SOME PROPERTIES OF AN OXALIC OXIDASE PURIFIED FROM BARLEY SEEDLINGS [1]

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During investigation of the degradation of diketogulonic acid to oxalic acid it was necessary to find a specific enzyme that could be used for testing this reaction. It was found that barley seedlings, especially in the roots, have a soluble oxalic oxidase. Some of its properties are reported here. It was mentioned previously in 1930 that a crude extract of barley could metabolize oxalic acid, but only in the presence of a yeast kockshaft (Fodor and Frankenthal, 1930). The enzyme we purified is related to moss oxidases (Datta and Meeuse, 1955) and is activated not only by flavins but also other compounds and its more purified state does not need a cofactor.

The roots and sprouts of 10-day barley seedlings were homogenized at 50,000 rpm in water and centrifuged at 59,000 x g in a Spinco centrifuge. The purification was accomplished with heat treatment, Ammonium sulfate fractionation and DEAE cellulose chromatography using phosphate buffers and an automatic step gradient elution device (Teekell et al., 1962). The specific activity obtained in different batches varied between 4,000 and 7,000 and the fold of purification between 800 and 1600. Before using the enzyme it was exhaustively dialyzed against water. Details of the purification will be reported elsewhere.

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Oxalic oxidation was followed by assayed respiratory C-14 O_2 arising from oxalate C-14 and counted by liquid scintillation procedures (Chiriboga and Roy, 1962). Generally the system used was 2 µm of oxalic acid, 1.25 µg of protein, 0.4 ml of succinate buffer (0.1 M at pH 3.6), made up to a volume of 1 ml. The incubating period was 10 minutes at 38° C. In stoichiometric studies, 3.3 µg of protein were used and incubated at 22° C. The reaction was stopped by acid injected into the vessel through a hole occluded by a rubber band. The disappearance of oxalic acid was measured using a procedure described before (Chiriboga, 1962).

The enzyme has a pH optimum around 3.6 in succinate buffer. Other buffers have some inhibitory action relative to succinate.

Table 1. Effect of buffers on the oxalic oxidase activity.

Buffers 0.08 M Na form - pH 4.00	Enzyme activity CPM	% in relation with succinate
Succinate	3408	100.0
Acetate	2213	64.9
Phosphate	1303	38.2
Malate	2093	61.4
Citrate	1837	52.7

Stoichiometric amounts of oxalic acid disappear in relation with the CO_2 collected (Fig. 1). The reaction could be in two steps; first, decarboxy-

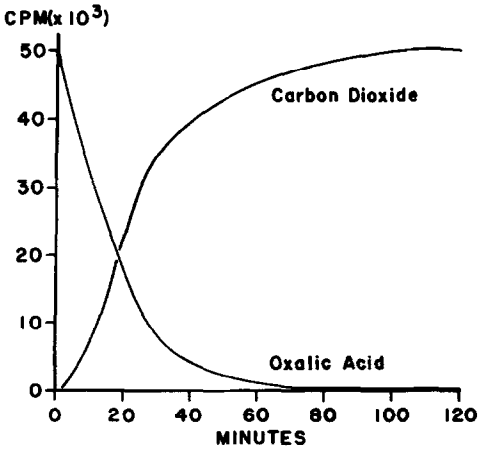

Fig. 1. Stoichiometry of the enzymatic oxidation of oxalic acid to carbon dioxide.

lation to formic acid with production of CO_2 and secondly, the oxidation of formic acid to CO_2. The latter is unlikely because the system did not metabolize formic C-14 and also because formic acid was not detected using the colorimetric method of Wood and Gest (1957). The system needs oxygen and the formation of H_2O_2 suspected is under study.

The system is very sensitive to changes in ionic strength (Fig. 2) probably due to changes in the dissociation of the substrate. Metals, especially copper and calcium, inhibit the enzymatic activity but probably by a different mechanism.

The inhibitors were tested in the system both with and without riboflavin. The results are shown in the following table:

Table 2. Effect of Inhibitors

Concentrations molar	Enzyme alone % of activity	Enzyme plus riboflavin* % of activity
Control	100.0	100.0
Iodoacetate		
5×10^{-4}	21.2	29.4
5×10^{-3}	1.7	5.5
2.5×10^{-2}	1.1	1.3
Fluoride		
5×10^{-4}	42.3	42.9
5×10^{-3}	3.4	19.2
2.5×10^{-2}	2.5	13.6
Cyanide		
5×10^{-4}	111.2	125.1
5×10^{-3}	133.1	107.8
2.5×10^{-2}	34.9	28.6
Arsenite		
5×10^{-4}	91.2	109.2
5×10^{-3}	143.3	108.5
2.5×10^{-2}	87.7	94.8

* Riboflavin 7.5×10^{-5}

The effect of iodoacetate is probably associated with the SH groups of the enzyme; the cyanide effect at lower concentration could be similar to the effect of EDTA and 8-hydroxyquinoline; that is, its metal binding activity. At higher concentrations the effect could be a salt effect or depression of some chain reaction. Flavin compounds activate the enzyme in the following order:

Table 3. Effects of Flavins.

Concentration 10^{-4} M	Activity CPM	%
No cofactor	3410	100.0
Riboflavin	9284	272.2
FMN	5797	170.0
FAD	5690	166.8

When using riboflavin or FMN we were not able to detect any quenching of the fluorescence with or without substrate present following the reaction in a Turner Fluorometer.

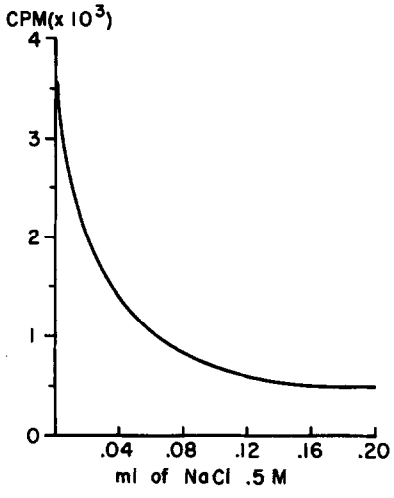

Fig. 2. Effect of ionic strength on enzymatic activity.

The solution of the enzyme is clear without any color and the lyophylized powder is pure white. The spectrum of the enzyme did not change after the treatment with acid. Precipitation at low pH with Ammonium sulfate in a cold environment and dialysis at a low pH did not yield an apoprotein. No evidence of binding of riboflavin, FMN, or FAD was observed in the presence or absence of substrate using ultrafiltration methods. When the enzyme was boiled for 20 minutes and then ultrafiltrated, its concentrate activated the enzymatic system. Chromatography of this product did not show any free flavin compounds but many ninhydrin positive spots. A microbiological analysis of this product is under way. EDTA and 8-hydroxyquinoline activated the enzyme. In many, but not all, comparative studies between riboflavin and 8-hydroxyquinoline they acted in a close pattern. When they were not at saturation levels, they acted

additively. When Fe^{+++} or Fe^{++} was added to the system, the effect of 8-hydroxyquinoline was not enhanced.

Table 4. Effects of synthetic chelates.

Concentration molar	Enzymatic activity CPM	%
Enzyme alone	1876	100.0
EDTA		
2×10^{-4}	2458	131.0
2×10^{-3}	2635	140.0
8-Hydroxyquinoline		
2×10^{-4}	8118	432.7
2×10^{-3}	10484	558.8
O-Phenantroline		
2×10^{-4}	1712	91.2
2×10^{-3}	1596	85.0
$\alpha\alpha'$ Dipyridyl		
2×10^{-4}	1448	79.1
2×10^{-3}	1605	85.5

The activation of 8-hydroxyquinoline and its similarity to riboflavin raises an interesting question and is under study. The effect could be by chelation of some metallic impurity that inhibits the system, the formation of some kind of chelate that could transport electrons, or some action of the active center of the enzyme.

Some quinones and related compounds activate the system, but methylene blue and menadione have some inhibitory effect.

Table 5. Effects of quinones and others.

Concentration 10^{-4} M	Enzymatic activity CPM	%
Enzyme alone	3595	100.0
Hydroquinone	15796	439.5
Quinhydrone	13416	373.2
P-quinone	10598	294.8
Quinoline	4823	134.1
Menadione	2884	80.2
Methylene blue	2840	79.0

Although it is true that flavins activated the oxalic oxidase purified from barley, no evidence was found that these are the native cofactors. Flavins according to our results acted in a free form in the system, whereas the na-

tive cofactor, if it exists, has a strong binding affinity for the enzyme. In the meantime, we do not know the nature of the native cofactor so it is difficult to understand the mechanism of the reaction.

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