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BOVINE MILK XANTHINE OXIDASE

PURIFICATION BY ULTRAFILTRATION AND CONVENTIONAL METHODS WHICH OMIT ADDITION OF PROTEASES

SOME CRITERIA FOR HOMOGENEITY OF NATIVE XANTHINE OXIDASE

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Summary

Methodological difficulties have been encountered when proteases were omitted from the conventional isolation of bovine milk xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2). The use of these conventional methods has been studied and modified to reduce the problems encountered. Some of the difficulties may be due to the presence of high concentrations of caseins, which exhibit a wide range of charges and sizes, thereby making separations based on charge and size more complicated. In addition, non-covalent interactions may occur between the caseins and xanthine oxidase leading to the formation of casein-xanthine oxidase micellar aggregates.

The difficulties encountered in this conventional isolation have been circumvented by purifying the enzyme directly from milk fat globule membranes that first have been washed free of most casein and other milk proteins. The xanthine oxidase is isolated by ultrafiltration through an Amicon XM-100A membrane at 5°C in 0.25 M sucrose/5 mM sodium salicylate. The largest molecular size of globular proteins which can penetrate this ultrafiltration membrane has been previously estimated to be around 100 000 daltons. Xanthine oxidase thus appears to be smaller than 100 000 daltons in its native state. The size observed for active xanthine oxidase previously isolated by other methods has been around 275 000—300 000 daltons.

Xanthine oxidase isolated by ultrafiltration appears similar to xanthine

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Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine; %T = (grams of acrylamide + grams of bis-acrylamide) per 100 ml of solution, %C = 100 × (grams of bis-acrylamide) per 100 ml of solution/T according to the nomenclature of Hjerten [42].

oxidase from conventional isolation methods according to empirical criteria of homogeneity based on size and also on the absorbances at 280 and 450 nm. Criteria based on charge were found to be less reliable.

Introduction

Xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2), a complex enzyme derived from the milk fat globule membrane [1,2], requires molybdenum, FAD and iron-sulfur for enzymatic hydroxylation of xanthine to urate [3]. Although the catalytic sequential transfer of electron equivalents from substrate to molybdenum to FAD to iron-sulfur centers of this enzyme reaction has been studied extensively, very little is known about the physical structure of the enzyme [4]. Xanthine oxidase, which has been isolated by procedures involving treatment with proteases, has previously been shown to have a protease-modified structure [5,6]. However, while several attempts have been made to isolate xanthine oxidase in the absence of proteases, methodological difficulties have been encountered in purifying the enzyme to homogeneity.

This paper describes the purification of xanthine oxidase to physical homogeneity by two quite different methods which yield similar products. The purifications also provide evidence relating to the possible in vivo structure of the enzyme and to probable causes of problems that have been encountered previously during the isolation of xanthine oxidase. Criteria for homogeneity of such preparations are also discussed. A preliminary account of a part of this work has appeared [7].

Experimental section

Materials

Triton X-100, Sepharose 6B, catalase (beef liver), γ-globulin (human, Cohn Fraction II), and xanthine (sodium salt, Grade III) were obtained from Sigma Chemical Co., St. Louis, Mo. Ammeline (2,4-diamino-6-hydroxy-s-triazine) was purchased from K and K Laboratories, Plainview, N.Y. Sephadex G-25, G-75 (Superfine) and G-200 and 1.5 × 90 cm columns were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Acrylamide, bis-acrylamide, and TEMED were obtained from Eastman Kodak Co., Rochester, N.Y. Acrylamide and bis-acrylamide were recrystallized twice from acetone [8]. TEMED was redistilled under vacuum at 22°C. Urea was recrystallized twice from hot ethanol. Hydroxyapatite was prepared as described by Tiselius et al. [9].

Methods

Gel filtration chromatography. Gel filtration chromatography was carried out with either Sepharose 6B or Sephadex gels swollen in the appropriate buffers. Prior to the addition of a protein sample, gel columns were equilibrated at 5°C with at least three column volumes of elution buffer or until the eluate had a constant absorbance at either 220 or 280 nm. A Mariotte flask maintained a pressure head of 20 cm during column chromatography.

Sedimentation velocity. Sedimentation velocity experiments were done at 20°C using Schlieren optics in a Beckman Model E Analytical Ultracentrifuge

using an AnD rotor. A Kodak 77-A filter was used over the mercury lamp of the centrifuge for most runs [10]. Samples were prepared for sedimentation velocity by dialysis for 24 h against three changes of buffer at 5° C. The final diffusate was used in the reference chamber of a double-sector cell. Peak positions were measured with a Nikon Shadowgraph. These data were used to calculate an observed sedimentation coefficient (S_{obs}) at 20° C.

Ultrafiltration. A 500 ml stirred chamber which accepted 72-mm diameter Amicon membrane filters was used for ultrafiltration. Amicon PM-10 and XM-100A membranes were obtained from Amicon Corp., Lexington, Mass. Ultrafiltrations were carried out at 5° C at pressures between 5 and 10 lbs/inch^2 with XM-100A membranes and 35 lbs/inch^2 with PM-10 membranes using either argon or nitrogen. The membranes were prepared for ultrafiltration according to the directions of the manufacturers and then pre-ultrafiltered with the solvent system that was to be used.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was done using a Buchler Polyanalyst which allowed temperature regulation of the lower chamber. Either 5%T and 2.6%C [11] or 7.2%T and 2.6%C [12] gels were prepared in 6-mm inner diameter glass tubes. Prior to polymerization the stock mixture of acrylamide/bis-acrylamide was deionized by filtration through a mixed-bed ion-exchange column. The buffer systems used were either those of Davis [12] or of our own formulation. Samples containing sucrose and bromophenol blue tracking dye were layered below the upper reservoir buffer onto the gel surface. Gels were polymerized with *n*-butanol on the top to achieve a flat gel surface [13]. Gels stained for xanthine oxidase activity [14] were immediately placed in assay mixtures after marking the position of the tracking dye front. Gels were stained for protein with either amido black in 7.5% acetic acid or with Coomassie Brilliant Blue in 10% isopropanol/10% acetic acid and destained in a Hoefer Diffusion Destainer containing mixed-bed ion-exchange resin for dye removal. The periodic acid-Schiff method of Fairbanks et al. [15] was used to detect the presence of glycoproteins in polyacrylamide gels.

Delipidation. Delipidation was carried out with either ethanol/diethyl ether (3 : 1, v/v) or $CHCl_3/CH_3OH$ (2 : 1, v/v) as described by Rouser et al. [16].

Absorption spectra. The absorption spectrum of xanthine oxidase preparations at both ultraviolet and visible wavelengths was recorded using a Cary 14 spectrophotometer with a 0—1.0 slidewire. Matched quartz cuvets with either 2- or 10-mm light paths were used. The absorption of xanthine oxidase at 450 nm (partly FAD, partly iron-sulfur centers) was also used to identify the elution position of the enzyme.

Enzyme assays. All enzyme assays were carried out at room temperature using either a Gilford 240 or Cary 14 spectrophotometer. A unit of xanthine oxidase activity was defined as the amount of enzyme which caused one unit change in absorbance/min at 295 nm in the presence of 0.15 mM xanthine [17]. The assay was carried out in 0.05 M potassium phosphate (pH 7.8)/ 0.005% EDTA. Alkaline phosphatase activity was measured using the method described in Sigma Bulletin 104 (Sigma Chemical Co., St. Louis, Mo.).

Preparation of dialysis tubing. All dialysis tubing was treated by heating twice to boiling in 0.01% EDTA solutions and washing with distilled water.

Complete recovery of xanthine oxidase activity was demonstrated after multiple dialyses in tubing prepared in this way.

Isolation of xanthine oxidase from milk or cream by conventional methods (Method A). This procedure, used with either milk or cream, does not include the addition of proteolytic agents contained in pancreatin. The procedure uses conventional techniques and was developed from earlier sources [17–21]. Other purifications omitting proteases and based on some of these sources have recently been published [22,23].

(1) Cooled (5° C) unpasteurized milk or cream pooled from genetically varied cows was obtained from a local dairy (Sealtest, Memphis, Tenn.). Sodium salicylate and EDTA were immediately added to concentrations of 2 mM salicylate and 0.01% EDTA. 1 l of this mixture was stirred into 1 l buffer containing 8 mM sodium salicylate, 4.0 mM cysteine and 0.20 M $K_2PO_4 \cdot 3H_2O$ at 50° C; the final pH was 7.8–7.9. This mixture was then incubated at 40° C for 2 h with constant stirring. All the remaining procedures were done at 5° C.

(2) Triton X-100 was added to the cooled mixture to a concentration of 1% (v/v). The mixture was stirred for 15 min.

(3) 200 g $(NH_4)_2SO_4$ per l was stirred into the chilled mixture. After 15 min the preparation was centrifuged at 12 000 × *g* for 30 min using a Sorvall GS-3 rotor. Several layers formed: an upper liquid layer covered a solid butter fat layer, below which was an opalescent yellow-green liquid layer. A white precipitate was packed in the bottom of the bottle. The liquid layers were filtered through a glass wool plug and combined; 70 g $(NH_4)_2SO_4$ per l filtrate was added. After stirring for 15 min the solution was centrifuged at 12 000 × *g* for 30 min. The pink-brown precipitate was dissolved in a minimal volume of 0.10 M sodium pyrophosphate (pH 7.15)/2 mM sodium salicylate/0.005% EDTA, yielding an orange-brown solution. The yield of xanthine oxidase activity was followed beginning with this solution.

(4) This solution was put through a column (5.0 × 72 cm) of Sephadex G-75 (Superfine) equilibrated with 0.10 M sodium pyrophosphate (pH 7.15), 0.005% EDTA and 2 mM sodium salicylate. Xanthine oxidase emerged in the void volume and was concentrated to about 10 ml by ultrafiltration using an Amicon XM-100A membrane.

(5) The concentrate was prepared for sorption to hydroxyapatite either by passage through a Sephadex G-25 column or by three dialyses against 1000 vol. 0.01 M potassium phosphate buffer (pH 6.8)/2 mM sodium salicylate. Sufficient hydroxyapatite (equilibrated with this buffer) was added to bind all xanthine oxidase. Xanthine oxidase bound to hydroxyapatite was recovered by low speed centrifugation, washed with 0.01 M potassium phosphate buffer and centrifuged. The enzyme was recovered by treatment with the smallest possible volumes of the following series of pH 6.8 phosphate buffers: 0.05 M twice, 0.10 M twice, 0.15 M twice, 0.20 M twice, 0.30 M once, and 0.40 M once. The exact volumes used varied and depended on the total amount of hydroxyapatite and xanthine oxidase in the preparation. 70–80% of the xanthine oxidase eluted in the 0.20 M step as an orange-brown solution. The remainder eluted in the 0.30 M step. The fractions with enzyme activity were concentrated either with an Amicon XM-100A membrane or by $(NH_4)_2SO_4$ precipitation.

(6) The concentrated solution was layered on a Sephadex G-200 column (2.5 × 195 cm) or on a Sephadex G-200 column connected in series to a column containing Sepharose 6B (both 1.5 × 90 cm). These columns were equilibrated with either 0.10 M potassium phosphate (pH 7.0)/0.005% EDTA, or 0.10 M sodium pyrophosphate (pH 7.15)/0.005% EDTA. This column series will be designated G-200/6B throughout the paper.

Isolation of xanthine oxidase from milk fat globule membranes by ultrafiltration methods (Method B). Milk fat globule membranes were isolated from fresh milk by the method of Dowben et al. [24] with some minor modifications. The isolation consisted of the following steps:

(1) Isolation: (a) Raw warm (35°C) milk was placed in an insulated container with sufficient sodium salicylate and EDTA to make final concentrations of 5 and 10 mM, respectively. (b) The raw milk was centrifuged with a swinging bucket rotor at 4600 × *g* for 30 min at 35°C within 30 min of milking time. The thin wafer of pink milk fat which appeared at the top of the bottle was recovered and gently dispersed in a volume of 0.25 M sucrose containing 5 mM sodium salicylate which was equal to the original milk volume. The solution was recentrifuged at 4600 × *g* for 30 min at 35°C. The pink fat, recovered as before, was washed four times by this procedure. The washed fat was put into a large flask and stored at 5°C overnight to cool the material for the next step. (c) Solidified fat globule material was disrupted by churning at low speed on a rotary shaker at 5°C until small balls of white fat were observed. The churned

TABLE I

PURIFICATION OF XANTHINE OXIDASE

Procedure	Total activity (units) [a]	Specific activity (I.U./mg) [b]	Yield (mg)	A_{280nm}/A_{450nm}
Conventional method (Method A) (based on a starting volume of 1 l of milk)				
Milk	—	—	16 400	—
After resuspension of 20—27% $(NH_4)_2SO_4$ precipitate in buffer	1248	—	—	10 [c]
After resuspension of hydroxyapatite fractions concentrated with $(NH_4)_2SO_4$	1011	—	—	5.0—6.0 [c]
Gel filtration chromatography				
(a) Sephadex G-200	505	—	—	5.0
(b) G-200/6B	500	1.96—2.6 [d]	7	4.35—4.60
Ultrafiltration method (Method B) (based on a starting volume of 1 l of raw milk)				
Milk	—	—	16 400	—
Fat globule membranes suspension (250 ml)	1000	—	—	—
XM-100A ultrafiltrate of 250 ml aliquot	450	2.6 [d]	8.6	4.6

[a] Units of activity defined as 1 unit change in absorbance at 295 nm/min in 0.15 mM xanthine [17].

[b] Specific activity (I.U./mg) was based on activity to flavin ratio values of 90—120 and conversion to I.U. [20].

[c] Obtained after removal of salicylate anion by dialysis to read the absorbance at 280 nm.

[d] By omitting salicylate from eluate or dialysis buffers at these stages the absorbances at 220 and 280 nm could be followed. However, since salicylate is used to protect the active site of xanthine oxidase, the omission of this anion leads to a decrease in activity. This was expected and observed.

material was centrifuged at 12 000 × *g* for 60 min at 5°C in a Sorvall GS-3 rotor, yielding a solid white upper milk fat layer and a light pink opalescent suspension containing milk fat globule membranes (Fraction A). This membrane material contained both xanthine oxidase and alkaline phosphatase activity [24—26]. The suspension was separated from the solidified milk fat layer by filtration through a glass wool plug. From approx. 8 l raw warm milk, about 2 l Fraction A were produced. The milk fat layer, warmed to 35°C and recentrifuged at 35°C at 12 000 × *g* for 30 min using a GS-3 rotor, yielded a smaller volume (400 ml) of pink-brown membrane suspension (Fraction B) with approximately twice the xanthine oxidase activity per unit volume of Fraction A. The pH of these suspensions was around 5.4.

(2) Isolation of xanthine oxidase from fat globule membranes by ultrafiltration: (a) Concentration of fat globule membranes by ultrafiltration through an Amicon XM-100A membrane at 5°C produced a light orange-brown ultrafiltrate with around 45% of the initial xanthine oxidase activity originally placed in the ultrafiltration cell. The ultrafiltrate lacked alkaline phosphatase activity. (b) The ultrafiltrate was concentrated by ultrafiltration at 5°C with an Amicon PM-10 membrane and produced a dark orange-brown solution.

All steps in the isolation of xanthine oxidase from fat globule membranes were carried out in 0.25 M sucrose/5 mM sodium salicylate. EDTA was omitted from all but the original warm milk since, at concentrations ordinarily used to protect xanthine oxidase, EDTA has been found to strip proteins from certain membranes [15].

Details of both purifications are presented in Table I.

Results

Isolation of xanthine oxidase from milk or cream by conventional methods (Method A)

The heterogeneity of peak fractions chromatographed on either Sephadex G-200 or Sepharose 6B in the presence of 2 mM sodium salicylate, and isolated by Method A up to step 3, was assessed both by polyacrylamide gel electrophoresis carried out in gels 7.2%T, 2.6%C using an alkaline buffer system [12] and by analytical ultracentrifugation. Of the two methods, only the centrifugation yielded clear evidence of heterogeneity. Fig. 1 gives the results of gel electrophoresis over a 100-fold range of protein concentration. High concentrations were used to detect low levels of impurities. At lower protein concentrations a single band (gels a—c) was observed which became more diffuse (gels d—h) at higher protein concentrations. Identical experiments were performed using an activity stain for xanthine oxidase in the absence and presence of 4 M urea in these gels. The results obtained were identical to those in Fig. 1. The same analyses were carried out with this preparation in which samples of enzyme were preincubated with xanthine, ammeline, allopurinol, nitrate, cyanide, and arsenite yielding identical results to Fig. 1. These substances have been found to bind within the active site of xanthine oxidase either reversibly or irreversibly [27].

Continuous polyacrylamide gel electrophoresis was also done with this material in gels 7.2%T, 2.6%C using 0.01 M potassium phosphate buffer (pH

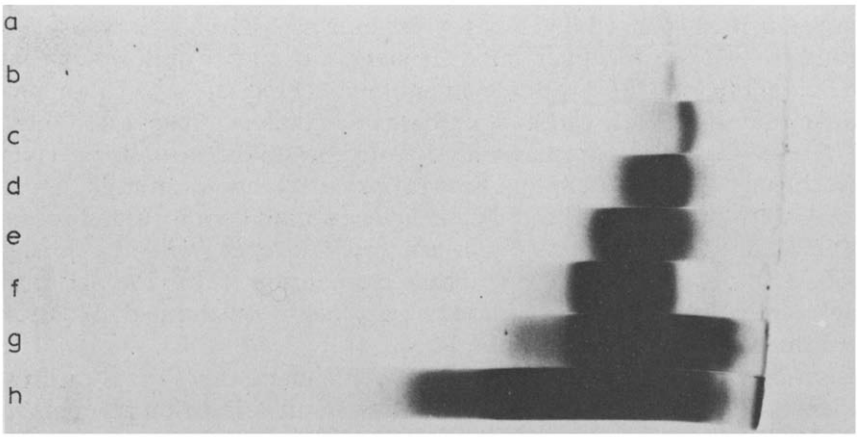


Fig. 1. Polyacrylamide gel electrophoresis of a xanthine oxidase preparation was carried out in gels 7.2%T, 2.6%C. The buffer system of Davis [12] was used for the resolving gel and in both buffer chambers and the stacking gel omitted. 2-Mercaptoethanol (0.01%, v/v), 0.005% bromophenol blue, and sucrose were added to the sample just prior to electrophoresis. The volume of the samples added to each gel was: gel a, 1 μl; gel b, 2 μl; gel c, 3 μl; gel d, 5 μl; gel e, 10 μl; gel f, 25 μl; gel g, 50 μl; and gel h, 100 μl. Each μl contained approx. 5 μg protein. Amido Black was used to stain for protein.

7.0). 0.005% EDTA and 2 mM sodium salicylate. None of the enzyme samples examined either with or without inhibitors demonstrated significant mobility with this buffer system. All gels displayed a single band near the top 1 cm of the gel.

In contrast, when sedimentation velocity experiments were carried out with the material used in the polyacrylamide gel electrophoreses illustrated in Fig. 1, three to four components could be observed (Fig. 2). The observed sedimentation coefficients were 5.9 S, 7.9 S—9.4 S, and 10.3 S. The 9.4 S component could be identified in the original plate as xanthine oxidase by a vertical line bisecting this peak between the two small peaks on the right [10].

Further purification of the material described above in Fig. 1 and 2 was carried out using hydroxyapatite batch methods. Removal of most of the contaminating material observed in sedimentation velocity (Fig. 2) was accomplished with hydroxyapatite prior to the 0.20 M step. Sedimentation velocity indicated that xanthine oxidase was 90% of the protein in the sample after one cycle of hydroxyapatite treatment. One main Schlieren peak (like that of Fig. 6a following) was observed with a vertical bisecting line and barely observable adjacent peaks on either side at diaphragm angles as low as 30°.

This material was chromatographed through a column containing Sephadex G-200 (Fig. 3). These results reflected the pattern observed during sedimentation velocity of this preparation, i.e. one major peak of xanthine oxidase activity and protein (at 280 nm) with adjacent smaller peaks. The fractions containing xanthine oxidase were pooled, concentrated by precipitation with $(NH_4)_2SO_4$, and dialyzed against buffer containing salicylate. Fraction B in Fig. 3 had an A_{280nm}/A_{450nm} ratio of about 5.0 across the peak. The elution pattern of Fraction B displayed a single symmetric peak and maintained a constant A_{280nm}/A_{450nm} ratio of 5.0 across the peak when rechromatographed through the G-200/6B system (Fig. 4). A further rechromatography of this

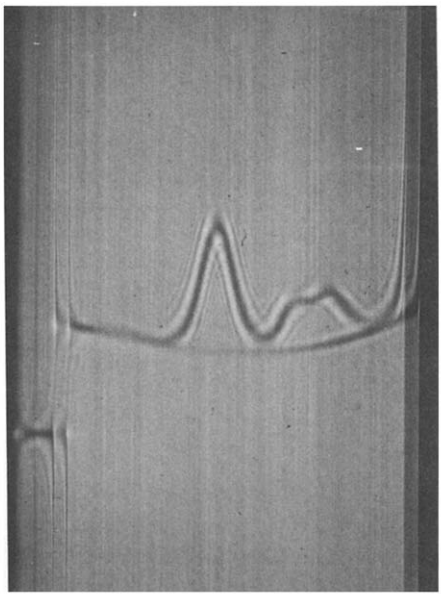


Fig. 2. Sedimentation velocity of material electrophoresed in Fig. 1 was carried out at 20°C at 56 100 rev./min with the diaphragm angle set at 60°. Protein concentration was approx. 12 mg/ml. The buffer contained 0.10 M potassium phosphate (pH 7.0)/0.005% EDTA.

same material in G-200/6B produced identical results.

The absorption spectrum of the pooled and concentrated material from these gel filtrations is shown in Fig. 5. The A_{280nm}/A_{450nm} ratio was 4.6. This spectrum was essentially similar to others which have been reported [20,21].

Sedimentation velocity of rechromatographed Fraction B samples (Fig. 4) was measured at 20°C (Fig. 6). The observed sedimentation coefficients were

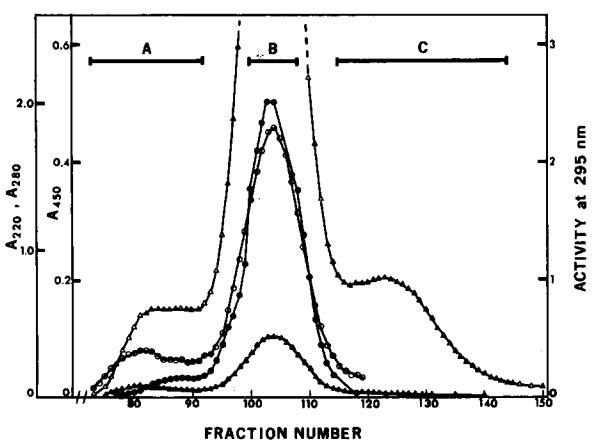


Fig. 3. Sephadex G-200 gel filtration chromatography of a xanthine oxidase preparation after hydroxyapatite treatment was carried out at 5°C in a 2.5 X 195 cm column which was equilibrated and eluted with 0.10 M potassium phosphate (pH 7.0)/0.005% EDTA. The blue dextran (void volume) fractions were 72—82. Absorbance at 220 and 280 nm were read in 2-mm path length cuvets. The absorbance at 450 nm was read in a 10 mm path length cuvet. Symbols: △, A_{220nm}; ▲, A_{280nm}; ○, A_{450nm}; ●, xanthine oxidase activity at 295 nm.

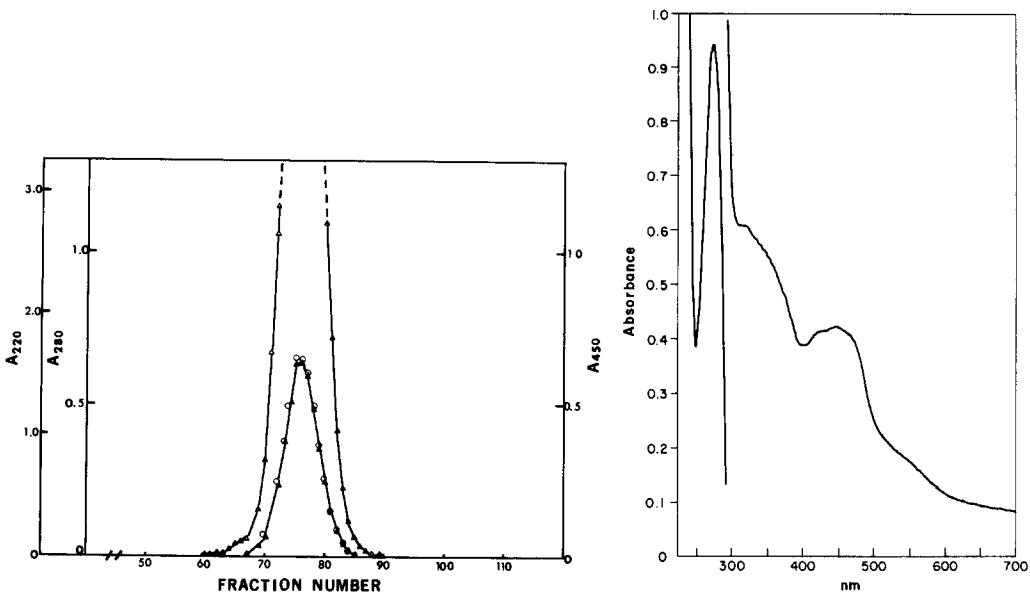


Fig. 4. Sephadex G-200/Sepharose 6B (G-200/6B) gel filtration chromatography of pooled material under B in Fig. 3 was carried out at 5° C. The columns were equilibrated and eluted with 0.10 M potassium phosphate (pH 7.0)/0.005% EDTA. The peak position of blue dextran (void volume) was at fraction 51 and the peak position for dinitrophenol-alanine (total volume) was fraction 133. Absorbances at 220 and 280 nm were read in 2-mm path length cuvets. The absorbance at 450 nm was read in a 10 mm path length cuvet. Symbols: △, A_{220nm}; ▲, A_{280nm}; ○, A_{450nm}.

Fig. 5. The absorption spectrum of homogeneous xanthine oxidase was scanned in a Cary 14. The buffer contained 0.10 M potassium phosphate (pH 7.0)/0.005% EDTA. The blank cuvet contained buffer. The absorbance scale (ordinate) was 1.0—2.0 up to 300 nm and 0—1.0 from 300 to 700 nm.

11.3 S at a protein concentration of 10 mg/ml (Fig. 6a) and 10.4 S at 20 mg/ml (Fig. 6b). Each of the peaks in Fig. 6 displayed a single symmetric component.

Polyacrylamide gel electrophoresis was also carried out with the material displayed in Fig. 6 using a gel 5%T, 2.6%C and an alkaline system [12]. A single diffuse biconvex band was observed (Fig. 7). Glycoprotein was not found when the gel was stained by the periodic acid-Schiff procedure. The material was found to contain small amounts of neutral lipid. Discontinuous polyacrylamide gel electrophoresis was also carried out for an extended period of time with an annular preparative gel. A vertical strip of this annular gel after 72 h running time demonstrated the same type of diffuse protein-stained pattern observed with the analytical gels in Fig. 7.

Isolation of xanthine oxidase from fat globule membranes by ultrafiltration (Method B)

The isolation of xanthine oxidase from either Fraction A or Fraction B by ultrafiltration through an Amicon XM-100A membrane yielded different distributions of xanthine oxidase activity in the retentate and the ultrafiltrate. When 100 ml of Fraction A was concentrated to a volume of 10 ml, the retentate contained about 55% and the ultrafiltrate contained 45% of the initial xanthine oxidase activity. When 100 ml of Fraction B was concentrated in the

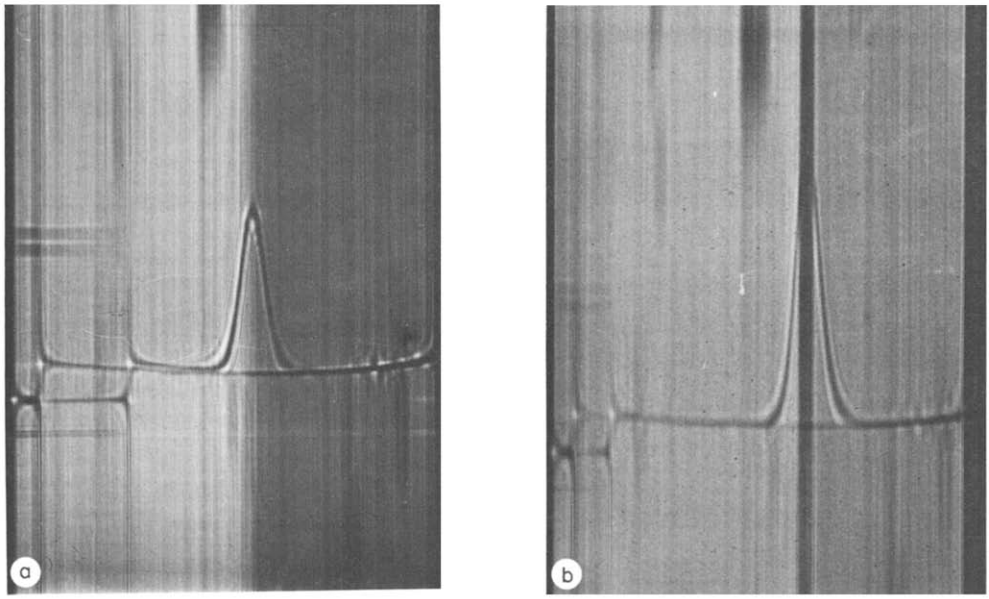


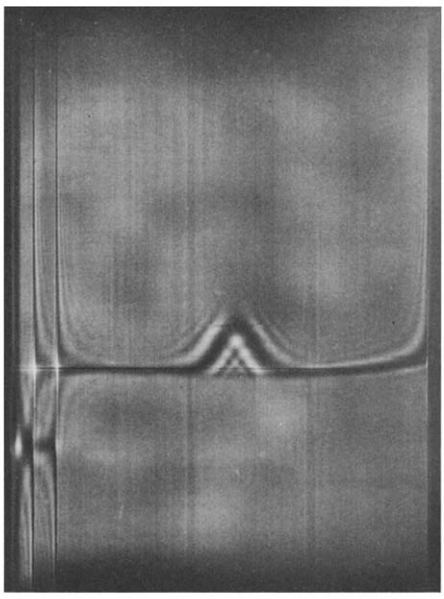
Fig. 6. Sedimentation velocity of homogeneous xanthine oxidase. (a) Sedimentation velocity was carried out at 20°C at 42 040 rev./min with the diaphragm angle set at 70°. Protein concentration was 10 mg/ml. The buffer contained 0.10 M potassium phosphate (pH 7.0)/0.005% EDTA. (b) Sedimentation velocity was carried out under the same conditions described in a except that the protein concentration was 20 mg/ml.

same way, the retentate contained approx. 75% and the ultrafiltrate contained 25% of the initial xanthine oxidase activity. Alkaline phosphatase activity was detected only in the retentates of Fraction A and Fraction B. Refiltration of xanthine oxidase in the ultrafiltrates showed that all of the activity retained its capacity to pass through XM-100A membranes.

Recent observations using gel filtration chromatography of buttermilk with Sepharose 2B [2] demonstrate the distribution similar to what we found with Fraction A. These investigators found about 42% of the original xanthine oxidase activity in their membrane fractions (BM_1) and 46% in their membrane-free fractions (BM_2) but also found significant alkaline phosphatase activity in BM_2. We observed about 55% of the xanthine oxidase activity in the membrane concentrate (retentate) and 45% in the ultrafiltrate without alkaline phosphatase activity.

The sedimentation velocity Schlieren patterns of xanthine oxidase isolated from Fraction A and Fraction B were identical and the observed sedimentation coefficient in 0.15 M NaCl was 11.3 S (Fig. 8). Chromatography of the ultra-

Fig. 7. Polyacrylamide gel electrophoresis of homogeneous xanthine oxidase was carried out in a gel 5%T, 2.6%C with the same buffers described in Fig. 1. 2-Mercaptoethanol (0.01%, v/v), 0.005% bromophenol blue, and sucrose were added to this 50 μg sample just prior to electrophoresis. Coomassie Brilliant Blue was used to stain for protein.



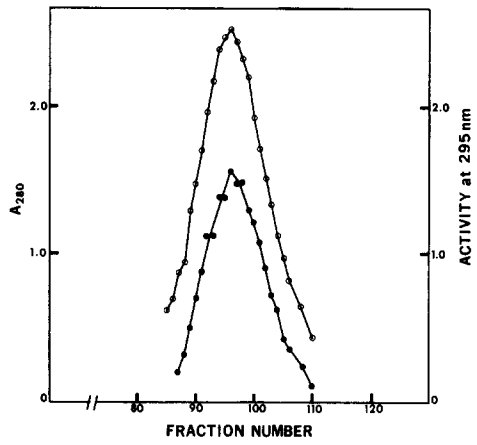


Fig. 8. Sedimentation velocity of this xanthine oxidase ultrafiltrate was carried out at 20°C at 42 040 rev./min with the diaphragm ange set at 45°. The concentration of the protein was approx. 5 mg/ml. Protein was dialyzed extensively against 0.10 M NaCl/0.005% EDTA to remove 0.25 M sucrose prior to ultracentrifugation.

Fig. 9. Sepharose 6B gel filtration chromatography of the xanthine oxidase ultrafiltrate was carried out at 5°C in a 1.5 X 90 cm column equilibrated and eluted with 0.10 M sodium pyrophosphate (pH 7.15)/ 0.005% EDTA. Symbols: ○, A_{280nm} and ●, xanthine oxidase asctivity at 295 nm. This is the same material observed in Fig. 8.

filtrates on Sepharose 6B demonstrated one coincident peak of protein and enzyme activity (Fig. 9). The absorption spectrum of this material was determined and found to be similar to Fig. 5. The A_{280nm}/A_{450nm} ratio of the pooled and concentrated fractions from this xanthine oxidase peak was 4.6.

Separation of xanthine oxidase from fat globule membranes by gel filtration with Sepharose 6B was also attempted and found to be much slower and less effective than ultrafiltration. Therefore it was not further tested.

Discussion

Criteria of homogeneity

Not until recently have several criteria of homogeneity begun to be applied to a xanthine oxidase preparation isolated in the absence of the proteases [22]. Ideally the criteria needed to establish the purity of xanthine oxidase would be a demonstration of both charge and size homogeneity.

Charge homogeneity would be demonstrated when a single sharp band was observed during polyacrylamide gel electrophoresis using a discontinuous buffer system in a series of gels with decreasing %T at constant %C, or when a single coincident peak of protein and enzyme activity was eluted from an ion-

exchange column. In Fig. 1 a single, but diffuse, band was observed with polyacrylamide gel electrophoresis over a 100-fold protein concentration range. An identical band was also observed with activity stain using the same xanthine oxidase preparation and gel buffer system.

Size homogeneity would be indicated if a single component with a symmetric peak were observed in repeated gel filtrations and in sedimentation velocity at various protein concentrations. Sedimentation velocity of the electrophoretically homogeneous preparation shown in Fig. 1 yielded several components but only one could be identified as xanthine oxidase (Fig. 2). The coincidence of the 220- and 280-nm peaks with FAD and iron-sulfur center absorption at 450 nm was also a useful indication of homogeneity (Fig. 4). In addition, a constant A_{280nm}/A_{450nm} ratio of 5.0 was observed across this symmetric peak in Fig. 4. When the material used in gel filtration in Fig. 4 was subjected to sedimentation velocity at two different protein concentrations, a single symmetric Schlieren peak was observed in both cases (Fig. 6). This same material did not migrate as a sharply defined band during polyacrylamide gel electrophoresis (Fig. 7) and instead displayed diffuse leading and tailing edges similar to the patterns observed in gels in Fig. 1. Thus for the preparations described here charge homogeneity proved to be a less demanding criterion than size homogeneity.

The observations that the band migrated with diffuse leading and tailing edges during polyacrylamide gel electrophoresis suggest either that association-dissociation phenomena occurred during electrophoresis or that charge heterogeneity was present [28]. The diffuse pattern in Fig. 7 is similar to the patterns observed during polyacrylamide electrophoresis of κ-casein, which is known to have charge heterogeneity [29]. The starting material used to isolate xanthine oxidase in Method A was pooled milk from genetically diverse cows. These cows might produce slightly different xanthine oxidase polypeptides with different net charges. Furthermore, since xanthine oxidase is a membrane protein, lipids or micellar proteins (e.g caseins) might bind, thereby masking or preventing ionization of potentially charged sites. We did find that neutral lipids were bound to the material shown in Fig. 7.

One further criterion of homogeneity generally applied to enzymes is the measurement of specific activity. In addition, the activity to flavin ratio (AFR) is a second type of activity parameter used for xanthine oxidase because of its convenience [30]. Although the AFR values ranged from 90 to 120 [6] we have not used the AFR or analogous measurements as a criterion of homogeneity for xanthine oxidase because the loss of cofactors such as FAD or molybdenum was not clearly related to the question of physical homogeneity of xanthine oxidase.

The reason we preferred the use of size as the primary criterion of homogeneity was that the problems associated with either charge or activity as a criterion were minimized. Heterogeneity due to genetic variants was less likely to cause significant differences in size. The forces that produce movement of xanthine oxidase through gel filtration media or in free solution during sedimentation velocity should have been independent of charge at the ionic strengths used in these experiments. In like manner the loss of a cofactor, e.g. molybdenum or FAD, should not have changed the molecular size significantly.

Isolation of xanthine oxidase from milk or cream by conventional methods (Method A)

The present purification differs from previous methods which omit proteases in the following ways:

Triton X-100, a non-ionic detergent, replaced *n*-butanol in steps 2 and 3. We chose Triton X-100 as a solubilizing agent because more complete information was available about the interactions of this detergent [31,32] with proteins and membranes than was known for *n*-butanol [33,34]. Triton X-100 was also more convenient and safe to use because it did not lead to progressive damage of polycarbonate centrifugation materials, was non-flammable and less volatile, and could be used over a wider temperature range than *n*-butanol without risking protein denaturation. However, it was also found that the two agents were equally effective in the purification process when solutions containing *n*-butanol were maintained at 5°C. The elution patterns from G-200/6B were essentially the same when *n*-butanol replaced Triton X-100.

In the salt fractionation step (step 3), the concentrations of $(NH_4)_2SO_4$ used were the same as those used in isolations including proteases [20,21]. These are not absolute limits and required readjustment with different sources of milk or cream. This was accomplished by carrying out preliminary isolations on each batch. Such a practice was feasible because milk or cream containing 2 mM sodium salicylate and 0.01% EDTA could be stored frozen without subsequent loss of xanthine oxidase activity.

Most of the contaminating proteins, e.g. the caseins, were removed in the first $(NH_4)_2SO_4$ step (0—20%). The selective removal of all caseins was not accomplished because the two $(NH_4)_2SO_4$ fractionation steps for xanthine oxidase (0—20% and 20—27%) overlapped with the $(NH_4)_2SO_4$ step required for complete precipitation of the caseins (0—27%) [35]. The effect of the use of pancreatin in earlier procedures was the degradation of most of the caseins by proteolysis prior to ammonium sulfate fractionation.

There are several additional reasons why a major problem in the non-proteolytic isolation of milk xanthine oxidase involves removal of the caseins. First, 80% of milk protein was casein [35]. Second, three major classes of caseins exist and each is genetically heterogeneous [35]. Therefore, much variability exists in their net charge distributions. Third, no consistent set of aggregation states exists because the caseins form micelles of different sizes and this is a function of a number of variables, e.g. the presence of Ca^{2+}, the temperature, and the ionic strength [36]. Thus, the distribution of casein molecular weights under non-denaturing conditions is also variable. Fourth, the detergent-like properties of the caseins, i.e. micelle formation, may introduce special problems in the isolation of xanthine oxidase since this enzyme probably belongs in the class of 'loosely associated' membrane proteins which, when released by non-denaturing solvents, retain their functional properties [37–39]. The membrane-binding property of xanthine oxidase may lead to similar non-covalent interactions with the caseins resulting in the formation of micelles. Certainly, the formation of micelles and mixed micelles is a well-known phenomenon in milk chemistry [36]. Thus the $(NH_4)_2SO_4$ precipitation of the caseins may also cause removal of micelle-bound xanthine oxidase. Therefore, it is possible that the requirement for an agent such as *n*-butanol

or Triton X-100 in this procedure is not only to release the enzyme from fat globule membrane sites but also to minimize non-covalent interactions with caseins. The observation that xanthine oxidase was absent from the 20—27% $(NH_4)_2SO_4$ precipitate (step 3) when neither *n*-butanol nor Triton X-100 was present is consistent with this suggestion.

In step 4 gel filtration chromatography with Sephadex G-75 rather than Sephadex G-25 was used to exchange buffers. An additional protein separation step occurred simultaneously since xanthine oxidase eluted in the void volume emerging before casein monomers (molecular weight range 16 000—28 000) [36].

The size of pooled fractions from gel filtration chromatography (step 4) or material from larger volumes of dissolved $(NH_4)_2SO_4$ precipitate (step 3) containing xanthine oxidase was reduced by ultrafiltration with an Amicon XM-100A membrane instead of either an Amicon PM-10 or PM-30. Concentration with the XM-100A membrane was faster. An additional separation of proteins which are not retained by the XM-100A membrane could also be effected with this membrane during concentration. Complete recovery of xanthine oxidase activity was usually observed with this procedure. However, at times the ultrafiltrate was found to contain about 10% of the total xanthine oxidase activity originally placed in the chamber. The possible significance of this observation is discussed below.

The use of hydroxyapatite was one of the most effective methods for removal of almost all protein contaminants. Between 70 and 80% of the xanthine oxidase eluted in the 0.20 M step as an orange-brown solution. The remaining xanthine oxidase eluted in the 0.30 M step. Most other proteins eluted prior to the 0.20 M step. Our experience with hydroxyapatite and the buffers used was essentially identical to that of others [18] who used xanthine oxidase isolated in the presence of pancreatin. The sedimentation velocity pattern in Fig. 2 represents the pre-hydroxyapatite stages of purification. A number of contaminating proteins were observed. By comparison, Fig. 6a is representative of the post-hydroxyapatite stage in which one major xanthine oxidase peak was observed. The yields in this step were often as high as 80—90%, depending on whether the 0.30 M step was carried out.

Previously, Sephadex G-200 gel filtration chromatography was used for xanthine oxidase purification [17,19,21]. In the present work the use of Sephadex G-200 alone was replaced with a column series of Sephadex G-200 connected to Sepharose 6B (G-200/6B). Larger aggregates, alleged to be polymers of xanthine oxidase, have been reported [17]. Therefore, it was important to have a gel filtration method which simultaneously resolved proteins smaller than xanthine oxidase, i.e. Sephadex G-200, and larger than xanthine oxidase, i.e. Sepharose 6B, whether or not aggregates of xanthine oxidase existed. This column arrangement also increased the effective column length [40]. Xanthine oxidase eluted in a more central position between the total and void column volumes (Fig. 4) when compared to the use of Sephadex G-200 alone (Fig. 3). In fact, the beginning of the elution position of xanthine oxidase in Fig. 3 (fractions 85—90) appeared very close to the void volume (fractions 72—82).

The G-200/6B system was also used to establish one of the main criteria of homogeneity. For example, Fig. 4 shows a symmetric xanthine oxidase peak

and displays a constant A_{280nm}/A_{450nm} ratio equal to 5.0 throughout. When the fractions from this peak were pooled, concentrated, dialyzed, and rechromatographed in the same G-200/6B system the same elution pattern was obtained.

The sedimentation velocity of the material from the peak shown in Fig. 4 displayed Schlieren patterns (Fig. 6) consistent with this elution pattern. A single symmetric peak was observed in either case.

Isolation of xanthine oxidase from fat globule membranes by ultrafiltration (Method B)

Purification of xanthine oxidase using ultrafiltration through an Amicon XM-100A membrane yielded essentially the same product as Method A. The observed sedimentation coefficients were the same and the Schlieren patterns both displayed single symmetric peaks.

Purification by ultrafiltration differed in several ways from Method A. First, the isolation was carried out with fresh, warm milk placed in a thermally insulated container to maintain the temperature at 35° C. This material was processed within 30 min of milking. Second, caseins and other milk proteins were removed by repeatedly washing the fat globule membranes at 35° C with 0.25 M sucrose containing 5 mM sodium salicylate. Third, neither membrane-solubilizing agents, e.g. *n*-butanol or Triton X-100, nor, extrinsic buffers were used. Ion-exchange methods, sorption methods, $(NH_4)_2SO_4$ fractionations, and gel filtration chromatography were eliminated. Fourth, cysteine was absent.

This purification appeared to depend primarily on the dissociation of xanthine oxidase from fat globule membranes at 5° C. The key to this isolation method was the use of fresh, warm milk which had not been chilled. Milk or cream obtained from commercial dairies is always cooled to 5° C for storage and yields white fat globule membranes lacking xanthine oxidase activity. Warming cream from commercial sources back to 35° C did not cause reassociation of xanthine oxidase with fat globule membranes; pink-colored membranes were not obtained and xanthine oxidase was absent from the membrane preparations. Our observations that xanthine oxidase was released from cooled fat globule membranes confirm those described in a much earlier publication [41].

A significant conclusion derived from the isolation of xanthine oxidase by comparison of these two quite different methods was that active xanthine oxidase (or components of xanthine oxidase which could reassociate after ultrafiltration) appeared smaller in size than the molecular size cutoff of the XM-100A membrane, i.e. 100 000 daltons. If this is so, then in vivo xanthine oxidase may be smaller than the reported size of 275 000—300 000 daltons [17,23]. In addition, the observation that about 10% of the xanthine oxidase passed through the XM-100A membrane during concentration of material from steps 3 or 4 of Method A is consistent with this proposal. This evidence suggests that residual Triton X-100 remaining bound to xanthine oxidase may have prevented complete aggregation to the larger form usually observed. An alternative possibility would be that a molecule as large as 275 000—300 000 daltons could pass through this ultrafiltration membrane if it had some particular shape. However, no protein passed into the ultrafiltrate in tests with human γ-globulin (150 000 daltons) or beef liver catalase (250 000 daltons).

The central issue now under investigation is to reconcile the observation of a sedimentation coefficient of 11.3 S for xanthine oxidase in 0.15 M NaCl isolated from fat globule membranes with the passage of this enzyme through an XM-100A membrane (consistent with a molecular weight less than 100 000). This sedimentation coefficient is consistent with a molecular weight in the range of 300 000 and is similar to the value previously reported for this enzyme when isolated either with proteases [21] or without proteases [17,23]. Clearly, a number of uncertainties about the structure of xanthine oxidase remain to be resolved. Paramount among them is the unequivocal resolution of the molecular size and shape of the native form of xanthine oxidase isolated by ultrafiltration.

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