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Oxalate Decarboxylase from *Collybia velutipes*

PURIFICATION, CHARACTERIZATION, AND cDNA CLONING*

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The oxalate-degrading enzyme oxalate decarboxylase (EC 4.1.1.2), which is inducible by oxalic acid, was purified to homogeneity from a crude extract of *Collybia velutipes*, a basidiomycetous fungus. Two forms of the enzyme were resolved on chromatofocusing. The two isozymes were shown to be related by amino acid composition, peptide mapping, and immunological cross-reactivity. Peak A, eluting at pH 3.3, was used for further study; the K_m was found to be 4.5 mM, and the V_{max} was 166 μmol/min/mg. The subunit molecular mass of the glycosylated enzyme was 64 kDa, whereas the mass of the deglycosylated protein was 55 kDa. The enzyme showed an acidic pI, was very stable over a wide pH range, and was moderately thermostable.

The cDNA encoding the enzyme was obtained by immunoscreening a λgt11 expression library. *In vitro* translation of hybrid-selected mRNA gave a 55-kDa protein. Genomic Southern hybridization indicated that oxalate decarboxylase is encoded by a single gene. The cDNA probe hybridized to a single 1.5-kilobase pair species of mRNA. The mRNA was shown to be induced by oxalic acid. A temporal relationship between enzyme activity and mRNA levels was observed, thus suggesting that the expression of oxalate decarboxylase is regulated at the transcriptional level.

Much of the oxalate from animals including humans originates from the oxalate ingested with plant material. Some green leafy vegetables (*e.g. Amaranthus*, spinach, rhubarb) are rich sources of vitamins and minerals, but they contain oxalic acid as a nutritional stress factor. Such plants, when consumed in large amounts, become toxic to humans because oxalate chelates calcium, and precipitation of calcium oxalate in the kidney leads to hyperoxaluria and destruction of renal tissues (1, 2). Apart from this, at least two other instances can be cited where oxalic acid is involved in an indirect manner. In one case, the production of oxalic acid is an important attacking mechanism utilized by *Whetzelinia sclerotiorium*, a fungus that causes serious damage to crops like sunflower. Oxalic acid accumulates in the infected tissues early in pathogenesis, and its concentration increases during the time the pathogen is colonizing the host tissues. The accumulation of oxalic acid in leaves causes symptoms of wilting and eventually leaf death. Thus, oxalic acid functions as a mobile toxin that moves from the base of stems to xylem sap and leaves (3).

In another case, consumption of *Lathyrus sativus* (chickling vetch) causes neurolathyrism, which is characterized by spasticity of leg muscles, lower limb paralysis, convulsions, and death. *L. sativus* is a protein-rich hardy legume that grows under extreme conditions such as draught and water-logging and does not require complex management practices. The neurotoxin β-*N*-oxalyl-L-α,β-diaminopropionic acid (ODAP)[1] is present in various parts of the plant. ODAP synthesis is a two-step reaction in which oxalic acid is an essential starting substrate. ODAP acts as a metabolic antagonist of glutamic acid, which is involved in transmission of nerve impulses in the brain. Hence, despite its rich protein content, the legume cannot be used as a food source (4).

A study of the function of oxalic acid in the above-mentioned systems highlights its role as an important stress factor. Thus, the isolation of a new useful gene will serve as a tool to degrade oxalic acid in plants where oxalic acid accumulates as such or is a substrate in the synthesis of neurotoxin or is a medium for pathogenesis. This can be achieved by effecting single gene transfer to these plants. Moreover, oxalic acid-depleted transgenic plants would be useful to study the role of oxalic acid in the plant.

Mechanisms of microbial metabolism of oxalic acid have been reviewed earlier (5). Of the known oxalic acid-degrading enzyme systems, oxalate decarboxylase from the basidiomycetous fungus *Collybia velutipes* is of interest because an earlier report using partially purified enzyme showed a simple single step breakdown of oxalic acid to carbon dioxide and formic acid, a nontoxic organic acid, in the absence of any cofactor requirement (6).

As part of a long-term program to develop transgenic plants with low oxalic acid content, we report, for the first time, the purification of oxalate decarboxylase to homogeneity, the biochemical characterization of some of its properties, immunoscreening of a cDNA library with antibodies raised against the enzyme, and the isolation and characterization of immunopositive clones. Using this clone, the regulation of oxalate decarboxylase in *C. velutipes* was also studied.

MATERIALS METHODS[2]

RESULTS AND DISCUSSION

Purification—Oxalate decarboxylase was purified from an oxalate-induced crude extract of *C. velutipes*. Maximal activity was obtained 2 or 3 days after the addition of oxalic acid. The

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[1] The abbreviations used are: ODAP, β-*N*-oxalyl-L-α,β-diaminopropionic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase pair(s).

[2] Portions of this paper (including "Materials and Methods," Tables 1 and 2, and Fig. 2–7 and 9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

results of a typical purification procedure are given in Table 1. The enzyme resolved into two peaks on a chromatofocusing column: peak A eluted at pH 3.3, and peak B eluted at pH 2.5 (Fig. 1). Peak A was purified 1670-fold with 2.9% recovery, whereas peak B coeluted with two minor contaminants and was purified 614-fold with 16% recovery (Table 1). These contaminants could be removed after passage through a Sepharose 4B gel filtration chromatography column (data not shown), and this protein was used for determining amino acid composition. Because of the high purity of peak A, this protein was used for further work. The material in peak A eluted as a single peak on a fast protein liquid chromatography Superose 12 column (data not shown), and 10 μg of protein gave a single spot on two-dimensional gel electrophoresis (Fig. 2*B*). The serial 2-fold dilutions of the enzyme showed that at least 45 ng of protein can be detected by Coomassie Blue staining (Fig. 2*A*). The migration distance of enzyme activity (R_f = 0.35, gel slice 4) correlated with that of the single stained band on nondenaturing PAGE (Fig. 3). No protein bands or enzyme activity was found in any other part of the gel. Thus, the protein band corresponding to peak A had the oxalate decarboxylase activity. Enzyme preparations were stable at 4 or −20 °C, and >70% of the initial activity could be measured after 4 months of storage at 4 °C at 1 mg/ml in 0.02 M potassium acetate (pH 4.5).

Relationship of Peak A to Peak B—Amino acid composition data of the two peaks indicated the presence of a very high content of acidic amino acids (22%) (Table 2). This could account for their low pI values, although the proportion amidated in native protein was not determined. The two peaks had very similar amino acid compositions, except for a 2-fold higher methionine and tyrosine content in peak B and a 2-fold higher cysteic acid content in peak A. Further relatedness was indicated by the peptide map of the two peaks using *Staphylococcus aureus* V8 protease (Fig. 4). The affinity-purified antibodies directed against peak A cross-reacted with peak B protein. The amino acid composition, peptide maps, and immunological cross-reactivity indicate that the two peaks resolved on chromatofocusing are related to each other. The two forms with differences in pI may arise from different degrees of amidation of acidic amino acids or may be due to microheterogeneity in the constituent oligosaccharide chains.

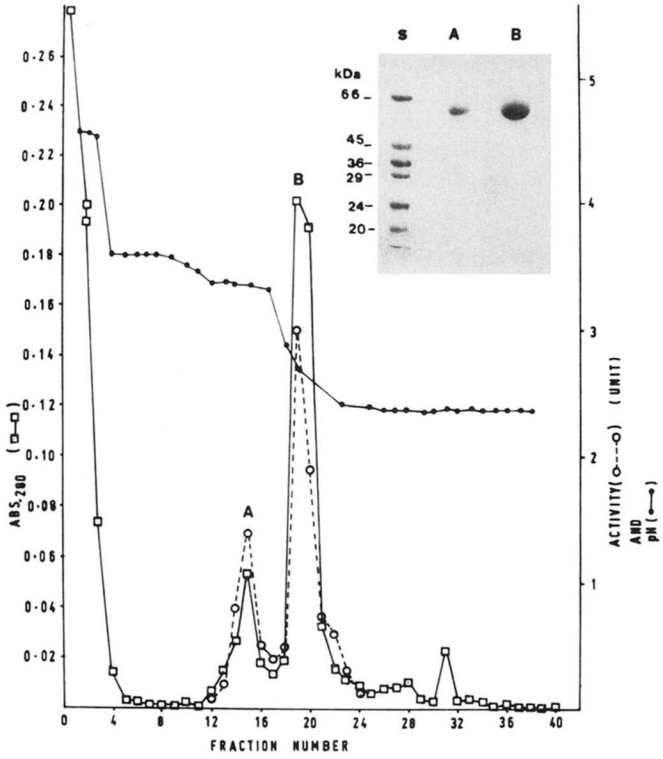

FIG. 1. **Elution profile of oxalate decarboxylase from chromatofocusing column.** The protein from the Acetone-IV step (see Miniprint) was loaded onto a DEAE-Sepharose CL-6B column (1 × 13 cm) equlibrated with 0.02 M potassium acetate (pH 4.5). Bound proteins were eluted with a decreasing pH gradient. The activity was associated with peak A eluting at pH 3.3 and with peak B eluting at pH 2.5. *Inset,* protein bands corresponding to the two peaks. Peaks A (*lane A*) and B (*lane B*) were resolved by 11% SDS-PAGE and stained with Coomassie Blue. *Lane S* shows molecular mass markers from Sigma (SDS-7).

Molecular Mass Determination—The molecular mass of the native enzyme estimated by gel filtration was 560 kDa (data not shown). Electrophoresis on 7–15% gradient SDS-polyacrylamide gel showed the presence of a single polypeptide of 64 kDa (Fig. 2*A*). This molecular size was consistently obtained with all different gel percentages used with the Laemmli buffer system (13). When the enzyme was treated with endo-β-*N*-acetylglucosaminidase H, the size of the major deglycosylated band was 55 kDa (Fig. 5). The enzyme was found to be glycosylated, and the high apparent molecular size obtained by gel filtration could be due to the tendency of certain glycoproteins to interact noncovalently in solution (7, 8).

Biochemical Properties—From Lineweaver-Burk plots, an apparent K_m value of 4.5 mM was calculated for potassium oxalate as the substrate. This gave a V_{max} of 166 μmol/min/mg. The enzyme was competitively inhibited by phosphate ions, and a K_i of 9 mM was obtained when 90 mM PO_4^{2-} was added to the reaction (data not shown). The enzyme was specific for oxalate as the substrate since citric acid, acetic acid, oxalacetic acid, succinic acid, and formic acid were not used as substrates.

The enzyme was not irreversibly inactivated over a wide range of pH values, and the pH optimum for decarboxylation is 3.0. The enzyme retained 78% of the initial activity after 20 min of incubation at 80 °C. Almost total inactivation occurred at 96 °C within 10 min of incubation. Enzyme activity was unaffected by sulfhydryl group reagents as the enzyme retained 95% of its activity in the presence of 50 mM *p*-chloromercuribenzenesulfonic acid or 50 mM iodoacetate. Oxalate decarboxylase retained 45% of its activity after incubation with 10% SDS for 30 min at room temperature. However, when heated to 60 °C in the presence of 10% SDS, almost all of the activity was lost. The glycoprotein nature of the protein was indicated by positive staining with periodate-Schiff base reagent; it bound to concanavalin A-Sepharose and was eluted with 0.5 M α-methylmannoside (data not shown). The neutral sugar content was estimated to be 15% by the phenol/sulfuric acid method.

Immunological Characterization—Immunotitration of the enzyme with 8 μl of crude anti-oxalate decarboxylase antiserum brought down >60% of the initial activity in the supernatant (Fig. 6). The antiserum against oxalate decarboxylase used at a dilution of 1:5000 could detect a minimum of 1.0 ng of peak A protein. The antiserum cross-reacted with all the peptides obtained from V8 protease digests (Fig. 4) and with the deglycosylated forms of peaks A and B of the enzyme (data not shown). The antiserum that was affinity-purified against peak A protein cross-reacted with peak B of oxalate decarboxylase and oxalyl-CoA decarboxylase (9) from *Oxalobacter formigenes* strain OxB. It did not cross-react with oxalate oxidase from *Hordeum vulgare* (barley).

Molecular Cloning of cDNA Encoding Oxalate Decarboxylase—For studies at the molecular level, a cDNA expression

library was constructed from 12-h oxalate-induced mRNA in λgt11. Approximately 47,000 recombinants were screened with the antibody pretreated with *Escherichia coli* lysate. Fifteen immunopositive plaques were obtained and plaque-purified; of these, 12 cross-hybridized (data not shown). These encoded fusion proteins of sizes comparable to insert sizes (Fig. 7*A*). The phage DNA from 15 immunopositive clones was immobilized onto a GeneScreen *Plus* membrane in duplicate and probed with oxalate-induced and -uninduced cDNA probes. Differential hybridization of the 15 immunopositive clones showed that 12 hybridized to the cDNA probe from oxalate plus mRNA and gave no signal with oxalate minus mRNA (Fig. 7*B*). Thus, the expression of 12 clones was induced by oxalate.

The pTZ18U subclone of the 1.2-kb insert from λ clone 3 was used to hybrid-select the mRNA. The *in vitro* translation of hybrid-selected RNA and immunoprecipitation of the translated product gave a band of 55 kDa (Fig. 8*B*, *lane 2*), which was similar to the size obtained with total poly(A$^+$) mRNA (*lane 3*) and corresponded to the size of the deglycosylated protein. This 55-kDa protein was not obtained when mRNA was omitted (Fig. 8*B*, *lane 1*) or with the nonrecombinant vector sequences (*lane 4*). The 55-kDa product was obtained with 12-h mRNA (Fig. 8*A*, *lane 2*) and not with uninduced 0-h mRNA (*lane 1*); the 55-kDa band was shown to be related to oxalate decarboxylase as the purified oxalate decarboxylase competed for antigen-binding sites and caused a decrease in the intensity of the 55-kDa band (*lane 3*) in the *in vitro* translation and immunoprecipitation experiments.

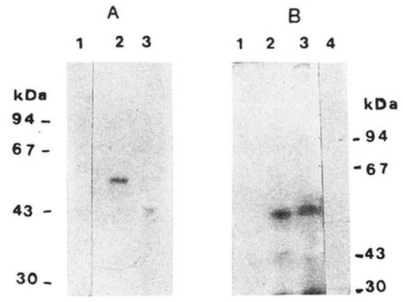

FIG. 8. *A, in vitro* translation. The translation products of *in vitro* translated total poly(A$^+$) RNA from uninduced, 0-h (*lane 1*), and 12-h oxalate-induced (*lane 2*) stages were immunoprecipitated and resolved by 10% SDS-PAGE. The gel was fluorographed and autoradiographed. *Lane 3* shows the disappearance of the 55-kDa band when purified oxalate decarboxylase was present as a competitor for binding to antibodies during immunoprecipitation. *B,* hybrid-selected translation. Poly(A$^+$) RNA (20 μg/ml) isolated from 12-h oxalate-induced *C. velutipes* was hybridized with plasmid DNA (1.2-kb cDNA insert in pTZ18U) bound to a GeneScreen *Plus* membrane. The RNA was eluted and translated in rabbit reticulocyte lysate, and the translated products were immunoprecipitated and resolved by 10% SDS-PAGE. *Lane 1,* no mRNA; *lane 2,* hybrid-selected mRNA from the 1.2-kb insert; *lane 3,* 12-h total poly(A$^+$) RNA; and *lane 4,* nonrecombinant vector sequences. The molecular mass markers are indicated.

Genomic Southern blots using the 1.2-kb insert as probe showed the presence of single bands with the *Bam*HI, *Eco*RI, *Hin*dIII, *Pvu*II, *Ssp*I, *Xba*I, and *Xho*I digests, indicating the presence of a single copy gene (Fig. 9*B*). The two bands of unequal intensities obtained with *Kpn*I and *Pst*I were due to the presence of internal sites (single site for each enzyme) in the 1.2-kb cDNA insert for these enzymes (data not shown). The 1.2-kb probe hybridized to a single species of mRNA of 1.5 kb from 12-h oxalate-induced poly(A$^+$) RNA, and no hybridization to RNA from the uninduced lane was seen (Fig. 9*A*).

Regulation of Oxalate Decarboxylase Expression Occurs at Transcriptional Level—From the same batch of cultures, samples were collected from different stages after induction and were analyzed for RNA levels, enzyme activity, and total protein. The Northern blot of total RNA showed that the 1.5-Kb band was absent at 0 h and peaked at 12 h. No RNA could be detected 3 days after induction (Fig. 10*B*). Enzyme activity was detected 12 h after the addition of oxalate and peak activity was seen on day 2, after which there was a steady decline. An associated increase or decrease in total protein was not observed (Fig. 10*C*). Hence, a temporal relationship was observed between the appearance of enzyme activity and the mRNA levels since the mRNA levels peaked 12 h after induction and the maximal enzyme activity was obtained 48 h after the addition of oxalic acid.

In conclusion, the largest cDNA clone (1.2 kb) was found to be related to most of the other immunopositive clones and is the presumptive oxalate decarboxylase clone because: 1) it hybridized to the cDNA probe from oxalate-induced mRNA

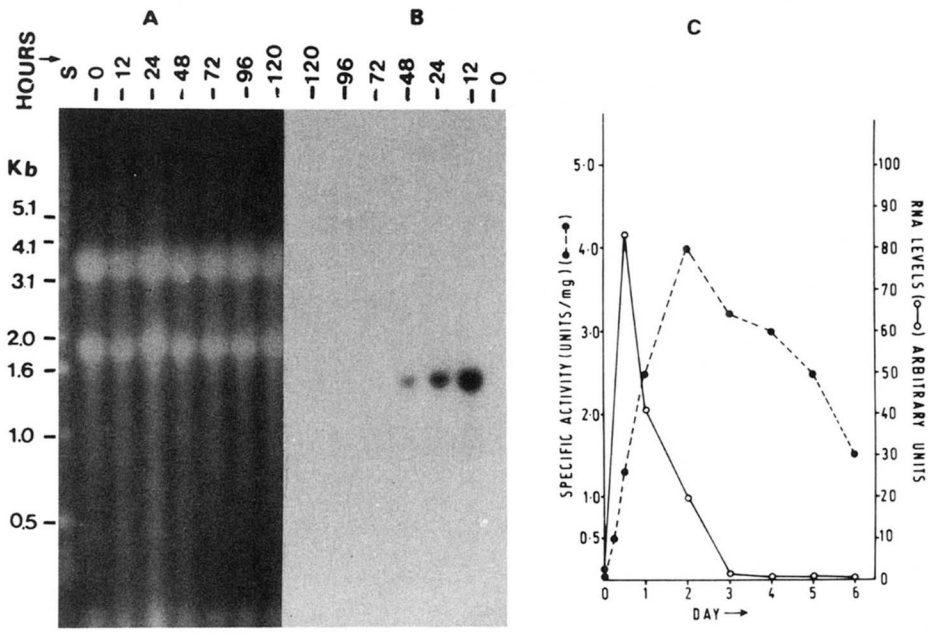

FIG. 10. **Regulation of oxalate decarboxylase gene expression.** Shown is total RNA (10 μg) isolated from cultures of *C. velutipes* at various time points after the addition of oxalic acid. *A,* ethidium bromide-stained 1.2% agarose gel showing relative loads of glyoxal-denatured RNA; *B,* total RNA transferred to GeneScreen *Plus* and hybridized to ^{32}P-labeled 1.2-kb cDNA insert; *C,* relative amount of poly(A$^+$) RNA encoding oxalate decarboxylase (○) from the integrated areas of the densitometric trace from the autoradiograph (*B*). The specific activity of the enzyme isolated from the same culture is also shown (●).

and not to uninduced cDNA, *viz.* it is differentially expressed (Fig. 7*B*); 2) the 1.5-kb mRNA to which it hybridized was induced by oxalate (Fig. 9*A*); and 3) it hybridized to mRNA, which, via *in vitro* translation and immunoprecipitation, gave a 55-kDa product (Fig. 8*A*, *lane 2*). This protein band was obtained with 12-h induced mRNA and not with 0-h uninduced mRNA and corresponded to the size of the deglycosylated protein. Further studies at the molecular level are being done with the genomic clone obtained by screening the genomic library with the 1.2-kb cDNA insert as the probe.

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Supplemental material to

Oxalate Decarboxylase: Purification, Characterization and cDNA cloning.

Anuradha Mehta and Asis Datta

MATERIALS AND METHODS

Organism and growth conditions. *C. velutipes* (strain S, A.T.C.C 13547) was grown on the surface of medium containing 5% dextrose, 1% peptone, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$ and 1% Difco malt extract at pH 5.2. The organism was grown from mycelial inoculation at 25°C in stationary cultures in a volume of medium one-fifth the volume of culture flasks. About 25 days after inoculation, the enzyme oxalate decarboxylase was induced by addition of 12.5 mM oxalic acid to each culture flask. The mycelium was harvested 2 to 3 days after the addition of oxalic acid and the mycelial pad was washed with cold distilled water and stored at -20°C. *C.velutipes* was maintained on slants of the same medium (10).

Purification

Step 1. Preparation of crude extract. The frozen mycelium was ground in Waring blender for 10 min with either dry ice or liquid nitrogen. The powder was extracted with three volumes of 0.1M potassium citrate buffer, pH 3.0 for 10 min. 4°C and the suspension centrifuged at 10,000 X g for 30 min at 4°C. The supernatant was filtered through a double layer of cheese cloth.

Step 2. Precipitation with acetone. The acetone concentrations were adopted from Shimazono and Hayaishi(6) except that the last two steps were not performed. The percentages quoted are on vol/vol basis assuming additive volumes. (a) Low temperature for acetone precipitation was maintained by an ice-salt bath at -10°C. The sample was chilled to 0°C and the first acetone precipitation at 33.3% was given by dropwise addition of chilled acetone to supernatant with constant mechanical stirring (Acetone-I). The mixture was equilibrated for 15 min and the precipitate formed was removed by centrifugation in a precooled rotor at 10,000 xg, 20 min, 2°C. The precipitate obtained from 33.33%-50% fractionation was dissolved in one-fifth the starting volume of cold 0.1M potassium acetate buffer at pH 4.5. The enzyme solution was dialyzed for 16 hours, 4°C against two changes of 0.02M potassium acetate buffer, pH 4.5 and a small precipitate formed during dialysis was removed by centrifugation (Acetone-II).
(b) Supernatant was brought to 40% acetone (Acetone-III) and the precipitate obtained was discarded. The precipitate from further addition to 50% (Acetone-IV) was dissolved in a small volume of 0.02 M potassium acetate, pH 4.5.

Step 3. Chromatofocusing. DEAE-Sepharose CL-6B (Pharmacia) was equilibrated in 0.02 M potassium acetate buffer, pH 4.5 and used to pack a 10 ml column (1X 13 cms bed). The precipitate from the last acetone precipitation was loaded at a flow rate of 10 ml/h. The column was washed with two column volumes of 0.02 M potassium acetate, pH 4.5 and the elutions effected by developing an internal pH gradient using 4 mM acidic buffer mix (4 mM each of DL-aspartic acid, L-glutamic acid and glycine, pH 2.3). The elution was done at a flow rate of 10 ml/h and 2 ml fractions collected; proteins were monitored by absorbance at 280 nm; the fractions were assayed for enzyme activity and the pH of each fraction determined. The fractions containing enzyme activity were pooled and dialyzed against water and concentrated in Amicon microconcentrator (30,000 M.W cut off). The enzyme was stored at 4°C.

Enzyme assay. The oxalate decarboxylase activity was also determined by measuring the liberation of $^{14}CO_2$ from (^{14}C)-oxalic acid (Amersham, 4.1mCi/mmol). The enzyme assay was carried out in small glass vials which contained 1 ml of the following reaction mixture : 0.2 M potassium citrate pH 3.0, 0.005 M potassium oxalate, pH 3.0, 5.6 nmoles (0.0227 µCi) of (^{14}C)-oxalic acid and 0.2 ml of enzyme solution. The tubes were preincubated for 5 min before the addition of enzyme. The tubes were sealed with rubber-stoppers and incubated at 37°C for 30 min in a shaking water bath. The reaction was terminated by injection of 0.2 ml of 50% v/v trichloroacetic acid through the rubber caps and the tubes were shaken for additional 60 min to trap all the $^{14}CO_2$ evolved in the 0.2 ml methylbenzethonium hydroxide(Sigma) placed in a plastic vial inside the glass tube. The plastic wells were withdrawn and the contents transferred to 5 ml of toluene based scintillation fluid and radioactivity determined in a liquid scintillation counter.Blank tubes were set up in which the 0.2 ml of 50% TCA was added before the enzyme or the enzyme was omitted. In kinetics experiments, the values were corrected for the radioactivity obtained from the boiled denatured enzyme.

Definition of a unit. One unit was defined as the amount of enzyme releasing 1µmole of $^{14}CO_2$ per min at 37°C under standard assay conditions. The overall assay efficiency was usually between 60-70%. Protein was determined by Lowry microassay method (11). V_{max} and K_m were determined by Lineweaver-Burk plot (12).

Molecular mass determination. The molecular mass of oxalate decarboxylase was determined by gel filtration chromatography. Purified enzyme (100 µg in 100 µl) was loaded on a FPLC gel permeation column (Superose 12 : 10 x 300 mm) at a flow rate of 0.5 ml/min, using 0.02 M potassium acetate buffer, 0.1M KCl pH 4.5 at room temperature. Proteins were detected at 280 nm. Standard proteins used were thryoglobulin (660 kDa Pharmacia), ferritin (440 kDa, Pharmacia) catalase (230 kDa, Pharmacia), aldolase (158 kDa, Pharmacia), alcohol dehydrogenase (150 kDa, Sigma) and carbonic anhydrase (29 kDa, Sigma). The subunit composition was determined by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis in 7% to 15% gradient gels using Laemmli buffer system (13). Proteins were stained with Coomassie brilliant blue R-250.

Criteria of purity. Homogeneity of purified oxalate decarboxylase was determined by resolving 10 µg of protein (peak A) on two-dimensional gel electrophoresis according to O'Farrell (14).

Amino acid composition. Samples were hydrolyzed in 6M HCl in evacuated and sealed tubes at 110°C for 22hr. The hydrolyzates were analyzed with an amino acid analyzer (LKB 4151 Alpha Plus). Cysteine and cystine were determined as cysteic acid after performic acid oxidation. Tryptophan was not determined. Digestion with V8 protease was done as described earlier (15).

Carbohydrate analysis. Glycoprotein staining was done by using the Periodate-Schiff base reagent. The natural sugar content was determined by phenol-sulfuric acid method (16) with glucose as standard. The enzyme was deglycosylated by Endo-β,N-acetylglucosaminidase H from *S.plicatus* (Boehringer Mannheim, 40 mU/µg) according to Trimble (17).

Preparation of antisserum. The oxalate decarboxylase (1 mg/ml) was heat denatured by boiling for 10 min in PBS in presence of 0.5% SDS. The protein antigen (150 µg) in PBS was emulsified with Freund's complete adjuvant and injected subcutaneously in a New Zealand White rabbit. Subsequent boosters were given in Freund's incomplete adjuvant subcutaneously after a period of three weeks. Fourth injection was given intravenously. Antibody titer was monitored using Ouchterlony immunodiffusion technique (18). The affinity purification of antibody was done according to Iwaki et al(19).

Western blotting and immunodetection. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) at 150 mA constant current for 3 hour at 15°C according to the procedure of Towbin et al (20). Immunodetection was done using 1:5000 dilution of anti-oxalate decarboxylase antibody and detected via alkaline phosphatase reaction (Amersham, Super-screen immunoscreening system).

Molecular Cloning. Total RNA was extracted from liquid nitrogen ground powder of *C. velutipes* according to the method of Chomczynski and Sacchi (21) and poly (A^+) RNA was selected by two cycles of chromatography on oligo (dT) cellulose. cDNA synthesis and cloning and immunoscreening of the library were performed following the instructions of Amersham. The cDNA was synthesized from mRNA of 12 hour oxalate induced culture by oligo(dT) priming of 5 µg of poly (A^+) RNA and cloned into the *Eco*RI restriction site of lambda gt11. The packaged phages were grown in *E.coli* host Y1090 r^- for immunoscreening. Anti-oxalate decarboxylase antibodies were preadsorbed with 1 mg/ml *E.coli* Y1090 cell lysate to remove background reactivity and used for immunoscreening. Goat anti-rabbit IgG alkaline phosphatase conjugate was used for detection of positive clones. The DNA was prepared from immunopositive phages according to Del Sal (22) and their insert size determined. Fusion proteins were characterized according to (23); relatedness of inserts was studied by using one of inserts as probe. The 1.2 kb insert from clone number 3 was subcloned in pT218U (USB) and used as a probe for other experiments.

Differential hybridization. Differential hybridization of immunopositive clones was studied by preparing single-stranded cDNA probes synthesized from mRNA isolated from mycelium at 0 hour and 12 hour of induction by oxalate. Recombinant phage DNA (0.5 µg) was bound to Gene Screen plus membrane in duplicate Hybri-slot™ Filtration manifold according to the instructions in Gene Screen Plus manual and hybridized to oxalate induced and uninduced cDNA probes. The specific activity of the probe was 2 x 10^8 cpm /µg cDNA.

Hybridization. Hybridization of DNA and RNA blots was at 42°C using the formamide procedure in Gene Screen Plus manual. An overnight prehybridization was done in 50% deionized formamide, 1% SDS, 1M sodium chloride, 10% dextran sulfate. The blots were hybridized to denatured probe (1-4 x 10^5 dpm/ml) at 42°C for 24 hour. The membranes were washed successively in 2 washes each of 2XSSC at room temperature, 2XSSC plus 1% SDS at 65°C for 30 min, 0.1XSSC at room temperature for 30 min. Damp membranes in plastic wraps were exposed to Kodak XAR films in presence of intensifying screen.

Probe preparation. The subcloned DNA in pTZ18U was digested with *Eco*RI and resolved on 2% low melting agarose gel insert DNA was incised and labeled with [α-^{32}P]dATP by the random primer labeling method of Feinberg and Vogelstein (24).

Genomic DNA isolation and Southern analysis. Genomic DNA was isolated from lyophilized *C.velutipes* by the method of Zolan and Pukkila (25). The DNA was banded twice on CsCl gradient to obtain DNA that could be digested with restriction enzyme. Four micrograms of genomic DNA was digested with various restriction endonucleases and resolved on 1.2% agarose gel. The DNA was transferred to Gene Screen Plus membrane by alkaline blotting procedure (26).

***In vitro* Translation.** Poly(A$^+$) RNA was translated using a rabbit reticulocyte lysate according to manufacturer's instructions (Promega). The translated proteins were precipitated by specific antisera and analyzed by SDS-polyacrylamide gel electrophoresis.

Hybrid selection. Hybrid selection of oxalate decarboxylase mRNA was performed by hybridizing poly(A$^+$)RNA (20 µg in 200 µl 65% formamide, 10 mM PIPES pH 6.4, 0.4 M NaCl, 8 mM EDTA, 0.5% SDS, 100 µg/ml yeast tRNA) at 50°C 4hour, to Gene Screen plus membrane on which denatured cDNA (4 µg) had been bound. Filters were prepared according to Gene Screen Plus manual and hybridization, washing and elution of hybridized RNA was performed (27,28). The eluted RNA was extracted with phenol:chloroform (1:1), precipitated in ethanol, reconstituted directly in *in vitro* translation mix and immunoprecipitated according to Anderson and Blobel (29).

Northern blot analysis. One µg of poly (A$^+$) RNA or 10 µg of total RNA was denatured with glyoxal and resolved on 1.2% agarose gels according to Sambrook (30) and capillary blotted onto the Gene Screen Plus membrane as instructed in Gene Screen Plus manual. Filters were probed with ^{32}P-labeled 1.2 kb cDNA insert.

Table 1

Purification table from a typical experiment[a]

Purification step	Total Protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification fold	Yield %
1.Crude extract	4480	950	0.21	1	100
2.Acetone II	120	608	5.06	24	64
3.Acetone-IV	3.8	260	68.8	328	27.3
4.Chromatofocusing					
a. Peak A	0.08	28	350	1670	2.9
b. Peak B	1.24	160	129	614	16.8

[a] 150g of liquid nitrogen ground powder used

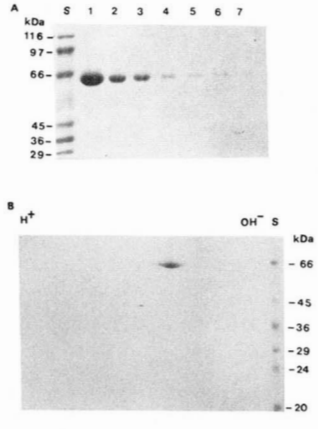


FIG.2 Criteria of purity.

(A) Two-fold serial dilutions (lanes 1 to 7) of oxalate decarboxylase starting with 3µg (lane 1) protein were resolved on 7-15% gradient SDS-PAGE.

(B) 10µg of oxalate decarboxylase (peak A) was resolved by isoelectric focusing (pH 2.5-5.0, ampholytes, Pharmacia) in first dimension and by 12% SDS-PAGE in the second dimension. Lane S shows SDS-7 (Sigma) calibration standards for molecular weight estimation. In (A) & (B) the gels were stained with Coomassie Blue.

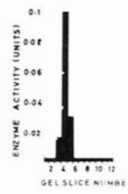


FIG.3 Activity-band correlation.

500 ng protein was electrophoresed in two lanes of 6% non-denaturing polyacrylamide gel; one lane was stained with Coomassie blue and the other was cut into twelve 4 mm sections. The gel slices were incubated in 200 µl of 0.1 M potassium acetate buffer, pH 4.5. The acrylamide was mashed and soaked overnight at 4°C. Enzyme activity was assayed and correlated to the band in stained lane. The migration distance (R_f of 0.35, gel slice no.4) of enzyme activity (0.2 units) correlated with that of the single stained band (500 ng). No protein band or enzyme activity was found in any other part of the gel.

Table 2
Amino acid Composition

	Peak A x[a]	Peak B %
Asx	10.57	10.31
Glx	11.75	11.52
Lys	3.2	2.96
Arg	2.84	2.89
His	2.68	2.74
Gly	8.44	9.44
Ser	7.23	7.19
Thr	8.56	8.48
Cys[c]	0.66	0.28
Tyr	0.84	1.85
Ala	11.03	10.92
Val	6.31	6.52
Leu	7.79	7.19
Ile	4.00	3.86
Pro	8.64	8.46
Phe	5.13	4.68
Met	0.34	0.69
Trp	ND[b]	ND
Ammonia	ND	ND

a: Mole percent
b: Not determined
c: Determined as cysteic acid

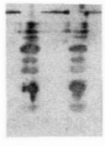


FIG.4. Comparison of Cleveland digest patterns from the two forms of oxalate decarboxylase.

Peptide maps were generated directly in 4.5% stacking gel of a 15% resolving gel. 10 µg of peak A and peak B polypeptides were cut from 11% SDS-polyacrylamide gel, and digested with 100 µg/ml V8 protease (*S. aureus*, Sigma). The peptides were electroblotted to a nitrocellulose membrane and immunodetected with 1:5000 dilution of anti-oxalate decarboxylase antibody. Lanes: 1, peak A protein and 2, peak B protein.

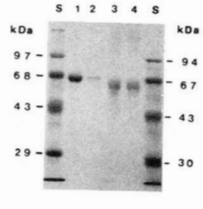


FIG.5 Deglycosylation of oxalate decarboxylase.

1 µg of the peak A enzyme was treated with 1mU (lane 3) and 10 mU (lane 4) of Endo H for 22 hours and resolved in 11% SDS-PAGE. Lanes 1 & 2 contain untreated controls for peak A. Lane S (right) shows the molecular weight markers from Pharmacia and the lane S (left) are the high molecular weight, prestained markers (BRL).

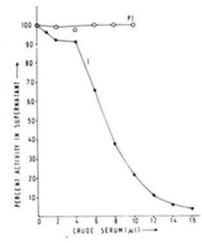
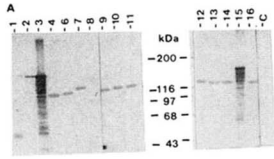


FIG.6 Immunotitration of enzyme activity.

1.5 µg of enzyme in 0.02 M potassium acetate buffer, pH 4.5 was incubated with different volumes of serum (I) at 25°C for 2 hour. Immunocomplexes were spun down at 12,000 xg for 10 min and residual activity detemined by standard assay. Control incubation was carried out with preimmune serum (PI).



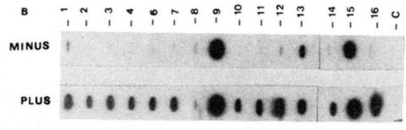


FIG.7 (A) Oxalate decarboxylase-β-Galactosidase fusion proteins.

IPTG-induced macroplaques were lysed in 1X Laemmli buffer and resolved on 10% SDS-PAGE, transferred to nitrocellulose and probed with anti-oxalate decarboxylase antibodies. The numbers above the lanes correspond to clone numbers. 'C' is the control lane with the macroplaque from nonrecombinant lambda gt11.

(B) Differential hybridization of the DNA from immunopositive plaques.

Phage DNA (500 ng) isolated from clones was bound to Gene Screen Plus membrane in duplicate and hybridized with oxalate uninduced (minus) and oxalate-induced (plus) cDNA probes (2.5×10^8 cpm/µg cDNA). Filters were washed as described in 'Materials and Methods'. 'C' refers to control nonrecombinant lambda gt11 DNA.

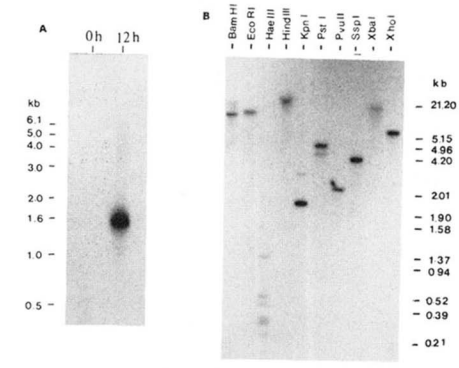


FIG.9 (A) Northern blot showing 1.5kb mRNA for *C. velutipes* oxalate decarboxylase.

1 µg poly (A^+) RNA from uninduced (0h) and oxalate induced (12h) stages was glyoxal-denatured and separated on a 1.2% agarose gel, blotted to Gene Screen plus membrane and probed with [α^{32}P]-labeled cDNA (9×10^7 cpm/µg) for oxalate decarboxylase. 1 kb ladder (BRL) was used as the size standard.

(B) Southern blot of genomic DNA from *C. velutipes*.

Genomic DNA (4 µg) was digested with restriction enzymes, resolved on 1.2% agarose gel, blot transferred to Gene Screen Plus membrane and hybridized to [α^{32}P]-labeled cDNA insert. The lambda *Hind* III/*Eco*RI and pUC 19 *Hin*fI digests were used as molecular size standards.