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PURIFICATION AND PROPERTIES OF GLYCOLLATE OXIDASE FROM *PISUM SATIVUM* LEAVES

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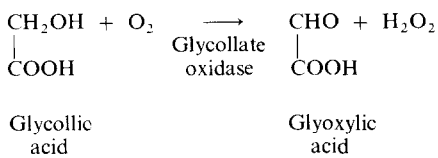
Abstract—A rapid and efficient method for the isolation of glycollate oxidase from pea leaves is described. The method utilizes the unusually high isoelectric point (pH 9·6) which has been determined for the enzyme using isoelectric focusing. The enzyme is apparently homogeneous by polyacrylamide gel electrophoresis and has a MW of *ca* 100000. Some properties of the enzyme are described.

INTRODUCTION

Glycollate oxidase (glycollate:oxygen oxidoreductase E.C. 1.1.3.1) is a flavoprotein, catalysing the oxidation of glycollic acid to glyoxylic acid [1].



It is found in relatively high concentration (about 2–3 units/g fr. wt) in green leaves where it is located in the peroxisomes [2]. The enzyme is inducible either by light [3] or by glycollate [4] and is believed to play a vital role in the process of photorespiration [5].

Although glycollate oxidase was first isolated from tobacco leaves more than 20 years ago [6,1] and subsequently crystallized from spinach [7] the techniques used were cumbersome by current standards. An attempt to improve the isolation by using the affinity chromatography principle [8] suffered the disadvantage that it was necessary to remove the FMN cofactor from the crude enzyme. This can be time consuming and often results in loss of enzyme activity.

A first requirement for a detailed study of this important and interesting enzyme was therefore, a quick and reliable isolation procedure.

RESULTS AND DISCUSSION

Purification procedure

The initial stages were based on those of Frigerio and Harbury [7]. Preliminary experiments had shown the enzyme to be most stable at 0–4° and pH 8·3. These conditions were therefore maintained unless otherwise stated. Precipitates were separated by centrifuging at 17000 *g* for 10 min.

(A) *Homogenization.* Pea leaves (200 g) were homogenized using full speed in an MSE "Atomix" blendor for 1 min in 800 ml of 0·1 M NaH_2PO_4, pH 8·0. The homogenate was strained through four layers of muslin and centrifuged.

(B) *Precipitation at pH* 5·3. The supernatant from step A was carefully adjusted to pH 5·3 with 10% v/v HOAc and after stirring for 10 min the precipitate was removed by centrifugation.

(C) *Ammonium sulphate fractionation.* The protein fraction precipitating from the supernatant from B between 25 and 43% saturation with $(NH_4)_2SO_4$ was redissolved in 10–15 ml of 0·02 M Tris–HCl pH 8·3.

(D) *Protamine sulphate precipitation.* Protamine sulphate solution (2 ml, 2%) was added to the protein solution slowly with stirring. The protamine: nucleic acid complex was removed by centrifugation. If this step was omitted, some of the glycollate oxidase complexed with the nucleic acid resulting in poor yields from subsequent steps.

(E) *Sephadex G25–DEAE cellulose chromatography.* The supernatant from D was applied to

Table 1. Summary of a purification of glycollate oxidase from pea leaves

Procedure	Volume (ml)	Total activity (units)	Total protein (mg)	Sp. act. (units/mg)	Yield (%)	Purification factor (×)
(A) Centrifuged brei	845	500	10100	0·05	(100)	
(B) pH 5·3 precipitation	870	495	4350	0·11	99	2·2
(C) Ammonium sulphate fractionation	12·7	280	590	0·47	56	4·3
(D) Protamine sulphate precipitation	13·0	248	445	0·56	50	1·2
(E) Sephadex G25/DEAE-cellulose	30·0	131	10·5	12·5	26	22·4
(F) Bio-gel A1·5 filtration				30·0		2·5

a 3·4 × 10 cm column of Sephadex G25 fine equilibrated in 5 mM Tris–HCl pH 8·3. The protein band emerging immediately after the void volume, was pumped (48 ml/hr) on to a 2·5 × 6 cm column of DEAE-cellulose (DE 52) equilibrated in the same buffer. The eluate was monitored A at 280 nm and the first absorbing band, containing the glycollate oxidase activity, was collected. Under the conditions used, most contaminating proteins were adsorbed on to the DEAE-cellulose, glycollate oxidase with a pI above the prevailing pH of 8·3 was positively charged and was therefore not adsorbed. To the enzyme solution, an equal volume of satd $(NH_4)_2SO_4$ solution pH 8·3 was added. At this stage the purified enzyme could be conveniently stored overnight in the refrigerator. The suspension was then centrifuged and the pellet redissolved in 0·4 ml of 50 mM Tris–HCl pH 8·3.

(F) *Agarose chromatography.* The concentrated protein solution was applied to a 1·5 × 30 cm column of 8% agarose gel (Biogel A 1·5) equilibrated in 50 mM Tris–HCl pH 8·3. Fractions of *ca* 2·5 ml were collected and assayed for glycollate oxidase activity and protein concentration. Table 1 summarizes a typical experiment using 200 g of starting material. The highest specific activity obtained from step F was 30·4 units/mg, if fractions with specific activities > 25 units/mg were bulked, a yield of *ca* 50% was obtained for this step.

Larger scale preparation

The preparative method could be scaled up 5 times by modifying the procedure as follows:

(a) One kilogram of pea shoots were deep frozen (−20°) and minced through an electric meat grinder. The frozen powder was mixed with 2 l. of 0·1 M NaH_2PO_4 pH 8 at room temp. using an overhead blendor. The brei was strained through nylon net using a basket centrifuge and treated as in steps B and C above.

(b) The redissolved $(NH_4)_2SO_4$ pellet was dialysed against 0·01 M Tris–HCl pH 8·3 in a hollow fibre device (Biofibre 50 beaker dialyser, Bio-rad Laboratories) until the diffusate contained no detectable sulphate or UV absorbing material. The dialysed protein solution could then be pumped on to a DEAE-cellulose column as in (E), the Sephadex column being omitted.

Properties

Storage. The purified enzyme was stable for several months if mixed with an equal volume of glycerol and stored at −15°. Prolonged storage as an ammonium sulphate pellet led to loss of the FMN cofactor.

K_m *values.* Using the procedure of Lineweaver and Burk [9] a K_m of $2{\cdot}5 \times 10^{-4}$ M was determined for glycollate. This compared with a recently published figure of $2{\cdot}62 \times 10^{-4}$ M [10] for the crude pea enzyme and the original value of $3{\cdot}8 \times 10^{-4}$ M for the pure spinach enzyme [1].

An estimate of the K_m for oxygen was made by measuring the slope of the oxygen consumption traces from normal assays at various points corresponding to known oxygen concentrations. The Lineweaver–Burk plot gave a straight line and indicated a K_m of $1{\cdot}33 \times 10^{-4}$ M for oxygen. This is considered to be a reasonable approximation since the enzyme does not show significant product inhibition and the glycollate concentration after all the

oxygen is consumed is still $0{\cdot}78 \times 10^{-3}$ M or more than three times the K_m.

Substrate specificity. In addition to glycollate the enzyme will oxidize glyoxylate (to yield oxalate), L-lactate (K_m $6{\cdot}6 \times 10^{-3}$ M) and other straight chain L-α-hydroxyacids up to α-hydroxycaproate (K_m for D/L racemic mixture is 7×10^{-3} M). The branched chain α-hydroxy*iso*butyric acid is not a substrate for the enzyme.

Isoelectric point. Crude enzyme after Sephadex G25 filtration was subjected to isoelectric focusing firstly over a broad pH range of 3–10 and subsequently over a nominal pH 8–10 range. Over the narrow pH gradient, the highest specific activity was in the fraction with pH 9·6, but the sharply rising pH profile at this point means that an accurate pI is difficult to measure. Higher pH range ampholines were not available at the time. The protein is clearly very basic with a pI not less than 9·6.

Amino acid analysis. Table 2 gives the amino acid composition of a sample of enzyme after purification step F. From this data using the method described by Schachman [11] it was possible to calculate a value of 0·74 cm^3 g^{-1} for the partial specific volume of the enzyme.

Table 2. Amino acid composition of glycollate oxidase

Amino acid	Molar ratio (half-cystine = 1)
Aspartic acid	37·1
Threonine	39·1
Serine	30·7
Glutamic acid	40·8
Proline	37·0
Glycine	42·8
Alanine	45·5
Valine	58·1
Half-cystine	1·0
Methionine	14·1
Isoleucine	35·9
Leucine	57·3
Tyrosine	17·2
Phenylalanine	25·1
Ammonia	38·5
Lysine	29·2
Histidine	7·1
Arginine	29·8

SDS-polyacrylamide gel electrophoresis. This showed a single protein band for the enzyme after purification step (F) and two main protein bands for the enzyme after step (E). Comparing the distances migrated with those of a series of proteins of known MW [12] allowed an estimate of the MWs of these proteins to be made. The proteins from step E enzyme had MWs of 100000 ± 5000 and 50000 ± 4000. The single protein from step (F) enzyme corresponded with the smaller of these two.

The single protein band shown on SDS-polyacrylamide gel electrophoresis of the pure enzyme is taken as a good indication of homogeneity. If the enzyme is run in its native form on normal gels at pH 7 it is necessary to have the negative electrode at the bottom. Under these conditions, a single protein band is again seen but this is less significant since any contaminating proteins would be unlikely to enter the gels under these conditions. Treatment with SDS will confer a negative charge on all proteins, at neutral pH.

Gel filtration. Chromatography of the enzyme after step E on Sephadex G75 showed a main band of enzyme activity eluting immediately after the void volume and a second, well separated band of lower activity. The same pattern was found on three separate runs. If material from the main peaks was bulked; concentrated and re-run, the identical pattern of two bands of activity was again seen. This implies that the higher MW species may be dissociating to give the smaller species.

Chromatography on Sephadex G100 showed the main activity peak to be somewhat retarded by the gel, this allowing an estimate of its MW to be made [13]. The elution value of the main peak of enzyme activity corresponded to a MW of 88000 ± 5000. The smaller peak was too diffuse to allow an accurate estimate of its size.

Ultracentrifugation. Samples of pure enzyme after step (F) were studied in the analytical ultracentrifuge by the meniscus-depletion, sedimentation equilibrium procedure of Yphantis [14]. From this data a minimum MW of 48500 was estimated. These data, together with those from gel-filtration and SDS-polyacrylamide gel electrophoresis combine to give us a picture of the composition of glycollate oxidase. It seems to have a minimum MW of 48500 as seen in the analytical ultracentrifuge, by SDS-polyacrylamide gel electrophoresis and on Sephadex G75 and G100 columns as the smaller component. The most active form seems to be a dimer which can be seen on Sephadex G75 and G100 columns and on some SDS-polyacrylamide gels. It is not known whether the monomer form is active or whether it slowly

dimerizes to give the activity seen after its resolution by Sephadex chromatography. Part of the increased specific activity after agarose gel-filtration may be due to isolation of the dimer from less active forms of the enzyme. We have on occasion, observed a low level of glycollate oxidase activity associated with a protein band of higher MW coexisting with the dimer and monomer bands. Frigerio and Harbury [7] originally proposed that the enzyme may exist as a mixture of monomer, dimer and tetramer. Our results would tend to confirm their suggestions.

EXPERIMENTAL

Materials. Pea plants (*Pisum sativum* cv Suttons Phenomenon) were grown in John Innes No. 1 compost in a greenhouse with supplementary heating and light in the winter. Leaves or whole shoots were harvested 2–3 weeks after planting.

Protein determinations. For crude samples the biuret method [15] was used. Where less protein was available either the direct spectrophotometric method [16] or, for more accurate measurements, the method of Lowry *et al.* [17] was used.

Enzyme assays. These were carried out in an O_2 electrode at 30°. The reaction mixture (3·2 ml) contained: Tris–HCl buffer pH 8·3 150 μmol, FMN 0·2 μmol, NaN_3 (to inhibit any residual catalase activity) 3 μmol, enzyme < 0·5 unit, sodium glycollate 3 μmol. The reaction was started by adding the sodium glycollate. Light was excluded from the reaction vessel to prevent photo-oxidation of the FMN. The reaction was followed by the uptake of O_2, the activity value of Chappell [18], 0·445 μg atoms O_2 per ml at 30° for air-saturated buffer, was used.

Isoelectric focusing. This was carried out using a 110 ml LKB "ampholine" column, according to the manufacturers' instructions.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. This was carried out by the general procedure of Shapiro *et al.* [12]. SDS-treated protein samples were run on 7·5% acrylamide gels, pH 7·5 for 4·5 hr at 4 mA per tube. Gels were fixed overnight in HOAc–EtOH–H_2O (1:1:8), stained for 3 hr in 0·2% Coomassie brilliant blue soln in HOAc–EtOH–H_2O (2:9:9) and destained in the fixing soln.

Ultracentrifuge studies. These were carried out on a Beckman model E analytical ultracentrifuge equipped with interference optics. The rotor speed was 19850 rpm and the temp. 16·25°.

Amino acid analyses. These were carried out using the Technicon Auto-Analyser system.

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