Isolation and Characterization of Sulfhydryl Oxidase from Bovine Milk*

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SUMMARY

A method is described for purification of sulfhydryl oxidase from bovine milk which consistently yields preparations with greater than 3000-fold purification over skim milk. A concentration-dependent association-dissociation of the enzyme was adapted to the development of an isolation procedure. Purified preparations exhibited two zones, both of which displayed activity, upon polyacrylamide disc gel electrophoresis, but only one zone following disc gel electrophoresis in sodium dodecyl sulfate. Its mobility indicated a subunit weight of 89,000.

Several lines of evidence suggest that iron is an integral part of the enzyme. Treatment of the enzyme with EDTA resulted in complete loss of activity which could be subsequently restored by dialysis against 1 μM ferrous sulfate. Furthermore, atomic absorption analysis and neutron activation analysis of separate enzyme preparations each indicated 0.5 atom of iron per subunit.

Chemical analyses of sulfhydryl oxidase accounted for 97% of the sample weight, of which 89% could be attributed to amino acid residues and 11% to carbohydrate residues. Five half-cystine residues per subunit were indicated by cysteic acid analysis and by sulfhydryl group determination. Comparison of this value to the total sulfhydryl groups without reduction tentatively suggests the presence of one disulfide bond.

Sulfhydryl oxidase was found to catalyze the oxidation of sulfhydryl groups in both small compounds and proteins, using O₂ as an oxidant and producing, in equimolar quantities, H₂O₂ and the corresponding disulfide. A Michaelis constant of 90 μM was obtained using reduced glutathione as substrate, under conditions of optimal pH and temperature, viz., pH 7.0 and 35°C. In this communication we describe a method for purification which yields a preparation having a 3000-fold increase in activity over skim milk. Activity was measured using both small thiol compounds (glutathione) and protein substrates. Results of chemical and subunit molecular weight analyses are also given.

EXPERIMENTAL PROCEDURE

Materials—Fresh raw milk was collected directly in glass containers at the afternoon milking from individual cows at the University Dairy Research Farm and immediately cooled and stored at 4°C. Enzyme grade sucrose and ammonium sulfate were obtained from Schwarz/Mann. Acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, 2-mercaptoethanol, and sodium dodecyl sulfate were purchased from Eastman (Rochester, N. Y.). α-Galactose, β-galactosamine (grade I), L-fucose, N-acetylgalactosaminic acid, EDTA, o-dianisidine, GSH, DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

1 The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Experiment Station of the products named, nor criticism of similar ones not mentioned.

2 The abbreviation used here is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).
and glutathione reductase (type III) were products of Sigma (St. Louis, MO.). Horseradish peroxidase (Worthington peroxidase D), RNase A (crystallized five times), and yeast RNA were purchased from Worthington (Freehold, N. J.), and crystalline rennin was obtained from Pierce (Rockford, III.). All metal and buffer salts were Baker reagent grade products.

**Activity Assay**—Two methods of assay were developed, one based on the disappearance of sulfhydryl groups, and the other on O₂ depletion. In the first, the concentration of sulfhydryl groups was measured by reaction with DTNB following Elman's procedure (4). In a typical assay, the reaction mixture contained 1.5 ml of 0.8 mM GSH in 0.047 M sodium phosphate at pH 7.0 (0.1 ionic strength) and 0.2 ml of enzyme solution. The control was identical except for the substitution of 0.2 ml of previously boiled enzyme solution for the native enzyme. Following incubation at 35°C, aliquots of 0.3 ml were removed at various times and added to 0.7 ml of 0.017 M sodium phosphate at pH 8.0. From the resulting solutions, 3 ml were removed and mixed with 20 μl of 0.01 M DTNB in 0.047 M sodium phosphate, pH 7.0. The absorbance of this solution was measured at 412 nm after 2 min. The rate of the enzymatically catalyzed reaction was determined from the linear portion of a plot of sulfhydryl concentration versus time.

In the second method of assay, the rate of O₂ consumption was measured with an oscillating platinum electrode using a Gilson model K Oxygraph. In a typical assay, 0.2 ml of enzyme solution was added to 1.5 ml of 0.8 mM GSH which had been equilibrated at 35°C in the electrode cell. Controls containing boiled enzyme exhibited no O₂ consumption. Enzymic reaction rates were calculated from the initial slopes obtained.

**Stoichiometry and Product Determination**—Two procedures were used to measure the formation of product, H₂O₂. In the first, the concentration of H₂O₂ was determined by horseradish peroxidase-catalyzed oxidation of o-dianisidine. A standard curve relating the 400 nm absorbance of oxidized o-dianisidine to the concentration of H₂O₂ was obtained by the addition of 2-ml volumes of standard solutions of H₂O₂ to 2 ml of the peroxidase-o-dianisidine reagent (5). Standard solutions of H₂O₂ were prepared from a stock solution whose concentration was assessed from its absorbance at 240 nm. Concentrations of H₂O₂ formed in the sulfhydryl oxidase-catalyzed reactions were determined from measurements of 400 nm absorbance following reaction with the peroxidase-o-dianisidine reagent.

Hydrogen peroxide was also quantitatively measured using an iodometric method. Iodine, produced by oxidation of iodide by H₂O₂, was titrated with 0.01 N Na₂S₂O₃ solution standardized against KIO₃ (6) using a starch indicator. The method used was a modification of those previously described (6, 7), and consisted of the addition of 1 ml of the unknown H₂O₂-containing solution, followed by flushing with N₂ for 5 min, addition of 0.25 ml of 50% KI solution, incubation in the dark for 15 min, addition of 12.5 ml of degassed water, addition of 1.0 ml of 2% starch solution, and finally, titration with Na₂S₂O₃.

In each case, the stoichiometry of the reaction was determined by measuring the sulfhydryl groups of the remaining substrate by the method described in the preceding section.

**Reductive Denaturation and Reactivation of RNase—Ribonuclease A** was reduced with 2 mercaptoethanol in the presence of 8 μM urea according to the procedure described by Anfinsen and Haber (8). Approximately 20 mg of RNase were dissolved in 3 ml of freshly prepared 8 μM urea. Immediately before use, the urea solution was passed through a column of Resin I-300 (Fisher Chemical Co.), an anion exchange resin, to remove any cyanate which may have formed. Following the reduction procedure (8), the protein was separated from the reagents by gel filtration through Sephadex G-25, and the completeness of reduction was assessed by reaction with DTNB.

Renaturation of reductively denatured RNase was accomplished using a modified procedure of Haber and Anfinsen (8). Reduced protein in the column effluent was diluted to a concentration of 1 mg/ml with 0.1 M acetic acid, and the pH adjusted to 7.0 with a saturated solution of Tris. This solution was divided into two equal portions; to one part, a solution of sulfhydryl oxidase (2 8 units of activity) in 0.047 M sodium phosphate, pH 7.0, was added, and to the other part, the same volume of phosphate buffer was added in place of sulfhydryl oxidase solution. These solutions were permitted to stand exposed to air at room temperature. Duplicate samples were removed periodically for determination of sulfhydryl concentrations using DTNB, and RNase activity using yeast RNA at pH 5.0 as described by Kalnitsky et al. (10), wherein the amount of acid-soluble oligonucleotides liberated under specified conditions was measured.

**Gel Electrophoresis—**Assessment of purification during enzyme isolation was made with polyacrylamide disc gel electrophoresis. Samples were subjected to electrophoresis in a Bio-Rad model 150 electrophoresis cell using a 2.5% stacking gel (4:1 ratio, acrylamide to methylenebisacrylamide) and a 7.5% separation gel (38:1 ratio) with the discontinuous buffer system of Davis (11). Protein bands were visualized by staining with Coomassie brilliant blue in a methanol-acetic acid-water solution (12) and destaining in 7% acetic acid using a Bio-Rad diffusion destainer.

**Sodium Dodecyl Sulfate Gel Electrophoresis—**Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Weber and Osborn (12, 13), except that a spacer gel was used as described by Pavelich and Hammes (14). Proteins were incubated at 37°C for 2 hours in 0.01 M sodium phosphate at pH 7.0, containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Calibration curves were obtained using as standard proteins: cytochrome c (type VI, Sigma), hemoglobin (Schwarz/Mann), β-lactoglobulin (crystallized twice), 4 trypsin (type III, Sigma), pepsin (crystallized twice, Sigma), horse liver alcohol dehydrogenase (Schwarz/Mann), glutamate dehydrogenase (type I, Sigma), bovine serum albumin (Fraction V, Sigma), and myoglobin (type A, Worthington Biochemicals). A Beckman Model 116 amino acid analyzer was used for amino acid analysis.

**Amino Acid Analysis**—Lyophilized samples were weighed into heavy walled ignition tubes and constant boiling (approximately 6 n HCl) was added; the resulting solutions were frozen, desalted, and the tubes were evacuated and sealed as described by Moore and Stein (16). Percentage of moisture in the lyophilized samples was determined by drying to constant weight at 75°C in vacuo over P₂O₅. Hydrolysis was effected in a refluxing toluene bath for 16, 24, 48, and 72 hours, and hydrolysates were analyzed using a Beckman Model 116 amino acid analyzer.

**Metal Ion Activation Studies**—Solutions of the enzyme were adjusted to 1 mM in EDTA, allowed to stand overnight at 4°C, and dialyzed to remove chelated metal ions. One-milliliter samples of the enzyme thus inactivated (30% protein) were dialyzed against 1 M solutions of ferrous sulphate, copper sulphate, zinc sulphate, cobalt chloride, manganous (II) chloride, and molybdenum dibromide, each adjusted to 10 mM in EDTA, and dialyzed to remove chelated metal ions. Activation analysis was performed on a 38.5-μg sample by irradiation in the N.C.S.U. Pulstar reactor at a neutron flux of 1 × 10¹⁴ neutrons/cm²-sec. Following a 14-day decay, the sample was counted for 90,000 s with a 110-μm² Ge(Li) detector coupled to a computerized ND 2200 multichannel analyzer.
Sulphydryl and Disulphide Analysis—The number of reactive sulphydryl groups was determined by reaction with DTNB according to Ellman’s procedure (4). The total number of sulphydryl groups was measured by reaction in the presence of 1% sodium dodecyl sulfate or 8 M urea. An estimation of number of disulfide bonds was obtained by a modification of Ellman’s procedure (23). Enzyme (2 to 10 mg) was dissolved in 1 ml of 0.05 M sodium phosphate at pH 7.4 and dissociated by addition of 1.44 g of urea, 0.2 ml of 0.5 M EDTA, and 2 µl of 1-octanol. Disulfide bonds were reduced by treating the sample with 1 ml of 2.5% NaBH₄ for 30 min at 40°C. Excess NaBH₄ was destroyed by adding 1 ml of pH 2.7 acetal sodium (10.4 g of NaH₂PO₄·H₂O per 100 ml of 0.1 M HCl) and 2 ml of acetone, followed by purging with N₂ for 6 to 8 min at 25°C. Finally, 1 ml of 2 M DTNB was added and the volume adjusted to 7.0 ml with H₂O. The reaction was allowed to proceed for 40 min before reading the absorbance at 412 nm.

Protein Concentration—A micro-Kjeldahl microdiffusion method was used to determine nitrogen. Triplicate analyses of the purified enzyme gave a value of 13.93 ± 0.35 at 95% confidence limits for the percentage of nitrogen.

Measurement of the absorption spectrum using a Cary 15 spectrophotometer, and correction for light scattering (24), indicated an absorbivity of $E_{278}^{	ext{max}} = 7.41$ for the maximum at 278 nm. However, the aggregated state of the enzyme causes considerable scattering, and thus relatively large corrections, so that estimation of concentrations based on nitrogen determinations was considered more reliable.

RESULTS

Enzyme Purification—In each case, a crude enzyme fraction, prepared in a manner similar to that previously described (1), was used as a starting material. Accordingly, skim milk was prepared from whole raw milk by centrifugation at 4,080 × g for 30 min at 30°C. Whey (Fraction B) was obtained from the skim milk by coagulation of the casein fraction with rennin. Approximately 2 mg of rennin were added per 100 ml of skim milk, and the reaction was allowed to proceed for 30 min at 30°C. Under these conditions, only the Fleu 106-Met 100 bond of ß-casein is hydrolyzed (25). The resulting curd was removed by centrifuging at 16,300 × g for 45 min at 30°C. The whey was cooled and immediately adjusted to one-half saturation in ammonium sulfate at 4°C. After standing overnight at 4°C, the precipitate (crude enzyme, Fraction C) was removed by centrifugation at 16,300 × g for 60 min at 4°C.

Ascending gel chromatography of the crude enzyme fraction on a column of Bio-Gel A-5m (1.5 × 80 cm), 6% agarose (Bio-Rad) at 4°C in 0.017 M sodium phosphate at pH 7.0 yielded three fractions. Only that protein eluting in the void volume (Fraction 1) exhibited enzymatic activity (14-fold purification). This represents a 1.5-fold greater purification than that obtained by chromatographing the crude enzyme fraction (Fraction C) on Sephadex G-200, which had been previously reported (26). Subsequent density gradient centrifugation of Fraction 1 at 25,000 rpm for 4 hours at 4°C in a linear 5 to 20% sucrose gradient following the method described by Martin and Ames (27) resulted in a pellet (Fraction 2) which contained most of the activity. A 2-fold increase in specific activity was attained in this step. The apparent state of high molecular association suggested that Fraction 1 could be purified by centrifugation in the absence of a gradient. Accordingly, a 10 mg/ml solution of Fraction 1 was centrifuged at 6037 × g for 30 min at 4°C to give a pellet whose specific activity indicated a 2-fold purification (Fraction 3).

Preliminary centrifugal investigations suggested that the state of molecular association was concentration-dependent; e.g., centrifugation of a dilute solution of crude enzyme (roughly the concentration of enzyme as it occurs in milk) yielded the activity in the supernatant fraction which, when concentrated and recentrifuged under the same conditions, produced a pellet containing most of the activity. Thus, this property was utilized in developing a procedure for isolation of the enzyme.

Crude enzyme (Fraction C) was dissolved in 0.047 M sodium phosphate at pH 7.0 to give a concentration of 3% protein, and dialyzed against the same buffer at 4°C. This solution was diluted with the buffer to 0.15% protein and allowed to stand overnight at 4°C. Rapidly sedimenting impurities (Fraction D) were removed by centrifuging at 2000 × g for 30 min at 4°C, and the resulting supernatant liquid (Fraction E) was concentrated in a 4°C cold room to approximately 3% protein with an Amicon TC-10 ultrafiltration system using an Amicon PM-10 membrane. This solution was again centrifuged at 2000 × g for 30 min at 4°C, but this time the enzymatic activity appeared in the pellet (Fraction F). Smaller proteins present in Fraction F were removed by dissolving the pellet in twice the volume of the previous solution, allowing the solution to stand overnight at 4°C to promote dissociation, and repeating the centrifugation at 2000 × g for 30 min at 4°C. The resulting pellet (Fraction H) was taken as the purified enzyme.

Characteristics of fractions obtained in various steps of this procedure are listed in Table I. Fraction F displayed considerably more activity than Fraction 1 from gel chromatography or Fractions 2 and 3 from sucrose gradient centrifugation and direct centrifugation of Fraction 1; in fact, a 60-fold purification was obtained in this single step. Gel chromatography of Fraction F gave only the fraction eluting in the void volume; its gel electrophoretic pattern was also very similar to that of Fraction 1. However, these fractions are not homogeneous as indicated by their gel electrophoretic patterns. Assays of protein eluted from the positions of each of the bands in an unstained gel showed that enzymic activity occurred only at the top of the spacer gel and at the interface of the spacer and separation gels. Those proteins from Fraction F which appeared in the separation gel were effectively removed by centrifuging a slightly more dilute solution. Only two bands, both of which were enzymically active, were detectable upon gel electrophoresis of Fraction H. Furthermore, only one protein-staining band was visible following disc gel electrophoresis of this fraction in sodium dodecyl sulfate.

This method of purification has been repeated numerous times.
solution containing NADPH, GSH, glutathione reductase, and decrease in absorbance at 340 nm with time was observed when a activity could be assayed by measuring either the rate of O2 de-
pletion or the decrease in sulfhydryl concentration, as suggested products was substantiated by incorporating GSH and sulfhydryl oxidase in the assay procedure for glutathione reductase. A linear and rotor speed).

sulfhydryl oxidase was measured against a blank which contained each of these except for sulfhydryl oxidase. However, the correla-
tion of rates of sulfhydryl group oxidation to O2 depletion, using the two assay procedures, did not correspond to the stoichi-
ometry depicted by Equation 1. For example, in one experiment, the respective rates measured were 0.025 μmol of GSH oxidized per min per ml and 0.0139 μmol of O2 consumed per min per ml. Thus, the observed stoichiometry is close to that of Equation 2 (2:1), and not that proposed by previous authors (4:1). Subse-
quent experiments established H2O2 as the other product of the sulfhydryl oxidase-catalyzed reaction. Both the horseradish peroxidase-catalyzed oxidation of o-dianisidine and the chemical oxidation of iodide to iodine, which were found to occur upon addition of sulfhydryl oxidase assay mixtures at appropriate intervals, demonstrate the production of H2O2 during GSH oxi-
dation to GSSG. Moreover, the stoichiometry, as revealed in the data presented in Table II, indicates that the actual reaction catalyzed by sulfhydryl oxidase is that shown in Equation 2.

Since preliminary results had suggested that the observed specific activity might depend upon the concentration of the enzyme stock solution, this possibility was investigated by storing stock solutions of sulfhydryl oxidase, ranging in concentration from 25 to 200 μg per ml, at 4° overnight prior to enzymatic assay. As shown by the data given in Fig. 1, the observed specific activity decreased with increasing enzyme concentration in the stock solution. A 1.7-fold decrease in specific activity was ob-
erved for the 200 μg per ml solution as compared to the 25 μg per ml stock solution. Similar results were also obtained for the crude enzyme preparation.

Enzymatic activity is shown as a function of GSH concentra-
tion in Fig. 2. The inset in Fig. 2 illustrates the double reciprocal plot of the same data. Substrate inhibition becomes apparent at concentrations above 0.8 mM. A K_m value of 90 μM was obtained. Similar data obtained using the crude enzyme preparation yielded the same value for K_m.

Both the temperature and pH dependence of the activity of the purified enzyme were examined using GSH as substrate. Maximum activity was observed at a temperature of 35°. A symmetrical, bell-shaped dependence of activity on pH was ob-
erved, with a pH optimum of 6.8 to 7.0, and apparent pK_a val-

### Table II

<table>
<thead>
<tr>
<th>Minutes of sulfhydryl oxidase reaction</th>
<th>GSH consumed/μl</th>
<th>H2O2 produced/μl (peroxidase)</th>
<th>H2O2 produced/μl (iodometric)</th>
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<tr>
<td>6</td>
<td>0.230 ± 0.014</td>
<td>0.938</td>
<td>0.118</td>
</tr>
<tr>
<td>12</td>
<td>0.313 ± 0.011</td>
<td>1.142</td>
<td>0.159</td>
</tr>
<tr>
<td>18</td>
<td>0.374 ± 0.005</td>
<td>0.177</td>
<td>0.183</td>
</tr>
<tr>
<td>24</td>
<td>0.446 ± 0.022</td>
<td>0.194</td>
<td>0.224</td>
</tr>
<tr>
<td>30</td>
<td>0.492 ± 0.010</td>
<td>0.224</td>
<td>0.245</td>
</tr>
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</table>

### Fig. 1. Effect of concentration of the enzyme (Fraction H) stock solution on the observed specific activity. Assays were performed by adding 0.2 ml of stock enzyme solution, ranging in concentration from 25 to 200 μg per ml, to 1.5 ml of substrate solution; O2 consumption was followed at 35° in pH 7.0 phosphate using 0.8 mM GSH as substrate. (Concentrations identified by the abscissa represent final concentrations of enzyme in the assay mixtures.)

### Fig. 2. Activity of sulfhydryl oxidase as a function of GSH concentration. O2 consumption was followed at 35° in pH 7.0 phosphate using 40 μg of purified enzyme. The inset, double reciprocal plot of the data. (The dotted line indicates the position of V_max/2 on the ordinate, and thus 1/K_m on the abscissa.)
use of 5.5 and 8.1 governing the ascending and descending limbs, respectively.

Complete reduction of the disulfide bonds in RNase A was achieved during the reductive denaturation of that enzyme as shown by reaction with DTNB of 7.84 mol of sulfhydryl per mol of protein. Rates of sulfhydryl group oxidation and regain of RNase activity in the presence and in the absence of sulfhydryl oxidase are shown in Fig. 3. In the presence of enzyme, complete oxidation and reactivation of RNase occurred within 1 hour, whereas more than 20 hours was required in its absence. As shown by the data in the inset of Fig. 3, a 1:1 relationship between sulfhydryl group oxidation and RNase activity was not observed. For example, when one-half of the —SH groups of the preparation had been reoxidized, only 15% of the RNase activity had been regained.

Subunit Molecular Weight Studies—Segrest et al. (15) have shown that the apparent molecular weights of glycoproteins obtained by sodium dodecyl sulfate disc gel electrophoresis are not independent of the gel concentration. Therefore, sulfhydryl oxidase was examined using a series of acrylamide concentrations. Average apparent molecular weights from a number of experiments with the purified enzyme were determined to be 91,200 ± 1,200, 89,800 ± 800, 89,000 ± 400, and 89,000 ± 1,400 at 5.0, 7.5, 10.0, and 12.5% acrylamide concentrations, respectively. For each concentration, the calibration plot of the logarithm of the molecular weight versus mobility was essentially linear. At 10 and 12.5% acrylamide concentrations the values obtained for four independent preparations of the enzyme were in very good agreement, yielding an average subunit weight of 89,000 ± 900. In each case, a single protein band was observed at varying gel concentrations and enzyme loadings.

Chemical Composition Studies—Results of amino acid and carbohydrate analyses are listed in Table III. These data indicate that essentially all of the sample weight (97%) is accounted for by amino acid and carbohydrate residues, of which 89% is represented by amino acid residues and 11% by carbohydrate.

Results of sulfhydryl group analyses of sulfhydryl oxidase were compared with those of β-lactoglobulin (28) and RNase A.

<table>
<thead>
<tr>
<th>Component</th>
<th>umoles/mg</th>
<th>g/100 g</th>
<th>Number/89,000 g</th>
<th>Nearest Integer</th>
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</thead>
<tbody>
<tr>
<td><strong>Amino acids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>0.504 ± 0.014</td>
<td>6.46</td>
<td>44.9</td>
<td>45</td>
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<tr>
<td>His</td>
<td>0.124 ± 0.004</td>
<td>1.70</td>
<td>11.0</td>
<td>11</td>
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<tr>
<td>Arg</td>
<td>0.332 ± 0.012</td>
<td>5.19</td>
<td>29.5</td>
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<tr>
<td>Asp</td>
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<td>8.03</td>
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<tr>
<td>Thr</td>
<td>0.475</td>
<td>4.80</td>
<td>42.3</td>
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</tr>
<tr>
<td>Ser</td>
<td>0.519</td>
<td>4.52</td>
<td>46.2</td>
<td>46</td>
</tr>
<tr>
<td>Glu</td>
<td>0.811 ± 0.018</td>
<td>10.47</td>
<td>72.2</td>
<td>72</td>
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<tr>
<td>Pro</td>
<td>0.416 ± 0.010</td>
<td>4.04</td>
<td>37.0</td>
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<tr>
<td>Gly</td>
<td>0.845 ± 0.035</td>
<td>4.83</td>
<td>75.2</td>
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<tr>
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<td>0.967 ± 0.026</td>
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<td>86.1</td>
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<td>Half-Cys</td>
<td>0.061 ± 0.001</td>
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<tr>
<td>Val</td>
<td>0.630</td>
<td>6.24</td>
<td>56.1</td>
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<tr>
<td>Met</td>
<td>0.082 ± 0.004</td>
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<td>7.3</td>
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<tr>
<td>Ile</td>
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<td>4.08</td>
<td>32.0</td>
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<tr>
<td>Leu</td>
<td>0.680</td>
<td>7.70</td>
<td>60.5</td>
<td>61</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.203</td>
<td>3.32</td>
<td>18.1</td>
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</tr>
<tr>
<td>Phe</td>
<td>0.326 ± 0.012</td>
<td>4.80</td>
<td>29.0</td>
<td>29</td>
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<tr>
<td>Trp</td>
<td>0.050 ± 0.005</td>
<td>1.08</td>
<td>5.2</td>
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<tr>
<td><strong>Carbohydrates</strong></td>
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<tr>
<td>Fucose</td>
<td>0.030</td>
<td>0.44</td>
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<td>3</td>
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<tr>
<td>Total hexose</td>
<td>0.444 ± 0.002</td>
<td>7.19</td>
<td>39.5</td>
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</tr>
<tr>
<td>N-Acetyleneuraminic acid</td>
<td>0.143 ± 0.002</td>
<td>2.90</td>
<td>12.7</td>
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<td>N-Acetylneuraminic acid</td>
<td>0.013</td>
<td>0.41</td>
<td>1.2</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 3. Oxidation and reactivation of reductively denatured RNase A by sulfhydryl oxidase. Disappearance of available —SH groups, as measured by reaction with DTNB, is shown for both the presence (●) and absence (▲) of sulfhydryl oxidase. Similarly, the appearance of RNase activity is shown for both the presence (●) and absence (▲) of sulfhydryl oxidase. The relationship between available —SH groups and RNase activity is illustrated in the inset for both the presence (●) and absence (▲) of sulfhydryl oxidase.
Effect of metal ions on sulfhydryl oxidase activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme activity (µmol O₂ consumed/min)</th>
</tr>
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<tbody>
<tr>
<td>Enzyme (control)</td>
<td>0.368</td>
</tr>
<tr>
<td>+ EDTA (1.0 mM)</td>
<td>0.012</td>
</tr>
<tr>
<td>+ Ferrous sulfate</td>
<td>0.256</td>
</tr>
<tr>
<td>+ Copper sulfate</td>
<td>0.109</td>
</tr>
<tr>
<td>+ Manganese (II)</td>
<td>0.085</td>
</tr>
<tr>
<td>+ Cobalt chloride</td>
<td>0.021</td>
</tr>
<tr>
<td>+ Zinc sulfate</td>
<td>0.029</td>
</tr>
<tr>
<td>+ Molybdenum dibromide</td>
<td>0.018</td>
</tr>
</tbody>
</table>

Although molybdenum dibromide was used to prepare the solution, it most likely oxidized to higher oxidation states.

Values obtained for the total number of sulfhydryl groups (half-cystine residues) in the reduced proteins were: 4.5 ± 0.5 for β-lactoglobulin, 7.6 ± 0.2 for RNase A, and 4.1 ± 0.2 per sulfhydryl oxidase subunit. The respective values are, thus, 5 ± 10% below the theoretical number of 5 for β-lactoglobulin and 8 for RNase A; accordingly, the number of half-cystine residues per sulfhydryl oxidase subunit is most likely 5. There were 2.0 ± 0.6 “available” sulfhydryl groups per subunit, measured by reaction of the enzyme with DTNB in phosphate buffer containing 5.7 mM EDTA, and 2.4 ± 0.2 “total” sulfhydryl groups, measured under the same conditions except for the inclusion of 8 M urea. Tentatively, these data suggest the presence of three sulfhydryl groups and one disulfide bond in the enzyme’s subunit structure.

Studies of enzyme activity and chemical analyses indicate that iron is an integral part of sulfhydryl oxidase. Treatment of the enzyme with EDTA resulted in complete loss of activity as illustrated by the data in Table IV. Dialysis against 1 mM Fe⁺ restored 70% of the original activity. Some activity could also be restored by Cu⁺ (30%) or Mn⁺ (20%). The electronic absorption spectrum of the native enzyme exhibited a typical protein maximum at 278 nm (E₃₀₅ = 7.41), but no maxima at longer wavelengths; thus, it appears that iron is not present in the form of a heme prosthetic group. Calculations based on atomic absorption spectral analyses indicated the presence of 0.48 g-atoms of iron and approximately 0.15 g-atoms of copper per 89,000. Neutron activation analysis of a separate preparation gave 11.1 ± 0.3 µg of iron for a 38.5-mg sample, which corresponds to 0.40 g-atoms of iron per 89,000. Copper could not be determined under the conditions employed, and analysis for other metals indicated only traces of zinc and cobalt. No molybdenum or manganese could be detected. Thus, iron appears to be required for the enzymatic activity of sulfhydryl oxidase, although it is not presently known whether the iron functions directly in the active site of the enzyme or indirectly by stabilizing the proper molecular conformation.

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