Resolution of sulphydryl oxidase from γ -glutamyltransferase in bovine milk by covalent chromatography on cysteinylsuccinamidopropyl-glass

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(Received 2 September 1982/Accepted 15 October 1982)

1. Sulphydryl oxidase from bovine milk was purified by covalent affinity chromatography on cysteinylsuccinamidopropyl-glass. Selective immobilization of the oxidase occurs through formation of a mixed disulphide between the enzyme and the substrate cysteinyl-glass matrix. Reductive elution of the bound protein can be accomplished with small thiols such as reduced glutathione (GSH), dithiothreitol or cysteine. This method leads to approx. 4000-fold purification of the enzyme from whey. Furthermore, complete resolution of sulphydryl oxidase from γ -glutamyltransferase was achieved with this procedure. 2. Antibodies prepared against this purified enzyme quantitatively precipitated 95% of the GSH-oxidative activity from detergent-solubilized skim-milk membranes, whereas 100% of the transferase activity remained in the supernatant fraction; these findings confirmed the distinction between these two enzymes. 3. Reverse-phase high-pressure-liquid-chromatographic analyses of assay mixtures containing both enzymes revealed an array of GSH derivatives generated by a combination of the oxidative and hydrolytic activities. However, purified sulphydryl oxidase yielded only GSSG with concomitant stoichiometric loss of GSH. 4. The chromatographic method described is simple and reproducible, and may be applicable to isolation of sulphydryl oxidase from other tissues.

Abbreviations used: GSH and GSSG, reduced and oxidized forms of glutathione; TNBS, 2,4,6-trinitrobenzenesulphonate; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); ABTS, 2,2'-azinodi-(3-ethylbenzthiazoline sulphonic acid); SDS, sodium dodecyl sulphate; h.p.l.c., high-pressure liquid chromatography; C₁₂E₉, n-dodecyl penta-(ethylene glycol) monoether.

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¶ The designation 'sulphydryl oxidase', which has been used consistently since the discovery of this enzymic activity by Kiermeier & Petz (1967), serves to distinguish this enzyme from that designated as 'thiol oxidase' (EC 1.8.3.2), which is totally different both in its molecular properties and the nature of the reactions it catalyses. Thiol oxidase catalyses oxidation of ethylenic-linked thiols to their corresponding disulphides while molecular oxygen is reduced to water. This enzyme also catalyses oxidation of catechol, contains copper, and is not inhibited by EDTA or thiol-specific reagents (Aurbach &

Sulphydryl oxidase¶ isolated from bovine skim milk is a metalloglycoprotein that catalyses the reaction:

The activity was first identified in milk by Kiermeier & Petz (1967) and the enzyme was isolated and partially characterized by Janolino & Swaisgood (1975). The nature of the enzyme-catalysed reaction has also been characterized with respect to protein folding (Janolino *et al.*, 1978; Swaisgood & Horton, 1980) and the conversion of xanthine dehydrogenase into xanthine oxidase (Clare *et al.*, 1981). Recently, the possibility that milk sulphydryl oxidase is a functional analogue of kidney glutathione oxidase has been considered (Sliwowski *et al.*, 1981).

Co-purification of milk sulphydryl oxidase with other proteins, most notably γ -glutamyltransferase and xanthine oxidase, has been encountered previously (Kitchen, 1974). The situation has been

Jakoby, 1962). Furthermore, it is a soluble enzyme having an $s_{25,w}$ of 2.42 S at pH 7.5.

complicated further by recent reports attributing the GSH-oxidizing activities of kidney (Griffith & Tate, 1980) and milk (Iwami *et al.*, 1981) to γ -glutamyltransferase. The transferase was postulated to initiate oxidation through formation of cysteinylglycine which then catalyses net disulphide synthesis through autoxidation and a series of thiol-disulphide interchange reactions (Griffith & Tate, 1980). Alternatively, Iwami *et al.* (1981) have suggested that a single bifunctional protein is responsible for both the transferase and oxidase activities of milk. However, more recent studies (Sliwowski *et al.*, 1981) have shown that the ratio of oxidase to transferase activities increased over 700-fold during isolation of milk sulphydryl oxidase using the method of Janolino & Swaisgood (1975). Resolution of the kidney enzymes has also been reported (Ashkar *et al.*, 1981; Schmelzer *et al.*, 1982).

A similar controversy existed until recently in the area of disulphide-bond formation in proteins. This dispute centred around the nature of the enzymic activities of protein disulphide-isomerase and glutathione-insulin transhydrogenase. Hillson & Freedman (1980) resolved the matter through separation of the two enzymes by covalent chromatography on a thiopropyl-Sepharose 6B matrix.

In the present paper, we describe the complete resolution of sulphydryl oxidase from γ -glutamyltransferase of bovine milk by covalent affinity chromatography on cysteinylsuccinamidopropyl-glass. The nature of sulphydryl oxidase-catalysed GSH oxidation is contrasted with that of γ -glutamyltransferase-initiated GSH oxidation.

Experimental

Synthesis of cysteinylsuccinamidopropyl-glass

Controlled-pore glass beads [200nm (2000 Å) pore diam., 80/120 mesh; Sigma Chemical Co., St. Louis, MO, U.S.A.] were cleaned with concentrated HNO_3 at 100°C for 45 min and washed with distilled water until the wash pH was neutral (Hawk *et al.*, 1972). It should be noted that, after any of the derivatization steps described below, the glass beads can be dried in an oven at 125°C, and the dry derivatized matrix can be stored indefinitely.

Aminopropyl-glass was synthesized by the aqueous method of Taylor & Swaisgood (1974). After washing and drying the beads, derivatization was qualitatively monitored with TNBS (Cuatrecasas, 1970). Succinylation (carboxypropionylation) of the aminopropyl derivative was performed by a modification of the method of Merrifield (1963), with 10% (v/v) triethylamine in acetone rather than chloroform. The degree of reaction completion was ascertained using the TNBS procedure.

L-Cysteine (50 ml of 0.1 M-cysteine/20 g of beads)

was covalently attached to the succinamidopropyl matrix by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide to activate carboxy groups in either a simultaneous activation-immobilization or a sequential activation-immobilization procedure (Cho & Swaisgood, 1974; Janolino & Swaisgood, 1982). Both procedures functioned equally well, although the latter method may be preferable, since it eliminates the possibility of polymer formation. The derivatized glass was washed thoroughly with water and the cysteine 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 a column of the beads. The concentration of covalently attached thiol groups was determined by adding 12 ml of 0.1 mM-Nbs₂ in sodium phosphate, pH 8, to 0.5 ml (220 mg) of glass beads and reading the absorbance of the supernatant at 412 nm.

Isolation of sulphydryl oxidase

Fresh bovine milk was obtained from the University dairy herd and skimmed by centrifugation at 4000 g for 30 min at 30°C. Whey was prepared by incubating skim milk with chymosin [2 mg of chymosin (rennin; Sigma Chemical Co.) per litre of skim milk] for about 30 min at 30°C and centrifuging at 16 300 g for 45 min at 4°C to remove the curd. Crude skim-milk membranes were obtained from whey either by precipitation at 60%-satd. $(\text{NH}_4)_2\text{SO}_4$ (Janolino & Swaisgood, 1975) or by a rapid ultrafiltration method using a Millipore Pelli-con-Cassette system equipped with a 100 000-M_w exclusion membrane. For the latter method, approx. 1 litre of whey was diafiltered with 6 litres of 0.047 M-sodium phosphate, pH 7, and the turbid solution retained by the membrane was concentrated to about 150 ml. Diafiltration and concentration were completed within roughly 1 h at room temperature. The diafiltered concentrated protein solution was solubilized with a non-ionic detergent [1% (w/v) C₁₂E₉ or 30 mM- β -octyl D-glucopyranoside; Sliwowski *et al.*, 1982]. After incubation with the detergent for at least 30 min at 4°C, the mixture was centrifuged at 100 000 g for 1 h at 4°C and the resulting clear supernatant fraction was removed with a syringe, care being taken to avoid contamination by the white material floating on the surface.

Sulphydryl oxidase was covalently bound to the affinity matrix by recirculating solubilized skim-milk membranes through a 60-ml column of cysteinylsuccinamidopropyl-glass beads for 4 h at 33°C. Adsorbed materials were removed by washing the beads with 1 litre of 0.047 M-sodium phosphate, pH 7, containing the solubilizing detergent. Finally, the enzyme was reductively released from the matrix by recirculating 100 ml of 2 mM-GSH in the phosphate buffer until no thiol groups could be detected with

Nbs₂. The sulphhydryl oxidase solution was adjusted to 0.5 M with enzyme-grade sucrose to help stabilize the activity. The matrix can be prepared for re-use by reduction as described above.

Enzymic assays

Three methods were used to monitor sulphhydryl oxidase activity. The standard assay was performed at 35°C with 0.8 mM-GSH in 0.047 M-sodium phosphate, pH 7. Depletion of thiol groups was measured by reaction with Nbs₂; accordingly, 85 μ l of assay solution was added to 1.0 ml of 100 μ M-Nbs₂ containing 10 mM-EDTA in sodium phosphate buffer, pH 8.0. The absorbance was measured at 412 nm (Ellman, 1959). Alternatively, O₂ consumption was measured with a Yellow Springs Instruments oxygen monitor equipped with a Clark electrode. Buffers were gassed with compressed air while being stirred at 35°C for at least 8 min before assay. Enzyme was added with continued stirring while a stable baseline was established for 3 min. Reaction was initiated by addition of a small volume of concentrated substrate solution (50 μ l of 48 mM-GSH). The initial concentration of dissolved O₂ was determined directly using the phenazine methosulphate/NADH method of Robinson & Cooper (1970). The product, H₂O₂, was also measured by using horseradish peroxidase and ABTS. A 0.5 ml portion of the standard assay mixture was treated with 0.2 ml of 20 mM-N-ethylmaleimide in sodium acetate, pH 5.5, for 10 min to block thiol groups that had not reacted. Peroxide was measured by addition of 0.5 ml of the resulting solution to 2.5 ml of the acetate buffer containing 3 μ mol of ABTS and 75 μ g of peroxidase. The absorbance of the dye was measured at 436 nm.

Glutathione assay mixtures were also analysed for all substrates and products using h.p.l.c. The progress of the reaction was monitored by h.p.l.c. analysis for GSH, GSSG, cysteinylglycine, cystinylbisglycine and the mixed disulphide using the procedure reported previously (Sliwowski *et al.*, 1981). Glutathione reductase-coupled assays were conducted as described by Griffith & Tate (1980).

γ -Glutamyltransferase activity was measured as described by Griffith & Tate (1980) using 1.0 mM- γ -glutamic acid *p*-nitroanilide and 20 mM-glycylglycine in 0.1 M-Tris/HCl, pH 8.0. Activity is expressed as μ mol of *p*-nitroaniline released per min (units). Alkaline phosphatase activity was measured (Kitchen, 1974), with *p*-nitrophenyl phosphate as substrate. Xanthine oxidase was assayed spectrophotometrically with 60 μ M-xanthine as substrate as described by Rowe & Wyngaarden (1966). Lactoperoxidase activity was determined as described in the *Boehringer Mannheim Manual* (1973) with 23.5 mM-ABTS and 0.26 mM-H₂O₂ as substrates.

Protein concentration was determined by the

dye-binding method of Bradford (1976) with a Bio-Rad (Richmond, CA, U.S.A.) kit. Samples containing detergent were precipitated with 4 vol. of cold acetone and the centrifuged pellets were dissolved in phosphate buffer. Standards were treated similarly.

Gel electrophoresis and molecular-sieve chromatography

Electrophoresis was conducted in 7.5% (w/v) acrylamide slabs with 2.6% cross-linking and 0.1% SDS or 3.5% acrylamide with similar cross-linking in the presence of β -octyl glucoside. The phosphate buffer system and Coomassie Blue-staining procedure of Weber *et al.* (1972) were used. Silver staining of 0.75 mm-thick vertical slabs was performed by a modification of the procedure of Merrill *et al.* (1981). Gels were washed overnight in solutions of decreasing alcohol and acid concentration (40–10% ethanol; 10–5% acetic acid) and stained by incubation for 30 min in potassium dichromate followed by 60 min in AgNO₃.

Dual protein-separation columns I-250 and I-125 (Waters Associates) were connected in tandem and eluted with 0.05 M-sodium phosphate, pH 7.0, containing 1% (w/v) C₁₂E₉ at a flow rate of 1.0 ml/min. Thyroglobulin and GSH were used to determine the mobile- and stationary-phase volumes respectively.

Immunization and immunoprecipitation

Purified sulphhydryl oxidase (approx. 600 μ g) was concentrated to 1.5 ml by ultrafiltration using an Amicon XM-50 membrane. This material was emulsified with an equal volume of Freund's complete adjuvant and injected intradermally into the flank area of a New Zealand White rabbit at 10–12 different sites. Similar injections were given after 2 weeks. At 1 month after the initial injections, an additional 600 μ g of enzyme was administered in three intravenous injections on alternate days. In the subsequent week, 30 ml of blood was collected and the anti-(sulphhydryl oxidase) titre was determined by the precipitin test using whey or dialyzed whey.

Immunoglobulin G was purified by batch treatment of the antisera with DEAE-Sephadex in 50 mM-sodium phosphate, pH 6.5. The resin was removed by centrifugation and the supernatant fraction, containing immunoglobulin G, was stored at –20°C.

Quantitative immunoprecipitation was performed by incubating 250 μ l of solubilized skim-milk membranes with various amounts of purified antibody. Control incubations omitting membranes were also prepared. The mixtures were held overnight at 4°C and then centrifuged for 20 min at 2850 rev./min in an IEC model CL table-top centrifuge (in a cold-room at 4°C). Resulting supernatant fractions were assayed for sulphhydryl oxidase activity (peroxide assay) and γ -glutamyltransferase.

Results

The structure of the cysteinylsuccinamidopropyl-glass derivative and an outline of the covalent-affinity-chromatographic steps employed in the present study are illustrated in Scheme 1. Several other glass matrices, including mercaptopropyl-, cysteinylamidopropyl-, cysteinylamidohexanamido-succinamidopropyl- and cysteinylglycylglycylamidopropyl-glass derivatives, were also synthesized and tested for specific covalent interaction with sulphhydryl oxidase. These matrices were chosen on the basis of previously determined substrate requirements of the enzyme (Swaisgood & Horton, 1980; Clare, 1981). All derivatives examined appeared to function equally well, with the notable exception of mercaptoethanol is not a substrate). The cysteinylsuccinamidopropyl matrix was chosen for subsequent studies, since it was relatively easy to synthesize with a high degree of derivatization as measured by Nbs₂ titration. Thus 3 μ mol of thiol groups per g of porous glass were obtained corresponding to 6.8 μ mol per ml of settled support.

A possible mechanism by which sulphhydryl oxidase binds to cysteinylsuccinamidopropyl-glass is shown in Scheme 1. Formation of at least a binary complex between the matrix and enzyme is postulated. A possible ternary complex involving an active form of oxygen might also be envisioned. The covalent nature of the bond is substantiated by the

observations that enzyme is selectively eluted by thiols and that the bond is stable to solutions containing non-ionic detergents, 4 M-urea or 1 M-NaCl.

In the present study, crude skim-milk membranes were prepared either by precipitation with (NH₄)₂SO₄ or by ultrafiltration through large-pore membranes (Scheme 2). After solubilization, re-circulation of these preparations through a column of cysteinyl-glass for 4 h was sufficient to allow maximum binding of sulphhydryl oxidase. Adsorbed proteins were subsequently removed by washing with a non-ionic detergent solution. Covalently bound enzyme could be reductively eluted with a variety of thiols: GSH, cysteine, or dithiothreitol.

Choice of detergent, C₁₂E₉ or β -octyl D-glucopyranoside, used for initial solubilization or desorption, did not affect the purity of the final product as judged by SDS/polyacrylamide-gel electrophoresis. However, stability of purified sulphhydryl oxidase to further manipulations such as concentration was enhanced by addition of octyl glucoside (30 mM) as well as sucrose (0.5 M).

An approx. 4000-fold purification of sulphhydryl oxidase activity from whey was achieved by using skim-milk membranes prepared from diafiltered whey, whereas the purification realized by precipitation with (NH₄)₂SO₄ preparations was somewhat less (Table 1). The relative purification obtained with respect to various contaminating activities was also dependent upon the starting material (Table

Scheme 1. Covalent affinity chromatography of sulphhydryl oxidase on cysteinylsuccinamidopropyl-glass

Scheme 2. Isolation of sulphhydryl oxidase from bovine milk by covalent chromatography

2). Use of $(\text{NH}_4)_2\text{SO}_4$ for preparation of skim-milk membranes causes precipitation of the 'globulin' fraction of whey (McKenzie, 1971), thus giving rise to large amounts of the soluble enzyme lactoperoxidase in these preparations (Table 2). Lactoperoxidase, which has been observed to interact with both soluble and immobilized thiols (Pommier & Cahnmann, 1979), binds to the cysteinylsuccinamido-propyl-glass, and a small portion is subsequently eluted during GSH treatment. However, covalent chromatography of diafiltered concentrated whey circumvents this problem, since diafiltration using a 100000- M_r -exclusion membrane removes 99% of the lactoperoxidase activity normally precipitated by 60% saturation with $(\text{NH}_4)_2\text{SO}_4$. Additionally, γ -glutamyltransferase is completely resolved from sulphhydryl oxidase by covalent chromatography of solubilized diafiltered whey.

Electrophoresis and molecular-sieve chromatography indicated substantial homogeneity for the enzyme prepared by covalent affinity chromatography. In SDS/polyacrylamide-gel electrophoresis, over 75% of the protein migrated as an 80000 Da peptide. Several minor contaminants of both larger and smaller size were detected by silver staining. Electrophoresis under non-denaturing conditions in 3.5% acrylamide gels containing β -octyl glucoside revealed only a single slowly migrating silver-staining band. More than 90% of the u.v.-absorbing material eluted in a single peak in the void volume of

h.p.l.c. protein-separation columns in the presence of the non-ionic detergent C_{12}E_9 . These observations may be reflections of the tendency of sulphhydryl oxidase to aggregate (Janolino & Swaisgood, 1975), even in detergent solutions.

The importance of complete assay of all possible products of the action of sulphhydryl oxidase and γ -glutamyltransferase on GSH when both enzymes are known or suspected to be present has been previously demonstrated (Sliwkowski *et al.*, 1981). Thus solubilized skim-milk membranes and the corresponding enzyme purified by covalent chromatography were assayed by analytical h.p.l.c. monitoring of GSH and its reaction products. All of the intermediate compounds expected from mutual catalysis by sulphhydryl oxidase and the GSH hydrolase activity of the transferase were observed for membrane preparations. The concentrations of two of these compounds, GSSG and cysteinylglycine, were found to proceed through a maximum and then decrease during the time course of the reaction. The mixed disulphide of GSH and cysteinylglycine accumulated slowly but steadily throughout the observation period. Cystinylbisglycine, the ultimate product expected from this mixed catalysis, was observed to accumulate after an initial lag period. Additionally, glutathione reductase-coupled assays resulted in non-linear progress curves with pronounced lag periods for preparations containing both enzymes, in agreement with observa-

Table 1. *Purification of sulphhydryl oxidase by covalent affinity chromatography*
Assay mixtures contained 0.8 mM-GSH in 0.05 M-sodium phosphate, pH 7.0. All assays were performed at 35°C.

Fraction	Volume (ml)	Total protein (mg)*	Depletion of SH†			Formation of H ₂ O ₂ ‡			Consumption of O ₂		
			Total units	Specific activity (units/mg)	Purification (fold)	Total units	Specific activity (units/mg)	Purification (fold)	Total units	Specific activity (units/mg)	Purification (fold)
From diafiltered whey											
Whey	910	8190	40	0.005	1	18.2	0.002	1	21	0.003	1
Solubilized skim-milk membranes	156	565	17	0.031	43	8.6	0.015	8	6.6	0.012	4
Covalent-chromatography-purified enzyme	99	0.545	13	24.2	33	4.0	7.27	22	6	10.9	3640
From 60%-satd.-(NH ₄) ₂ SO ₄ pellet											
Solubilized crude skim-milk membranes	20	377	4.2	0.011	1						
Covalent-chromatography-purified enzyme	20	0.624	2	3.2	48						

* Protein was determined by Bradford's (1976) method using Bio-Rad reagents. Fractions containing detergent were first precipitated with 4 vol. of cold acetone and the pellets dissolved in phosphate buffer. Standards were treated similarly.

† Depletion of SH groups was monitored by using the Nbs₂ reaction as described in the Experimental section.

‡ H₂O₂ formation was measured with horseradish peroxidase using ABTS after prior reaction with *N*-ethylmaleimide as described in the Experimental section.

Fig. 1. *Glutathione reductase-coupled assays of sulphhydryl oxidase-containing fractions*

Incubation mixtures (1.0 ml) contained glutathione reductase (0.15 unit), NADPH (0.350 mM) and 85 μ l of enzyme. A, whey; B, skim-milk membranes; C, fraction which did not bind to the Cys-glass column; D, enzyme purified by covalent chromatography. Reactions were initiated by adding 50 μ l of 48 mM-GSH. The curves represent tracings from the spectrophotometric output.

tions by Griffith & Tate (1980) for transferase-associated GSH-oxidative activity (Fig. 1).

In contrast, with sulphhydryl oxidase purified by covalent chromatography, h.p.l.c. analysis showed that all of the GSH consumed was converted directly into GSSG and no further degradation was observed. Furthermore, glutathione reductase-coupled assays conducted with purified sulphhydryl oxidase produced linear progress curves exhibiting no lag periods.

Stoichiometry of sulphhydryl oxidase-catalysed GSH oxidation, as described by Janolino & Swaisgood (1975), was confirmed for the enzyme purified by covalent chromatography. The formation of H₂O₂ (Table 1) was substantiated using horseradish peroxidase-catalysed ABTS oxidation after removal of interfering thiols (Randall, 1946) by *N*-ethylmaleimide treatment. In addition, the rate of O₂ consumption observed in the presence of both catalase and purified sulphhydryl oxidase was one-

Table 2. *Enzymic activities measured in various fractions obtained during sulphhydryl oxidase purification*(I) Isolation from solubilized 60%-satd.-(NH₄)₂SO₄ pellet of whey

Enzyme	Soluble skim-milk membranes [60%-satd.-(NH ₄) ₂ SO ₄ pellet]		Non-binding fraction		Fraction eluted by GSH	
	Sp. activity (units/mg)	Total units	Sp. activity (units/mg)	Total units	Sp. activity (units/mg)	Total units
γ -Glutamyltransferase	12.1	242	6.7	134	0.93	0.58
Lactoperoxidase	46.4	928	0.012	0.24	0.34	0.21
Xanthine oxidase	0.08	1.6	0.039	0.79	0.000	0.00
Alkaline phosphatase	0.06	1.3	0.000	0.00	0.000	0.00

(II) Isolation from solubilized diafiltered whey

Enzyme	Whey		Solubilized membranes		Non-binding fraction		Fraction eluted by GSH	
	Sp. activity (units/mg)	Total units	Sp. activity (units/mg)	Total units	Sp. activity (units/mg)	Total units	Sp. activity (units/mg)	Total units
γ -Glutamyltransferase	0.239	1950	2.36	1330	0.838	384	0.00	0.00
Lactoperoxidase	*	*	0.004	2.1	0.000	0	0.00	0.00
Xanthine oxidase	*	*	0.002	1.1	0.014	6.27	0.00	0.00
Alkaline phosphatase	*	*	0.016	9.2	0.002	0.94	0.00	0.00

* Not determined.

half the rate observed for catalysis by sulphhydryl oxidase alone.

Perhaps the most convincing evidence for existence of a distinct oxidase in bovine milk is shown in Fig. 2. Quantitative immunoprecipitation of sulphhydryl oxidase activity resulted when solubilized skim-milk membranes were treated with antibody raised against the enzyme which had been purified by covalent chromatography. In marked contrast, none of the γ -glutamyltransferase activity of the membrane fraction was lost upon treatment with the antibody (Fig. 2).

Discussion

The present work was undertaken to develop a method for sulphhydryl oxidase purification that would effectively resolve it from γ -glutamyltransferase. A previously described procedure for isolation of the enzyme from bovine milk (Janolino & Swaisgood, 1975) involves a lengthy three-step centrifugal method starting with (NH₄)₂SO₄ precipitation of crude skim-milk membranes. Also, the final product contains small amounts of γ -glutamyltransferase (Sliwkowski *et al.*, 1981). A preliminary indication that covalent affinity chromatography might be useful in purifying sulphhydryl oxidase was obtained in earlier studies with a GSH-matrix (Abraham, 1979); however, these

experiments were compromised by an inability to regenerate the thiol-matrix and by low yields.

A re-evaluation of various contaminating activities in milk, together with studies of the substrate specificity of sulphhydryl oxidase, led to design of the matrix employed in the present investigation. Thus covalent chromatography of detergent-solubilized skim-milk membranes on cysteinylsuccinamidopropyl-glass was found to yield a highly purified preparation of enzyme. Most significantly, this chromatographic procedure results in the first complete separation of sulphhydryl oxidase from γ -glutamyltransferase.

The specific activity of sulphhydryl oxidase purified by affinity covalent chromatography is comparable with, and indeed somewhat higher than, that of this enzyme isolated by the earlier procedure (Janolino & Swaisgood, 1975). This may reflect the tendency of covalent chromatography to select for active forms of the enzyme, as has been reported in the case of isolation of papain and other thiol proteinases on mercuri-agaroses (Mole & Horton, 1973; Brocklehurst *et al.*, 1974).

CNBr-activated agaroses have been more frequently employed than controlled-pore glass as matrices for covalent as well as affinity chromatography. However, porous glass provides several advantages as a chromatographic medium. The inherent rigidity of glass is amenable to high flow

Fig. 2. Quantitative immunoprecipitation reaction of sulphydryl oxidase

Rabbit antibodies were raised against sulphydryl oxidase that had been purified by covalent chromatography. Incubations were performed with solubilized skim-milk membranes. Membranes (250 μl) in 47 mM-phosphate, pH 7.0, containing 1% C₁₂E₉ were incubated with various amounts of anti-(sulphydryl oxidase), in phosphate buffer, pH 6.5, overnight at 4°C. The γ-globulin fraction, obtained from serum of the immunized rabbit by batch treatment with DEAE-Sephadex, contained approx. 5 mg of protein/ml. The samples were then centrifuged for 20 min. The supernatants were assayed for sulphydryl oxidase (peroxide method) and γ-glutamyltransferase as described in the Experimental section. Symbols: O, oxidase activity; □, transferase activity.

rates and large pressure drops across a column of beads. Also, the use of glass beads eliminates the hydration steps required with agarose, and the glass surface can withstand rapid and drastic changes in solvent composition. Non-specific adsorption of protein is frequently mentioned as a disadvantage of controlled-pore glass; however, derivatized glass surfaces do not share the characteristics of 'naked' glass. In the present studies, rinsing the column with a non-ionic detergent was sufficient to overcome possible problems associated with non-specific protein adsorption. On a volume basis, ligand loading achieved with glass was probably lower than that obtained with agarose, but it is comparable with respect to surface area.

Elimination of interfering thiols from the reaction

mixture by treatment with *N*-ethylmaleimide permitted use of the horseradish peroxidase/dye assay for measuring the H₂O₂ produced during sulphydryl oxidase-catalysed GSH oxidation. Further confirmation of the stoichiometry proposed earlier by Janolino & Swaisgood (1975) was provided by the observation that the presence of excess catalase reduced the rate of O₂ consumption by one-half. Thus the reaction catalysed by the milk oxidase results in H₂O₂ formation, as has also been found for the kidney enzyme known as 'glutathione oxidase' (Ormstad *et al.*, 1979), in contrast with γ-glutamyltransferase-initiated oxidation (Tate *et al.*, 1979), findings that support the mechanistic distinction between these two activities.

The possibility that sulphydryl oxidase and γ-glutamyltransferase exist as a covalently linked multienzyme complex, as suggested previously (Tate *et al.*, 1979; Tate & Orlando, 1979; Iwami *et al.*, 1981), now appears unlikely. In the present study, solubilization was achieved with non-ionic detergents as opposed to limited proteolysis, thereby diminishing the likelihood of an 'artefactual' separation of the two activities. Specific immunoprecipitation of the solubilized oxidase from crude solubilized membrane preparations without decreasing γ-glutamyltransferase activity further substantiates the existence of two distinct proteins.

The data presented here, in conjunction with the findings of others (Griffith & Tate, 1980; Iwami *et al.*, 1981; Ashkar *et al.*, 1981), strongly imply the existence of two types of thiol-oxidative mechanisms: (1) a direct, enzymic oxidation catalysed by sulphydryl oxidase, and (2) a non-enzymic, γ-glutamyltransferase-initiated oxidation presumably mediated by autoxidation of enzymically formed cysteinylglycine and subsequent transhydrogenation reactions. The discrepancy between the immunoprecipitation results presented here and those of Iwami *et al.* (1981) can also be explained by such a dual oxidative mechanism. Thus if only transferase-initiated oxidative activity had been isolated in that study, such activity would be expected to be precipitated by transferase-directed antibodies.

The development of a new, relatively simple, and yet reproducible procedure for isolation of γ-glutamyltransferase-free sulphydryl oxidase and the preparation of antibodies specific for the purified enzyme should assist in the elucidation of the physiological significance of this membrane-associated protein.

This is Paper 8167 of the Journal Series of the North Carolina Agricultural Research Service. This investigation was supported in part by the North Carolina Dairy Foundation and by National Institutes of Health Biomedical Sciences Support Grant RR07071.

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