Arch. Pharm. Res. Vol. 17, No. 5, pp. 318-322, 1994

An Active Monomeric Form of Bovine Milk Xanthine Oxidase

Chu Hee Lee, Doo Hyun Nam and Keun Huh

College of Pharmacy, Yeungnam University, Kyongsan 712-749, Korea

(Received May 4, 1994)

Upon gel filtration, the commercial bovine milk xanthine oxidase preparation was fractionated into two preparations showing enzyme activity. Native polyacrylamide gel electrophoresis showed that one was in a dimeric form and the other was a monomer having molecular weight of 150 kDa. It was also found that this commercial enzyme existed mostly in an active monomeric form without loss of enzyme activity. The rabbit antisera produced against two enzyme preparations cross-reacted well each other. In SDS-polyacrylamide gel electrophoresis, however, both enzyme preparations yielded two smaller protein bands below 150 kDa, which appeared to bind with both antisera with high affinity but not to retain enzyme activity. It implies that bovine milk xanthine oxidase can lose its activity when monomeric subunit is further degraded.

Key words: Bovine milk xanthine oxidase, Active monomeric subunit

INTRODUCTION

Even though xanthine oxidase (XO, EC 1.1.3.22) has been recognized as the rate-limiting enzyme in purine metabolism, it also participates in the oxidation of many other substrate including endogenous and exogenous compounds (Parks and Granger, 1986). However, there are still many questions about the main physiological functions of the enzyme in spite of its long and complicated history.

The enzyme is widely distributed among a variety of species (from bacteria to man), and found in most of mammalian tissues, especially within the mammary tissue and body fluids including milk (Kaetzel *et al.*, 1984; Krentitsky *et al.*, 1986). Bovine milk is one of the richest sources for this enzyme, containing as much as 160 mg/l both in the skim milk and in the cream fractions (Briley and Eisenthal, 1974; Mather *et al.*, 1977; Bruder *et al.*, 1982).

It is well known that mammalian XO consists of two subunits of which molecular mass is about 150 kDa, and that it contains four oxidation-reduction active cofactors per subunit: one FAD, one molybdopterin, and two iron-sulfur centers of the spinach ferredoxin type (Rajagopalan and Handler, 1967; Zikakis and Silver, 1984). However, it is still obscure if the activity of the enzyme can be maintained by the only one subunit (Cheng *et al.*, 1988).

We describe here several evidences that a single subunit of the bovine milk XO has a full enzyme activity.

MATERIALS AND METHODS

Enzyme Purification

Partially purified bovine milk XO was purchased from Sigma Chemical Co. (Catalog No. X4500, St. Louis, MO, USA). The enzyme suspension was dialyzed against 10 mM sodium phosphate buffer (pH 7.8) containing 1 mM ethylenediamine tetraacetate (EDTA) and 1 mM sodium salicylate. After concentrating by Freeze Dry System (Labconco Corporation, Kansas City, MO, USA), the enzyme preparation was applied on a Sephadex G-200 column (1.5×50 cm) preequilibrated with 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and eluted with the same buffer at the flow rate of 0.25 ml per minute. The fractions having enzyme activity were divided into two preparations following elution profile, and lyophilized separately. These preparations were stored at −70℃ and used for the further studies.

Gel Electrophoresis and Immunoblotting

Electrophoresis in native or sodium dodecyl sulfate (SDS)-denatured polyacrylamide gels was performed

Correspondence to: Doo Hyun Nam, College of Pharmacy, Yeungnam University, Kyongsan 712-749, Korea

according to the procedure of Laemmli (1970). Gels were stained with Coomassie blue for protein bands, or with nitroblue tetrazolium cholride (NBT) and xanthine for enzyme activity (Waud and Rajagopalan, 1976). Immunoblotting was performed using rabbit anti-xanthine oxidase antibody as a primary antibody, goat anti-rabbit IgG antibody-alkaline phosphatase conjugate as a secondary antibody and NBT with 5-bromo-4-chloro-3-indolylphosphate (BCIP) as chromogen (Towbin *et al.*, 1979).

Preparation of Antibody

Antibodies aganist the two enzyme preparations were raised in rabbits by subcutaneously injecting enzymes (100 μg) at multiple sites in the back (Madara *et al.*, 1990). The titer of antibodies was measured by solid-phase enzyme-linked immunosorbent assay (ELISA) using the same system in immunoblotting procedure.

Assay of Enzyme Activity and Protein Content

XO enzyme activity was measured by the method of Stirpe and Della Corte (1969), in the assay mixture of 4 ml containing 100 mM potassium phosphate (pH 7.8), 60 μM xanthine and 20-100 μl of samples. One unit of enzyme was defined as the amount of protein producing 1 nmole of uric acid from xanthine per minute. The amount of protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

RESULTS

Purification of Bovine Milk Xanthine Oxidase

When XO preparation from Sigma Chemical Co. (Catalog No. X4500) was separated on 7.5% SDS-polyacrylamide gels to verify its purity, two smaller protein fragments as well as a single band around 150 kDa having enzyme activity were observed together in protein staining. In order to remove these inactive proteins, the enzyme preparation was further purified through Sephadex G-200 column. During fractionation of eluents, the interesting fact was found that the fractions having enzyme activity were divided into two separate peaks as seen in Fig. 1. The specific activity and the ratio of A_{280}/A_{450} (absorption for protein vs. flavin prosthetic group; Massey *et al.*, 1969) demonstrated that the second fraction was much more pure enzyme than the first one. The result of the purification was summarized in Table I. The purified enzyme has a specific activity of 720 units/mg protein with 1.4-fold purification.

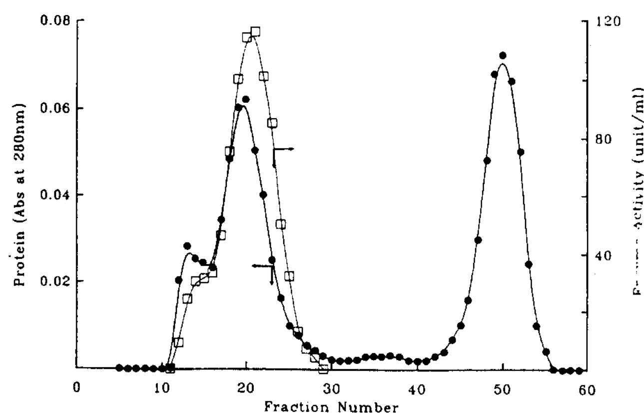


Fig. 1. Gel filtration pattern of commercial bovine milk XO enzyme on Sephadex G-200. Bovine milk XO enzyme preparation from Sigma Chemical Co. was loaded on Sephadex G-200 column and eluted with 100 mM potassium phosphate buffer (pH 7.8) cotaining 1 mM EDTA and 0.1 mM PMSF at the rate of 0.25 ml/min. —●—: amount of protein (A_{280}); —□—: activity for xanthine oxidase.

Electrophoretic Behavior of the two Enzyme Preparations

Two enzyme preparations from Sephadex G-200 column chromatography were subjected to the native (nondenatured) polyacrylamide gel electrophoresis. By protein staining and activity staining, the first peak was confirmed as the dimeric form of the enzyme having molecular weight of about 300 kDa. The other fraction showing enzyme activity was identified as its monomeric subunit of 150 KDa (Fig. 2). From this, it could be confirmed that the two preparations of gel filtration were attributed to two conformational forms of the enzyme, the dimeric and the monomeric. Furthermore,

Table I. Purification of the commercial bovine milk XO enzyme through gel filtration

	fraction number	enzyme form[a]	total protein (mg)	specific activity (unit/mg)	total activity (unit)	recovery yield (%)	A_{280}/A_{450} ratio
After dialysis			6.7104	518.15	3477.0		7.2
After gel filtration	11-13	D	0.0546	289.69	15.8	0.5	5.8
	14-17	D+M	0.1196	707.07	84.6	2.4	
	18-30	M	3.4387	720.16	2476.4	71.2	4.0

[a]D: Enzyme fractions having dimeric form of XO (the first peak), M: enzyme fractions having monomeric form of XO (the second peak), D+M: enzyme fractions overlapped between the first peak and the second peak on chromatogram.

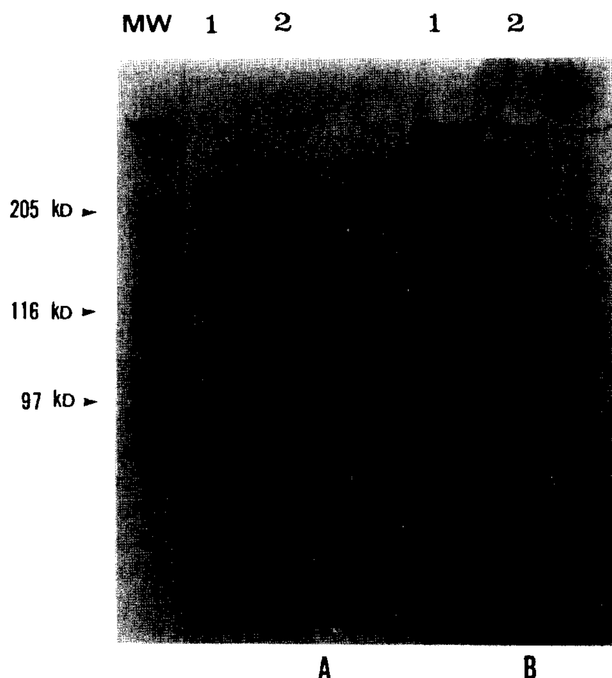


Fig. 2. Native (nondenatured) polyacrylamide gel (7.5%) electrophoretic pattern of the two XO preparations separated during gel filtration. The two enzyme fractions, the first peak (lane 1) and the second peak (lane 2), were subjected to native gel electrophoresis, and stained for protein bands (A) or for activity detection (B).

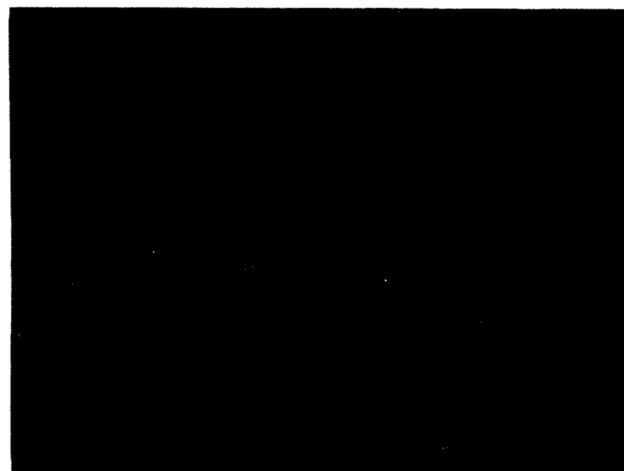
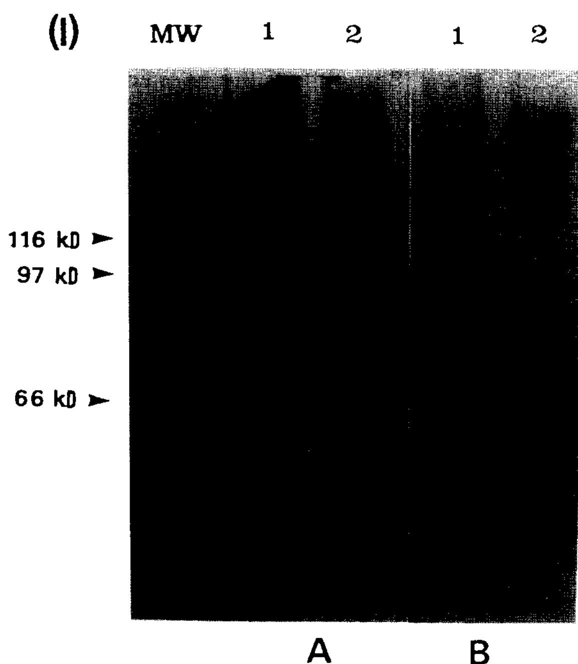


Fig. 3. Immunodiffusion analysis of antisera against the two XO enzyme preparations. D': Antiserum against dimeric form of enzyme, M': antiserum against monomeric form of enzyme, D: enzyme preparation in dimeric form, M: enzyme preparation in monomeric form.

it was an interesting finding that major peak of gel filtration was the second one, i.e. the active monomeric species, which could be stained by NBT in the presence of xanthine and molecular oxygen.

Immunochemical Analysis



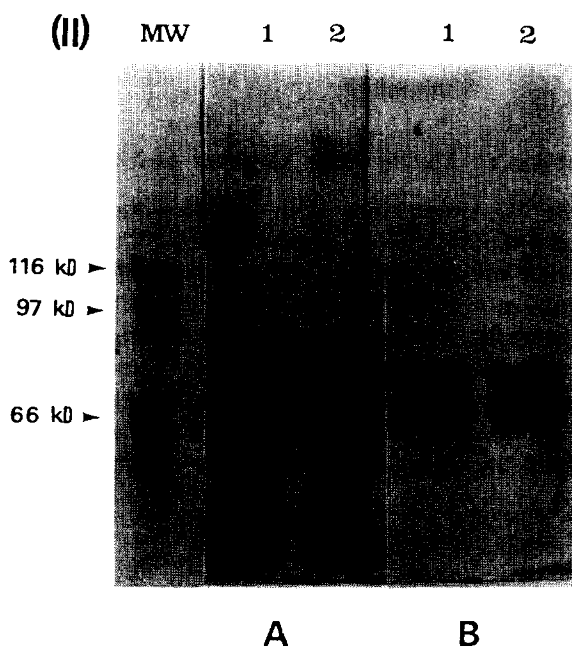


Fig. 4. Western blot analysis of the two enzyme preparations on 7.5% native and SDS-denatured polyacrylamide gels. In native gels (I), enzyme samples of dimeric XO (lane 1) and monomeric XO (lane 2) were treated by the dyes excluding SDS and 2-mercaptoethanol and unboiled. In SDS-denatured gels (II), enzyme samples of dimeric (lane 1) and monomeric XO (lane 2) were treated with the dyes containing SDS and 2-mercaptoethanol and boiled. After electrophoresis, protein bands were transferred to the nitrocellulose paper and incubated with either anti-dimer antibody (A) or anti-monomer antibody (B).

Antisera aganist two forms of XO were obtained from rabbits immunized with each of the above preparations. The titers of the antisera were determined as 10^7 using 5 μg of the dimeric form and 10^5 for the monomeric form, respectively. In immunodiffusion test, these two antibodies showed the identical immunological reactivity against both of the two forms of XO (Fig. 3), which implies that these two forms retain the same epitopes. The immunological analysis of the enzyme preparations on native (nondenatured) and SDS-polyacrylamide gels using both antisera gave the same results with protein staining (Fig. 4). However, on SDS-polyacrylamide gels, two smaller and inactive fragments capable of binding with antisera were observed instead of monomeric subunit of XO (Fig. 4). This result shows that the monomeric XO enzyme can lose its activity when degraded into its fragments of 72 kDa and 81 kDa by strong denaturating circumstances such as in the presence of SDS.

DISCUSSION

In spite of its long history, the molecular structure of XO has not been clearly elucidated. It has been generally accepted that this enzyme has an apparent molecular weight of 300 kDa in its native state, constituted of two identical subunits of 150 kDa each. Previous researchers reported that the enzyme preparation of XO by proteolytic procedure resolved as three bands corresponding to 92 kDa, 42 kDa, and 20 kDa fragments in SDS-polyacrylamide gel electrophoresis (Cheng *et al.*, 1988; Waud *et al.*, 1975). Based on this finding, they postulated that this enzyme consists of two monomeric subunits by forming hydrogen bonds between 92 kDa fragments, which are linked with other two fragments by the basic amino acids labile to proteolytic attack (Nagler and Vartanyan, 1976).

It is still unclear whether this enzyme exists only in a dimeric form and whether each subunit is catalytically active. Parks and Granger (1986) asserted that the enzyme could be dissociated into two inactive subunits, even though Cheng *et al.* (1988) reported that each monomeric subunit could retain its oxidase activity in 6 M urea. In the present study, it was observed that bovine milk XO was separated through Sephadex G-200 column into two different conformational forms, the dimeric and the monomeric, both of which could exert the enzyme activity, and that monomeric form was a predominant fraction rather than dimeric in phosphate buffer, as evidenced by both activity staining and immunological detection, on either native (nondenatured) or SDS-polyacrylamide gels. Our data are consistent with the results by Clare and Lecce (1991), who reported that commercial preparations of XO enzymes from Sigma Chemical Co. and Boeringer Mannheim GmBH were resolved into two different bands by activity staining and Western blotting after running native gel electrophoresis, although they failed to address this.

Our finding of two smaller and inactive protein bands on SDS-denatured gels suggests that this enzyme could be degraded into 72 kDa and 81 kDa fragments by denaturation, differently from proteolytic degradation of enzyme. The location of cleaved site is not clear, but the determination of amino-terminal sequences of each fragments and comparison with those predicted from mouse XO cDNA clone (Terao *et al.*, 1992) will give a clue for the denaturation mechanism of XO enzyme by SDS.

ACKNOWLEDGEMENT

This work was kindly supported by Ministry of Education (Academic Promotion Funds) for the stimulation of research institutes in university.

REFERENCES CITED

Briley, M. S. and Eisenthal, R., Association of xanthine oxidase with the bovine milk-fat-globule membrane: Catalytic properties of the free and membrane-bound enzyme. *Biochem. J.*, 143, 149-157 (1974).

Bruder, G., Heid H., Jarasch, E. D., Keenan, T. W. and Mather, I. H., Characteristics of membrane-bound and soluble forms of xanthine oxidase from milk and endothelial cells of capillaries. *Biochem. Biophys. Acta*, 701, 357-369 (1982).

Cheng, S. G., Koch, U. and Brunner, J. R., Characteristics of purified cows' milk xanthine oxidase and its submolecular characteristics. *J. Dairy Sci.*, 71, 901-916 (1988).

Clare, D. A. and Lecce, J. G., Copurification of bovine milk xanthine oxidase and immunoglobulin. *Arch. Biochem. Biophys.*, 286, 233-237 (1991).

Kaetzel, C. S., Mather, I. H., Bruder, G., and Madara, P. J., Characterization of a monoclonal antibody to bovine xanthine oxidase. *Biochem. J.*, 219, 917-925 (1984).

Krentitsky, T. A., Spector, T., and Hall, W. W., Xanthine oxidase from human liver: Purification and characterization. *Arch. Biochem. Biophys.*, 247, 108-119 (1986).

Laemli, U. K., Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685 (1970).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with folin phenol reagent. *J. Biol. Chem.*, 193, 265-275 (1951).

Madara, P. J., Banghart, L. R., Jack, L. J. W., Neira, L. M., and Mather, I. H., Affinity purification of polyclonal antibodies from antigen immobilized *in situ* in sodium dodecyl sulfate polyacrylamide gels. *Anal.*

Biochem., 187, 246-250 (1990).

Massey, V., Brumby, P. E., Komai, H., and Palmer, G., Studies on milk xanthine oxidase; some spectrum and kinetic properties. *J. Biol. Chem.*, 244, 1682-1691 (1969).

Mather, I. H., Weber, K., and Keenan, T. W., Membranes of mammary gland. XII. Loosely associated proteins and compositional heterogeneicity of bovine milk fat globule membrane. *J. Dairy. Sci.*, 60, 394-402 (1977).

Nagler, L. G. and Vartanyan, L. S., Subunit structure of bovine milk xanthine oxidase: Effect of limited cleavage by proteolytic enzymes on activity and structure. *Biochim. Biophys. Acta*, 427, 78-90 (1976).

Parks, D. A. and Granger, D. N., Xanthine oxidase: biochemistry, distribution and physiology. *Acta Phys. Scand.*, 548, 87-99 (1986).

Rajagopalan, K. V. and Handler, P., Purification and properties of chicken liver xanthine dehydrogenase. *J. Biol. Chem.*, 242, 4097-4107 (1967).

Stirpe, F. and Della Corte, E., Regulation of rat-liver xanthine oxidase: Conversion *in vitro* of the enzyme activity from dehydrogenase (Type D) to oxidase (Type O). *J. Biol. Chem.*, 244, 3855-3863 (1969).

Terao, M., Cazzaniga, G., Ghezzi, P., Bianchi, M., Falciani, F., Perani, P., and Garattini, E., Molecular cloning of a cDNA coding for mouse liver xanthine dehydrogenase. *Biochem. J.*, 283, 863-870 (1992).

Towbin, H., Staehelin, T., and Gordon, J., Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, 76, 4350-4354 (1979).

Waud, W. R., Brady, F. O., Wiley, R. D., and Rajagopalan, K. V., A new purification procedure for bovine milk xanthine oxidase: Effect of proteolysis on the subunit structure. *Arch. Biochem. Biophys.*, 169, 695-701 (1975).

Waud, W. R. and Rajagopalan, K. V., Purification and properties of the NAD^+-dependent (Type D) and O_2-dependent (Type O) forms of rat liver xanthine dehydrogenase. *Arch. Biochem. Biophys.*, 172, 354-364 (1976).

Zikakis, J. P. and Silver, M. R., Bovine milk xanthine oxidase: Effect of limited proteolysis on kinetics and structure. *J. Agric. Food Chem.*, 32, 340-343 (1984).