[Chem. Pharm. Bull. 27(9)2003—2007(1979)]

UDC 547.158.61.07 : 581.192 : 582.542.1

Purification and Properties of Oxalate Oxidase from Barley Seedlings[1)]

Mamoru Sugiura, Hirohiko Yamamura, Kazuyuki Hirano,[2a)] Masanori Sasaki,[2b)] Masako Morikawa, and Minoru Tsuboi[2c)]

Gifu College of Pharmacy,[2a)] Niigata College of Pharmacy[2b)] and Tokyo College of Pharmacy[2c)]

(Received October 26, 1978)

Oxalate oxidase from barley seedlings (*Hordeum vulgare* L. var. *distichon* Alefeld) was purified to homogeneity as determined by disc gel electrophoresis.

The enzyme hydrolyzed oxalate, but no other related acids. The optimum pH was around pH 3.5, and this enzyme was stable at acidic pHs. The enzyme appears to consist of two identical subunits. Various other properties were investigated.

Keywords——barley seedlings; oxalate oxidase; oxalic acid; purification; properties

Since oxalate oxidase was discovered in mold by Houget and Mayer,[3)] this enzyme has been found in various sources[4)]; in some cases it has been partially purified and its properties studied. However, oxalate oxidase has not yet been investigated in detail and has not been obtained in a homogeneous state.[5)]

In this paper, we report the purification of oxalate oxidase from barley seedlings (*Hordeum vulgare* L. var. *distichon* Alefeld) and describe its properties.

Materials and Methods

Materials——Peroxidase was purchased from Sigma Co. Ltd., and ampholine was obtained from LKB Co. Ltd. Other reagents used were of reagent grade.

Assay of Enzyme Activity——Oxalic acid (18 mg) dissolved in 100 ml of 0.1 M succinate buffer (pH 3.5) was used as a substrate. The developing solution was prepared as follows; 8 mg of 4-aminoantipyrine, 8 mg of peroxidase (152 U/mg) and 20 μl of N,N-dimethylaniline were dissolved in 100 ml of 0.1 M phosphate buffer (pH 5.5). The substrate solution (1.5 ml) was preincubated at 37° for 5 min, and then the enzyme solution (2—100 μl) was added. After 10 min, the enzyme reaction was stopped by the addition of 0.3 ml of 0.1 M Tris solution and 1 ml of the developing solution. The absorbance of the resulting solution was measured at 550 nm. One unit of the enzyme activity was defined as the amount of the enzyme which formed 1 μmol of hydrogen peroxide per min in the standard assay system.

Determination Protein——Protein was determined by the method of Lowry *et al.* using albumin as a standard protein.[6)]

Disc Gel Electrophoresis——Electrophoresis was carried out with 7.5% polyacrylamide gel (pH 9.4) at a constant current (3.5 mA/tube) for 100 min at 4°. The protein in the gel was stained with Amide black 10B.

SDS-Gel Electrophoresis——Electrophoresis was performed with 5% polyacrylamide gel containing 0.1% SDS according to the method of Weber and Osborn.[7)]

1) This paper forms Part CLIX of "Studies on Enzymes," by M. Sugiura.
2) Location: *a) 5-6-1 Mitahorahigashi, Gifu, 502, Japan*; *b) 5829 Kamishinei-cho, Niigata, 950-21, Japan*; *c) 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.*
3) J. Houget and A. Mayer, *Compt. Rend.*, **185**, 304 (1927).
4) *a)* A. Foder and L. Frankenhal, *Biochem. Z.*, **225**, 417 (1930); *b)* J. Chiriboga, *Biochem. Biophys. Res. Commun.*, **11**, 4 (1963); *c)* A.E. Leek and V.S. Butt, Proc. *Biochem. J.*, **128**, 87 (1973); *d)* E.B. Vaisey and V.H. Cheldeline, *Arch. Biochem. Biophys.*, **95**, 66 (1961); *e)* Y. Suzuki and J.D. Meesue, *Plant Cell Physiol.*, **6**, 25 (1965).
5) J. Chiriboga, *Arch. Biochem. Biophys.*, **116**, 516 (1966).
6) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
7) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).

Isoelectric Focusing——Electrofocusing was performed with 1% carrier ampholyte (pH 2.5—5.0) at a constant potential of 700 V for 40 hr at 4° according to the method of Vesterberg and Svensson.[8]

Crude Enzyme——Barley seed (*Hordeum vulgare* L. var. *distichon* ALEFELD) was supplied by the Aichiken Agricultural Research Center. Seedlings were used as an enzyme source after 10 days.

Results

Purification of Oxalate Oxidase

Barley seedlings (1.6 kg) were homogenized with a polytron and oxalate oxidase was extracted with 1.6 l of chilled distilled water. The extract was centrifuged at $1000\times g$ for 30 min at 4°. The resulting supernatant was heated at 80° for 3 min and then centrifuged at $1000\times g$ for 15 min at 4°. Solid ammonium sulfate was added to the supernatant to give a final 0.7 saturation and the solution was allowed to stand for 20 min at 0°. The resulting precipitate was redissolved in distilled water. The solution was dialyzed overnight against 3 l of distilled water at 4°.

The dialyzed enzyme solution was fractionated with ammonium sulfate, and the fraction corresponding to 0.3—0.5 saturation of ammonium sulfate was collected and dissolved in

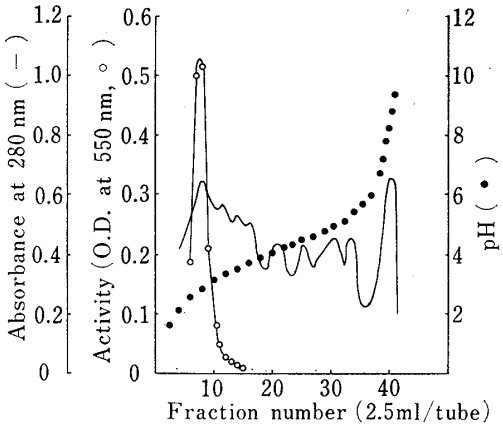

Fig. 1. Isoelectric Focusing Pattern of Oxalate Oxidase from Barley Seedlings

The enzyme was applied to the isoelectric focusing column (110 ml) with 1% carrier ampholyte (pH 2.5—5.0). Electrophoresis was performed for 40 hr with a constant potential of 700 V at 4°.

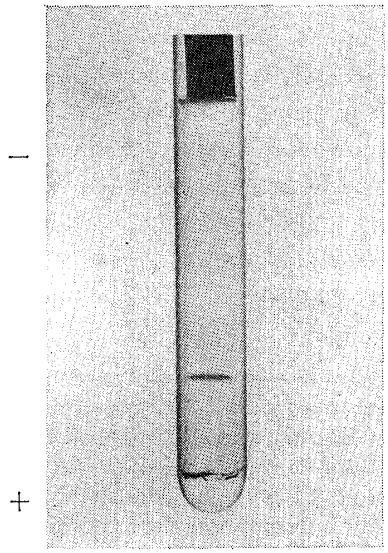

Fig. 2. Disc Electrophoretic Pattern of Purified Oxalate Oxidase from Barley Seedlings

Conditions: 7.5% polyacrylamide gel, pH. 9.4, 3.5 mA/tube, 4°, 100 min.

TABLE I. Purification of Oxalate Oxidase from Barley Seedlings

Purification step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude enzyme extract	53980	47.2	8.7×10^{-4}	—
Heat treatment	43278	264.0	6.1×10^{-3}	100
Ammonium sulfate precipitation (0.75 satn.)	2203	138.8	6.3×10^{-2}	52.6
Ammonium sulfate fractionation (0.3—0.5 satn.)	346.9	180.4	5.2×10^{-1}	68.3
Isoelectric focusing	9.69	82.4	8.5	31.2

8) O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).

distilled water. The enzyme solution was dialyzed overnight against distilled water at 4° and concentrated with a membrane filter to a small volume. The concentrated enzyme was fractionated by electrofocusing as shown in Fig. 1.

The enzyme gave a single peak at around pH 2.8. The purification procedure is summarized in Table I. The recovery of activity was 31% during the purification; the specific activity of the purified enzyme was 8.5 units/mg with 1396-fold purification compared with the crude enzyme. The homogeneity of the purified enzyme was confirmed by disc gel electrophoretic pattern shown in Fig. 2.

Properties of Oxalate Oxidase

Molecular Weight——The molecular weight was estimated by the Sephadex G-200 gel filtration technique using γ-globulin, bovine serum albumin, ovalbumin and cytochrome C as standard proteins. The molecular weight of oxalate oxidase from barley seedling was found to be 1.5×10^5. The subunit molecular weight of this enzyme was also investigated by SDS-electrophoresis (Fig. 3). The subunit molecular weight was calculated to be 7.5×10^4. Thus, the enzyme appears to consist of two identical subunits.

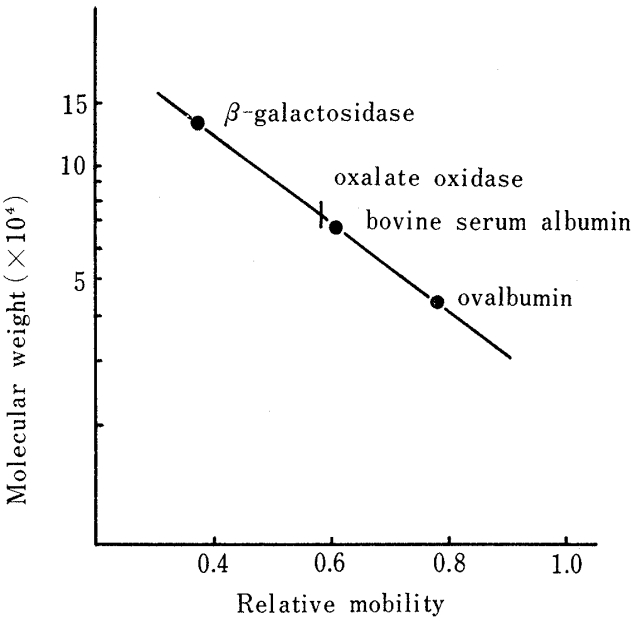

Fig. 3. Determination of the Molecular Weight of Oxalate Oxidase from Barley Seedlings

7.5% polyacrylamide gel containing 1% sodium dodecyl sulfate was used.

Table II. Substrate Specificity of Oxalate Oxidase from Barley Seedlings

Substrate (0.6 mM)	Relative activity (%)
Oxalic acid	100
Malonic acid	0.66
Succinic acid	0
Glutaric acid	0.72
Citric acid	0.44
Glycolic acid	0.55
Glycoxylic acid	0.54
α-Ketoglutaric acid	0.66
Maleic acid	0.73
Oxalacetic acid	0.97
Oxamide	0.73
L-Aspartic acid	0.36
L-Glutamic acid	0.66
Glycine	0.66
Ascorbic acid	0.36
Oxalsuccinic acid[a)]	1.09
Malic acid	0.73

a) 0.06 mM.

Substrate Specificity

The substrate specificity of the oxalate oxidase was investigated, and the results are shown in Table II. Oxalate oxidase has high activity towards oxalic acid but not towards other acids.

pH Stability and Optimum pH

The optimum pH of oxalate oxidase was around pH 3.2, and more than 80% of the activity remained after treatment at 37° for 30 min in the pH range from pH 2 to pH 4.

Optimum Temperature and Heat Stability

The enzyme has maximum activity at 35°. The heat stability of oxalate oxidase was investigated by heating the enzyme in 0.2 M succinate buffer (pH 3.5) at various temperatures

for 30 min. More than 80% of the activity remained at 75°; this enzyme was extreamly stable below 70°.

Effect of Various Reagents on the Activity

As shown in Table III, oxalate oxidase was activated by 8-hydroxyquinoline, but not by EDTA or *o*-phenanthroline The activity of oxalate oxidase was strongly inhibited by 2-mercaptoethanol, iodine and N-bromosuccinimide.

Effect of Various Metal Ions and Anions on the Activity

Oxalate oxidase activity was inhibited by Hg^{2+} and halogen ions such as fluoride, chloride and iodide, but not by other metal ions, as shown in Table IV.

Table III. Effects of Various Reagents on Oxalate Oxidase Activity

Reagent	Remaining activity (%)		
	10^{-3} M	10^{-4} M	10^{-5} M
8-Hydroxyquinoline	186	259	164
o-Phenanthroline	72	75	101
EDTA	103	83	84
L-Cysteine	5	55	105
2-Mercaptoethanol	13	20	72
KCN	165	103	97
N-Bromosuccinimide	—	3	66
Diisopropylfluorophosphate	126	121	106
Monoiodoacetate	16	77	93
p-Chloromercuribenzoate	72	79	98
Iodine	4	13	36

Table IV. Effects of Cations and Anions on Oxalate Oxidase from Barley Seedlings

Salt	Remaining activity (%)		
	10^{-3} M	10^{-4} M	10^{-5} M
None	100	100	100
CH_3COOAg	78	102	101
$(CH_3COO)_2Ca$	114	104	103
$(CH_3COO)_2Cu$	136	106	119
$(CH_3COO)_2Hg$	27	66	114
$(CH_3COO)_2Mn$	97	100	116
CH_3COONa	89	109	117
$(CH_3COO)_2Ni$	151	121	84
$(CH_3COO)_2Pb$	112	64	83
$(CH_3COO)_2Zn$	136	137	107
NaF	40	87	83
NaCl	43	72	76
NaBr	114	77	93
NaI	14	51	80
Na_2SO_4	75	86	78
$NaNO_3$	16	39	67
Na_2CO_3	52	69	66
NaN_3	2	11	33

Discussion

A homogeneous preparation of oxalate oxidase from barley seedlings was obtained by the techniques of heat treatment, ammonium sulfate fractionation and isoelectric focusing. The specific activity of the purified oxalate oxidase was 8.5 units/mg.

From the results of molecular weight and subunit molecular weight measurements, this enzyme appears to consist of 2 subunits. The isoelectric point of this enzyme is pH 2.8, which is very acidic, so the enzyme may contain acidic groups as phosphoryl or sulfonyl groups.

Optimum pHs of oxalate oxidase from mold,[3,4e] barley seedlings,[5] spinach beet[4c] and fungi[4d] were pH 5.0, pH 3.5, pH 4.0 and pH 2.6, respectively, suggesting that oxalate oxidase from barley seedlings is generally different from the former three. Our enzyme was inhibited by nitrate, halogen ions and sodium azide, like the mold oxidase.

Chiriboga[5] had demonstrated that oxalate oxidase from barley seedlings is activated by 8-hydroxyquinoline and EDTA, but the enzyme obtained from barley seedlings (*Hordeum vulgare* L. var. *distichon* Alefeld) was activated only by 8-hydroxyquinoline.

This oxalate oxidase from barley seedlings is very stable in the acidic pH range, and its substrate specificity is characteristic.

We are now investigating its application for the determination of oxalate in serum for clinical diagnostic tests.