BOVINE MILK XANTHINE OXIDASE FRACTIONS OF IMPROVED POTENCY; ISOLATION OF MOLYBDENUM-FREE, IRON-POOR, ACTIVE PREPARATIONS[1]

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Bovine milk xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) was previously reported (Bray, 1963; Avis et al., 1956; Bray et al., 1966) to contain 8 atoms of iron, 1 to 2 atoms of molybdenum, and 2 molecules of flavin adenine dinucleotide (FAD) per protein molecule of 308,000 molecular weight (as determined from the FAD content); all these three constituents were considered to be intimately involved in the catalytic reaction and, on the basis of electron spin resonance studies, a sequence was suggested (Bray et al., 1964) involving electron transfer from the substrate, xanthine, first to molybdenum, then to flavin, then to iron (Ehrenberg and Bray, 1965), and finally to oxygen. Iron-free, active enzyme preparations were recently reported (Bayer and Voelter, 1966; Uozumi et al., 1967), indicating that the iron is obviously not essential for the oxidation of xanthine by molecular oxygen; however, these reports have not been confirmed (Johnson et al., 1967). The most active fractions of bovine intestinal xanthine oxidase were found (Roussos and Morrow, 1966; Roussos, 1967) to be devoid of molybdenum and contain an average of 17.4 atoms of iron, 4.2 atoms of copper, and 1.0

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molecule of FAD per minimal molecular weight (based on FAD content) of 236,000 ± 82,000.[2] The results of the present studies on the xanthine oxidase from bovine milk indicate that, as in the case of the intestinal enzyme, molybdenum is not essential in the xanthine:O_2 oxidoreductase activity, favor the idea that at least a major portion of the iron is also not important for the latter activity, disagree seriously with the composition of the enzyme as derived from earlier work, cast doubt on the general validity of the above-mentioned intramolecular electron transfer sequence, and suggest that all investigations on this enzyme have been thus far carried out with samples which had become at least 60—70% inactivated during preparation, storage or other treatment.

The enzyme was purified at 0—4° by a procedure which incorporates various techniques (Roussos, 1967) for controlling contamination by extraneous metals. Commercial xanthine oxidase (Worthington Biochemical Corp.; Code: XO) was dialyzed for 23 hours against two 1000 volume-changes of 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and then centrifuged. The supernatant fluid (Fraction I; 201 mg protein) was applied to a DEAE-Sephadex column (4.9 cm^2 X 1.6 cm) which was pre-equilibrated and then washed (Fig. 1) with the above dialysis-buffer (400 ml) until the effluent was free of enzymatic activity, protein, and $\underline{E}_{280}$. A linear gradient of elution was applied with 10 mM (400 ml) and 100 mM (400 ml) of phosphate buffer (pH 7.4) containing 10 μM EDTA as limiting concentrations. The eluates with the highest specific activity were pooled (Fraction II) and applied to a Chelex 100 column (4.2 cm^2 X 2 cm) which was pre-equilibrated and then washed with 20 mM phosphate buffer (pH 7.4) containing 1 mM EDTA (5 ml). The combined effluent was dialyzed for 6 hours against two 50 volume-changes of 100 μM phosphate buffer (pH 7.4) containing 10 μM EDTA (Fraction III). Both disc electrophoresis on polyacrylamide gel at pH 9.5 and zone

[2] Arithmetic mean ± standard deviation.

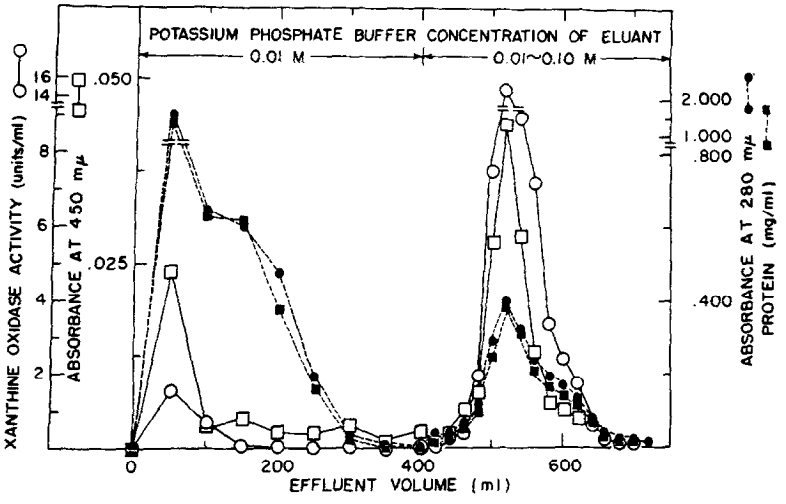

Fig. 1. Chromatography of Fraction I on a DEAE-Sephadex column. For details and definitions see the text and Table I. Protein was determined by the method of Lowry et al. (1951). Enzymatic activity was assayed at 25°. The respective specific activities of Fraction I, the peak "wash fraction" (Fraction W), Fraction II, and Fraction III isolated during this purification were: "APR" = 4.76, 0.68, 40.2, and 59.2; "AFR" = 75.0, 43.8, 357, and 344.

electrophoresis on cellulose polyacetate at pH 7.6—10.6 gave rise with several of the final fractions to a single migrating protein band.

The following conclusions are based on the data of Table I. Despite their higher "PFR" values (Avis et al., 1955; Gilbert and Bergel, 1964) and except for the "APR" of sample 6, the purified fractions possessed specific activities which were significantly higher than those of hitherto reported preparations, being about 3-fold as high in the case of sample 4 (Bray et al., 1966; Avis et al., 1955; Gilbert and Bergel, 1964). The properties of Fraction W, which probably consists of the "inactive" form of the enzyme (Bray, 1963; Avis et al., 1955), resemble roughly those of the previously reported (Bray et al., 1966) "high-molecular-weight fraction". At least 78% of the flavin of Fraction III is present in the form of FAD. The minimal molecular weight of the enzyme, calculated on the basis of the FAD content of seven purified preparations, is 354,000 ± 94,100;[2] this is 2.3-fold higher

Table I. COMPOSITION OF MILK XANTHINE OXIDASE PREPARATIONS

Sample[a]	Fraction[b]	"PFR"[c]	Specific activity[d]		μmoles/gm protein[e]		microgramatoms/gm protein[f]			Fe/Mo/Cu/FAD (gramatoms/gramatoms/gramatoms/mole)	Minimal molecular weight[g]
			"APR"	"AFR"	Total flavin	FAD	Fe	Mo	Cu		
1	I	30	2.33	68.8	——[i]	——[i]	1.74	0.11	0.74		
2	W	27	1.59[h]	42.8[h]	1.15	0.97	2.30	0.17	0.06	2.4/0.18/0.06/1.0	1,030,000
3	III	10	18.8	188	5.56	4.31	5.28	<0.34[j]	2.32	1.2/<0.079[j]/0.54/1.0	233,000
4	III	5.8	51.5	300	3.24	3.41	6.88	0.81	0.61	2.0/0.24/0.18/1.0	294,000
5	III	9.7	17.7	171	2.77	2.40	25.7	10.3	4.61	10.7/4.28/1.92/1.0	417,000
6	III	12	12.6	153	2.23	1.96	12.9	<0.049[j]	1.67	6.6/<0.025[j]/0.85/1.0	511,000

[a] Sample 1 served as the starting material for both samples 2 and 3.

[b] See the text and Fig. 1 for the description of the purification step represented by each fraction.

[c] I.e., E_{280}/E_{450} (Avis et al., 1955).

[d] A unit of xanthine oxidase was defined according to Fridovich (1962), except that the assay was carried out at 23.5°, unless otherwise specified. For definition of symbols "APR" and "AFR" see Avis et al.(1955).

[e] Total flavin and FAD contents were determined fluorometrically by the procedure of Burch (1957); riboflavin, carried through the entire procedure, was used as standard. $E^{1\%}_{1cm}$ (280 mμ) values of 11.5 and 13.1 were used

Table I — Continued

in calculating the protein content of samples possessing "PFR" values of <9.4 and >9.4, respectively (Avis et al., 1956); protein data thus calculated for fifteen different preparations were found to be 13.6 ± 8.5% lower than the corresponding figures determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin (Sigma Chemical Co.) as the standard; the protein content of Fraction III determined by the latter method was found to be 1.5% lower than its dry weight content determined by drying at 110° and correcting for ash weight.

Fe, Mo, and Cu contents of either diluted, undiluted, or concentrated samples and blanks were determined by atomic absorption spectroscopy (Willis, 1963) with a Beckman model 979 spectrophotometer (laminar flow burner; air-acetylene flame, fuel-rich in the case of Mo; triple pass; hot operation mode; wavelength setting (mμ): Fe, 248.3; Mo, 313.3; Cu, 324.8) used with a linear/log Varicord model 43 strip chart recorder. The standard addition (two additions per determination) method (Willis, 1963; Ramírez-Muñoz et al., 1966) was used throughout, dialysis fluids serving as both blanks and diluents for both samples and standards. The detection limits and coefficients of variation of Fe, Mo, and Cu were 0.01—0.05, 0.01—0.02, and 0.01—0.02 ppm and <6.5, 3.0, and <6.2%, respectively. No chemical interference effects could be detected in these analyses, and known quantities of all three metals added to a crude enzyme extract (14 mg protein/ml) were quantitatively recoverable. The same analytical results for Fe, Mo, and Cu were obtained whether or not the samples were subjected to dry ashing at 400° for 24 hours prior to analysis.

[g] Calculated on the basis of one mole of FAD per mole of enzyme.

[h] Enzymatic activity was determined at 25°.

[i] Not determined.

[j] Not detected; any Mo present was there only at a level lower than the limit cited. Metal analyses of samples 3 and 6 and of their corresponding blanks were carried out both before and after 5- and 16-fold respective concentration by lyophilization.

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than the value of the crystalline preparation (Avis et al., 1956). The iron content of Fraction III did not always go parallel with its specific activity and, in the case of samples 3 and 4, it was about 4—5-fold lower than the content of previous preparations (Avis et al., 1956; Bray et al., 1966); moreover, the molar Fe:FAD ratio of 4 previously observed has not been confirmed. The molybdenum content and specific activity of the purified enzyme were not directly proportional to one another, and the most active fraction (sample 4) contained 5 times less molybdenum than previously reported (Avis et al., 1956; Bray et al., 1966); moreover, there was no appreciable change in enzymatic activity in the absence of this metal (samples 3 and 6). In contrast to an earlier report (Avis et al., 1956), the purified fractions

contained significant amounts (relatively to the Mo content) of copper, but at a level considerably lower (on the basis of both protein and FAD contents) than that of the intestinal enzyme (Roussos and Morrow, 1966); the significance, if any, of this observation is not known.

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