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PURIFICATION AND CHARACTERIZATION OF XANTHINE OXIDASE FROM LIVERS OF VITAMIN E DEFICIENT RABBITS

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Summary

Xanthine oxidase which increases in activity during vitamin E deficiency was purified from livers of deficient rabbits. The procedure incorporates preparative sucrose gradient centrifugation and yields a homogeneous preparation on acrylamide gel electrophoresis. The purified enzyme exhibits a pH optimum of 8.1 and a K_m value of 22 μM. Gel filtration chromatography gave the molecular weight of 280 000. Acrylamide gel electrophoresis in the presence of sodium dodecylsulphate reveals two types of subunits of molecular weights 52 000 and 99 000.

Introduction

Increase in liver xanthine oxidase activity (xanthine: oxygen oxidoreductase, EC 1.2.3.2) in vitamin E deficient rabbits has been reported [1]. This phenomenon was re-examined [2] in view of findings of Della Corte and Stirpe [3] that in freshly prepared soluble fractions from rat liver the activity is present mostly as an NAD^+-dependent dehydrogenase. Storage of liver or liver extracts at −20° C resulted in loss of the dehydrogenase activity with concommitant increase in oxidase activity. These two activities represent two interconvertible forms of the enzyme [4]. Vitamin E deficient and normal rabbit livers both exhibit the dehydrogenase activity which was found to undergo conversion to the oxidase form, the activities of the vitamin E deficient animals being 4—12 fold higher than corresponding controls [2].

In order to study the mechanism by which vitamin E deficiency increases the activity of this purine-oxidizing enzyme it is necessary to obtain purified xanthine oxidase. This paper describes the preparation of purified xanthine oxidase from livers of vitamin E deficient rabbits.

Materials and Methods

Reagents. Biogel P-300 was purchased from Bio-Rad Laboratories, bovine liver catalase from Calbiochem, horse liver alcohol dehydrogenase, rabbit muscle lactate dehydrogenase and phosphorylase *a* from Sigma Chemical Co., bovine serum albumin, ovalbumin, myoglobin from Schwarz/Mann and Blue Dextran 2000 from Pharmacia Fine Chemicals. Acrylamide gel reagents were obtained from Canalco and the dietary ingredients from ICN Nutritional Biochemicals Corporation.

Animals. Male New Zealand White rabbits obtained from a local breeder and weighing approximately 500 g at the beginning of the experiments were used. The vitamin E deficient animals had free access to the diet described by Young and Dinning [5] and were sacrificed by cervical dislocation when the deficient animals showed marked signs of muscular dystrophy. Usually 4—6 weeks feeding with the experimental diets was necessary to attain this stage of vitamin E deficiency.

Enzyme assays. The xanthine oxidase activity was assayed at 292 nm as described by Rowe and Wyngaarden [6] by determining spectrophotometrically the amount of uric acid formed from xanthine as substrate. Xanthine dehydrogenase activity was similarly determined in the presence of NAD^+ according to the method of Della Corte and Stirpe [3]. The activity is expressed in μmoles of uric acid formed in 1 min by 1 mg of protein.

Protein was determined by the colorimetric method of Miller [7] using bovine serum albumin as a standard or spectrophotometrically according to Waddell [8]. All spectrophotometric assays including the A_{280}/A_{450} ratio were performed on a Gilford Model 2400.

Molecular weight determination by gel filtration. The molecular weight was estimated according to Andrews [9]. A 0.9 cm × 60 cm column of Biogel P-300 was poured to a height of 50 cm and equilibrated with 0.1 M Tris—HCl buffer pH 8.0 containing 0.1 M KCl and adjusted to a flow rate of 0.5 ml per h.

Two mg of each protein were dissolved in 0.5 ml of the equilibrating buffer to which 2 drops of a 30% (w/v) glucose solution were added and applied to the column. 0.5 ml of 0.5% solution of Blue Dextran 2000 in equilibrating buffer was used to obtain the void volume of the column before each protein was applied to the column. 0.33 ml fractions were collected and monitored at 230 nm for protein and assayed for xanthine oxidase activity.

Sucrose gradient centrifugation. 5—20% (w/v) sucrose gradients were prepared according to the method of Toft and Spelsberg [10]. 0.2 ml of the enzyme preparation (approx. 600 μg) was layered on top of each gradient and centrifuged at 35 000 rev./min for 14 h using a Beckman Spinco rotor SW 50.1. 15-drop fractions were collected, read at 230 nm for protein and assayed for xanthine oxidase.

Polyacrylamide gel electrophoresis. Analytical gel electrophoresis (7.5% (w/v) acrylamide) was performed using a Buchler Polyanalyst apparatus at a constant current (3 mA per gel) at pH 8.9 in Tris—glycine buffer at 4°C according to the method of Davis [11] and at pH 7.1 (4 mA per gel) in imidazole—glycylglycine buffer in keeping with the method of Igarashi and Hollander [12]. Xanthine oxidase was detected by staining as described by Yen and

Glassman [13]. The quantity of enzyme applied to each gel ranged from 1 to 7.5 μg; the actual amounts are detailed in the legend of the appropriate figure. The polyacrylamide gels were stained for protein as described by Weber and Osborn [14].

Subunit molecular weight determination. Sodium dodecylsulphate acrylamide gel electrophoresis was performed by the method of Weber and Osborn [14]. 40 μg of purified enzyme previously incubated in 1% (v/v) mercaptoethanol and 1% (w/v) sodium dodecylsulphate were applied on an acrylamide gel. The purified xanthine oxidase was also incubated prior to electrophoresis in the presence of 6 M urea.

Results

Purification of rabbit liver xanthine oxidase. The rabbit liver xanthine oxidase was purified by a modification of the procedure of Rowe and Wyngaarden [6] used for purification of the enzyme from rat liver. Though the A_{280}/A_{450} ratio of 5.18 reported by these authors for the rat liver enzyme was indicative of a high purity, it has been reported [15] that some rat liver preparations isolated by this method showed impurities.

Acrylamide gel electrophoresis of the rabbit enzyme at this stage showed a single impurity. The necessary additional purification was accomplished by preparative sucrose gradient centrifugation.

12 ml (4.44 mg protein) of enzyme solution (Step V, Table I) was concentrated to 1.5 ml with a Model 12 ultrafiltration cell using a XM-50 membrane (Amicon Corporation), under 30 lb/inch^2 of nitrogen. The solution then was dialyzed overnight against 1000 vol. of 0.1 M potassium phosphate buffer, pH 7.8. Approximately 600 μg of the non-diffusible material (0.2 ml) was layered on each of six 5—20% (w/v) sucrose gradients, centrifuged and fractionated. The typical gradient profile is shown in Fig. 1. The fraction from each gradient containing the highest specific activity was pooled and yielded a specific activity of 11.34, a 1.2 fold purification over the previous step.

Fig. 2 illustrates the migration pattern of purified rabbit liver xanthine oxidase in 7.5% (w/v) polyacrylamide gel at pH 8.9 and pH 7.1 when stained

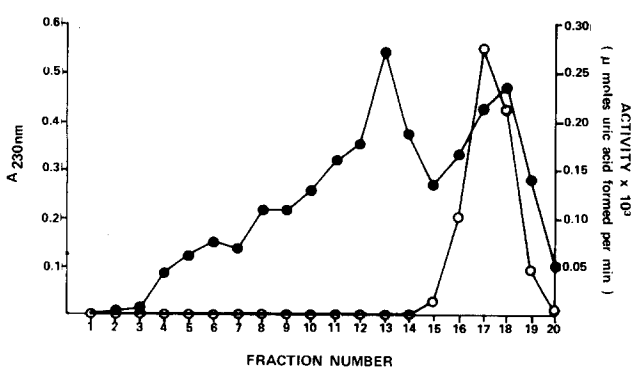


Fig. 1. Sucrose gradient centrifugation of xanthine oxidase. ●———●, absorbance at 230 nm; ○———○, xanthine oxidase activity. Top of the gradient is to the left. For details see the text.

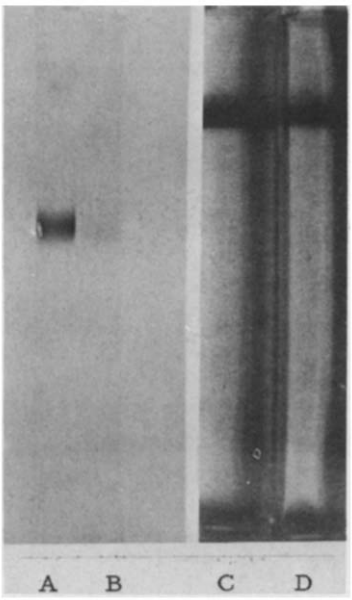


Fig. 2. Analytical polyacrylamide gel electrophoresis of purified rabbit liver xanthine oxidase. The enzyme was subjected to electrophoresis as described in the Materials and Methods. The staining method and quantities of enzyme applied were: A, pH 8.9 (1 μg) stained for activity; B, pH 8.9 (4 μg) stained for protein; C, pH 7.1 (2 μg) stained for activity; D, pH 7.1 (7.5 μg) stained for protein. Electrophoresis was toward the anode (bottom of the figure).

for protein and activity. The overall yield and purification ratio is listed in Table I. An apparently homogeneous preparation of the enzyme was obtained and gave an A_{280}/A_{450} ratio of 5.28.

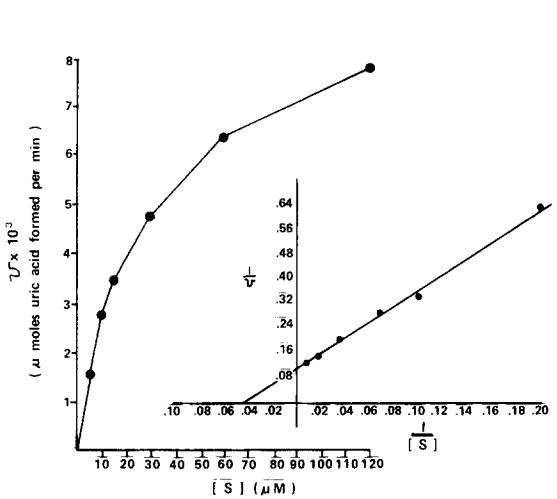
Properties of the purified xanthine oxidase. Due to the fact that storage of the frozen preparations was thought to convert or abolish the xanthine dehydrogenase activity [2,3] no xanthine dehydrogenase activity was expected to be present in the purified preparation. Suitable assay confirmed this.

TABLE I

PURIFICATION OF RABBIT LIVER XANTHINE OXIDASE

For details see the text.

Purification steps		Vol (ml)	Protein (mg/ml)	Specific activity ($10^3 \cdot \mu$moles/min/mg protein)	Recovery (%)	Purification factor
I.	High-speed supernatant	1980	15.00	0.83	100	—
II.	Heating	1460	6.85	2.22	86	2.6
III.	$(NH_4)_2 SO_4$ precipitation	708	5.10	6.00	86	7.1
IV.	Acetone fractionation	456	2.15	22.90	86	27
V.	Hydroxyapatite	100	0.37	236.00	35	280
VI.	Sucrose gradient centrifugation	14.4	1.00	278.8	16	330



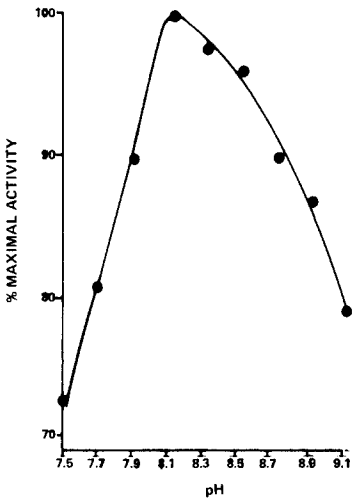
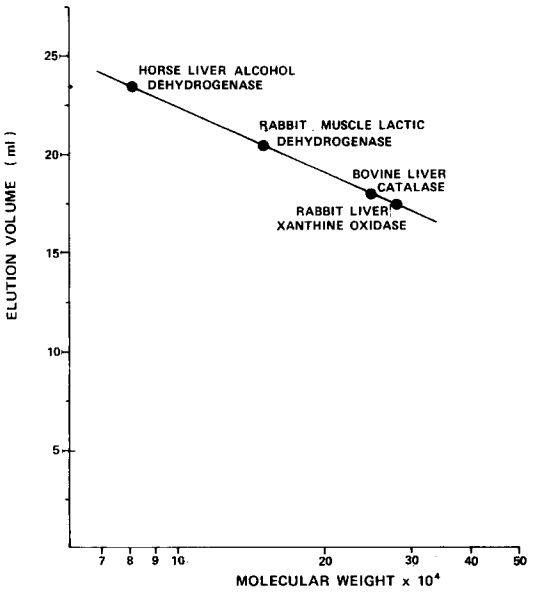


Fig. 3. Effect of substrate concentration on rabbit liver xanthine oxidase. Purified enzyme (2.2 μg per assay) was assayed as described in Materials and Methods at pH 8.1 with substrate concentrations varying from 10 to 120 μM. Results are presented in a double reciprocal plot where v is expressed as $10^3 \cdot \mu$moles of uric acid formed per min.

Fig. 4. Effect of pH on rabbit liver xanthine oxidase activity. Purified enzyme (2.2 μg) was assayed as described except that the pH varied from 7.5 to 9.1 using 0.1 M Tris buffer.



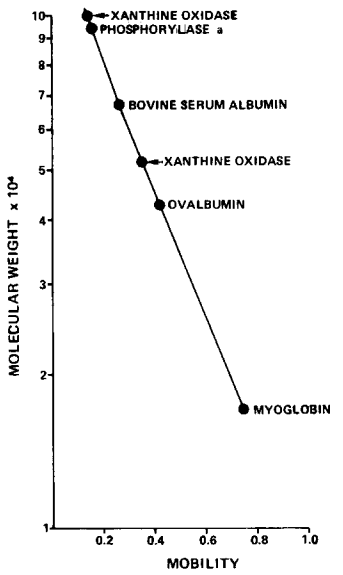


Fig. 5. Molecular weight of rabbit liver xanthine oxidase estimated by gel filtration. Elution volumes of the enzyme and of three marker proteins (horse liver alcohol dehydrogenase, mol. wt. 81 000, rabbit muscle lactate dehydrogenase, mol. wt. 150 000 and bovine liver catalase, mol. wt. 250 000) are plotted against the molecular weights (log. scale). Xanthine oxidase molecular weight was estimated at 280 000.

Fig. 6. Subunit molecular weight of rabbit liver xanthine oxidase estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. Mobilities of the enzyme and four marker proteins (myoglobin, mol. wt. 17 200; ovalbumin, mol. wt. 43 000; bovine serum albumin, mol. wt. 68 000 and phosphorylase *a*, mol. wt. 94 000) were plotted against the molecular weight. Xanthine oxidase gave two bands corresponding to mol. wt. of 52 000 and 99 000.

Effect of substrate concentration. The effect of substrate concentration on xanthine oxidase activity is given in Fig. 3. The enzyme appears to follow normal Michaelis—Menten kinetics and gives a K_m value of 22 μM. The activity of the purified preparation was linear from 1 to 4 μg (not tested further) and at enzyme concentrations of 2.2 μg of the activity was linear for a minimum of 10 min.

Optimum pH. Fig. 4 illustrates the pH profile of xanthine oxidase and reveals a pH optimum of 8.1.

Molecular weight. Purified xanthine oxidase, when applied to the Biogel P-300 column was eluted as a single peak as determined by assaying for protein and activity.

Fig. 5 depicts a plot of elution volume versus molecular weight for xanthine oxidase and for the 3 marker proteins. An approximate molecular weight for the xanthine oxidase of 280 000 was obtained.

Subunit molecular weight. Two major bands were observed when the acrylamide gels were stained for protein and their migrations represented apparent molecular weights of 97 000—99 000 and 52 000—51 000 (2 separate experiments). A third minor band of an apparent molecular weight of 63 000 was also detected. Fig. 6 shows a plot of mobility versus molecular weight using phosphorylase *a*, bovine serum albumin, ovalbumin and myoglobin as marker proteins. The presence of 6 M urea had no additional effect on dissociation.

Discussion

Xanthine oxidase has been purified from a variety of mammalian sources including hog liver [16], rat liver [4,6,15] and bovine liver [17], intestine [18] and milk [19]. To this we add rabbit liver, the purification procedure based on that of Rowe and Wyngaarden [6]. Whereas rat liver xanthine oxidase can be purified by this method to homogeneity [15], sucrose gradient centrifugation had to be added as an additional step to obtain a homogeneous rabbit enzyme. The yield of the purified enzyme is similar to that from the rat liver by the method of Rowe and Wyngaarden [6].

The molecular weight of the rabbit liver enzyme of 280 000 is similar to that for milk xanthine oxidase, 300 000 [20] and hog liver xanthine oxidase, 288 000 [16]; the Michaelis constant, as well as pH optimum, is similar to preparations from other sources.

Attempts to define the subunit molecular weights of milk xanthine oxidase by analytical ultracentrifugation methods in sodium dodecylsulphate [21] or in 4 M guanidine, 0.1 M mercaptoethanol [20] have been made. The smallest polypeptide chain dissociable with sodium dodecylsulphate gave an approximate molecular weight of 150 000 indicating that the native protein is probably dimeric. Molecular weights varying from 100 000 to 150 000 resulted when 4 M guanidine and 0.1 M mercaptoethanol were used.

On the other hand, when rabbit liver xanthine oxidase was treated with sodium dodecylsulphate and mercaptoethanol, the enzyme dissociated giving subunits of molecular weights of approximately 99 000 and 52 000. These are the smallest dissociable units for xanthine oxidase reported from any source and indicate the enzyme is comprised of non-identical subunits. The possibility

of further dissociation of 99 000 units into 52 000 units cannot, however, be excluded. The presence of a minor band at approximately 63 000 may indicate further dissociation of 99 000 subunits, although no smaller unit within the range of 35 000 to 40 000 was observed.

Since the activity of xanthine oxidase from control animals is substantially less than that of the vitamin deficient animal [2], the purification of this enzyme was carried out with deficient tissue only. Specific antibodies to this enzyme produced in sheep allowed the comparison of the enzyme to that of the control animal.

The immunochemical identity of liver xanthine oxidase from control and vitamin E-deficient rabbits and elucidation of the mechanism by which the activity of this enzyme is elevated during vitamin E deficiency have been reported [22]. Immunochemical titration of liver xanthine oxidase from control and vitamin E-deficient animals, demonstrated that the enzyme from both sources was immunologically identical. According to the theoretical consideration of Feigelson and Greengard [23] for immunochemical analysis of changes in enzyme activities the titration likewise revealed that the enzyme from both sources exhibited identical catalytic efficiency. It was thus shown by this method that the increase in activity of rabbit liver xanthine oxidase during vitamin E deficiency was the direct result of the accumulation of this enzyme [22].

An increased incorporate of [^{14}C]leucine into immunochemically reactive xanthine oxidase was also observed during deficiency. The data was interpreted as indicating, that the accumulation of xanthine oxidase was due at least in part to the increased rate of synthesis de novo of this enzyme. To our knowledge, the data represents the first direct evidence that vitamin E influenced the synthesis of a specific protein [22].

Acknowledgements

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