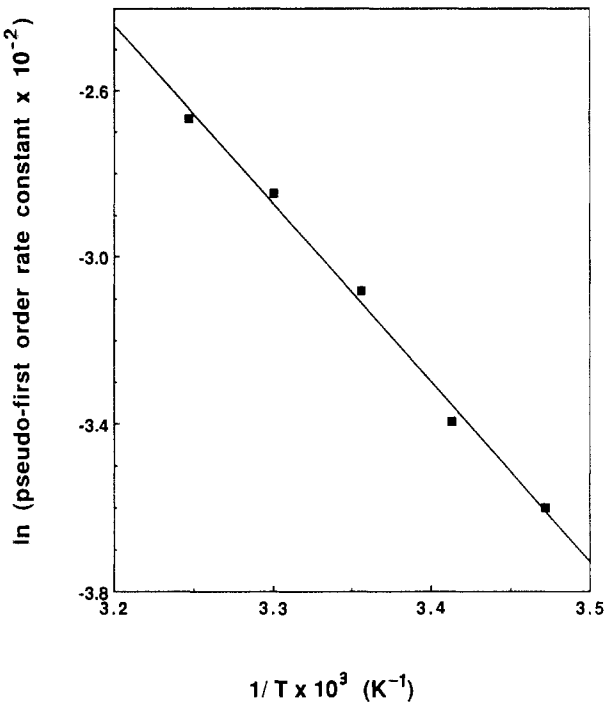

Figure 5. Effect of temperature on the pseudo-first-order rate constant of sulfitolysis of 1% BSA at pH 6.5 and 0.1 M sodium sulfite.

Table I. Thermodynamic Calculations of BSA Sulfitolysis Using Souble- and Solid-State Copper Catalyst at 25 °C, 0.16% BSA, 0.1 M Sulfite, and pH 7.0

	solid-state copper	soluble-state copper[a]
$\Delta G^{\ddagger}$, kcal/mol	21.7	22.9
$\Delta H^{\ddagger}$, kcal/mol	7.9	13.2
$\Delta S^{\ddagger}$, cal/mol·K	-46.2	-32.7
E_a, kcal/mol	8.5	13.8
k,[b] min^{-1}	10.4×10^{-2}	5.3×10^{-3}

[a] pH 6.5; Kella et al. (1985). [b] Pseudo-first-order rate constant.

increased by about 1.6-fold for every 10 °C increase in the temperature.

The activation energy (E_a) for the sulfitolysis reaction, obtained from the slope of the Arrhenius plot (Figure 5), was about 8.5 kcal/mol. Other thermodynamic parameters for the copper carbonate catalyzed sulfitolysis reaction were obtained from the equations $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, $\Delta H^{\ddagger} = E_a - RT$, and $\Delta G^{\ddagger} = -RT \ln [kh/k_bT]$ where $\Delta H^{\ddagger}$, $\Delta G^{\ddagger}$, and $\Delta S^{\ddagger}$ are activation enthalpy, free energy, and entropy, respectively; k_b, h, and R are the Boltzmann, the Planck, and the gas constants, respectively; and k is the rate constant (s^{-1}) at temperature T. These values for copper carbonate catalyzed sulfitolysis at pH 6.5 and 25 °C are summarized in Table I.

The effect of pH on the pseudo-first-order rate constant of sulfitolysis is shown in Figure 6. The pH versus rate constant profile exhibited two transition regions, that is, between pH 8 and 9 and between 6.5 and 7.5. As the pH was decreased from 9 to 8.5, the rate constant apparently increased rapidly; between pH 8.5 and 7.5, no appreciable change in the rate constant was observed. However, when the pH was decreased further, the rate constant increased dramatically between 7.5 and 6.5 and decreased slightly at 6.0. Experiments below pH 6.0 were not possible because of precipitation of BSA near its isoelectric pH 5.3. Thus, the maximum rate of sulfitolysis was observed at pH 6.5.

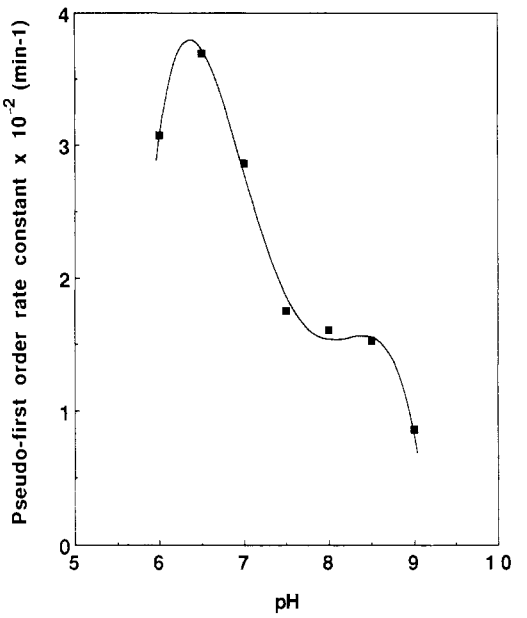

Figure 6. Effect of pH on the pseudo-first-order rate constant of sulfitolysis of 1% BSA at 25 °C and 0.1 M sodium sulfite.

DISCUSSION

The mechanism of the sulfitolysis reaction in the presence of copper as the oxidizing agent has been studied before (Cecil and McPhee, 1955; Cole, 1967; Friedman, 1973; Kella and Kinsella, 1985). When copper is used in the soluble form as copper sulfate, the pseudo-first-order rate constant for the sulfitolysis reaction at 25 °C was about 0.53×10^{-2} min^{-1} (Kella and Kinsella, 1985). However, under similar experimental conditions (i.e., 0.16% protein concentration, 0.1 M sulfite, pH 6.5, 25 °C) the pseudo-first-order rate constant for the sulfitolysis reaction catalyzed by the solid-state copper is about 10.4×10^{-2} min^{-1} (Figure 4). This represents approximately about 20-fold increase in the reaction rate. The activation energy of the reaction in the presence of solid-state copper is 8.5 kcal/mol, which is considerably smaller compared to 13.79 kcal/mol reported for soluble copper (Kella and Kinsella, 1985). This suggests that the higher rate of sulfitolysis in the presence of solid copper catalyst is apparently due to a decrease in the activation energy of oxidation of thiol groups by copper. The dramatic difference in the activation energies might be related to differences in the degree of solvation of Cu^{2+} in the soluble and solid forms. When Cu^{2+} is in the solid state, the absence of the hydration shell may facilitate rapid flow of electrons from the thiolate anion to oxygen via cupric ion, thus resulting in a higher rate of oxidation of the liberated thiol groups; however, when Cu^{2+} is in the soluble hydrated form, the transfer of electrons from the thiolate ions to copper may require additional energy to reorient the water molecules in the hydration shell, thus resulting in decreased rate of flow of electrons from the thiolate anions to oxygen via Cu^{2+}.

A maximum rate of sulfitolysis was observed at pH 6.5. Above pH 6.5, the pH dependency of the rate constant followed two distinct transitions (Figure 6). This may be due to variations in both the charge on the side chain groups in the vicinity of disulfide bonds and the active concentration of SO_3^{2-} as a function of pH. It has been shown that presence of positively charged groups in the vicinity of disulfide bonds increased the rate of sulfitolysis (McPhee, 1956). Although the concentration of the active SO_3^{2-} ion increases when the pH is

increased from 6.5 to 7.5, simultaneous increase in the electronegativity of the protein would cause electrostatic repulsion between the protein and the sulfite ion and thus decrease the rate of the sulfitolysis reaction. Relatively small changes in the rate between pH 7.5 and 8.5 might be attributed to minor changes in the negative charge of the protein because of the presence of very few ionizable side chains in this pH range. The rapid decrease in the rate constant between pH 8.5 and 9.0 might be due to ionization of sulfhydryl groups (pK = 8.8), tyrosine (pK = 9.6), and side-chain amino groups of lysine (pK = 10.2), which might further increase the negative charge of the protein.

Although several other oxidizing agents, such as iodosobenzoate, tetrathionate (Bailey and Cole, 1959), and pure oxygen (Chan, 1968), have been used to catalyze the sulfitolysis reaction, copper has been found to be the best because of its high specificity to thiol groups (Swan, 1957; Pechere et al., 1958; Kella and Kinsella, 1985). However, in most of these studies copper has been used in the soluble form as copper sulfate. This approach has several disadvantages: First of all the rate of the sulfitolysis reaction catalyzed by soluble copper is very slow and usually takes several hours to reach completion even at very low protein concentration in the medium (Kella and Kinsella, 1985). Furthermore, stoppage of the reaction requires addition of chelating agents such as EDTA, and the purification of the protein from the reaction mixture involves extensive dialysis and/or chromatographic procedures. The method described here has several advantages, such as higher rate of reaction, conservation of the catalyst, easy separation of the product, greater control over the extent of modification, and the possibility of continuous-flow operation in a column reaction vessel packed with $CuCO_3$–silica beads. The method can be adapted to industrial scale for sulfonation of food proteins to improve their functional properties.

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