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CHARACTERISTICS OF SULFHYDRYL OXIDASE ISOLATED BY A SIMPLE CHROMATOGRAPHIC PROCEDURE *

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Summary

Permeation chromatography of whey or centrifugally clarified solutions of $(NH_4)_2SO_4$-whey precipitates on controlled-pore glass (3000 Å pore diameter) yielded a substantially purified form of the enzyme which eluted in the void volume This fraction obtained directly from whey appeared to have the least protein contamination as demonstrated by various types of gel electrophoresis, lack of detectable reactivity with rabbit antibovine xanthine oxidase antibody and high specific activity.

Sulfhydryl oxidase, which catalyzes the oxidation of thiols to disulfides using molecular oxygen as the electron acceptor, is highly aggregated in milk and its state of molecular association is concentration-dependent [1—4]. This property was utilized in development of a centrifugation procedure for isolation of enzyme from whey [1] Purified preparations exhibited two zones, both of which displayed activity, following polyacrylamide electrophoresis but only one zone following electrophoresis in sodium dodecyl sulfate (SDS).

In the present investigation, we have characterized sulfhydryl oxidase preparations (void volume fractions) obtained from permeation chromatography of whey and half-saturated $(NH_4)_2SO_4$-whey precipitate using controlled-pore glass This method allows preparation of sulfhydryl oxidase in a substantially

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purified form using a single step, thus greatly reducing the number of operations and the time previously required.

The starting material for chromatography was various concentrations of either rennin-whey or a crude enzyme fraction. The latter (Fraction C) was precipitated from rennin-whey by half-saturation with $(NH_4)_2SO_4$ [1] Following dialysis against sodium phosphate (pH 7, $\Gamma/2 = 0.1$), the preparation was diluted to a protein concentration of 0.12% which is roughly the concentration of the enzyme in whey. Prior to chromatography this solution was filtered, using a 0.45 μm filter, and in some cases, concentrated with an Amicon TCF-10 ultrafiltration assembly using a PM-10 membrane.

A typical elution profile for direct chromatography of whey, shown in Fig. 1, indicates fractionation into a major void volume fraction (peak I), a 'shoulder' (peak II) and a large peak in the internal volume Identical results were obtained for the Fraction C proteins.

Earlier studies indicated that most of the enzymic activity is located in the void volume fraction [4]. Accordingly, this fraction (Fraction V, including peaks I and II indicated in Fig. 1) as obtained from whey, concentrated whey and various concentrations of Fraction C was assayed for sulfhydryl oxidase. Typical results are listed in Table I. The percent of the total activity recovered

TABLE I

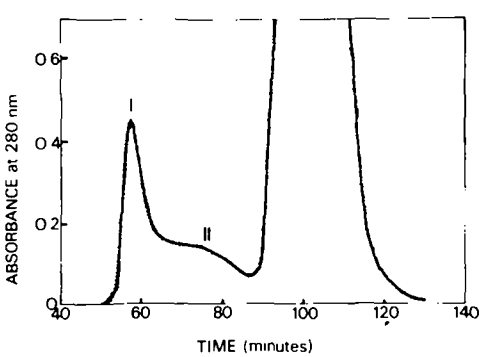
Sulfhydryl oxidase activity, purification, percent recovery and total amino acid content of Fraction V obtained from whey and Fraction C by permeation chromatography using CPG-3000 columns Activity was assayed by following the depletion of -SH groups, measured by reaction with 5,5′-dithiobis(2-nitrobenzoic acid), during enzyme-catalyzed oxidation of reduced glutathione [1] One unit of activity is defined as the oxidation of 1 μmol/min GSH under assay conditions Fraction V, void volume fraction, Fraction C, $(NH_4)_2SO_4$-whey precipitate

	Total units (l whey)	Spec Act (units/mg E)	Purification	Recovery (%)	Percent of total weight represented by amino acid residues [a]
Whey	79 9	0 012	1	100	
CPG-3000 (I)	34 0	3 5	298	46	50 9
CPG-3000 (II)	31 9	4 0	343	43	69 1
Whey, conc (10X)					
CPG-3000 (I)	37 4	1 9	161	51	
CPG-3000 (II)	32 1	1 7	146	43	
Fraction C (0 12%)					
CPG-3000 (I)	32 4	2 6	225	44	39 3
CPG-3000 (II)	17 3	2 4	203	23	48 9
Fraction C, conc (25X)					
CPG-3000 (I)	38 0	2 1	177	51	
CPG-3000 (II)	19 8	2 0	172	27	
Fraction C, centrifuged					
CPG-3000 (I)	29 8	3 3	288	40	66 0
CPG-3000 (II)	24 9	3 7	316	34	81 2

[a] Excluding half-cystine and tryptophan It should also be recalled that sulfhydryl oxidase is a glycoprotein containing 10—15% carbohydrate [1] The quantity of protein in these preparations (g total amino acid residues/100 g enzyme preparation) was determined by amino acid analysis following the procedure of Moore and Stein [5]

in the void volume fraction increased with increasing concentration of whey or Fraction C These observations are consistent with the previously noted concentration-dependency of the degree of aggregation However, such a relationship was not observed for specific activities. Accordingly, Fraction V obtained by chromatography of the least concentrated crude enzyme fractions (whey or Fraction C) gave the highest specific activity Thus, association apparently also results in the inclusion of non-enzyme protein or other constituents in the enzyme particles

It must be emphasized that these preparations contain non-protein contaminants, most likely lipid, in addition to other proteins Consequently, purification results from removal of either or both of these contaminants Centrifugation of a dilute solution of Fraction C was earlier reported to yield the activity in the supernatant fraction [1], thus, rapidly sedimenting impurities were removed by sedimentation at $2000 \times g$ for 30 min at 4°C. Centrifugation of Fraction C prior to chromatography yielded resulting specific activities for void volume fractions which were 30—50% greater than that obtained from Fraction C directly (Table I). Furthermore, the percent of the total weight of these preparations attributable to amino acid residues increased by more than 50% Most likely lipid material, perhaps membrane fragments, are removed by the centrifugation. Essentially all of the weight of peak II of this fraction, which



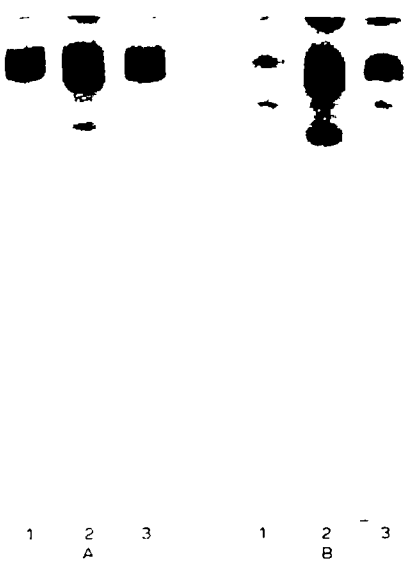


Fig 1 Elution profile for controlled-pore glass chromatography of whey The column (2 5 X 200 cm) contained 3000 Å mean pore diameter glass (CPG-3000, 120/200 mesh, obtained from Electro-Nucleonics, Inc) and was operated at a flow rate of 10 ml/min Proteins were eluted with 0 047 M sodium phosphate, pH 7, following calibration with DNA and riboflavin

Fig 2 Polyacrylamide gel electrophoretic pattern for Fraction V in SDS Tube gels (7 5% acrylamide with 1% β-mercaptoethanol) were electrophoresed in a Bio-Rad Model 150 Electrophoresis Cell using an average loading of 75 μg protein per gel A CPG-3000(I) from (1) whey, (2) dilute Fraction C and (3) centrifuged Fraction C B CPG-3000(II) from (1) whey, (2) dilute Fraction C and (3) centrifuged Fraction C

has the highest specific activity, is accounted for by amino acid residues and the carbohydrate moiety of this enzyme [1,6].

Standard polyacrylamide gel electrophoresis [7] of Fraction V obtained from either whey, Fraction C (0 12%) or centrifuged Fraction C gave essentially the same pattern, namely, a protein band occurred only on top of the spacer gel. However, not all of these preparations are homogeneous as was shown by polyacrylamide gel electrophoresis in SDS [8,9] (Fig. 2). Nevertheless, preparations CPG-3000(I) from whey and CPG-3000(I) from centrifuged Fraction C each gave a single visible protein-staining band in the separating gel

Results of Ouchterlony double-diffusion studies, using rabbit antibodies, indicated that the void volume fractions CPG-3000(I) and CPG-3000(II) all formed precipitation bands when incubated with antibodies against sulfhydryl oxidase Some preparations of the enzyme also displayed precipitation bands against xanthine oxidase antibodies indicating contamination of these preparations with this enzyme However, CPG-3000(I) from whey and CPG-3000(I) from centrifuged Fraction C did not exhibit a second precipitation band resulting from reactivity with xanthine oxidase antibodies.

Thus, sulfhydryl oxidase can be obtained with roughly 300-fold purification from whey or centrifuged Fraction C using a single chromatographic pass through CPG-3000. This substantially purified preparation can be obtained in a few hours, whereas, the previous method requires one week of carefully performed isolation steps. Furthermore, these preparations appear to be of similar purity to that previously reported with respect to protein impurities. This one-step physical method of isolation allows preparation of the enzyme while minimizing the possible formation of artifacts due to the isolation procedure.

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