한국농화학회지 Vol. 21, No. 2. June 1978
J. Korean Agricultural Chemical Society

Studies on Xanthine Oxidase from Bovine Thyroid Glands I)

[Part 1] Purification and Substrate Specificity

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(Received May 13, 1978)

소의 갑상선에 있는 크산친 옥시다아제에 관한 연구

[제 1 보] 효소의 정제와 기질특이성

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(1978년 5월 13일 수리)

SUMMARY

Xanthine oxidase from bovine thyroid glands was purified to apparent homogeneity when judged by analytical disc gel electrophoresis. The purification procedures include pancreatin digestion, butanol extraction, ammonium sulfate precipitation, calcium phosphate gel adsorption, ultrafiltration, calcium phosphate gel-cellulose column chromatography, gel filtration, preparative Sephadex G-25 column electrophoresis, and preparative polyacrylamide gel electrophoresis. The enzyme was enriched 1,000-fold. However, its specific activity was markedly low as compared with highly purified milk enzyme.

Thyroidal xanthine oxidase exhibited a low specificity for substrates and electron acceptors. The kinetic properties of thyroid xanthine oxidase were found to be similar to those of the milk enzyme on the basis of Michaelis constants for common substrates.

INTRODUCTION

It has been previously reported that xanthine oxidase (E.C. 1.2.3.2.) is present in the cytosol fraction of bovine thyroid glands (1) and that thyroidal xanthine oxidase may play an important role of the generation of the hydrogen peroxide required for thyroxine biosynthesis (2). Xanthine oxidase is the enzyme which catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to form uric acid and hydrogen peroxide as the final products. It has been recognized that xanthine oxidase can utilize NADH, NADPH, acetaldehyde, purines, pyrimidines and pterines as substrates. The enzyme has also a low electron acceptor specificity such as molecular oxygen, cytochrome c, ferricyanide and artifical dyes (3,4). Xanthine oxidase is widely distributed in tissues. The isolation and purification of the enzyme have been extensively studied with different mammalian tissues (5-10).

The present report describes the purification of xanthine oxidase from bovine thyroid glands and the experimental results pertaining to the specificities of substrates and electron acceptors and kinetic properties of thyroid xanthine oxi-

dase. Studies involving composition and physicochemical properties are reported in the following paper.

MATERIALS AND METHODS

Materials. Bovine thyroid glands were obtained from a local slaughter house. Acrylamide, bisacrylamide and N,N,N,'N'-tetra methylethylenediamine(TEMED) were procured from Eastman. Sephadex G-25 and G-200 were supplied by Pharmacia. 2,6-Dichloroindophenol was obtained from Matheson, Coleman and Bell: molecular weight standard proteins from Schwarz and Mann Research Laboratories. All other biological chemicals were purchased from Sigma Chemical Co.

Assays. Xanthine oxidase assays in which oxygen was the electron acceptor have been described previously (1). However, when artificial acceptors were used, the activity of xanthine oxidase was meausred as follows in the presence of 66 μM xanthine and 50 μM phosphate, pH 7.8: dichloroindophenol (40 μM) reduction, by the decrease in absorbance at 600 nm; cytochrome c (20 μM) reduction, by the increase in absorbance at 535 nm; phenazine methosulfate (50 μM) reduction, by the aerobic formation of uric acid as determined at 292 nm (11).

Protein determination. Protein contents were determined by either of two methods. For the higher concentrations, the protein was measured by the Biuret Method (12) and lower protein concentration determinations were estimated by the method of Lowry *et al.* (13). Bovine serum albumin was used as standard protein. All spectrophotometric determinations were performed on a Perkin-Elmer Coleman, Model 124 Recording Spectrophotometer.

Gel electrophoresis. Analytical gel electrophoresis was performed by a slightly modified procedure of Ornstein and Davis (14) in a Buchler Poly-Analyst apparaturs. The gel concentration employed for the purity test was 7.5% acrylamide. Usually, a pair of the same samples of 100 μl containing 20-50 μg protein were simultaneously electrophorized at 4°C with a constant current of 2.5 ma per tube. One of the gels was stained with aniline blue black dye for protein stain, while the other gel for the enzyme activity was stained by the incubation of the gel in a reaction medium containing 0.05M phosphate, pH 7.8, 50 μM xanthine and 0.1mM nitro blue tetrazolium.

RESULTS

Isolation and purification of bovine thyroidal xanthine oxidase. Fresh bovine thyroid glands were homogenized as a 30% suspension in 0.2 M $NaHCO_3$-1 mM salicylate-5 mM EDTA-0.1 mM dithioerythritol. The crude homogenate was passed through four layers of cheesecloth and the filtrate collected. A supernatant fraction was prepared from the crude homogenate by centrifugation at 45,000×g for 1 hr in a Sorvall RC 2-B. After centrifugation, the pellets were discarded and the supernatant saved for the purification. Unless specified otherwise, all procedures were conducted at 4°C. The progressive purification was also followed by the following measurements: optical densities at 280 nm(Protein) and 450 nm(Flavin). Pancreatin treatment: For each 100 ml supernatant fraction, 165 mg of pancreatin was added and the mixture was incubated at 37°C for 3.5 hr. The incubated mixture was kept in cold room overnight.

Butanol-Ammonium sulfate fractionation: With vigorous stirring 20ml of *n*-butanol which was previously cooled to 10°C was slowly added to each 100ml of the incubated digest and stirred for an additional 20 min. The mixture was centrifuged at 12,000×g for 10 min to saparate denatured protein, fat, and *n*-butanol. The lower aqueous layer containing the enzyme fraction was siphoned off and saved. The upper layer was discarded. Thirty-five percent ammonium sulfate saturation of the aqueous solution was attained by adding crystals of the salt. The resulting protein suspension was stirred for an extra 15 min before centrifugation at 12,000×g for 10 min. After centrifugation, the inactive

protein floating on the top of the aqueous solution was adjusted to 55% saturation by adding additional ammonium sulfate with stirring and allowed to stand for 1 hr in cold room until the protein floated to the top and then the majority of the aqueous portion was siphoned off and discarded. The top layer of the floating precipitate was collected and centrifuged at 12,000×g for 10 min. The top butanol layer and the lower aqueous layer were discarded. The middle layer of the protein was dissolved in the minimum volume of 0.1 M phosphate buffer, pH 6.2, containing 1 mM salicylate 0.3 mM EDTA. The dissolved protein was dialyzed against two changes of the same buffer.

Calcium phosphate gel treatment: Calcium phosphate gel was prepared by the method of Wood (15). The gel suspension of 20 mg/ml was added to the dialyzed enzyme at a ratio of 1 mg of gel for each 2 mg of protein. The mixture was allowed to sit for 5 min and centrifuged at 10,000×g for 5 min. The sediment was washed once with 5 ml of 0.1 M phosphate buffer, pH 6.2, containing 1 mM salicylate and 0.3 mM EDTA per 100 mg of the gel. The supernatant was discarded and the enzyme then eluted from the gel by suspending the gel in 5 ml of the same buffer containing 5% ammonium sulfate for each 100 mg of gel used and was centrifuged at 10,000×g for 5 min. The bulk elution was repeated twice and the eluates combined and concentrated by an Amicon ultrafiltration apparatus with an XM-100 membrane. The concentrated enzyme was dialyzed overnight against 0.1 M phosphate buffer, pH 6.2, containing 1 mM salicylate and 0.3mM EDTA. Calcium phosphate-cellulose chromatography: The mixture of calcium phosphate and cellulose was prepared by mixing 500ml of calcium phosphate gel (dry weight, 20 mg/ml) and 67 g of cellulose (Whatman CF 11) in 500 ml of water. The dialyzed enzyme obtained from 2 l of supernatant fraction was applied to calcium phosphate-cellulose column (1×30 cm), which was previously equilibrated with 0.1 M phosphate, pH 6.2, containing 1mM salicylate and 0.3mA EDTA. The column was washed with the column equilibration buffer until all the colorless impurities had been eluted. The enzyme was then eluted by the same buffer containing 5% ammonium sulfate. The fractions with high enzyme activity were combined and concentrated by an Amicon ultrafiltration using PM-10 membrane.

Sephadex G-200 chromatography: The concentrated enzym fractions were applied to Sephadex G-200 column(1×79 cm) which had been equilibrated with 0.1 M phosphate, pH 7.4. The enzyme was eluted with the same buffer. The fractions containing enzyme activity were pooled and concentrated again by ultrafiltration through PM-10 membrane.

Preparative column electrophoresis on Sephadex G-25: The above concentrated enzyme was applied to a Sephadex G-25 column (1.1×40cm) equilibrated with 0.06 M Tris-glycine buffer, pH 8.3, and electrophorized under the continuous buffer syetem, 0.06 M Tris-glycine buffer, pH 8.3, at 490 V and 4 ma in cold room with an apparatus designed originally by Whitehead *et al.* (15). After a period of 14 hr, the colored

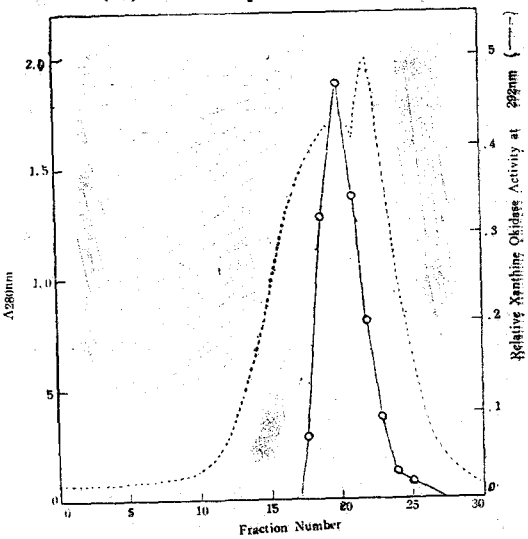

Fig. 1. Elution pattern of thyroid xanthine oxidase from preparative Sephadex G-25 electrophorsis column (2.2×50cm). The enzyme activity was determined under the condition described in the text. Each fraction contained 1.5ml.

Table 1. Summary of Results for the Purification of Thyroid Xanthine Oxidase

Fraction	Total Volume ml	Total Protein mg	Total Units[a]	Specific Activity[b] $\times 10^{-4}$	AFR[c]	PFR[d]	Recovery %	Fold Purification
Supernantant	4,000	198,000	19.8	1	0.03	25.8	100.0	1
Pancreatin Digest 20% n-BuOH 35-55% $(NH_4)_2SO_4$ Dialysis	395	5,580	10.5	19	0.60	41.6	53.0	19
Cal-P-Gel	130	342	6.4	187	3.1	40.1	32.3	187
UF XM-100	11	274	6.5	237	2.7	28.9	32.8	237
Cal-P-Cellulose	16	143	4.7	330	10.8	50.2	23.7	330
G-200 Column	25	83	4.9	590	22.2	42.2	24.8	590
Prep G-25 Electrophoresis	15	64	4.8	743	23.6	38.5	24.2	743
Prep PAG Electrophoresis	4.5	24	2.5	1,030	24.0	10.6	12.6	1,030

[a] A unit of enzymatic activity was defined as the number of μmoles of urate formed per min at 25°C with 66.6μM xanthine as substrate.

[b] Specific activity of the enzyme was expressed as units per mg of protein.

[c] AFR value was obtained by dividing the change in absorbancy per min at 292 nm by the absorbancy at 450 nm of the xanthine oxidase employed in the assay (18).

[d] PFR=E_{280}/E_{450} was initially designated by Avis *et al.* (18).

band of xanthine oxidase was eluted with the electrophoresis buffer and 1 to 2 ml fractions were collected. The elution pattern of a typical electrophoresis experiments (Fig. 1) showed two major protein peaks. But the enzyme activity was observed only with the electrophoretically fast moving protein. The fractions containing xanthine oxidase were combined and concentrated by ultrafiltration using PM-10 membrane.

Preparative polyacrylamide gel electrophoresis: The above concentrated fraction was subject to preparative acrylamide gel electophoresis with a Buchler Poly-Prep apparatus. The electrophoresis system was essentially identical to the system used for analytical disc gel electrophores is described previously. After an electrophoresis period of 12 hr at 40 ma and 0°C, the resolving gel containing the enzyme was extruded from the column and a thin vertical strip was cut from the extruded gel. The location of xanthine oxidase was identified by the purple color stain on the gel when the strip of the gel was incubated in reaction medium containing 0.1 mM nitro blue tetrazolium dye and 50 μM xanthine and 50 mM phosphate, pH 7.8. The remaining portion of the gel containing the active xanthine oxidase was macerated in 0.1 M Tris-HCl, pH 8.1. The eluted enzyme was separated from the gel by filtration through fine glass wool. The filtrate was concentrated in an Amincon ultrafiltration using PM-10 membrane. The res-

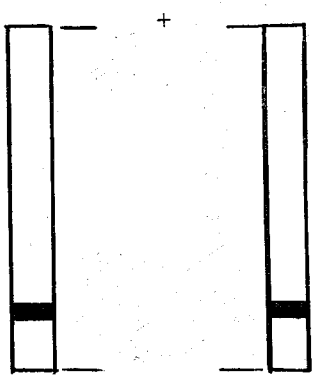

Fig. 2. Diagram of polyacrylamide gel electrophoresis of thyroid xanthine oxidase. P: Analytical gel electrophoresis of the enzyme obtained from preparative electrophoresis; stained for protein with aniline blue black dye. A: Analytical gel electrophoresis of the same enzyme preparation stained with nitro blue tetrazolium dye for xanthine oxidase activity. The protein was migrated from cathode to anode.

Table 2. Apparent Michaelis Constant(Km) of Thyroidal Xanthine Oxidase

Substrate	Acceptor	Km (M)
Xanthine	Oxygen	1.6×10^{-6}
	Dichloroindophenol (40μM)	2.8×10^{-5}
	Cytochrome c (20μM)	1.7×10^{-6}
	Phenazine methosulfate (50μM)	2.5×10^{-6}
	Nitro blue tetrazolium (40μM)	6.7×10^{-6}
Hypoxanthine	Oxygen	3.4×10^{-6}
	Dichloroindophol (40 μM)	4.3×10^{-6}
NADH	Dichloroindophol (40 μM)	1.2×10^{-4}
NADPH	Dichloroindophenol (40 μM)	
Acetaldehyde	Dichloroindophenol (40 μM)	3.2×10^{-1}

All values except acetaldehyde were detrmined with 0.1mg of thyroid xanthine oxidase purifiedy hrough Sephadex G-200 column chromatograph. When acetaldehyde was the substrate, the enzyme concentration was increased 5-fold.

ults of a typical purification of thyroidal xanthine oxidase are presented in Table 1.

Disc gel electrophoresis. The electrophoresis results shown in Fig. 2 indicate that the enzyme obtained from preparative polyacrylamide gel electrophoresis was electrophoretically homogeneous and that the single protein band contained the enzyme activity.

Substrate specificity and apparent Michaelis constants. The most common substrates and electron acceptors for xanthine oxidase were examined to determine the substrate specificity of thyroidal xanthine oxidase. The apparent Km values obtained from a double reciprocal plot of the data according to the method of Lineweaver and Burk (17) are given in Table 2.

DISCUSSION

The isolation and purification of thyroid xanthine oxidase was followed initially by the method of Massey *et al.* (7). However, the enzyme preparations were not homogeneous when subjected to analytical disc gel electrophoresis. Further resolution of the enzyme preparations on Sephadex G-200 was not satisfactory although the specific enzyme activity increased two fold. The subsqeuent preparative electrophoresis steps were necessary to obtain electrophoretically pure enzyme. The specific activity of the most pure thyroidal xanthine oxidase was surprisingly very low as compared with highly purified milk xanthine oxidase (Table 3).

Table. 3. Comparison of Enzyme Activity Between Highly Purified Thyroid and Milk Xanthine Oxidase

Enzyme Activity Unit	Throid	Milk[a]	Ratio=$\frac{\text{Milk}}{\text{Thyroid}}$
Specific Activity[b] (i.u./mg)	0.1	3.5	35
AFR	24	160	6.6
PFR	10.6	5.4	0.5

[a] Analytical data for milk xanthine oxidase were obtained from Massey *et al.* (7).

[b] i.u. is defined as the number of μmoles of product formed per min at 25°C.

This result is comparable to the findings obtained from purified xanthine oxidase of rat brain (9) and mouse skeletal muscle (10). The low specific activity of thyroid enzyme may not be due to selective inactivation of an active enzyme during purification since the purification procedures for active milk enzyme preparation (7) was employed in the early purification steps for thyroid enzyme. It is noted that the ratio of AFR value of milk to thyroid enzyme is 6.6 while the ratio of the specific activity between them is 35. Those ratios suggest that the thy-

roid enzyme may contain less FAD than milk enzyme. However, the ratio of PFR value of milk to thyroid enzyme indicates that thyroid enzyme possesses one half as much content of FAD for milk enzyme, if the assumption are correct that protein impurities other than the enzyme are negligibly little in both enzyme preparations and that the enzymes are constituted with the same or very similar protein moiety. Xanthine oxidase isolated from bovine thyroid glands conforms to a low specificity for substrates and electron acceptors (Table 2) as observed with the enzyme from other sources. The thyroid enzyme functions as an oxidase and has no dehydrogenase activity as NAD^+ will not accet electrons from xanthine or hypoxanthine. Neither of the pyridine nucleotides, NADH or NADPH, nor acetaldehyde was oxidized by molecular oxygen as found for the bovine intestine xanthine oxidase (19). However, the milk enzyme is able to oxidize NADH or acetaldehyde with oxygen (20). The Km of 1.6×10^{-6} M with xanthine for thyroid enzyme is of the same magnitude reported by Bray (21) and Fridovich and Handler (22). Thyroidal xanthine oxidase has a Km for xanthine somewhat smaller than its Km for hypoxanthine as reported for milk enzyme (23). With dichloroindophenol as electron acceptor, unlike milk enzyme (20) the affinity of hypoxanthine and xanthine for thyroid enzyme is greater than that of NADH.

It can be concluded from this study that xanthine oxidase from thyroid glands has a broad substrate specificity and kinetic properties similar to the enzyme isolated from other sources but with some differences. The enzyme purification results suggest that bovine thyroid glands may have a different form of xanthine oxidase from an active form of milk enzyme. It is of interest to determine whether the enzyme isolated from thyroid is a tissue specific isozyme.

요 약

소의 갑상선에서 추출한 Xanthine oxidase를 disc gel eleectrophoresis로서 정제도(Purity)를 측정하여 Xanthine oxidase 이외의 다른 불순 단백질이 나타나지 않을 때까지 정제하였다. 그 정제 과정은 Pancreatin digestion, butanol 추출, ammonium sulfate 단백질 침전, calcium phosphate gel-cellulose column chromatography, gel filtration, preparative Sephadex G-25 column electrophoresis와 Preparative polyacrylamide gel electrophoresis 등을 포함하고 있다. 이러한 과정을 통하여 갑상선 Xanthine oxidase는 1,000배 정도 정제되었다. 그러나 효소의 비활성도(Specific activity)는 우유에서 추출한 이 효소에 상응하는 정도로 정제된 효소의 비활성도와 비교 되었을 때 지극히 낮았다.

갑상선 Xanthine oxidase도 효소 반응에 필요한 기질과 electron acceptor의 특수성(Specificity)이 어느 특수한 한 기질에 한정되지 않았음을 보였고 Kinetic 성질도 우유에서 추출된 Xanthine oxidase와 비교하였을 때 가장 일반적인 Xanthine oxidase 기질에 대한 Michaelis 상수(Km)가 약간의 예외도 있었으나 상당히 비슷하였다.

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