PROTEOLYTIC ACTIVITY IN BOVINE MILK XANTHINE OXIDASE PREPARATIONS

Gene R. Nathans* and E. P. Kirby Hade

The Department of Biochemistry, The University of Tennessee, Center for the Health Sciences, Memphis, TN 38163

Received June 25,1975

Bovine milk xanthine oxidase isolated in the presence of pancreatin was found to copurify with proteases from pancreatin. The effect of these proteases was observed by incubating azo-albumin with different xanthine oxidase preparations and by the observation of polypeptide degradation products with sodium dodecyl sulfate polyacrylamide gel electrophoreses. A commercial preparation of xanthine oxidase exhibited significant proteolytic activity.

Bovine milk xanthine oxidase (xanthine: oxygen oxidoreductase; E.C. 1.2.3.2.), derives from milk fat globule membranes (1), and has been extensively investigated because of its unique enzymatic properties (22). However, the structural properties of this enzyme are not well understood (2). Almost all of the isolations of XO from milk or cream have been carried out in the presence of pancreatin which contains proteases from the pancreas (8-10, 15). During the investigation of the subunit structure of XO significant proteolytic activity was found to copurify with XO isolated in the presence of pancreatin (3). This communication will present evidence demonstrating that proteases from pancreatin copurify with XO. Their presence in a commercial preparation of XO is also demonstrated.

MATERIALS AND METHODS

Xanthine (sodium salt, Grade III), pancreatin (hog pancreas, Grade VI), PMSF, azo-albumin (Type I), Trizma Base, 2-mercaptoethanol and Sepharose 6B were obtained from Sigma Chemical Co., St. Louis, Mo. Sephadex G-200 was obtained from Pharmacia Fine Chemicals Inc., Piscataway, N.J. Coomasie Brilliant Blue (R-250) was obtained from Wilson Diagnostics, Glenwood, IL. Pyronin Y, acrylamide, bis-acrylamide, and N,N,N',N',-tetramethylenediamine were obtained from Eastman Kodak Co., Rochester, N.Y. and further purified if necessary (4). SDS, Sequenal Grade, was obtained from Pierce Chemical Co., Rockford, IL. All other salts and reagents were the best available grade.

Gel filtration was carried out at 4°C with two 1.5 x 90 cm columns attached in series (Sephadex G-200 first, Sepharose 6B second) (5). Protein concentration of the column fractions was followed at 220 nm.

---

*Present address: The Franklin McLean Memorial Research Institute (operated by the University of Chicago for the U.S. Energy Research and Development Administration), 950 East 59th Street, Chicago, IL 60637; to whom all enquiries should be addressed.

Abbreviations: XO, bovine milk xanthine oxidase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

SDS (1%) polyacrylamide gel electrophoreses were carried out in 5% gels in a temperature regulated chamber maintained at 21°C (6). Samples were prepared for electrophoresis by at least three dialyses in 20 mM Tris-HCl, 2 mM EDTA (pH 8.0), sample buffer. After dialysis a volume of 2% SDS, 2% 2-mercapto-ethanol, 20% sucrose, and 20 µg per ml Pyronin Y was mixed with an equal sample volume, immediately heated in boiling water for several minutes, cooled, and layered on each gel for electrophoresis. Gels were stained for protein with Coomasie Brilliant Blue.

XO was isolated from cooled (5°C) unpasteurized milk or cream donated by Sealtest in Memphis, TN. A complete description of the methods used in this investigation for isolation of XO from milk or cream is described elsewhere (3). This procedure is similar to published methods (7-9). The main steps in this isolation involve two successive ammonium sulfate steps (0 to 20% w/v and 20% to 27% w/v) in the presence of 1% Triton X-100. Resuspension of the 20% to 27% precipitate yielded a preparation which displayed the characteristic absorption spectrum of XO from 360 nm to 700 nm (8,9). At this point in the purification the XO activity has increased 500-fold with an activity to flavin ratio ranging from 90 to 120 (8). The 280 nm/450 nm ratio of this preparation was less than or equal to 10.

Xanthine oxidase (Code: XOP), obtained from Worthington Biochemical Co., Freehold, N.J. was found to be fully active after storage at 4°C for about two years. The Worthington preparation used in these experiments is described in their Manual to be chromatographically prepared from their less pure Code: XO preparation, to have a 280 nm/450 nm ratio of approximately 6.0, and to be $\geq$ 90% homogeneous on polyacrylamide gel electrophoresis. The method of purification of XO described by Worthington involved the use of pancreatin (10).

One unit of XO activity was expressed by the conventional units used in XO literature as the amount of enzyme which changed the absorbance of 0.15 mM xanthine in 0.05 M potassium phosphate, pH 7.8, containing 0.005% EDTA by one absorbance unit at 295 nm in one minute at room temperature (11).

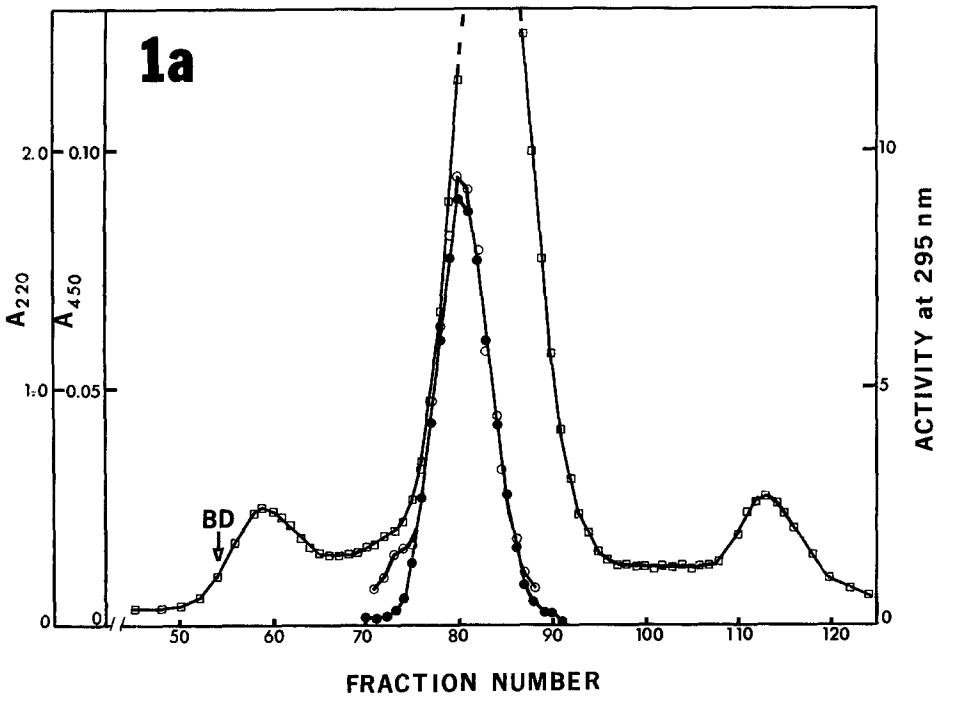
Proteolytic activity was studied at room temperature with azo-albumin as the substrate (12). The two buffers employed are those generally used in the isolation of XO: (a) 0.10 M potassium phosphate, pH 7.8, 2 mM sodium salicylate, 0.005% EDTA; and (b) 0.10 M sodium pyrophosphate, pH 7.15, 2 mM sodium salicylate, 0.005% EDTA.

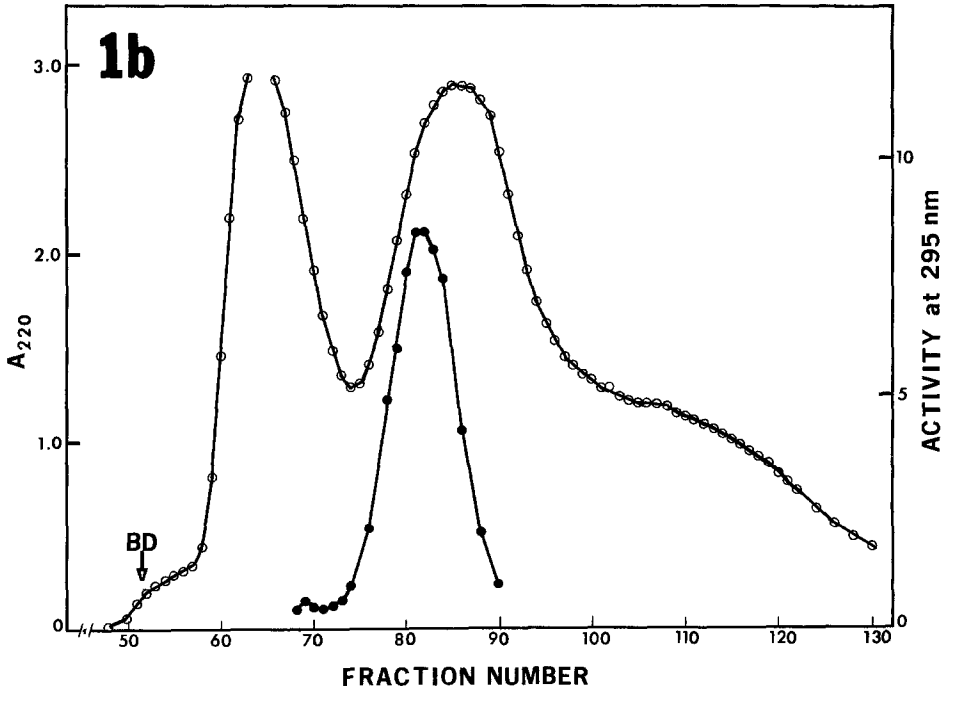
The preparations of XO applied to the Sephadex G-200/Sepharose 6B columns, to SDS polyacrylamide gels, and used to examine proteolytic activity, derived either from the 20% to 27% ammonium sulfate precipitate or from the Worthington preparation.

RESULTS

The elution patterns from Sephadex G-200/Sepharose 6B gel filtration of XO isolated in the absence and presence of pancreatin are presented in Figures 1a and 1b. Symmetric peaks of XO activity eluted at the same relative positions with both preparations and indicated similar Stokes' radii. More protein was observed (A_{220nm}) in the elution pattern (relative to XO activity) in the preparation which did not contain pancreatin.

SDS polyacrylamide gel electrophoretograms of the above preparations are presented in Figure 2. The major observation in gels c and d, was the loss of all the bands representing the larger molecular sized components observed in gels a and b. Gel e contained XO obtained from Worthington. A dark smear


1a
2.0
1.0
0
0.10
0.05
0
A220
A450
BD
50
60
70
80
90
100
110
120
10
5
0
ACTIVITY at 295 nm
FRACTION NUMBER


1b
3.0
2.0
1.0
0
A220
BD
50
60
70
80
90
100
110
120
130
10
5
0
ACTIVITY at 295 nm
FRACTION NUMBER

representing lower sized degradation products is observed at the bottom of this gel. The larger sized components are also absent in gel e.

One of the gels in Figure 2 (gel d) contained XO isolated in the presence of both 3 mM PMSF and pancreatin. Pancreatin contains several serine and non-serine proteases (13). Therefore, PMSF, a serine-protease inhibitor, was added at saturating concentrations(14) in the initial isolation step with pancreatin to inhibit these proteases and to observe the effect of the addition of PMSF on the isolation of XO. No real difference was apparent between the SDS gels in which pancreatin or PMSF and pancreatin were present during purification. The major observation again is the loss of the larger components described above.

The results of the experiments with azo-albumin as a substrate are presented in Table I. These results indicated that preparations with pancreatin included during isolation of XO exhibited significant proteolytic activity over the pH range 7.15 to 7.8. Proteolytic activity did not appear to be inhibited completely by 3 mM PMSF. This observation is consistent with the pattern observed in gel d in Figure 2 in which PMSF did not prevent degradation of protein either during dialysis in 20 mM Tris-HCl, 2 mM EDTA (pH 8.0) sample buffer, or during SDS denaturation. The majority of the proteolytic activity probably occurred when the sample of XO (100 µl) was dialyzed against three changes of 50 ml of 20 mM Tris-HCl, 2 mM EDTA (pH 8.0) buffer. Polypeptide degradation products were dialyzed away under these conditions and therefore less protein was actually added to the SDS gels than initially dialyzed.

DISCUSSION

Pancreatin was introduced into the purification of XO to remove large concentrations of the caseins found in milk or cream (15). However, the above evidence indicates that proteases from pancreatin copurify with XO through the final ammonium sulfate step. In general, in those purifications in which pancreatin is included, the incubation period extends from several hours to overnight (8,9). Further steps in the purification of XO are often carried

---

Figure 1a and 1b: Sephadex G-200/Sepharose 6B Gel Filtration Chromatography of Xanthine Oxidase Isolated by Proteolytic (Figure 1a) and Non-proteolytic (Figure 1b) Methods.

1a: A_{220nm} (□-□), A_{450nm} (○-○), and activity at 295 nm (●-●).

1b: A_{220nm} (○-○) and activity at 295 nm (●-●).

Buffer: 0.10 M sodium pyrophosphate, pH 7.15, 0.005% EDTA. Fraction volumes were 2.05 ml. BD is the position of the elution peak of Blue Dextran. The position of peak elution of DNP-alanine is fraction number 138 in Figure 1a and 140 in Figure 1b.

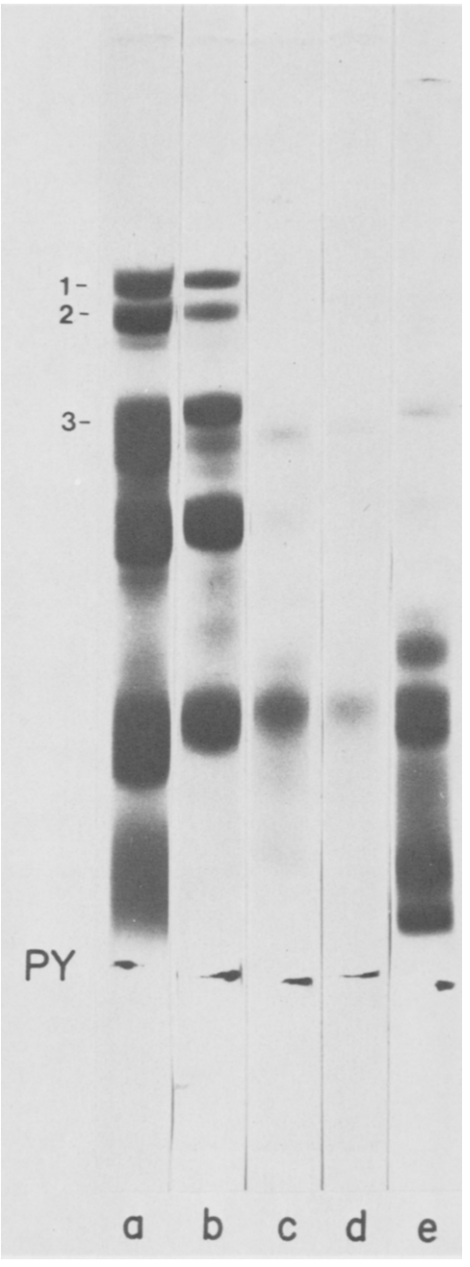


Figure 2: SDS (1%) Polyacrylamide Gel Electrophoresis of Xanthine Oxidase. *a.* Nonproteolytic preparation; *b.* nonproteolytic preparation precipitated with $CaCl_2$; *c.* XO isolated in the presence of pancreatin; *d.* XO isolated in the presence of PMSF and pancreatin; and *e.* Worthington XO. PY indicates the position of the Pyronin Y tracking dye at the end of electrophoresis. Estimated molecular size: 1 = 155,000, 2 = 125,000, and 3 = 85,000.

Table I

Proteolytic Activity of Xanthine Oxidase Preparations with Azo-albumin as Substrate

XO Preparation	$A_{440\ nm}$ of TCA Supernatant	
A. Buffer: 0.10 M potassium phosphate, pH 7.8, 2 mM sodium salicylate, 0.005% EDTA		
	No PMSF	With PMSF
Control	0.093	0.075
No pancreatin*	0.081	0.072
With pancreatin	2.858	2.100
Worthington XO	2.863	2.368
B. Buffer: 0.10 M sodium pyrophosphate, pH 7.15, 2 mM sodium salicylate, 0.005% EDTA		
	No PMSF	With PMSF
Control	0.080	.....
No pancreatin**	0.065	.....
With pancreatin	2.847	.....
Worthington XO	2.843	.....

*These values are the average of six duplicate assays.
**These values are the average of two duplicate assays.
The above values are the average of duplicate assays except where indicated. PMSF was added to each tube prior to the addition of XO at a final concentration of 3 mM. Proteolysis was initiated by pipetting XO preparations into each tube and mixing. Approximately 0.15 mg total protein or more was contained in each aliquot of XO. Control tubes contained the same volume of buffer as the XO preparation. The tubes were incubated overnight at room temperature before precipitation with TCA.

out with hydroxylapatite or tricalcium phosphate chromatography (8,9). However, the effect of these proteases on XO may have already occurred by this point in the isolation procedure.

Earlier studies with XO obtained from Worthington, purified with pancreatin, suggested that proteolytic modification of XO might be present (16). Evidence that proteases were still present and active was not presented at that time.

The observation that proteases may have either modified the structure of XO or are still present in certain XO preparations has several implications. First, the real subunit structure of XO will be obscured due to proteolytic modification. The observations that the apparent size of XO is around 275,000

to 300,000 (17-19), whether isolated with or without pancreatin, and that the catalytic activity is still maintained, suggest that despite the presence of proteases the tertiary-quaternary structure remains intact. Studies on the resolution of the subunit structure of XO isolated without pancreatin have recently been carried out (3,20). Second, commercial preparations of XO have been used in investigations concerning the proposed relationship between XO and atherosclerosis (21). These experiments involved, in part, the injection of such preparations into experimental animals to produce antibodies to XO. If proteases are present, and if the XO is proteolytically modified, then the presence of both active proteases in the injection site and the introduction of modified XO molecules could introduce complications into the formation of antibodies to native XO.

ACKNOWLEDGMENTS

We thank Dr. Robert M. Macleod, Dr. Eugene Goldwasser, and Dr. Thomas F. Deuel for their valuable criticisms concerning this manuscript. This work was supported by American Cancer Society Institutional Research Grant IN-85G-6.

REFERENCES

1. Morton, R. K. (1954). Biochem. J. 57, 231-237.
2. Bray, R. C. (1973) in Chemistry and Uses of Molybdenum (Mitchell, P.C.H., ed.), pp. 216-223, Climax Molybdenum Co., Ltd., London.
3. Nathans, Gene R. (1974). Doctoral Dissertation, University of Tennessee, Memphis, TN. 75-10830, University Microfilms, A Xerox Co., Ann Arbor, MI.
4. Rodbard, D. and Chrambach, A. (1971) Anal. Biochem. 40, 95-134.
5. Joustra, M. K. (1966). Protides of the Biological Fluids 14, 533-541.
6. Fairbanks, G., Steck, T. L., and Wallach, D.F.H. (1971). Biochemistry 10, 2606-2616.
7. Brady, F. O. (1970). Doctoral Dissertation, Duke University, Durham, NC. 70-21985, University Microfilms, A Xerox Co., Ann Arbor, MI.
8. Hart, L. I., McGartoll, M. A., Chapman, H. F., and Bray, R. C. (1970). Biochem. J. 116, 851-864.
9. Massey, V., Brumby, P. E., Komai, H., and Palmer, G. (1969). J. Biol. Chem. 244, 1682-1691.
10. Gilbert, D. A. and Bergel, F. (1964). Biochem. J. 90, 350-353.
11. Nelson, C. A. and Handler, P. (1968). J. Biol. Chem. 243, 5368-5373.
12. Tomarelli, R. M., Charney, J., and Harding, M. L. (1949). J. Lab. Clin. Med. 34, 428-433.
13. Uriel, J. and Avrameas, S. (1965). Biochemistry 4, 1740-1749.
14. Prouty, W. F. and Goldberg, A. L. (1972). J. Biol. Chem. 247, 3343-3352.
15. Ball, E. G. (1939). J. Biol. Chem. 128, 51-67.
16. Carey, F. G., Fridovich, I., and Handler, P. (1961). Biochim. Biophys. Acta 53, 440-442.
17. Andrews, P., Bray, R. C., Edwards, P., and Shooter, K. V. (1964). Biochem. J. 93, 627-632.
18. Hedrick, J. L. and Smith, A. J. (1968). Arch. Biochem. Biophys. 126, 155-164.
19. Wongchai, V. (1974). Doctoral Dissertation, University of Tennessee, Memphis, TN. 75-2505, University Microfilms, A Xerox Co., Ann Arbor, MI.
20. Nathans, G. R. and Hade, E. P. K. Manuscript in preparation.
21. Oster, K. A., Oster, J. B., and Ross, D. J. (1974). Amer. Lab. 6, 41-47.
22. Olson, J. S., Ballou, D. P., Palmer, G., and Massey, V. (1974). J. Biol. Chem. 249, 4363-4382.