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RESOLUTION OF RENAL SULFHYDRYL OXIDASE FROM γ-GLUTAMYLTRANSFERASE BY COVALENT CHROMATOGRAPHY ON CYSTEINYLSUCCINAMIDOPROPYL-GLASS[1]

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SUMMARY: Sulfhydryl oxidase from bovine kidney cortex was purified 2500-fold by covalent chromatography using cysteinylsuccinamidopropyl-glass. GSH oxidation catalyzed by the resulting preparation was found to be totally enzymatic, as judged by the inability of the preparation to reduce nitro blue tetrazolium, and H_2O_2 was found to be a product, as had been previously observed with milk sulfhydryl oxidase. No GSH peroxidase activity could be detected, using either H_2O_2 or *t*-butylhydroperoxide. The chromatographically purified renal sulfhydryl oxidase was resolved from γ-glutamyltransferase as evidenced by a 12,000-fold increase in ratio of the two enzymatic activities over that exhibited by crude kidney homogenates, and antibodies raised against purified milk sulfhydryl oxidase cross-reacted with the kidney oxidase, but not the kidney transferase.

Sulfhydryl oxidase, as isolated from bovine skim milk (1), is a membrane-associated glycoprotein which catalyzes the oxidation of GSH according to the equation:

$$2\ GSH + O_2 \longrightarrow GSSG + H_2O_2 \qquad (1)$$

In addition to catalyzing disulfide bond formation in glutathione, the enzyme is active in catalyzing the oxidation of a number of other low molecular weight thiol substrates, and can also catalyze disulfide bond formation in a variety of proteins (1-3). The enzyme is highly aggregated, and emerges in the void volume of columns of Bio-Gel A-150m or 3000 Å controlled pore glass beads (4).

GSH can also be oxidized by a "glutathione oxidase" activity which is present in the plasma membrane fraction of kidney (5, 6). Preliminary immunological studies, employing an indirect fluorescent antibody technique,

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have revealed that antibodies prepared against bovine milk sulfhydryl oxidase bind not only to the plasma membranes of mammary tissue cells, but also to the plasma membranes of kidney proximal tubules (7). Many efforts to separate kidney GSH-oxidizing activity from γ-glutamyltransferase (EC 2.3.2.2) have been unsuccessful (8, 9), and recent reports have attributed the glutathione-oxidizing activity of kidney (10) and milk (11) to the transferase. In the case of the kidney, it was proposed that γ-glutamyltransferase catalyzes the formation of cysteinylglycine which, in turn, catalyzes nonenzymatic oxidation of GSH through autoxidation and thiol-disulfide interchange (10). In the case of milk, it was suggested that a single bicephalic enzyme is responsible for both the glutamyltransferase and oxidase activities (11).

Although Ashkar *et al.* (12) have recently reported separating a portion of rat kidney GSH-oxidizing activity from glutamyltransferase activity by gel filtration of Triton X-100-treated plasma membranes through Bio-Gel P-200, unlike milk sulfhydryl oxidase, the activity penetrated the bed volume of the column. Unfortunately, the possibility of metal ion-catalyzed autoxidation was not ruled out as the source of glutathione-oxidizing activity which penetrated the gel matrix.

Successful separation of bovine milk sulfhydryl oxidase from most of the γ-glutamyltransferase activity present in skim milk membranes (increasing the ratio of oxidase:transferase activities by 700-fold over that in crude membranes) through successive ammonium sulfate fractionation steps, led us to conclude that the oxidase and the transferase are distinct and separable entities, at least in milk (13). Preliminary investigations revealed that the two enzymes can be even more effectively and reproducibly separated by covalent chromatography of detergent-solubilized skim milk membranes on cysteinylsuccinamidopropyl-glass columns (14).

In view of the controversy surrounding the mechanism of renal glutathione oxidation and the question regarding the existence of a sulfhydryl oxidase in mammalian kidney (6, 8-10), we sought to determine whether covalent

chromatography could resolve a kidney sulfhydryl oxidase from the γ-glutamyltransferase. In this communication, we report the successful resolution of the two activities through selective binding of the oxidase to a column of cysteinylsuccinamidopropyl-glass, followed by elution of the oxidase with GSH.

MATERIALS AND METHODS

Controlled-pore glass beads (2000 Å pore diam., 80/120 mesh) were obtained from Sigma Chemical Co., and cleaned for 45 min with reagent grade nitric acid at 100 °C, then rinsed with distilled water to neutral pH, and dried in an oven at 125 °C. The beads were derivatized with aminopropyltriethoxysilane (15), and then succinylated (16). The degree of reaction was monitored using 2,4,6-trinitrobenzenesulfonate (17). L-Cysteine (50 ml of 0.1 M cysteine/20 g beads) was covalently attached to the succinamidopropyl-glass matrix using 1-ethyl-3-(dimethylaminopropyl)carbodiimide to activate the succinic carboxyl groups. The resulting cysteinylsuccinamidopropyl-glass beads were washed thoroughly with water, and the cysteinyl residues were completely reduced by recirculating 50 ml of 0.1 M dithiothreitol in 8 M urea, 1 M NaCl, and 0.1 M acetic acid through the bead column.

Bovine kidneys, obtained from Randolph Meat Packing Co., Asheboro, NC, were either used on the day of slaughter or were frozen for later use. The cortex was carefully dissected from each kidney, and 90 g was homogenized with 450 ml of 47 mM sodium phosphate, pH 7.0, for 2 min in a Waring blender. The cortical homogenate was clarified by centrifuging at 1080 $\times g$ for 20 min at 4 °C, and the resulting supernatant fraction (referred to as "crude homogenate") was centrifuged at 102,000 $\times g$ for 1 h at 4 °C. The pellet was solubilized by stirring for 1 h with 1 % (w/v) polyoxyethylene-9-lauryl ether (P-9-L)[2]. The suspension was again centrifuged at 102,000 $\times g$ for 1 h, and the supernatant ("solubilized pellet") fraction was subjected to covalent chromatography by recirculating 30 ml for 12-14 h at room temperature through a 1.0 × 34-cm column of L-cysteinylsuccinamidopropyl-glass beads. Nonbinding protein was removed by washing the column with at least 8 column volumes of 1 % P-9-L, 1.0 M NaCl, pH 7.0; the detergent was then removed by washing the column with 47 mM sodium phosphate, pH 7.0. Bound protein was then eluted from the column by recirculating 30 ml of 2 mM GSH in 47 mM sodium phosphate, pH 7.0, overnight at room temperature.

Sulfhydryl oxidase activity toward GSH was determined using the DTNB assay previously reported (13), and γ-glutamyltransferase activity was measured using the γ-glutamyl *p*-nitroanilide assay of Griffith and Tate (10). Nonenzymatic (metal ion-mediated) oxidation of GSH was monitored by measuring the rate of reduction (increase in A_{560}) of nitro blue tetrazolium at 35 °C (18); a typical assay mixture contained, in addition to enzyme, 1.45 mM GSH and 0.244 mM NBT. The production of H_2O_2 was measured using horseradish peroxidase and ABTS. Protein concentrations were estimated by the dye-binding assay of Bradford (19) using a Bio-Rad kit (Richmond, CA); when present, detergent was removed using Bio-Beads SM-2 (Bio-Rad) before samples were subjected to protein assays.

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[2]Abbreviations used: P-9-L, polyoxyethylene-9-lauryl ether; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NBT, nitro blue tetrazolium; ABTS, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid); EDTA, ethylenediaminetetraacetic acid.

TABLE I. SEPARATION OF KIDNEY SULFHYDRYL OXIDASE FROM γ-GLUTAMYLTRANSFERASE BY COVALENT CHROMATOGRAPHY [a]

Fraction	Protein Conc. (mg/ml)	SH Oxidase $\left(\frac{\mu mol}{min \cdot mg}\right)$	Purification (Fold)	SH Oxidase / γ-Glu Tr'ase (Activity Ratio)
Crude Cortical Homogenate	17.2	0.0022	1	0.011
Solubilized Pellet	0.68	0.15	68.2	0.047
Glutathione-Eluting Enzyme From Cys-Column	0.018	5.56	2530	131.2

[a] Abbreviations: SH Oxidase, sulfhydryl oxidase; γ-Glu Tr'ase, γ-glutamyltransferase; Cys-Column, cysteinylsuccinamidopropyl-glass column.

RESULTS AND DISCUSSION

The immobilized cysteinyl-glass matrix, which appeared to be successful in the isolation of sulfhydryl oxidase from bovine milk and its resolution from γ-glutamyltransferase activity (14), is also effective in separating sulfhydryl oxidase from the glutamyltransferase activity in bovine kidney. As can be seen from the results presented in Table I, such covalent chromatography leads to some 2500-fold purification of renal sulfhydryl oxidase and a 12,000-fold increase in ratio of oxidase to transferase activities over that present in the crude cortical homogenate.

Nonenzymatic, metal ion-mediated autoxidation of sulfhydryl groups can lead to substantial formation of disulfide bonds *in vitro* with the production of superoxide anion (18). The latter can be detected by incubation with nitro blue tetrazolium which, upon reduction, leads to an increase in absorbance at 560 nm (18). NBT assays revealed considerable nonenzymatic oxidation of GSH in the presence of crude kidney homogenates or solubilized pellet fractions; however, the purified sulfhydryl oxidase preparation, eluted from the cysteinylsuccinamidopropyl-glass column with glutathione, is virtually devoid of such nonenzymatic autoxidative activity (Fig. 1). In contrast, H_2O_2 was observed as a product of the reaction, at levels approaching the stoichiometry given in equation 1: glutathione oxidation measured by the DTNB

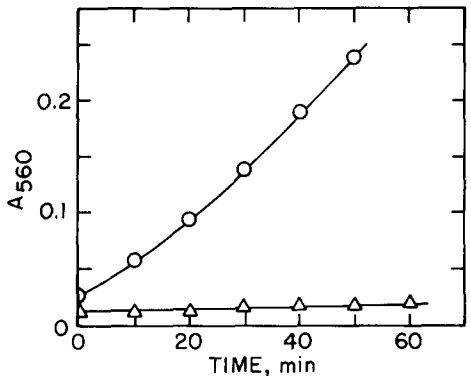

FIG. 1. Nonenzymatic oxidation of GSH as determined by the ability to reduce nitro blue tetrazolium. Circles, solubilized pellet; triangles, glutathione-eluting material from cysteinyl-derivatized column.

assay as 0.082 μmol/min.ml corresponded with 0.032 μmol H_2O_2/min ml measured by incubation with ABTS and horseradish peroxidase.

The chromatographically isolated renal sulfhydryl oxidase was found to be fully inhibited by 3 mM EDTA, as had been previously found with milk sulfhydryl oxidase (1) preparations. GSH-oxidizing activity was also lost (>99% inactivation) by heating the glutathione-eluted enzyme preparation at 100 °C for 60 min. Glutathione peroxidase activity could not be detected using either H_2O_2 or *t*-butylhydroperoxide.

Antisera were prepared by immunizing rabbits with purified bovine milk sulfhydryl oxidase which had been prepared by covalent chromatography on cysteinylsuccinamidopropyl-glass columns (14). These were found to exhibit cross-reactivity with the purified kidney enzyme as evidenced by immunoprecipitin lines formed on Ouchterlony plates. Moreover, the immunoglobulin fraction quantitatively precipitated sulfhydryl oxidase activity from solubilized pellet fractions of kidney cortical homogenates, without inhibiting γ-glutamyltransferase activity of these preparations. Thus, it appears that kidney sulfhydryl oxidase (or "glutathione oxidase") is antigenically similar to (and may be identical with) milk sulfhydryl oxidase. Like the milk enzyme, the renal oxidase can be separated from γ-glutamyltransferase without prior proteolytic digestion. Thus, the concept that, in the kidney, glutathione-oxidizing activity is due solely to

"nonenzymatic oxidation and transhydrogenation reactions of cysteinylglycine," formed from GSH by γ-glutamyltransferase (10), no longer appears tenable.

The choice of cysteinylsuccinamidopropyl-glass as a matrix for the covalent chromatography of sulfhydryl oxidase provides a simple and rapid method for separating the enzyme from the glutamyltransferase contained in both milk and kidney membranes. By contrast, a sulfhydrylcellulose column, prepared according to the procedure of Feist and Danna (20), failed to bind sulfhydryl oxidase activity when solubilized preparations were recirculated through the column. Thus, the nature of the thiol-containing ligand appears to be important in the binding of the oxidase by covalent chromatography, and may reflect the specificity of bovine sulfhydryl oxidase for cysteine-containing substrates, including peptides and proteins (1-3), and its lack of activity in catalyzing the oxidation of simpler thiols such as 2-mercaptoethanol, dithiothreitol, or lipoic acid (7).

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