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PURIFICATION AND CHARACTERIZATION OF DIAMINE OXIDASE FROM CLOVER LEAVES

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Abstract—Diamine oxidase (DAO, EC 1.4.3.6) was purified 135-fold from leaves of subterranean clover (*Trifolium subterraneum* L. cv Seaton Park) and three isoenzymes were identified. The native enzyme has an M_r of *ca* 150 000 and comprises two subunits both having an M_r of 80 000. Clover DAO has a broad specificity range and is inhibited by copper ligands and reagents reactive towards carbonyl groups. Copper is essential for enzyme activity with the apoenzyme being reactivated specifically by copper. The enzyme has a broad pH optimum from pH 7–8 and an activation energy of 47 kJ/mol with 1,4-diaminobutane as substrate.

INTRODUCTION

Diamine oxidases (DAO, EC 1.4.3.6) are widespread in nature and have been purified from several animal [1] and plant [2–11] species. All of these contain copper as a cofactor, unlike the amine oxidases of cereals [12, 13]. DAOs oxidize amines by the following reaction.

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$$

In clover, DAO synthesis occurs during early leaf development and its synthesis appears to be regulated by copper [14]. We have now purified and characterized clover DAO extending the earlier work of Werle and Hartung [15]. DAOs purified from some legume species have shown multiple forms consisting of one major and one or several minor forms [5, 7, 8]. It is not established that these were isoenzymes and they may have resulted from a proportion of the enzyme being modified by the extraction procedure, protease action or phenolics. In addition, different parts of the same plant can possess DAOs with different properties [16] and this may explain why some workers have found multiple DAO forms when using whole tops of seedlings as the starting material [7, 17].

In this paper we report that three isoenzymes of DAO were co-purified from a defined plant part (young folded leaves of clover) with the two major forms being of about equal abundance.

RESULTS AND DISCUSSION

Purification of DAO

DAO was purified 135-fold from a leaf homogenate of clover leaves (Table 1) and judged pure by native gel electrophoresis at two pHs and SDS-gel electrophoresis. In native gel electrophoresis, both the protein and enzyme stains showed the presence of three isoenzymes of DAO that migrated closely to one another and consisted of one minor band separating two major bands (R_f 0.19–0.24, pH 9, 7% gel). The following observations confirm that the multiple DAO bands were isoenzymes and had not resulted from the action of proteases or phenolics leading to a fraction of the DAO being modified: (i) polyvinylpolypyrrolidone was included in the extraction buffer to bind phenolics released when the leaves were homogenized, (ii) a sample of freshly prepared crude homogenate electrophoresed at pH 9 yielded the multiple bands even when the protease inhibitor phenylmethylsulphonyl fluoride (1 mM) was added and also when bovine serum albumin was added to the extraction buffer at 10 times the concentration of total leaf protein in the extract. The excess bovine serum albumin would have protected the leaf proteins from both protease action and phenolics, (iii) the relative amounts of DAO isoenzymes in native gels did not change with each step of the purification and was reproduced when DAO was purified from different batches of leaves.

Table 1. Purification of DAO from clover leaves

Step	Protein (mg)	Activity (μkat)	Specific activity (μkat/mg protein)	% Yield
Crude homogenate	990	8.05	0.008	100
$(NH_4)_2SO_4$ 30–70% fraction	427	5.98	0.014	74
Phosphocellulose chromatography	4.35	4.38	1.01	54
Hydroxylapatite chromatography	3.38	3.73	1.10	46

Properties of clover DAO

Clover DAO had similar properties to other legume DAOs [4–11] with regard to substrate specificity, copper content, absorption spectra, amino acid composition and M_r. The enzyme catalysed oxidation of a wide range of

amines but acted preferentially towards 1,4-diaminobutane, 1,5-diaminopentane and spermidine and had K_m of 38, 19 and 74 μM, respectively, for these substrates. The range of substrates oxidized by clover DAO was similar to that of pea DAO [4] but was less specific than that of *Euphorbia* [3] and lentil DAO [10]. Copper ligands (NaN_3, Na diethyldithiocarbamate, NH_4 pyrrolidinedithiocarbamate) and reagents that react with carbonyl groups (phenylhydrazine, semicarbazide, hydroxylamine) inhibited the enzyme. With 1,4-diaminobutane as a substrate, clover DAO showed a broad pH optimum from pH 7 to 8 in 67 mM KPi and had an activation energy of 47 kJ/mol as calculated from the Arrhenius plot (DAO assayed in range of 6–40°).

The spectrum of clover DAO showed a broad absorption band centred at 490 nm (E^{M}_{1cm} = 2 × 10^3) and a band at 280 nm (E^{M}_{1cm} = 1.29 × 10^5). The band at 490 nm disappeared after adding phenylhydrazine but was replaced by a larger peak at 446 nm (E^{M}_{1cm} = 3.9 × 10^4). Titration of clover DAO with phenylhydrazine showed the presence of one carbonyl group per enzyme dimer as found for other copper DAOs [1, 2, 9, 10, 18].

Although three isoenzymes could be identified from native gel electrophoresis, only a single band with an M_r of 80 000 resulted from gel electrophoresis run under denaturing conditions. This indicated that all three isoenzymes consisted of the same subunit size. Since the native M_r as determined by gel filtration was *ca* 150 000 the enzyme can be described as a dimer of identically sized subunits.

The copper content of clover DAO was found to be 0.063%, which, based on an M_r of 150 000 for the enzyme, suggests 1.5 copper atoms per dimer. Other copper DAOs contain 2 copper atoms per dimer [1, 3, 8–10]. Copper may have been lost during the purification of DAO or the amount of DAO overestimated in the protein assay. Apo-DAO prepared *in vitro* was reactivated specifically by Cu^{2+} with reactivation being maximal after 20 min when *ca* 80% of the original activity of the holoenzyme was regained. Table 2 shows the amino acid composition of clover DAO which is similar to that of other legume DAOs in that a high proportion of acidic residues are present.

Table 2. Amino acid composition of clover DAO

Amino acid	Number of residues relative to Met
Asp	41
Thr	25
Ser	26
Glu	34
Gly	22
Ala	14
Val	24
Met	1
Ile	24
Leu	27
Tyr	12
Phe	16
His	24
Lys	23
Arg	18

EXPERIMENTAL

Plant material. Subterranean clover (*Trifolium subterraneum* L. cv. Seaton Park) was grown in soln culture with all nutrients supplied as described in ref. [19] except that copper as $CuSO_4$ was supplied at 1 μM. Young folded leaves were collected because they contained the highest DAO activity per mg protein [14]. Leaves were stored at −80°.

Purification of DAO. The whole purification procedure was carried out at 4°.

Step 1. About 100 g of young leaves were powdered using a food processor. To the powdered leaves 0.1 M KPi pH 7 containing 25 mM mercaptoethanol was added with polyvinylpolypyrrolidone (5 g/100 ml) and acid-washed sand. The mixture was ground with a mortar and pestle, filtered through cheese cloth then centrifuged at 10 000 *g* for 40 min. *Step 2.* Solid $(NH_4)_2SO_4$ was added to the supernatant of step 1. Ppts that formed at 30–70% satn were collected and dissolved in 10 mM KPi pH 6.4, then dialysed against 4 l of the same buffer overnight. *Step 3.* The dialysed soln was clarified by centrifugation before being applied to a phosphocellulose column (2 × 10 cm) previously equilibrated with 10 mM KPi pH 6.4. When $A_{280\,nm}$ had dropped to zero, DAO was eluted with a continuous convex gradient of 1 M NaCl in 10 mM KPi pH 6.4. *Step 4.* The active fractions of step 3 were combined and concentrated, and after overnight dialysis against 4 l of 10 mM KPi pH 7, the sample was applied to a hydroxylapatite column (1.6 × 20 cm) equilibrated with 10 mM KPi pH 7. Protein was eluted with a continuous convex gradient where the limit buffer was 0.4 M KPi pH 7. The fractions containing DAO activity were combined, concentrated and used as the pure enzyme. The purified enzyme in KPi (0.1 M, pH 7) was stable at −20° for many months.

Polyacrylamide gel electrophoresis. Native and SDS gel electrophoresis were carried out as described in ref. [20]. To stain for DAO activity, the gel was incubated at 30° in 67 mM KPi pH 7 with 100 μg horseradish peroxidase, 50 μM guaiacol and 5 mM 1,4-diaminobutane. Regions of the gel with DAO activity stained brown.

Copper analysis. Samples of pure DAO in soln were analysed for copper by atomic absorption spectrophotometry with a heated graphite atomizer. Analysis was performed using the 324.7 nm spectral line together with a deuterium lamp for correction of background absorption.

DAO assay. DAO was assayed by measuring the uptake of O_2. For routine assays, DAO in 2 ml of 67 mM KPi buffer pH 7 was preincubated for 2 min at 30° in a Hansatech O_2 electrode. The reaction was started by adding 10 μmol 1,4-diaminobutane dissolved in 50 μl buffer.

Amino acid analysis. About 0.5 mg of DAO was sealed in a glass tube with 6 M HCl and hydrolysed by incubating overnight at 110°. After the HCl was evaporated with a stream of N_2, the sample was made up to 1 ml with water for analysis using a Varian 5560 HPLC equipped with Varian amino acid analysis columns.

Preparation of apo-DAO. Apo-DAO was prepared by overnight dialysis of pure DAO against 0.01 M Na diethyldithiocarbamate. The dialysed soln was centrifuged at 20 000 *g* for 40 min, then redialysed overnight against KPi buffer (10 mM, pH 7) that had been purified on a Chelex-100 column. After dialysis the sample was centrifuged at 20 000 *g* for 40 min and used for reactivation studies.

Gel filtration. The M_r of native DAO was estimated by gel filtration on a Sephacryl S-300 column (1.6 × 100 cm) calibrated

with proteins of known M_r. The column was run at 20 ml/hr with a KPi buffer (0.1 M, pH 7) containing 0.5 M KCl.

Protein assay. Protein was assayed as described in ref. [21] using bovine serum albumin as standard.

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